Candida albicans Mitochondrial Protein Import Assay

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[Abstract] 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*, *C. albicans* is not subject to the Crabtree effect and 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 the protocol: Preparation of Mitochondria from *Candida albicans*) (Hewitt *et al*., 2013) produces a more simplified system that can be interrogated using the standard tools of molecular biology. In addition, the import of radiolabelled proteins described in this protocol is a sensitive technique that can be used to determine details of kinetics and interactions of imported proteins.

Materials and Reagents

1. Rabbit Reticulocyte Lysate System, Nuclease Treated (Promega Corporation, catalog number: L4960)
2. ^{35}S labeled cysteine and methionine (PerkinElmer, catalog number: 072007MC)
3. SUPERase-In (Life Technologies, Ambion®, catalog number: AM2696)
4. Mitochondria
5. Sorbitol
6. Valinomycin
7. Oligomycin
8. Antimycin
9. Soybean Trypsin Inhibitor (SBTI) (Sigma-Aldrich)
10. Amino acids
11. Coomassie Brilliant Blue G250

12. Digitonin (Calbiochem, catalog number: 11024-24-1)
13. RNA for *in vitro* translation (see Recipes)
14. Import buffer (IB) (see Recipes)
15. Blue native lysis buffer (see Recipes)
16. 10x BN (Blue Native) loading dye (see Recipes)
17. RNA (see Recipes)
18. ATP (see Recipes)
19. NADH (see Recipes)
20. AVO (see Recipes)
21. Proteinase K (PK) (see Recipes)
22. Apyrase (see Recipes)
23. Phenylmethanesulfonylfluoride (PMSF) (see Recipes)
24. Trypsin (see Recipes)

Equipment

1. Water bath with temperature control
2. Centrifuge
3. Filter – 0.45 µm Acrodisc syringe filter (Pall Life Sciences)

Procedure

A. Translation Reaction (30 °C for 50 min) – 15 µl reaction, scale as needed.
   1. 10 µl rabbit reticulocyte lysate.
   2. 1 µl amino acids mix (1 mM but no cysteine or methionine).
   3. 1 µl RNA.
   4. 1 µl ^{35}S labeled cysteine and methionine.
   5. 1 µl SUPERase-In (add a maximum of 2.5 µl).

B. Mitochondria Preparation
   1. 100 µl of Import buffer (IB) per 50 µg of mitochondria.
   2. Standard conditions for 100 µl IB:
      a. ATP (0.4 M) 1 µl
      b. NADH (0.5 M) 0.4 µl
      c. Mix by inversion and leave at import temperature (25 °C standard) for 5 min to equilibrate.

d. Add 5 µl translation reaction per 100 µl import (reaction mix can be diluted by addition of water and 2.4 M sorbitol to a final sorbitol concentration of 0.6 M so up to 15 µl of this solution can be added to 100 µl of import buffer which can help increase signal if translation efficiency is low) and incubate at import temperature removing samples at suitable time points as described in part 3.

3. Optional additions:
   a. AVO mix of mitochondrial inhibitors for dissipation of membrane potential (1 µl per 100 µl).
   b. Apyrase- 2 µl per 100 µl IB – treat lysate and mitochondria for 10 min at 25 °C.

C. Stopper solutions
1. 100 µl cold IB per 100 µl import in standard conditions.
2. Additions include the following but concentrations may need to be optimized:
   a. Trypsin: 1 µl per 100 µl of stopper solution
   b. SBTI: 4 µl per 200 µl of mix
   c. PK: 2 µl per 100 µl of stopper solution
   d. PMSF: 1 mM final (vortex when added)
   e. PK/trypsin containing solutions need 20 min before addition of inhibitor
3. At each time point remove 95 µl if adding 5 µl translation reaction/100 µl IB or 105 µl if adding 15 µl/100 µl IB.

D. Sample Treatment
1. Once inhibitor added to all samples spin at 4 °C/8,000 *x g*/10 min.
2. Remove and discard supernatant.
3. Add 100 µl cold IB (do not resuspend pellet – add protease inhibitor if protease used in stopper solution).
4. Spin at 4 °C/8,000 *x g*/5 min.
5. Remove and discard supernatant.
   (For SDS resuspend pellet in appropriate amounts of water and SDS loading dye)
   For blue native PAGE:
6. Resuspend pellet in 50 µl cold blue native lysis buffer (see Recipes) per 2 µl of mitochondria.
7. Leave samples on ice for 15 min with gentle vortex every 5 min.
   (FOR ANTIBODY SHIFT: Add 2 µl antibody and leave on ice for 1 h – gentle vortex every 20 min – then continue as per method below)
8. Spin samples at 4 °C/14,000 *x g*/10 min (or 15,000 if available).
9. Remove 45 µl supernatant to new tube with 5 µl blue native loading dye.

Recipes

1. RNA from *in vitro* transcription reaction using 5 µg linear plasmid DNA (or 1.5 µg of PCR product with SP6 promoter)
2. Import buffer (IB)
   0.6 M sorbitol
   50 mM HEPES (adjust to pH7.4 with KOH)
   2 mM KPi (pH 7.4 made by mixing 1 M KH_2PO_4 with 1 M K_2HPO_4)
   25 mM KCl
   10 mM $MgCl_2$
   0.5 mM EDTA
   1 mM DTT stored at RT (room temperature)
3. ATP
   Aliquots of 0.4 M stored at -20 °C (pH to 7)
4. NADH
   Aliquots of 0.5 M stored at -20 °C
5. AVO
   100x stock frozen in EtOH & foiled (1 µM valinomycin, 20 µM oligomycin, 8 µM antimycin) stored at -20 °C
6. PK (Proteinase K)
   5 mg/ml aliquots in IB stored at -20 °C
7. PMSF
   0.2 M in EtOH stored at RT for up to 1 month
8. Trypsin
   Use 10 mg/ml stock (to give 0.1 mg/ml final) for 20 min (stored at -20 °C)
9. SBTI
   50 mg/ml (for 5 min on ice) (stored at -20 °C)
10. Apyrase
    500 units/ml in 50% glycerol stored at -20 °C
11. 10x BN (Blue Native) loading dye
    0.5 M aminocaproic acid
    0.1M Bis Tris pH 7.0
    10% Coomassie Brilliant Blue G250 – filter and store at room temperature
12. Blue native lysis buffer
    20 mM Tris-HCl
    0.1 mM EDTA
    50 mM NaCl

10% glycerol (store at room temperature)
Add PMSF and 5% w/v digitonin (Calbiochem – heat to dissolve and store at 4 °C) to give a final concentration of 1% digitonin and 1 mM PMSF just before use.

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