MitoPrep Protocol

Fiumera protocol - modified from Broadley/Neupert/Martinez protocols
Mitosis purified here are beautifully active in IOTL experiments

Buffers:
Tris-DTT buffer: 0.1M Tris-SO4 pH 9.4, 10mM DTT (Tris-SO4 means pH adjusted w/ H2SO4)
MP2
  1.2M sorbitol
  20 mM KH2PO4 pH 7.4
  Use MP2 buffer in a volume of 6.7 ml/g ww of yeast cells.
  Add 3 mg zymolyase/ g ww (20T from Assoc. of Cape Cod
  (Seikagaku America) 1-800-237-4512 1G #120491
Dounce Buffer:
  10 mM Tris pH 7.4
  1 mM EDTA
  0.2% BSA (fatty acids free)
  1 mM PMSF
  0.6 M sorbitol
  Use at 13.4 ml/g ww
SEH
  20 mM HEPES pH 7.4
  0.6 M Sorbitol
  1 mM EDTA
2x SEH:
  60 mM HEPES pH 7.4
  1.2 M Sorbitol
  2 mM EDTA
Nycodenz:
50% solution in H2O
Other:
30C shaking water bath
SS-34 rotor, reserve Sorvall
2 preweighed falcon tubes
40 ml dounce

Harvest cells:
Spin 1-2L down in big boy rotor, 4C, 10 minutes, 4000K
Wash 1x with cold H2O (1/10 volume ~200 mL)
Spin.
Transfer to preweighed Falcon tubes (2 tubes/Lculture)
Repellet in tabletop Sorvall. (4200, 4C, 5’)
Weigh wet cell weight.
Spheroplasts (cells without cell wall)
Resuspend Tris-DTT buffer (2 ml/g wet weight)
Rock 10’, 30C
Pellet 5’ 5000 rpm SS-34
Wash pellet with MP2
5 min, 5000 rpm SS-34
Resuspend pellet in MP2 (6.7 ml/g ww)- Add zymolyase (3 mg/g ww)
Rock 30-60 min, 30C till sphereoplasts form
2 ml H2O + 50 ul cells vs. 2 ml 1.2 M sorbitol + 50 ul cells. Look for clearing under phase contrast microscope. Spheroplasts bloat and lyse in hypotonic solution.
Pellet 5’ 3000 rpm SS-34

**Dounce:**
Resuspend each tube in a small amount of Dounce Buffer, bring up to volume in dounce
Dounce ~10 times in 40 mL dounce

**Collect Crude Mitochondria**
Pellet 5’, 4000 rpm SS-34
save supernatant- (This first pellet contains nuclei and cellular debris.)
Respin sup’ 4000 rpm SS-34
Save sup-
Spin sup 12 min 12000rpm SS-34 (Pellet=mitochondria, supernatant=cytoplasm)
Resuspend pellet in 10 mL SEH (small volume 1st)
Spin 5 min 4000 SS-34
[optional respin sup 5’ 4000 rpm SS-34][I skip this if doing gradients]
Collect sup, spin 12 min 12000 rpm SS-34
Resuspend mitos in 300 ul SHE
spec quantify: 10 ul to 990ul 0.6% SDS, vortex briefly, 15K 4’
OD 280 of 0.21 = 10 mg/mL

**Gradient Purification**
1. Prepare 15ml each of 25, 20, 15, 10, 5% (w/v) nycodenz in dounce buffer.
   25% = 7.5ml 50% Nycodenz + 7.5 ml 2x SEH Buffer
   20% = 6 ml 50% Nycodenz + 7.5 ml 2xSEH buf + 1.5ml H2O
   15% = 4.5 ml 50% Nycodenz + 7.5 ml 2xSEH buf + 3.0ml H2O
   10% = 3 ml 50% Nycodenz + 7.5 ml 2xSEH buf + 4.5 ml H2O
   5% = 1.5 ml 50% Nycodenz + 7.5 ml 2xSEH buf + 6.0ml H2O
2. Make 2 gradients per culture. Overlay 2ml: 2 mL of 20, 15, 10, 5 (in that order).
3. Overlay 1-2 ml crude mt (50-75mg) onto each gradient. Centrifuge 40,000rpm for 30min in SW-41 rotor (timing begins after rotor is up to speed)
   Four bands will(should) be seen:
   1. 7-5% mt fragments (white or tan)
   2. 12% mitoplasts (pinkish)
   3. 16% mitochondria (pink-brownish)
   4. 19% cell debris (broad, granular)
4. Remove the mt band with needle and syringe.
5. Dilute the mitochondria with SEH (~25ml total). Spin 10 minutes at 12,000xg.
   Resuspend (250ul/gradient) gently.
6. Estimate concentration:
   10 ul to 990ul 0.6% SDS, vortex, spin 3’ 4C, measure OD 280
   A280 of 0.12=10mg/ml protein in the undiluted mix.
Freeze 1 mg aliquots in SEH buffer.
(can be beneficial to dilute to 10 mg/ml if mitos are too concentrated)

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<tbody>
<tr>
<td>strain</td>
<td>Y4-17 (1-109,cox18Δ)</td>
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<tr>
<td>OD 600/Klett</td>
<td>1.56</td>
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<tr>
<td>Wet weight</td>
<td>12g</td>
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<tr>
<td>ml crude mitos</td>
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<tr>
<td>OD 280 (crude)</td>
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<tr>
<td>Mg/ml crude mitos</td>
<td>20.9</td>
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<td>Total crude mitos</td>
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Gradient profile

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<tbody>
<tr>
<td>ml pure mitos</td>
<td>0.55 ml</td>
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<tr>
<td>OD 280 (pure)</td>
<td>.27</td>
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<tr>
<td>mg/ml</td>
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<tr>
<td>total mgs</td>
<td>12.4</td>
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<tr>
<td>0.5 mg aliquot</td>
<td>22.1ul</td>
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