OptiPrep™ Application Sheet S17

Purification of yeast spheroplast mitochondria

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.

1. Introduction

This text is primarily concerned with the application of iodixanol gradients to the purification of yeast spheroplast mitochondria. However because of the popularity and efficacy of the Nycodenz®-based method however, first published by Glick and Pon [1] in 1995, this methodology is summarized in Section 3. Section 4 is devoted to the use of iodixanol gradients. These sections are prefaced by Section 2, which is devoted to the preparation of spheroplasts from cultured yeast cells and the subsequent isolation of a crude mitochondrial fraction.

2 Isolation of a crude mitochondrial fraction from yeast spheroplasts

2a. Solutions required (see Section 2d, Notes 1and 2)

A. Spheroplast buffer: 1.2 M sorbitol, 20 mM phosphate buffer, pH 7.4 (see Box 1)

B. Spheroplast lysis medium: 0.6 M sorbitol, 20 mM MES-KOH, pH 6.0 (see Box 1)

Box 1

Keep the following stock solutions at 4°C:
100 mM Hepes; 2.38 g per 100 ml water
1 M MES, purchased ready-made solution
100 mM KH_2PO_4; 1.36 g per 100 ml water
100 mM $K_2HPO_4 \bullet 3H_2O$; 2.28 g per 100 ml water

Solution A: Mix 40 ml of $K_2HPO_4 \bullet 3H_2O$ with 10 ml of KH_2PO_4; check the pH is 7.4 and adjust if necessary. Dissolve 21.8 g of sorbitol in 50 ml of water, add 20 ml of the phosphate buffer and make up to 100ml

Solution B: Dissolve 10.9 g of sorbitol in 50 ml of water; add 2 ml of the MES stock; adjust to pH 6.0 with 1 M KOH and make up to 100 ml.

2b. Spheroplast production (adapted from ref 1)

The protocol is designed for a 5 l culture of yeast cells, which should yield 35-40 g of packed yeast cells.

1. Use 2.5 mg of Zymolase 20T per g of cells and dissolve in Solution A (2 ml per g of cells).

2. Wash the cells in 40 ml of Solution A and centrifuge at 2000 *g* for 5 min.

3. Decant the supernatant and suspend the cell pellets in the Zymolase solution and incubate at 30° for 30 min with gentle shaking.

2c. Production of crude mitochondria (adapted from ref 1)

Carry out all operations at 0-4°C. The protocol can be scaled down proportionately as required.

1. Centrifuge the spheroplast suspension at 4000 *g* for 5 min.

2. Suspend the spheroplasts in Solution A (Box 1) at 40 ml per spheroplast pellet from approx 35 g wet weight of yeast cells and centrifuge at 4000 *g* for 5 min.

3. Remove the supernatant and repeat steps 1 and 2.

4. Resuspend each spheroplast pellet in 50 ml of Solution B (Box 1) and homogenize in a tight-fitting Dounce homogenizer (Wheaton Type A) using 15 strokes of the pestle (see Section 2d, Note 3).

5. Dilute the homogenate with Solution B (1.5 vol.) and centrifuge at 1500 *g* for 5 min to pellet the nuclei and unbroken cells.

6. Decant and retain the supernatants; as the pellets may be loosely packed it may be better to aspirate the supernatants using 20 ml syringe and metal cannula.

7. Resuspend each pellet in Solution B and repeat Steps 4-6.

8. Combine the supernatants and centrifuge at 12,000 *g* for 10 min.

9. Decant the supernatants and resuspend the crude mitochondrial pellets in Solution B using 3-4 gentle strokes of the pestle of a loose-fitting (Wheaton Type B) Dounce homogenizer (see Note 4).

10. Centrifuge the suspensions at 1500 *g* for 5 min to remove any aggregates and debris.

11. Using a syringe and metal cannula, aspirate the supernatants carefully; repeat step 8 and resuspend the pellets in one of the solutions described in Sections 3-5.

2d. Notes

1. It is normal practice to add protease inhibitors to all the solutions used in the lysis of the spheroplasts and in all subsequent operations. The chosen inhibitors should be added from routine stock solutions just prior to use. Because of the variable hydration of powdered MES, a commercially available 1 M solution is more convenient.
2. Spheroplasts may be prepared from yeast by zymolase digestion and disrupted in a Dounce homogenizer [1] as described in this Application Sheet; there are however a number of variations. In the method use by Ishihara et al [2] for example, Solution A contained 1.4 M sorbitol, 20 mM Tris-HCl, pH 7.5, in SD(-N) medium, while Solution B contained 1M sorbitol, 0.5% polysucrose (Ficoll) and 1 mM $MgCl_2$.
3. Alternative methods of homogenization of the spheroplasts are passage of the spheroplast suspension through a 3 μm pore size polycarbonate filter [2] or repeated passage through a fine syringe needle.
4. Resuspension of any crude mitochondrial pellets must be carried out as gently as possible.

3. Nycodenz® gradients

3a Introduction

For yeast grown in a semi-synthetic lactate medium, a top-loaded discontinuous gradient of 14.5% and 18% (w/v) Nycodenz® was devised by Glick and Pon [1]; the mitochondria that band at the interface of the two solutions are highly purified and metabolically active. Glick and Pon [1] however pointed out that the density of the mitochondria depends on the yeast strain and on the growth conditions. Thus if the 14.5%/18% Nycodenz® system was found to be unsatisfactory it was necessary to determine the true density of the mitochondria either in a shallow discontinuous gradient or a continuous gradient first. Once the banding density of the mitochondria, and of the contaminating organelles, has been established, it may be possible to devise a simpler discontinuous gradient. In some instances however, it may be desirable to use a more discriminating continuous gradient if other organelles in the crude mitochondrial fraction are to be studied at the same time. Section 3b will summarize the discontinuous Nycodenz® gradient methodology for wild-type yeast strains (D273-10B, MATα; ATCC 25657) grown in a semi-synthetic lactate medium.

3b. Gradient methodology (see ref 1)

1. Prepare the 14.5% and 18% (w/v) Nycodenz® solutions by diluting 14.5 ml and 18 ml of a 50% Nycodenz® stock solution with 25 ml of 1.2 M sorbitol, 40 mM MES-KOH, pH 6.0 and make up each to 50 ml with water.

2. Resuspend the crude mitochondria pellets in lysis medium (approx. 1 ml per 10 g of original yeast wet weight) using a small volume loose-fitting Dounce homogenizer.

3. In 13 ml tubes for a swinging-bucket rotor (approx 13 ml tubes, e.g. Beckman SW 41Ti, Sorvall TH641 or equivalent), underlayer 6 ml of the 14.5% Nycodenz® solution with the same volume of 18% Nycodenz® and layer 1 ml of the crude mitochondrial suspension on top.

4. Centrifuge at 200,000 g_{av} for 30 min and collect the mitochondria from the interface of the two Nycodenz® solutions; dilute the suspension with approx 4-5 vol. of 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4 Solution C. Pellet the mitochondria at 12,000 *g* for 10 min and resuspend in the same buffer.

♦ If the density of these Nycodenz® solutions is unsuitable, Glick and Pon [1] recommended investigating the following alternatives 15% and 21%, 14% and 20%, 13% and 19% or 12% and 18% (w/v).

4. Iodixanol gradients

Solutions of iodixanol of the same concentration as those of Nycodenz® (described above) can be substituted; indeed Lindahl et al [3] reported that this was successful. Although the solutions will have the same density, if prepared in the same way as described in Section 3b (step 1) they will have a lower osmotic pressure. Meeusen et al [4] was the first to describe the use of a customized discontinuous gradient of iodixanol in which the crude mitochondrial fraction is layered beneath the gradient, so that the organelles float to their banding density. Continuous gradients of iodixanol have also been developed; see section 4b.

4a. Flotation in a discontinuous iodixanol gradient

4a-1. Solutions required (see Section 4a-4, Note 1)

A. OptiPrep™

B. OptiPrep™ dilution buffer: 0.8 M sorbitol, 60 mM Hepes-KOH, pH 7.4 (see Box 2)

C. Iodixanol (40% w/v) working solution: 2 vol. of Solution A + 1 vol. Solution B

D. Mitochondria suspension buffer: 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4 (see Box 2)

Box 2

Stock solutions of 100 mM Hepes: see Box 1.

Solution B: Dissolve 14.6 g of sorbitol in 60 ml of Hepes stock; adjust to pH 7.4 with 1 M KOH and make up to 100 ml.

Solution D: Dissolve 10.9 g of sorbitol in 50 ml of water; add 20 ml of the Hepes stock; adjust to pH 7.4 with 1 M KOH and make up to 100 ml.

4a-2. Ultracentrifuge rotor requirements

38 ml swinging-bucket rotor: e.g. Beckman SW28 **OR** 17 ml swinging-bucket rotor: e.g. Beckman SW28.1 **OR** 13 ml swinging-bucket rotor: e.g. Beckman SW 41Ti

4a-3. Protocol (adapted from ref 2)

Carry out all operations at 0-4°C. The protocol can be scaled down proportionately as required.

1. Suspend crude mitochondria in approx. 40 ml of Solution D using two or three gentle strokes of the pestle of a loose-fitting Dounce homogenizer and recentrifuge at 12,000 *g* for 10 min (see Section 4a-4, Note 2).

2. Suspend the washed crude mitochondrial pellet in 20 ml of Solution C (see Section 4a-4, Notes 3 and 4).

3. Prepare iodixanol solutions of ρ = 1.10 and 1.16 g/ml by diluting Solution C with Solution D (3 + 7 and 6.25 + 3.75 v/v respectively).

4. In tubes for the 38 ml swinging-bucket rotor layer 10 ml of the mitochondrial suspension and approx 14 ml each of the ρ = 1.10 and 1.16 g/ml solutions. Scale down all volumes proportionately if smaller volume rotors are used .

5. Centrifuge at 80,000 *g* for 3 h.

6. Collect the band of mitochondria (see Figure 1); dilute with four volumes of Solution D and harvest the purified organelles at 10,000 *g* for 10 min (see Section 4a-4, Note 5).

7. Remove any aggregated material by recentrifugation at 3,000 *g* for 5 min.

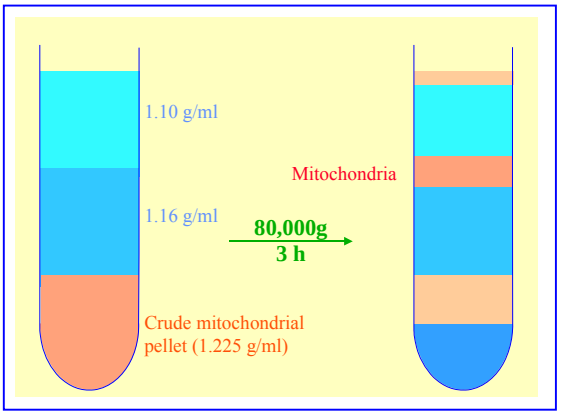

Figure 1 Banding of yeast mitochondria in a discontinuous gradient of iodixanol

4a-4. Notes

1. Yeast spheroplast lysates are usually adjusted to an osmolality of 500-600 mOsm using sorbitol as the principal osmotic balancer. Preparation of solutions of approx 560 mOsm and 750 mOsm using a 40% iodixanol working solution are described in **Application Sheet S02**. If solutions of a higher iodixanol concentration are required, it is strongly advised that the osmolality of the solutions is checked by using an osmometer and Solution A adjusted as required so that Solutions B has the required osmolality.
2. Resuspension of any crude mitochondrial pellets must be carried out as gently as possible.
3. In the method of Meeusen et al [4], the crude mitochondrial pellet was resuspended in 50% iodixanol rather than 40% iodixanol. This should not affect the efficacy of the separation.
4. It is worth noting that the discontinuous gradient could be adapted to a median loading of the mitochondrial pellet in the 1.16 g/ml solution; a strategy that was found to give better preservation of function than bottom loading in the case of mammalian mitochondria in similar Nycodenz® gradients. It is believed that the lower hydrostatic pressure experienced by the median-loaded mitochondria is responsible. For more information see **Application Sheet S14**.
5. The method was also used by Tamura et al [5] and Chatterjee et al [6].

4b. Sedimentation in a continuous iodixanol gradient

4b-1. Solutions required

See Section 4a-1 for details.

4b-2. Rotor requirements

Although any of the rotors described in Section 4a-2 may be used, the procedure that is presented in Section 4b-3 uses only 12,000 *g* so many swinging-bucket rotors for high-speed centrifuges are adequate. Note however that continuous gradients may be used for a more comprehensive fractionation of spheroplast homogenates that will also involve analysis of membranes of the ER, endosomes and vacuole; in these instances much higher *g*-forces are used (see Section 4b-5)

4b-3. Protocol (adapted from ref 7)

Carry out all operations at 0-4°C. The protocol can be scaled down proportionately as required.

1. Prepare two iodixanol solutions of 2% and 25% (w/v) by mixing Solutions C and D (See Section 4a-1) at 2:38 and 25:15 v/v ratios respectively.

2. Construct a linear gradient from equal volumes of the two solutions using a two chamber gradient maker or a Gradient Master™. In a 13 ml tube, the total gradient volume should be approx 11 ml; in a 17 ml tube approx 14 ml and in a 38 ml tube approx 30 ml (see Section 4b-4, Notes 1-3).

3. Suspend the crude mitochondria in approx 40 ml of Solution D using two or three gentle strokes of the pestle of a loose-fitting Dounce homogenizer and recentrifuge at 12,000*g* for 10 min.

4. Suspend the washed crude mitochondrial pellet in 10-20 ml of Solution D and layer on top of the linear gradients, filling the tubes according to the manufacturer's specifications.

5. Centrifuge at 12,000 *g* for 2 h.

6. Harvest the gradient (see Figure 2) in 0.5-2.0 ml fractions (depending on gradient volume) by tube puncture, upward displacement with a dense medium or aspiration from the meniscus. For more information on the harvesting of gradients see **Application Sheet S08**

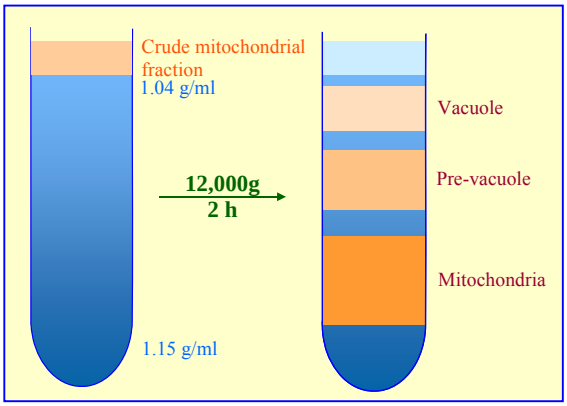

Figure 2: Fractionation of a crude yeast spheroplast mitochondrial fraction in a linear iodixanol gradient: approximate distribution of organelles

4b-4. Notes

1. If a gradient maker is not available, the gradient may be prepared by allowing a discontinuous gradient of equal volumes of 2%, 8%, 14%, 20% and 25% iodixanol to diffuse. For more information on making gradients see **Application Sheet S03.**

2. Chen and Kaplan [7] quoted a 0-25% iodixanol gradient, but the 2-25% gradient, which will not affect the subsequent separation, is easier to load with the sample.

3. This gradient has been used [7-12] in studies on the accumulation of Fe by yeast mitochondria.

4b-5. Other continuous gradients

The gradient used by Ishihara et al [13] comprised the following iodixanol (w/v) solutions: 10% (1.5 ml), 15% (2 ml), 20% (2 ml), 25% (1.5 ml), 30% (1 ml), 40% (1 ml) and 50% (0.5 ml) in Solution B (see Section 2a). It was centrifuged at 174,000g for 16 h and will become continuous during the centrifugation. In this gradient endosomes and vacuoles banded in the top third of the gradient and the mitochondria peaked quite sharply at approx 25%. The final continuous density profile of the gradient would reflect both diffusion of iodixanol across the interfaces and sedimentation of iodixanol molecules close to the bottom of the tube.

Narrower range continuous gradients were used by Kerssen et al [14]; the 15.5-36% (w/v) iodixanol gradient, which also contained 18% sucrose, was primarily used for a study of the peroxisome import receptor (Pex5p) but produced a very clear mitochondrial band as well. Gradients of 2.25-24% (w/v) iodixanol centrifuged in a vertical rotor for 90 min at 30,000 *g* [15] or 48,000 *g* [16] also gave very good separation of mitochondria from peroxisomes.

♦ For more recent papers that have reported the use of iodixanol gradients gor the isolation of yeast mitochondria see refs 17-23

5. References

1. Glick, B. J. and Pon, L. A. (1995) *Isolation of highly purified mitochondria from Saccharomyces cerevisiae* Meth. Enzymol., **260**, 213-223
2. Ishihara, N., Hamasaki, M., Yokota, S., Suzuki, K., Kamada, Y., Kihara, A., Yoshimori, T., Noda, T. and Ohsumi, Y (2001) *Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion* Mol. Biol. Cell, **12**, 3690-3702
3. Lindahl, P.A. Garber Morales, J., Miao, R. and Holmes-Hampton, G. (2009) *Isolation of Saccharomyces cerevisiae mitochondria for Mössbauer, EPR, and electronic absorption spectroscopic analyses* Methods Enzymol., **456**, 267-285
4. Meeusen, S., Tieu, Q., Wong, E., Weiss, E., Schieltz, D., Yates, J. R. and Nunnari, J. (1999) *Mgm101p is a novel component of the mitochondrial nucleoid that binds DNA and is required for the repair of oxidatively damaged mitochondrial DNA* J. Cell Biol., 145, 291-304
5. Tamura, Y., Harada, Y., Nishikawa, S-I, Yamano, K., Kamiya, M., Shiota, T., Kuroda, T., Kuge, O., Sesaki, H., Imai, K., Tomii, K. and Endo, T. (2013) *Tam41 is a CDP-diacylglycerol synthase required for cardiolipin biosynthesis in mitochondria* Cell Metab., **17**, 709–718
6. Chatterjee, N., Pabla, R. and Siede, W. (2013) *Role of polymerase η in mitochondrial mutagenesis of Saccharomyces cerevisiae* Biochem. Biophys. Res. Comm., **431**, 270–273

7. Chen, O. S. and Kaplan, J. (2000) *CCC1 suppresses mitochondrial damage in the yeast model of Friedreich's ataxia by limiting mitochondrial iron accumulation* J. Biol. Chem., **275**, 7626-7632
8. Radisky, D. C., Babcock, M. C. and Kaplan, J. (1999) *The yeast frataxin homologue mediates mitochondrial iron efflux* J. Biol. Chem., **274**, 4497-4499
9. Yun, C-W., Ferea, T., Rashford, J., Ardon, O., Brown, P. O., Botstein, D., Kaplan, J. and Philpott, C.C. (2000) *Desferrioxamine-mediated iron uptake in Saccharomyces cerevisiae. Evidence for two pathways of iron uptake* J. Biol. Chem., **275**, 10709-10715
10. Chen, O. S. and Kaplan, J. (2001) *YFH1-mediated iron homeostasis is independent of mitochondrial respiration* FEBS Lett., **509**, 131-134
11. Chen, O. S., Hemenway, S. and Kaplan, J. (2001) *Genetic analysis of iron citrate toxicity in yeast: implications for mammalian iron homeostasis* Proc. Natl. Acad. Sci. USA, **99**, 16922-16927
12. Crisp, R. J., Pollington, A., Galea, C., Jaron, S., Yamaguchi-Iwai, Y. and Kaplan, J. (2003) *Inhibition of heme biosynthesis prevents transcription of iron uptake genes in yeast* J. Biol. Chem., **278**, 45499-45506
13. Ishihara, N., Hamasaki, M., Yokota, S., Suzuki, K., Kamada, Y., Kihara, A., Yoshimori, T., Noda, T. and Ohsumi, Y (2001) *Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion* Mol. Biol. Cell, **12**, 3690-3702
14. Kerssen, D., Hambruch, E., Klaas, W., Platta, H.W., de Kruijff, B., Erdmann, R., Kunau, W-H. and Schleibs, W. (2006) *Membrane association of the cycling peroxisome import receptor Pex5p* J. Biol. Chem., **281**, 27003-27015
15. Oeljeklaus, S., Reinartz, B.S., Wolf, J., Wiese, S., Tonillo, J., Podwojski, K., Kuhlmann, K., Stephan, C., Meyer, H.E., Schliebs, W., Brocard, C., Erdmann, R. and Warscheid, B. (2012) *Identification of core components and transient interactors of the peroxisomal importomer by dual-track stable isotope labeling with amino acids in cell culture analysis* J. Proteome Res. 2012, 11, 2567−2580
16. Welker, S., Rudolph, B., Frenzel, E., Hagn, F., Liebisch, G., Schmitz, G., Scheuring, J., Kerth, A., Blume, A., Weinkauf, S., Haslbeck, M., Kessler, H. and Buchner, J. (2010) *Hsp12 is an intrinsically unstructured stress protein that folds upon membrane association and modulates membrane function* Mol. Cell, **39**, 507–520
17. Suzuki, K., Nakamura, S., Morimoto, M., Fujii, K., Noda, N.N., Inagaki, F. and Ohsumi, Y. (2014) *Proteomic profiling of autophagosome cargo in Saccharomyces cerevisiae* PloS One, **9**: e91651
18. He, C.H., Xie, L.X., Allan, C.M., Tran, UP.C. and Clarke, C.F. (2014) *Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast coq null mutants* Biochim. Biophys. Acta, **1841**, 630–644
19. Wang, Y., Lilley, K.S. and Oliver, S.G. (2014) *A protocol for the subcellular fractionation of Saccharomyces cerevisiae using nitrogen cavitation and density gradient centrifugation* Yeast, **31**, 127–135
20. Gold, V.A.M., Ieva, R., Walter, A., Pfanner, N., van der Laan, M. and Kühlbrandt, W. (2014) Visualizing active membrane protein complexes by electron cryotomography Nat. Commun., **5**: 4129
21. Lahiri, S., Chao, J.T., Tavassoli, S., Wong, A.K.O., Choudhary, V. et al (2014) *A conserved endoplasmic rrticulum membrane protein complex (EMC) facilitates phospholipid transfer from the ER to mitochondria* PLoS Biol., **12**: e1001969
22. He, C.H., Black, D.S., Nguyen, T.P.T., Wang, C., Srinivasan, C. and Clarke, C.F. (2015) *Yeast Coq9 controls deamination of coenzyme Q intermediates that derive from para-aminobenzoic acid* Biochim. Biophys. Acta, **1851**, 1227–1239
23. Sakakibara, K., Eiyama, A., Suzuki, S.W., Sakoh-Nakatogawa, M., Okumura, N., Tani, M., Hashimoto, A., Nagumo, S., Kondo-Okamoto, N. et al (2015) *Phospholipid methylation controls Atg32-mediated mitophagy and Atg8 recycling* EMBO J., **134**, 2703-2719