OptiPrep™ Application Sheet S14

Purification of mammalian mitochondria in a discontinuous gradient

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- **OptiPrep™ Application Sheet 14a "Purification and analysis of mammalian mitochondria"** provides a referenced protocol review.
- **To access other Application Sheets** referred to in the text: return to the **2020SMemapp** file and select the appropriate S-number.
- For a bibliography of all published papers return to the initial list of Folders and select "**Reference Lists**" to open **Reference List RS03** which provides a complete list of published papers.
- In a **Methodological Supplement (Section 8)** a summary of methods from recent papers (2011-2018) is given in this Application Sheet; it includes its own reference section.
- Methods for the isolation of peroxisomes (**Application Sheets S11-S13**) or lysosomes (**Application Sheet S55**) may also provide enriched mitochondrial fractions in the same gradient. All of these organelles may be purified from a "light" or "heavy+light" mitochondrial pellet or from a post-nuclear supernatant (see **Application Sheet S07**).

1. Background

Fractionation of particles is often improved by bottom-loading of a sample and this technique was used by Zhou et al [1] who described the flotation of liver mitochondria through a discontinuous iodixanol gradient of 1.175 and 1.079 g/ml, from a dense solution (approx 1.2 g/ml). The method provides very good resolution of the mitochondria from both lighter and denser organelles. Choi et al [2] used a similar flotation strategy for HEK293 mitochondria but based the method on one for the purification of yeast mitochondria by Meeusen et al [3].

Top-loading may however be preferred because of the known sensitivity of mitochondria to hydrostatic pressure [4]. Graham et al [5] first reported the use of a compromise median-loaded Nycodenz® gradient in which the LMF in 25% Nycodenz®, was underlayered by 40% Nycodenz® and overlayered by 23%, 20%, 15% and 10% Nycodenz®. The gentle centrifugation conditions of 52,000 *g* for 1.5 h might also be well suited to the isolation of organelles. While there was good separation of mitochondria from lysosomes and lysosomes from Golgi membranes, resolution of mitochondria from peroxisomes was relatively poor. This was remedied by the introduction of additional layers of 30% and 34% Nycodenz® [6], which allowed the peroxisomes to band at the 34%/40% Nycodenz® interface, later confirmed by Teoh et al [7]. Material at the 25%/34% interface was rich in cytochrome oxidase but completely lacking in peroxisome and ER markers. The strategy is satisfactory for both tissues and cultured cells and it has been adapted to the use of iodixanol [8]. For convenience the methodology below is based on the use of Optiprep™.

2. Solutions required

A. OptiPrep™ (60% w/v iodixanol)
B. Homogenization medium: 0.25 M sucrose, 1 mM EDTA, 20 mM Hepes-NaOH, pH 7.4.
C. Diluent: 0.25 M sucrose, 6 mM EDTA, 120 mM Hepes-NaOH, pH 7.4.
D. Working Solution of 50% iodixanol (ρ = 1.272 g/ml): 5 vol of solution A + 1 vol of solution C

Keep the following stock solutions at 4°C:
500 mM Hepes (free acid), 11.9 g per 100 ml water.
100 mM EDTA ($Na_2 \bullet 2H_2O$), 3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2.0 ml and 8.0 ml respectively of EDTA and Hepes stocks; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml.
Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 6 ml of EDTA stock and 24 ml of Hepes stock; adjust to pH 7.4 with 1 M NaOH; make up to 100 ml.

Protease inhibitors may be included at the operator's discretion. For more information on gradient solution preparation **see Application Sheet S01**.

3. Ultracentrifuge rotor requirements

Any swinging-bucket rotor with 14 ml tubes (e.g. Beckman SW41TI or Sorvall TH641) or 38 ml tubes (e.g. Beckman SW28, Sorvall AH629 or equivalent) may also be used.

4. Preparation of a crude mitochondrial fraction

Keep all solutions, and carry out all operations, at 0-4°C.

1. *Liver or other tissue*: Mince the tissue very finely with scissors (or with a tissue chopper) and transfer to a Potter-Elvehjem (Teflon and glass) homogenizer with Solution B (use 10 ml medium for every 2.5 g tissue). Homogenize using approx 6 strokes of the pestle (500-700 rpm). For more information about homogenization of tissues **see Application Sheet S05**.

2. *For cells:* Wash 1-3x10^8 cells in 5 ml of phosphate buffered saline and again with 5 ml of Solution B. Suspend the cells in 3 ml of Solution B and homogenize in a ball-bearing homogenizer using five passes or homogenize in a tight-fitting Dounce homogenizer (Wheaton Type A) using 20 strokes of the pestle. For more information about homogenization of cells **see Application Sheet S06**.

3. Centrifuge the homogenate at 1000 g_{av} in a fixed-angle rotor for 10 min to pellet the nuclei.

4. Aspirate and retain the supernatant.

5. Re-homogenize the pellet gently in Solution B using 2-3 gentle strokes of the pestle of a loose-fitting Dounce homogenizer and the centrifugation repeated.

6. Combine the supernatants and centrifuge the supernatant(s) at 10-17,000 g_{av} for 10-15 min to produce a crude heavy + light mitochondrial fraction. For more information on differential centrifugation **see Application Sheet S07 and also Section 8c**.

7. Resuspend this pellet in 2.0-3.0 ml of Solution B using a loose-fitting (Wheaton Type B) Dounce homogenizer (2-3 strokes of the pestle).

5. Bottom or median loaded gradients

5a. Gradient solutions and centrifugation (adapted from ref. 1)

Table 5-1: Preparation of gradient solutions

Density (g/ml)	% (w/v) iodixanol	Solution D (vol)	Solution B (vol)
1.079	10	10	40
1.100	14.5	14.5	35.5
1.127	20	20	30
1.150	25	25	25
1.160	27	27	23
1.175	30	30	20

For bottom loading adjust the crude fraction from Step 7 (**Section 4**) to 36% (w/v) iodixanol (1.204 g/ml) by mixing with Solution D (1.4 + 3.6 vol. respectively). In some cases the sample may be adjusted to a higher density, for example 44% (w/v) iodixanol [9]. In approx. 14 ml tubes layer 4.0-4.5 ml of this suspension and overlay with 4.5 ml of each of the chosen gradient solutions (see Table 1): 1.079 and 1.175 g/ml [1]; 1.10 and 1.16 g/ml [2] or 1.127 and 1.150 g/ml [9]; the latter was used for mouse spinal cord. Mitochondria from KB-V1 and MCF7 cells have also been purified in the 1.079-1.175 g/ml format [10].

For median loading adjust the crude fraction from Step 7 (**Section 4**) to 25-30% (w/v) iodixanol and layer between 36% (w/v) iodixanol and one of the lower density solutions.

Gradients may be completed with an optional 1-2 ml of the homogenization medium on top. This facilitates the collection of the lowest density material. Centrifuge at approx 50,000 g_{av} for 3-4 h. Use a slow deceleration program or allow the rotor to decelerate from 2000 rpm without the brake.

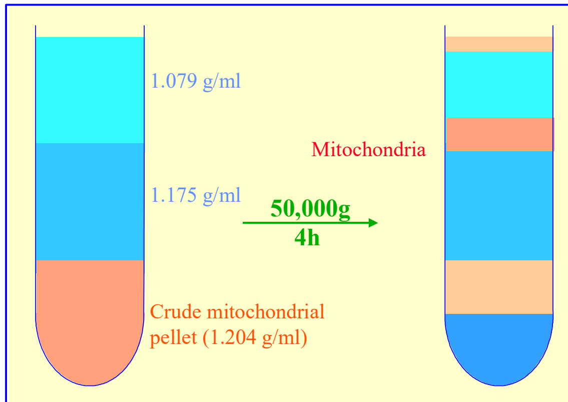

Figure 1 Purification of mammalian liver mitochondria in a discontinuous iodixanol gradient, according to ref 1.

For larger volumes of crude mitochondrial fraction, in other rotors (36-38 ml tubes), the volumes should be scaled up proportionately. For more details on the preparation of pre-formed discontinuous iodixanol gradients **see Application Sheet S03**.

5b. Analysis

Harvest the mitochondria which band just above the upper interface (see Figure 1) or collect the gradient in 1 ml fractions either low density end first by upward displacement or dense end first by carefully introducing a narrow metal cannula (connected to a peristaltic pump) to the bottom of the tube. For more information on gradient harvesting **see Application Sheet S08**.

Iodixanol does not significantly inhibit any enzyme so far tested. Standard spectrophotometric methods (carried out above 340 nm), for measuring organelle enzyme markers can be performed directly on gradient fractions [11]. Protein can also be measured directly by any Coomassie blue-based method [11]. If it is necessary to remove the gradient medium, fractions can be diluted with an equal volume of buffer; pelleted at approx 30,000g_{av} for 10 min and resuspended in a suitable buffer.

The 1.127 and 1.150 g/ml format [9] produced a particularly good separation of endoplasmic reticulum at the top of the 1.127 g/ml layer from the mitochondria at the next interface.

6. Top-loaded gradients

A multi-step iodixanol gradient of 10, 15, 20, 25 and 30% (w/v) iodixanol centrifuged at 100,000 *g* for 3 h [12] gave an excellent resolution of several membrane compartments (plasma membrane, Golgi, endoplasmic reticulum + lysosomes and mitochondria + peroxisomes) from a rat liver post-nuclear supernatant (see Figure 2). The mitochondria + peroxisome fraction was re-run on a second gradient of 20%, 25%, 30% and 35% iodixanol, centrifuged under the same conditions to resolve these organelles completely.

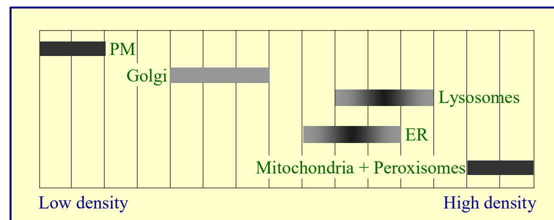

Figure 2: Distribution of rat liver membranes in a top-loaded discontinuous iodixanol gradient, data from ref 12. See text for more details.

Cardiac monocytes mitochondria are often purified on a composite discontinuous gradient of 6% (v/v) Percoll® and 17% and 35% (w/v) iodixanol, centrifuged at 50,000 *g* for 30 min [13-15]. The mitochondria band at the interface between the two iodixanol solutions. It is not known if the Percoll® layer could be replaced by an iodixanol solution of identical density (approx. 1.04 g/ml or 3% (w/v) iodixanol).

Brain mitochondria: More recently Islinger et al [16] reported the use of free-flow electrophoresis as the optimal method for removing synaptosomal contamination of brain mitochondria and the authors recommended the use of a top-loaded discontinuous gradient run in a vertical rotor for preparing the mitochondria. The gradient used was prepared by dilution of OptiPrep™ with a hypoosmotic medium of 5 mM HEPES-KOH, 1 mM EDTA, 2 mM PMSF, 1 mM DTT, 1 mM ε-aminocaproic acid, pH 7.4. We have adapted this to gradient solution preparation by dilution with a routine 0.25 M sucrose-containing buffer. Tubes for a Beckman VTi50 vertical rotor, are loaded with 3 ml 25%, 10 ml, 21%, 13 ml 14.5% and 4 ml 8.5% (w/v) iodixanol; the crude mitochondrial suspension is loaded on top, to fill the tube. The rotor is centrifuged at 33,000 *g* for approx 35 min. The mitochondria band close to the bottom of the tube.

7. References

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8. Application Sheet S14 – Methodological Supplement

♦ **This supplement is divided into method type and includes its own reference section (8g).**

8a Removal of myelin from spinal cord material

If contamination of mitochondria is a serious problem, a simple short spin density barrier centrifugation might be of use. Parone et al [1] suspended a 12,000 *g* – 10 min pellet from a spinal cord post-nuclear supernatant in 12% iodixanol in 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2 and centrifuged it at 17,000 *g* for 10 min. The myelin layer at the top is separated from the organelle pellet.

8b Homogenization of cells

As the use of an EDTA containing homogenization medium may destabilize nuclei, HEK cells were first homogenized in 150 mM $MgCl_2$, 10 mM KCl, 20 mM HEPES pH 7.4; this was adjusted to 8.5% (w/v) sucrose and the nuclei removed at 1000 *g* for 10 min. A 5000 *g* – 10 min pellet was washed once in the same buffered sucrose/Mg/K medium and finally resuspended in a routine buffered sucrose/EDTA solution [2].

An interesting method was adopted by Bhowmick et al [3] for monkey kidney cells that avoided the use of any shearing forces that might be potentially damaging to mitochondria. The cells were

suspended in 1-2% (w/v) Triton X100, 0.01-0.03 % Nonidet P40 and 0.4-0.6% CHAPS for 30 min on ice. A 7000 *g* – 10 min pellet, prepared from a post-nuclear supernatant, was then suspended in a routine buffered 0.25 M sucrose solution prior to the gradient purification.

8c Pre-gradient centrifugation

A standard approach is to remove the nuclei from the homogenate and then to pellet a heavy + light mitochondrial fraction as described in Section 4. However many authors prefer to reduce the presence of more slowly sedimenting particles by using just a heavy mitochondrial fraction by using 5000 *g* for 10-20 min

8d Three-layer bottom-loaded gradients

A commonly used flotation format first used by Choi et al [4,5] in which the crude mitochondrial fraction is suspended in 36% (w/v) iodixanol; overlaid with solutions of 30% and 10% and centrifuged at approx 50,000 *g* for 4 h. The solutions are prepared by dilution of OptiPrep™ with the homogenization buffer and the mitochondria band at the 10%/30% interface. This has been used for brain/spinal cord [6,7], kidney [8], osteosarcoma cells [9] and HEK cells [2,10]. In a variant of this format, the sample layer was adjusted to 50% iodixanol with centrifugation at 80,000 *g* for 3 h [10]. Variants of this format were developed by Wood-Allum et al [11] of 44%, 25% and 20% iodixanol (also employed for HEK cells, monkey kidney cells and neuroblastoma cells [3]); 36%, 25%, 17.5% iodixanol gradient for liver [12] and a 36%, 25%, 20% iodixanol gradient centrifuged at 100,000 *g* for 4 h for HeLa cells [13]. More recently, mitochondria from colon cancer cells [14] and lymphoma cells [15] were purified by flotation from a 36% (w/v) iodixanol solution overlaid by 30% and 10% iodixanol, with centrifugation at 80,000 *g* [14] or 50,000 *g* for 3 h [15]. The method as shown in Figure 1 was used for both HEK and HeLa cells [16]

In all these flotation gradients the mitochondria band at an interface between the two lower density iodixanol solutions, a layer of homogenization medium on top is thus not strictly required, but should be included if top-banding organelles are also to be analyzed.

8e Top-loaded gradients

A crude mitochondrial fraction in 15% iodixanol (w/v) from neuroblastoma cells and fibroblasts [17], layered on a 30, 27, 23, 20 and 17% iodixanol gradient centrifuged at 145,000 *g* for 4 h resolves mitochondria (23-27% interface) from lysosomes (top of gradient). This discontinuous 17%-30% iodixanol gradient, (with the crude fraction in 10% or 15% iodixanol), centrifuged for 2 h, has also been used for mouse hepatic tissue [18] Caco-2 cells [19] and endothelial cells [19] . Similar top-layered gradients of 10, 15, 20, 25, 30% iodixanol, centrifuged at 100,000 *g* for 1 h, have been used for HEK cells [20], brain, kidney, liver, heart and skeletal muscle [21]. In this gradient the mitochondria banded around the mid-point of the gradient.

8f Median-loaded gradients

In an analysis of the organelles from PHA-blast cells generated from human peripheral blood mononuclear cells, Schmidt et al [22,23] used a discontinuous gradient of 27%, 22.5%, 19%, 16%, 12%, 8% (v/v) Optiprep™, with the sample median loaded in the 19% (v/v) layer and centrifuged at 150,000 *g* for 5 h. This gradient covers a much lower density range than that which is regularly used; whether this is a requirement of the specific cell type is not known. The lowest and highest density layers are equivalent to approx. 5% and 16% (w/v) iodixanol. The gradient was primarily used to purify secretory lysosomes.

8g References

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OptiPrep™ Application Sheet S14 9th edition, January 2020