OptiPrep™ Application Sheet S12

Purification of mammalian peroxisomes on a discontinuous gradient or density barrier

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- **OptiPrep™ Mini-Review MS03 "The purification of peroxisomes – a bibliography"** covers published papers reporting the use of OptiPrep™ for the isolation of these organelles from all mammalian cells and tissues and from non-mammalian sources. **OptiPrep™ Application Sheet S55 deals with their isolation from yeast.**
- To access the Mini-Review return to the initial list of Folders and select "**Mini-Reviews**"
- 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. Background

Peroxisomes can be purified in iodixanol gradients in high yield (80-90%) with no detectable contamination from any other organelle [1,2]. This is a property unique to iodixanol because the densities of other organelles, particularly that of mitochondria (approx ρ = 1.14 g/ml) and endoplasmic reticulum (approx ρ = 1.13 g/ml) are much lower than that of peroxisomes (approx ρ = 1.19-1.23 g/ml). In Nycodenz® mitochondria have a significantly higher density (approx ρ = 1.165 g/ml) than in iodixanol because only the latter can provide an iso-osmotic medium at densities above ρ = 1.15-1.16 g/ml. The density of peroxisomes on the other hand is relatively little affected by the type of iodinated density gradient medium because of their lack of an osmotic space. In Percoll® both peroxisomes and endoplasmic reticulum have the same banding density and these two organelles cannot be resolved.

Ghosh and Hajra [3] developed a rapid density barrier method for the isolation of peroxisomes using Nycodenz® in which approx. 2 ml of the light mitochondrial fraction (LMF) is layered over 15 ml of 30% (w/v) Nycodenz® and centrifuged at 131,000 g_{av} for 1 h in a fixed-angle rotor. The peroxisomes form a loose pellet. This has been adapted to the use of OptiPrep™ in the protocol described in Section 2. The density of the barrier has been increased slightly to improve the resolution from the other organelles. Some alternative one- or two-layer gradients are described in Section 3.

2. Use of a density barrier

2a. Solutions Required (all iodixanol solutions are give as % w/v; **see also Section 2e, Note 1**)

A. OptiPrep™

B. OptiPrep™ diluent: 0.25 M sucrose, 10 mM EDTA, 1% (v/v) ethanol, 100 mM Mops-NaOH, pH 7.2

C. Working Solution of 54% (w/v) iodixanol (ρ = 1.291 g/ml): 9 vol. of OptiPrep™ + 1 vol. of Solution B.

D. Homogenization medium: 0.25 M sucrose, 1 mM EDTA, 0.1% (v/v) ethanol, 10 mM Mops-NaOH, pH 7.2.

E. Gradient solutions: Make up two dilutions of solution C, containing 47% iodixanol (ρ = 1.257 g/ml) and 32% iodixanol (ρ = 1.185 g/ml) by mixing solutions C and D, 8.7 + 1.3 and 6.0 + 4.0, v/v, respectively).

Keep the following stock solutions at 4°C:

500 mM Mops	10.45 g per 100 ml water
100 mM EDTA (Na_2•$2H_2O$)	3.72 g per 100 ml water

Solution B: Dissolve 8.5 g sucrose in 50 ml of water; add 1.0 ml, 10 ml and 20 ml respectively of ethanol, EDTA and Mops stocks; adjust to pH 7.2 with 1 M NaOH and make up to 100 ml.

Solution D: Dissolve 17 g sucrose in 100 ml water; add 0.2 ml, 2.0 ml and 4.0 ml respectively of ethanol, EDTA stock and Mops stocks; adjust to pH 7.2 with 1 M NaOH and make up to 200 ml.

2b. Ultracentrifuge rotor requirements

Any swinging-bucket rotor for an ultra-centrifuge capable of approx 100,000g with a tube capacity of approx 17 ml tubes (**see Section 2e, Note 2**)

2c. Protocol

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

1. Mince the liver (wet weight approx 10 g) very finely with scissors and transfer to a Potter-Elvehjem (Teflon and glass) homogenizer.

2. Homogenize the mince in solution D (use 10 ml medium for every 2.5 g tissue), using approx 6 strokes of the pestle (500-700 rpm).

3. Centrifuge the homogenate at 3000g_{av} in a fixed-angle rotor for 10 min to pellet the nuclei and heavy mitochondria (**see Section 2e, Notes 3 and 4**).

4. Centrifuge the supernatant(s) at 17,000g_{av} for 10-15 min to produce a light mitochondrial pellet.

5. Resuspend this pellet in approx 4.0 ml of solution D using a loose-fitting Dounce homogenizer (2-3 strokes of the pestle) and add an equal volume of 47% iodixanol. The refractive index of this suspension should be 1.3782; adjust to this value with Solutions C or D if necessary. The density of this suspension is approx 1.145 g/ml.

6. Transfer approx 14 ml of the suspension to a 17 ml tube for a suitable swinging-bucket rotor and underlayer with 2 ml of the 32% iodixanol. Top up the tube with suspension or solution D if necessary. Centrifuge at 110,000*g* for 2 h.

7. The peroxisomes band towards the bottom of the tube, partly within the 32% iodixanol layer and partly as a loose pellet. Aspirate and discard the liquid above the 32% layer, then harvest this layer and the pellet of peroxisomes.

Fraction	Catalase	SDH
Homogenate	58.6	nd
Light mit pellet	114.5	0.18
Peroxisomes	1442.8	0.02

Table 1 Enzyme analysis of fractions: catalase in µmoles hydrogen peroxide/ min/mg protein, SDH (succinate dehydrogenase) in µmoles succinate oxidized/min/mg protein. nd = not determined

2d. Analysis

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 [4]. Protein can also be measured directly by any Coomassie blue-based method [4].

A typical enzyme analysis is shown in Table 1. 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 (recoveries are >90%). The specific activity of catalase in the peroxisome harvest represents an approximately 25-fold purification over the homogenate and approx 37% of the total catalase activity of the LMF was recovered. Barrier techniques, while simple, are inevitably a compromise between purity and yield. Because of the density heterogeneity of all organelles it is impossible to choose a density for the suspending medium which is greater than that all of the mitochondria and less than that of all of the peroxisomes

2e. Notes

1. The density of Solution E can be adjusted to suit the operator's requirements, if it is reduced, more of the peroxisomes will pellet; if it is increased, the peroxisomes will band predominantly at the interface between the sample and the barrier. If it is preferable to band the peroxisomes at an interface then it may be advisable to underlayer the sample with an additional 2 ml layer of approx. 25% iodixanol. Protease inhibitors may be included in any or all of the media at the operator's

discretion. Strategies for preparing gradient solutions for mammalian tissues are given **in Application Sheet S01**.

2. Choice of rotor is not particularly critical; Ghosh and Hajra [3] used a fixed-angle rotor. The separation may be adapted to larger or smaller volume rotors.
3. This pellet may be rehomogenized in solution D and the centrifugation repeated.
4. For more information on homogenization of tissues and cells and differential centrifugation of an homogenate **see respectively Application Sheets S05, S06 and S07**.

3. Alternative gradient formats

Lamhonwah et al [5] adjusted the light mitochondrial suspension to a slightly higher concentration of iodixanol (27.5%) and omitted the denser cushion; 5 ml of this was centrifuged at 250,000 *g* for 4 h in a swinging-bucket rotor to pellet the peroxisomes. By contrast Mi et al [6] adjusted the LMF to 28% iodixanol (15 ml) and underlayered it with 2 ml of 50% iodixanol, so that after 2 h at 131,000 *g* the peroxisomes banded at the interface. This format can probably be applied to LMFs from any source – an identical two-layer format was used for mussel peroxisomes [7]. A three-layer format [8] in which the LMF in 22.5% iodixanol (4.5 ml) is sandwiched between layers of 20% and 27.5% iodixanol (2 ml each) resulted in the peroxisomes from chicken embryo liver banding in the densest layer.

4. References

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