

OptiPrep™ Application Sheet S13

Purification of mammalian peroxisomes in a self-generated gradient

- ◆ 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 all published papers reporting the use of OptiPrep™: to access 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.
- ◆ Methods for the isolation of mitochondria (**Application Sheets S14-S16**) or lysosomes (**Application Sheets S53 and S54**) may also provide enriched peroxisomal 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

Peroxisomes can be purified in self-generated iodixanol gradients in high yield (80-90%) with no detectable contamination from any other organelle. In iodixanol peroxisomes are the densest of the major subcellular organelles ($\rho = 1.18\text{-}1.20$ g/ml) present in the light mitochondrial fraction (LMF) from mammalian tissues and cells. Mitochondria have a median density of approx 1.145 g/ml and lysosomes approx 1.115 g/ml. Metrizamide or Nycodenz® gradients have been used previously to purify peroxisomes [1,2] but because mitochondria have a higher density in Nycodenz® or metrizamide than in iodixanol, the resolution of these two organelles is much easier in iodixanol gradients [3]. In Percoll® both peroxisomes and endoplasmic reticulum (ER) have the same banding density and these two organelles cannot be resolved; in iodixanol the ER has a much lower density.

The protocol below is for mammalian liver, but similar techniques using other tissues or cells have been published. Variations in centrifugation conditions and **a brief summary of some methodological variations is provided in Section 7**.

Formation of self-generated gradients requires ideally a vertical or a near-vertical rotor with a sedimentation path length of <25 mm. Some small volume fixed-angle rotors may be satisfactory, they tend to produce S-shaped gradients that are relatively shallow in the middle of the tube and steep at both ends in the centrifugation times which are valid for organelles (2-3 h). This particular protocol does require a slightly S-shaped gradient for its efficacy however, thus some fixed-angle rotors (<10 ml tube volume) may be satisfactory.

2. Solutions Required (see Section 6, Note 1)

- A. OptiPrep™
- B. Homogenization medium: 0.25 M sucrose, 1 mM EDTA, 0.1% (v/v) ethanol, 10 mM Mops-NaOH, pH 7.4
- C. Dilution medium: 0.25 M sucrose, 6 mM EDTA, 0.6% (v/v) ethanol, 60 mM Mops-NaOH, pH 7.4
- D. Working Solution of 50% iodixanol ($\rho = 1.272$ g/ml): mix 5 vol. of solution A with 1 vol. solution C

Keep the following stock solutions at 4°C:
 500 mM Mops 10.45 g per 100 ml water.
 100 mM EDTA ($\text{Na}_2 \cdot 2\text{H}_2\text{O}$) 3.72 g per 100 ml water

Solution B: 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.

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 0.6 ml, 6 ml and 12 ml respectively of ethanol, EDTA stock and Mops stocks; adjust to pH 7.2 with 1 M NaOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements

A vertical or near vertical rotor with a tube capacity of 10-14 ml capable of >180,000g or a fixed-angle rotor with a tube capacity of <10 ml (see Section 6, Note 2)

4. Protocol (adapted from ref 3)

Carry out all operations at 0-4°C.

1. Mince the liver very finely with scissors 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 (see Section 6, Note 3).
2. Centrifuge at 3000 g for 10 min to pellet the nuclei and heavy mitochondria. This pellet may be rehomogenized in solution B and the centrifugation repeated (see Section 6, Note 3).
3. Centrifuge the supernatant(s) at 17,000 g for 10-15 min to produce a "light mitochondrial pellet".
4. Resuspend this pellet in solution B using a loose-fitting Dounce homogenizer (2-3 strokes of the pestle). Adjust to a volume of about 15 ml per 10 g tissue; then mix with an equal volume of solution D (final iodixanol concentration = 25%: $\rho = 1.150$ g/ml).
5. Transfer to a suitable tube (10-14 ml) for a vertical, near-vertical or low-angle (less than 24°) fixed-angle rotor and centrifuge at a minimum of 180,000 g_{av} . The time required for formation of the gradient will depend on the rotor type; at 180,000g it will be about 3 h, at higher g-forces the time can be reduced (see Section 6, Notes 4-7).
6. Allow the rotor to decelerate from 3000 rpm without the brake and collect the gradient by upward displacement or by tube puncture, or with a syringe (see Section 6, Note 8). For more information on harvesting gradients see Application Sheet S08.

5. Analysis

Iodixanol does not significantly inhibit any enzyme so far tested. Spectrophotometric assays carried out above 340 nm, can be performed directly on gradient fractions: this includes the standard assays for catalase, succinate dehydrogenase and β -galactosidase [4]. Protein can also be measured directly by any Coomassie blue-based method [4].

If it is necessary to remove the gradient medium, fractions can be diluted with an equal volume of buffer; pelleted at approx 30,000 g_{av} for 10 min and resuspended in a suitable buffer.

A typical result is shown in Figure 1, which shows the distribution of marker enzymes across the gradient. The activity in each fraction is expressed as a % of the total in the tube before centrifugation. Fractions 1-7 contain more than 90% of the total catalase with no detectable contamination from mitochondria or lysosomes. The Golgi membranes (not shown) band at the top of the gradient (far right of figure).

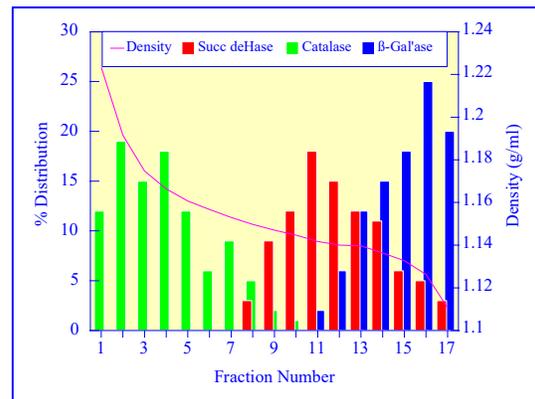


Figure 1 Isolation of peroxisomes in a self-generated gradient of iodixanol: enzyme distribution. Succ deHase = succinate dehydrogenase, β -Gal'ase = β -galactosidase.

6. Notes

1. Protease inhibitors may be included in any or all of the media at the operator's discretion. Strategies for preparing working solutions for mammalian tissues are given in Application Sheet S01.
2. The lower the angle of a fixed-angle rotor, the more suitable it is for the creation of self-generated gradients (ideally the angle should be <24°). Rather few modern fixed-angle rotors however conform to this specification and in such cases it may be necessary to use a smaller volume tube

(preferably a Beckman g-Max adapted tube, which reduces the height of the tube rather than its diameter) in order to restrict the sedimentation path length.

3. For more information on homogenization of tissues and cells and differential centrifugation of an homogenate **see respectively Application Sheets S05, S06 and S07.**
4. Although much higher g-forces are required to generate the gradient than to band the organelles in a pre-formed gradient; the use of vertical rotors, which have very short sedimentation path lengths, means that the hydrostatic pressure on the organelles is no greater than in a swinging-bucket rotor at a lower g-force.
5. The precise conditions required for peroxisome purification in self-generated gradients, depends very much on the rotor type. Any vertical rotor, with a sedimentation path length of <25 mm would provide a very simple and efficient system; at approx 350,000_{g_{av}} separation would take place in 1-2 h. **Vertical and near-vertical rotors can produce a range of density profile shapes; for more information see Application Sheet S04.**
6. Optimal separation of the mitochondria and peroxisomes depends on the formation of a relatively shallow gradient in the middle of the tube (see Fig 1) and a sharp gradient at the bottom to prevent the peroxisomes from hitting the wall of the tube.
7. Always check the gradient density profile that is generated in a particular rotor using a blank gradient before using it for any fractionation and adjust the centrifugation conditions as appropriate. Refractive index (RI) is the most accurate method for determination of density and density tables giving RI values are given **in Application Sheet S01.** If a refractometer is not available then absorbance measurement is a possible alternative, **see Application Sheet S09.**
8. Once the banding position of the peroxisomes has been established, the reproducibility of self-generated gradients is so high, that a syringe can be used to harvest a standard volume from the bottom of the gradient.

7. Methodological variations

1. McClelland et al. [5] adjusted the LMF to 25% (w/v) in the manner described in the Protocol and centrifuged at 180,000g for 2.5 h in a Beckman NVT65 near-vertical rotor. Kurochkin et al [6] used the same rotor but centrifuged for 3.5 h and the LMF was mixed with an equal volume a working solution containing 5 vol. of OptiPrep™ and 1 vol. of 0.16 M sucrose, 12% (w/v) PEG1500, 60 mM Mops, pH 7.4, 6 mM EDTA, 6 mM DTT and 0.6% ethanol.
2. He et al [7] used a Hepes buffer rather than Mops and centrifuged at 180,000g for 3 h in a Beckman 50.2Ti fixed-angle rotor to study the expression and to characterize bile acid CoA:amino acid *N*-acyltransferase in peroxisomes.
3. Morel et al [8] isolated the peroxisomes from human hepatoblastoma (HepG2) cells. The homogenization buffer contained 0.25 M sucrose, 10 mM triethanolamine-acetic acid, pH 7.8 and the centrifugation was carried out at 180,000g for 4.5 h. Characterization of the human glutathione *S*-transferase kappa gene and protein was carried out.
4. A post-nuclear fraction rather than an LMF from CHO cells was processed according to the usual protocol [9].
5. In a large volume fixed-angle rotor with 39 ml tubes (Beckman 50.2Ti) fractionation was achieved at 150,000 g for 4 h [10].

8. References

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