OptiPrep™ Application Sheet S11

Purification of mammalian peroxisomes in a continuous gradient

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- An **OptiPrep™ Reference List RS02 "Purification of peroxisomes – reference list"** lists published papers reporting the use of OptiPrep™ according to tissue or cell type: to access return to the initial list of Folders and select "**Reference Lists**"
- **To access other Application Sheets** referred to in the text: return to the **2020SMemapp** file and select the appropriate S-number.
- Methods for the isolation of mitochondria (**Application Sheets S14-S16**) or lysosomes (**Application Sheet S55**) 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

Nycodenz® and Percoll® gradients have been widely used for the purification of peroxisomes; both offered a big improvement in resolution from mitochondria and lysosomes compared to sucrose gradients. Hiltunen et al [1] commented however that to reduce contamination from mitochondria to a minimum Nycodenz® was the gradient medium of choice. Moreover in Percoll® gradients peroxisomes and endoplasmic reticulum (ER) have a very similar banding density; in Nycodenz® the ER has a much lower density. Since 1994 the use of iodixanol gradients has gained much popularity for peroxisome isolation. Iodixanol offers even more advantages than Nycodenz®. In both Nycodenz® and iodixanol the peroxisomes are the densest of the major subcellular organelles (ρ = 1.19-1.23 g/ml) and well separated from the other organelles in the light mitochondrial fraction. In iodixanol the density of these other organelles is lower than in Nycodenz®; mitochondria have a median density of approx 1.145 g/ml and lysosomes approx 1.12 g/ml; slightly lower than the figures in Nycodenz®; the separation from peroxisomes is thus potentially greater in iodixanol. The other big advantage of using iodixanol is that the gradient solutions are prepared simply by dilution of OptiPrep™.

Section 2 describes the OptiPrep™ method as applied to rat liver in detail; it is adapted from the method of Van Veldhoven et al [2,3]. Section 3 describes some of the procedural variations.

2. Iodixanol gradient methodology

2a. Solutions Required (see Section 2d, Note 1)

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 1mM EDTA, 0.1% (v/v) ethanol, 5 mM Mops pH 7.2.

C. 6 mM EDTA, 0.6% ethanol, 30 mM Mops, pH 7.2.

D. 1 M sucrose.

E. Gradient solutions: Make up from solutions A, C, D and water using respectively, these ratios by volume:
E1: 5 + 0.6 + 0.4 + 0.0 (50% iodixanol)
E2: 4 + 0.6 + 0.7 + 0.7 (40% iodixanol)
E3: 2 + 0.6 + 1.1 + 2.3 (20% iodixanol)

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 17 g sucrose in 100 ml water; add 0.2 ml, 2 ml and 2 ml respectively of ethanol, EDTA stock and Mops stock; adjust to pH 7.2 with 1 M NaOH and make up to 200 ml.

Solution C: Add 0.6 ml, 6 ml and 6 ml respectively of ethanol, EDTA and Mops stocks to 50 ml water; adjust to pH 7.2 with 1 M NaOH and make up to 100 ml.

Solution D: Dissolve 34.2 g sucrose in 50 ml water and make up to 100 ml.

2b. Ultracentrifuge rotor requirements

Any 30-50 ml fixed-angle rotor for an ultracentrifuge, capable of approx 100,000 *g* (**see Section 3**)

2c. Protocol

Carry out all operations at 0-4°C.

1. Mince the liver very finely with scissors and transfer to a Potter-Elvehjem 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) (**see Section 2d, Note 2**).

2. Centrifuge the homogenate at 3000 g_{av} in a fixed-angle rotor for 10 min to pellet the nuclei and heavy mitochondria. This pellet may be rehomogenized in solution B and the centrifugation repeated.

3. Centrifuge the supernatant(s) at 17,000 g_{av} for 10-15 min.

4. Resuspend the 17,000 *g* pellet in solution B using a loose-fitting Dounce homogenizer (2-3 strokes of the pestle). Adjust to a volume of about 0.5 ml per g of tissue.

5. Use a two chamber gradient maker or a Gradient Master to prepare a linear gradient from 9 ml each of gradient solutions E2 and E3 in thick-walled polycarbonate tubes for a 36-40 ml fixed-angle rotor and underlayer each gradient with 2 ml of gradient solution E1 (**see Section 2d, Note 3**).

6. Layer 3 ml of the suspension over each gradient and centrifuge at 105,000 g_{av} for 1 h.

7. Allow the rotor to decelerate from 1000 rpm without the brake, the collect the gradient in 1 ml fractions dense end first (**see Section 2d, Note 4**).

2d. Notes

1. The variable volume of 1 M sucrose maintains each solution isoosmotic. Keep these solutions, and carry out all subsequent operations, at 0-4°C. Protease inhibitors may be included in any or all of the media at the operator's discretion.
2. For more information on homogenization of tissues and cells and differential centrifugation of an homogenate **see respectively Application Sheets S05, S06 and S07**.
3. Thin-walled tubes can be used but may require some capping or sealing device. For more details on the preparation of pre-formed iodixanol gradients **see Application Sheet S03**.
4. Gradients can be unloaded dense end first by carefully introducing a narrow metal cannula (connected to a peristaltic pump) to the bottom of the tube. Thin-walled tubes can be collected by tube puncture. For more information on unloading gradients **see Application Sheet S08**.

2e. Analysis

A typical result with rat liver is shown in Figure 1, which shows the distribution of marker enzymes across the gradient. The catalase (peroxisome) band is well separated from all of the mitochondria (glutamate dehydrogenase) and lysosomes (acid phosphatase). The endoplasmic reticulum (glucose-6-phosphatase) is the least dense membrane type. This is in contradistinction to Percoll® gradients in which ER and peroxisomes always overlap. Similar separations of peroxisomes are obtained with mouse kidney [4] and hepatocytes [5].

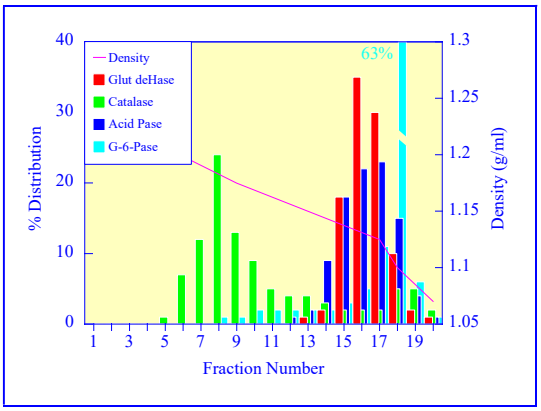

Figure 1 Isolation of peroxisomes: enzyme distribution in gradient. Glut deHase = glutamate dehydrogenase, Acid Pase = acid phosphatase; G-6-Pase = glucose-6-phosphatase**.** Adapted from ref 2 with kind permission of the authors and Academic Press.

The yield of peroxisomes is 80-90% with no detectable contamination from any other organelle. Yields of peroxisomes from Percoll® gradients are often low due to loss of material during the final

centrifugation step to remove the Percoll®. This is normally obligatory as Percoll® frequently interferes with subsequent analyses. If the concentration of peroxisomes in the gradient fraction(s) is sufficiently high for the analytical technique to provide reliable information, it is usually unnecessary to remove the gradient medium. Unlike Percoll®, iodixanol does not interfere with any spectrophotometric assays in the visible region of the spectrum nor does it affect the proper running of SDS-polyacrylamide gels. Standard spectrophotometric methods (carried out above 340 nm), for measuring organelle enzyme markers can be performed directly on gradient fractions [6]. Protein can also be measured directly by any Coomassie blue-based method [6]. If it is necessary to remove the gradient medium, dilute fractions with an equal volume of buffer; pellet at approx 30,000g_{av} for 10 min and resuspend in a suitable buffer. For SDS-PAGE proteins can be precipitated directly in TCA, iodixanol is soluble in acid solutions.

3. Alternative centrifugation/gradient formats for iodixanol gradient separations

1. Joly et al [7] used exactly the same iodixanol gradient as described in this OptiPrep™ Application Sheet but carried out the centrifugation at 125,000 *g* for 1 h in a Beckman SW41 swinging-bucket rotor. An almost identical distribution of organelle markers was reported. The authors investigated the localization of malonyl-CoA decarboxylase.
2. **Light and dense peroxisomes** have been resolved in a non-linear continuous gradient [8-12]. It was generated from a discontinuous one of 1.12, 1.155, 1.19, 1.225 and 1.26 g/ml (equivalent to 18.5%, 26%, 32.5%, 40.5% and 47.5% iodixanol) by freezing in liquid nitrogen (and storage at -20°C) followed by rapid thawing (approx. 30 min). The volumes of solution used were 10, 7, 6, 3 and 4 ml respectively and the crude fraction was layered on top. After centrifugation at 39,000 g_{max} for 30 min in a Beckman VTi50 rotor the light peroxisomes banded at approx 1.21 g/ml and the dense peroxisomes at approx. 1.24 g/ml. Islinger and Weber [10] showed that iodixanol gradients could even identify a very low density population. This gradient was also described in refs 11 and 12.
3. In a comprehensive methodological review paper Islinger et al [13] reserved the sigmoidal gradient for isolation of the peroxisomes from a light mitochondrial fraction; whilst the peroxisomes from a heavy mitochondrial fraction were isolated in a discontinuous gradient [13] – **see Application Sheet S14a.**
4. Antonenkov et al [14] used an iodixanol gradient as a final step for the production of highly purified peroxisomes, virtually devoid of any contamination. An initially discontinuous gradient comprised 6 ml each of 20%, 25%, 30% and 35% (w/v) iodixanol, together with 4 ml each of 40% and 50% iodixanol, in tubes for the Beckman VTi50 rotor. After being allowed to diffuse overnight at 4°C, 8-9 ml of crude peroxisomes was layered on top and centrifuged at 65,000 g_{av} for 1 h. The peroxisomes produced by this procedure were used in physical [14], membrane transport [15] and fatty acid binding protein studies [16]. In a more recent paper the centrifugation conditions were 100,000 *g* for 90 min [17].
5. Note that using a vertical rotor confers significant advantages for organelle isolation. The short sedimentation path lengths of such rotors, compared to either swinging-bucket or fixed-angle rotors permits both the use of lower *g*- forces and shorter times. This also means that the fragile organelles are exposed to much lower hydrostatic pressures.

5. References

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