OptiPrep™ Application Sheet S15

Fractionation of mammalian mitochondria, lysosomes and peroxisomes in continuous gradients

- 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.
- **Axis-Shield Mini-Reviews** provide a short protocol review and bibliography of all papers reporting the use of OptiPrep™ for the fractionation of a total, heavy or light mitochondrial fraction. **Mini-Review MS04** focusses on the isolation of **mitochondria**, **MS12 - lysosomes** and **MS02 - peroxisomes**: to access return to the initial list of Folders and select "**Mini-Reviews**"
- Centrifugation of a post-nuclear supernatant (PNS) at 3000 *g* for 10 min produces the heavy mitochondrial fraction (HMF) containing principally mitochondria plus some of the lysosomes and peroxisomes. The light mitochondrial fraction (LMF) is the material that sediments from the 3000 *g* supernatant at 12-20,000*g* for 10-20 min and contains mitochondria, lysosomes, peroxisomes and some of the microsomes. For a total mitochondrial fraction the 3,000*g* step is omitted.

1. Background

Both Nycodenz® and iodixanol have been widely used to analyze the organelles of the LMF or HMF+LMF. In iodixanol gradients the density of lysosomes is slightly lower than that in gradients of Nycodenz®; the density of mitochondria is much lower (ρ = 1.13-1.15 against 1.18-1.20 g/ml) while the density of peroxisomes is virtually the same [1]. The density of the endoplasmic reticulum (ER) present in the LMF is rather more variable and may overlap either the lysosomes or the mitochondria. Nevertheless each of the organelles has a distinctive banding pattern in both gradient media. In Percoll® gradients the ER co-bands with peroxisomes [2] and the two organelles cannot be resolved. Modulation of the density range of the gradient may be necessary to optimize a particular separation from a particular tissue or cell type.

- Section 2 of this Application Sheet describes the preparation of the LMF or HMF+LMF by differential centrifugation (for more information see **Application Sheet S07**.)
- Section 3 describes the use of iodixanol gradients (variants are given in Table 1 in Section 3e).

2. Preparation of LMF or HMF+LMF

2a. Solutions required (see Box 1)

Homogenization medium (HM): 0.25 M sucrose, 1 mM EDTA, 20 mM Hepes-NaOH, pH 7.4 (see Section 2c, Notes 1 and 2)

Box 1

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•$2H_2O$); 3.72 g per 100 ml water

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

2b. Protocol

Carry out all operations at 0-4°C.

To prepare the HMF+LMF omit steps 6-8 and use the combined 1000 *g* supernatants (from step 5) instead of the 3000 *g* supernatant in step 9.

1. *For soft tissues:* Mince the tissue very finely with scissors (or with a tissue chopper) and transfer to a Potter-Elvehjem (Teflon and glass) homogenizer with the HM (use 10 ml for every 2.5 g tissue). Homogenize using approx 6 strokes of the pestle at 500-700 rpm (see Section 2c, Note 3).

2. *For cells:* Wash 1-3x10^8 cells in 5 ml of phosphate buffered saline and again with 5 ml of HM. Suspend the cells in 3 ml of HM and homogenize in a ball-bearing homogenizer using five passes (see Section 2c, Note 4).

3. Centrifuge the homogenate at 1000 g_{av} for 5 min to pellet the nuclei (do not use the brake to decelerate the rotor); then carefully decant the supernatant or aspirate it using a syringe and metal cannula and retain on ice.

4. Resuspend the pellet in 10 ml (5 ml for cells) of HM using 2-3 gentle strokes of the pestle of a loose-fitting Dounce homogenizer (see Section 2c, Note 5).

5. Repeat the centrifugation and combine the supernatants.

6. To pellet the HMF centrifuge the suspension at 3000 g_{av} for 10 min, then aspirate or decant the supernatant and retain on ice.

7. Resuspend the 3000 *g* pellet (HMF) in about half the original volume of HM.

8. Gently homogenize the pellet using a loose-fitting Dounce homogenizer and repeat step 6.

9. Centrifuge the combined 3000 *g* supernatants at 17,000 g_{av} for 10-15 min.

10. Resuspend the pellet (LMF) in a small volume (approx. 2 ml) of HM using a loose-fitting Dounce homogenizer (see Section 2c, Notes 6 and 7).

2c. Notes

1. Protease inhibitors may be included in the HM at the operator's discretion.
2. Any suitable buffered isoosmotic solution may be used and there is considerable variation in the detailed composition of the HM in the literature. Media, which are most "mitochondria-friendly", are based on 0.25 mM mannitol rather than sucrose and the EDTA is often replaced with 0.1 mM EGTA for rat liver. Alternatively peroxisome-specific media often contain 0.1% (v/v) ethanol. MOPS is another frequently used buffer.
3. The described methodology applies to tissues such as rodent liver and kidney. Other tissues such as skeletal and cardiac muscle, intestine and brain require special treatments and the operator should consult relevant texts. For more information see **Application Sheet S05**.
4. The ball-bearing homogenizer (cell cracker) is generally regarded as one of the best devices for cultured cells; delicate organelles are best preserved by this technique. If one is not available, shearing by several passages through a syringe needle may be a reliable alternative. For more information see **Application Sheet S06**.
5. The nuclei may be very fragile since any homogenization medium containing EDTA is not well suited to the preservation of these organelles. The pellet must be washed by very gently.
6. The LMF may be washed to remove trapped microsomes by suspension to the original volume with HM and repeating steps 9 and 10.
7. If the suspension is layered beneath a gradient, it may be resuspended in the appropriate solution or mixed with a dense solution. In the latter case the total volume may be doubled or trebled.

3. Fractionation of LMF (or HMF+LMF) in a continuous iodixanol gradient

3a. Solutions required (see Section 3d, Note 1)

OptiPrep™

Homogenization medium (HM): see Section 2a

OptiPrep™ Diluent (OD): 0.25 M sucrose, 6 mM EDTA, 120 mM Hepes-NaOH, pH 7.4 (see Box 2)

Working Solution (WS) of 50% iodixanol (ρ = 1.272 g/ml): 5 vol. of solution OptiPrep™ + 1 vol. of OD

Box 2
See Box 1 for stock solutions

OD: Dissolve 8.5 g sucrose in 50 ml water; add 6 ml and 24 ml respectively of EDTA stock and Hepes stock; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3b. Ultracentrifuge rotor Requirements

Any swinging-bucket rotor for an ultracentrifuge capable of 100,000g with a tube capacity of approx 17 ml tubes, e.g. Beckman SW28.1 or Sorvall AH629 (see Section 3d, Note 2)

3c. Protocol

Bring gradients to 0-4°C and carry out all subsequent steps at this temperature.

1. Dilute WS with HM to produce gradient solutions with iodixanol concentrations of (1) 19% and 27% (w/v) iodixanol (see Section 3d, Notes 3 and 4).

2. Use a two chamber gradient maker or a Gradient Master™ to prepare a linear gradient from 6.0 ml each of the two gradient solutions in 17 ml tubes for the swinging-bucket rotor (see Section 3d, Note 5).

3. Adjust the LMF or HMF+LMF suspension to 30% iodixanol by mixing it with WS and layer 3-4 ml beneath the gradient.

4. Layer 1-2 ml HM on top of the gradient to fill the tube to 3-4 mm from the top of the tube and centrifuge in a suitable swinging-bucket rotor at approx 70,000 g_{av} for 1.5-2 h. Use a slow deceleration program, or if one is not available allow the rotor to decelerate from 2000 rpm without the brake.

5. 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, by tube puncture or aspiration from the meniscus. For more information on harvesting gradients see **Application Sheet S08**.

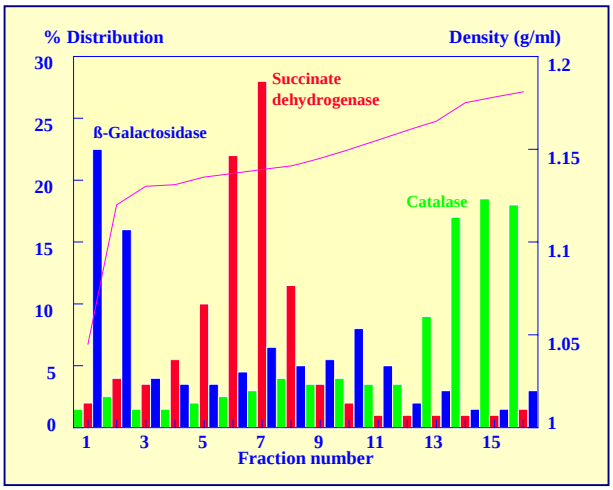

Figure 1 Fractionation of LMF on a pre-formed iodixanol gradient (19-27%): enzyme distribution; Succ deHase = succinate dehydrogenase; ß-Gal'ase = ß-galactosidase.

3d. Notes

1. Protease inhibitors may be included in OD at the operator's discretion. Strategies for preparing gradient solutions for mammalian tissues and cells are given in **Application Sheet S01**.
2. Rotors of other tube capacity are permissible, e.g. Beckman SW41Ti (approx. 13 ml) or SW28 (approx. 39 ml). Scale up or down all the volumes given in the Protocol proportionately. A vertical rotor such as the Beckman VTi50 may also be used. The short path length of such a rotor reduces the hydrostatic pressure on the organelles and allows a reduction in either *g*-force or centrifugation time (see Table 1).
3. The density of the 19% and 27% (w/v) iodixanol solutions is 1.124 and 1.162 g/ml respectively. Typical results with the gradient are shown in Figure 1.
4. Modulation of the density range of the gradient may be required for other tissue or cell types and for customization to individual requirements (see Section 3e).
5. If one of these devices is not available a continuous gradient can be prepared by diffusion of a discontinuous gradient. The 19-27% iodixanol gradient could be produced from equal volumes of 19%, 22%, 25%, and 27% iodixanol. For more details on the preparation of gradients see **Application Sheet S03**.

3e. Summary of published gradient conditions

Published papers reporting the use of the method described in this Application Sheet (or a modification of the method) have been sorted alphabetically according to tissue or cell source in Table 1 (see next page). Each entry has a summary of the iodixanol gradient and centrifugation conditions used; whether the LMF or LMF+HMF was loaded on top or beneath the gradient and also an indication of the organelles that were identified in the gradient.

Table 1 Pre-formed continuous iodixanol gradients for the fractionation of HMF, LMF or HMF+LMF[1]

Tissue/cell	Fraction	Gradient[2]	Load[3]	RCF/time	Organelles analyzed[4]	Ref #
Brain (mouse)	HMF+LMF	10-30	B	52,000 *g*/1.5h	Lys	4
Carcinoma	HMF & LMF	10-30	T	80,000 *g*/2h	Mit, lys, perox	5
	LMF	10-30	B	100,000 *g*/3h	Mit, perox, ER, Golgi, PM	13
	PNS[5]	10-30	B	90,000 *g*/1.5h	Lys	21
COS 7	HMF+LMF	0-22	B	70,000 *g*/2h	Lys	6
	HMF	10-30	B	52,000 *g*/1.5h	Mit	7
Glioblastoma	HMF+LMF	10-30	B	52,000 *g*/1.5h	Mit	8
Hepatoma	HMF+LMF	10-30	B	52,000 *g*/1.5h	Golgi, ER, mit	9
Human liver[1]	**LMF**	**13-45(v)**	**T**	**33,000 *g*/1.25h**	**Perox, mit, ER, (cytosol)**	**10,11**
Human epithelial	HMF+LMF	10-30	B	52,000 *g*/1.5h	Mit, lys, perox, ER	12
MDCK	HMF+LMF	10-30	T	100,000 *g*/1h	Lys, Golgi, ER	14
Mouse liver[1]	**LMF**	**13-45(v)**	**T**	**33,000 *g*/1.25h**	**Perox, mit, ER, (cytosol)**	**15,16**
	LMF	10-30	B	52,000 *g*/1.5h	Mit, lys, perox, ER, Golgi	1
	HMF+LMF	10-30	B	52,000 *g*/1.5h	Mit	22
Rat heart	LMF	19-27	B	70,000 *g*/1.5 h	Mit, ER	19
Rat liver	PNS[5]	20-40	T	ns[6]	Perox, mit, ER	17
	LMF	10-40	T	105,000 *g*/1 h	Perox, mit	20
Rat skeletal muscle	LMF	10-40	T	105,000 *g*/1 h	Perox, mit	20
T-lymphoblasts	HMF+LMF	5-20	B	70,000 *g*/1.5h	Mit	18

1. Bold text indicates methods that may also be used specifically for the purification of peroxisomes
2. Figures in % iodixanol (w/v), d = discontinuous, v = vertical rotor
3. B = bottom-loaded gradient, T = top-loaded gradient
4. Mit = mitochondria, lys = lysosomes, perox = peroxisomes, ER = endoplasmic reticulum, PM = plasma membrane
5. PNS = post nuclear supernatant
6. ns = not stated

5. Gradient 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 [3]. Protein can also be measured directly by any Coomassie blue-based method [3]. 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. Schmidt et al [23] noted that the extensive washing of organelles that was required for organelles purified in Percoll™ led to a serious loss of functionality. For more information on analyzing gradients see **Application Sheet S09**.

6. References

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