OptiPrep™ Application Sheet S18

Fractionation of rough and smooth endoplasmic reticulum in self-generated 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.
- An alternative protocol using a sedimentation velocity iodixanol gradient is described in Application Sheet S19
- An **OptiPrep™ Mini-Review (MS07)** "Analysis of membrane trafficking in mammalian tissues and cells: fractionation of ER, Golgi, TGN, PM and endosomes" provides a bibliography of all published papers reporting the use of OptiPrep™ for analysis of these membranes: to access return to the initial list of Folders and select "**Mini-Reviews**".

1. Background

For the analysis of protein synthesis and translocation, the widely-used routine method for separation of the smooth and rough endoplasmic reticulum (SER and RER), which was devised by Walter and Blobel [1], involves a simple 1.3 M sucrose density barrier. In this system the SER bands at the interface while the RER and ribosomes pellet. Call et al [2] noted however that for some cell types (these workers were using plasmacytoma cells) the method did not yield high activity ER microsomes. These workers consequently chose [2,3] the more efficacious self-generated iodixanol gradient protocol that is described in this Application Sheet. Morand et al [4] also observed that this method provided the highly purified SER and RER that was necessary for proteomic analysis.

The technique takes advantage of the ability of iodixanol to form reproducible self-generated gradients very rapidly in vertical or near-vertical rotors. It simply involves mixing a total microsome fraction with an iodixanol working solution followed by a 2 h centrifugation in a vertical rotor and thus it is actually easier to set up, and takes no longer to execute, than the sucrose density barrier method. Although the banded material in the gradient is normally recovered by collection of the gradient in 10-20 equal volume fractions, the major SER and RER zones are so clearly defined that they may also be retrieved using a syringe. Moreover, as the RER forms a band in the gradient, rather than a gelatinous co-pellet with the ribosomes, its recovery is rather easier.

The method was first described for analysis of the assembly of very low-density lipoproteins in rabbit hepatocytes [5]. It is thus a very useful means of providing a "snapshot" of the secretory process at a particular time following administration of appropriate radiolabelled precursors. The protocol is based on data from ref 5.

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

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4

C. OptiPrep™ Diluent: 0.25 M sucrose, 100 mM Tris-HCl, pH 7.4

D. 54% Iodixanol Working Solution (ρ = 1.291 g/ml): mix 9 vol. of Solution A with 1 vol. of Solution C

Keep the following stock solution at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water.

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2.0 ml of Tris stock; adjust to pH 7.4 with 1 M HCl and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 10 ml of Tris stock; adjust to pH 7.4 with 1 M HCl and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Vertical or near vertical rotor (10-14 ml tube capacity) capable of approx 350,000 g_{av}, such as the Beckman VTi65.1 or NVT65 or Sorvall 65V13 (**see Section 6, Notes 2 and 3**)

4. Protocols

Carry out all operations at 0-4°C

4a. Microsome Preparation

1. Prepare an homogenate of the tissue or cells; in this protocol the tissue (liver) is homogenized in solution B using a Potter-Elevehjem homogenizer with 5-6 gentle strokes of the pestle (**see Section 6, Note 4**).

2. Remove the nuclei by centrifugation at 1000 *g* for 10 min (**see Section 6, Note 5**).

3. Carefully decant the 1000 *g* supernatant and centrifuge it at 15,000 g_{av} for 20 min to pellet the mitochondria, lysosomes, peroxisomes and Golgi membranes.

4. Aspirate the 15,000 *g* supernatant carefully, using a syringe and metal cannula to avoid disturbing the sometimes loosely-packed upper zone of the pellet (**see Section 6, Note 6**).

5. Pellet the microsomes from the 15,000 *g* supernatant by centrifugation at 100,000 g_{av} for 45 min (**see Section 6, Notes 7 and 8**) and resuspend the pellet in solution B (30 ml per 10g liver).

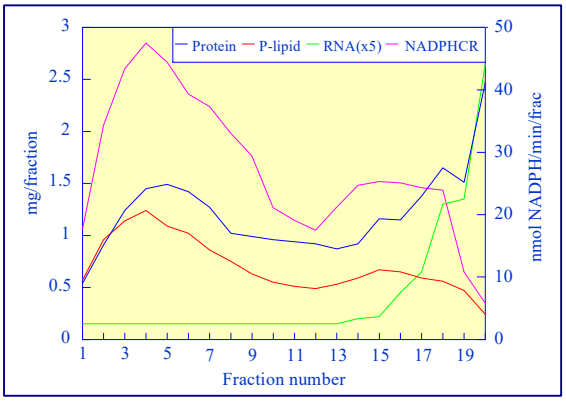
4b. Fractionation of microsomes

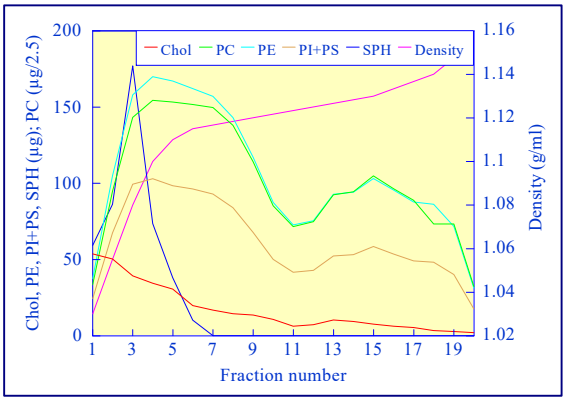
1. Mix 6.3 vol. of microsome suspension with 3.7 vol. of solution D (final iodixanol concentration 20%, w/v; ρ = 1.127 g/ml).

2. Transfer to tubes (11-14 ml) for a vertical rotor or near-vertical rotor.

3. Centrifuge at 350,000 g_{av} for 2 h, turning off the brake during deceleration from approx. 3000 rpm (**see Section 6, Note 9**).

4. Harvest the gradients by upward displacement with a dense solution or by tube puncture in 0.5-1.0 ml fractions for analysis (**see Section 6, Note 10**)





Figures 1 (left) and 2 (right) Distribution of hepatic microsomes in self-generated iodixanol gradient (low density on left).
Figure 1: Distribution of protein, phospholipids (P-lipid), RNA and NADPH cytochrome c reductase (NADPHCR).
Figure 2: Distribution of cholesterol (chol), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SPH).

5. Analysis

Protein, lipid and NADPH cytochrome c reductase profiles of the gradient (Figure 1) reveal two broad bands of material. RNA increases gradually in the lower third of the gradient, the very sharp increase in the bottom fraction being accompanied by a rapid fall in phospholipid and NADPH cytochrome c reductase. The SER is thus distributed broadly in the top half of the gradient, the RER in the bottom third and ribosomes band in the last fraction (**see Section 6, Note 11**).

A phospholipid profile of the gradient (Figure 2) shows that most types follow the same biphasic profile as the protein and total phospholipid; the sphingomyelin on the other hand peaks very sharply in the third fraction. This plasma membrane marker indicates that the lightest vesicles are either derived from the plasma membrane or are destined for incorporation into this membrane. The cholesterol profile is similar to that of the sphingomyelin, in that it increases towards the low-density end of the gradient but it is nevertheless quite distinctive in detail.

This system has been used to show that although the major site of lipid assembly into VLDL occurs in a discrete fraction of the SER, other distinct intracellular pools of lipid may be involved in apolipoprotein B transit into the RER lumen. See ref. 5 for more details.

- The method has been used for analysis of mammalian liver [4, 8-11], rabbit hepatocytes [5], rabbit enterocytes [12,13], human hepatoma [6], T-cell [3], plasmacytoma cells [2], mouse hybridoma [14] and human carcinoma cells [7].
- In a variation of the self-generated gradient, Wang et al [15,16] adjusted the microsomal fraction from carcinoma cell lines to 20% (w/v) iodixanol and centrifuged small volume fractions (1 ml) at 200,000 *g* overnight. This may be a useful option for laboratories that do not have access to the rotor/ultracentrifuge required as described above.

6. Notes

1. Protease inhibitors (PMSF, leupeptin, antipain, aprotinin etc) 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 sedimentation path length of the tube should be approx 17 mm. The preparation may be scaled down to approx 5 ml tubes with, for example, the Beckman VTi65.2 vertical rotor. It should be possible to reduce the centrifugation time to approx 1 h 45 min with this smaller volume rotor, although Morand et al [4] actually used just over 2 h. It may be feasible to use a small volume high-performance fixed-angle rotor but it will be necessary to modulate the centrifugation conditions in order to produce a gradient with the correct density profile. For more information on formation of self-generated gradients **see Application Sheet S04**.
3. The tubes of choice are Optiseal™ tubes, which are only available for Beckman rotors. Since they are sealed with a central plastic plus rather then heat- or crimp-sealed, they are easy to use and most importantly, gradients within them can be unloaded by any of the standard techniques available to open-topped tubes for swinging-bucket rotors.
4. Solution B may be any buffered isoosmotic sucrose solution. Alternatively, for some cultured cells, it may be necessary to swell them prior to homogenization. Call et al [2,3,] for example used 10 mM Hepes-KOH, pH 7.5 to swell plasmacytoma cells. Always return the homogenate to isoosmotic conditions as soon as possible. **For more information about homogenization of tissues and cells see respectively Application Sheets S05 and S06**.
5. The 1000 *g* step may be omitted, but retention of step 2 may improve yields. If the homogenate is treated directly as in step 4, large amounts of rapidly sedimenting nuclei and debris may trap a lot of other smaller particles.
6. The centrifugation conditions used to remove the light mitochondrial pellet organelles vary from 10,000 *g* to 17,000 *g* and from 10-20 min. **For more information on differential centrifugation see Application Sheet S07**.
7. Centrifugation conditions for pelleting microsomes vary; Higashi et al [6] used 138,000 *g* for 1 h
8. It is possible to omit the 100,000 *g* sedimentation of the microsomes and simply take the 12,000 *g* supernatant and adjust this to 20% iodixanol. Although this is quicker, it has the disadvantage that the cytosolic proteins contaminate the gradient fractions; these can however be removed subsequently by pelleting the membranes.
9. Higashi et al [6] used 100,000 *g* for 13 h, although rotor was not specified. Liao and Carpenter [7] also used an overnight centrifugation (at 200,000 *g*) in small volumes (1 ml/tube) but again the rotor was not given.
10. **For more information on the harvesting of gradients Application Sheet S08**.

11. The density profile (see Figure 2), which is steep at the top of the gradient, is not optimal for resolving vesicles in the low-density region. To study these vesicles more satisfactorily, or, for example, if it is required to study specifically either the SER or the RER, the density profile of the gradient can be altered, either by changing the starting concentration of the iodixanol or the centrifugation conditions, **see Application Sheet S04**.

7. References

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