OptiPrep™ Application Sheet S62

Isolation of plasma membrane from cultured cells by flotation through a discontinuous gradient

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- An Axis-Shield Mini-Review (MS05) "Analysis of membrane trafficking and signaling" provides a bibliography of all published papers reporting the use of OptiPrep™ for analysis of these membranes: to access **click here.** The references are listed according to cell type.
- 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

Using iodixanol gradients, isolation of a plasma membrane (PM) fraction is often achieved simultaneously with the purification of other subcellular membranes in either continuous (linear) or discontinuous iodixanol gradients, centrifuged for 1-3 h or overnight (see **Application Sheets S21-25**). This Application Sheet highlights examples in which the PM is particularly well separated from intracellular compartments.

The PM is usually the least dense of all the subcellular membranes. In such situations, flotation through one or more low-density solutions from a high-density suspension often provides improved recovery and/or resolution than does sedimentation from the top of the gradient. If the density at the top of the gradient is in the range 1.04-1.08 g/ml (equivalent to approx 2.5-10% w/v iodixanol), the PM is usually well resolved from slightly denser components such as Golgi and early endosomes. Additionally, overloading the gradient (instability of the sample/gradient interface) cannot occur in a flotation format and resolution of the PM from cytoplasmic proteins, which remain in the dense sample zone, is much improved. In top-layering cytoplasmic proteins move down the gradient by both sedimentation and diffusion and are likely to contaminate the least dense material.

This flotation technique has been used for mouse astrocytoma cells [1], PC12 pheochromocytoma cells [2] and MCF7 human breast adenocarcinoma cells [3]. All used a routine buffered solution of 0.25 M sucrose and 1 mM EDTA, although 2 mM $MgCl_2$ was included for the PC12 cells. The latter is included as an option in Section 2 (below). Whether this a specific requirement for PC12 cells or whether it would of more general benefit can only be determined experimentally. The protocol below is adapted from ref 1, while Sections 5.5.1 and 5.6 describe alternative gradients and centrifugation conditions from refs 2 and 3.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Homogenization buffer (HB): 0.25 M sucrose, 1 mM EDTA, 2 mM $MgCl_2$ (optional), 20 mM Hepes-NaOH, pH 7.4

C. Diluent: 0.25 M sucrose, 6 mM EDTA, 12 mM $MgCl_2$ (optional), 120 mM Hepes-NaOH, pH 7.4

D. Working Solution (WS) of 50% (w/v) iodixanol (ρ = 1.272 g/ml): 5 vol. of solution A + 1 vol. of solution C

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
1 M $MgCl_2$•$6H_2O$: 20.3 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2 ml of EDTA stock and 8 ml of Hepes stocks (optional: 0.4 ml $MgCl_2$ stock); adjust to pH 7.4 with 1 M NaOH and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 6 ml of EDTA stock and 24 ml of Hepes stock (optional: 1.2 ml $MgCl_2$ stock); adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements (see Section 5.2)
Any swinging-bucket rotor capable of 100,000-200,000g with tube volumes of approx. 14 ml (e.g. Beckman SW41 or Sorvall TH641) or 5 ml (e.g. Beckman SW55 or Sorvall AH650)

4. Protocol
Carry out all operations, except the washed with phosphate-buffered saline (Step 1) at 0-4°C.

1. Wash the cells twice in phosphate-buffered saline to remove the culture medium, and then once in Solution B.

2. Suspend the cells in a small volume of Solution B ($2.5x10^7$ cells in 0.5-1.0 ml)

3. Disrupt the cells by repeated passages through a fine syringe needle (e.g. up to 20x using a 25G needle), in a ball-bearing homogenizer (3-10 passes) or with 20 strokes of the pestle of a tight-fitting Dounce homogenizer (see Section 5.3).

4. Centrifuge the homogenate at 2000 *g* for 10 min and harvest the supernatant (see Section 5.4.1).

5. Centrifuge the supernatant at 100,000 *g* for 40-60 min (see Section 5.4.2).

6. Prepare solutions of 2.5%, 10%, 17.5, 25% and 30% (w/v) iodixanol solution by mixing Solutions D and B 2.5:47.5, 1:5, 17.5:32.5, 1:1 and 3:2 (v/v) respectively (see Section 5.5.1).

7. Suspend the pellet from Step 5 in 0.75 ml of 30% iodixanol (see Section 5.5.1).

8. In 13 ml tubes for the swinging-bucket rotor prepare a discontinuous gradient from 3 ml each of the 2.5%, 10%, 17.5% and 25% iodixanol by the underlayering technique, using a syringe and metal cannula and finally underlayer the sample in 30% iodixanol (see Section 5.5.2).

9. Centrifuge at 165,000 g_{av} for 3.5 h (see Section 5.6).

10. Collect the gradient in 0.5 ml fractions by tube puncture, upward displacement or aspiration from the meniscus. For more information on harvesting gradients see **Application Sheet S08**.

5. Technical Notes and Review
5.1. Homogenization media and gradient solutions

The homogenization medium often has to be tailored to the tissue or cell type and it is not known if the composition of the HM is relevant to the separation. All of the published methods using the type of protocol used in this OptiPrep™ Application Sheet have used a 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and often, but not always, containing 1 mM EDTA. Supplementation of the HM with inorganic salts is becoming increasingly common and can reduce ionic interactions, aggregation between membranes and combat any raised viscosity of the homogenate due to cytoskeletal proteins. Some media that omit sucrose entirely use either NaCl or KCl or both as the principal osmotic balancer(s). The composition of the HM should also be compatible with any subsequent analytical process. The inclusion of divalent cations can guard against nuclear breakage; stabilize membranes generally, but may lead to aggregation. Table 1 summarizes some of the other HMs that have been used. Other examples are given in **Application Sheets S05 (tissues) and S06 (cells).**

The preparation of a 50% iodixanol working solution (Solution D) ensures that the concentrations of EDTA (and $MgCl_2$, if included) and buffer are constant in all gradient solutions. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**. Protease inhibitors may be included in Solutions B and C at the operator's discretion.

5.2. Ultracentrifuge rotors

These separations have been performed either in 13 ml tubes or 5 ml tubes. Other swinging-bucket rotors or even vertical rotors may be used. Larger volume swinging-bucket rotors may require longer centrifugation times but smaller volume rotors and vertical rotors will need shorter times. Note however that the progressive change in gradient density profile (due to diffusion and sedimentation of the iodixanol molecules) may also be modulated in other rotors and affect the final resolution.

5.3. Homogenization

The homogenization protocol should be tailored to the cell (or tissue) type. Potter-Elevhjem or Dounce homogenization for tissues and Dounce homogenization for cells used to be the standard procedures. For cells however use of 12-20 passages through a syringe needle (the Gauge Number (G) varies from 21 to 25) sometimes preceded by Dounce homogenization, has become very common. The ball-bearing homogenizer ("cell cracker") is now widely regarded as one of the most effective and reproducible of devices.

Ideally the procedure should be as gentle and reproducible as possible, the aim being to cause at least 95% cell disruption without damage to the major organelles, particularly the nuclei and lysosomes. The type and severity of the homogenization process will have consequences for the integrity of the organelles and the size of the vesicles produced from tubular structures in the cytoplasm. Therefore the pattern of membrane banding in any subsequent gradient may not be easily predicted. Some hints on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells)**.

5.4. Differential centrifugation

5.4.1. Removal of nuclei

Nuclear pelleting may be carried out at 500-3000*g* for 5-10 min; the higher RCFs (g-forces) resulting in removal of some of the mitochondria, which can facilitate subsequent layering of the sample on the gradient. To recover any vesicles trapped in the pellet (more serious at the higher RCFs), the pellet is sometimes resuspended in HM, recentrifuged and the two supernatants combined. A possible disadvantage of this practice is that unless the resuspension of the pellet is carried out very gently, the nuclei may be damaged, with consequent leakage of DNA, which may lead to almost irreversible aggregation of the subcellular membranes.

5.4.2. Preparation of sample for gradient loading

If the size of the sample is sufficiently small this step may be omitted and the 2000*g* supernatant itself adjusted to a high density for loading under the gradient. Since the cytosolic proteins remain in at the bottom of the gradient during the subsequent centrifugation, this practice should not be detrimental to the purity of the recovered plasma membrane.

5.5. Density gradients

5.5.1. Alternative formats

Other gradients that have been used for plasma membrane flotation are:

- For PC12 cells [2], 5%, 10%, 15% and 20% (w/v) iodixanol, sample underlaid in 25% (w/v) iodixanol
- For MCF-7 cells [3], 20% and 24% (w/v) iodixanol, sample underlaid in 32% (w/v) iodixanol
- A three layer discontinuous gradient, which was originally designed for the isolation of PM from *Drosophila* [4], was also used for its isolation from mouse embryos [6]. The embryos were homogenized in 150 mM NaCl, 0.2 mM EGTA, 100 mM Tris-HCl, pH 7.4 and a post-nuclear supernatant was mixed with OptiPrep™ to 40% (w/v) iodixanol solution and overlaid by 30% and 5% iodixanol. After centrifugation at 100,000 *g* for 3 h the plasma membrane was harvested from the top interface [5].

5.5.2. Construction

Although underlayering is the recommended method for making discontinuous gradients, overlayering is also an option. For more information on gradient construction see **Application Sheet S03**. If necessary, adjust all volumes proportionately so that tubes (after sample application) are properly filled according to the manufacturer's instructions. Gradients and sample volume should be scaled up or down proportionately as required for larger or smaller rotors.

5.6. Centrifugation conditions

Other centrifugation conditions that have been used are: for PC12 cells [2], 88,000*g* for 17 h; for MCF-7 cells [3], and 83,000g for 2 h

5.7. Analysis

In a 5%, 10%, 15%, 25%, 30% iodixanol flotation gradient from PC12 cells [2] the plasma membrane, as identified by the EGF receptor banded at the 5/10% and 10/15% interface. The PM from MCF-7 cells also banded at the top of the 20% iodixanol layer (Figure 1, Panel a), while that from mouse astrocytoma cells (Figure 1, Panel b) was also the least dense membrane (banding mainly at approx. 10% iodixanol). In the latter case the PM marker (transferrin receptor) showed a distinct biphasic trait and interestingly the murine coronavirus spike protein (S-protein) was confined to the Golgi and the denser plasma membrane component. The implication is that this gradient may be able to resolve subfractions of the PM.

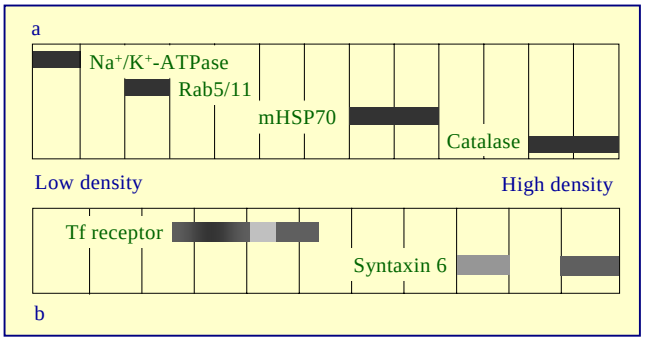

Figure 1 Panel a: Fractionation of MCF-7 human breast adeno-carcinoma cell membranes in a 20%, 24%, 32% iodixanol flotation gradient, approx. disposition of markers, adapted from ref 3, for more information see 5.7. Panel b: Fractionation of mouse astrocytoma cell membranes in a 2.5%, 10%, 17.5%, 25%, 30% iodixanol flotation gradient, approx. disposition of markers, adapted from ref 1, for more information see 5.7.

The plasma membrane overlapped with neither endosomes [2,3] nor Golgi markers [3]. Endosome markers EEA1 and Rab5B from PC12 cells were both recovered almost entirely from the 25/30% interface region (1.15-1.175 g/ml) and in the case of MCF-7 cells both Rab5 and Rab 11 (Figure 1, Panel a) banded at >20% iodixanol. The Golgi marker (Syntaxin 6) from mouse astrocytoma cells (Figure 1, Panel b) banded towards the bottom of the gradient in the 17.5-25% iodixanol zone. Thus compared to top-loaded gradients (see for example **Application Sheet S21**) the banding density of both endosomes and Golgi is unexpectedly high in these flotation gradients. On the other hand the position of mitochondria (mHSP70 marker) from MCF-7 cells (Figure 1, Panel a) was more or less as expected.

6. References

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