OptiPrep™ Application Sheet S37

Determination of the cytosolic or membrane location of a large protein complex

- 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.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5)

1. Background

Differential centrifugation or sedimentation through a sucrose gradient does not easily provide an unambiguous answer to the question of whether a particular protein shifts from a cytosolic location to a membrane location during subcellular processing or during some functional modulation. Differential centrifugation requires several washes of the membrane pellet to remove all traces of cytosolic proteins. In differential centrifugation and sedimentation through a sucrose gradient, the proteins are also moving in the same direction as the membrane vesicles by sedimentation and diffusion. **Application Sheets S35 and S36** describe systems in which the membranes are allowed to float through a density gradient from a dense load zone. This is an ideal format because:

- Proteins sediment, particularly as they are exposed to the g_{max}, while membranes migrate in the opposite direction
- The difference in density between the membranes and the proteins is much greater in iodixanol than in sucrose
- The lower viscosity of iodixanol gradients means that the particles move more quickly.

If the protein is part of a rapidly sedimenting large complex however, it may be possible to simplify the protocol considerably by avoiding bottom-loading of the sample. Instead, the sample is merely adjusted to a uniform concentration of iodixanol and centrifuged in a vertical or near-vertical rotor for approx 1 h at approx 350,000 *g* [1]. Plasma membrane vesicles (from any source) have a low density (<1.1 g/ml) in iodixanol, while proteins have a density of approx 1.26 g/ml and at 350,000 *g* rapidly sedimenting protein complexes will sediment through the gradient within 1 h in a short-sedimentation path length rotor. The gradient that is formed under these centrifugation conditions will have a wide density range with a shallow median section. Such a gradient is ideal for the separation of two particles with dissimilar densities, which will band at opposite ends of the gradient.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0 (see Note 3)

C. Diluent: 540 mM KOAc, 12 mM $Mg(OAc)_2$, 120 mM Hepes-KOH, pH 8.0.

D. Working Solution (50% iodixanol): mix 5 vol. of Solution A with 1 vol. of Solution C.

Keep the following stock solutions at 4°C:
1 M Hepes (free acid): 23.8 g per 100 ml water.
1 M KOAc: 9.81 g per 100 ml water
100 mM $Mg(OAc)_2 \bullet 4H_2O$: 2.15 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 4 ml, 18 ml and 4 ml of Hepes, KOAc and $Mg(OAc)_2$ stocks respectively; adjust to pH 8.0 with 1 M KOH and make up to 200 ml.

Solution C: Mix 12 ml, 54 ml and 12 ml of Hepes, KOAc and $Mg(OAc)_2$ stocks respectively; adjust to pH 8.0 with 1 M KOH and make up to 100 ml.

3. Rotor requirements (see Section 5.2)

Vertical rotor with 11-13 ml tubes (e.g. Beckman VTi65.1 or Sorvall 65V13, or near-vertical rotor (e.g. Beckman NVT65).

4. Protocol (adapted from ref 1)
Carry out all operations at 0-4°C.

1. Homogenize the cells in Solution B in a cell cracker (ball-bearing homogenizer) using 4-6 passages. Monitor the efficacy of the homogenization by phase contrast microscopy (see Section 5.3).

2. Centrifuge the homogenate at 1000 *g* for 5 min to pellet the nuclei.

3. Mix 2 vol. of post-nuclear supernatant (PNS) with 3 vol. of Solution D.

4. Centrifuge at approx 350,000 g_{av} for 1 h.

5. Unload the gradient by tube puncture, upward displacement with a dense medium or aspiration from the meniscus in 0.5-1 ml fractions. For more information on harvesting gradients see **Application Sheet S08**.

♦ A brief review of some of the publications that have used this methodology is given Section 5.4.

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. Organic osmotic balancers such as sucrose, mannitol and sorbitol were introduced for their compatibility in functional studies on subcellular membranes; moreover these low ionic strength HMs and gradient solutions permit the direct use of fractions for SDS-PAGE. Although 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and containing 1 mM EDTA is still a widely used HM, supplementation 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 (for example at 140 mM) 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. Some other examples are given in **Application Sheets S05 (tissues) and S06 (cells).**

Strategies for preparing working solutions (WSs) for mammalian tissues and cells are given in **Application Sheet S01**. These strategies were originally devised for simple HMs such as 0.25 M sucrose, 1 mM EDTA, 10 mM Hepes-KOH, pH.7.4. Diluting 5 vol. of OptiPrep™ with 1 vol. of 0.25 M sucrose, 6 mM EDTA, 60 mM Hepes-KOH, pH.7.4 produces a 50% iodixanol solution containing the same concentration of EDTA and buffer as the HM and more or less isoosmotic, thus when diluted with the HM all solutions will also contain these same concentrations and also be isoosmotic. Because OptiPrep™ itself behaves as an isoosomotic solution, it is unnecessary to raise the concentration of the osmotic balancer in the OptiPrep™ diluent. However inclusion of extra components at significantly higher concentrations (e.g. the 90 mM in Solution B) compromises the strategy slightly. The preparation of a 50% (w/v) iodixanol working solution (WS) from OptiPrep™ and Solution C ensures that when this is added to the PNS, the KOAc, $Mg(OAc)_2$ and buffer concentrations will not change but the osmolality will be raised slightly. Only experimentation will determine which is more important – keeping the KOAc concentration or the osmolality constant. Nürnberger et al [2] first prepared a 54% (w/v) iodixanol solution by diluting OptiPrep™ with $1/10^{th}$ vol. of 900 mM KOAc, 20 mM $Mg(OAc)_2$, 200 mM Hepes-KOH, pH 8.0

♦ Protease inhibitors may be included in any of the homogenization media and diluents at the operator's discretion.

5.2 Ultracentrifuge rotors and tubes

The manner in which iodixanol forms self-generated gradients and the rotor requirements for rapid formation of such gradients is described in ref 3 and also in **Application Sheet S04**. Other rotors that have been used for the analysis of membranes and cytosolic proteins are the small volume (3.3-3.9 ml tube volume) TLN100 near-vertical rotor [2] and also the MLN-80 (up to 8 ml) near-vertical rotor [4], both of which are accommodated into the Beckman Tabletop Ultracentrifuge. With Beckman rotors it is strongly recommended that Optiseal™ tubes be used for their ease of use, both when setting up and unloading the gradients.

5.3 Homogenization

The homogenization protocol should be tailored to the cell (or tissue) type. Potter-Elevhjem homogenization for tissues and Dounce homogenization for cells used to be the standard procedures. For cells use of 5-15 passages through a 27- or 25-gauge syringe needle, sometimes preceded by Dounce homogenization, is more 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. Some hints on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells).**

5.4 Analysis

Grindstaff et al [1] were able to demonstrate that the plasma membrane protein, E-cadherin banded almost exclusively in fractions 1-2 in the membranes at the top of the gradient in both contact-naive and differentiated MDCK cells. On the other hand the Sec6/8 complex changed its location from an exclusively cytosolic one in the contact-naive cells to a predominantly membrane-bound one in the differentiated cells (Figure 1). This protocol has also been used in the detection of membrane located Rac1:effector complexes [5,6] and cytosol located GFP tagged Sec3 fusion proteins [7] in MDCK cells.

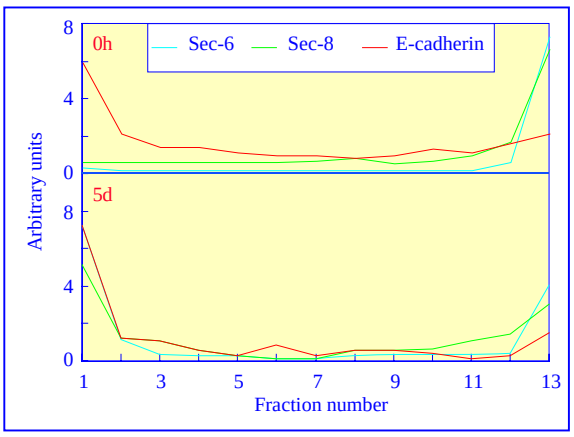

Figure 1 Analysis of post-nuclear supernatant (PNS) for Sec6/8 complex and E-cadherin in self-generated iodixanol gradients (low density of the left): Upper panel (0h) PNS from contact-naive MDCK cells; lower panel (5d) PNS from differentiated MDCK cells. Adapted from ref 1 with kind permission of the authors and Cell Press. See text for more information.

In a study of inversin complex formation with catenins and N-cadherin in polarized epithelial cells [2], immunoblotting of the gradient fractions with an antibody to inversin detected a 140 kDa protein distributed broadly in the gradient at a low level, while a 125 kDa protein was only detected in the low-density membrane fraction, along with pan-cadherin and β-catenin.

Clathrin coat components in Triton X-100 treated post-mitochondrial supernatants from stably transfected LLC-PK1 cells were virtually restricted to the cytoplasmic fraction, while from cells expressing transfected μ1B, clathrin components along with Sec8, Exo70 and the AP-1B cargo molecule TfnR were located in the low-density membrane fraction while the AP-1A cargo molecule furin remained in the high-density cytosol region [4].

♦ For other examples of the use of this methodology see refs 8-10.

6. References

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7. Acknowledgements

Axis-Shield PoC, AS wish to thank Dr Kent Grindstaff, Dr Charles Yeaman and Dr W James Nelson, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305-5345, for their kind cooperation in the preparation of this text.

Application Sheet S37; 8th edition, October 2016

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