OptiPrep™ Application Sheet S31

Analysis of plasma membrane domains and apical junctional complex from polarized epithelial cells in a self-generated gradient

- 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 **2020SMemapp** file and select the appropriate S-number.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5)

1. Background

The resolution of plasma membrane domains, primarily the basolateral and apical domains from polarized tissues such as intestine, liver and kidney and also from polarized cells such as human colon adenocarcinoma (Caco-2) cells and Madin-Darby canine kidney (MDCK) cells, is an important preliminary requirement for studies on how functional dichotomy at the cell surface is achieved. Methods often involve the use of divalent cations. Brush border preparations, from for example intestinal mucosa, treated with 10 mM $MgSO_4$, allow residual basolateral membrane and intracellular membranes to be removed by low-speed centrifugation [1]. The basolateral membranes are then prepared in a separate density gradient protocol. Ellis et al [2] used a modification of this procedure; basolateral and apical membranes from Caco-2 cells were separated in a sucrose gradient and 10 mM $MgCl_2$ was used to remove contaminating intracellular membranes from the basolateral domain band.

Musch et al [3] used the established divalent cation procedure to resolve the apical and basolateral domains of Caco-2 cells, but then used an iodixanol gradient to improve the subsequent purification of the basolateral domain: **see Application Sheet S29**. However ileal brush border and basolateral membranes from a total microsomal fraction can be well resolved in a single iodixanol gradient: **see Application Sheet S28**. Clearly there are important analytical advantages to the ability to separate the two domains in the same gradient. Moreover, the high resolving power of iodixanol gradients can achieve a separation of other plasma membrane microdomains that have hitherto been difficult to study with sucrose gradients. Biemesderfer et al [4] reported that shallow continuous iodixanol gradients are capable of resolving the microvillar and intermicrovillar domains from the renal brush border: **see Application Sheet S30**.

This Application Sheet describes the use of a self-generated gradient to analyze proteins in the apical and basolateral domains and the apical junctional complex from MDCK cells. Such gradients can be prepared simply by adjusting the sample (e.g. a post-nuclear supernatant) to a uniform concentration of iodixanol; transferring the suspension to a tube for a vertical or near-vertical rotor and centrifuging for 1-3 h at approx 350,000*g*. These gradients are highly reproducible, very simple to prepare and the absence of any interfaces contributes to a minimization of particle aggregation.

However if it is necessary to establish whether a protein might have a cytosolic or membrane location, the standard approach is to load the PNS in a dense solution beneath a discontinuous gradient so that the membranes float to their banding position while all of the soluble proteins and protein complexes (their density in iodixanol is >1.26 g/ml) remain in the load zone or sediment. For this methodology **see Application Sheet S35**. This strategy has now been combined with that of a self-generated gradient. The post-nuclear supernatant is adjusted to 30% (w/v) iodixanol and overlaid with equal volumes of 20% and 10% iodixanol and centrifuged in a vertical or near-vertical rotor [5-7]. Some variations on this format are described in Section 5. This discontinuous gradient format has one other interesting property; the density profile that is generated by a combination of diffusion and self-generation is more closely linear in the top 90% of the gradient volume than with the single concentration format. For information on the latter **see Application Sheet S04**.

2. Solutions required (**see Section 5.1**)

A. OptiPrep™

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

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:

500 mM Hepes (free acid)	11.9 g per 100 ml water
2 M KOAc	19.6 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 8 ml, 9 ml and 4 ml of Hepes, KOAc and $Mg(OAc)_2$ stocks respectively; adjust to pH 8.0 with KOH and make up to 200 ml.

Solution C: Mix 24 ml, 27 ml and 12 ml of Hepes, KOAc and $Mg(OAc)_2$ stocks respectively; adjust to pH 8.0 with KOH and make up to 100 ml.

3. Ultracentrifuge 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 refs 5-7)

Carry out all operations at 0-4°C. All iodixanol concentrations are given as % (w/v).

1. Wash the cell monolayer twice with Solution B and scrape the cells into the same solution.

2. Pellet the cells at 600 *g* for 10 min and resuspend them in 1-3 ml of Solution B.

3. Homogenize the cells in a cell cracker (ball-bearing homogenizer) using 4-10 passages or pass the suspension through a 22G needle followed by a 26G needle (six times through each). Monitor the efficacy of the homogenization by phase contrast microscopy (**see Section 5.3**).

4. Centrifuge the homogenate at 3000 *g* for 10 min to pellet the denser mitochondria, nuclei and cell debris (**see Section 5.4.1**).

5. Aspirate the 3000 *g* supernatant and adjust it to 30% (w/v) iodixanol by mixing with Solution D, volume ratio of 2:3 (**see Section 5.4.2**).

6. Make up solutions of 10%, 20% (w/v) iodixanol by mixing Solution D with Solution B, volume ratios of 1:4 and 2:3 respectively (**see Section 5.4.2**).

7. Transfer 3.75 ml of the three iodixanol solutions to an Optiseal™ tube (11.2 ml) for the vertical or near-vertical rotor by under- or over-layering (**see Section 5.5**).

8. Centrifuge at 353,000 g_{av} for 3 h using a slow acceleration program.

9. Allow the centrifuge to decelerate to rest from 2000 rpm without the brake or use a slow deceleration program.

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 from sealed tubes **see Application Sheet S08**.

♦ For information on analysis of the gradient fractions **see Section 5.6**

5. Technical Notes and Review

5.1 Homogenization media

The homogenization medium (HM) 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.

Supplementation of the HM with inorganic salts (containing K^+ or Na^+ ions) 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. The 0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0 described in this protocol was also used by Yeaman et al [8] for NRK-49F and NRK-52E rat kidney cells.

Solutions are buffered with Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and it is unlikely if the type of buffer significantly influences the fractionation, although triethanolamine does seem to offer some advantages in homogenization efficiency. Other examples of homogenization media are given in **Application Sheet S06**.

The preparation of a Working Solution as described, ensures that the concentrations of KOAc, $Mg(OAc)_2$ and buffer are constant throughout the gradient, while the sucrose and iodixanol act as osmotic balancers to maintain an approx. constant osmolality. If this is deemed unimportant the iodixanol solutions may be prepared simply by diluting OptiPrep™ with Solution B. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

In some cases the surface proteins are cross linked by dithiobis(succinimidylproprionate) prior to homogenization [5-7]. Protease inhibitors may be included in Solutions B and C at the operator's discretion.

5.2 Ultracentrifuge rotors

Other rotors with different sedimentation path lengths may be suitable but the optimal centrifugation conditions will require investigation; only vertical, near-vertical or low-angle small volume fixed-angle rotors can normally be used for self-generated gradients. For more information see **Application Sheet S04**.

5.3 Homogenization

Dounce (or sometimes Potter-Elvehjem) homogenization was the most widely used procedure at one time but the ball-bearing homogenizer or "cell cracker", with the standard 0.3747 in (9.52 mm) ball bearing, is now regarded as one of the most effective and reproducible of devices. If this is not available however 10-20 passages through a syringe needle (the Gauge Number (G) varies from 21 to 26) is usually an efficient alternative. Sometimes, as in this protocol, the efficacy of this method is improved by switching to a second finer syringe needle for half the passes. Occasionally use of a syringe needle is prefaced by Dounce homogenization.

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 other hints on homogenization are given in **Application Sheet S06**.

5.4 Differential centrifugation

5.4.1 Removal of nuclei

Nuclear pelleting may be carried out at 500-3000g for 5-10 min; the higher RCFs (*g*-forces) resulting in removal of some of the mitochondria. 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 3000*g* supernatant is inconveniently large, then the microsomes may need pelleting at 100,000*g* for 45 min and resuspension in a total volume of 1.5 ml of Solution B before adjusting to 30% (w/v) iodixanol. Note that most of the cytosolic proteins will be removed in the 100,000*g* supernatant. Yeaman [6] also suggested an alternative gradient made up from 10%, 15%, 20%, 25% and 30% (w/v) iodixanol, in which case the 3000*g* supernatant or the resuspended microsomes need to be adjusted to 35% (w/v) iodixanol. This format was better at resolving very slowly sedimenting low MWt proteins from the floating denser membranes.

The 10% and 20% iodixanol layers may also be prepared by mixing the 3000*g* supernatant with Solution D; this permits a greater sample loading per gradient but resolution of the membrane compartments from the smaller soluble proteins is probably less clear [6,8].

5.5 Setting up the gradient

Although underlayering with a syringe and metal cannula is the recommended method for making discontinuous gradients, overlayering maybe more convenient since the tubes need to be filled exactly to the bottom of the neck. For more information on gradient construction **see Application Sheet S03**. If necessary, adjust all volumes proportionately so that tubes are properly filled according to the manufacturer's instructions.

5.6 Analysis

Figure 1 describes the resolution of the apical and basolateral domains of MDCK cells. Using E-cadherin as a basolateral marker and gp135 as an apical marker the gradient was clearly very effective at resolving these domains [6]. The major bands of E-cadherin and gp135 from MDCK cells were separated by as much as 0.1 g/ml. Na^+/K^+ ATPase co-banded with the E-cadherin [7]. Note however that the major E-cadherin band from the epithelial-like NRK-52E cells was found at a much lower density than that from MDCK cells [9] emphasizing the difference in plasma membrane density that may be found when these gradients are used to fractionate the membrane compartments from different cell types. Each cell type may have its own distinctive membrane banding patterns.

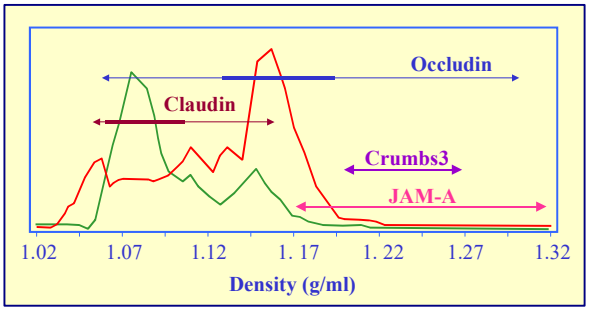

Figure 1 Distribution of markers from MDCK cells in self-generated iodixanol gradient: gp135 (—), E-cadherin (—), thickness of lines (occludin and claudin) indicates concentration of labeling; data adapted from refs 6 and 7, for more information see text.

Vogelmann and Nelson [7] found that occludin was widely distributed through the gradient but it was most heavily concentrated in the basolateral domain region of the gradient (Figure 1), while claudin was predominantly in the apical domain region. JAM-A and crumbs3 were confined to denser regions of the gradient. If however the densest region of the gradient is harvested; diluted to 10% (w/v) iodixanol; made part of a second 10%, 20%, 30% iodixanol gradient and re-centrifuged under the same conditions, the JAM-A now bands in the middle of the gradient and can be completely resolved from the much more densely-banding crumbs3 [7]. This may indicate that both JAM-A and crumbs3 reside

in particles that are denser than those of the basolateral or apical domains, but the JAM-A containing particles are much smaller.

This gradient system displays much finer resolving powers than is suggested by Figure 1. For example although the figure suggests occludin was widely distributed but mainly confined to the basolateral domain, its distribution actually revealed three distinct peaks [7]. Vogelmann and Nelson [7] used the gradient, in association with immunofluorescence microscopy, to reassess the distribution of a variety of proteins at the surface of MDCK cells and to study the temporal role of the epithelial apical junctional complex in development of surface polarity. Yeaman [6] compared the translocation and localization of the Sec6/8 complex in contact-naïve and polarized MDCK cells, while Amieva et al [5] used the gradient system to separate fractions containing intercellular junctions, apical and basolateral domains and cytosol from MDCK cells, in their study of the disruption of the epithelial cell apical-junctional complex by the CagA protein from *Helicobacter pylori.* In the study by Gromley et al [10] of centriolin anchoring of exocyst and SNARE complexes in MDCK cells, centriolin was mostly associated with membranes and furthermore co-fractionated with a fraction of Sec8 that was slightly less dense than the junction-associated peak. A clear separation of basolateral and apical domains from Golgi in these gradients showed the importance of the coupling of microtubules to post-Golgi trafficking in polarized cells [11]. Wang et al [12] increased the centrifugation time to 4h in their studies on E-cadherin trafficking. Using the alternative sample loading format in which the 3000 *g* supernatant is mixed into all three of the iodixanol layers, Yeaman et al [8] studied the redistribution of occludin and Sec8 during the induction of Ca^{2+} dependent cell adhesion of MDCK cells.

Self-generated iodixanol gradients have also been used to study the association of Par complex proteins with the tight junction of MDCK cells [13] and in the identification of occludin-containing vesicles from the medium surrounding the basolateral membrane [14]

- A methodological review by Vogelmann and Nelson [15] provides an excellent description of the use of these iodixanol gradients in combination with a variety of other techniques in the study of membrane trafficking and exocytosis.

5.7 Matrix adherence

Gerl et al [16] used a novel method for capturing the free-floating apical membranes from MDCK cells grown on permeable supports. They were overlaid with semidry filter paper (Whatman 3-MM) for 10 min and then rehydrated for 30 min in 150 mM ammonium bicarbonate and the surface scraped with a cell scraper. Supernatants, which contained membranes and filter paper remnants were underlaid with pure OptiPrep™ and centrifuged for 3 h at 160,000 *g*, in 13 ml tubes (e.g. Beckman SW 41Ti). The bulk of the supernatant was removed, leaving 1 ml and the membranes , which banded at the interface were harvested in the remaining 1 ml plus 2 ml of the OptiPrep™ (final iodixanol concentration of 40% (w/v) iodixanol) and overlaid with 30% iodixanol in ammonium bicarbonate and then with ammonium bicarbonate. After centrifugation for 90 min at 280,000 *g*, the membranes were harvested from the 30% iodixanol/ammonium bicarbonate interface.

5.8 Other plasma membrane domains

Bandyopadhyay et al [17] were able to resolve, from HepG2 cells homogenized in a Brij-containing solution, light and dense plasma membrane domains in a 2.5%, 10%, 20%, 30% iodixanol gradient (125,000 *g* for 12h). The light domain was enriched in Thy-1 (a GPI-anchored protein), while Lyn and glyceraldehyde phosphate dehydrogenase were in the denser domain.

6. References

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