OptiPrep™ Application Sheet S28

Fractionation of brush border and basolateral plasma membrane domains from intestinal (ileal) mucosa

- 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

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 isolation of the basolateral domain from intestinal mucosa cells, originally carried out in a variety of sucrose gradients, was transferred to Percoll® gradients by Scalera et al [2] and used subsequently by other workers, e.g. Cohen et al [1]. Distribution of the basolateral membrane within the Percoll® gradient is however inconveniently broad, the band extending over more than 80% of the gradient volume and, as with all Percoll® gradients, it is necessary to remove the colloidal silica before any analysis can be carried out. Moreover isolation of the brush border domain had to be carried out separately using the divalent cation precipitation technique. Although this is less of a problem for preparative work, for analytical work it is much less suitable.

Iodixanol gradients are however now being increasingly used to provide the high resolution necessary to purify both the apical and basolateral membranes away from other intracellular membranes, using a total membrane fraction sedimented from an homogenized ileal cell post-nuclear supernatant; thus permitting the use of a single separation technique [3].

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Homogenization buffer: 2 mM DTT, 1 mM EGTA/1.006 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5

C. OptiPrep™ dilution buffer: 150 mM NaCl, 50 mM NaF, 20 mM Hepes-NaOH, pH 7.2

3. Ultracentrifuge rotor requirements (See Section 5.2)

Swinging-bucket rotor with 13-14 ml tubes (e.g. Beckman SW41Ti, Sorvall TH641 or similar)

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
200 mM DTT: 3.08 g per 100 ml water
100 mM EGTA: 3.80 g per 100 ml water (pH 11-12)
100.6 mM $CaCl_2 \bullet 2H_2O$: 1.478 g per 100 ml water
1 M Hepes (free acid): 23.8 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water
500 mM NaF: 2.1 g per 100 ml water

Solution B: To 100 ml water add 2 ml each of DTT, EGTA and $CaCl_2$ stock solutions and 4 ml of Tris stock solution; adjust to pH 7.5 with 1 M HCl and make up to 200 ml.

Solution C: To 50 ml water add 15 ml, 10 ml and 2 ml respectively of NaCl, NaF and Hepes stock solutions; adjust to pH 7.2 with 1 M NaOH and make up to 100 ml

4. Protocol (adapted from refs 1 and 3)
Carry out all operations except Step 1 at 0-4°C.

1. Incubate the ileal segments in gassed Ringer's-bicarbonate buffer at 37°C as required.

2. Place the segments on a chilled glass plate and scrape off the mucosa gently with a glass slide.

3. Transfer the mucosa to a small beaker and pour on 10 vol. of Solution B and place in ice.

4. Homogenize using a Polytron™ homogenizer using 10 x 10 sec bursts, each burst being separated by a 20 sec rest. During the rest periods swirl the contents of the beaker in the ice to ensure efficient cooling (**see Section 5.3**).

5. Centrifuge the homogenate 3000 *g* for 5 min to pellet unbroken cells, nuclei and debris.

6. Carefully decant the supernatant repeat the centrifugation.

7. Centrifuge the supernatant from step 6 at 160,000 *g* for 1 h. Carry out Steps 8 and 9 during the centrifugation.

8. Prepare solutions of 10%, 12.5%, 15%, 17.5%, 20%, 22.5%, 25%, 27.5% and 30% (w/v) iodixanol by diluting OptiPrep™ with Solution C (**see Section 5.1**).

9. Using a syringe and metal cannula layer 1.25 ml of each solution in tubes for the swinging-bucket rotor (**see Section 5.4.1**).

10. Resuspend the pellet from Step 7 in Solution C and layer approx. 1.3 ml on top of each gradient, to fill the tube (**see Section 5.4.2**).

11. Centrifuge at approx 64,000 g_{av} for 90 min; allow the rotor to decelerate without the brake or use a slow deceleration program.

12. Unload the gradient in approx. 0.6 ml fractions using upward displacement, tube puncture or aspiration from the meniscus. For more information on unloading gradients **see Application Sheet S08**.

♦ **For details of the expected separation of the apical and basolateral domains Section 5.5**.

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

Additional protease inhibitors may be included in Solutions B and C at the operator's discretion. Preparation of the density gradient solutions by diluting OptiPrep™ with Solution C will mean that the concentration of NaCl, NaF and Hepes will fall as the iodixanol concentration increases. If this is deemed undesirable then first dilute 5 vol. of OptiPrep™ with 1 vol. of a solution containing 150 mM NaCl, 300 mM NaF, 120 mM Hepes-NaOH, pH 7.2 and then dilute this 50% (w/v) iodixanol Working Solution with Solution C to prepare the density gradient solutions. The NaF and Hepes concentrations will then be constant throughout the gradient. If the OptiPrep™ diluent also contains 6x150 mM NaCl, all the density solutions will be grossly hyperosmotic, which would probably be undesirable. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

Other homogenization media have been used: Scalera et al [2] used a simple one of 0.25 M sucrose, 10 mM triethanolamine-HCl, pH 7.6

5.2 Ultracentrifuge rotors

These separations have been performed in 13 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. All volumes should be scaled up or down proportionately. Note however that the progressive change in gradient density profile (due to diffusion of the iodixanol molecules) may also be modulated in other rotors and affect the final resolution.

5.3 Homogenization

Although the Polytron is the homogenizer of choice, less sophisticated rotating blades homogenizers such as the Waring blender have also been used [2].

5.4 Density gradients

5.4.1 Construction

Discontinuous gradients are normally most easily prepared by underlayering (i.e. low density first) using a syringe (1-2 ml) and a long metal cannula); overlayering small volumes is more difficult using either a syringe or Pasteur pipette. One alternative for overlayering the small volumes used in this protocol is to use a small volume (low-pulsating) peristaltic pump; first to take up the required volume of solution into the attached tubing and second, to reverse the flow, in order to expel it slowly on to a denser layer in the centrifuge tube. For more information on gradient construction **see Application Sheet S03**.

5.4.2 Tube loading

In swinging-bucket rotors of different tube volumes scale up or down the volumes proportionately. The separation achieved in this protocol is probably based at least partly on sedimentation velocity, so the sample volume should never be more than 10% of the gradient volume. If necessary, adjust all volumes proportionately so that tubes are properly filled according to the manufacturer's instructions.

5.5 Gradient analysis

If it is necessary to concentrate a fraction or to remove the iodixanol before analysis, **see Application Sheet S09**.

In this gradient system, the banding of the apical and basolateral domains of the ileal mucosal cell plasma membrane is shown in Figure 1. The apical domain was identified using sucrase as a marker and the basolateral marker was Na^+/K^+ ATPase; EEA1 was used as an early endosomes marker. Li et al [3,4] were able to demonstrate that the Na^+-H^+ exchanger (NH3) was localized to the apical domain and to the early endosomes. More recently the Ser/Thr kinase Akt has also been localized principally to the apical domain [5]. The gradient has also been used for the fractionation of Caco-2 cell membranes [5]; for more information **see Application Sheet S29**.

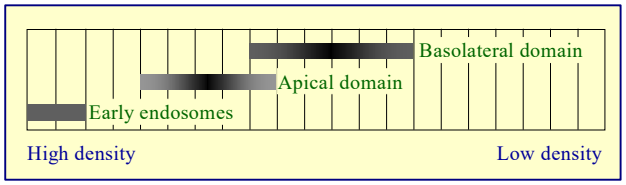

Figure 1 Approx. disposition of rabbit ileal subcellular membrane markers in the iodixanol gradient. Data adapted from ref 3

The gradient may be applicable to the resolution of basolateral and apical plasma membrane domains from other tissues and cells but this can only be determined experimentally.

- The OptiPrep™ method has been applied to ileal cells from rabbit [3-6], mouse jejunal mucosal cells [7], mouse ileal cells [8] and frozen human jejunal specimens [9].
- Excellent methodological reviews are provided in refs 6 and 10
- Refs 11 and 12 review the function of the Na/H exchanger NH3 from data obtained in OptiPrep™ fractionations
- Ref 13 reviews proteomic analysis associated with inflammatory bowel disease

6. References

1. Cohen, M.E., Wesolek, J., McCullen, J., Rys-Sikora, K., Pandol, S., Rood, R.P., Sharp, G.W.G. and Donowitz, M. (1991) *Carbachol- and elevated Ca^{2+}-induced translocation of functionally active protein kinase C to brush borders of rabbit ileal Na^+ absorbing cells* J. Clin. Invest., **88**, 855-863
2. Scalera, V., Storelli, C., Storelli-Joss, C., Haase, W. and Muer, H. (1980) *A simple and fast method for the isolation of basolateral plasma membranes from rat small intestinal epithelial cells* Biochem. J., **186**, 177-181
3. Li, X., Galli, T., Leu, S., Wade, J.B., Weinman, E.J., Leung, G., Cheong, A., Louvard, D. and Donowitz, M. (2001) *Na^+-H^+ exchanger 3 (NHE3) is present in lipid rafts in the rabbit ileal brush border: a role for rafts in trafficking and rapid stimulation of NHE3* J. Physiol., **537**, 537-552
4. Li, X., Zhang, H., Cheong, A., Leu, S., Chen, Y., Elowsky, C.G. and Donowitz, M. (2004) *Carbachol regulation of rabbit ileal brush border Na^+-H^+ exchanger 3 (NHE3) occurs through changes in NHE3 trafficking and complex formation and is Src dependent* J. Physiol., **556.3**, 791-804
5. Li, X., Leu, S., Cheong, A., Zhang, H., Baibakov, B., Shih, C., Birnbaum, M.J. and Donowitz, M. (2004) *Akt2, phosphatidylinositol 3-kinase, and PTEN are in lipid rafts of intestinal cells: role in absorption and differentiation* Gastroenterol., **126**, 122-135
6. Li, X. and Donowitz, M. (2008) *Fractionation of subcellular membrane vesicles of epithelial and nonepithelial cells by OptiPrep™ density gradient ultracentrifugation* In Methods Mol. Biol., **440**, Exocytosis and Endocytosis (ed. Ivanov, A.I.) Humana Press, Totowa, NJ, pp 97-110
7. Tobin, V., Le Gall, M., Fioramonti, X., Stolarczyk, E., Blazquez, A.G., Klein, C., Prigent, M., Serradas, P., Cuif, M.H., Magnan, C., Leturque, A., and Brot-Laroche, E. (2008) *Insulin internalizes GLUT2 in the enterocytes of healthy but not insulin-resistant mice* Diabetes, **57**, 555-562
8. Sullivan, S., Alex, P., Dassopoulos, T., Zachos, N.C., Iacobuzio-Donahue, C., Donowitz, M., Brant, S.R., Cuffari, C., Harris, M.L., Datta, L.W., Conklin, L., Chen, Y. and Li, X. (2009) *Down-regulation of sodium transporters and NHERF proteins in IBD patients and mouse colitis models: potential contributors to IBD-associated diarrhea* Inflamm. Bowel Dis., **15**, 261–274
9. Ait-Omar, A., Monteiro-Sepulveda, M., Poitou, C., Le Gall, M., Cotillard, A., Gilet, J., Garbin, K., Houllier, A., Château, D., Lacombe, A., Veyrie, N., Hugol, D., Tordjman, J., Magnan, C., Serradas, P., Clément, K., Leturque, A. and Brot-Laroche, E. (2011) *GLUT2 accumulation in enterocyte apical and intracellular membranes a study in morbidly obese human subjects and ob/ob and high fat–fed mice* Diabetes **60**, 2598–2607
10. Li, X. and Donowitz, M. (2014) *Fractionation of subcellular membrane vesicles of epithelial and non-epithelial cells by OptiPrep™ density gradient ultracentrifugation* In Exocytosis and Endocytosis, Methods in Molecular Biology, **1174** (ed. Ivanov, A,I.) Springer Science+Business Media New York 2014, pp 85-99
11. Donowitz, M. and Li, X. (2007) *Regulatory binding partners and complexes of NHE3* Physiol. Rev., **87**, 825-872
12. Donowitz, M., Mohan, S., Xinjun Zhu, C., Chen, T-E., Lin, R., Cha, B., Zachos, N.C., Murtazina, R., Sarker, R. and Li, X. (2009) *NHE3 regulatory complexes* J. Exp. Biol., **212**, 1638-1646
13. Alex, P., Gucek, M. and Li, X. (2009) *Applications of proteomics in the study of inflammatory bowel diseases: current status and future directions with available technologies* Inflamm. Bowel Dis., **15**, 616–629

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