OptiPrep™ Application Sheet S29

Fractionation of apical and basolateral plasma membrane domains from Caco-2 cells

- 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 (PM) 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]. 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.

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. This Application Sheet describes the use of $CaCl_2$ to separate the apical domain from the basolateral domain + endomembranes from a post-mitochondrial supernatant of Caco-2 cells and then subsequently an iodixanol gradient to resolve the basolateral domain from endomembranes [3]. Methods for the resolution of some other plasma membrane domains may be accessed via the Index.

2. Solutions required (see Section 5.1)

A. OptiPrep™
B. Phosphate-buffered saline (PBS)
C. Homogenization buffer: 3 mM EDTA, 10 mM Tris-HCl, pH 7.4
D. OptiPrep™ dilution buffer: 18 mM EDTA, 60 mM Tris-HCl, pH 7.4
E. Working Solution (50% w/v iodixanol): mix 5 vol. of Optiprep™ with 1 vol. of Solution D
F. Ca^{2+} solution: 100 mM $CaCl_2$

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
100 mM EDTA (Na_2•$2H_2O$): 3.72 g per 100 ml water
100 mM $CaCl_2$•$2H_2O$: 1.47 g per 100 ml water

Solution C: To 50 ml water add 3 ml of EDTA and 1 ml of Tris stock solutions; adjust to pH 7.4 with 1 M HCl and make up to 100 ml.

Solution D: To 50 ml water add 18 ml of EDTA and 6 ml of Tris stock solutions; adjust to pH 7.4 with 1 M HCl and make up to 100 ml.

3. Ultracentrifuge rotor requirements (see Section 5.2)

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

4. Protocol (adapted from ref 3)

Carry out all operations except Step 1 at 0-4°C.

1. Grow Caco-2 cells on permeable supports as required.

2. Scrape off the cells from the filter using a policeman into ice-cold PBS.

3. Pellet the cells at 600 *g* for 10 min (or 20 sec in a microfuge) and wash the cells once with PBS.

4. Suspend the cells in 5 ml of Solution C and homogenize in a tight-fitting Dounce (glass-glass) or Potter-Elvehjem (Teflon-glass) homogenizer (**see Section 5.3**).

5. Centrifuge the homogenate 1000 *g* for 5 min to pellet unbroken cells, nuclei and debris (see Section 5.4).

6. Carefully aspirate the supernatant and centrifuge it at 10,000 *g* to pellet the mitochondria and most of the lysosomes and peroxisomes.

7. Carefully aspirate the supernatant; add 1/10th of the volume of Solution F and leave on ice for 30 min.

8. During the incubation on ice prepare solutions of 40%, 30% and 5% (w/v) iodixanol by diluting Solution E with Solution C at volume ratios of 4:1, 3:2 and 1:9 respectively (**see Section 5.1**).

9. Centrifuge the suspension (Step 7) at 10,000 *g* for 15 min.

10. Aspirate and retain the supernatant and resuspend the pellet containing the basolateral PM domain in 1-2 ml of 40% iodixanol.

11. Pellet the apical PM domain from the supernatant at 100,000 *g* for 30 min.

12. During this centrifugation prepare 11-12 ml continuous gradients from equal volumes of the 30% and 5% (w/v) iodixanol solutions using either a two chamber gradient maker or a Gradient Master™ in tubes for the swinging-bucket rotor (**see Section 5.5.1**).

13. Using a syringe and metal cannula underlay the gradient with the basolateral PM domain suspension from Step 10 (**see Section 5.5.2**).

14. Centrifuge the gradients at approx. 165,000 g_{av} for 3 h and allow the rotor to decelerate without the brake below 2000 rpm or use a controlled deceleration program.

15. Unload the gradient in 0.5-1.0 ml fractions using upward displacement, tube puncture or aspiration from the meniscus. For more information on unloading gradients **see Application Sheet S08**; for more information on gradient analysis **see Section 5.6**.

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 described in this Application Sheet. 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. The most commonly used isoosmotic HMs contain 0.25 M sucrose buffered either with 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). Some other examples of homogenization media for cultured cells are given in **Application Sheet S06**.

Often however, as in this protocol, a frankly hypoosmotic medium is used to swell the cells and so facilitate homogenization. This may be important to the plasma membrane domain isolation but it may

also cause some organelles such mitochondria and lysosomes to fragment. Only experimentation can determine if other homogenization media are permissible with Caco-2 cells or with other polarized cells.

By using the strategy of first preparing a 50% (w/v) iodixanol Working Solution (Solution E) containing 3 mM EDTA, 10 mM Tris-HCl, pH 7.4, the concentration of EDTA and Tris will be constant throughout the gradient. This will not be the case if the gradient solutions are simply prepared by diluting OptiPrep™ with Solution C. Strategies for preparing working solutions (WSs) for mammalian tissues and cells are given in **Application Sheet S01**.

- Protease inhibitors may be added to Solutions C and D at the operator's discretion.

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 Musch et al [3] used 20 strokes of the pestle of a tight fitting Potter-Elvehjem homogenizer this may not be the only homogenization protocol that is valid. Moreover the method may need customizing to other polarized cells. Dounce and Potter-Elvehjem homogenization were the most widely used procedures at one time but the ball-bearing homogenizer ("cell cracker") 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 25) is usually an efficient alternative. 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 hints on homogenization are given in **Application Sheet S06**.

5.4 Differential centrifugation

Musch et al [3] omitted this step and centrifuged the homogenate directly at 10,000*g*. Nuclei in particular will sediment very rapidly under these conditions and this may lead to entrapment and loss of smaller particles.

5.5 Density gradients

5.5.1 Construction

If neither of these gradient-making devices is available then a continuous gradient can be prepared by diffusion of a discontinuous gradient (use equal volumes of 5%, 10%, 15%, 20%, 25% and 30% (w/v) iodixanol). For more information on gradient construction **see Application Sheet S03**.

5.5.2 Tube loading

In swinging-bucket rotors of different tube volumes scale up or down the volumes proportionately. If necessary, adjust all volumes (also proportionately) so that tubes are properly filled according to the manufacturer's instructions.

5.6 Gradient analysis

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

The basolateral membrane bands in the 10-15% (w/v) iodixanol region [4], this is at slightly higher density than that reported for in other plasma membrane fractionation gradients (5-10% iodixanol) but it may reflect the binding of the $CaCl_2$, unique to this type of isolation.

- An excellent review of the iodixanol methodology as applied to epithelial cells is given in ref 5.

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. Ellis, J.A., Jackman, M.R. and Luzio, J.P. (1992) *The post-synthetic sorting of ensogenous membrane proteins examined by the simultaneous purification of apical and basolateral plasma membrane fractions from Caco-2 cells* Biochem. J., **283**, 553-560
3. Musch, M.W., Walsh-Reitz, M.M. and Chang, E.B. (2006) *Roles of ZO-1, occludin, and actin in oxidant-induced barrier disruption* Am. J. Physiol. Gastrointest. Liver Physiol., **290**, 222-231
4. Musch, M.W. (2006) *Personal communication*
5. 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

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