OptiPrep™ Application Sheet S33

Purification of lipid rafts from cells and tissues (detergent-free method)

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **OptiPrep™ Mini-Review (MS09)** "The purification of lipid rafts (detergent-free method) – a bibliography" lists all published papers reporting the use of OptiPrep™: to access return to the initial list of Folders and select "**Mini-Reviews**". **The references are divided into cell or tissue type and also highlight the analytical content**.
- 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 and information regarding methodological variations and analysis are contained in the "Technical Notes" section (Section 5)

1. Background

The importance of lipid-rich microdomains of the plasma membrane in signal-transduction events, in lipid transport, in various internalization processes and in the regulation of plasma membrane-cytoskeleton interactions have become well established over the last six years. A number of important cholesterol and sphingolipid-rich structures have been identified and studied, notably caveolae and lipid rafts. The isolation of caveolae using OptiPrep™ is the subject of **Application Sheet S34**.

Isolation of the two types of lipid-rich particle developed along quite distinct lines. Caveolae were purified from a plasma membrane fraction after sonication by sequential flotation in continuous and discontinuous iodixanol gradients (e.g. refs 1 and 2). Lipid rafts on the other hand were isolated, also by flotation through a discontinuous iodixanol gradient, as detergent resistant membranes (e.g. refs 3 and 4), generally from a post-nuclear supernatant (PNS). Usually the PNS and gradient contained Triton X100 as the detergent, but sometimes other detergents such as CHAPS or Brij were used. Lipid rafts, which like caveolae have a relatively low density, float away from detergent soluble proteins and detergent-insoluble cytoskeleton-associated proteins, which remain in the load zone. The method is described in the companion **Application Sheet S32**.

Both methodologies have been widely applied and both appear in a huge number of publications. The sonication method is rather lengthy and yields are rather poor. The use of detergents may however introduce artifacts, notably the coalescence of smaller lipid domains. In spite of this, the detergent-containing iodixanol gradients have been able to resolve lipid-rich domains of different densities (e.g. refs 5 and 6). In 2005 Macdonald and Pike [7] introduced a detergent-free flotation method, which incorporated a continuous iodixanol gradient very similar to that used by Smart et al [1], but used a PNS as the source rather than a purified plasma membrane fraction. The following protocol for monolayer-grown cells is adapted from ref 7.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Isolation medium: 0.25 M sucrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.8

C. OptiPrep™ diluent: 0.25 M sucrose, 120 mM Tris-HCl, pH 7.8

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

E. Working Solution diluent: 0.25 M sucrose, 20 mM Tris-HCl, pH 7.8

F. Phosphate-buffered saline (PBS)

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
100 mM $MgCl_2 \bullet 6H_2O$: 2.03 g per 100 ml water
100 mM $CaCl_2 \bullet 2H_2O$: 1.47 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml of water; add 4 ml of Tris stock and 2 ml each of the $MgCl_2$ and $CaCl_2$ stock solutions; adjust to pH 7.8 with 1 M HCl and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 50 ml of water; add 12 ml of Tris stock solution; adjust to pH 7.8 with 1 M HCl and make up to 100 ml.

Solution E: As Solution B minus $MgCl_2$ and $CaCl_2$

3. Ultracentrifuge rotor requirements (**see Section 5.2**)
Swinging bucket rotor for an ultracentrifuge with a tube volume of approx 13 ml (e.g. Beckman SW41Ti or Sorvall TH641)

4. Protocol
Carry out all operations, except the PBS washes in Step 3, at 0-4°C

1. Prepare a 20% (w/v) solution of iodixanol from 2 vol. of Solution D and 3 vol. of Solution E.

2. In tubes for the swinging-bucket rotor prepare 8-9 ml gradients from equal volumes of the 20% iodixanol and Solution B using a two-chamber gradient maker or Gradient Master™ (**see Section 5.3**) and keep at 4°C.

3. Wash the cell monolayer once in PBS and twice Solution B; then scrape the cells into Solution B.

4. Pellet the cells at 250 *g* for 2 min and then resuspend the cell pellet in 1 ml of Solution B.

5. Homogenize the cells by 20 passages through a 22G syringe needle (**see Section 5.4**)

6. Centrifuge the homogenate at 1000 *g* for 10 min.

7. Carefully aspirate and retain the supernatant.

8. Resuspend the pellet in 1 ml of Solution B and repeat the homogenization by repeated passage through the 22G syringe needle.

9. Centrifuge the suspension at 1000 *g* for 10 min.

10. Aspirate the supernatant and mix the combined supernatants with an equal volume of Solution D.

11. Using a syringe and metal cannula underlayer the gradient with the dense sample to fill the tube (**see Section 5.3**).

12. Centrifuge at 52,000 *g* for 90 min (**see Section 5.5**).

13. Collect the gradient in approx 0.5-0.75 ml fractions by tube puncture, upward displacement with a dense medium or aspiration from the meniscus and analyze the fractions as required (**see Sections 5.6 and 5.7**).

5. Technical Notes
5.1 Homogenization media and gradient solutions

The solutions, as described, were used by Macdonald and Pike [7] and Pike et al [8] for CHO cells. The same strategy was also used for HeLa cells [7]. Detergent-free lipid raft fractions have also been prepared from renal brush border membranes [9] and from neuroblastoma cells [10] homogenized in Tris-buffered 150 mM NaCl, 5 mM EDTA. The relevance of the type of homogenization medium to the efficacy of the technique is not known.

The preparation of a Working Solution as described, ensures that the concentration of buffer is constant throughout the gradient, while the sucrose and iodixanol act as osmotic balancers to maintain an approx. constant osmolality. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

Protease inhibitors such as PMSF, leupeptin, antipain, aprotinin etc should be included in all of the media.

5.2 Ultracentrifuge rotors

The method can probably be scaled down to smaller volume swinging-bucket rotors, but this will need experimental verification. Since the *g*-force and time are both quite modest, the resolution may in part be based on rate of flotation, if this is so then shorter tubes may require shorter times.

5.3 Forming the gradient and loading the sample

If neither of these devices is available then a continuous gradient may be prepared from diffusion of a discontinuous gradient. Equal volumes of Solution B and 5%, 10%, 15% and 20% iodixanol would be suitable. For more information on gradient construction see **Application Sheet S03**. In the non-detergent preparation of rafts from brush border membranes [9] and neuroblastoma cells [10] the gradients were discontinuous rather than continuous.

Formation of discontinuous gradients and underlayering the gradient with the sample are best achieved using a syringe with metal cannula (i.d. approx 0.8 mm). Metal filling cannulas can be obtained from most surgical instrument supply companies. Make sure that the tubes are filled in accordance with the manufacturer's instructions.

5.4 Homogenization

For some suspension culture cells it may be necessary to use a smaller gauge needle (27G or 25G). The ball-bearing homogenizer ("cell cracker") is now widely regarded as one of the most effective and reproducible of devices. Choice of a suitable device will require experimentation. Brush border membranes were fragmented using a Dounce homogenizer [9]. Some hints on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells)**.

5.5 Centrifugation conditions

Centrifugation times and *g*-forces used by other workers have generally been more severe: 170,000 *g* for 4 h [9] or 200,000 *g* for 18 h [10].

5.6 Gradient analysis

For more information on harvesting gradients **see Application Sheet S08**. Always check on the distribution of raft and non-raft markers in the gradient to confirm that the centrifugation has achieved a satisfactory resolution and recovery of rafts. Once the pattern of marker banding in the gradient has bee confirmed as being reproducible, then it may be possible to retrieve the raft and other fractions using a syringe and metal cannula or an automatic pipette.

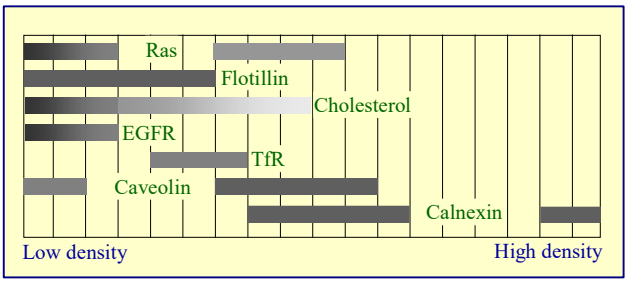

Figure 1 Approx. distribution of markers in iodixanol gradient. Data adapted from ref 7, for more information see text. EGFR = epidermal growth factor receptor, TfR = transferrin receptor

5.7 Technical review

Macdonald and Pike [7] reported that 70% of the EGFR and 40-50% of the ras and flotillin were found in the major raft fraction at the top of the gradient (Figure 1). Caveolin distribution was not dissimilar to that found by Smart et al [1] in the first continuous iodixanol gradient, i.e. it was present in the low-density raft fractions but most was detected in denser fractions that also contained calnexin. Because the gradient is continuous rather than discontinuous as in the detergent-containing strategy (**see Application Sheet S32**) the marker distribution displays not unexpectedly a considerably greater complexity. For example, flotillin, an established raft marker protein is not confined to the topmost three fractions but extends to higher densities rather like the cholesterol and although they both overlap non-raft markers such as the transferrin receptor, they have a distribution that suggests that the gradient may be able to resolve partially a slightly denser raft fraction.

A study of the effect of glycosphingolipids, an α-linolenic acid-containing GM1a (C18:3-GM1a), a stearic acid-containing molecule (C18:0-GM1a) and a lyso-GM1a, on the distribution of neural cell adhesion molecule (NCAM) in neuroblastoma cells [10], compared detergent-containing sucrose gradients and detergent-free iodixanol gradients. Whilst the lyso-GM1a totally removed NCAM from the low-density raft fraction in both gradients, C18:3-GM1 only shifted the NCAM to a high density in the detergent-free iodixanol gradients. Although these observations emphasize the need for more studies in comparisons between the various lipid-rich domain isolation strategies, there are some clear indications that detergent and non-detergent methods can provide similar data. Inoue et al [9] investigated the type IIa sodium/phosphate co-transporter protein (NaP_i) in brush border membranes and reported simple detergent extraction of the membranes and detergent-free iodixanol gradients both showed the protein to have a raft location.

6. References

1. Smart E. J., Ying, Y-S., Mineo, C. and Anderson, R. G. W. (1995) *A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane* Proc. Natl. Acad. Sci., USA, **92**, 10104-10108
2. Uittenbogaard, A., Ying, Y. and Smart, E. J. (1998) *Characterization of a cytosolic heat-shock protein-caveolin chaperone complex* J. Biol. Chem., **273**, 6525-6532
3. Oliferenko, S., Paiha, K., Harder, T., Gerke, V., Schwarzler, C., Schwarz, H., Beug, H., Gnthert, U. and Huber, L.A. (1999) *Analysis of CD44-containing lipid rafts: Recruitment of Annexin II and stabilization by the actin cytoskeleton* J. Cell Biol., **146**, 843-854
4. Lafont, F., Verkade, P., Galli, T., Wimmer, C., Louvard, D. and Simons, K. (1999) *Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells* Proc. Natl. Acad. Sci., USA, **96**, 3734-3738
5. Lindwasser, O.W. and Resh, M.D. (2001) *Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains* J. Virol., **75**, 7913-7924
6. Opekarova, M., Malinska, K., Novakova, L. and Tanner, W. (2005) *Differential effect of phosphatidylethanolamine depletion on raft proteins. Further evidence for diversity of rafts in Saccharomyces cerevisiae* Biochim. Biophys. Acta, **1711**, 87-95
7. Macdonald, J.L. and Pike, L.J. (2005) *A simplified method for the preparation of detergent-free lipid rafts* J. Lipid Res., **46**, 1061-1067
8. Pike, L.J., Han, X. and Gross, R.W. (2005) *Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids* J. Biol. Chem., **280**, 26796-26804
9. Inoue, M., Digman, M.A., Cheng, M., Breusegem, S.Y., Halahiel, N., Sorribas, V., Mantulin, W.W., Gratton, E., Barry, N.P. and Levi, M. (2004) *Partitioning of NAP_i cotransporter in cholesterol-, sphingonyelin-, and glycophingolipid-enriched membrane domains modulates NAP_i protein diffusion, clustering, and activity* J. Biol. Chem., **279**, 49160-49171
10. Nakagawa, T., Morotomi, A., Tani, M., Sueyoshi, N., Komori, H. and Ito, M. (2005) *C18:3-GM1a induces apoptosis in Neuro2 cells: enzymatic remodeling of fatty acyl chains of glycosphingolipids* J. Lipid Res., **46**, 1103-1112

Application Sheet S33; 6th edition, January 2018