OptiPrep™ Application Sheet S32

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

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **OptiPrep™ Reference List RS06** "**Analysis of lipid rich detergent-resistant domains**" provides a protocol review and list of all published papers reporting the use of OptiPrep™: to access return to the initial list of Folders and select "**Reference Lists**". **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 **2020SMemapp** file and select the appropriate S-number.
- Important technical notes and information regarding methodological variations are contained in the "Technical Notes and Review" 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. 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**.

A widely used method for the isolation of lipid rafts is based on the insolubility of these structures in a non-ionic detergent (usually TritonX-100). Either the intact cells are treated with a detergent-containing solution or a post-nuclear supernatant is prepared from a cell homogenate and then detergent is added to this supernatant. The former approach was adopted by Oliferenko et al [1] for EpH4 cells; the latter by Lafont et al [2] for MDCK cells. The detergent-treated material is then adjusted to a high density and layered under a discontinuous iodixanol gradient (also usually containing the detergent). The lipid rafts, which have a relatively low density, float away from soluble proteins and detergent-soluble cytoskeleton-associated proteins, which remain in the load zone. An alternative detergent-free method is provided in **Application Sheet S33**.

This protocol is based on refs 1 and 2. Lafont et al [2] adjusted the post-nuclear fraction to either 35% iodixanol (overlayered by 30% and 0%) or 40% iodixanol (overlayered by 30%, 20% and 5%). Oliferenko et al [1] used more steps 35%, 30%, 25%, 20% and 0% (with a total cell extract in 40%) iodixanol. In all cases however the lipid rafts band close to the top of the gradient. In a more recent modification to the gradient format Lindwasser and Resh [3] adjusted the sample to 50% iodixanol and overlayered with 40%, 30%, 20% and 10% iodixanol. By harvesting the modified gradient in smaller volume fractions the authors were able to identify subfractions of these lipid-rich domains, which displayed a heterogeneous cholesterol, GM1 glycolipid and caveolin-1 content. The gradient might therefore be usefully modified in the low-density region to be able to discriminate different low-density sub-domains.

2. Solutions required (see Section 5.1)

A. OptiPrep™
B. Isolation medium: 150 mM NaCl, 5 mM dithiothreitol (DTT), 5 mM EDTA, 25 mM Tris-HCl, pH 7.4 supplemented with a cocktail of protease inhibitors
C. Triton X-100
D. Solution B + 1% (w/v) Triton X100
E. Phosphate-buffered saline (PBS)

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water
100 mM EDTA (Na_2•$2H_2O$): 3.72 g per 100 ml water
100 mM DTT: 1.54 g per 100 ml water

Solution B: To 100 ml water; add 5 ml, 30 ml, 10 ml and 10 ml, respectively of Tris, NaCl, EDTA and DTT stock solutions; adjust to pH 7.4 with 1 M HCl and make up to 200 ml.

Solution D: Add 2 ml of Triton X100 to Solution B before adjusting to pH 7.4.

3. Ultracentrifuge rotor requirements (**see Section 5.2**)
Any small volume (approx 4 ml) swinging bucket rotor for an ultracentrifuge (e.g. Beckman SW60Ti or Sorvall TH660)

4. Protocol
Carry out all operations at 0-4°C

4a. Isolation from a total cell lysate
1. Wash the cell monolayer twice with PBS and scrape into this medium.

2. Pellet the cells and resuspend in 0.2 ml of Solution D; then leave on ice for 30 min (**see Section 5.3**).

4b. Isolation from a post-nuclear supernatant
1. Homogenize the cells in Solution B (**see Section 5.3**) and centrifuge the homogenate at 1000 *g* for 10 min.

2. Adjust the supernatant to 1% Triton X-100 and leave on ice for 30 min.

4c. Gradient separation
1. Add 2 vol. of OptiPrep™ to 1 vol. of either the homogenate or 1000 *g* supernatant.

2. Dilute OptiPrep™ with Solution D to give 35%, 30%, 25% and 20% (w/v) iodixanol (**see Sections 5.4 and 5.5**)

3. In tubes for the swinging-bucket rotor layer 0.6 ml each of the sample, the four gradient solutions and Solution D to fill the tube.

4. Centrifuge at 160,000 g_{av} for 4 h (**see Section 5.5**).

5. Collect the lipid rafts from the top interface (**see Figure 1**) or harvest the gradient in a number of equal volume fractions and analyze as required (**see Section 5.6**).

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

If the cells are homogenized in the absence of detergent (Protocol 4B), then the homogenization medium may be tailored to the tissue or cell type. A medium containing NaCl, EDTA and a buffer is perhaps the most popular for the isolation of rafts, but there are variations. For example the level of DTT used by Oliferenko et al [1] was 1 mM rather than 5 mM and EDTA was omitted. Some proteins, which associate with lipid rafts, exhibit a Ca^{2+}-dependence, so inclusion of a chelating agent may be detrimental to the study. DTT may be omitted and the concentration of Triton X-100 may be as low as 0.1% and as high as 2%. Sometimes the level of detergent in the discontinuous gradient is lower than in the sample layer. CHAPS or Brij may replace Triton X100 as the detergent. Just a few of the variations in isolation media are given in Table 1.

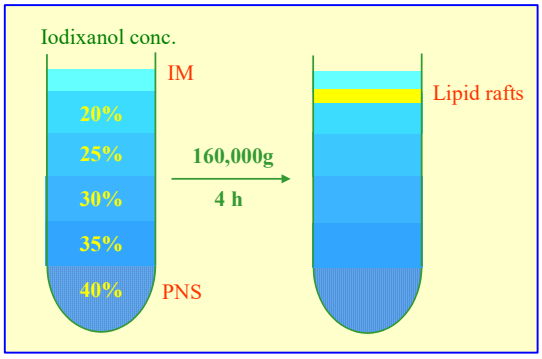

Figure 1 Isolation of lipid rafts by flotation from a post-nuclear supernatant (PNS) in a discontinuous iodixanol gradient. IM = isolation medium. See text for more details.

Protease inhibitors such as PMSF, leupeptin, antipain, aprotinin etc should be included in Solutions B and D as required.

5.2 Ultracentrifuge rotors

Many of these separations have been performed in relatively small volume swinging-bucket rotors (4-5 ml) but the gradients and sample volume may be scaled up or down proportionately as required.

5.3 Homogenization

To achieve complete cell lysis in the presence of detergent (Protocol 4a) it may be necessary to supplement this with some mechanical means, such as repeated passage through a syringe needle. If the cells are to be homogenized prior to addition of detergent (Protocol 4b) then use an homogenization protocol 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 however 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 since these are to be removed from the homogenate before detergent is added. Some hints on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells).**

- Note that for yeast cells it is common to break them by vortexing with glass beads using the same NaCl, EDTA, Tris buffer
- Note that sometimes, particularly with a tissue, a plasma membrane or other organelle is isolated using standard techniques prior to detergent extraction. For isolation of specific membranes see the Index

Table 1 Some methodological variations in the isolation of lipid rafts

Source material	EDTA; DTT; TX100 [1]	Iodixanol gradient[2]	RCF (time)	Ref #
MDCK TGN vesicles	5mM; 5mM; 0.1%	30%, 20%, 10%, 5%	160,000*g* (4 h)	2
MDCK cells	2mM; 2mM; 2.0%	43%, 35%, 30%, 25%, 20%, 0%	250,000*g* (2.5 h)	4
	1 mM; ------; 2.0%	40%, 30%, 5%	78,000*g* (4 h)	6
	5mM[3]; 5mM; 1.0%	40%, 25%, 0%	160,000*g* (4 h)	10
	5mM[3]; 1 mM; 1.0%	40%, 30%, 25%, 5%[5]	100,000*g* (4 h)	15
	------; 1 mM; 1.0%	40%, 35%, 30%, 25%, 20%, 0%	120,000*g* (12 h)	18
Drosophila	0.2 mM[3]; ---; 2.0%	24%, 21%, 15%, 6%	130,000*g* (5 h)	5
Human breast carc.	5 mM; ----; 0.2%	35%, 30%, 0%	170,000*g* (4 h)	7
HEK293	5 mM; ----; 0.1%	35%; 30%	160,000*g* (4 h)	8
	5 mM; 1 mM; 2.0%[4]	45%, 35%, 30%, 20%, 0%	180,000*g* (4 h)	11
Yeast	5 mM; ----; 1.0%	40%, 30%, 0% (0.1% TX100)	200,000*g* (2 h)	9
	5 mM; ----; 1.0%	35%, 30%, 0%	147,000*g* (16 h)	19
Jurkat	5 mM; ----; 1.0%	40%, 35%, 30%, 25%, 0%	150,000*g* (7 h)	12
Oligodendrocytes	5 mM; ----; 1.0%[6]	40%, 30%, 0%	200,000*g* (2 h)	13
Neurons	2 mM; 5 mM; 1%	40%, 30%, 5%	150,000*g* (5 h)	14
Neuroblastoma cells	5 mM, ----, 0.1%	35%, 30%, 0%	200,000*g* (4 h)	16
Ciliary ganglion[7]	-----; 1 mM; 0.1%	35%, 30%, 0%	285,000*g* (4 h)	17
COS-1	5 mM; ----; 0.5%	50%, 40%, 30%, 20%, 10%	170,000*g* (4 h)	3

1. Cell lysis media also contained NaCl and a Tris buffer, in most instances
2. The first figure in each series gives the % iodixanol in the sample
3. EGTA was used in place of EDTA
4. Density solutions contained Ca^{2+} and no DTT
5. All solutions contained 10% sucrose, the density layers did not contain DTT
6. CHAPS (20 mM) was also used in this study
7. Membrane fraction first obtained in preliminary OptiPrep™ gradient

5.4 Forming the discontinuous gradient

Discontinuous gradients are normally most easily prepared by underlayering (i.e. low density first) using a 1 ml syringe and a long metal cannula; overlayering small volumes is more difficult using either a syringe or Pasteur pipette. One alternative for overlayering 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**. If necessary, adjust all volumes proportionately so that tubes are properly filled according to the manufacturer's instructions.

5.5 Gradient and centrifugation conditions

There are wide variations in the details of the discontinuous gradient; sometimes it is a relatively simple three-layer gradient (including the dense sample), sometimes there may be up to 6 layers. The latter are more likely to provided resolution of different detergent-resistant domains. Some commonly used formats are summarized in Table 1.

As far as is known the optimal centrifugation time and RCF have not been thoroughly investigated. Oliferenko et al [1] used a longer centrifugation time of 12 h at a slightly lower RCF ($120{,}000g_{av}$). Because of the relatively short sediment path length of the rotor, 4 h at the higher RCF is probably satisfactory, but the centrifugation conditions may vary with the mode of preparation and there is evidence from other work that for optimal resolution long centrifugation times (>12 h) at relatively low *g*-forces (<100,000*g*) are recommended. Some commonly used regimes are summarized in Table 1.

- Adapting a protocol developed by Yeaman et al [20], Lynch et al [21] set up a discontinuous gradient of 10%, 20% and 30% (w/v) iodixanol (each layer containing the sample) in tubes for Beckman VTi90 vertical rotor and centrifuged it at 350,000 *g* for 3 h. During that time a linear gradient will form by a self-generation and diffusion. It provided a means of distinguishing multiple membrane domains.

5.6 Gradient analysis

Depending on the resolution that is required it may be sufficient to use an automatic pipette to collect the gradient in four or five broad zones. Alternatively for higher resolution the gradient should be unloaded either by tube puncture, upward displacement or automatic aspiration from the meniscus. For more information 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.

6. References

1. 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
2. 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
3. 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
4. Lafont, F., Lecat, S., Verkade, P. and Simons, K. (1998) *Annexin XIIIb associates with lipid microdomains to function in apical delivery* J. Cell Biol., **142**, 1413-1427
5. Rietveld, A., Neutz, S., Simons, K. and Eaton, S. (1999) *Association of sterol- and glycosylphosphatidylinositol-linked proteins with Drosophilia raft lipid microdomains* J. Biol. Chem., **274**, 12049-12054
6. Benting, J.H., Rietveld, A.G. and Simons, K. (1999) *N-glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells* J. Cell Biol., **146**, 313-320
7. Manes, S., Mira, E., Gomez-Mouton, C., Lacalle, R.A., Keller, P., Labrador, J.P. and Martinez-A, C. (1999) *Membrane raft microdomains mediate front-rear polarity in migrating cells* EMBO J., **18**, 6211-6220

8. Bruckner, K., Labrador, J.P., Scheiffele, P., Herb, A., Seeburg, P.H. and Klein, R. (1999) *EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains* Neuron, **22**, 511-524
9. Bagnat, M., Keranen, S., Shevchenko, A., Shevchenko, A. and Simons, K. (2000) *Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast* Proc. Nat. Acad. Sci. USA, **97**, 3254-3259
10. Plant, P.J., Lafont, F., Lecat, S., Verkade, P., Simons, K. and Rotin, D. (2000) *Apical membrane targeting of Nedd4 is mediated by an association of its C2 domain with annexin XIIIb* J. Cell Biol., **149**, 1473-1483
11. Gimpl, G. and Fahrenholz, F. (2000) *Human oxytocin receptors in cholesterol-rich vs. cholesterol-poor microdomains of the plasma membrane* Eur. J. Biochem., **267**, 2483-2497
12. Harder, T. and Kuhn, M. (2000) *Selective accumulation of raft-associated membrane protein LAT in T cell receptor signaling assemblies* J. Cell Biol., **151**, 199-207
13. Simons, M., Kramer, E-M., Thiele, C., Stoffel, W. and Trotter, J. (2000) *Assembly of myelin by association of proteolipid protein with cholesterol and galactosylceramide-rich membrane domains* J. Cell Biol., **151**, 143-153
14. Ledesma, M.D., Da Silva, J.S., Crassaerts, K., Delacourte, A., De Stropper, B. and Dotti, C.G. (2000) *Brain plasmin enhances APP α-cleavage and Aβ degradation and is reduced in Alzheimer's disease brains* EMBO Rep., **1**, 530-535
15. Lecat, S., Verkade, P., Thiele, C., Fiedler, K., Simons, K. and Lafont, F. (2000) *Different properties of two isoforms of annexin XIII in MDCK cells* J. Cell Sci., **113**, 2607-2618
16. Tansey, M.G., Baloh, R.H., Milbrandt, J. and Johnson, E.M. (2000) *GFRα –mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival* Neuron, **25**, 611-623
17. Bruses, J.L., Chauvet, N. and Rutishauser, U. (2001) *Membrane lipid rafts are necessary for the maintenance of the α7 nicotinic acetylcholine receptor in somatic spines of ciliary neurons* J. Neurosci., **21**, 504-512
18. Hanwell, D., Ishikawa, T., Saleki, R. and Rotin, D. (2002) *Trafficking and cell surface stability of the epithelial Na^+ channel expressed in epithelial Madin-Darby canine kidney cells* J. Biol. Chem., **277**, 9772-9779
19. Lee, M.C., Hamamoto, S. and Schekman, R. (2002) *Ceramide biosynthesis is required for the formation of the oligomeric H^+ -ATPase Pma1p in the yeast endoplasmic reticulum* J. Biol. Chem., **277**, 22395-22401
20. Yeaman, C., Grindstaff, K. K. and Nelson, W. J. (2003) *Mechanism of recruiting Sec6/8 (exocyst) complex to the apical juntional complex during polarization of epithelial cells* J. Cell Sci., **117**, 559-570
21. Lynch., R.D., Francis, S.A., McCarthy, K.M., Casas, E., Thiele, C. and Schneeberger, E.E. (2007) *Cholesterol depletion alters detergent-specific solubility profiles of selected tight junction proteins and the phosphorylation of occluding* Exp. Cell Res., **313**, 2597-2610

7. Acknowledgements

We wish to thank Dr Lukas Huber, IMP, Research Institute of Molecular Pathology, A-1030, Vienna, Austria and Dr Kai Simons EMBL, Cell Biology and Biophysics Program, 69117 Heidelberg, Germany for their kind cooperation in the preparation of this text.

serumwerk bernburg

Serumwerk Bernburg AG • Hallesche Landstraße 105 b • 06406 Bernburg • Germany
phone: +49 3471 860 429 • diagnostic.serumwerk.com • diagnostic@serumwwerk.com • serumwerk.de