OptiPrep™ Application Sheet S44

Endocytosis of ligands: analysis in sedimentation velocity gradients

- 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)
- See Section 5.7 for a brief review of methodology which includes a buoyant density separation of fractions from an initial velocity gradient; this technology maximizes resolution of compartments
- For a more detailed methods review see **OptiPrep™ Application Sheet S42 "Endocytosis analysis – a review of density gradient methods".**
- See also **RS12 "Endocytosis – a bibliographical review"** which lists all the relevant papers reporting the use of OptiPrep™: to access this file return to the initial list of Folders and select "**Reference Lists**".

1. Background

There are many examples of the use of both Nycodenz® and iodixanol gradients for the analysis of the endocytic process. The procedure described in this Application Sheet fractionates endocytic compartments in 0-45% Nycodenz® gradients at 85,000 *g* for 45 min i.e. the compartments are resolved by sedimentation rate (rather than density). It demonstrates that as the endocytic process progresses, ligands are associated with endosomal compartments of increasing sedimentation velocity [1-7]. This strategy is also able to identify a "pre-lysosomal compartment" which bands at a slightly lower density than the lysosomes. It has been used for the analysis of the internalization of asialoglycoproteins [1,2,4-7] and LDL [3]. The protocol has been developed primarily for rat hepatocytes but it can be used for any cell type or perfused tissue, although only the use of cultured monolayers of cells is ideally suited to some of the shorter ligand internalization times.

The denser parts of the Nycodenz® gradient are hyperosmotic. When iodixanol is substituted for Nycodenz®, the gradient can be isoosmotic throughout its entire density range. In all other respects however, the properties of the two gradient media are essentially identical (solutions of the same % w/v concentration give the same density). What effect the osmolality has on the resolution in the densest part of the gradient is not known. However, because of the ease of use of iodixanol (OptiPrep™ can be simply diluted with the homogenization medium), the Nycodenz® methodology described in refs 2 and 4 has been adapted to iodixanol in this Application Sheet. he banding of endocytic compartments might vary with the type of cell or tissue and the type of ligand under investigation.

2. Solutions required (see Section 5.1)

2a. Homogenization medium (HM): 0.25 M sucrose, 1 mM EGTA, 10 mM Hepes-NaOH, pH 7.2 (**see Box**)

2b. Iodixanol stock solution (**see Box →**)

Mix 4.5 vol. with 1.5 vol. of OptiPrep™ diluent

Add protease inhibitors as required to any of the solutions as required.

Keep the following stock solutions at 4°C:

1 M Hepes (free acid)	23.8 g per 100 ml water
100 mM EGTA (free acid)	3.80 g per 100 ml water (pH 11-12)

HM: Dissolve 17 g sucrose in 100 ml water; add 2 ml of EGTA stock and 20 ml of Hepes stock; adjust to pH 7.2 with 1 M NaOH and make up to 200 ml.

OptiPrep Diluent: Dissolve 8.5 g sucrose in 50 ml water; add 4 ml each of Hepes and EGTA stocks; adjust to pH 7.2 with 1 M NaOH and make up to 100 ml

3. Ultracentrifuge rotor requirements (see section 5.2)

Any swinging-bucket rotor for an ultracentrifuge capable of 100,00 *g* with a tube capacity of approx 38 ml tubes (e.g. Beckman SW28 or Sorvall AH629)

4. Protocol

Suspensions of hepatocytes are allowed to bind ^{125}I-labelled asialofetuin at 4°C for 60 min in a suitable incubation buffer. The ligand is then internalized during subsequent incubation at 37°C for periods of 30 sec, 60 sec, 2.5 min, 15 min or 30 min (see Note 1). At the end of each time period, the internalization is stopped by addition of ice-cold incubation buffer containing EGTA.

Perform all of the following at 4°C

1. Wash the cells in solution HM to remove any surface-bound ligand and then homogenize them in this solution using 20 strokes of the pestle of a Dounce homogenizer (**see Section 5.3**).

2. Pellet debris and nuclei by centrifugation for 5 min at 2000 g_{av}; then decant and retain the supernatant.

3. Resuspend the pellet in HM; re-centrifuge at 2000 *g* and combine the two supernatants.

4. Use either a standard two-chamber gradient maker or a Gradient Master to prepare 34 ml continuous 0-45% (w/v) iodixanol gradients from equal volumes of the stock solution and the HM in 36-40 ml tubes for a suitable swinging bucket rotor (**see Section 5.4**).

5. Load approx 4 ml aliquots of each sample on to each gradient (**see Section 5.5**).

6. Centrifuge the gradients at 85,000 g_{av} for 45 min at 4°C; allow the rotor to decelerate from 2000 rpm without the brake.

7. Unload the gradients in 2 ml fractions by upward displacement and analyze the fractions (**see Section 5.6**). For more information on harvesting gradients **see Application Sheet S08**.

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. 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. Although 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) is still a widely used HM, supplementation 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). 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.

If a hypoosmotic medium is used to swell the cells to achieve an adequate degree of homogenization, it is important to return the homogenate to isoosmotic conditions as soon as possible. Other examples of homogenization media are given in **Application Sheets S05 (tissues) and S06 (cells).**

The use of the OptiPrep™ Diluent keeps the concentration of EGTA and buffer constant through the gradient. If this is not regarded as critical the OptiPrep™ may be diluted with the HM. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

5.2 Ultracentrifuge rotors

Smaller volume rotors or vertical (or near-vertical) rotors may be used but since this method relies on sedimentation velocity for its efficacy, the separations will certainly need shorter times in rotors with smaller sedimentation path lengths. In vertical or near-vertical rotors, the sample occupies a very narrow zone after reorientation in the tube and thus should provide the ideal format.

5.3 Homogenization

The homogenization protocol should be tailored to the cell (or tissue) type. Potter-Elvehjem homogenization for tissues and Dounce homogenization for cells used to be the standard procedures. For cells 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 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 Sheets S05 (tissues) and S06 (cells).**

5.4 Gradient construction

If neither a two-chamber gradient maker nor a Gradient Master™ is available for making continuous gradients then these may be formed from diffusion of discontinuous gradients. In this case layer equal volumes of 0%, 10%, 20%, 30% and 40% (w/v) iodixanol. For more information on gradient construction see Application Sheets S03. If necessary, adjust all volumes proportionately and make sure that, after loading of the sample, tubes are properly filled according to the manufacturer's instructions.

5.5 Sample layering

As with all sedimentation velocity density gradients the volume of sample should not exceed approx 10% of the total gradient volume. Scale down all sample and gradient volumes proportionately.

5.6 Analysis

Figure 1 shows that (in Nycodenz® gradients) with increasing times of incubation at 37°C (internalization) after binding of the ligand to the cell surface, the peak of radiolabel shifts progressively to higher densities.

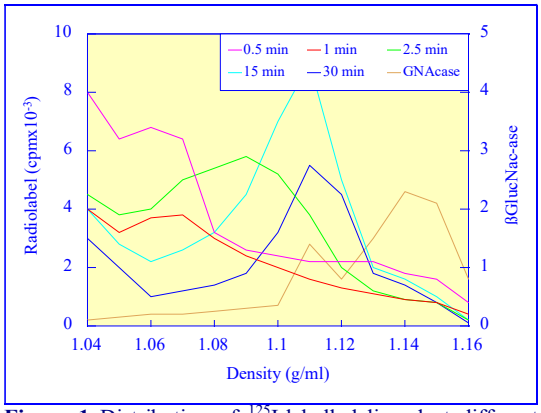

Figure 1 Distribution of ^{125}I-labelled ligand at different incubation times in Nycodenz® gradients. Also shown is the banding position of lysosomes (GNAcase = ß-NAcetylglucosaminidase). Figure adapted from ref 4.

After 30 sec of internalization, the main peak of activity is found near the top of the gradient, at a density of about 1.06 g/ml. After 60 sec, the total amount of internalized ligand increases, with the main peak at a slightly increased density, but with more of the label distributed further into the gradient. After 2.5 min incubation, total internalized ligand increases by about 5 times, with the main peak at 1.09 g/ml, but widely spread to a density of 1.12 g/ml. At 15 min incubation the density of the main peak shifts to 1.11 g/ml with approx the same amount of internalized ligand as after 2.5 min, while after 30 min, the amount of ligand had decreased by half, with the peak still at 1.11 g/ml. The 1.11 g/ml material is clearly resolved from the main lysosome band, which peaks around 1.15 g/ml.

The pattern of labelling is interpreted in the following manner. Upon uptake of the ligand from the cell surface, it is contained in small, slowly sedimenting particles, which, with time, increase in size as shown by their increased sedimentation rate. After 15 min, the particles increase to a size sufficient to allow them to reach their buoyant density within the 45 min centrifugation. Under the recommended centrifugation conditions only the largest endosomes reach their buoyant density. If the centrifugation time is increased to 3 h, the radiolabel at the early time points also peaks close to 1.11 g/ml. The particles, which band at this density after a 45 min centrifugation are probably a pre-lysosomal compartment from which the ligand exits and is degraded between 2.5 and 30 min.

The use of tyramine-cellobiose attached to the ligand is recommended for the study of the degradation steps. Tyramine-cellobiose promotes the retention of degradation products in the cell. In this way the degradation process from a pre-lysosomal compartment through primary lysosomes to secondary lysosomes can be studied by comparing acid soluble and acid precipitable radiolabel [8].

5.7 Combined sedimentation velocity and buoyant density analysis

A study of nerve growth factor-mediated signal transduction that investigated the role of the TrkA receptor tyrosine kinase and associated proteins (APPL1 and GIPC1) implicated a special population of endosomes [9,10]. A post-nuclear fraction of PC12 cells was first layered over a 0-30% (w/v) iodixanol gradient and centrifuged at 133,000 *g* for 90 min. The gradient was collected in five sequential fractions and to facilitate the subsequent processing they were adjusted to an iodixanol concentration of at least 32.5% (w/v) iodixanol and overlaid with a continuous 0-30% (w/v) iodixanol gradient (or the fractions might be underlaid previously formed gradients) and recentrifuged for approx.17 h. From the second fraction of the first gradient two completely distinct populations of membrane vesicles were resolved: the lighter population (approx 1.12 g/ml) contained predominantly pTrk and smaller amounts of APPL1 and GIPC1; the denser population (approx. 1.20 g/ml) contained almost exclusively APPL1. The methodology was later applied to human neuroblastoma cells [11].

In a detailed account of the methodology and the analytical procedures McCaffrey et al [12] showed that Rab5 and Rab4 and Rab7, respectively markers for primary endocytic vesicles, recycling endosomes and multivesicular carrier vesicles, showed distinctive distributions through the gradients.

6. References

1. Berg, T., Ford, T., Kindberg, G. M., Blomhoff, R. and Drevon, C. (1985) *Intracellular transport of asialoglycoproteins in rat hepatocytes* Exp. Cell Res., **156**, 570-574
2. Berg, T., Kindberg, G. M., Ford, T. and Blomhoff, R. (1985) *Intracellular transport of asialoglycoproteins in rat hepatocytes. Evidence for two subpopulations of lysosomes* Exp. Cell Res., **161**, 285-296.
3. Gudmundsen, O., Nenseter, M. S. and Berg, T. (1993) *Endocytosed LDL and β-VLDL follow different intracellular pathways in rat liver* Biochim. Biophys. Acta, **1210**, 63-72.
4. Kindberg, G. M., Ford, T., Blomhoff, R., Rickwood, D. and Berg, T. (1984) *Separation of endocytic vesicles in Nycodenz gradients* Anal. Biochem., **142**, 455-462.
5. Kindberg, G. M., Refsnes, M., Christofferson, T., Norum, K. R. and Berg, T. (1987) *The relationship between autophagy and the intracellular degradation of asialoglycoproteins in cultured rat hepatocytes* J. Biol. Chem., **262**, 7066-7071.
6. Kindberg, G. M., Tolleshaug, H., Gjoen, T. and Berg, T. (1991) *Lysosomal and endosomal heterogeneity in the liver: a comparison of the intracellular pathways of endocytosis in rat liver cells* Hepatology, **13**, 254-259.
7. Malaba, L., Smeland, S., Senoo, H., Norum, K. R., Berg, T., Blomhoff, R. and Kindberg, G. M. (1995) *Retinol –binding protein and asialo-orosomucoid are taken up by different pathways in liver-cells* J. Biol. Chem., **270**, 15686-15692.
8. Pittman, R. C., Green, S. R., Attie, A. D. and Steinberg, D. (1979) *Radiolabeled sucrose covalently linked to protein. A device for quantifying degradation of plasma proteins catabolized by lysosomal mechanisms* J. Biol. Chem., **254**, 6876-6879
9. Lin, D.C., Quevedo, C., Brewer, N.E., Bell, A., Testa, J.R., Grimes, M.L., Miller, F.D. and Kaplan, D.R. (2006) *APPL1 associates with TrkA and GIPC1 and is required for nerve growth factor-mediated signal transduction* Mol. Cell. Biol., **26**, 8928-8941
10. Pryor, S., McCaffrey, G., Young. L.R. and Grimes, M.L. (2012) *NGF causes TrkA to specifically attract microtubules to lipid rafts* PLoS One **7**: e35163
11. Xin, X., Gfeller, D., Cheng, J., Tonikian, R., Sun, L., Guo, A., Lopez, L., Pavlenco, A., Akintobi, A., Zhang, Y., Rual, J-F., Currell, B., Seshagiri, S., Hao, T., Yang, X., Shen, Y.A., Salehi-Ashtiani, K., Li, J., Cheng, A.T., Bouamalay, D., Lugari, A., Hill, D.E., Grimes, M.L., Drubin, D.G., Grant, B.D., Vidal, M., Boone, C., Sidhu, S.V. and Bader, G.D. (2013) *SH3 interactome conserves general function over specific form* Mol. Systems Biol., **9**: 652
12. McCaffrey, G., Welker, J., Scott, J., van der Salm, L. and Grimes, M.L. (2009) *High-resolution fractionation of signaling endosomes containing different receptors* Traffic, **10**, 938–950

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