OptiPrep™ Application Sheet S47

Analysis of exocytosis, exocyst function and plasma membrane domain targeting in (A) self-generated and (B) pre-formed 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.
- **Application Sheet S47a "Intracellular exocytic vesicle trafficking and exocyst complex – a short methodological summary"** provides a brief protocol review of papers reporting the use of OptiPrep™ which complements this Application Sheet.
- Important technical notes and information regarding alternative methodologies are contained in the "Technical Notes and Review" section (Section 5); for non-mammalian cells see Section 5.8

A. Self-generated gradients

1. Background

Self-generated gradients offer an important advantage for the fractionation of membrane compartments involved with trafficking and signaling; they are highly reproducible and very simple to prepare. In a vertical or near-vertical rotor with a sedimentation path length of <25 mm, gradient formation takes place in 1-3 h at approx 350,000 *g*. They can be prepared simply by adjusting the sample (usually a post-nuclear supernatant) to a uniform concentration of iodixanol and then transferring the suspension to the centrifuge tube. Some of the density profiles that can be obtained in this manner are described in **Application Sheet S04**.

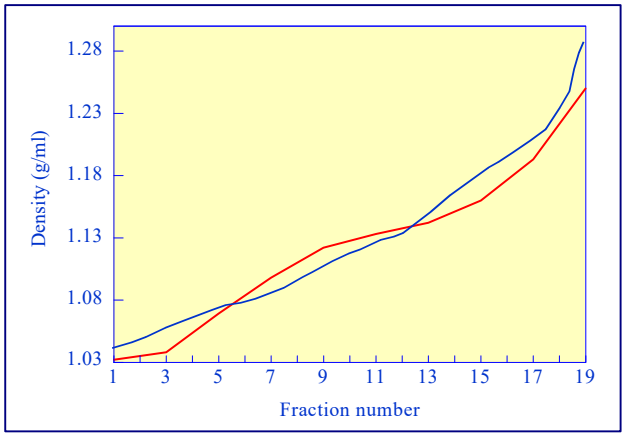

Figure 1 Density profiles: comparison of starting format: 25% (w/v) iodixanol (red line); 10%, 20%, 30% (w/v) iodixanol (blue) in Beckman VTi65.1 vertical rotor, 353,000*g* for 3 h at 4°C

In the example in Figure 1, centrifugation of a 25% (w/v) iodixanol solution for 3 h centrifugation at 353,000 g_{av} (at 4°C) produces a gradient that is more or less linear from 1.02 g/ml to 1.15 g/ml but is steeper on the densest region and clearly retains the slight sigmoidal form more prominent in shorter spin gradients. To overcome the latter, Yeaman et al [1] used a starting format of a discontinuous gradient, comprising equal volumes of 10%, 20% and 30% (w/v) iodixanol. The gradient formed under these conditions retains its steeper densest portion and the sigmoidal form is lost (Figure 1).

This gradient is very effective for the study of the transfer of proteins or protein complexes to the surface or the secretion of virus and virus-like particles (**see Section A5.6**).

2. Solutions required (see Section 5.1 for important information)

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0

Keep the following stock solutions at 4°C:

500 mM Hepes (free acid)	11.9 g per 100 ml water
1 M KOAc	9.8 g per 100 ml water
100 mM $Mg(OAc)_2 \bullet 4H_2O$	2.15 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 8 ml, 18 ml and 4 ml of Hepes, KOAc and $Mg(OAc)_2$ stocks respectively; adjust to pH 8.0 with KOH and make up to 200 ml.

3. Ultracentrifuge rotor requirements (see Section 5.2)

Vertical rotor with 11-13 ml tubes (e.g. Beckman VTi65.1 or Sorvall 65V13, or near-vertical rotor (e.g. Beckman NVT65)

4. Protocol (adapted from ref 1)

Carry out all operations at 0-4°C. All iodixanol concentrations are given as % (w/v).

1. Homogenize the cells in Solution B in a cell cracker (ball-bearing homogenizer) using 4-6 passages. Monitor the efficacy of the homogenization by phase contrast microscopy (**see Section 5.3**).

2. Centrifuge the homogenate at 800 *g* for 5 min to pellet the nuclei.

3. Mix the post-nuclear supernatant (PNS), OptiPrep™ and solution B in the following volume ratios (3:3:0, 3:2:1 and 3:1:2) to produce three suspensions containing 30%, 20% and 10% iodixanol respectively (**see Sections 5.4 and 5.7**).

4. Layer equal volumes the three suspensions in tubes for the vertical or near-vertical rotor; 11.2 ml Optiseal™ tubes for the Beckman VTi65.1 or NVT65 rotor are the recommended ones (**see Sections 5.5 and 5.7**).

5. Centrifuge at 353,000 g_{av} for 3 h using a slow acceleration program.

6. Allow the centrifuge to decelerate to rest from 2000 rpm without the brake or use a slow deceleration program.

7. Collect the gradient in 0.5 ml fractions either by tube puncture, aspiration from the meniscus or upward displacement; the latter two options are only permissible with Beckman Optiseal™ tubes. For more information on harvesting gradients **see Application Sheet S08**.

♦ A brief summary of the analytical capabilities of this gradient system is given in Section 5.6.

5. Technical Notes and Review

5.1 Homogenization media

The homogenization medium (HM) 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.

Supplementation of the HM with inorganic salts (containing K^+ or Na^+ ions) 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. Solutions are buffered with Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and it is unlikely if the type of buffer significantly influences the fractionation, although triethanolamine does seem to offer some advantages in homogenization efficiency [2]

The 0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0 described in this protocol was used by Yeaman et al [1] for NRK-49F and NRK-52E rat kidney cells. Kolesnikova et al [3] who used the same gradient homogenized human hepatoma (HUHT-7) and HeLa cells in a standard 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, while Leblanc et al [4] replaced the 1 mM EDTA with 1.5 mM $MgCl_2$ for mouse embryo fibroblasts (3T3 cells). Other examples of homogenization media are given in **Application Sheet S06**.

Protease inhibitors may be included in Solution B at the operator's discretion.

5.2 Ultracentrifuge rotors

Other rotors with different sedimentation path lengths may be suitable but the optimal centrifugation conditions will require investigation; only vertical, near-vertical or low-angle small volume fixed-angle rotors can normally be used for self-generated gradients. For more information **see Application Sheet S04**.

Interestingly Leblanc et al [4] used a small volume (4 ml maximum) swinging-bucket rotor at a lower g-force (260,000*g*). This rotor type is not normally used for self-generated gradients; diffusion may be sufficiently rapid to form the correct continuous gradient with this small volume rotor. Moreover, sedimentation of iodixanol molecules will occur at *g*-forces >180,000*g*, thus gradient self-generation is likely even in this rotor.

5.3 Homogenization

The homogenization protocol should be tailored to the cell type. Dounce homogenization was the most widely used procedure 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 [3,4]. 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 Gradient solutions

If it is considered that it is important to maintain one or more of the reagents present in the homogenization medium at the same concentration throughout the gradient, then make up a Working Solution of 50% iodixanol first. For example, if the homogenization buffer is 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, then dilute 5 vol. of OptiPrep™ with 1 vol. of 0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCl, pH 7.5. If this is used in place of the OptiPrep™ in Step 3, then the concentrations of Tris and EDTA will be the same in all three suspensions. The concentration of the osmotic balancer (0.25 M sucrose) is normally not changed in the Working Solution otherwise the solutions would become significantly hyperosmotic. The volume ratios for the three suspensions using a 50% iodixanol working solution instead of OptiPrep™ would need to be changed to 2:3:0; 2:2:1 and 2:1:2, respectively. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

5.5 Gradient set-up and formation

Sealed tubes require complete filling; a small volume of Solution B may be added to fill the tube to the required level if necessary.

The ability of a two or three-layer starting format to modulate the density profile obtained by self-generation is often a useful means of achieving a more linear gradient [5]. Although it may be considered that one of the important advantages of using a single uniform iodixanol concentration format (i.e. the lack of any interfaces) is lost in the layered format, this is ameliorated by the fact that the particles in the post-nuclear supernatant are diffusely and uniformly spread through the gradient. Diffusion of iodixanol across the interfaces will, moreover, rapidly "soften" the original density discontinuities. This happens more effectively in a vertical rotor than in a swinging-bucket rotor because of the large interfacial surface area. Although Yeaman et al [1] developed this methodology using a vertical rotor, a near-vertical rotor is probably the rotor of choice since any soluble proteins sediment towards the bottom of the tube. In a vertical rotor the proteins will sediment towards the entire length of the wall of that part of the tube furthest from the rotor axis. Subsequently Andersen and Yeaman [6] used a near-vertical rotor.

♦ It is important to note that although a three-layer discontinuous gradient was often constructed, the selection of vertical, near-vertical or small volume high-performance fixed-angle rotors, centrifuged at approx. 300,000 *g* will permit the formation of a self-generated continuous (but not necessarily linear) gradient.

5.6 Gradient analysis

The density of the fractions from a blank gradient can be checked by refractometry. Absorbance measurements are an alternative method. For more information see **Application Sheet S09**.

Figure 2 summarizes the distribution of plasma membrane, endosomes (and TGN) and cytosolic proteins in the gradient. The endosome marker (panel a) used by Yeaman et al [1] was syntaxin13, present in early and recycling endosomes. Late endosomes, ER and lysosomes were detected in denser fractions overlapping the lighter cytosolic protein region. On the other hand, late endosomes from human hepatoma cell (panel b) were clearly less dense than their NRK counterparts. Although these gradients can be used for a variety of cell types, the fine detail of the membrane fractionation patterns in these gradients is clearly cell-dependent.

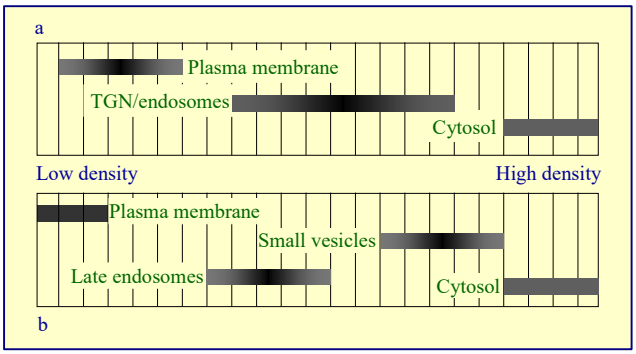

Figure 2 Approximate distribution of subcellular membranes in iodixanol gradients Panel a: NRK cells, data adapted from ref 1; Panel b: Marburg virus VP40 expressing human hepatoma cells, data adapted from ref 3

Yeaman et al [1] compared the distribution of the Sec6/8 complex, which is involved with the functioning of the exocytic pathway, in two forms of NRK cells: NRK-49F, which formed fibroblast-like junctions and NRK-52E, which formed epithelial-like junctions. The authors were able to show that in NRK-52E cells the Sec6/8 complex co-fractionated principally with the plasma membrane, while relatively little was detected in the TGN/endosomes or in cytosolic protein regions. In NRK-49F cells on the other hand significant amounts of the complex were detected in the cytosolic proteins and in two distinctive fractions in the TGN/endosome region. The pattern in the latter was quite different to that of the syntaxin13 or VAMP4 profiles.

Kolesnikova et al [3] were able to use the gradient to monitor the translocation of the VP40 matrix protein of Marburg virus in infected cells. At 7 h post-infection most of the VP40 was associated with the small vesicle fraction but as the infection progressed (up to 24 h) the gradient permitted the demonstration of a shift through the endosomal/ER zone to the plasma membrane (Figure 2, panel b).

HEK cells [7,8] and endothelial cells [9] also show the distinctive patterns of plasma membrane, endosomes, small vesicles and soluble proteins shown in Figure 2 (panel b); while if the primary objective is a simple and convenient preparation of plasma membrane, the crude fraction can simply be adjusted to 30% iodixanol; this has been executed with such diverse material as mouse brain [10] and MDCK cells [11].

♦ Ref 12 reviews the use of self-generated iodixanol gradients in the analysis of the way that exocytic trafficking is involved with the establishment of distinctive plasma membrane domains in epithelial cells.

5.7 Alternative gradient formats

Sometimes the PNS is only present in the densest of the gradient layers; this was the case in the analysis of HeLa cells [13] of recycling and early endosomes from COS cells [14]; a detailed study of MDCK cells, which also included a 15% (w/v) iodixanol layer [15] and an investigation into the membrane association of paxillin in prostate cancer cells in which the PNS in 30% (w/v) iodixanol

underlaid 25%, 20%, 15% and 10% (w/v) iodixanol [16]; a near-vertical rotor was used, thus the gradient formed remains a self-generated one.

5.8 Non-mammalian cells

Drospophila exocyst function has been analyzed using the method as described in Sections 2-4 [17] and polarized secretory vesicle delivery in yeast cells has been down-scaled to a TLA120.2 fixed-angle rotor; the spheroplast lysate was adjusted to 40% (w/v); underlaid beneath a 35% (w/v) layer and centrifuged at 100,000 *g* for 3 h [18]. The low *g*-force used to create this gradient is a consequence of the very small volumes used – a sample volume of 0.1 ml was overlaid by 1 ml of the 35% iodixanol solution in a small volume TLA120.2 fixed-angle rotor. Chang et al [19] and Caballero-Lima et al [20] used the same small volume rotor to generate the gradient in studies of yeast secretory vesicles.

B. Pre-formed gradients

The following table summarizes the cell types and gradient formats that have been used; a short description of the analysis is also included.

Cell type	Centrifugation conditions: %(w/v) iodixanol: *g*-force/time	Analysis	Ref #
HEK/T/dendritic	6-18: 200,000*g*/75 min	Resolution of HIVexosomes and HIV	21
HeLa	6-18: 250,000*g*/90 min	Resolution of HIV-Nef exosomes/progeny virions	22
HeLa	10,29,30: 60,000*g*/3 h*	Sx16, Sec5, Sec6, Sec10, Rab11, Rab35, VAMP2	23
MDCK	10,20,25,30,40: 90,000*g*/16 h	TGFα-containing basolaterally targeted exosomes	24
MDCK	10-40: 90,000*g*/18 h	TGFα-containing exosomes	25
Neutrophils	5-30: 34,000*g*/18.5 h	Leukotriene B release from cells	26
PC12 cells	14.5,50: 190,000*g*/5 h	Dense core vesicles	27

* Post-nuclear supernatant in densest layer

C. References

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