OptiPrep™ Application Sheet S25

Analysis of ER, plasma membrane, endosomes, Golgi, ERGIC and TGN from cells and tissues in sedimentation velocity gradients

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- An **OptiPrep™ Reference List (RS05)** "Analysis of membrane trafficking in mammalian tissues and cells: fractionation of ER, Golgi, TGN, PM and endosomes" provides a bibliography of all published papers reporting the use of OptiPrep™ for analysis of these membranes: to access return to the initial list of Folders and select "**Reference Lists**".
- **To access other Application Sheets** referred to in the text: return to the **2020SMemapp** file and select the appropriate S-number.
- Section 5 of this Application Sheet is a short review of some of the variations in the methodology according to cell or tissue type and indicates the type of membranes that were analyzed.

1. Background

Using modern OptiPrep™ technology, fractionation and subfractionation of membrane compartments have generally been carried out by buoyant density banding in either pre-formed or self-generated gradients. Fractionation on the basis of sedimentation velocity however, may provide increased resolving power, particularly of the multiple compartments of relatively low density membranes (ERGIC, Golgi, TGN etc). Both sucrose and glycerol gradients have been used for sedimentation velocity analysis of membrane vesicles but their usefulness is compromised by the increasing osmolality of the gradients, as a result of which, osmotically-active vesicles progressively decrease in size during their progress through the gradient. Since the size of the vesicle is the predominant factor in determining its sedimentation velocity, hyperosmotic gradients are less than ideal for these separations. Iodixanol gradients on the other hand can be made isoosmotic throughout their length.

Sedimentation velocity separations are traditionally carried out in continuous linear gradients, such gradients are essential, for example, for the sedimentation analysis of proteins. For the membrane analysis described herein however such strictures have been relaxed and both discontinuous and continuous iodixanol gradients have been used. Moreover, by manipulation of the density of each layer of a discontinuous gradient, gradients can easily and reproducibly adjusted to optimize the separation of both slowly and rapidly sedimenting particles. Furthermore by making the difference in density between adjacent layers and the volume of these layers relatively small, a discontinuous gradient will, more or less, approach a continuous format.

The protocol described in this Application Sheet provides discontinuous (adapted from ref 1) and continuous (adapted from ref 2) gradient alternatives.

- Two types of solution preparation are provided:
- Type I is a standard general-purpose sucrose-based homogenization medium containing a buffer and EDTA
- Type II contains KCl as the major osmotic component instead of sucrose. KCl-containing solutions may be useful for any homogenate that contains a lot of microfilament-derived proteins, which tend to form a gel in the absence of salt.
- In some instances it may be necessary to use a hypoosmotic solution in order to achieve a satisfactory homogenization of a particular cell type. Some of these variations are described in Section 5.1

2. **Solutions required** (**see Section 5.1**)

Type I

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 1 mM EDTA 10 mM Hepes-NaOH, pH 7.4

C. Diluent: 0.25 M sucrose, 6 mM EDTA, 60 mM Hepes-NaOH, pH 7.4

D. Working Solution of 50% (w/v) iodixanol (ρ = 1.272 g/ml): 5 vol of solution A + 1 vol of solution C

Keep the following stock solutions at 4°C:
500 mM Hepes (free acid): 11.9 g per 100 ml water.
100 mM EDTA (Na_2•$2H_2O$): 3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2 ml of EDTA stock and 4 ml of Hepes stock; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 6 ml of EDTA stock and 12 ml of Hepes stock; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

Type II

A. OptiPrep™

B. Homogenization medium: 130 mM KCl, 25 mM NaCl, 1 mM EGTA, 25 mM Tris-Cl, pH 7.4

C. Diluent: 130 mM KCl, 25 mM NaCl, 6 mM EGTA, 25 mM Tris-Cl, pH 7.4

D. Working Solution of 50% (w/v) iodixanol: 5 vol. of solution A + 1 vol. of solution C.

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
1 M KCl: 7.45 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water
100 mM EGTA (free acid): 3.80 g per 100 ml water (pH 11-12)

Solution B: To 100 ml water; add 5.0 ml, 26 ml, 5.0 ml and 2.0 ml, respectively of Tris, KCl, NaCl and EGTA stocks; adjust to pH 7.4 with 1 M HCl and make up to 200 ml.

Solution C: To 20 ml water; add 2.5 ml, 13 ml, 2.5 ml and 6.0 ml, respectively of Tris, KCl, NaCl and EGTA stocks; adjust to pH 7.4 with 1 M HCl and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Any swinging-bucket rotor capable of approx 200,000 *g* with a tube volume of 13 ml, e.g. Beckman SW 41 or Sorvall TH641 (**see Section 5.2**)

4. Protocol

Carry out all operations at 0-4°C.

1. Produce an homogenate from the chosen tissue or cells using Solution B (Type I or II). **See Section 5.3 for more information**.

2. Centrifuge the homogenate at 1000 *g* for 10 min (**see Section 5.4**).

3. Centrifuge the supernatant at 3,000 *g* for 10 min and keep the supernatant for subsequent gradient separation (**see Section 5.4**).

4. **For a discontinuous gradient**: Prepare 10 ml each of 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 30% (w/v) iodixanol solution by mixing the appropriate volumes of Solutions B and D (Type I or II). **For a continuous gradient**: Prepare 20 ml of the 2.5% and 30% solutions (**see Section 5.5.1**).

5. In 13 ml tubes for the swinging-bucket rotor **(discontinuous gradient)**: layer 1.2 ml each of the nine gradient solutions (see Notes 10 and 11); **(continuous gradient)**: use a two-chamber gradient maker or Gradient Master™ to make an approx. 12 ml gradient from equal volumes of the 10% and 30% iodixanol solutions (**see Section 5.5.2**).

6. Layer the vesicle suspension on top of the gradient and centrifuge at 55,000 *g* for 90 min or 126,000 *g* for 25 min (**see Section 5.6**).

7. Collect the gradient in 0.5 fractions by tube puncture, aspiration form the meniscus or upward displacement. For more information on harvesting gradients **see Application Sheet S08**.

8. Analyze the fractions as appropriate (for examples of analyses from the published literature **see Section 5.7**)

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. Although 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and containing 1 mM EDTA 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. Rather more unusually hamster embryo fibroblasts were homogenized in phosphate-buffered saline [3].

If a hypoosmotic medium is required to swell the cells in order to achieve an adequate degree of homogenization it is important to return the homogenate to isoosmotic conditions as soon as possible. Schroder et al [2] for example washed CHO cells first in an isoosmotic sucrose buffer (0.25 M sucrose, 1 mM EDTA, 10 mM triethanolamine-HOAc, pH 7.4); suspended $3x10^6$ cells in 0.5 ml of 83 mM sucrose (in the same EDTA-buffer) and allowed them to swell for 3 min at room temperature. This step is often carried out on ice, but more efficient swelling may be obtained at the higher temperature. Homogenization (see 5.3) was performed by two passages through a 28G needle. Isoosmolality was reestablished by adding an equal volume of 415 mM sucrose before three additional passages through the needle was carried out. Human meningioma cells were swollen in 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 for 15 min on ice before being disrupted in a in a Dounce homogenizer [4]. Other examples of homogenization media are given in **Application Sheets S05 (tissues) and S06 (cells).**

The composition of Solution C is designed so that when Solution D is diluted with Solution B the concentrations of important additives such as EDTA or EGTA are constant. Protease inhibitors may be included in Solutions B and C at the operator's discretion. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

5.2. Ultracentrifuge rotors

Many of these separations have been performed in 13 ml tubes for a Beckman SW41 type rotor. Other swinging-bucket rotors or even vertical rotors may be used. Larger volume swinging-bucket rotors may not require longer centrifugation times (they normally have similar sedimentation path lengths to the SW41) but smaller volume rotors and vertical rotors will certainly need shorter times. Gradients and sample volume should be scaled up or down proportionately as required. Note however that the progressive change in gradient density profile (due to diffusion and sedimentation of the iodixanol molecules) may also be modulated in other rotors and affect the final resolution.

5.3. Homogenization

The homogenization protocol should be 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 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. Differential centrifugation

Nuclear pelleting may be carried out at 500-3000g for 5-10 min; sometimes the homogenate is centrifuged sequentially at 1000g/10 min and 3000g/10 min [1]. Exposure of the homogenate to 3000g will remove some of the mitochondria, which can facilitate subsequent layering of the sample on the gradient. In one instance a higher RCF (g-force) was used (17,000g for 15 min) prior to gradient loading [5].

To recover any vesicles trapped in the pellet (more serious at the higher RCFs), the pellet is sometimes resuspended in HM, recentrifuged and the two supernatants combined. A possible disadvantage of this practice is that unless the resuspension of the pellet is carried out very gently to avoid damage to the organelles. If the nuclei are damaged consequent leakage of DNA may lead to almost irreversible aggregation of the subcellular membranes.

As the separation is based on sedimentation velocity, it is important that the volume of sample applied to the top of the gradient should be <10% of the gradient volume. This is why there is a tendency to keep the homogenate volume to an absolute minimum. If the homogenate volume is inconveniently large then the membrane vesicles might be sedimented at approx 100,000g for 30 min prior to resuspension in a smaller volume of buffer, but this has rarely been reported for this method. The necessary re-homogenization of the pellet may lead to further reductions in vesicle size – with unpredictable consequences for the subsequent gradient separation.

5.5. Density gradients

5.5.1. Alternative formats

The discontinuous gradients that have been reported in published papers all conform to the format described in this OptiPrep™ Application Sheet. Continuous gradients are slightly more variable; for example, for CHO cells a 10-22.5% (w/v) iodixanol gradient has been used [2] and a 0-40% gradient for MDCK cells [5]. A summary is given in Table 1.

5.5.2. Construction

Discontinuous gradients are normally most easily prepared by underlayering (i.e. low density first) using a syringe (1-2 ml) 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. 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. 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.

5.6. Centrifugation conditions

As far as is known the optimal centrifugation time and RCF have not been thoroughly investigated. Commonly used regimes are summarized in Table 1.

Table 1 Centrifugation conditions for various cell and tissue types

Cell/tissue type	Gradient (% iodixanol)	Centrifugation	Ref #
Brain (mouse)	2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30%	126,000g/30 min	6, 7
CHO	10-22.5% continuous	54,000g/90 min	2
Embryo fibroblasts (hamster)	2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30%	126,000g/25 min	8
		126,000g/30 min	3, 9
MDCK	0-40% continuous	85,000g/45 min	5
Meningioma (human)	2.5-30% continuous	126,000g/30 min	4
Neuroblastoma	2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30%	126,000g/25 min	10
Vero	2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30%	126,000g/25 min	1
Xenopus laevis pituitary	10-30% continuous	26,000g/90min	11

5.7. Analysis

In most cases these gradients have been used as a simple, rapid means of separating ER and Golgi, which band predominantly in the bottom third and between one third and halfway down the gradient respectively, although these positions may vary with the cell type and/or the gradient. When the iodixanol concentration top of the gradient was 10% (w/v) the Golgi and ER banding from CHO cells was predictably shifter upwards [2]. MDCK microsomes run on a 0-40% (w/v) iodixanol continuous gradient (85,000g for 45 min) display in addition a small vesicle fraction at the top of the gradient that contained early endosomes and TGN [5]. Interestingly when a light mitochondrial fraction was substituted for the microsomes, the Golgi banded at the top, while the ER was approx. half way down the gradient.

These gradients can also achieve further resolution. An example of this is given in Figure 1, taken from ref 10. Blots of SDS-PAGE gels of gradient fractions were probed with calnexin (ER marker), βCOP (*cis*-/medial-Golgi marker), rab8 (TGN vesicle marker). Early endosomes, as identified by rab5 show a unique biphasic distribution, locating to both low- and high- density fractions. There is evidence however that fine detail of distribution in sedimentation velocity gradients may vary with the cell type or with the homogenization protocol (or both).

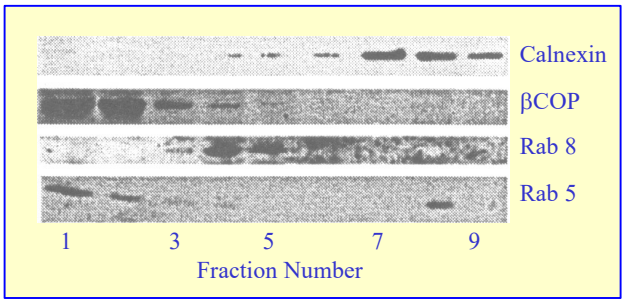

Figure 1 Subcellular fractionation of neuroblastoma (N2a) cells on a 2.5-30% discontinuous iodixanol gradient: immunoblotting of SDS-PAGE on gradient fractions (high density on right). See text for more details. Reproduced from ref 10 with kind permission of the authors and Blackwell Publishing

With HEK293 cells for example both βCOP and rab8 overlapped at the top of the gradient (Figure 2a), although as with neuroblastoma cells, the βCOP tended to be associated with the least dense compartment [3]. Membrin was used as a Golgi marker in this study and although the vast majority was (as expected) in a compartment less dense than the ER, a minor fraction partly co-banded with the ER. This may reflect the function of membrin as an ER-Golgi SNARE. The biphasic distribution of membrin was even more pronounced in fractions from meningioma cells (Figure 2b). In this study Protein 4.1B (ΔU2) was identified at the top of the gradient (plasma membrane) while an E^{465}-S^{851} mutant of this protein was confined to the denser part of the gradient [4].

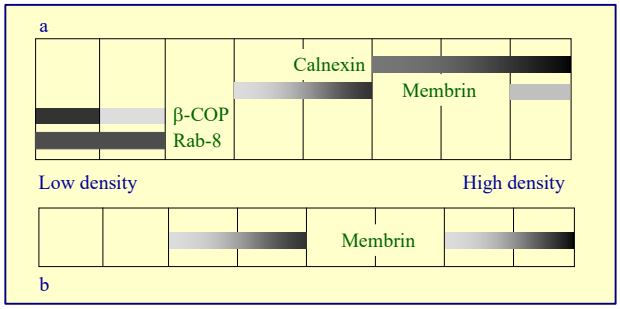

Figure 2 Approximate distribution of markers in iodixanol density gradients: Panel a, HEK293 cell membranes in a 2.5-30% (w/v) discontinuous gradient, data adapted from ref 3; Panel b, human meningioma cells in a 2.5-30% continuous gradient, data adapted from ref 4. For more information see text.

- The fractionation pattern from *Xenopus laevi* [11] was broadly similar to the mammalian examples, in a 10-30% iodixanol gradient, the Golgi banded close to the top and the ER approx. in the middle of the gradient. Interestingly distinctive banding patterns (in the Golgi region) were displayed for p24 putative cargo receptors and for the pro-opiomelanocortin prohormone from white-adapted and black-adapted animals.

6. References

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