# OptiPrep™ Application Sheet S43

## Endocytosis of ligands: fractionation of clathrin-coated vesicles, endosomes and lysosomes by buoyant density in a self-generated gradient

- 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 Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5)
- There are two relevant **Axis-Shield Mini-Reviews: MS13 "Endocytosis – a bibliographical review"** which lists all the relevant papers reporting the use of OptiPrep™ and **MS14 – "Endocytosis analysis – a review of density gradient methods"**: to access these files return to the initial list of Folders and select "**Mini-Reviews**".

### 1. Background

By using the ability of iodixanol to form self-generating gradients, the resolution of endosomes can be accomplished simply by mixing a heavy-mitochondrial supernatant (or any other suitable fraction containing endosomal vesicles) with iodixanol to an appropriate starting concentration and centrifuging in a vertical rotor for 1-2 hours. From homogenization to collection of fractions takes less than 3 hours. Self-generated gradients offer the potential for high resolution since the migration of particles within the centrifugation tube is not impeded by any interface, thus the possibilities for particle aggregation are minimized.

### 2. Solutions required (see Box→ and Section 5.1)

**A.** OptiPrep™

**B.** Diluent: 0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCl, pH 7.4

**C.** Working solution of 50% iodixanol (ρ = 1.272 g/ml): 5 volumes of Solution A + 1 volume of Solution B.

**D.** Homogenization medium: 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4

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 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.

Solution D: 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.

### 3. Ultracentrifuge rotor requirements (see Section 5.2)

A vertical or near vertical rotor capable of approx 350,000g (e.g. Beckman VTi65.1, VTi65.2, NVT65, NVT90 or Sorvall TV1665, 65V13 or 70V6), or a high-performance fixed-angle rotor with a tube capacity of <6ml (e.g. Beckman 80Ti with 4.2 ml g-Max tubes)

### 4. Protocol

Carry out any ligand binding, uptake and processing, as required using the tissue or cell system of choice. Subsequently all operations must be carried out at 4°C.

1. Homogenize the tissue (or cells) in Solution D: for mammalian liver use 6-8 strokes of the pestle of a Potter-Elvehjem homogenizer (500 rpm). Use about 4 ml per gram of tissue (see Section 5.3).

2. Centrifuge the homogenate in a swinging-bucket rotor at 3000 $g_{av}$ for 10 min. The pellet may be washed with Solution D if necessary and the two supernatants combined.

3. Make a 20% iodixanol solution (ρ = 1.127 g/ml) by diluting Solution C with Solution D.

4. Dilute the 3000 *g* supernatant with Solution C, 3:1 (final concentration = 12.5% iodixanol).

5. Transfer approx 9 ml of the suspension to a suitable tube (10-12 ml) for a vertical or near-vertical rotor; underlay with 1.5 ml 20% iodixanol and overlay with Solution D to fill the tube (see Section 5.4).

6. Centrifuge at approx 350,000 $g_{av}$ for 1.5 h (slow acceleration program to 800 rpm). Use a slow deceleration program (or no brake) from 800 rpm.

7. Collect the gradient by tube puncture, upward displacement with a dense medium or aspiration from the meniscus in approx 0.5 ml fractions and analyze as required (see Section 5.5).

8. If it is necessary to remove cytosolic proteins from the fractions and/or to concentrate them, dilute with an equal volume of buffer and sediment the membranes at approx 350,000*g* for 15 min (see Section 5.6).

♦ Information regarding the expected resolution of endocytic compartments can be found in Sections 5.7 and 5.8

## 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) and containing 1 mM EDTA is still a widely used HM for both tissues and cultured cells, for the latter in particular, 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 required to swell the cells in order to achieve adequate 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)**.

Protease inhibitors may be included in Solutions B and C at the operator's discretion. Methods for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

### 5.2 Ultracentrifuge rotors

The sedimentation path length of the tube should be <24 mm. The protocol provides centrifugation times and g-forces for 11 ml Optiseal™ tubes for the Beckman VTi65.1, they may need to be optimized to produce the required iodixanol density gradient in other rotors. Smaller volume rotors can be used with little or no modification to the protocol, but larger volumes may require significantly longer centrifugation times. For more information on the rotor requirements for self-generated gradients see Application Sheet S04.

### 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 Sealed tube preparation for vertical and near-vertical rotors

Beckman Optiseal™ tubes are certainly the tubes of choice for these rotors. Note that all sealed tubes must be filled in accordance with the manufacturer's recommendations and volumes should be scaled up or down proportionately for larger or smaller volume tubes. If a near-vertical rotor is used, the 20% iodixanol cushion may be omitted; it is present in tubes for vertical rotors to prevent any dense particle reaching the wall of the centrifuge tube. Use of an overlay is a convenient way of filling the tube.

### 5.5 Unloading sealed tubes

Heat sealed tubes may only be conveniently unloaded by tube puncture, unless the top of the tube is cut off. The latter procedure may however cause considerable disturbance to the gradient and cannot be recommended. Beckman Optiseal™ tubes however may be unloaded by any of the routine methods. For more information on harvesting gradients **see Application Sheet S08**.

### 5.6 Cytosolic proteins

By removing cytosolic proteins after fractionation, endosomes need not be pelleted and resuspended prior to separation. Small volume open-topped thick-walled tubes for a microultracentrifuge are a convenient way of recovering sedimented membrane fractions. Do not use more than 15 min at 350,000 *g* or 1-1.5 h at 100,000 *g* otherwise sedimentation of the iodixanol molecules themselves may interfere with pellet formation.

### 5.7 Gradient analysis

$^{99m}$Tc labeled neogalactosylalbumin, a ligand that is taken into hepatocytes by the asialoglycoprotein receptor, was injected into a perfused rat liver system for 1 min and then chased with cold medium, for periods up to 20 min. After homogenization of the blanched liver, a 3000 *g* supernatant was processed according to the protocol described in this Application Sheet [1].

The gradients were analyzed for radiolabel (see Figure 1). After a 1 min chase the radiolabel peaked at a density of approx 1.105 g/ml and after a further 1 min chase moved to a marginally lower density (approx 1.10 g/ml). The fractions in this region contained clathrin (not shown). After a 10 min chase there was a pronounced shift in the main radiolabel-containing material to a much lower density (approx 1.075 g/ml) region, which was devoid of clathrin. After a 20 min chase the radiolabel had moved into a denser compartment, which was coincident with the acid phosphatase.

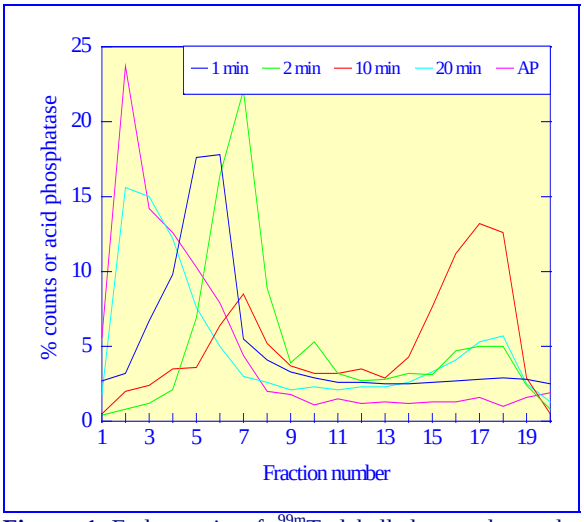


**Figure 1** Endocytosis of $^{99m}$Tc-labelled neogalactosyl-albumin by perfused rat liver: effect of chase time of perfusion. Distribution of radiolabel and acid phosphatase (AP) in self-generated iodixanol gradients. High density on left.

The data is interpreted as follows: (1 min) the ligand is found initially in a dense clathrin-coated vesicle; (2min) uncoating of the vesicle reduces its density; (10 min) the ligand has been transferred to a low density endosome and (20 min) the ligand appears in a lysosome or pre-lysosomal compartment. The data is discussed in more detail in ref 1.

Under the recommended conditions, the gradient contains a central shallow region that separates light and dense endosomes. Lysosomes band in the sharp gradient formed at the bottom of the tube, while

mitochondria and peroxisomes band below the lysosomes. Plasma membrane (not shown) bands between the lysosomes and the densest endosomes (Figure 1). For a more linear gradient use longer centrifugation times. The minimum *g*-force required for efficient self-generation of iodixanol gradients is 180,000 $g_{av}$ but linear gradients are difficult to achieve at this low g-force.

To subfractionate the early clathrin-coated vesicles and the plasma membrane, which tend to have a high density, the starting concentration of iodixanol should be increased to 15% or 17.5% (w/v). The plasma membrane, which has been identified in this region, (see ref 1) also contains clathrin and accounts for its relatively high density. To analyze more effectively the low-density endosomes, the starting concentration of iodixanol might be reduced to 10% (w/v).

A second example of the use of this gradient system is taken from Molinari et al [2], who separated late endosomes, vacuoles and lysosomes in their studies on the vacuolation induced in HBK cells by *Helicobacter pylori* vacuolating toxin. In Figure 2 the β-glucosaminidase and Rab7 profiles identify the lysosomes in the dense region of the gradient (principally fractions 1-3) while the late endosomes peaked at a much lower density around fraction 9. CI-M6PR is a late-endosome and *trans*-Golgi network marker, and shows that the latter bands at a slightly lower density (peak fraction 4) than the lysosomes. This separation was carried out in a Beckman NVT90 near-vertical rotor.

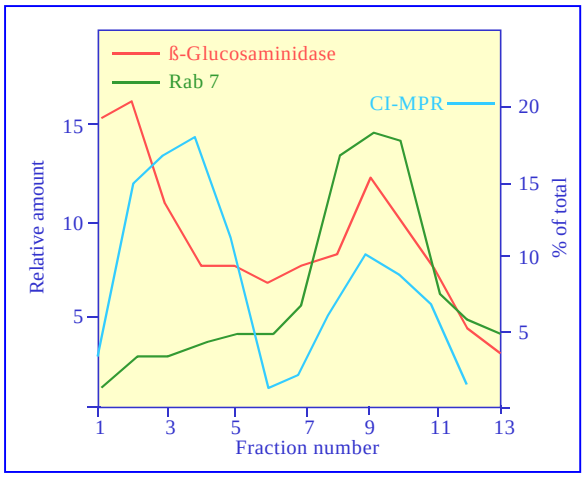


**Figure 2** Distribution of markers from BHK cell post-nuclear supernatant in self-generated iodixanol gradient: 12.5% iodixanol at 387000$g_{av}$ for 130 min, dense end on left. β-Glucosaminidase and Rab7 expressed as relative amount, CI-M6PR as % of total. For more information see text. Adapted from ref 2 with kind permission of the authors and the American Society of Biochemistry and Molecular Biology.

### 5.8 Other self-generated gradient methods

Gradients comprising three layers rather than two have also been reported: 1.2 ml of 10%, 1.3 ml of 20% and 2.4 ml of 30% (w/v) iodixanol, the latter containing a 3000 *g*–10 min supernatant from CHO cells was used to create a gradient that was very close to linear in a Beckman NVT90 near-vertical rotor centrifuged at 350,000 *g* for 3 h. The gradient was used to analyze early and recycling endosomes [3]. A more standard strategy of starting with a solution of uniform density (30% iodixanol) centrifuged for 4 h at 365,000 *g* separated the ER and TGN from endosomes in a study of progression of endosomal transport [4].

Landry et al [5] used a similar gradient system to that described by Chen et al [3]; 2.5 ml of a HeLa cells post-nuclear supernatant (PNS) was adjusted to 30% iodixanol (2.5 ml total); layered under approx. 1.2 ml each of 20% and 10% iodixanol and centrifuged at 360,000 *g* for 3 h. The system was used to demonstrate that cell death signaling is associated with a diversion of recycling endosomes trafficking to the Golgi. In a simpler gradient system the PNS from a population of brain cells was simply adjusted to 13% iodixanol; the self-generated iodixanol gradient was used to separate early and late endosomes in a study of norepinephrine transporter trafficking [6]. Self-generated gradients have also been used in a study of the uptake by macrophages of the virulence antigen during infection by *Yersinia pestis* [7].

Lampugnani et al [8] used a strategy first described by Yeaman et al [9] to create a virtually linear gradient by using equal volumes of 10%, 20% and 30% (w/v) iodixanol, in tubes for the Beckman Vti65.1 vertical rotor, centrifuged at 350,000 *g* for 3 h. The gradients analyzed the internalization of vascular endothelial cadherin by endothelial cells and were able to provide distinctive banding patterns for plasma membranes, clathrin-coated vesicles and early endosomes.

A low-density membrane fraction from 3T3-L1 adipocytes, adjusted to 14% (w/v) iodixanol, was fractionated in a gradient, self-generated at 295,000 *g* for 1 h in a Beckman TLN100 rotor [10]. The gradient was able to resolve the constitutive recycling pool (endosomal recycling compartments) and the insulin-sensitive GLUT4 storage vesicles.

## 6. References


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