OptiPrep™ Application Sheet S35

Separation of membrane-bound and cytosolic proteins (flotation through a discontinuous gradient)

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **OptiPrep™ Reference List RS13** "**The resolution of soluble cytosolic proteins from membrane vesicles and organelles: a bibliography**" provides a 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 particle type.**
- **To access other Application Sheets** referred to in the text: return to the **2020SMemapp** file and select the appropriate S-number.
- **Sections 5.1-5.5** contain some important technical notes for processing mammalian cells
- **Section 5.6.1** lists some of the gradient variations used with different mammalian cells; **5.6.2.** and **5.6.3** deal with bacteria and yeast cells respectively. **For plant cells see Application Sheet S61**.

1. Background

There are many situations where it is necessary to provide an efficient separation of membrane vesicles from the cytosol in order to distinguish the location of a protein to one or other compartment. Permeabilization of cultured cells to release cytoplasmic vesicles of the exocytic pathway and the isolation of vesicles budded from the plasma membrane by selective hypoosmotic cell disruption also require subsequent resolution of these vesicles from the cytosol which is also released.

A number of protocols have been published which all rely on the same strategy; i.e. flotation of the vesicles through a gradient (usually discontinuous, but sometimes continuous) of either Nycodenz® or iodixanol from a dense load zone. An early example of this flotation strategy was elaborated and widely used for isolation of budded vesicles from yeast. The crude vesicle containing fraction was adjusted to approx 37% (w/v) Nycodenz®; 7 ml of this overlaid with 3 ml each of 35%, 25% and 15% Nycodenz® and centrifuged at 100,000 *g* for 12 h [1-3]. Later the Nycodenz® flotation strategy was applied more widely to the separation of vesicles and cytosol [4-7].

More recently the method has been adapted to the use of OptiPrep™ and because of ease of use of this medium compared to Nycodenz®, it has become the method of choice and it is the one that is described in this Application Sheet. Another advantage of iodixanol gradients is that because of the lower osmolality of iodixanol solutions, compared to those of Nycodenz®, the difference in density between proteins and vesicles is enhanced. In iodixanol proteins have a density of approx. 1.26 g/ml, while that of membrane vesicles is generally < 1.13 g/ml, thus if the crude vesicle-containing fraction is adjusted to 30% (w/v) iodixanol (approx 1.16 g/ml) and overlayered with 25% iodixanol (approx 1.14 g/ml) then the vesicles will float through the lower density layer and the proteins will tend to sediment from the load zone. This is an ideal way of separating the two compartments, which are separated by the median-density layer. A typical fractionation is described diagrammatically in Figure 1. Generally higher *g*-forces and shorter centrifugation times are used.

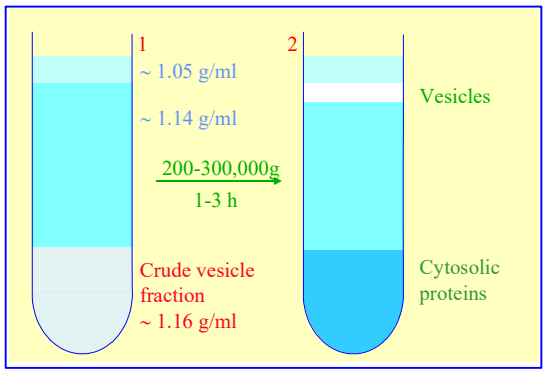

Figure 1 Separation of vesicles and cytosolic proteins from mammalian cultured cells by flotation in a discontinuous iodixanol gradient [8,9]

2. Solutions required

Choose Type I or Type II (**see Section 5.1**)

2a. Type I

A. OptiPrep™

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

C. OptiPrep™ 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

2b. Type II

A. OptiPrep™

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

C. OptiPrep™ Diluent: 130 mM KCl, 25 mM NaCl, 6 mM EGTA, 150 mM Tris-HCl, 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:

Type I

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

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 15 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

Swinging-bucket rotor (e.g. Beckman SW55 or Sorvall TH660; the procedure can be scaled down or up as a required. For smaller volumes use the Beckman TLS55; for larger volumes use the Beckman SW41 or Sorvall TH641.

4. Protocol

Carry out all operations at 0-4°C.

If the gradient is being used to isolate budded vesicles or cytoplasmic vesicles from permeabilized cells then remove the cells from the vesicle-containing suspension either in a microcentrifuge or by centrifugation at 1000 *g* for 5 min in a low-speed centrifuge and use the supernatant for Step 3.

1. Suspend the washed cells in Solution B and disrupt them by Dounce homogenization, repeated passages through a fine syringe needle or a ball-bearing homogenizer (**see Section 5.2**).

2. Centrifuge the homogenate at 1000 *g* for 15 min and aspirate the supernatant (**see Section 5.3**).

3. Adjust the supernatant to 30% (w/v) iodixanol by thorough mixing with Solution D (volume ratio of 2:3 respectively).

4. Prepare solutions containing 25% and 5% (w/v) iodixanol by diluting Solution D with Solution B (volume ratios of 1:1 and 1:9).

5. In tubes for a swinging-bucket rotor layer 2 ml each of the crude vesicle fraction in 30% iodixanol and the 25% iodixanol and fill the tube by overlayering with 5% iodixanol (**see Section 5.4**).

6. Centrifuge at approx 250,000 *g* for 3 h and collect the vesicles which band at the top interface and the bottom layer containing cytosolic proteins (**see Section 5.5**). Alternatively collect the gradient in 0.5 ml fractions by tube puncture, upward displacement or aspiration from the meniscus. For more information on harvesting gradients see **Application Sheet S08**.

♦ **Section 5.6 lists some of the cell types and gradient formats that have been used**.

5. Technical Notes and Technological Review

5.1 Solution strategies for mammalian cells

The solutions that are recommended are general purpose ones for the homogenization of cultured cells and tissues. The homogenization medium often has to be tailored to the tissue or cell type but it is unlikely that the composition of the HM is relevant to the separation described in this OptiPrep™ Application Sheet. Organic osmotic balancers such as sucrose **(Type I)** 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. The most commonly used isoosmotic HMs contain 0.25 M sucrose buffered either with Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and often, but not always, containing 1 mM EDTA or EGTA.

Supplementation of the HM 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 **(Type II)** that omit sucrose entirely use either NaCl or KCl or both as the principal osmotic balancer(s). Often a frankly hypoosmotic medium is needed used to swell the cells and so facilitate homogenization. Some other examples of homogenization media for cultured cells are given in **Application Sheet S06**.

In cases where the gradient is being used to isolate either budded vesicles or cytoplasmic vesicles released by the permeabilization of cultured cells special solutions may be used. A 140 mM KCl, 10 mM Hepes-KOH, pH 7.2 solution containing either 2 mM EGTA and 1 mM DTT or 2.5 mM $Mg(OAC)_2$ was used for budded vesicles [1] or vesicles from permeabilised cells [2] respectively.

The preparation of a Working Solution (Solution D) using an OptiPrep™ diluent as described, ensures that the concentrations of reagents such as EDTA, EGTA and the buffer are constant throughout the gradient. The concentrations of the osmotic balancers such as sucrose, KCl or NaCl are not similarly raised in the OptiPrep™ diluent; if they were, the solutions would be grossly hyperosmotic. If the maintenance of constant EDTA, EGTA and buffer concentrations is deemed unimportant, the gradient solutions may be prepared directly from OptiPrep™. For more information about preparing gradient solutions **see Application Sheet S01**.

Protease inhibitors may be included in Solutions B and C at the operator's discretion.

5.2 Homogenization

Dounce (or sometimes Potter-Elvehjem) homogenization was the most widely used procedure at one time but the ball-bearing homogenizer or "cell cracker", with the standard 0.3747 in (9.52 mm) ball bearing, 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 26) is usually an efficient alternative. Sometimes the efficacy of this method is improved by switching to a second finer syringe needle for half the passes. 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 other hints on homogenization are given in **Application Sheet S06**.

5.3 Differential centrifugation

Although centrifugation at 1000*g* will remove the nuclei, there is no obvious reason why this step should not be carried out at 3000*g* to remove some of the mitochondria as well.

5.4 Preparing the discontinuous gradient

As with all flotation methods a small volume of buffer or 5-10% iodixanol is always layered on top of the density barrier to prevent banding of the vesicles at an air/liquid interface. The latter often leads to adherence of particles to the wall of the tube and aggregation. Sometimes the layer of 5% iodixanol is replaced by the lysis buffer. For information on preparing gradients **see Application Sheet S03**.

5.5 Collection of fractions from the gradient

Small volume gradients may be divided into three of four zones simply by very careful aspiration using a syringe and metal cannula (i.d. approx 0.8 mm); an automatic pipette may be used but the end of the pipette tip should be cut off to enlarge the orifice.

5.6 Review of methodology

5.6.1 Mammalian cells

Some of the cells and tissues that have been analyzed by this flotation strategy, the gradient format and centrifugation conditions are listed in Table 1. There is no obvious reason why more than three layers are required for a simple separation of the total membrane vesicle fraction from the cytosol, unless it is important to discriminate between vesicles of different density.

Cell type	% iodixanol gradient[1]	RCF (time)	Ref#
Brain tissue	30%, 25%, 5%	200,000 *g* (2 h)	10
	36%, 31%, 5%	199,000 *g* (3 h)	11
293T	50%, 40%, 10%	200,000 *g* (2 h)	12
	50%, 40%, 30%, 20%	170,000 *g* (4 h)	13
	40%, 28%, 0%	165,000 *g* (3 h)	14
CHO	30%, 25%, 10%	170,000 *g* (3 h)	9
COS7	30%, 25%, 5%	200,000 *g* (2 h)	15
N2A	14%, 12%, 6%	52,000 *g* (1.5 h)	16
	36%, 31%, 5%	199,000 *g* (3 h)	11
MCF-7	30%, 25%	166,000 *g* (1 h)	17
Huh-7	35%, 30%, 25%, 5%	160,000 *g* (4 h)	18
MDCK	30%, 25%, 5%	170,000 *g* (3 h)	8
PC3	36%, 31%, 5%	186,000 *g* (3 h)	19
SH-SY5Y	40%, 30%, 5%	200,000 *g* (2 h)	20
	35%, 30%, 5%	200,000 *g* (2 h)	21
Vero	35%, 30%, 0%	165,000 *g* (4 h)	22
	35%, 30%, 0%	128,000 *g* (4 h)	23

Table 1: Selected papers describing the use of pre-formed discontinuous iodixanol gradients for separation of membranes and cytosolic proteins from mammalian tissues and cells

[1] The sample is always in the highest density iodixanol solution

In an analysis of vesicles budded from NRK cells Joglekar et al (2003) top-loaded a 7%, 50% discontinuous gradient (100,000 *g* for 40 min), the top layer containing cytosolic proteins was then removed; the remainder mixed and layered under a 5-25% (w/v) continuous iodixanol gradient centrifuged at 100,000 *g* for 90 min to separate the ER and Golgi [24].

A top-loaded discontinuous gradient of 2% and 50% (w/v) iodixanol (100,000 *g* for 1 h) was used to establish that IL-32 was almost exclusively membrane-bound [25]

5.6.2 Bacteria

De Leeuw et al [26] devised a basically similar strategy for separating the membranes and soluble protein fractions from *E. coli*. A membrane pellet in 500 mM KOAc, 5 mM $Mg(OAc)_2$, 50 mM Hepes-KOH, pH 7.6 was mixed with 0.25 M sucrose, 50% iodixanol in the same buffer (volume ratio of 0.15:1.05) and overlaid with 5.8 vol. of 125 mM sucrose, 30% iodixanol in the same buffer and 3 vol. of buffer. The gradient was centrifuged at 166,000 *g* for 3 h. This system has been frequently used [e.g. 26-28].

5.6.3 Yeast

Although in most cases yeast cells are homogenized via spheroplast formation, they are occasionally homogenized with glass beads with a lysis buffer of 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4 [29]. The clarified lysate, adjusted to 40% (w/v) iodixanol, overlaid by 30% iodixanol and the lysis buffer was centrifuged at 55,000 *g* for 2 h.

Medkova et al [30] prepared spheroplast lysates in a 20 mM triethanolamine-acetate buffer, pH 7.2, containing 0.8 M sorbitol and 1 mM EDTA, which were clarified at 10,000*g* for 10 min. The lysates were adjusted to 40% (w/v) iodixanol and overlaid by 35% iodixanol. The iodixanol solutions

contained 0.4 M sorbitol and 1 mM EDTA in the same buffer and the gradients were centrifuged in a small volume fixed-angle rotor (Beckman TLA120.2) at 100,000 *g* for 3 h. Medkova et al [30] included 50 mM MES, pH 6.5 in the lysate and 20 mM MES in the 35% iodixanol solution to stabilize the membrane-protein association.

Although sorbitol is a common component of yeast lysates, it is by no means universally included. Wang et al [31] used the same buffered NaCl/EDTA solution as Ge et al [29]. The lysate was adjusted to 40% (w/v) iodixanol by simple addition of half the volume of Optiprep™, overlaid with 30% iodixanol and lysis buffer and centrifuged at 200,000 *g* for 5 h in a small volume Beckman TLS55 rotor.

More complex discontinuous iodixanol gradients have also been used [32]; the spheroplast lysate was adjusted to 37% (w/v) iodixanol and layered beneath, 30%, 25%, 19%, 0% iodixanol and centrifuged at 75,000 g_{av} for 4 h to separate cytosolic and vacuolar fractions. A continuous gradient was generated in a Gradient Master™ from equal volumes of lysis medium (400mM Sucrose, 100mM NaCl, 20mM HEPES pH7.2) and a lysate (adjusted to 27% w/v iodixanol) was centrifuged for 16 h in a Beckman SW41 rotor at 190,000 *g*. It resolved low density lysed vacuole membranes from denser membranes and soluble cytosolic proteins at the bottom of the gradient [33].

6. References

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