OptiPrep™ Application Sheet S38

Isolation of secretory granules from pancreatic cells

- 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 (see Section 4)
- Cultured murine βTC6-F7 cells, mouse pancreatic β cells and insulinoma cells have been used in the study of insulin secretion; all three are presented in this Application Sheet.
- See Section 4.9 for information on exocrine (acinar) cell granules

1. Background

A crude mitochondrial/secretory granule fraction from pancreatic islet β cells can be obtained as a pellet by a simple centrifugation of a post-nuclear supernatant over a 0.3 M sucrose barrier at 15,000 rpm for 12 min [1]. Sucrose gradients are however not particularly successful for preparing secretory granules at relatively high purity and yields, principally because the high osmolality of the gradients causes all osmotically active organelles to approach a limiting density.

Protocols using Percoll® gradients are able to resolve intact granules from mitochondria, peroxisomes, endoplasmic reticulum and plasma membrane [2], all of which are significantly less dense than the granules in isoosmotic Percoll® gradients. In these gradients however, lysosomes have a significantly higher density than the other major organelles and consequently tend to overlap the granule fraction significantly. Hutton et al [2] ameliorated this problem by carrying out two Percoll® gradients, the first low-density gradient to obtain a granule/lysosome fractions and then a second denser gradient to remove the lysosome contamination. Even so, the final granule fraction contained 12% of the total lysosomes and was enriched over the homogenate in the lysosome marker enzyme, aryl sulphatase, some nine-fold. Another disadvantage of using Percoll® is that it is necessary to wash the pelleted granule fraction from the final gradient three times to remove the colloidal silica particles.

2. Gradient selection

Continuous iodixanol gradients appear to be the most successful in resolving the insulin containing granule fraction from both a cultured murine cell line, βTC6-F7 [3] and also from mouse pancreatic β (islet) cells [4,5]. Buchanan et al [3] found that the Percoll® based technology was unsatisfactory for cultured cells and developed a single-step iodixanol gradient purification, which provides both a purer product and also avoids repeated centrifugations to remove the gradient medium.

3. Solutions required (see Box and Section 4.1)

A. OptiPrep™

B. Homogenization medium: 0.3 M sucrose, 1 mM K-EDTA, 1 mM $MgSO_4$, 10 mM MES-NaOH, pH 6.5

C. OptiPrep™Diluent: 0.3 M sucrose, 6 mM K-EDTA, 6 mM $MgSO_4$, 60 mM MES-NaOH, pH 6.5

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

4. Ultracentrifuge rotor requirements

Any swinging-bucket rotor capable of approx 200,000 *g* with tube volumes of 13 ml, e.g. Beckman SW 41 or Sorvall TH641 (see Section 4.2)

Keep the following stock solutions at 4°C:

100 mM MES*	3.90 g per 200 ml water
100 mM $MgSO_4 \bullet 7H_2O$	2.46 g per 100 ml water
100 mM $EDTA \bullet K_2$	3.68 g per 100 ml water

Solution B: Dissolve 20.4 g sucrose in 100 ml water; add 20 ml, 2 ml and 2 ml, respectively of MES, $MgSO_4$ and EDTA stock solutions; adjust to pH 6.5 with 1 M KOH and make up to 200 ml.

Solution C: Dissolve 10.2 g sucrose in 60 ml of MES stock; add 6 ml each of $MgSO_4$ and EDTA stock solutions; adjust to pH 6.5 KOH and make up to 100 ml.

* Note that MES is available with variable states of hydration: the weight of MES above is calculated for the anhydrous molecule.

5. Protocol (adapted from refs 3-5)
Carry out all operations at 0-4°C.

1. Homogenize the cells in Solution B using a ball-bearing homogenizer (see Section 4.3).

2. Centrifuge the homogenate at 500 *g* for 10 min and retain the post-nuclear supernatant (PNS).

3. Prepare 20 ml each of 8% and 19% (w/v) iodixanol by mixing Solutions D and B using volume ratios of 8:42 and 19:31 (see Section 4.4 for alternative gradients).

4. Use a two-chamber gradient maker or Gradient Master™ to make an approx. 12 ml gradient from equal volumes of the 8 and 19% iodixanol solutions tubes for the swinging-bucket rotor (see Section 4.5).

5. Layer the PNS on top of the gradient and centrifuge at 160,000 g_{av} for 16 h (see Section 4.6).

6. Collect the gradient in 0.5-1.0 ml fractions either by tube puncture, upward displacement or aspiration from the meniscus. For more information on harvesting gradients see **Application Sheet S08**.

- For information on analysis of the gradient fractions see Section 4.7.

4. Technical Notes and Review
4.1 Homogenization media

The homogenization medium (HM) often has to be tailored to the tissue or cell type. The most common osmotic balancer is 0.25 M sucrose, although slightly higher concentrations, as in this protocol, are quite common. The use of 0.3 M sucrose in the homogenization medium and in the OptiPrep™ diluent (Solution C), slightly increases the density and osmolality of the gradient solutions, compared to those given in **Application Sheet S01**, which are based on 0.25 M sucrose.

Supplementation of the HM with inorganic salts (containing K^+ or Na^+ ions) is becoming increasingly common in the analysis of endoplasmic reticulum, plasma membrane and Golgi in iodixanol gradients; it can reduce ionic interactions, aggregation between membranes and combat any raised viscosity of the homogenate due to cytoskeletal proteins. Whether the fractionations reported in this OptiPrep™ Application Sheet would benefit from such modifications can only be assessed by experimentation. The inclusion of divalent cations guards against nuclear breakage and stabilizes membranes generally, but in some cases can lead to aggregation.

The preparation of a Working Solution as described, ensures that the concentrations of EDTA, $MgSO_4$ and buffer are constant throughout the gradient. If this is deemed unimportant the iodixanol solutions may be prepared simply by diluting OptiPrep™ with Solution B. The sucrose concentration in Solution C should not be increased 6x, in line with the other components. If it were, the Working Solution would be grossly hyperosmotic. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

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

4.2 Ultracentrifuge rotors

Smaller or larger volume swinging-bucket rotors may be used for smaller or larger amounts of material. It may also be possible to use a vertical or near-vertical rotor; use of these rotors would speed up the separation considerably at the same *g*-force, because of the much shorter sedimentation path length.

4.3 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, as in this protocol, 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. Some other hints on homogenization are given in **Application Sheet S06**.

4.4 Density gradient selection

In a later publication the use of a 12-24% (w/v) iodixanol gradient was described [6]. Most gradient separations are based on buoyant density, but see Section 4.9 for a sedimentation rate option.

4.5 Density gradient preparation

If neither of these gradient-making devices is available then a continuous gradient can be prepared by diffusion of a discontinuous gradient (use equal volumes of 8%, 12%, 16% and 20% (w/v) iodixanol). For more information on gradient construction see **Application Sheet S03**.

Application of this technique to the isolation of granule fractions from other cells may need modification to the density range of the gradient.

4.6 Tube loading

In swinging-bucket rotors of different tube volumes scale up or down the volumes proportionately. If necessary, adjust all volumes (also proportionately) so that tubes are properly filled according to the manufacturer's instructions.

4.7 Gradient analysis

If it is necessary to concentrate a fraction or to remove the iodixanol before analysis, see **Application Sheet S09**.

Figure 1 shows the distribution of granule markers (insulin and amylin), a mitochondrial marker (citrate synthase) and a lysosomal marker (aryl sulphatase) from βTC6-F7 cells in the iodixanol gradient. Both the mitochondria and lysosomes are well resolved from the granule fraction in the steep gradient that is formed towards the bottom of the tube. Only 2% of the lysosomes and 0.9% of the mitochondria were recovered in the granule fraction. Although the continuous gradient originally formed is essentially linear, some sharpening of the gradient at the top and bottom of the tube will always occur at the RCF and time used in this purification, due to sedimentation of the iodixanol molecules themselves (i.e. some degree of self-generation will occur). Note that mitochondria are denser in iodixanol than they are in Percoll®; this accounts for the differences in banding patterns of lysosomes and mitochondria in the two media.

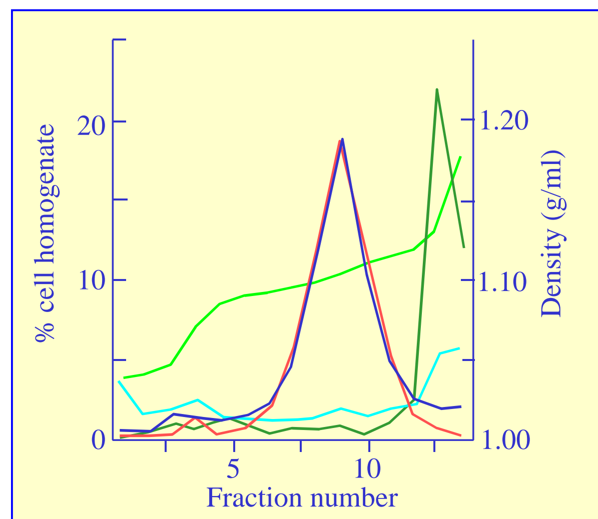

Figure 1 Banding of insulin granules, mitochondria and lysosomes in a continuous iodixanol gradient. Insulin (—), amylin (—), citrate synthase (—), aryl sulphatase (—), density (—); reproduced from ref 3, with kind permission of the authors, © The Biochemical Society.

Using an iodixanol gradient, Buchanan et al [3] investigated the functions and properties of these granules more fully and to identify a new peptide involved in regulating glucose-mediated insulin secretion.

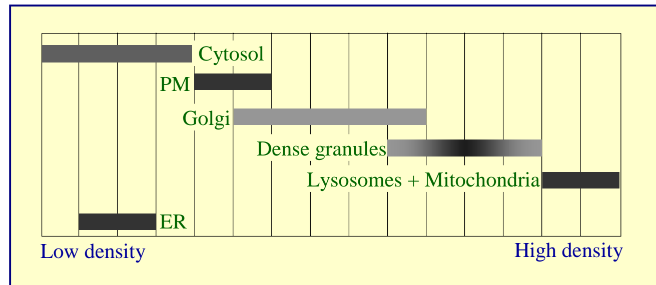

Figure 2 Approx. distribution of membrane compartments from mouse pancreatic β cells in an iodixanol gradient. Data adapted from ref 9: PM = plasma membrane, ER = endoplasmic reticulum

The large dense-core insulin containing vesicles of mouse pancreatic β cells banded in a similar position to the insulin granules of βTC6-F7 cells (Figure 2), close to, but well-resolved from the lysosomes and mitochondria [4]. Varadi et al [4] also identified the cytosolic, plasma membrane (PM) and Golgi containing regions that banded in the lower density regions of the gradient. The only significant overlap of the insulin containing vesicles was observed with the denser Golgi membranes. Endosomes banded close to the top of the gradient. The banding of the various non-granule membrane compartments was more or less in line with that observed by other workers, i.e. the density increased in the order endosomes<PM<Golgi<lysosomes<mitochondria; the ER however was less dense than the PM and Golgi; the reverse is normally the case. Varadi et al [4] identified the SUR1 and Kir6.2 subunits of the ATP-sensitive K^+ channel in the large dense-core insulin containing vesicles and in ref 10 the involvement of myosin Va in secretory vesicle transport.

The original iodixanol gradient described by Buchanan et al [3] was also used by Jurczyk et al [7] in a study on the secretion of insulin granules from pancreatic β cells. A review by Cooper [8] includes observations made on granules isolated by different methodologies.

More recently Chen et al [9] used a discontinuous gradient of 8.8%, 13.2%, 17.6%, 23.4% and 30 % (w/v) iodixanol, the top-loaded sample was centrifuged at 100,000 *g* for 75 min. Granules banded on top of and below the 17.6% layer; the denser granules were more mature [9].

4.8 Sedimentation velocity separation

Although the majority of fractionations are achieved on the basis of particle buoyant density (and there is evidence such separations are more effective when carried out for more than 6 h), the alternative sedimentation velocity strategy, which requires very short centrifugation times, may have advantages if the functions being studied are rather labile. Cao et al [10] homogenized INS-1E insulinoma cells in 0.3 M sucrose, 1 mM EDTA, 1 mM $MgSO_4$, 10 mM MES-KOH, pH 6.5 and layered a PNS on a discontinuous gradient of 30%, 23.4%, 17.6%, 13.2% and 8.8% (w/v) iodixanol (10 ml total volume) and centrifuged it at 100,000 *g* for 75 min. The authors used the gradient to show that PICK1 co-localizes with insulin granules that band approx. in the middle of the gradient.

4.9 Pancreatic exocrine cells

Secretory granules from pancreatic exocrine tumour cells have also been isolated and separated from the lighter ER and from the cytosol in 10-30% (w/v) iodixanol gradients (137,000 *g* for 2h) in a small volume rotor (0.2 ml of PNS on top of a 2 ml gradient) [11,12].

Recently granules have been isolated from cultured pancreatic exocrine cells in a 0-30% (w/v) iodixanol gradient centrifuged at 20,000 *g* for 3 h [13]. Granules banded in the bottom half of the gradient, with a sharp peak at the high density end; treatment of the cells with β-D xyloside broadened the band towards lower densities.

5. References

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