Fractionation of plasma membrane microdomains of the brush border from renal cortex tissue and from glomeruli (slit diaphragms)

- 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.

1. Renal cortex

1a. Background

The resolution of plasma membrane domains, primarily the basolateral and apical domains from polarized tissues such as intestine, liver and kidney and also from polarized cells such as human colon adenocarcinoma (Caco-2) cells and Madin-Darby canine kidney (MDCK) cells, is an important preliminary requirement for studies on how functional dichotomy at the cell surface is achieved. Methods often involve the use of divalent cations. Brush border preparations from, for example intestinal mucosa, treated with 10 mM $MgSO_4$, allow residual basolateral membrane and intracellular membranes to be removed by low-speed centrifugation [1]. The basolateral membranes are then prepared in a separate density gradient protocol. Ellis et al [2] used a modification of this procedure; basolateral and apical membranes from Caco-2 cells were separated in a sucrose gradient and 10 mM $MgCl_2$ was used to remove contaminating intracellular membranes from the basolateral domain band.

Iodixanol gradients are however now being increasingly used to provide the high resolution necessary to purify both the apical and basolateral membranes away from other intracellular membranes, as part of single procedure, sometimes without the use of divalent cations. Methods for the resolution of some other plasma membrane domains may be accessed via the Index.

The high resolving power of iodixanol gradients can however achieve a separation of other plasma membrane microdomains that have hitherto been difficult to study with sucrose gradients. Biemesderfer et al [3] reported that shallow continuous iodixanol gradients are capable of resolving the microvillar and intermicrovillar domains from the renal brush border and it is this technology is presented in this Application Sheet.

- Important technical notes and a summary of the analyses that have been carried out on the membrane fractions are given in **Section 1e**

1b. Solutions required (see Section 5.1)

A. OptiPrep™

B. OptiPrep™ dilution buffer: 0.25 M sucrose, 120 mM Tricine-NaOH, pH 7.8

C. Working Solution of 50% (w/v) iodixanol: mix 5 vol. of OptiPrep™ with 1 vol. of Solution B.

D. Homogenization buffer: 0.25 M sucrose, 20 mM Tricine-NaOH, pH 7.8

E. Gradient solutions: Dilute Solution C with Solution D to produce solutions containing 5%, 15% and 25% (w/v) iodixanol

> Keep Tricine as a 1 M stock solution at 4°C; 17.9g per 100 ml water.
>
> Solution B: Dissolve 8.5 g of sucrose in 50 ml water; add 12 ml of Tricine stock solution; adjust to pH 7.8 with 1 M NaOH and make up to 100 ml.
>
> Solution D: Dissolve 17 g of sucrose in 100 ml of water; add 4 ml of Tricine stock solution; adjust to pH 7.8 with 1 M NaOH and make up to 200 ml.

1c. Ultracentrifuge rotor requirements (see Section 5.2)

Swinging-bucket rotor with 13-14 ml tubes (e.g. Beckman SW41Ti, Sorvall TH641 or similar)

1d. Protocol (adapted from ref 1)
Carry out all operations at 0-4°C.

1. Separate the renal cortex from the excised kidney.

2. Homogenize the tissue in 35 ml of Solution D using a loose-fitting Potter-Elvehjem homogenizer and centrifuge the homogenate at 1900 *g* for 15 min.

3. Aspirate the supernatant and centrifuge at 21,000 *g* for 20 min.

4. The lower well-packed layer of the bipartite pellet contains the major organelles while the upper loosely-packed layer contains the denser microsomes.

5. Aspirate the supernatant plus the loosely packed microsomes using a syringe and metal cannula and centrifuge at 48,000 *g* for 30 min.

6. Resuspend the pellet in the 5% iodixanol solution (10-20 mg protein/ml).

7. Using a two-chamber gradient maker or Gradient Master® prepare linear 15-25% iodixanol gradients (12-13 ml), then layer approx 0.5 ml of the microsome suspension on top to fill the tube (**see Section 5.3**).

8. Centrifuge at 100,000 *g* for 3 h and collect the gradient either by tube puncture, aspiration from the meniscus or upward displacement in 0.5-1.0 ml fractions (**see Section 5.4**).

1e. Technical Notes and Review

Homogenization media and gradient solutions

Protease inhibitors may be included in Solutions B and D at the operator's discretion. The preparation of a Working Solution as described, ensures that the concentration of buffer is constant throughout the gradient. If this is deemed unimportant the 5%, 15% and 25% iodixanol solutions may be prepared by diluting OptiPrep™ with Solution D. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

Ultracentrifuge rotors

These separations have been performed in 13 ml tubes. Other swinging-bucket rotors or even vertical rotors may be used. Larger volume swinging-bucket rotors may require longer centrifugation times but smaller volume rotors and vertical rotors will need shorter times. All volumes should be scaled up or down proportionately. 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.

Density gradients

If neither of these devices is available, form a continuous iodixanol gradient by allowing a discontinuous one to diffuse. 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.

Harvesting the gradient

Methods for the efficient harvesting of density gradients are described in **Application Sheet S08**. If it is necessary to concentrate a fraction or to remove the iodixanol before analysis, **see Application Sheet S09**.

Gradient Analysis

Two major areas of microdomain markers have been identified; one narrow band close to the top of the gradient contains typical microvillar markers such as villin and the Na-P_i co-transporter NaPi-2. Towards the middle of the gradient is broader band that was highly enriched in the Na^+/H^+ exchanger isoform NH3, which was only a very minor component of the villin-containing band [3]. Megalin (and clathrin) were present in both regions [3,4]. The median NH3-containing band was identified as the intermicrovillae microdomain, which was later shown to be highly enriched in myosin VI [5]. Interestingly the distribution of villin in the median region of the gradient was distinctive from that of the myosin. This data suggests that a shallow 15%-25% (w/v) iodixanol gradient (covering the approx. density range 1.10-1.14 g/ml is capable of very fine discrimination and may be applicable to plasma domain resolution from other tissues. More recently megalin processing in the brush border [6], processing of the Na^+/H^+ exchanger [7]; its down-regulation by dipeptidyl peptidase IV inhibition [8] and its reduction in spontaneously-hypertensive rate [9] have been reported. Studies on the Type IIc Na-P_i exchanger have used an identical method [10].

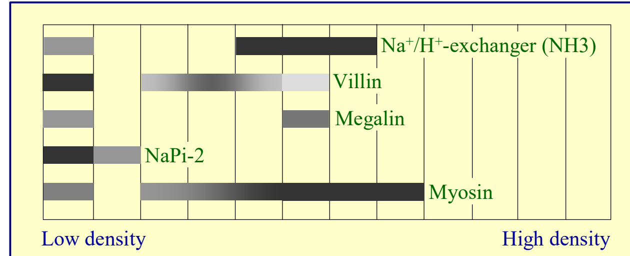

Figure 1 Distribution of markers from rat microsomal fraction in iodixanol gradient, data adapted from refs 1 and 2. For more information see text in 5.4.2

2. Glomeruli (slit diaphragms)

The self-generated gradients, produced from a three-iodixanol layer discontinuous gradient, developed by Yeaman [11] and Vogelmann and Nelson [12] (**see Application Sheet S31**) have also been used in the analysis of junctional proteins from glomeruli [13]. A PNS fraction from the glomerular homogenate is mixed with OptiPrep™ is adjusted to 30% (w/v) iodixanol and overlaid with equal volumes of 20% and 10% iodixanol. Self-generated gradients are normally run in either vertical or near-vertical rotors, since for efficient formation a rotor with a short sedimentation path length is required, but Fukasawa et al [13] used a Beckman SW60 Ti (4 ml tubes) at 350,000 *g* for 3 h. It is however known that in the absence of a vertical or near vertical rotor, if a two layer starting format is used (rather than a uniform concentration of iodixanol), effective gradients can be generated in, for example, a 10 ml fixed-angle rotor [14].

The gradient displayed remarkable resolving power: for example, occludin, cadherins, claudin-5 and crumbs-3 all had very distinctive distributions through the gradient. For more information see ref 13.

3. References

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