OptiPrep™ Application Sheet S34

Purification of caveolae from cells and tissues by sonication of a plasma membrane fraction (detergent-free)

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **OptiPrep™ Mini-Review (MS10)** "The purification of caveolae – a bibliography" lists all published papers reporting the use of OptiPrep™: to access return to the initial list of Folders and select "**Mini-Reviews**". **The references are divided into cell or tissue type and also highlight the analytical content**.
- 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 and information regarding methodological variations are contained in the "Technical Notes and Review" section (Section 5)

1. Background

Although the existence of caveolae has been recognized in the electron microscope since the early fifties, it is only relatively recently that their importance in a number of internalization processes has been established in a variety of epithelial and endothelial cells (e.g. see ref 1 for more information). This in turn has led to the development of isolation processes in order to study their composition and to define their functions at the molecular level and now it is widely recognized that lipid-rich domains, such as caveolae and lipid rafts, in the plasma membrane are critical in cell signaling and virus processing.

Early methods to purify these surface membrane domains relied on the relative detergent-resistance of caveolae compared to that of rest of the plasma membrane. Although exposure of membranes to Triton X-100 does lead to the selective solubilization of the bulk of the plasma membrane, loss of some proteins from the caveolae themselves is also liable to occur (see ref 1).

Smart et al [1] therefore developed a method that avoids the use of Triton X-100. After isolation of a plasma membrane fraction from either human skin fibroblasts or MA104 cells, the caveolae are released by sonication in a standard cell homogenization medium. The first part of the isolation procedure is a flotation through a continuous iodixanol gradient; this gradient is essentially a resolving gradient in which the caveolin-rich vesicles are concentrated in the top third of the gradient, while the predominantly caveolin-poor vesicles band in denser regions. A second discontinuous gradient is essentially a concentration gradient to band the caveolin-rich vesicles sharply at an interface. Figure 1 summarizes this procedure.

The protocol in this Application Sheet has been adapted from refs 1 and 2.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Diluent: 0.25 M sucrose, 6 mM EDTA, 120 mM Tricine-NaOH, pH 7.6

C. Working solution of 50% iodixanol (ρ = 1.282 g/ml): mix 5 vol. of solution A with 1 vol. of solution B

D. Suspension medium: 0.25 M sucrose, 1 mM EDTA, 20 mM Tricine-NaOH, pH 7.6

Keep the following stock solutions at 4°C:
500 mM Tricine: 8.95 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 24 ml of Tricine stock; adjust to pH 7.6 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 8 ml of Tricine stock; adjust to pH 7.6 with 1 M NaOH and make up to 200 ml.

3. Ultracentrifuge rotor requirements (see Section 5.2)

Any 13-17 ml swinging bucket (e.g. Beckman SW41 or SW28.1, Sorvall TH641 or AH629).

4. Protocol

Carry out all operations at 0-4°C.

1. Prepare a plasma membrane fraction by a suitable technique (**see Section 5.3**).

2. Suspend the plasma membrane in 2 ml of solution D in a suitable tube (approx 1.5 cm diameter).

3. Place the tube in ice and introduce the tip of a sonicator probe (approx diameter 3 mm) to a point equidistant from the top and bottom of the suspension (**see Section 5.3**).

4. Sonicate twice for 6 sec at a total power of 50 J/W per sec, then allow to rest for 2 min before repeating the sonication procedure twice more, i.e. a total of six sonication bursts.

5. Add 1.84 ml of solution C and 0.16 ml of solution D. The final iodixanol concentration is 23% (w/v).

6. Produce two gradient solutions of 10% (w/v) iodixanol (ρ = 1.076 g/ml) and 20% (w/v) iodixanol (ρ = 1.125 g/ml) by diluting solution C with solution D (1 + 4 and 2 + 3 v/v respectively).

7. Using a standard two chamber gradient former or a Gradient Master™ produce a 9 ml linear 10-20% iodixanol gradient in an approx 13 ml tube for the swinging-bucket rotor (**see Section 5.4**).

8. Underlayer the gradient with 4 ml of the sample and centrifuge at 53,000 g_{max} for 90 min.

9. Collect the top 5 ml of the gradient (see Figure 1); transfer to a new centrifuge tube and mix with 4 ml of solution C (**see Section 5.5**).

10. Produce two new gradient solutions of 5% and 15% (w/v iodixanol by diluting solution C with solution D (1 + 9 and 3 + 7 v/v respectively)

11. Layer 1.0 ml of 15% and 0.5 ml of 5% iodixanol over the sample and centrifuge at 52,000 *g* for 90 min (**see Section 5.6**).

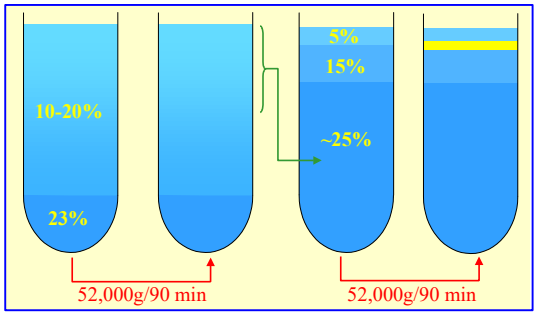

Figure 1 Flow chart for isolation of caveolae

12. Collect the caveolae-rich opaque layer that forms above the 15% iodixanol layer (see Figure 1).

5. Technical Notes and Review

5.1 Homogenization media and gradient solutions

Protease inhibitors (PMSF, leupeptin, antipain, aprotinin etc) may be included in Solutions B and D at the operator's discretion. Strategies for preparing gradient solutions for mammalian tissues are given in **Application Sheet S01**.

5.2 Ultracentrifuge rotors

Most of the published papers quote the use of swinging-bucket rotor of the tube volume specified, but there are examples in which the method has been scaled up larger volume rotors (such as the Beckman SW28) but rather rarely to smaller volume rotors.

5.3 Plasma membrane preparation and release of caveolae

It is beyond the scope of this Application Sheet to cover the precise methodology for preparing the plasma membrane from cultured cells. The vast majority of published papers reporting the use of the protocol described in this OptiPrep™ Application Sheet for the isolation of caveolae have also cited a Percoll® gradient method for initial isolation of the plasma membrane, as described by Smart et al [1].

However, unless the colloidal silica particles of the Percoll® contribute to the success of the sonication and the subsequent gradient separation, there is no obvious reason why other methods for the isolation of the plasma membrane should not be used. See "Plasma membrane" in the Index for some examples of OptiPrep™ based methods.

There is moreover an interesting alternative approach for the isolation of the plasma membrane that uses the binding of cationic silica to the surface of intact cells, first described by Chaney and Jacobson [3], the silica raises the density of the plasma membrane and thus permits its simple isolation. Kincer et al [4] perfused the cationic silica through the mouse vasculature to isolate the plasma membrane of the endothelial cells lining the femoral arteries. The method may however be used less elaborately for the density perturbation of the plasma membrane from any cultured cells. The methodology is described in **Application Sheet S27**.

Schnitzer et al [5] also used the colloidal silica method for rat lung endothelial cells. The size and structural disposition in the membrane of the caveolae abrogates their ability to bind the colloidal silica, thus they can be recovered as a detergent-resistant membrane [5]. Subsequently the colloidal silica may be detached by raising the ionic strength of the medium and non-caveolae lipid-rich domains isolated, also as detergent-resistant membranes [5].

Song et al [6] introduced another option to the use of detergent; MDCK cells were homogenized in 500 mM sodium carbonate sequentially in a Dounce homogenizer, a Polytron homogenizer and by sonication before harvesting the caveolin-rich membranes by flotation through a sucrose gradient. In the case of vascular smooth muscle cells Ishizaka et al [7] used the same strategy but omitted the Polytron homogenization; HEK cells were disrupted by the same Dounce homogenization/sonication strategy but the caveolae were isolated by the double iodixanol gradient described in this OptiPrep™ Application Sheet [8].

Caveolin-rich low-density membranes have also been isolated from hepatoma cells without sonication or detergent [9]. After an initial homogenization in a routine homogenization medium (137 mM NaCl, 50 mM Hepes, pH 7.4, containing vanadate) by 20 passages through a 27G syringe needle, the post-nuclear supernatant was loaded on to a 2%, 10%, 15% and 18% (w/v) iodixanol gradient and centrifuged at 235,000*g* for 90 min. The caveolin-rich domain banded at the 2%/10% iodixanol interface. Yanase and Madaio [10] used a similar strategy, also for hepatoma cells, but omitted the 2% iodixanol from the gradient.

5.4 Iodixanol gradient preparation

A gradient volume needs to be used so that the tube is properly filled (according to the manufacturers instructions) when the 4 ml sample is underlayered. An approx 9 ml gradient was used by Smart et al [1]. The method can be scaled up or down as required, adjusting the volumes of sample and gradient proportionately. If the volume of the gradient is increased, the volume occupied by the caveolae-containing fractions may also increase. As an approximation the top third-to-half of the gradient should be removed. If the relative volumes of gradient and sample are significantly different it may be advisable to check the distribution of the caveolae by assaying fractions for caveolin by electroblotting. For more information on gradient preparation **see Application Sheet S03**.

5.5 Collecting the continuous gradient

It is probably sufficient simply to collect the top 5 ml of the gradient with a syringe attached to a flat-tipped metal cannula (i.d. approx 0.8 mm) or an automatic pipette, whose tip has been cut to enlarge the orifice. Either way the gradient must be withdrawn very slowly to avoid aspiration denser regions. The gradient may alternatively be collected by tube puncture, upward displacement with a dense medium or aspiration from the meniscus for a more complete analysis. More information about harvesting material from gradients may be found in **Application Sheet S08**.

5.6 The discontinuous gradient

The volumes used in the second gradient may also have to be modified for larger volume tubes; it is probably good practice to increase the volumes of the sample and the two iodixanol layers, proportionally. For more information on gradient preparation **see Application Sheet S03**. Collect interfacial band with a syringe attached to a flat-tipped metal cannula (i.d. approx 0.8 mm) or an automatic pipette, whose tip has been cut to enlarge the orifice.

6. References

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7. Acknowledgements

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