OptiPrep™ Application Sheet S49

Isolation of optical system membranes and structures

- 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.

This Application Sheet covers the purification of the following particles:

1. Retinal rod outer segments (ROS) and ROS disks

- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 1.5).
- Section 1.5 includes information on some variations to the given format and some of the more recent references

1.1 Background

The use of OptiPrep™ for the isolation of mouse retinal rod outer segments (ROS) was first published in 1998 [1]. After removal of the retinas, they are repeatedly vortexed in a Ringer's solution containing iodixanol (usually, but not always, 8% v/v/ OptiPrep™) and centrifuged at low speed. During this procedure the ROS are released into the supernatants and subsequently banded in a two-step 10%, 18% (v/v) OptiPrep™ gradient. The method was extended without modification to the isolation of ROSs from human tissue [2]. Howes et al [3] and Calvert et al [4] replaced the two-layer iodixanol gradient with a three layer one for the purification of the ROS, although the densities of the three layers were slightly different and Ringer's solution was replaced by Locke's solution.

More recently Liang et al [5] introduced a continuous iodixanol gradient for the purification of the ROSs and subsequently, after osmotic lysis of the ROSs, the optical disks were recovered on a second continuous iodixanol gradient. For convenience, the methodology described in this Application Sheet is adapted from ref 5 but the extraction and gradient variations for ROS purification are presented in Section 5.3.

1.2 Solutions required (see Section 5.1)

A. OptiPrep™

B. Ringers Solution: 130 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.02 mM EDTA, 10 mM Hepes-NaOH, pH 7.4

C. Lysis buffer: 2 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
10 mM EDTA (Na_2•$2H_2O$): 0.372 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml of water
100 mM KCl: 0.745 g per 100 ml of water
100 mM $MgCl_2$•$6H_2O$: 2.03 g per 100 ml of water
100 mM $CaCl_2$•$2H_2O$: 1.47 g per 100 ml water
100 mM Tris (free base): 1.21 g per 100 ml

Solution B: Mix together the following volumes of the above stocks, **except the Tris** (in order), 4 ml, 0.4 ml, 26 ml, 7.2 ml, 4.8 ml and 2.4 ml; adjust to pH 7.4 with NaOH and make up to 200 ml

Solution C: Add 4 ml of Tris stock solution to 100 ml of water; adjust to pH 7.4 with 1 M HCl; make up to 200 ml

1.3 Centrifuge requirements

Swinging-bucket rotor for a high-speed centrifuge with a tube volume of approx 15 ml

1.4 Protocol

1. Make an 8% (v/v) OptiPrep™ solution by diluting 8 ml of Solution A to 100 ml with Solution B.
2. Suspend 12 mouse retinas in 0.12 ml of this solution. Carry out this and the following steps at room temperature.
3. Vortex for 1 min and then centrifuge at 200 *g* for 1 min.

4. Carefully recover the supernatant (without disturbing the pellet) and retain.

5. Resuspend the pellet in 0.12 ml of the 8% OptiPrep™ solution and repeat steps 3-4.

6. Repeat this procedure five or six times.

7. Prepare two gradient solutions of 10% and 30% (v/v) OptiPrep™ by diluting 1 ml and 3 ml of Solution A to 10 ml with Solution B respectively.

8. Using a two-chamber gradient maker or a Gradient Master™ prepare a 10-12 ml continuous gradient from these two solutions in tubes for the swinging-bucket rotor (**see Section 5.2**).

9. Layer the combined supernatants (approx. 1.5 ml) on top and centrifuge at 26,500 *g* for 30 min.

10. Collect the band of ROSs (about two thirds of the way from the top of the gradient).

11. Dilute with 3 vol. of Solution B and centrifuge at 500 *g* for 3 min to pellet the nuclei.

12. Harvest the ROSs by centrifuging at 26,500 *g* for 30 min.

13. To prepare the optical disks, lyse the ROSs by suspending them in 2 ml of Solution C and maintain them at 0°C for 15 h.

14. Prepare two gradient solutions of 15% and 40% (v/v) OptiPrep™ from 1.5 ml and 4 ml of Solution A, each diluted to 10 ml with Solution B respectively.

15. Using a two-chamber gradient maker or a Gradient Master™ prepare a 10-12 ml continuous gradient from these two solutions in tubes for the swinging-bucket rotor (**see Section 5.2**).

16. Layer the lysed ROSs on top of the gradient and centrifuge at 26,500 *g* for 30 min.

17. Harvest the disks that band about two thirds of the way from the top of the gradient

1.5 Technical Notes and Review

1.5.1 Homogenization media and gradient solutions

All iodixanol solutions in this Optiprep™ Application Sheet are prepared simply by diluting Optiprep™ with Ringers Solution, which will mean that the levels of KCl, $MgCl_2$, $CaCl_2$, EDTA and buffer will be reduced. If it is considered important that these levels should be maintained in all density solutions then a working solution of 50% (w/v) iodixanol should be first prepared by diluting 5 vol. of Optiprep™ with 1 vol. of 130 mM NaCl, 21.6 mM KCl, 14.4 mM $MgCl_2$, 7.2 mM $CaCl_2$, 0.12 mM EDTA, 60 mM Hepes-NaOH, pH 7.4. The working solution would thus contain the same concentrations of KCl, $MgCl_2$, $CaCl_2$, EDTA and Hepes as the Ringers buffer. Further dilutions with Ringers buffer should then need to be adjusted appropriately from those given in the protocol to give gradient solutions of the correct density. Note that if the concentration of NaCl in the diluent were also raised then the osmolality of the 50% (w/v) iodixanol will be unacceptably high. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

1.5.2 Construction of gradients

If neither of these gradient-making devices is available then a continuous gradient can be prepared by diffusion of a discontinuous gradient. For example, the 10-30% (v/v) Optiprep™ gradient (step 7) might be generated from a discontinuous gradient of equal volumes of 10%, 18%, 24% and 30% Optiprep™. Likewise the 15-40% (v/v) Optiprep™ gradient (step 14) might be generated from a discontinuous gradient of equal volumes of 15%, 21%, 27%, 33% and 40% Optiprep™. For more information on gradient construction **see Application Sheet S03**.

1.5.3 Variations in gradient and centrifugation format

Continuous gradients

Continuous iodixanol gradients normally conform to the above protocol [5-13], although Peshenko et al [14,15] used a 20-30% (v/v) OptiPrep™gradient and a higher *g*-force of 75,000 *g*, for 40 min.

Discontinuous gradients

The commonly used discontinuous gradient format involves layering the extracted ROSs in 8% (v/v) Optiprep™ over a two layers of 10% and 18% (v/v) Optiprep™; the centrifugation conditions are however somewhat diverse: 3,300 *g* for 10 min [1,2], 50,000 *g* for 1 h [16] 70,000 *g* for 1 h [17]. Variants in the density of the gradient layers include 8.3% and 20% with the sample in 6.7%, centrifuged at only 1,425 *g*, for 15 min [4]. Gradients comprising three layers: 10, 20 and 30% (sample in 8%), after centrifugation at 17,000 *g* for 50 min the ROSs banded at the 20%/30% interface [3] and four layers: 8%, 12%, 16% and 20% (sample in 2%) [18,19].

- Occasionally after repeated extractions with 8% (v/v) Optiprep™ no gradient is used [20].
- Refs 21-23 are meetings abstracts, reporting the use of Optiprep™ but no details are provided.
- **Refs 24-35 all report the use of iodixanol gradients prepared from OptiPrep™ for ROS isolation using methods identical or very similar to those described above**

2. Photoreceptor outer segments (POS)

Jiang et al [36] and Hazim et al [37] described homogenizing retinas in 130 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.02 mM EDTA, and 10 mM HEPES, pH 7.4; then after centrifugation at 100 *g* for 1 min, the supernatant was layered over 8, 10 and 15% (w/v) iodixanol and centrifuged at 12,000 *g* for 20 min. The outer segments banded at the 10-15% interface. In an interesting variation Pelkonen et al [38] described homogenizing the tissue in 8% iodixanol (in a bicarbonate buffer). After an initial centrifugation at 720 *g* for 3 min (repeated five times); the suspension was diluted to 2% iodixanol and layered over 10% and 20% iodixanol, banding the POS at 14,000 *g* for 30 min. Rao et al [39] used a similar approach to that described in ref 38 but the gradient was a continuous one (10-30% iodixanol).

3. References

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Application Sheet S49; 7th edition, February 2018

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