OptiPrep™ Application Sheet S40

Isolation of a granule fraction from human platelets

- 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" sections (Section 5)

1. Background

Platelet-rich plasma (PRP) may be prepared by centrifugation of whole blood (anticoagulated by citrate) at 200 *g* for 20 min [1] or 320 *g* for 15 min [2]. The platelets are then pelleted from the PRP by centrifugation at 2000 *g* for 15 min after addition of EDTA to a final concentration of 0.7 mM [2] and then washed several times in a suitable buffer. A very efficient alternative is to purify the platelets on a low-density cushion of iodixanol (1.063 g/ml) from whole blood. This is described in **Application Sheet C12**. The band of platelets may be recovered with, or separately from, the plasma supernatant.

Any incubation with labeling reagents etc are normally carried out in the PRP before the platelets are subjected to a washing procedure prior to homogenization. Gogstad [2] originally disrupted the platelets in 0.27 M sucrose, 2 mM EDTA, pH 6.5 in a French pressure cell, but more recently this has been replaced by nitrogen cavitation [1,3]. A metrizamide gradient was used by Gogstad [2] and Feng et al [1] but this has now been replaced by the easier-to-use and more "particle-friendly" iodixanol [3, 4].

2. Solutions required

- See Section 5.1 for important information about the composition of the following solutions

A. OptiPrep™

B. Homogenization buffer: 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 0.5 mM EGTA, 25 mM Hepes-NaOH, pH 7.4

C. OptiPrep™ diluent: 145 mM NaCl, 30 mM KCl, 6 mM $MgSO_4$, 60 mM glucose, 3 mM EGTA, 25 mM Hepes-NaOH, pH 7.4

D. Working Solution (50%, w/v iodixanol): Mix 5 vol. of Solution A with 1 vol. of Solution C

E. 100 mM EDTA

Keep the following stock solutions at 4°C
1 M Hepes (free acid): 23.8 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water
1 M KCl: 7.45 g per 100 ml water
100 mM $MgSO_4 \bullet 7H_2O$: 2.46 g per 100 ml water
100 mM EGTA: 3.80 g per 100 ml water (pH 11-12)
100 mM EDTA($Na_2 \bullet 2H_2O$): 3.72 g per 100 ml water

Solution B: Dissolve 0.36 mg glucose in 100 ml of water; add 29 ml, 1 ml, 2 ml, 1 ml and 5 ml respectively of the NaCl, KCl, $MgSO_4 \bullet 7H_2O$, EGTA and Hepes stocks; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml with water.

Solution C: Dissolve 1.08 g glucose in 50 ml water; add 14.5 ml, 3 ml, 6 ml, 3 ml and 2.5 ml respectively of the NaCl, KCl, $MgSO_4 \bullet 7H_2O$, EGTA and Hepes stocks; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml with water.

3. Ultracentrifuge rotor requirements

Swinging-bucket rotors with tube volumes of 17 ml (e.g. Beckman SWS28) or 13 ml (e.g. Beckman SW 41)

4. Protocol (adapted from refs 1-3)

Carry out Steps 1 and 2 at room temperature and all subsequent steps at 4°C

1. Prepare a PRP from whole blood by centrifugation at 320*g* for 15 min or purify the platelets from whole blood using **Application Sheet C12**. If using the latter aspirate all of the plasma supernatant with the band of platelets.

2. Add 100 mM EDTA to the PRP (volume ratio of 0.7:100) and harvest the platelets by centrifugation at 2000 *g* for 15 min.

3. Wash the platelet pellet by sequential suspension and re-centrifugation in Solution B.

4. Suspend the washed platelets in 50 ml of Solution B.

5. Subject the platelet suspension to nitrogen cavitation at 100 atmos. for 20 min (see Section 5.2).

6. Carefully collect the cavitate and centrifuge it at 4000 *g* for 20 min; aspirate and discard the supernatant (see Section 5.3).

7. Resuspend the pellet in 50 ml of Solution B and repeat Step 5.

8. Centrifuge the cavitate at 4000 *g* for 20 min; aspirate and retain the supernatant.

9. Add Solution D to the cavitate (volume ratio of 11:39) to adjust the iodixanol concentration to 11% (w/v) iodixanol and transfer 11 ml or 15 ml to 13 ml or 17 ml tubes respectively for the chosen swinging-bucket rotor (see Section 5.4).

10. Prepare a solution of 30% (w/v) iodixanol from Solution D and Solution B (3:2 volume ratio).

11. Underlayer each cavitate with 2 ml of the 30% iodixanol from a syringe and long metal cannula (i.d. approx 0.8 mm).

12. Centrifuge at 38,000 *g* for 3 h.

13. Aspirate and discard all of the supernatant above the band, then collect the interfacial band and the 30% iodixanol layer and dialyze it to remove the iodxanol (see Section 5.5).

14. Prepare 11%, 15%, 20%, 25% and 30% (w/v) solutions of iodixanol from Solutions D and B (volume ratios of 11:39 1.5:3.5, 2:3, 1:1 and 3:2 respectively).

15. Construct discontinuous gradients in 13 ml tubes from 2 ml each of each of the iodixanol solutions by underlaying with a syringe and metal cannula and then layer the dialyzed sample (approx 2.5 ml containing approx 2 mg protein/ml) on top (see Section 5.6).

16. Centrifuge at 38,000 *g* for 3 h. Allow the rotor decelerate from 2000 rpm without the brake, or use a controlled deceleration program.

17. Collect the material which bands at each interface using a syringe and metal cannula (the α-granules band at the 25%/30% interface) or collect the entire gradient in 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 more information on analysis of the gradient fractions see Section 5.7.

5. Technical Notes and Review

5.1 Homogenization media

- Note that Flaumenhaft et al [3] reported that in their studies Solution B contained prostaglandin (2.5 μM PGE_1), 100 μg/ml apyrase and 100 μM aspirin.

The additions to Solution B described above was clearly important to the functional analysis of the granules [3]. It is probable but not certain that these additions are not integral to the fractionation procedure since Gogstad [2] used a simple solution containing sucrose and EDTA. Supplementation or complete replacement of sucrose containing media with NaCl and/or KCl is becoming increasingly common in the analysis of various membrane compartments in iodixanol gradients. Ionic solutions can

reduce ionic interactions, aggregation between membranes and combat any raised viscosity of the homogenate due to cytoskeletal proteins.

The preparation of a working solution (Solution D) as described, ensures that the concentrations of KCl, $MgSO_4$, glucose and EGTA are constant throughout the gradient. If this is deemed unimportant the iodixanol solutions may be prepared simply by diluting OptiPrep™ with Solution B. The NaCl and buffer concentrations in Solution C were not increased 6x, in line with the other components; if they were then 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.

5.2 Homogenization

The disruption of most mammalian cells can usually be adapted to the use of a number of homogenization devices, but the disruption of platelets is a more stringent process and methods other than the one described here or the French Press method reported by Gogstad [2] are probably not valid in view of the much smaller size of these particles. Nitrogen pressure vessels are commercially produced by Artisan Industries in the USA and Baskerville and Lindsay in the UK.

5.3 Collection of the cavitate

When collecting the cavitate open the delivery valve of the pressure vessel very slowly to avoid an explosive expulsion of the contents of the vessel. Allow the foam in the cavitate to disperse by gentle agitation before continuing.

5.4 Loading the rotor

There is no obvious reason why a larger volume rotor such as the Beckman SW28 (39 ml tube) should not be used for the initial ultracentrifugation, which is primarily a concentration process that avoids pelleting the platelet membranes. The latter may leady to serious aggregation of the material.

5.5 Dialysis

To remove iodixanol by dialysis, large pore size dialysis tubing is recommended. Alternatively the Maxi GeBAflex (www.geba.org) dialysis tube (highest MWt cut off), may be used or a G25 Sephadex column. For more information on removal of iodixanol see **Application Sheet S09**.

5.6 Discontinuous gradient set up

Although overlayering is the most common method for construction of a discontinuous gradient, underlayering is both more effective and easier to execute. For more information on gradient construction see **Application Sheet S03**.

A variation that, to our knowledge has not been tested, is to load the sample in a dense medium below the gradient rather than in a low density medium on top. With this strategy it would be possible to eliminate the dialysis step altogether, since the material recovered from the 30% iodixanol cushion (Step 13 of the Protocol) would simply be layered beneath the subsequent 11-25% discontinuous gradient. If the centrifugation conditions (Step 16) allow the particles to reach their banding density then the position of the sample is irrelevant. If however the separation is based on sedimentation velocity then bottom-layering would not be an option.

5.7 Analysis of gradients

Analysis of the banded material in the gradient showed [3] that the material at the 11%/15% iodixanol interface contained plasma membrane (the platelets were biotin-labelled prior to cavitation); that at the 15%/20% interface was lysosomal; α-granules banded at the 25/30% interface, while dense granules were recovered from the 30% iodixanol layer. The gradient was used to show that the α-granules were specifically coated with F-actin.

6. References

1. Feng, D., Crane, K., Rozenvayn, N., Dvorak, A.M. and Flaumenhaft, R. (2002) *Subcellular distribution of 3 functional platelet SNARE proteins: human cellubrevin, SNAP-23 and syntaxin2* Blood **99**, 4006-4014
2. Gogstad, G.O. (1980) *A method for the isolation of α-granules from human platelets* Throm. Res., **20**, 669-681
3. Flaumenhaft, R., Dilks, J.R., Rozenvayn, N., Monahan-Earley, R., Feng, D. and Dvorak, A.M. (2005) *The actin cytoskeleton differentially regulates platelet α-granule and dense-granule secretion* Blood, **105**, 3879-3887
4. Woronowicz, K., Dilks, J.R., Rozenvayn, N., Dowal, L. Blair, P.S., Peters, C.G., Woronowicz, L. and Flaumenhaft, R. (2010) *The platelet actin cytoskeleton associates with SNAREs and participates in R-granule secretion* Biochemistry, **49**, 4533–4542

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