OptiPrep™ Application Sheet S39

Isolation of a granule fraction from eosinophils

- 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 **2020SMemapp** file and select the appropriate S-number.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" sections (Section 5)

1. Background

A number of published protocols for the isolation of secretory or storage granules from eosinophils use a Nycodenz® gradient. Some of these were prepared from Nycoprep™ 1.15, an isoosmotic solution of density 1.15 g/ml containing 27.6% Nycodenz®. The latter is no longer commercially available, thus all Nycodenz® solutions must be made from the powder. The Nycodenz® method for the isolation of crystalloid granules from human peripheral blood eosinophils was first described by Levi-Schaffer et al [1] in the study of their association with granulocyte-macrophage colony-stimulating factor. Subsequently this methodology was also applied to study their release of interleukin-2 [2] and interleukin-6 [3] in inflammatory reactions, the mobilization of the chemokine RANTES in response to γ-interferon [4], the release of interleukin-13 [5] and the mechanism of the eosinophil respiratory burst in asthma [6].

More recently the method has been adapted to an iodixanol gradient covering approximately the same density range [7-14]. It is this method that is described below and it is adapted from ref 12.

2. Solutions required (see Section 5.1)

A. Homogenization medium: 0.25 M sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 10 mM Hepes-NaOH, pH 7.4

B. OptiPrep™ diluent: 0.25 M sucrose, 6 mM EGTA, 60 mM Hepes-NaOH 7.4

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

D. Gradient solution: 45% (w/v) iodixanol; mix 4.5 vol. of Solution C with 0.5 vol. of Solution A

- Note that Solution A may also adjusted to 1 mM ATP before use [11]. Protease inhibitors may be included in Solutions A-C at the operator's discretion [11].

Keep the following stock solutions at 4°C
1 M Hepes (free acid): 23.8 g per 100 ml water
100 mM EGTA: 3.80 g per 100 ml water (pH 11-12)
1 M $MgCl_2 \bullet 6H_2O$; 20.3 g per 100 ml water

Solution A: Dissolve 8.5 g sucrose in 50 ml water; add 1ml each of the Hepes and EGTA stock solutions and 0.2 ml of the $MgCl_2$ stock solution; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml with water.

Solution B: Dissolve 8.5 g sucrose in 50 ml water; add 6ml each of the Hepes and EGTA stock solutions; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Any swinging-bucket rotor with tube volumes of approx. 13 ml (e.g. Beckman SW 41 or Sorvall TH641)

4. Protocol

4a. Cell preparation

Prepare polymorphonuclear leukocytes (PMNs) from fresh human blood (anticoagulant EDTA) as described in **Application Sheet C12**, but carry out the centrifugation at 4°C. In **Application sheet C12** the method is described as being carried out at room temperature; it may be necessary to increase the centrifugation time to allow for the increased liquid viscosity at the lower temperature.

Wash and resuspend the PMNs harvested from the gradient in a buffered saline and then purify the eosinophils using immunomagnetic beads by negative selection. Anti-CD16 will remove neutrophils;

anti-CD14 and anti-CD3 will remove any residual mononuclear cells. The use of immunomagnetic beads to negatively select the eosinophils has become the method of choice and it should be carried out in accordance with the manufacturer's recommendations and ref 15. Simple discontinuous gradients are however still in use for the subsequent separation of normodense and hypodense eosinophils. **For more information see ref. 15**.

4b. Isolation of granules

Carry out all steps at 4°C

1. Wash the cells twice in Solution A and then suspend the eosinophils in this solution; typically, $5x10^7$ eosinophils are used at approx. $1x10^7$ cells/ml.

2. Homogenize the cells by nitrogen cavitation at 600 psi for 10 min (**see Section 5.2**).

3. Centrifuge the homogenate at 200 *g* for 10 min (**see Section 5.2**).

4. Prepare gradients in tubes for the SW41 rotor from 4 ml of Solution A and 5 ml of Solution D using either a two-chamber gradient maker or Gradient Master™. Finally underlayer with 0.5 ml of Solution D. (**see Section 5.3**).

5. Layer the 200 *g* supernatant on top of the gradient (approx 2 ml per gradient) and centrifuge at 100,000 *g* for 1 h. Allow the rotor to decelerate using a slow deceleration program or turn off the brake below 2000 rpm.

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 S09**. For information on analysis of the gradient fractions **see Section 5.4**.

5. Technical Notes and Review

5.1. Solutions

Supplementation of the homogenization medium 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 Application Sheet would benefit from such modifications can only be assessed by experimentation. Levi-Schaffer et al [1] adjusted Solution A to 2mM $MgCl_2$ and 1 mM ATP for the final suspension of the eosinophils prior to homogenization, to enhance the retention of granule functional activity.

The preparation of a Working Solution as described, ensures that the concentrations of EGTA and buffer are constant throughout the gradient. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

5.2. Homogenization and production of a post-nuclear supernatant

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; it was used by Levi-Schaffer et al [1], while nitrogen cavitation was used by Spencer et al [2]. If neither of these devices is available however 10-20 passages through a syringe needle (the Gauge Number (G) varies from 21 to 26) can be an efficient alternative, but its efficacy must be tested before using it as an alternative for granule preparation. 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. Neves et al [11] used 1 ml of cell suspension. Some other hints on homogenization are given in **Application Sheet S06**.

Neves et al [11] resuspended the pellet from step 3 in 1ml of Solution A; repeated the cavitation and 200 *g* centrifugation (step 3) and combined the two supernatants. In earlier methods the first low-speed centrifugation was carried out at 400 *g* for 10 min.

5.3. Density gradients

Note that the earlier Nycodenz® gradients the low-density gradient solution was 2% (w/v) and a permissible alternative is to use 2% (w/v) iodixanol in this method (prepare this from a mixture of Solution D (2 vol.) and Solution A (43 vol.). If the top of the gradient is 0% (w/v) iodixanol, layering of the post-nuclear supernatant might be difficult, hence a 2% low-density solution may be preferable. If neither of the gradient-making devices is available then a continuous gradient may be generated from a discontinuous one by diffusion: use 1 ml of 2% (or 0%) and equal volumes of 5%, 15%, 25%, 35%, 40% and 45% (w/v) iodixanol. For more information on gradient construction **see Application Sheet S03**.

It is also worth noting the use of a very simple discontinuous gradient in which a post-nuclear supernatant from a HL60 cells is mixed with an equal volume of a dense solution (ρ = 1.12 g/ml) and 14 ml layered between 14 ml of 1.05 g/ml and 5 ml of 1.12 g/ml solutions [16]. The two densities are approx equivalent to 19% and 4% (w/v) iodixanol. The gradient was centrifuged at 37,000 *g* for only 35 min to purify **azurophilic granules**.

5.4. Analysis of the gradients

The granules band close to the bottom of the gradient at approx 1.20 g/ml, while cytosolic proteins remain at the top of the gradient. Under the short centrifugation time condition soluble proteins will sediment and diffuse only a short distance from the sample layer. The plasma membrane will also band close to the top of the gradient. Spencer et al [7] confirmed the identity and purity of the granule fraction by light and electron microscopy and also by Major Basic Protein and CD63 staining in flow cytometry analysis. The authors were able to demonstrate the existence of preformed cytokine receptors within the granules. Iodixanol gradients described in this Application Sheet are isoosmotic, it is not possible to make Nycodenz® with similar osmotic properties and this is thought to be responsible for the iodixanol gradient's unique ability to resolve eosinophil vesicles from the granules [11]. **See Application Sheet S09 for more general information on gradient analysis**.

♦ For a more detailed account of the methodology see ref 17

6. References

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