OptiPrep™ Application Sheet C30

Isolation of rat alveolar type II pneumocytes, lymphoid and myeloid cells

- 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 Cell Index; key Ctrl "F" and type the C-Number in the Find Box
- For other pulmonary cell methods **see Application Sheet C29**
- See **Reference List RC09** for a list of published papers on all pulmonary cells

1. Background

Alveolar (pneumocyte) type II pneumocytes are widely studied because they synthesize and secrete the phospholipid-rich lung surfactant, which lines the air-alveolar interface and prevents alveolar collapse by lowering surface tension at low lung volumes. Isolation of these cells from both adult and foetal lung is an important prerequisite for the culture and study. Viscardi et al [1-6], who successfully developed a Nycodenz® gradient technique, emphasised the importance of the non-toxic, non-invasive properties of this gradient medium and pointed out that although Percoll® gradients had been used previously for purifying these cells, the potentially toxic nature of a polyvinyl-pyrollidone-coated silica colloid was of considerable concern in studies of their function.

Viscardi et al [1] reported that that the purity of type II pneumocytes from adult lung on Nycodenz® gradients was over 80%; very similar figures were obtained from foetal lung tissue. The recovery of these cells from adult lung was over 70%, while for foetal lung this figure was rather lower at 45-50%. Both the purity and the viability of the cells (approx. 97%) from Nycodenz® gradients were considerably higher than that obtained from cells purified by IgG panning. The cells from Nycodenz® gradients moreover had almost four times the plating efficiency. Other workers [7-9] have reported an even higher purity of >90% for the cells from foetal lung tissue. The same gradient system has also been used by Driscoll et al [10,11] and Johnston et al [12].

- Recently discontinuous iodixanol gradients have been used to purify these cells [13-19]

2. Preparation of cell suspension

The treatment of the lung tissue prior to gradient centrifugation is a complex operation, the detail of which varies from laboratory to laboratory and is outside the scope of this Application Sheet. For detailed information see refs 1 and 7-13. Briefly the preparation of the cell suspension involves five steps:

1. Rats are anaesthetized and heparin injected as an anticoagulant.

2. After tracheostomy the vasculature is perfused with a buffered saline medium such as 140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 10 mM HEPES, 2.0 mM $CaCl_2$, 1,3 mM $MgSO_4$, pH 7.4 to remove blood cells [1]. Sometimes this is supplemented with glucose, nystatin and an antibiotic-antimycotic [12].

3. To remove macrophages, the lungs are lavaged several times with 140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 10 mM HEPES, 6 mM glucose, 0.2 mM EGTA, pH 7.4 followed by lavage with the Step 2 buffer [1]. Sometimes the lavage procedure uses the Step 2 buffer (minus divalent cations) followed by Step 2 buffer [12].

4. Partial disaggregation of the cells by lavage with either elastase [1] or pronase [12]

5. After removal of the trachea, the lung tissue is minced in a solution containing DNase I (in Step 2 buffer) and foetal bovine serum and then incubated at 37°C for 5 min in a trypsinizing flask [1].

3. Cell fractionation in Nycodenz®
The following protocol is adapted from ref 1.

3a. Reagents and solutions required (see Note 1)

1. Nycodenz® powder
2. Nycodenz® solvent: 3 mM KCl, 0.3 mM EDTA, 5 mM HEPES-NaOH, pH 7.5 (Solution 1)
3. Working Solution Diluent: 0.75% (w/v) NaCl, 3 mM KCl, 0.3 mM EDTA, 5 mM HEPES-NaOH, pH 7.5 (Solution 2)

Keep the following stock solutions at 4°C
100 mM HEPES (free acid): 2.38 g per 100 ml water; adjust to pH 7.4 with 1 M NaOH before making up to final volume.
100 mM EDTA (Na_2•$2H_2O$) 3.72 g per 100 ml water
1 M KCl: 7.46 g per 100 ml water

Solution 1: To 50 ml of water add 5 ml of the HEPES stock solution and 0.3 ml of both the KCl and EDTA stock solutions; adjust to pH 7.4 and make up to 100 ml with water

Solution 2: Dissolve 0.75 g NaCl in 50 ml of water; add 5 ml of the HEPES stock solution and 0.3 ml of both the KCl and EDTA stock solutions; adjust to pH 7.4 and make up to 100 ml with water

3b. Protocol

1. Prepare a 27.6% (w/v) Nycodenz® stock solution in Solution 1. Place 50 ml of Solution 1 in a 150 ml beaker on a magnetic stirrer set at approx. 50°C and add the Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and then make up to 100 ml with Solution 1. Filter sterilize if required.

2. Dilute the Nycodenz stock solution with Solution 2 to produce 4.6% (w/v) Nycodenz® solution for a continuous gradient or three solutions of 20.7%, 13.8% and 4.6% Nycodenz® for a discontinuous gradient (see Note 2).

3. Filter the lung tissue (in approx 25 ml buffer) sequentially through 2- and 4-ply cotton gauze, followed by 100-, 37- and 15-μm nylon mesh.

4. Dilute with cell suspension buffer to 50 ml and centrifuge at 130 *g* for 10 min (see Note 3).

5. Suspend the cell pellets in DMEM containing 2% foetal bovine serum.

6. Produce a continuous Nycodenz® gradient of 1.03-1.15 g/ml using a two-chamber gradient maker or a Gradient Master™ using 4 ml each of the 27.6% and 4.6% Nycodenz® solutions.

7. **Alternatively** produce a discontinuous gradient from 2 ml each the 27.6%, 20.7%, 13.8% and 4.6% Nycodenz® solutions (see Step 2) by underlayering from a syringe and metal cannula and allow a continuous gradient to form by diffusion (see Notes 4 and 5).

8. Layer 3 ml of the cell suspension (approx 1 x 10^7 cells/ml) on top of the gradient.

9. Centrifuge at 1500 *g* for 20 min at 15°C.

10. Harvest the band of cells (at approx. 1.056 g/ml) just below the sample/gradient interface (see Note 6).

4. Cell fractionations using OptiPrep™
4a Separation of Alveolar Type II cells

In recent publications [13-28] a simple two layer gradient of iodixanol of densities 1.040 and 1.080 g/ml was described for the partial purification of Type II alveolar cells from human lung tissue prior to further purification by negative selection with magnetic beads. As iodixanol is available as a sterile solution of density 1.32 g/ml (OptiPrep™) it considerably simplifies gradient solution preparation. If an ordinary balanced salt or buffered saline solution is used to dilute the OptiPrep™, then these two densities are equivalent to approx 6.5% and 14% (w/v) iodixanol, i.e. OptiPrep™:diluent ratios of 6.5:53.5 and 14:46.

4b Separation of mononuclear/lymphoid/haematopoietic type cells

The separation of myeloid and lymphoid cells has also been achieved using a two-layer iodixanol gradient of 4% and 14.5% (w/v), centrifuged at 600 *g* for 20 min [29]. This gradient was also used to separate haematopoietic cells [30].

Leukocytes (mononuclear cells) from lung tissue have been separated on a 1.079 g/ml barrier prepared from OptiPrep™ (diluted with RPMI containing 10% FCS) [31,32].

5. Notes

1. As all of the solutions used are isoosmotic, it is highly likely that iodixanol can be substituted for Nycodenz® in the methods in Section 3; certainly its availability as a sterile 60% (w/v) solution (OptiPrep™) would make solution preparation much easier. OptiPrep™ would simply be diluted with a suitable buffered saline to the same % (w/v) iodixanol as the Nycodenz® % (w/v). **See Application Sheet C01 for more information.**
2. See Steps 6 and 7 for information about choice of gradient solution preparation.
3. This pelleting step is sometimes carried out at higher g-forces, e.g. 500 *g* [12]
4. Metal "filling" cannulas can be obtained from any hospital supplies company.
5. If the tube is capped and carefully rotated to a horizontal position, diffusion to form a linear gradient will occur in about 1 h at room temperature. If the tube is maintained in a vertical position this process will take at least 6 h. It is always advisable to check that the density gradient is linear by unloading the gradient in a series of equal volume fractions and determining their density by measuring the refractive index or absorbance. For more information about the preparation gradients see ref 29 **and Application Sheet C02**.
6. The broad band at approx. 1.086 g/ml contains fibroblasts, endothelial cells and macrophages, while any erythrocytes band close to the bottom of the gradient.

6. References

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