

OptiPrep™ Application Sheet C19

Purification of leukocyte fractions from rodent/rabbit peritoneal exudates

- ◆ 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

1. Background

Hattori et al [1] harvested the cells from the peritoneal fluid; lysed the residual erythrocytes in 0.2% NaCl and restored isotonicity by adding an equal volume of 1.6% NaCl. The MCs and PMNs were then separated on Nycoprep™ 1.077A. This medium, which was primarily produced for the separation of MCs and PMNs from rodent blood, is no longer commercially available but may be prepared from Nycodenz® powder (14.1% (w/v) Nycodenz®, 0.30% (w/v) NaCl, 5 mM Tricine-NaOH, pH 7.2). A solution of the same density and osmolality ($\rho = 1.077 \pm 0.001$ g/ml; osmolality 265 mOsm) may more easily be prepared from OptiPrep™, see [Application Sheet C45](#).

Frevert et al [2] also used a method that was worked out for the separation of MCs and PMNs from rodent blood. In this method, developed by Freeman et al [3], an isoosmotic solution (NycoPrep™ 1.15) was used, which is no longer commercially available. This solution containing 27.6% (w/v) Nycodenz® in 3 mM KCl, 0.3 mM CaNa₂-EDTA 5 mM Tris-HCl, pH 7.5 (density = 1.15 g/ml) was subsequently diluted with the same KCl, EDTA, Tris solution containing 0.75 g NaCl to produce solutions of 18.4% and 13.8% Nycodenz® ($\rho = 1.098$ and 1.075 respectively). The peritoneal cell suspension (2-6 ml) was layered on top of 2.5 ml of each of the density solutions and centrifuged at 400 g for 30 min at 26°C. The PMNs banded around the lower interface.

Fisker et al [4] developed a single Nycodenz® barrier to separate MCs and PMNs; the density and osmolality of which was modulated according to the type of peritoneal exudates (from a thioglycolate-stimulated or an unstimulated animal). For cells from stimulated rats the optimum density and osmolality of the barrier was 1.106 g/ml and 400 mOsm respectively, for non-stimulated rats 1.091 g/ml and 325 mOsm. Sawant and McMurray [5] used the same strategy for guinea pig peritoneal exudates. The density barrier solutions were again prepared from NycoPrep™ 1.15. The MCs banded at the interface and the PMNs formed a pellet.

- ◆ An identical solution to NycoPrep™ 1.15 may be prepared from Nycodenz® powder or from OptiPrep™. Both options are given (see Note 1).

2. Reagents and solutions required (see Note 2)

- OptiPrep™ (shake the bottle gently before use) **OR** Nycodenz® powder
- Diluent (OptiPrep™): 0.85% (w/v) NaCl, 6 mM KCl, 10 mM HEPES-NaOH, pH 7.4 **OR**
- Diluent (Nycodenz®): 6 mM KCl, 10 mM HEPES-NaOH, pH 7.4
- Hyperosmotic diluent: 3 mM KCl, 0.3 mM Na₂-EDTA, 0.3 mM CaCl₂, 1.245 M NaCl, 5 mM HEPES-NaOH, pH 7.4.
- Lavage solution: Krebs Ringer salt solution buffered with 25 mM HEPES to pH 7.4 containing 1% (w/v) bovine serum albumin.

Keep the following stock solutions at 4°C:

100 mM HEPES (free acid)	2.38 g per 100 ml water
100 mM EDTA (Na ₂ •2H ₂ O)	3.72 g per 100 ml water
100 mM KCl	0.75 g per 100 ml water
100 mM CaCl ₂ •2H ₂ O	1.47 g per 100 ml water

Solution B: Dissolve 0.85 g NaCl in 50 ml water; add 10 ml of HEPES stock solution and 6 ml of KCl stock solution; adjust to pH 7.4 with NaOH and make up to 100 ml. Solution C, omit the NaCl, start with 50 ml water.

Solution D: Dissolve 7.2 g NaCl in 50 ml water; add 5 ml, 3 ml, 0.3 ml and 0.3 ml respectively of HEPES KCl, CaCl₂ and EDTA stock solutions; adjust to pH 7.4 with NaOH and make up to 100 ml.

3. Protocol

1. **OptiPrep™**: Dilute 4.6 ml of solution A with 5.4 ml of solution B to produce a Working Solution of approx 1.15 g/ml. **OR**
2. **Nycodenz®** To make 100 ml of the 1.15 g/ml Working Solution place approx. 50 ml of Solution C in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 27.6 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and then make up to 100 ml with water. Filter sterilize if required.
3. To make up a 1.106 g/ml and 400 mOsm density barrier, mix the chosen Working Solution with Solution D and water in the volume ratio of 0.7:0.08:0.22 respectively; to make up a 1.091 g/ml and 325 mOsm density barrier use ratios of 0.6:0.06:0.34.
4. Collect the peritoneal cells in the Solution E and centrifuge at 300 g for 10 min at 20°C.
5. Wash the cells in Solution E once and resuspend the cells in this medium to about 10⁷ cells/ml.
6. Underlayer 7 ml of the cell suspension with 3 ml of density barrier and centrifuge at 700 g for 20 min at 20°C.
7. The mononuclear cells and PMNs separate across the density barrier (see Note 3).

4. Notes

1. Making up the 1.15 g/ml working solution from OptiPrep™ is certainly easier than it is from Nycodenz® powder and the two solutions will have the same physical properties. It is therefore very unlikely that the separation will differ but it should be stressed that the use of OptiPrep™ has not been validated.
2. Nycoprep™ 1.15 contained 3 mM KCl, 5 mM Tris-HCl, pH 7.5 and 0.3 mM CaNa₂EDTA. The latter was included to improve the long-term stability of the solution. It has been omitted from the working solutions. If the concentration of EDTA is important to the separation however, 0.6 mM CaNa₂EDTA or Na₂EDTA may be included in Solutions B or C. For more information about preparing density gradient solutions for mammalian cells see [Application Sheet C01](#).
3. The authors reported ca. 95% purity of the mononuclear cells (top band) and PMNs (bottom band).

5. References

1. Hattori, H., Imai, H., Hanamoto, A., Furuhashi, K. and Nakagawa, Y. (2005) *Up-regulation of phospholipid hydroperoxide glutathione peroxidase in rat casein-induced polymorphonuclear neutrophils* Biochem. J., **389**, 279-287
2. Frevert, C.W., Huang, S., Danace, H., Paulauskis, J.D. and Kobzik, L. (1995) *Functional characterization of the rat chemokine KC and its importance in neutrophil recruitment in a rat model of pulmonary inflammation* J. Immunol., **154**, 335-344
3. Freeman, G.E., Dalton, C.A. and Brooks, P.M. (1991) *A Nycodenz gradient method for the purification of neutrophils from the peripheral blood of rats* J. Immunol. Meth., **139**, 241-249
4. Fisker S., Kudahl, K. and Sonne, O. (1990) *Isolation of rat peritoneal mononuclear and polymorphonuclear leucocytes on discontinuous gradients of Nycodenz* J. Immunol. Meth., **133**, 31-38
5. Sawant, K.V. and McMurray, D.N. (2007) *Guinea pig neutrophils infected with Mycobacterium tuberculosis produce cytokines which activate alveolar macrophages in non-contact cultures* Infect. Immun., **75**, 1870-1877

Application Sheet C19; 6th edition, May 2016

