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™.

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 $CaNa_2$-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® (ρ = 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)

A. OptiPrep™(shake the bottle gently before use) **OR** Nycodenz® powder

B. Diluent (OptiPrep™): 0.85% (w/v) NaCl, 6 mM KCl, 10 mM HEPES-NaOH, pH 7.4 **OR**

C. Diluent (Nycodenz®): 6 mM KCl, 10 mM HEPES-NaOH, pH 7.4

D. Hyperosmotic diluent: 3 mM KCl, 0.3 mM Na_2-EDTA, 0.3 mM $CaCl_2$, 1.245 M NaCl, 5 mM HEPES-NaOH, pH 7.4.

E. 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•$2H_2O$)	3.72 g per 100 ml water
100 mM KCl	0.75 g per 100 ml water
100 mM $CaCl_2$•$2H_2O$	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, $CaCl_2$ 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 10^7 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 easier than it is from Nycodenz® powder and the two solutions will have the same physical properties. It is 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 $CaNa_2EDTA$. 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 $CaNa_2EDTA$ or Na_2EDTA 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., Furuhama, K. and Nakagawa, Y. (2005) *Up-regulation of phospholipid hydroperoxide gluthatione 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 acitivate alveolar macrophages in non-contact cultures* Infect. Immun., **75**, 1870-1877

Application Sheet C19; 6th edition, January 2018

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