OptiPrep™ Application Sheet C12

Isolation of human polymorphonuclear leukocytes (granulocytes) from a leukocyte rich plasma in a discontinuous iodixanol gradient

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- **OptiPrep™ Application Sheet C03** "Purification of mononuclear cells, monocytes and polymorphonuclear leukocytes – a methodological review" compares all of the currently available methodologies. For isolation of PMNs from **experimental animals** see **Application Sheet C44**.
- **OptiPrep™ Reference List RC01** "Purification of mononuclear cells, monocytes and polymorphonuclear leukocytes" provides a comprehensive list of all the published papers reporting the use of OptiPrep™
- To access **RC01** return to the initial list of Folders and select "**Reference Lists**".
- 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

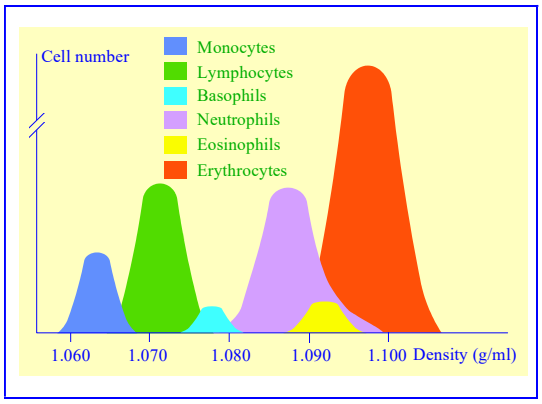

Figure 1: Density of human blood cells

1. Background

With the exception of basophils, the polymorphonuclear leukocytes (PMNs) or granulocytes from human peripheral blood have densities predominantly above 1.080 g/ml, while mononuclear cells (MCs) have densities below 1.077 g/ml (see Figure 1). Since the density of erythrocytes significantly overlaps that of the denser neutrophils there is only one means by which PMNs may be isolated from whole human blood using a single step method and that is to use the density gradient medium called Polymorphprep™. It contains diatrizoate and a polysaccharide [1] and has a high density (1.113 g/ml) and osmolality (445 mOsm). The high osmolality causes loss of water from the erythrocytes; creating a continuous gradient in which the MCs and PMNs band according to their buoyant densities. It is a very reliable method but the donor must be healthy; not presenting with any mild anemia and the separation must be carried out as soon as possible after the collection.

There are two alternative strategies that involve the use of a simple density barrier (ρ = 1.077 g/ml) solution, rather than a two-layer density gradient. (1) Whole blood is layered over the density barrier; after centrifugation the MCs band at the interface, while the erythrocytes and PMNs sediment through the barrier to form a pellet. The latter is then recovered and the erythrocytes selectively lysed in isotonic NH_4Cl or ice-cold water to leave a pure PMN fraction. Neither of these alternatives may be regarded as ideal. (2) In the other alternative a leukocyte-rich plasma (LRP), prepared from whole blood by polysucrose-aggregation of the erythrocytes (as described in this Application Sheet), is layered over the density barrier which separates the MCs and PMNs. The latter form a pellet at the bottom of the tube. These two alternatives may be considered less suitable because the formation of a pellet of the cells of interest, may lead to some aggregation of the PMNs with consequent impairment of function.

Because of the possible undesirable effects of pelleting the PMNs, it is strongly recommended that an LRP is used; a dense cushion (1.090-1.095 g/ml) may then be placed beneath the 1.077 g/ml layer in order to band the PMNs rather than pellet them. The two density gradient solutions can be easily prepared from OptiPrep™ and it is this method that is described in the Application Sheet.

♦ Preparation of the leukocyte-rich plasma is achieved by adjusting the blood to 0.6% (w/v) polysucrose and allowing the aggregated erythrocytes to sediment at 1 *g*. There are however several popular alternatives that are described in Notes 1 and 2.

2. Solutions required

A. OptiPrep™ (shake gently before use).
B. Diluent: 0.85% (w/v) NaCl, 1 mM EDTA, 20 mM HEPES-NaOH, pH 7.4
C. Polysucrose: 6% (w/v) polysucrose (M_r = 400-500 x 10^3) in 0.85% (w/v) NaCl
D. Lysis buffer: 0.83% (w/v) NH_4Cl, 10 mM HEPES-NaOH, pH 7.0
E. 1.8 (w/v) NaCl, 20 mM Hepes-NaOH, pH 7.4

♦ Strategies for preparing density solutions for mammalian cells are described in **Application Sheet C01.**

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

Solution B: Dissolve 0.85 g NaCl in 50 ml water, add 20 ml and 1 ml of HEPES and EDTA stock solutions respectively; adjust to pH 7.4 with 1 M NaOH; make up to 100 ml.

Solution C: Add the polysucrose **slowly** to the rapidly stirred NaCl solution.

Solution D: Dissolve 0.83 g NH_4Cl in 50 ml water; add 10 ml of HEPES stock solution, adjust to pH 7.0 with 1 M NaOH and make up to 100ml.

Solution E: Dissolve 1.8 g NaCl in 50 ml water, add 20 ml of HEPES stock solution; adjust to pH 7.4 with 1 M NaOH; make up to 100 ml

3. Protocol

1. To 9 vol. of freshly drawn blood (containing 2 mM EDTA as anticoagulant) add 1 vol. of Solution C (see Notes 1 and 2).

2. Allow the aggregated erythrocytes to settle to the bottom (20-40 min at room temperature); then aspirate the entire supernatant.

3. Prepare the following density solutions from OptiPrep™ and Solution B (respectively): 1.077 g/ml, 5 vol. + 17 vol. and **EITHER** 1.090 g/ml, 8 vol. + 22 vol.; **OR** 1.095 g/ml, 17 vol. + 43 vol. (see Notes 3 and 4).

4. Underlayer 5 ml of LRP with 2.5-3.0 ml of 1.077 g/ml solution and the same volume of **EITHER** 1.090 g/ml **OR** 1.095 g/ml (see Fig. 2 and Note 5).

5. Centrifuge at 18-22°C for 25 min at 800 *g*.

6. Harvest the PMNs from the lower interface and the mononuclear cells from the upper interface (see Fig. 2).

7. Dilute the PMN suspension with an equal volume of Solution B and collect the PMNs by centrifugation at 250-350 *g* for 10 min.

8. Resuspend the pellet in a suitable medium for analysis.

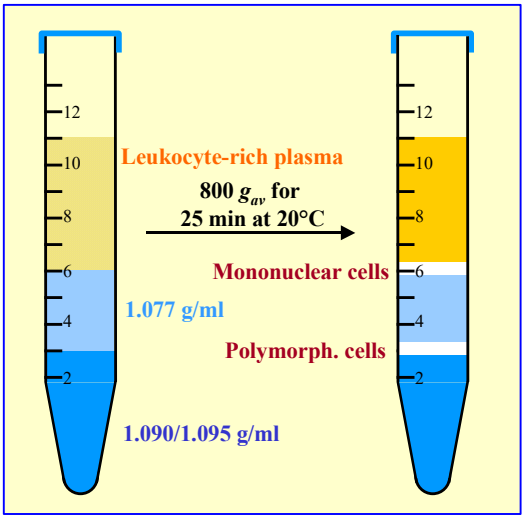

Figure 2 Separation of polymorphs from mononuclear cells from a leukocyte-rich plasma.

9. To remove residual erythrocyte contamination of the PMNs, resuspend the cell pellet in 3 ml of Solution D and incubate at 37°C for 7 min **OR** resuspend the PMNs in 3 ml ice-cold distilled water, then after 30 sec add an equal volume of Solution E.

10. Harvest the PMNs by centrifugation and resuspend in a suitable medium (see Note 6).

4. Notes

1. If exposure of the cells to polysucrose is deemed undesirable (there is evidence that this macromolecule can adsorb to the surface of leukocytes) then centrifuge the blood at 200 g_{av} for 15-20 min at 18-22°C and harvest the buffy coat in the plasma supernatant from the top of the packed erythrocytes and use this in Step 4.
2. Methylcellulose can be added to the blood is an alternative to polysucrose for erythrocyte aggregation. A variant of the method for removing the erythrocytes, adapted from Boyum [2], is to layer the blood over 12% (w/v) iodixanol in 130 mM NaCl, containing 1.66% methylcellulose. After standing at 1g the aggregated erythrocytes sediment to the bottom of the tube [3,4].
3. If the density of this cushion is 1.090 g/ml a small percentage of the neutrophils and most of the eosinophils will sediment through this layer. If a density of 1.095 g/ml is chosen, virtually all of the PMNs will be retained by the high-density barrier. On the other hand, fewer of the residual erythrocytes in the LRP will contaminate the PMN band using the lower density cushion. See section 5 for some examples.
4. Occasionally the low-density 1.077 g/ml iodixanol layer is replaced with a routine commercial peripheral blood mononuclear (PBMC) isolation medium such as Lymphoprep™. There may be some merit in the use of this solution as one of its components, 5.7% (w/v) polysucrose, may cause the residual erythrocytes to aggregate further and thus assist their sedimentation into a pellet. On the other hand this concentration of polysucrose is almost ten times higher than that to which the blood is adjusted in Step 2 of the protocol. See Section 5 for some examples.
5. For more information about preparing discontinuous gradients see **Application Sheet C02**.
6. In a recent study of twelve healthy males [5], the two-layer iodixanol gradient consistently gave a yield of ≥ 95%.

5. Methodological review

Some of the gradient and centrifugation conditions described in published papers reporting the use of the method described in this Application Sheet to purify PMNs from primate peripheral blood are summarized in Table 1.

Table 1 Primate PMN isolation using OptiPrep™

Species	Gradient and centrifugation comments	Ref. #
Human	Iodixanol solutions of 1.077 + 1.090 g/ml, 1200 *g* for 25 min	6
	Iodixanol solutions of 1.077 + 1.090 g/ml, 800 *g* for 30 min	7
	Iodixanol solutions of 1.077 + 1.090 g/ml, 600 *g* for 20 min	8
	PBMC isolation medium + iodixanol solution of 1.095 g/ml, 400/500 *g* for 25/20 min	9-16
Monkey	Iodixanol solutions of 1.077 + 1.095 g/ml	17

The methodology has been used, with minor variations, for the isolation of PMNs from bovine, guinea pig, mouse, rabbit and rat sources. For references see **Reference List RC01**.

6. References

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