OptiPrep™ Application Sheet C10

Separation of monocytes from a leukocyte-rich plasma by flotation through 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
- **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 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
- An alternative flotation method from **whole blood** is described in **Application Sheet C11 (see index)**. A **sedimentation method** (from a leukocyte-rich plasma) is also available for the isolation of a monocyte-rich fraction (**see Application Sheet C46)**

1. Background

The monocytes in human peripheral blood, account for, on average, about 8% of the leukocyte population. They tend to be larger (15-20 µm) than lymphocytes (6-20 µm) and they also have a slightly lower density (Figure 1). These properties allow some scope for their separation by centrifugation. Boyum [1] introduced a Nycodenz® density barrier (ρ = 1.068 g/ml) for resolving monocytes and lymphocytes from a leukocyte-rich plasma. This is commercially available as Nycoprep™ 1.068. It has a slightly raised osmolality (335 mOsm); this enhances the density difference between the monocytes and the osmotically-sensitive lymphocytes, whose density is increased. The method is very effective and the purity of the monocytes is greater than 90% but the monocytes do not form a distinct band; they are concentrated in the upper half of a broad turbid zone within the Nycoprep™ 1.068.

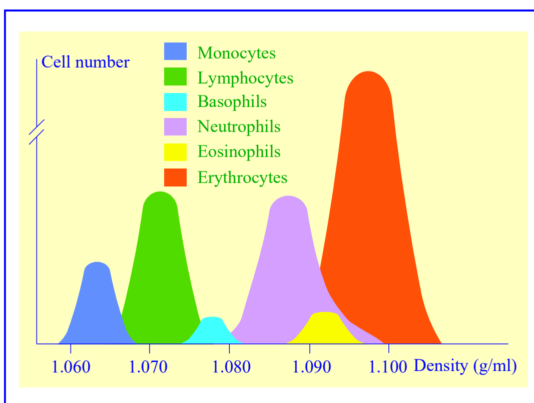

Figure 1: Density of human blood cells

In the alternative strategy developed by Graziani-Bowering et al [2], which is the subject of this Application Sheet, OptiPrep™ is added to a leukocyte-rich plasma (LRP) to raise its density to approx 1.1 g/ml and overlaid by two lower density layers of 1.084 and 1.068 g/ml (Figure 2-1). The leukocytes rapidly float to the top of the plasma layer when the tube is centrifuged and initially form a narrow band at the interface between the sample and the 1.084 g/ml solution layer (Figure 2-2). The monocytes (because of their size and low density) migrate upwards through this layer and into the 1.068 g/ml layer. The smaller and denser lymphocytes tend to float more slowly, and in this way a separation between the two types of cells is effected (Figure 2-3). Because of the heterogeneity of the monocyte population the monocyte band is

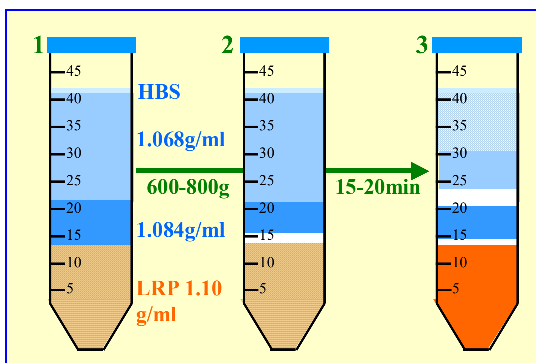

Figure 2: Isolation of human monocytes: LRP = leukocyte-rich plasma; HBS = Hepes-buffered saline; See text for more information.

diffuse and may occupy at least a 10 ml zone below the HBS. Polymorphonuclear leukocytes (granulocytes) from the LRP tend to remain at the top interface of the sample zone.

2. Solutions required

A. OptiPrep™ (shake gently before use)

B. Diluent: 1.0% (w/v) NaCl, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4, containing 0.5% (w/v) bovine serum albumin (make up fresh)

C. HEPES-buffered saline: 0.85% (w/v) NaCl, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4, containing 0.5% (w/v) bovine serum albumin (make up fresh)

Keep the following stock solutions at 4°C:
100 mM HEPES (free acid) 2.38 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$) 3.72 g per 100 ml water.

Solution B: Dissolve 1.0 g NaCl and 0.5 g BSA in 50 ml water, add 10 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: as Solution B using 0.85 g NaCl

3. Protocol (for 50 ml of blood)

1. Prepare two solutions of 1.068 g/ml and 1.084 g/ml by mixing OptiPrep™ and solution B using the following volume ratios: 1 vol. + 4 vol. and 1 vol. + 3 vol. respectively (see Note 1).

2. Centrifuge freshly drawn, whole blood (anti-coagulant 1.5 mM EDTA final concentration) at 400 g_{av} in a swinging-bucket rotor, at about 20°C, for 10-15 min.

3. Harvest the buffy coat in approx 10 ml of the plasma supernatant (LRP). Some erythrocytes will also be collected but try to keep them to a minimum. Over 80% of the leukocytes are recovered in this manner.

4. Mix the LRP with OptiPrep™ (10 ml + 4 ml respectively) and in a 50 ml centrifuge tube overlayer with 7.5 ml of the 1.084 g/ml solution and 20.0 ml of the 1.068 g/ml solution and then layer a small volume of Solution C (approx. 0.5 ml) on top (see Notes 3-5).

5. Centrifuge at 600-800 g_{av} in a swinging-bucket rotor for 20-25 min at 20°C. Do not use the brake during deceleration (see Notes 6 and 7).

6. Collect the monocytes that float into the 1.068 g/ml layer (see Figure 2 and Notes 8 and 9).

4. Notes

1. As this method separates the monocytes and lymphocytes on the basis of density and size, small differences in run conditions from laboratory to laboratory may influence its success. Improved recoveries of monocytes may be obtained by adjusting the density of the 1.084 g/ml layer within the range 1.079-1.089 g/ml. There is evidence that it may be preferable to prepare the two density gradient solutions by diluting the OptiPrep™ Stock with a culture medium (RPMI or DMEM) containing 10% serum. The small increase in the density of two solutions (an increase of approx 0.002 g/ml) may also be beneficial. For density tables see **Application Sheet C01**.
2. The method only works satisfactorily on fresh blood (used within 2 h of drawing) from healthy individuals.
3. For smaller amounts of LRP use a 15 ml tube and scale down all volumes to maintain the geometry of the interfaces observed in a 50 ml tube. For more information about preparing discontinuous gradients for cell fractionation see **Application Sheet C02**.
4. The topmost layer of HEPES-buffered saline is important - cells reaching the top of the 1.068 g/ml in the absence of the saline tend to adhere to the wall of the tube.
5. In the preparation of the density solutions, a 1% NaCl solution is used rather than a 0.85% NaCl solution because of the sensitivity of monocytes to reductions in ionic strength. It may therefore be useful dilute the LRP with a medium other than OptiPrep™ itself. Dilution of OptiPrep™ (4.5 vol.) with 0.5 vol. of 8% NaCl, 10 mM Hepes-NaOH, pH 7.4 will produce a solution containing 54% iodixanol and 0.8% NaCl, with a density of 1.293 g/ml. Dilution of the LRP (10 ml) with 4.5 ml of this solution will raise the density of the LRP in the same way as adding 4 ml of OptiPrep™

Surface Marker	% events
CD3+	3.4
CD14+/CD4-	1.6
CD14-/CD4+	6.9
CD14+/CD4+	84.1
All monocytes	92.6

Table 1: Flow cytometry analysis of monocyte band Figures are % of cells exhibiting the cell surface marker, adapted with permission from ref 2.

but the ionic strength will be raised. This modification has not been investigated but it may improve monocyte viability.

6. Increasing the time of centrifugation will increase yields but decrease purity. If lymphocyte contamination is unacceptable, try reducing the centrifugation time or harvest less of the 1.069 g/ml layer or make the middle density barrier 1.079 g/ml rather than 1.084 g/ml.
7. Note that in **Application Sheet C11**, the centrifugation is carried out at 4°C; this may improve monocyte viability, but the lower temperature has not been investigated with the method described in this Application Sheet.
8. Because of small variations in tube sizes and centrifugation conditions the precise position of the monocyte band may vary. The monocyte band isolated by this technique has been analyzed by flow cytometry and contains at least 90-95% monocytes with only 3-5% contamination from T-cells. Results from a typical experiment are given in Table 1.
9. A comparison of the production of cytokines from monocytes isolated from the blood of pregnant women by iodixanol flotation and elutriation showed no statistically significant difference between the two methods [3]. Although the method was developed primarily for the isolation of monocytes, it has been used for the simultaneous recovery of lymphocytes [4] and both lymphocytes and PMNs [5].

♦ Ovine monocytes have also been isolated by this method [6]

5. References

1. Bøyum, A. (1983) *Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium* Scand. J. Immunol., **17**, 429-436
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3. Nutt, J.C., Willis, C.C., Morris, J.M. and Gallery, E.D.M. (2004) *Isolating pure populations of monocytes from the blood of pregnant women: comparison of flotation in iodixanol with elutriation* J. Immunol. Meth., **293**, 215-218
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