OptiPrep™ Mini-Review MC01

Mononuclear cells, monocytes and polymorphonuclear leukocytes: a methodological review

- **Mini-Review MC02** is a bibliography listing all the references reporting the use of iodixanol for mononuclear cells, according to species, cell type and research topic

1. Iodinated density gradient media

In the early nineteen-sixties Arne Bøyum, who was working in Oslo on the fractionation of blood leukocytes, recognized that the derivatives of triiodobenzoic acid that were being synthesized as X-ray imaging agents (for human intravenous injection) would also make ideal density gradient media for mammalian cell fractionation. The modern version of the medium that he devised for the purification of human peripheral blood mononuclear cells (PBMCs), which is marketed by Alere Technologies AS under the trade-name Lymphoprep, is almost identical to that described

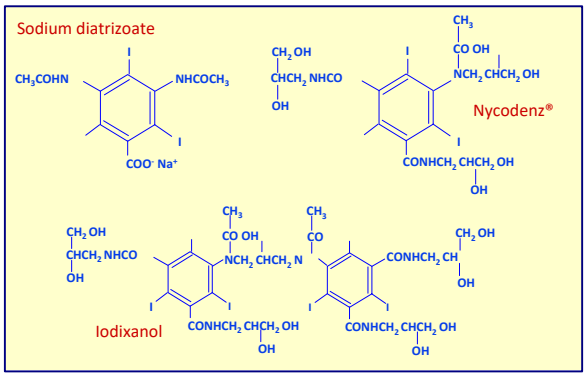


Figure 1: Molecular structure of iodinated density gradient media

in Boyum's seminal paper published in 1968 [1]. It contains the ionic compound sodium diatrizoate (also known as Hypaque™); its molecular structure is shown in Figure 1. Later non-ionic derivatives, which are better tolerated by cells, were produced as X-ray imaging agents. These included iohexol (known under the commercial name Nycodenz®) in the early nineteen-eighties and about ten years later iodixanol, which is more or less a dimer of Nycodenz® (see Figure 1). Iodixanol is available commercially as a sterile 60% (w/v) solution called OptiPrep™. Axis-Shield density gradient media are produced in facilities that operate under strict EU cGMP compliance and to the European Pharmacological Standard of <1.0 endotoxin unit/ml. The actual measured levels of endotoxin are regularly <0.13 units/ml. This information, together with density and osmolality data, is available on the Certificate of Analysis that accompanies each batch of medium.

- Because of their use as X-ray imaging agents, these compounds have been clinically tested; no other density gradient media conform to this high standard.

2. Density barrier isolation of human PBMCs

2a. Lymphoprep™

The isolation of human PBMCs is undoubtedly the most frequently performed of any density gradient technique.

- The composition of Lymphoprep™ is: 9.1% (w/v) sodium diatrizoate and 5.7% (w/v) polysaccharide; density = 1.077 ± 0.001 g/ml, osmolality = 290 ± 15 mOsm (<0.13 endotoxin units/ml).

The polysaccharide, which contributes to the overall density of the medium, also aggregates the erythrocytes to enhance their rate of sedimentation. The standard protocol is to dilute blood with an equal volume of saline; layer 6 ml over 3 ml of Lymphoprep™ and centrifuge at 800 *g* for 20 min. Typical results are shown in Figure 2.

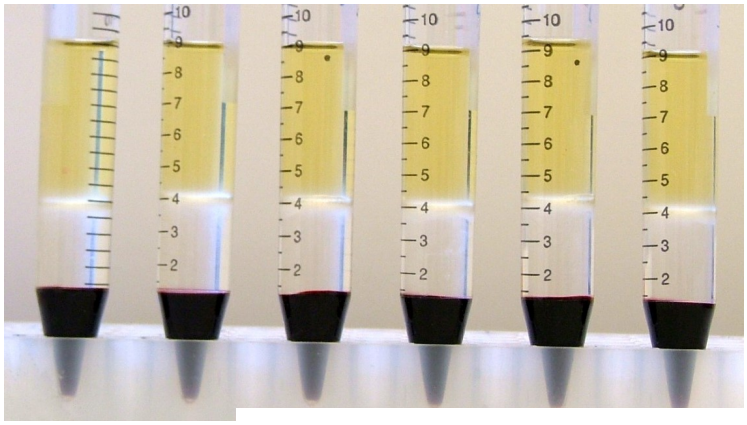


Figure 2: Purification of human PBMCs from six donors on Lymphoprep

For frequent processing of large numbers of blood samples the **Lymphoprep™ Tube** offers a time-saving option. Tubes are pre-filled with Lymphoprep™, contained below a porous plastic frit, thus permitting the diluted blood to be poured onto the frit. During centrifugation the erythrocytes pellet through the frit; displacing the medium upwards, allowing the PBMCs to band at the plasma/medium interface above the frit. The PBMCs may be recovered simply by pouring off the liquid from the tube. The procedure is illustrated in Figure 3. **Lymphoprep™ Tubes** containing 2 ml or 10 ml of Lymphoprep™ are available.

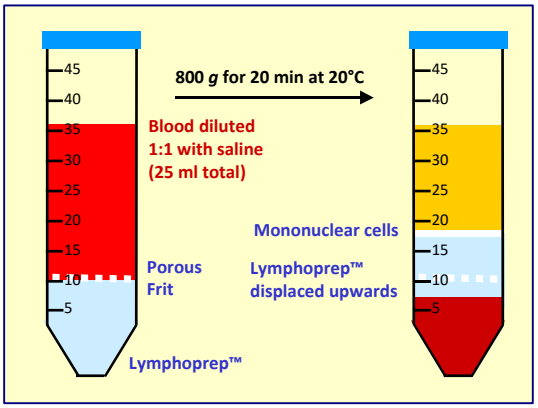


Figure 3: Purification of human PBMCs in a Lymphoprep™ Tube: diagrammatic representation

2b. Nycoprep™ 1.077

There is evidence that the polysaccharide in any of the commercial PBMC isolation media can be adsorbed on to the surface of lymphocytes and affect their mitogenic stimulation [2]. The only customized polysaccharide-free medium for the isolation of human PBMCs is Nycoprep™ 1.077. It has the same density, osmolality and low endotoxin levels as Lymphoprep™; it contains 14.1% (w/v) Nycodenz®, 0.44% (w/v) NaCl, 5 mM Tricine-NaOH, pH 7.0.

2c. From OptiPrep™

The 1.077 g/ml solution for human PBMC isolation may also be prepared by dilution of 5 vol. of OptiPrep ™ with 17 vol. of any suitable isoosmotic medium. The methodology is described in OptiPrep™ Application Sheet C03 (see Section 8)

2d. Removal of platelets from PBMCs isolated on a density barrier

A drawback of any sedimentation on to a density barrier is that the platelets co-band with the PBMCs. The routine procedure to remove platelets is to dilute the interface harvest with saline and centrifuge at a speed (approx. 300 *g*) and time (approx. 5 min) that will loosely pellet the PBMCs but leave most of the platelets in the supernatant. After very careful removal of the majority of the supernatant, the dilution with saline and centrifugation is repeated twice. The procedure is tedious and inefficient.

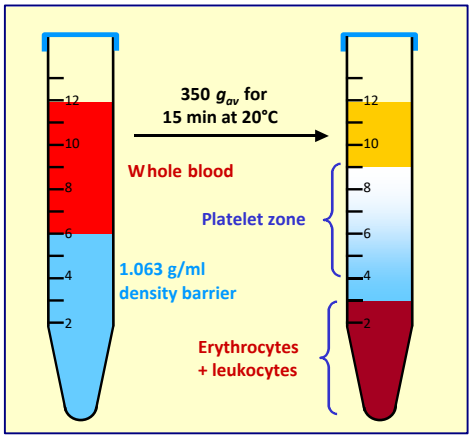


Figure 4: Removal of platelets from PBMCs harvested from a 1.077 g/ml barrier.

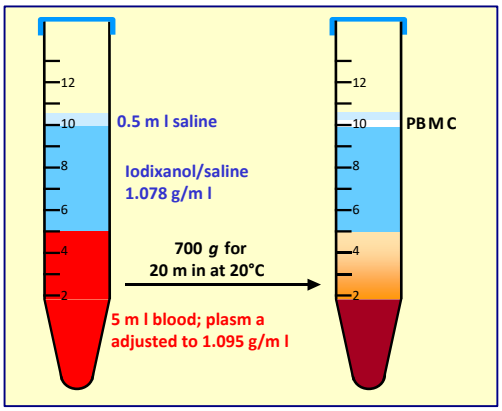
A simple sedimentation velocity separation was developed to prepare platelets from whole blood for functional studies. It is equally efficacious for the removal of platelets from a PBMC preparation. The PBMC harvest from above 1.077 g/ml the barrier is diluted with saline and layered over a 1.063 g/ml solution prepared from OptiPrep™ and centrifuged as described in Figure 4. All of the PBMCs sediment to the bottom of the tube, while the platelets form a broad band just beneath the interface. The method was originally worked out using Nycodenz® [3]. ♦ The methodology is described in OptiPrep™ Application Sheet C12 (see Section 8).

3. Flotation isolation of human PBMCs

3a. Mixer strategy

In 1990 Ford and Rickwood [4] published a method in which the plasma itself became the density barrier. A 19% (w/v) Nycodenz® solution (ρ = 1.100 g/ml) was added to an equal volume of whole blood to raise the density of the plasma to 1.077 g/ml. During centrifugation at 1500 *g* for 30 min at 20°C the erythrocytes and polymorphonuclear leukocytes (PMNs) sediment while the PBMCs float to the top and are recovered from the meniscus and the medium below it. In the modern version OptiPrep™ is simply mixed with the blood. An advantage of the method is that if the blood is mixed with the OptiPrep™ upon collection, the centrifugation may be carried out up to 24 h later. A small disadvantage is that the final density of the plasma depends on the haematocrit of the blood.

♦ The methodology is described in OptiPrep™ Application Sheet C04



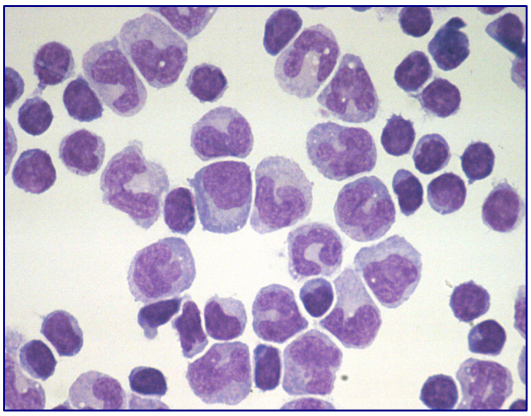


Figure 5a (left): Separation of platelet-free PBMCs by flotation, **5b (right)**: Photomicrograph of PBMC harvest

3b. Platelet-free PBMCs

Platelet contamination can be avoided entirely in a barrier flotation strategy. The plasma in the blood is adjusted to 1.095 g/ml (by addition of a 40% iodixanol solution); a solution of 1.077 g/ml (OptiPrep™ diluted with buffered saline) and a small volume of saline are layered on top. The PBMCs float to the top interface; all of the other cells and platelets remain at the bottom of the tube (see Figures 5a and 5b).

- The methodology is described in OptiPrep™ Application Sheet C05 (see Section 8)

4. Purification of monocytes from human blood

All monocyte purification methods use a leukocyte-rich plasma (LRP) rather than whole blood. The LRP may be prepared as a buffy coat by low speed centrifugation (400 *g* for 10-15 min) of whole blood or by allowing the erythrocytes to aggregate and sediment at 1 *g* in the presence of 0.6% (w/v) polysucrose.

4a. Sedimentation on to a density barrier

Boyum [5,6] introduced a Nycodenz® density barrier (ρ = 1.068 g/ml) for resolving monocytes and lymphocytes from a leukocyte-rich plasma (LRP). It had a slightly raised osmolality (335 mOsm) to enhance the density difference between the monocytes and the osmotically-sensitive lymphocytes (whose density is increased preferentially). 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 in the concentrated upper half of a broad turbid zone within the density barrier (see Figure 6). In the modern version of this method the density barrier is prepared by dilution of OptiPrep™ with a hyperosmotic buffered saline of 1.05% (w/v) NaCl, 10 mM Tricine-NaOH, pH 7.0.

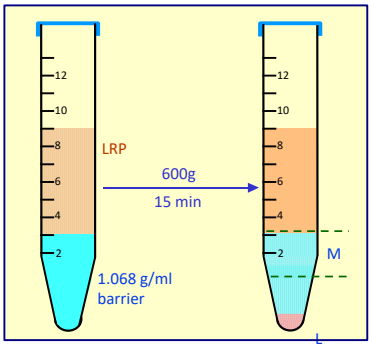


Figure 6 Separation of human monocytes from a leukocyte-rich plasma (LRP) on a density barrier: M = monocytes, L = lymphocytes

- The methodology is described in OptiPrep™ Application Sheet C51 (see Section 8)

4b. Flotation through a discontinuous gradient

In the alternative strategy developed by Graziani-Bowering et al [9], OptiPrep™ is added to the LRP to raise its density to approx 1.1 g/ml. The leukocytes will rapidly float to the top of this dense plasma (Figure 7:1-3) when this suspension is centrifuged. In this way the mononuclear cells initially form a narrow band at the interface between the sample and a 1.084 g/ml solution layered on top. The monocytes (because of their size and density) migrate upwards through this layer and through a second low-density barrier (ρ=1.068 g/ml). 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 7:2-3). Polymorphonuclear leukocytes (granulocytes) from the LRP tend to remain at the top interface of the sample zone.

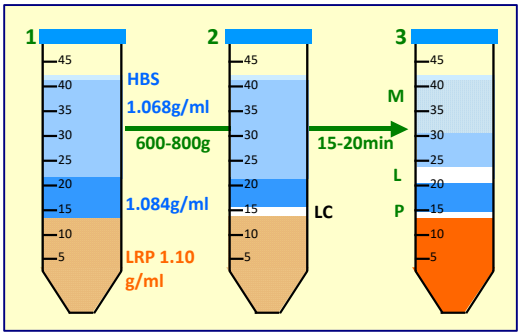


Figure 7: Isolation of human monocytes: LRP = leukocyte-rich plasma; LC =leukocytes; HBS = Hepes-buffered saline; M = monocytes, L= lymphocytes; P= polymorphonuclear leukocytes.

Flow cytometry analysis of the monocyte-rich band showed that only 3.4% of cells were $CD3^+$ (i.e. T-cells); 1.6% of cells were $CD14^+/CD4^-$, 6.9% were $CD14^-/CD4^+$ and 84.1% were $CD14^+/CD4^+$, i.e. a total of 92.6% were identified as monocytes [7].

The methodology is described in OptiPrep™ Application Sheet C09 (see Section 8)

- It has also been adapted to the use of whole blood in OptiPrep™ Application Sheet C10 (see Section 8)

5. Purification of human polymorphonuclear leukocytes (PMNs)

5a. From whole blood [8]

Polymorphprep™ contains 13.8% (w/v) sodium diatrizoate and 8% (w/v) Dextran 500; it has a density of 1.113 g/ml, a raised osmolality of 445 mOsm. It is the only medium capable of separating PBMCs and PMNs in one step from whole blood. **The use of whole blood is essential**: water in the dextran-aggregated erythrocytes, which sediment ahead of the leukocytes, passes into the Polymorphprep™ under the influence of the osmotic pressure gradient, effectively diluting the medium. As a consequence the osmotic pressure inside the erythrocytes increases; thus as they continue to sediment through the medium the osmotic pressure gradient between the cell and the medium and the loss of water from the cells progressively decline. The end result is the creation of a continuous density gradient in the medium. It is in this continuous gradient that the PBMCs and PMNs are resolved (see Figure 8). The efficacy of the method relies on the use of fresh blood from healthy donors.

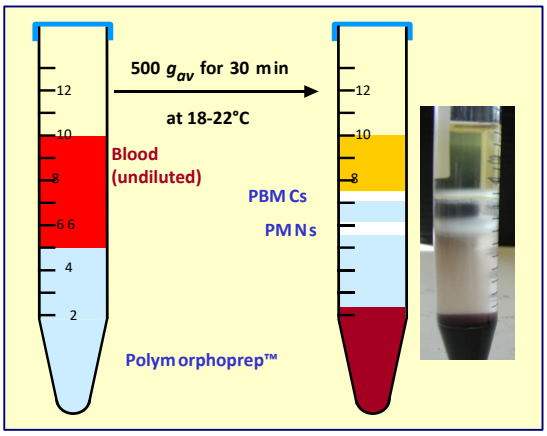


Figure 8: Purification of PMNs using Polymorphprep™ PBMCs = peripheral blood mononuclear cells; PMNs = polymorpho-nuclear leukocytes. Inset right shows the typical result of a separation in a 15 ml centrifuge tube from 5 ml of blood and 5 ml of Polymorphprep™

- The methodology is described in the Polymorphprep™ Application Sheet (see Section 8).

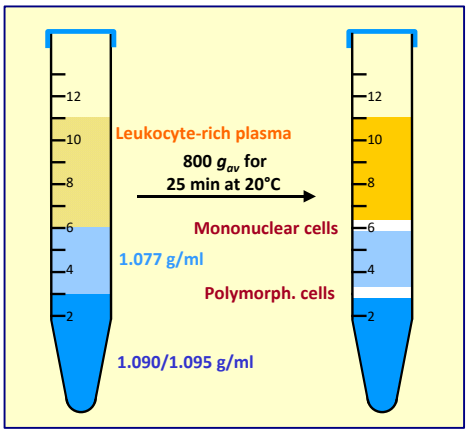


Figure 9: Purification of PMNs from an LRP.

5b. From a leukocyte-rich plasma (LRP)

The LRP is best prepared from whole blood by allowing the erythrocytes to aggregate and sediment at 1 *g* in the presence of 0.6% (w/v) polysucrose. If this is then layered over a solution of density 1.077 g/ml (for example Lymphoprep™) and centrifuged at 600-700 *g* for 20 min, then the PBMCs will band at the interface and the PMNs will pellet. This is quite a common approach. However, the pelleting and consequent aggregation of PMNs at the bottom of the tube disturbs the functional integrity of the cells. Pelleting can be avoided by including a high-density cushion beneath the 1.077 g/ml layer. The easiest strategy is to prepare both layers by dilution of OptiPrep™ with a buffered saline (see Figure 9). The method is more robust than the Polymorphprep™ method; it is less dependent on the time from drawing the blood.

- The methodology is described in OptiPrep™ Application Sheet C11 (see Section 8).

6. Mononuclear cells (MCs) and neutrophils from experimental animals

6a. Using a 1.077 g/ml density barrier

Although commercial media designed for isolation of human blood PBMCs (see Section 2a) such as Lymphoprep™ or Histopaque™ 1.077 have been used for rodent and rabbit blood, the yields are lower because lymphocytes from these species have a higher median density than those of human blood. Consequently there are some commercial media (e.g. Histopaque™ 1.083), which address this problem simply by raising the density of the medium from 1.077 g/ml to 1.083 g/ml. This effectively improves the yield of MCs but significantly increases the contamination from neutrophils. Bøyum et al [9] overcame this serious problem by using a 1.077 g/ml of slightly reduced osmotic pressure (265 mOsm). Lymphocytes are osmotically-sensitive, neutrophils are not; thus reduction of the osmotic pressure effectively reduces the density of lymphocytes but has no effect at all on the density of the neutrophils. A 1.077 g/ml, 265 mOsm density barrier is thus the only means of obtaining rodent and rabbit MCs in high yield without neutrophil contamination.

- The methodology has also been used for MCs from canine, porcine and bovine blood.
- The reduced osmolality barrier is no longer available commercially as Nycoprep™ 1.077A; it is however prepared very easily from OptiPrep™; the methodology is described in Application Sheet C43 (see Section 8).
- The reduced osmolality barrier is also used for the purification of MCs from a variety of animal tissues; the methodology is described in OptiPrep™ Application Sheet C40 (see Section 8).

If a leukocyte-rich plasma (LRP) is used instead of whole blood the same reduced osmolality 1.077 g/ml barrier may be used for the simultaneous isolation of neutrophils, which will pellet. The pellet will also contain erythrocytes not aggregated by the polysucrose during the preparation of the LRP. After removal of the MCs and all of the liquid above the neutrophil pellet, the latter is suspended in isotonic ammonium chloride to lyse the erythrocytes selectively. Finally the neutrophils are pelleted and resuspended in saline.

- The methodology is described in OptiPrep™ Application Sheet C45 (see Section 8).

6b Using a mixer flotation strategy

The method described in Section 3a has also been adapted to rat, mouse and bovine blood described in OptiPrep™ Application sheets C06, C07 and C08 respectively (see Section 8).

7. Clinical trials

There are now several papers from groups that have cultured the PBMCs purified in iodixanol gradients for administration to groups of patients with cancer [10-14].

8. References

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COSMO BIO USA
2792 Loker Ave W, Suite 101
Carlsbad, CA 92010

TEL: 760-431-4600
FAX: 760-431-4604
email : info@cosmobiousa.com
web : www.cosmobiousa.com