OptiPrep™ Application Sheet C44

Isolation of polymorphonuclear leukocytes (granulocytes) from rat, mouse, guinea pig and rabbit blood

- 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

A common approach to the problem of isolating rodent and rabbit polymorphonuclear leukocytes (PMNs) was to use a density barrier of Nycoprep™ 1.077A (ρ = 1.077 g/ml, osmolality = 265 mOsm). The method was first published by Bøyum et al [1]. This customized medium separated the mononuclear cells (MCs), which band at the interface, from the PMNs and the erythrocytes, which sediment through the barrier to form a pellet. To retrieve the PMNs, the erythrocytes may be selectively lysed in isotonic ammonium chloride solution or ice-cold distilled water. An alternative approach is to remove the erythrocytes from whole blood first by aggregation with dextran [2-4], methylcellulose [5,6] hetastarch [7] or Plasmagel [8,9] and then layer the resulting leukocyte-rich plasma (LRP) over the Nycoprep™ 1.077A, so that the PMN pellet contains only a very small percentage of residual erythrocytes. Nycoprep™ 1.077A is no longer commercially available but an iodixanol solution of the same density and osmolality can be easily prepared from OptiPrep™ and it is this method that is described in Section 2.

There are also a few examples of the use of other density barriers and two-layer discontinuous gradients, prepared from both Nycodenz® and OptiPrep™. A few examples are given in Section 3. Polymorphprep™, a medium normally restricted to the use of human blood, has also been used in a few cases (see Section 4).

2. Reduced osmolality density barrier

2a. Solution preparation (see Box)

A. OptiPrep™ (60%, w/v iodixanol) – shake the bottle gently before use

B. Buffered saline (isoosmotic): 0.85% (w/v) NaCl, 10 mM Tricine-NaOH, pH 7.0

C. Buffered saline containing EDTA

D. 20% (w/v) polysucrose (dextran), MWt ≥450,000 in Solution B (see Section 2c, Note 1)

E. Erythrocyte lysis solution

Keep the following stock solutions at 4°C:
100 mM Tricine: 1.79g per 100 ml water.
100 mM EDTA(K_2•$2H_2O$) 4.40 g per 100 ml water

Solution B: Dissolve 0.85g NaCl in 50 ml water; add 10 ml of Tricine stock solution; adjust to pH 7.0 with 1 M NaOH and make up to 100 ml.

Solution C: Dissolve 0.85 g NaCl in 50 ml water, add 10 ml and 3 ml of Tricine and EDTA stock solutions respectively; adjust to pH 7.0 with 1 M NaOH; make up to 100 ml.

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

2b. Protocol

1. Start by preparing the hypoosmotic 1.077 g/ml density barrier: dilute Solution B with water (2.5 vol. + 0.5 vol.) and then mix 2.7 vol. of OptiPrep™ with 9.3 vol. of the diluted saline solution (see Section 2c, Note 2).

2. Collect the blood by cardiac puncture into a syringe containing a volume of Solution C equal to that of the expected blood volume (see Section 2c, Note 3).

3. Mix 9.25 vol. of blood with 0.75 vol. of Solution D (see Section 2c, Notes 4-6).

4. Allow the blood to stand at room temperature for 30 min then remove the leukocyte-rich plasma (LRP) from above the aggregated erythrocyte pellet.

5. Layer 1 vol. of the leukocyte-rich plasma over 0.5 vol. of the 1.077 g/ml density barrier prepared in step 1 (see Section 2c, Note 7).

6. Centrifuge at 700 *g* for 20 min at room temperature and remove all of the liquid above the pellet by aspiration (see Section 2c, Note 8).

7. Suspend the pellet in 5 vol. of Solution E and lyse the erythrocytes by incubation at 37°C for 7 min (see Section 2c, Note 9).

8. Sediment the PMNs at 250-300 *g* for 15 min and resuspend in Solution B or process as required (see Section 2c Note 10).

2c. Notes

1. Polysucrose is best dissolved by slowly adding the liquid to the solid, stirring with a glass rod after addition of each 1-2 ml aliquot.
2. If an osmometer is available check the osmolality of these solutions; the osmolality of the barrier solution should be 265±10 mOsm. The dilutions should be prepared as accurately as possible. OptiPrep™ is quite viscous and when dispensing volumes with an automatic pipette the liquid should be aspirated into, and ejected from, the pipette tip slowly. If there is significant loss of mononuclear cells to the pellet, try increasing the density of the barrier slightly (2.8 vol. of OptiPrep™ and 9.2 vol. of the diluted saline).
3. Compared to human blood collected by venepuncture, both mouse and rat blood (collected by cardiac puncture) is very prone to clotting. Even if anticoagulant is present in the syringe, coagulation can still be problematical. This is minimized if the blood is also diluted directly by pre-charging the syringe with a volume of saline containing EDTA (4 mM) at twice the required final concentration (Solution C). **If this approach is not used, then dilute the blood with an equal volume of Solution B before proceeding to Step 3**. We have found that EDTA is the most reliable and use it at 2 mM final concentration; other workers use concentrations as high as 4 or 5 mM or other anticoagulants such as citrate or heparin. See Section 1 above for more information on preparing the LRP.
4. It is also possible to use whole blood rather than an LRP in this separation and to remove the erythrocytes from the pellet (see step 6) by aggregation in polysucrose. Although this approach is rather uncommon, it does reduce the time before the gradient separation of the PMNs from the mononuclear cells and platelets (see ref 10).
5. If the blood is not diluted during cardiac puncture (see Note 3) it is common to add an equal volume of 6% (w/v) polysucrose [2,3], although half this concentration is often effective. On the other hand if the blood has been diluted during cardiac puncture, the use of a small volume of a high concentration polysucrose solution (20%) minimizes any further dilution of the blood; make sure that the viscous polysucrose is mixed well with the blood by repeated gentle inversion. If the aggregation is unsatisfactory, double the volume of 20% polysucrose.
6. Other erythrocyte aggregating agents may be used: 2% (w/v) methylcellulose is added at a volume ratio to whole blood of 1:10 [6]; 2-3 ml of Plasmagel was added to 5 ml of heparinized whole blood by Song et al [8]; equal volumes of whole blood and 6% (w/v) hetastarch in 0.9% NaCl is a third option [7].
7. Because of the functional problems that can arise from the pelleting of PMNs, some workers include a small volume of a dense cushion to prevent this; a cushion of density 1.11 g/ml can be simply produced by diluting 1 vol. of OptiPrep™ with 2 vol. of the saline solution prepared in Step 1. A typical separation in a 15 ml conical tube therefore might be 5 ml of LRP/saline, 2.5 ml of the 1.077 g/ml density barrier and 1.0 ml of cushion. For small volumes of mouse blood use a smaller volume (narrower) tube.
8. If a cushion is used remove all of the liquid above the PMN band and, using a syringe attached to a length of Teflon tubing, remove as much of the cushion as possible.
9. Lysis using ice-cold distilled water for 30 sec before adding an equal volume of double strength saline is an alternative.

10. For a selection of papers reporting the use of NycoPrep 1.077A for rabbit PMNs and rodent PMNs see refs 11-13 and 2,4,6,14-19 respectively. Rat PMNs have been prepared using this OptiPrep™-based method [20]

♦ For more recent papers reporting the use of the OptiPrep™ methodology see refs 21-25

3. Other density barriers and two-layer discontinuous gradient

Rodent PMNs have also been separated from mononuclear cells by pelleting through a 1.09 g/ml barrier at 600 *g* for 15 min [26]. This was prepared from Nycodenz® but could easily be formed by dilution of OptiPrep™ with a regular buffered saline solution and is equivalent to an iodixanol concentration of 16% (w/v). From a functional point of view, it would be better to include a small dense cushion of about 1.11 g/ml (20% iodixanol) to prevent the pelleting of the PMNs.

The use of a two layer discontinuous gradient was developed by Freeman et al [27] who started with an isoosmotic solution of 27.6% (w/v) Nycodenz® in 3 mM KCl, 0.3 mM $CaNa_2$-EDTA 5 mM Tris-HCl, pH 7.5 (this isoosmotic solution, which was available as NycoPrep 1.15 is no longer produced commercially). It was diluted with the same KCl, EDTA, Tris solution containing 0.75 g NaCl/100 ml to produce solutions of 18.4% and 13.8% Nycodenz® (ρ = 1.098 and 1.075 respectively). A rat leukocyte-rich plasma (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. The diluent was slightly hypoosmotic (see Section 2). Iodixanol solutions of the same % (w/v) concentration and very similar osmolality could be produced by a two-step dilution of OptiPrep™:

1. Dilute OptiPrep™ with an equal volume of normal buffered saline (Solution B in Section 2a) to produce an isoosmotic 30% (w/v) iodixanol solution
2. Dilute this further to produce 18.4% and 13.8% (w/v) iodixanol solutions with a 0.75% (w/v) NaCl solution in 10 mM Tricine-NaOH, pH 7.0

Rabbit PMNs have also been isolated from an LRP using a two-layer iodixanol gradient (density 1.085 and 1.086 g/ml) with centrifugation at 500 *g* for 35 min [28,29]

4. Polymorphprep™

There are a few reported cases of the use of Polymorphprep™ for rat [30-33], mouse [34-36] and rabbit [37-40] PMNs. Although the rationale behind the use of this medium for human PMNs is that the use of whole blood is essential to the separation, see **Polymorphprep™ Application Sheet (on home page, select "Products")** for more details, Shibata et al [39] used a PMN-rich suspension from which the erythrocytes had been removed. McCartney-Francis et al [31] diluted the Polymorphprep™ 5:1 for rat PMNs and the use of a high concentration of EDTA (7.7 mM) may reflect the aggregation problems sometimes encountered with rat blood [32]. Wen et al [35] used this medium for the removal of PMNs from mouse leukocytes prior to isolation of macrophages and monocytes. In a variant of the two-layer discontinuous gradient the dense cushion layer was replaced by Polymorphprep™, the high osmolality of this layer presumably allowing the majority of the erythrocytes to sediment through it [41].

5. Other species

PMNs have been isolated from bovine [42,43] and guinea pig blood [44] in iodixanol gradients

6 References

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