C45 Isolation of polymorphonuclear leukocytes (granulocytes) from rat, mouse and rabbit blood

- **OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml**
- For links to other relevant files click on the double blue arrow in the following text

1. Background

A common approach to the problem of isolating rodent and rabbit polymorphonuclear leukocytes (PMNs) is to use a medium that primarily is used for the isolation of mononuclear cells (MCs) from these animals. This is described in Application Sheet C43 ➔➔. Briefly, the method involves layering blood (diluted 1:1 with buffered saline) over half its volume of a density barrier (ρ = 1.077 g/ml, osmolality = 265 mOsm). After centrifugation the MCs are recovered from the interface. Because the PMNs and erythrocytes sediment into the pellet, one commonly used option (Strategy A) is firstly to remove the erythrocytes from whole blood by aggregation with dextran [1-3], methylcellulose [4,5] hetastarch [6] or Plasmagel [7,8] and then layer the resulting leukocyte-rich plasma over the density barrier. A modification of this, which is incorporated into this protocol, is to underlay the barrier with a dense cushion that will retain the PMNs and thus prevent the PMNs from forming a pellet. To retain the best functional integrity of PMNs, avoidance of pelleting is widely regarded as important.

A second option is to use whole blood; recover the erythrocyte + PMN pellet and then selectively lyse all the erythrocytes in isotonic ammonium chloride solution or ice-cold distilled water (Strategy B). Some other options are discussed in Section 5.

2. Strategy A

2a. Solutions required

A. OptiPrep™ (60%, w/v iodixanol)
B. Buffered saline: 0.85% (w/v) NaCl, 10 mM Tricine, pH 7.0
C. Buffered saline + EDTA: 0.85% (w/v) NaCl, 2 mM EDTA, 10 mM Tricine-NaOH, pH 7.0 (see Note 1)
D. 20% (w/v) polysucrose (dextran), MWt ≥450,000 in Solution B (see Notes 2-4)
E. Lysis buffer: 0.83% (w/v) NH_4Cl, 10 mM Tricine-NaOH, pH 7.0

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 2 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. Dilute Solution B with water (volume ratio 2.5:0.5 respectively); this solution has an osmolality of approx 242 mOsm (see Note 5).

2. Produce the density barrier solution by diluting OptiPrep™ with the 242 mOsm solution (volume ratio 2.7: 9.3 respectively); this solution has a density of 1.077 g/ml and osmolality approx 265 mOsm (see Note 5).

3. Produce a cushion solution of density approx. 1.10 g/ml by diluting 1.8 vol. of OptiPrep™ with 4.2 vol. of Solution B (see Note 6).

4. Collect the blood by cardiac puncture into a syringe anticoagulant; EDTA, citrate, ACD or heparin is usually satisfactory (see Note 1).

5. Dilute the blood with an equal volume of Solution C (see Note 1).

6. Mix 9.25 vol. of the blood with 0.75 vol. of Solution D (see Notes 3 and 4).

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

8. Layer 1 vol. of the LRP over 0.5 vol. of 1.077 g/ml density barrier (see Steps 1 and 2) and 0.5 vol. of the cushion. Underlayering the sample using a syringe and metal cannula is an alternative (see Notes 7 and 8).

9. Centrifuge at 700 *g* for 20 min at room temperature and harvest the PMNs from the lower interface (see Figure 1).

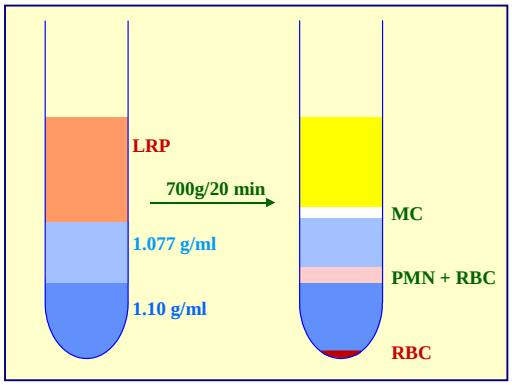

Figure 1 Separation of rodent PMNs in a discontinuous iodixanol gradient: LRP = leukocyte-rich plasma, MC = mononuclear cells, PMN = polymorphonuclear leukocytes, RBC = red blood cell pellet (see Note 9)

10. Dilute the suspension with at least 2 vol. of Solution B and sediment the PMNs at 250-300 *g* for 15 min.

11. Suspend the PMNs in Solution E and lyse any residual erythrocytes by incubation at 37°C for 7 min (see Note 10).

12. Sediment the PMNs at 250-300 *g* for 15 min and resuspend in Solution B or process as required.

3. Strategy B

3a. Solutions required

See Section 2a, but omit Solution D.

3b. Protocol

Execute Steps 1, 2, 4 and 5 of Section 2b and then continue with the following:

1. Layer 1 vol. of the diluted blood over 0.5 vol. of 1.077 g/ml density barrier.

2. Centrifuge at 700 *g* for 20 min at room temperature and remove all of the liquid above the pellet by aspiration (see Note 11).

3. Suspend the pellet in 5 vol. of Solution E and lyse the erythrocytes by incubation at 37°C for 7 min (see Notes 10 and 12).

4. Sediment the PMNs at 250-300 *g* for 15 min and resuspend in Solution B or process as required.

4. Notes

1. 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 Solution C (equal to the expected volume of blood) containing EDTA at twice the required final concentration (4 mM in this case). **If this approach is used do not dilute the blood further with Solution C in Step 5**. 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. Whatever anticoagulant is chosen, use the same rationale for solution handling as with EDTA.

2. 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.
3. If the blood is not diluted during cardiac puncture (see Note 1) it is common to add an equal volume of 6% (w/v) polysucrose [1,2], 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.
4. Other erythrocyte aggregating agents may be used: 2% (w/v) methylcellulose is added at a volume ratio to whole blood of 1:10 [5]; 2-3 ml of Plasmagel was added to 5 ml of heparinized whole blood by Song et al [7]; equal volumes of whole blood and 6% (w/v) hetastarch in 0.9% NaCl is a third option [6].
5. If an osmometer is available check the osmolality of these solutions. The dilutions should be prepared as accurately as possible. If the density of the barrier solution is too high poor sedimentation of the erythrocytes may be observed, particularly in Strategy B.
6. The cushion may be omitted but the PMNs will pellet in the subsequent centrifugation step. If too many of the densest PMNs are lost to the pellet in the subsequent centrifugation, it is permissible to increase the density of the cushion solution. Reducing the density of the cushion to 1.095 g/ml will reduce the contamination by erythrocytes but more of the denser PMNs will be lost to the pellet. Recipes for solutions of different density can be found in Application Sheet C01 ➔➔
7. A typical separation in a 15 ml tube might be 5 ml of sample, 2.5 ml of the 1.077 g/ml density barrier and 2.5 ml of cushion. For small volumes of mouse blood use a smaller volume (narrower) tube.
8. Flat-tipped metal cannulas can be purchased from many surgical instrument companies.
9. An essentially similar strategy was used by Freeman et al [9] 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 stock was 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). 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.
10. Lysis using ice-cold distilled water for 30 sec before adding an equal volume of double strength saline is an alternative.
11. Take care not to remove any of the PMNs which may be laying on top of the erythrocytes.
12. Theoretically it should also be possible to remove the erythrocytes by aggregation in polysucrose but the use of this technique at this stage of the protocol has not been documented.

5. Other options

Polymorphprep ™

There are a few reported cases in which the Axis-Shield medium developed specifically for the isolation of PMNs from human blood (Polymorphprep™) has also been used for rat [10-13], mouse [14-16] and rabbit [17-20]. Although the rationale behind the use of this medium is that the use of whole blood is essential to the separation, see Polymorphprep™ Application Sheet ➔➔ for more details, Shibata et al [19] used a PMN-rich suspension from which the erythrocytes had been removed. McCartney-Francis et al [11] 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 [12]. Wen et al [15] used this medium for the removal of PMNs from mouse leukocytes prior to isolation of macrophages and monocytes. In a variant of Strategy B 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 [21].

Other OptiPrep ™ methods

Two-layer discontinuous iodixanol gradients have also been used by Matute-Bello [22] and Frevert et al [23] for rabbit blood PMNs.

6. References

- Abstracts of the following references can be accessed in the linked Abstract File (CA45). ➔➔ Each abstract has the same number as the reference.

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