This section describes procedures for the preparation of human mononuclear cell populations from peripheral blood (UNIT 7.1) and tissues (UNIT 7.8). Additional protocols detail the isolation of specific subpopulations of T cells (UNITS 7.2-7.4), B cells (UNIT 7.5), monocytes/macrophages (UNIT 7.6), and lymphokine-activated killer/natural killer cells (LAK/NK; UNIT 7.7).

Isolation of Whole Mononuclear Cells from Peripheral Blood and Cord Blood

Peripheral blood is the primary source of lymphoid cells for investigations of the human immune system. Its use is facilitated by Ficoll-Hypaque density gradient centrifugation—a simple and rapid method of purifying peripheral blood mononuclear cells (PBMC) that takes advantage of the density differences between mononuclear cells and other elements found in the blood sample. Mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer because they have a lower density; in contrast, red blood cells (RBC) and granulocytes have a higher density than Ficoll-Hypaque and collect at the bottom of the Ficoll-Hypaque layer (Fig. 7.1.1). Platelets are separated from the mononuclear cells by subsequent washing or by centrifugation through a fetal-bovine-serum (FBS) cushion gradient that allows penetration of mononuclear cells but not platelets. The mononuclear cell sample can be purified from monocytes by adherence or by exposure to l-leucine methyl ester (see Support Protocol 1 and Support Protocol 2). Cord blood and peripheral blood from infants contain immature cells, including nucleated red cells, that can result in significant contamination of the mononuclear cell layer. Removal of these cells requires additional steps (see Support Protocol 3). The isolation procedures described here can also be applied to cell populations derived from tissues (UNIT 7.8).

CAUTION: When working with human blood, cells, or infectious agents, biosafety practices must be followed (see Chapter 7 introduction).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.
ISOLATION OF MONONUCLEAR CELLS BY FICOLL-HYPAQUE GRADIENT CENTRIFUGATION

**Materials**

- Heparinized blood or heparinized cord blood (Appendix 3F)
- PBS (Appendix 2)
- Ficoll-Hypaque solution (density 1.077 g/liter; see recipe)
- Hanks balanced salt solution (HBSS; Appendix 2)
- FBS (e.g., HyClone), with or without heat inactivation (1 hr, 56°C; Appendix 2)
- Complete RPMI-10 medium (Appendix 2)
- 15- or 50-ml conical centrifuge tubes
- Beckman GPR centrifuge with GH-3.7 horizontal rotor (or equivalent temperature-controlled centrifuge)

Additional reagents and equipment for counting cells and trypan blue exclusion for determining viability (Appendix 3A & 3B), flow cytometry (Unit 5.4; optional), and depletion of contaminating cells from mononuclear fractions (see Support Protocol 3; optional)

1. Place fresh heparinized blood into 15- or 50-ml conical centrifuge tubes. Using a sterile pipet, add an equal volume of room-temperature PBS. Mix well.

   *When isolating cells from a leukapheresis donor, dilute blood with PBS (1:4 blood/PBS).*

   *Cord blood is readily available in 10-ml volumes; larger volumes can be obtained if required. Cord blood is prone to clotting and it is therefore helpful to add to each 10-ml aliquot 1 ml PBS containing 250 U heparin to supplement the heparin already contained in heparin-coated tubes. The blood should immediately be gently mixed, and should be examined when it reaches the laboratory for small blood clots. Because the clotting reaction releases proteins that can affect lymphocyte phenotype and function, samples containing clots should be discarded.*

2. Slowly layer the Ficoll-Hypaque solution underneath the blood/PBS mixture by placing the tip of the pipet containing the Ficoll-Hypaque at the bottom of the sample tube. Use 3 ml Ficoll-Hypaque per 10 ml blood/PBS mixture.

   *To maintain the Ficoll-Hypaque/blood interface, it is helpful to hold the centrifuge tube at a 45° angle.*

   *Alternatively, the blood/PBS mixture may be slowly layered over the Ficoll-Hypaque solution.*

3. Centrifuge 30 min in a GH-3.7 rotor at 2000 rpm (900 × g), 18°C to 20°C, with no brake.

4. Using a sterile pipet, remove the upper layer that contains the plasma and most of the platelets (Fig. 7.1.1). Using another pipet, transfer the mononuclear cell layer to another centrifuge tube. Wash cells by adding excess HBSS (~3 times the volume of the mononuclear cell layer) and centrifuging 10 min at 1300 rpm (400 × g), 18°C to 20°C. Remove supernatant, resuspend cells in HBSS, and repeat the wash once to remove most of the platelets.

   *The washing steps described above usually remove most of the platelets from mononuclear cell suspension. There are certain disease states associated with increased platelet concentrations (mononuclear cell to platelet cell ratio >10:1) in the peripheral blood, and additional steps are needed to remove the extra platelets in these cases. Add 3 ml FBS to a centrifuge tube for each milliliter of mononuclear cells. Layer the cell suspension (1–2 × 10^7 cells/ml) over the FBS (alternatively, carefully layer the FBS under the cell suspension, which will rise as FBS is added). Centrifuge 15 min at 800 rpm (200 × g), 18°C to 20°C. Discard the supernatant containing the platelets. Resuspend cell pellet in complete RPMI-10 and proceed as in step 5.*
5. Resuspend mononuclear cells in complete RPMI-10. Count cells (APPENDIX 3A) and determine viability by trypan blue exclusion (APPENDIX 3B).

Cord blood, and to a lesser extent peripheral blood from infants, gives a population of cells that is contaminated with erythrocytes and their precursors. Pure mononuclear cell populations may be obtained either by subjecting the cells to a second cycle of Ficoll-Hypaque gradient separation as described here, or by lysing the erythrocytes (see Support Protocol 3).

6. If desired, determine the purity of the PBMC population by flow cytometry (UNIT 5.4).

DEPLETION OF MONOCYTES/MACROPHAGES FROM MONONUCLEAR CELLS USING ADHERENCE METHOD

Approximately 40% of the isolated mononuclear cells obtained in the Basic Protocol are monocytes and macrophages. Monocytes can be depleted from the isolated mononuclear cell suspension by taking advantage of the fact that monocytes adhere to plastic whereas lymphocytes do not.

Additional Materials (also see Basic Protocol)
- Mononuclear cell suspension (see Basic Protocol)
- Complete RPMI-20 medium (APPENDIX 2), room temperature
- 150-cm\(^2\) tissue culture flasks, slanted-neck

1. Centrifuge mononuclear cells for 10 min at 1400 rpm \((300 \times g)\), 18° to 20°C. Remove supernatant and resuspend cell pellet in complete RPMI-20 to a final concentration of \(2 \times 10^6\) cells/ml. Transfer 50 ml cell suspension to a 150-cm\(^2\) tissue culture flask. A biological-grade petri dish can be used, but adherence is increased when a tissue culture flask is used.

2. Incubate horizontally for 1 hr in a 37°C, 5% CO\(_2\) humidified incubator.

3. Decant nonadherent lymphocytes into a centrifuge tube. Rinse tissue culture flask gently with 37°C complete RPMI-10; add this wash to the centrifuge tube. Centrifuge 10 min at 1400 rpm \((300 \times g)\), 18° to 20°C. Repeat steps 1 to 3 one time.

4. Discard supernatant. Resuspend cells in 5 to 10 ml complete RPMI-10. Count cells (APPENDIX 3A) and determine viability by trypan blue exclusion (APPENDIX 3B).

Cells should be >90% viable. The adherent cells can be recovered using the procedures outlined in UNIT 7.6.

5. If desired, determine the purity of the PBMC population by flow cytometry (UNIT 5.4).

DEPLETION OF MONOCYTES/MACROPHAGES FROM MONONUCLEAR CELLS USING L-LEUCINE METHYL ESTER

Monocytes/macrophages can also be depleted from PBMC suspensions by a procedure that takes advantage of their rich lysosomal enzyme content (Thiele et al., 1983). This procedure employs a lysosomotropic agent—L-leucine methyl ester—that is taken up by phagocytic and cytotoxic cells and is concentrated in lysosomes, where it is converted by lysosomal enzymes to L-leucyl-L-leucine methyl ester, which is toxic to the cells. B cells and most T cells (cells not rich in lysosomes) are unaffected by exposure to L-leucine methyl ester. However, this agent does eliminate natural killer (NK) cells and cytotoxic T cells (UNIT 7.7).

Additional Materials (also see Basic Protocol)
- 1 mM L-leucine methyl ester (Sigma) in complete RPMI medium (serum-free and filter sterilized; APPENDIX 2)
Additional reagents and equipment for nonspecific esterase staining (APPENDIX 3C) or for flow cytometry (UNIT 7.9) and preparation of monocyte-specific fluorescence-labeled antibodies (UNIT 5.3)

1. Incubate mononuclear cells in 1 mM l-leucine methyl ester prepared in complete serum-free RPMI medium at $5 \times 10^6$ cells/ml for 40 min at room temperature.

2. Wash cells twice in HBSS (see Basic Protocol, step 4) and resuspend in complete RPMI-10.

3. Confirm that remaining lymphocytes are not contaminated by monocytes (large cells with eccentric, crescented nuclei) by light microscopy or by nonspecific esterase staining (APPENDIX 3C), which results in staining of monocytes but not other cells. Alternatively, examine the remaining lymphocytes by flow cytometry (UNIT 7.9) for the presence of cells staining with monocyte-specific fluorescence-labeled antibodies (UNIT 5.3).

**DEPLETION OF CONTAMINATING CELLS FROM MONONUCLEAR CELL FRACTIONS FROM CORD OR INFANT PERIPHERAL BLOOD**

When blood from very young infants is fractionated under the same conditions as adult blood, the mononuclear cell layer is often visibly contaminated with red blood cells (Fig. 7.1.2). In addition to this visible contamination, the mononuclear cell layer contains nucleated red cell precursors. Flow cytometric analysis shows that the proportion of cells that fail to react with CD45 (a leucocyte-common marker) can exceed 50%, even after mature red cells have been excluded on the basis of light-scatter parameters (Fig. 7.1.3). The contaminating cells can be removed by lysis in hypotonic ammonium chloride. This treatment does not affect the viability or most functions of mononuclear cells, but may affect antigen processing function, because ammonium ions inhibit lysosomal function. For studies that may be adversely affected by hypotonic ammonium chloride, the contaminating cells may be removed almost as efficiently by using two rounds of Ficoll-Hypaque density gradient separation. The improvement in purity produced by this second round of Ficoll-Hypaque density centrifugation suggests that the conditions (e.g., cell concentration and viscosity) in the first round do not allow all the cells to reach their isopycnic position.

**Figure 7.1.2** Separation of adult (A) and cord (B) blood under identical conditions. The cord blood sample shows significant contamination of the interface layer with red blood cells.
**Additional Materials** *(also see Basic Protocol)*

ACK lysing buffer *(UNIT 3.1)*

1. Centrifuge the cell suspension obtained from the Ficoll-Hypaque separation 10 min at 400 \( \times \) g, room temperature, and remove the supernatant fluid.

2. Resuspend the cell pellet in ACK lysing buffer, using 5 ml solution per 10 ml of original blood volume. Allow to stand 5 min at room temperature, add 25 ml PBS, mix, and centrifuge 15 min at 300 \( \times \) g, room temperature. Remove supernatant, resuspend cell in 30 ml RPMI-10 or PBS, and centrifuge once more at 300 \( \times \) g, room temperature.

Alternatively, centrifuge the cell suspension obtained from the Ficoll-Hypaque separation 10 min at 400 \( \times \) g, room temperature, remove the supernatant fluid, and resuspend the cell pellet in a volume of PBS equal to the original blood volume. Separate this suspension on a Ficoll-Hypaque density gradient (see Basic Protocol).

3. Determine the purity of the mononuclear cell population by flow cytometry using a CD45 (leucocyte common antigen) monoclonal antibody (Chapter 5).

**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 5.*

**Ficoll-Hypaque solution** *(density 1.077 g/liter)*

- 64.0 g Ficoll (molecular weight 400,000; e.g., Sigma)
- 99.0 g sodium diatrizoate (e.g., Sigma)
- 0.7 g NaCl

Dissolve Ficoll and NaCl in 600 ml water using a magnetic stirrer at low speed. Add sodium diatrizoate. When all components are in solution, add water to 1 liter. Filter sterilize with a 0.22-\( \mu \)m filter unit (Nalgene). Store at 4° to 25°C and protect from direct light.

Alternatively, Ficoll-Hypaque may be purchased already in solution (Ficoll-Paque, Pharmacia Biotech).

**COMMENTARY**

**Background Information**

Ficoll-Hypaque gradient centrifugation allows rapid and efficient isolation of mononuclear cells from human peripheral blood (Boyum, 1968). As such, this technique is the starting point of most studies of human lymphoid cells. In general, Ficoll-Hypaque centrifugation does not change either the phenotype or the function of the isolated mononuclear cell population. However, it may be best to verify this in studies of cells from patients with various diseases (Kaplan et al., 1982).

Monocytes/macrophages are removed from the mononuclear cell population in order to study lymphocyte function in the absence of monocytes/macrophages, or monocyte function in the absence of lymphocytes.

The use of a single Ficoll-Hypaque density separation gives variable results with cord blood and peripheral blood from infants. The mononuclear cell layer is frequently contaminated with erythrocytes, usually visible as cell clumps sticking to the underside of the mononuclear layer (Fig. 7.1.2). Flow cytometric analysis (Fig. 7.1.3) shows that even when mature erythrocytes are excluded on the basis of light scatter, a significant number of CD45-negative cells remain. These cells include glycoporphin-positive red cell precursors, although the number of glycoporphin-positive cells is less than the number of CD45-negative cells (Ridings et al., 1996). The number of CD45-negative cells varies between cord preparations, from \( \leq 10\% \) to \( \geq 50\% \). Cord blood also contains a small number of CD34-positive stem cells. These cells are too few to affect phenotype significantly but may nonetheless affect functional cells. It should also be borne in mind that the number of CD45-negative cells is not fully accounted for by red cell precursors; this implies...
that there are uncharacterized additional cells in cord blood which may affect functional cells.

**Critical Parameters and Troubleshooting**

The yield of mononuclear cells from peripheral blood depends on the percentage of contaminating granulocytes and platelets and the efficiency of erythrocyte removal. For maximum yield and purity, it is essential to remove all the material at the Ficoll-Hypaque interface and to ensure that no Ficoll-Hypaque solution or supernatant is removed with the sample. Including Ficoll-Hypaque will increase the granulocyte contamination; including supernatant will increase the platelet contamination. Erythrocytes can aggregate and trap lymphocytes in the clumps. These clumps will sediment into the pellet and reduce the yield of lymphocytes. Diluting the blood with PBS before Ficoll-Hypaque centrifugation will reduce the clumping. In addition, the temperature of the centrifuge can affect the yield of lymphocytes. At low temperatures, lymphocyte yields are reduced because a longer centrifugation time is required. At high temperatures, lymphocyte viability is decreased and erythrocyte aggregation is increased. During the gradient centrifugation, the temperature should be maintained at 18°C to 20°C.

Occasionally, it is necessary to isolate mononuclear cells from clotted blood. This has been successfully accomplished by using streptokinase to dissolve the blood clots. Lymphocytes isolated from clotted blood function in some respects similarly to those isolated from heparinized blood, although the lymphocyte yield is only 60% of the yield from heparinized blood (Niku et al., 1987).

**Anticipated Results**

When blood is obtained from a normal donor, the yield should be 1–2 × 10^6 mononuclear cells/ml of blood. Approximately 60% to 70% of the mononuclear cells are lymphocytes with a viability of >95%. The platelet count is <0.5% of the total platelet content of the original blood sample. After depletion of monocytes/macrophages by plastic adherence, ~95% of the mononuclear cells are lympho-
cytes with a viability of >95%. After depletion using L-leucine methyl ester, >99% of the mononuclear cells are lymphocytes with a viability of >95%. Yields of cells from cord blood tend to be somewhat higher than those from adult peripheral blood, ranging from 0.7 to 4.2 × 10^6 cells per ml. Viabilities of cord blood cells range from 86% to 100%, with most samples giving 100%.

**Time Considerations**

Isolating mononuclear cells from peripheral blood takes ~1 hr. This is dependent on the initial volume of blood obtained; the larger the blood volume, the longer the preparative time before the samples are ready for centrifuging. Lysis of erythrocytes followed by washing adds approximately 20 min to the procedures, while the alternative procedure of introducing a second round of Ficoll-Hypaque separation adds 40 min. Decreasing the concentration of platelets from the isolated mononuclear cells takes 30 min. If the isolated mononuclear cells are to be stored, platelets should be removed before freezing the cells. Removing monocytes/macrophages from the mononuclear cell population takes 2 to 3 hr using the adherence method, and 1 to 2 hr using L-leucine methyl ester. If the isolated mononuclear cells are frozen, further purification can be done later.

**Literature Cited**


**Key References**


Excellent publication that describes the theory and application of Ficoll-Paque for isolation of lymphocytes.


Excellent resource with clear and simple description (with pictures) about how to collect peripheral blood.

Contributed by Marjorie E. Kanof
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