

# Chapter 2

## Isolation of Human Neutrophils from Venous Blood

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### Abstract

Venous blood provides a ready source of large numbers of unstimulated granulocytes and mononuclear cells. Exploiting the differences in the relative densities of the leukocytes circulating in venous blood, one can separate leukocytes from erythrocytes as well as isolate the individual leukocyte populations in high purity for use in ex vivo studies.

**Key words** Granulocytes, Mononuclear cells, Ficoll-Hypaque, Dextran sedimentation

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### 1 Introduction

Under normal conditions, there are  $\sim 7.4 \text{ K/mm}^3$  (4.5–11.0) white blood cells in circulation, approximately 59 % are PMN, 2.7 % eosinophils, and 4 % monocytes. The different densities of the circulating hematopoietic cells are exploited in order to separate erythrocytes from leukocytes and then to isolate the individual leukocyte populations. Although a variety of methods for rapid, one-step isolation of PMN have been developed recently and used successfully, we routinely use one of two variants of a method first described by Bøyum in 1968 [1]. PMN isolated in this way do not consume oxygen and maintain their secretory vesicles intracellularly, two features that suggest that the PMN are bona fide resting cells and thus not activated by the isolation procedure.

Two major steps are involved, each using specific conditions to separate cells based on their intrinsic density: sedimentation in dextran at  $1 \times g$  and differential sedimentation in a discontinuous density gradient of Ficoll-Hypaque. In dextran, erythrocytes form rouleaux and thus sediment more rapidly than do granulocytes in suspension. In the Ficoll-Hypaque, granulocytes and erythrocytes pellet to the bottom, whereas mononuclear cells (i.e., lymphocytes and monocytes), basophils, and platelets remain at the interphase between plasma/buffer and the Ficoll-Hypaque. Because we routinely process relatively large quantities of blood (e.g.,  $\geq 200 \text{ ml}$ )

and are primarily interested in recovering PMN (not monocytes), we generally perform dextran sedimentation first, followed by Ficoll-Hypaque sedimentation. When smaller volumes of blood are processed or when monocytes are the targeted cell of interest, the sedimentation in Ficoll-Hypaque can be performed first. Both approaches are described below.

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## 2 Materials

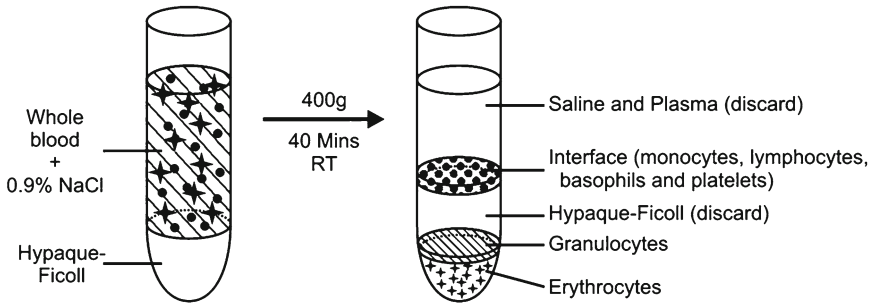
1. Anticoagulant: The preferred anticoagulant is preservative-free sodium heparin (1,000 units/mL). Lithium heparin, EDTA, or citrate are also acceptable.
2. Dextran: 3 % (w/v) Dextran-500 (average molecular weight 200,000–500,000) in endotoxin-free, sterile 0.9 % NaCl. Dissolving 30 g/L of dextran in sterile saline (*see Note 1*). Heat the solution if necessary to promote dissolution of the dextran and handle the solution using sterile technique.
3. Ficoll-Hypaque solution: originally prepared by mixing Ficoll 400 and isopaque to create a solution with a density of 1.077 g/mL [1]. Currently premixed, sterilely prepared Ficoll-Hypaque plus can be purchased. Each 100 mL contains 5.7 g Ficoll 400 and 9.0 g diatrizoate solution with .0231 g of disodium calcium EDTA in endotoxin-free water. Protected from light, the solution is stable for 3 years when stored at 4–30 °C. The appearance of yellow color or particulate material indicates deterioration.
4. Endotoxin-free, sterile water.
5. 1.8 % (w/v) NaCl solution.
6. Sterile, endotoxin-free, Hanks' balanced salt solution (HBSS) without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ .

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## 3 Methods (*See Note 2*)

### **3.1 Dextran Sedimentation Followed by Ficoll-Hypaque Density Centrifugation**

1. Draw blood into a syringe containing sufficient preservative-free heparin to have a final concentration of 20 U/mL in the blood sample.
2. In a 50 mL conical tube containing the blood, add an equal volume of 3 % dextran.
3. Mix tubes by repeated inversion (ten times), and set tubes upright for 18–20 min at room temperature (*see Note 3*).
4. Aspirate the straw-colored, leukocyte-rich, erythrocyte-poor upper layer with a sterile plastic pipette or a 10–15 mL sterile syringe and transfer the aspirate to a sterile 50 mL conical tube (*see Note 4*).



**Fig. 1** Ficoll-Hypaque gradient separation of granulocytes and peripheral blood mononuclear cells. Ficoll-Hypaque gradient before (*left side*) and after (*right side*) centrifugation. Reproduced from ref. 4 by permission of Humana Press©2007

5. Pellet leukocytes by centrifugation at  $500\times g$  for 10 min at  $4^{\circ}\text{C}$ ; aspirate and discard the supernatant.
6. Resuspend pellets in 10 mL, shake well but *do not* vortex. Three resuspended pellets can be pooled into a single conical tube to achieve a final volume of 35 mL. Top off with sterile saline if needed to reach 35 mL.
7. Carefully underlay the leukocyte suspension with 10 mL of Ficoll-Hypaque using a sterile, plastic 10-mL pipette (Fig. 1).
8. Centrifuge at  $400\times g$  for 40 min at room temperature (*see Note 5*).
9. After centrifugation, two bands should be apparent in the conical tube (Fig. 1). The lighter band contains mononuclear cells, whereas the denser band has both granulocytes and erythrocytes. If monocytes are desired, aspirate the less dense band using a sterile plastic pipette and mix with an equal volume of cold sterile saline in a separate conical tube.
10. Aspirate and discard supernatant above the PMN-erythrocyte layer, taking care not to lose any of the PMN-rich pellet.
11. Resuspend each pellet in sterile water and mix well (but *do not* vortex) for 28 s. Promptly restore tonicity by adding an equal volume of 1.8 % saline and mixing (*see Note 6*).
12. Centrifuge  $500\times g$  for 5 min at  $4^{\circ}\text{C}$ . Discard supernatant and repeat **step 11** if more lysis is needed, but repeat **step 11** only *once* (*see Note 6*).
13. Resuspend cells in HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at  $\leq 3 \times 10^7$  cells/mL.
14. Determine the cell concentration by manually counting using a hemocytometer. The differential of leukocytes can be assessed by examining a stained slide microscopically (*see Note 7*).

**3.2 Ficoll-Hypaque  
Density  
Centrifugation-  
Mononuclear Cell  
Recovery**

1. Draw blood into a syringe as above and dilute with 1 volume of 0.9 % NaCl at room temperature to a total volume of 40 mL in a sterile 50 mL conical plastic tube (*see Note 8*).
2. Carefully underlay the diluted blood with 10 mL of Ficoll-Hypaque.
3. Centrifuge at  $400\times g$  for 40 min at room temperature.
4. After centrifugation, remove the HBSS and plasma above the mononuclear band at the interface (*see Fig. 1*) and discard.
5. Recover the fraction containing mononuclear cells, combining bands from no more than two gradients into a separate 50 mL conical tube.
6. Bring each of the pooled fractions to 50 mL with sterile HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to dilute the Ficoll-Hypaque (*see Note 9*).
7. Pellet mononuclear cells at  $600\times g$  for 10 min at 4 °C.
8. Discard supernatant, pool, and wash pellets twice more with HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ .
9. Resuspend final pellet in 5 mL of HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and count.

**3.3 Ficoll-Hypaque  
Density Centrifugation  
First-PMN Recovery**

1. After **step 5** in Subheading **3.2**, when the supernatant and the mononuclear band have been removed, aspirate and discard the remaining gradient above the PMN-erythrocyte pellet (*see Fig. 1*).
2. Resuspend the pellet in a volume up to 25 mL with HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ .
3. Add 25 mL of 3 % dextran, mix by inverting the tube ten times, and let stand upright at room temperature for 18–20 min.
4. Aspirate the straw-colored, leukocyte-rich, erythrocyte-poor upper layer with a sterile plastic pipette or a 10–15 mL sterile syringe and transfer the aspirate to a sterile 50 mL conical tube (*see Note 4*).
5. Pellet leukocytes by centrifugation at  $500\times g$  for 10 min at 4 °C; aspirate and discard the supernatant.
6. Resuspend each pellet in sterile water and mix well (but *do not* vortex) for 28 s. Promptly restore tonicity by adding an equal volume of 1.8 % saline and mixing (*see Note 6*).
7. Resuspend cells in HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at  $\leq 3\times 10^7$  cells/mL.
8. Determine the cell concentration by manually counting using a hemacytometer. The differential of leukocytes can be assessed by examining a stained slide microscopically.

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## 4 Notes

1. The concentration and the molecular weight range of the dextran is a critical determinant in the speed with which cells sediment [2]. The preparation of 3 % in the 200,000–500,000 weight range provides excellent sedimentation of erythrocytes within 18–20 min, with rates slower using either the lower molecular weight dextrans (<20,000) or the high molecular weight (>7,000,000) preparations. Given the range in size of the components in the dextran being used, one can anticipate corresponding variation in the rate of sedimentation (reflected as differences in the number of cells recovered). For example, we currently allow sedimentation to occur for 30 min at room temperature to obtain maximal number of granulocytes. Be prepared to modify the time allotted for sedimentation accordingly. The volume of the dextran–blood mixture has no effect on the efficiency of sedimentation. In the event that sedimentation has proceeded for longer than the intended time, the sample can be mixed again and the sedimentation repeated with no adverse effect on yield or purity. Whenever alternative agents are used for sedimentation, it is important to determine if the recovered PMN are primed, as occurs with gelatin [3].
2. In general, always use polypropylene tubes, as glass and polyethylene may activate the PMN during isolation. Pipettes should be sterile and buffers should be prepared with sterile, endotoxin-free reagents, as endotoxin will prime PMN. Cell suspensions should never be vortexed.
3. Adequate mixing of the sample is essential for reproducible sedimentation, presumably to distribute the erythrocytes throughout the suspension and provide maximal opportunities for rouleaux formation. When directly examined, ten mixing inversions were sufficient to guarantee reproducible and maximum sedimentation; fewer inversions compromised the reproducibility in yield.
4. Once the leukocyte-rich supernatant from the dextran sedimentation has been centrifuged, proceed as quickly as possible with the isolation procedure. Do not leave cell pellets at intermediate steps. Aspirates and cell pellets are biohazard waste and should be handled accordingly.
5. The centrifugation of the Ficoll-Hypaque gradient should be at 20 °C and with no brake on the centrifuge. When performed at 4 °C, there is more PMN contamination of the mononuclear band than seen when centrifugation is at 20 °C (2.3 vs 0.1 %, respectively), and more lymphocyte and mononuclear contamination of the PMN pellet (6.9 and 0.6 % vs 1.4 and 0.1 %).

It is best to have a dedicated room-temperature centrifuge for this use; alternatively, have the refrigeration in the centrifuge turned off overnight before running the gradient the following morning.

6. The hypotonic lysis of erythrocytes exploits the relative resistance of leukocytes to osmotic stress. This difference is relative and prolonged or repeated hypotonic lysis will damage PMN. Attention to limiting the time of exposure to hypotonicity to 18–20 s should be strictly maintained. More than two cycles of hypotonic lysis must be avoided, as they will not lyse additional erythrocytes but will begin to damage PMN.
7. When counting the PMN suspension, one can directly count the PMN by diluting the cell suspension 1:20 in 3 % acetic acid and observe under 40X objective. In the acetic acid solution, the nuclear morphology of PMN is clearly identified, allowing direct counting of the PMN in suspension. Alternatively, PMN suspension can be diluted 1:20 in HBSS and counted to determine the leukocyte concentration. A separate sample (e.g., 10  $\mu$ L) can be diluted 1:5 in HBSS or saline and placed on a slide (either using a cytospin or simply maneuver the slide to obtain a thin layer) and allowed to air-dry. The slide can then be stained with Wright stain (or an equivalent) and a differential performed. With the total number of leukocytes and the percent of PMN on differential staining, the number of PMN isolated can be calculated. In general, the yield should be 2–4 million PMN/mL blood drawn and the suspension should be ~98 % PMN with a few contaminating eosinophils.
8. Efficient separation is achieved by 1:1 dilution of blood with saline prior to centrifugation in the Ficoll-Hypaque gradient. The tendency of lymphocytes to be trapped in erythrocyte-granulocyte aggregates decreases the yield of lymphocytes while simultaneously contaminating the granulocyte pellet with lymphocytes. Dilution of whole blood at the start of the isolation will decrease both problems.
9. Neither mononuclear cells nor granulocytes tolerate long incubation in Ficoll-Hypaque, so it is best to dilute the gradient matrix in the isolated cell fractions as soon as feasible.

## References

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Neutrophil Methods and Protocols

Quinn, M.T.; DeLeo, F.R. (Eds.)

2014, XVI, 551 p. 93 illus., 28 illus. in color., Hardcover

ISBN: 978-1-62703-844-7

A product of Humana Press