PMN- 01a - Isolation of Human PMN from Buffy Coat V.3

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ABSTRACT

Separation of Human Neutrophils (PMN) from Buffy Coat: list of published papers using this protocol


MATERIALS

- Ethylenediaminetetraacetic acid disodium salt dihydrate Sigma Aldrich Catalog #ED2556
- Ficoll Paque PLUS Ge Healthcare Catalog #17144003-500 ml
- Fetal Bovine Serum (FBS) EuroClone Catalog #ECS0180L-500 ml
- RPMI 1640 EuroClone Catalog #ECM 0495L-500 ml
- NaCl Sigma Aldrich Catalog #S9625
- NH4Cl Merck Serono GmbH Catalog #1.01145.1000
- KHCO3 Merck Serono GmbH Catalog #1.04854.500
- Acetic Acid 100% Sigma Aldrich Catalog #A6283
- Genitian violet 1% Marco Viti Catalog #not available

Optical Microscope (for manual cell count)

BEFORE START INSTRUCTIONS

All reagents used in this protocol must be at room temperature

1. Place 5 ml of venus blood from BUFFY COAT into 10 ml volume centrifuge tube.

2. Add 2 mL of Dextran solution and mix well drawing in and out of a pipette.
3  Incubate in the **DARK** for **00:45:00** at **37 °C**

4  Place **3 mL** of **Fycoll-HyPaque** media solution into a 10 ml volume centrifuge tube.

5  Slowly and carefully layer the supernatant from blood/dextran mixture onto the Fycoll-HyPaque media solution.

**Note**

Important: when layering the sample, do not mix the Fycoll-HyPaque media solution and supernatant

6  Centrifuge at **400 x g**, **Room temperature, 00:30:00**, **no break**
7 Draw off the mononuclear cell layer at the Ficoll/plasma interface along with plasma and Ficoll media, leaving the white cell layer of granulocytes above the red blood cell layer undisturbed.

8 Resuspend the remaining cell layer in 5 mL of NaCl 0.15 Molarity (M) and centrifuge at 400 x g, Room temperature, 00:05:00
9 Aspirate the supernatant with a plastic pipette Pasteur.

10 Lyse remaining red blood cells in \(5\,\text{mL}\) of hypotonic Lysis Buffer for \(00:05:00\).

11 Centrifuge at \(400\,\text{x}\,\text{g},\,\text{Room temperature,}\,00:05:00\)
Aspirate the supernatant with a plastic pipette Pasteur.

Resuspend the pellet in 5 mL NaCl 0.15 Molarity (M).

Centrifuge at 400 x g, Room temperature, 00:05:00.
15 Aspirate the supernatant with a plastic pipette Pasteur.

16 Resuspend the cell pellet in $5 \text{ mL} \ NaCl$ 0.15 Molarity (M) for cell counting.

17 Mix $10 \mu\text{L}$ of cell suspension with an equal amount of Türk solution (dilution factor=2) allow mixture 00:03:00 at Room temperature (RT).
Take 10 µL of the mixture and place it inside a Bürker chamber and view under an optical microscope using 40x magnification.

Count cells in each square found in the four corners and in the central square (figure 1 below), including those that lie on the bottom and left-hand perimeters, but not those that lie on the top and right-hand perimeters (see figure 2 below).

**Total number of cells per ml = mean number of cells x dilution factor x 10^4 (hemacitometer volume)**

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**Figure 1**
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here
in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

18  OPTIONAL STEP

For automatic cell count with **Cellometer machines** use **Trypan Blue**. The machine will calculate the number of cells /ml and the % of viability.

Take [10 µL] of cell suspension and add an equal amount of Trypan Blue. Use all the volume to place it in a counting chamber. Place the chamber inside Cellometer and count.
OPTIONAL STEP

If needed, check the purity of PMN suspension by using morphological parameters of the flow cytometer.

For this test 0.5 x10^6 PMN in 500 µL of PBS are enough.
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<th>NAME</th>
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<td>BD FACS Celesta</td>
<td>Flow Cytometer</td>
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### EXPECTED RESULTS

**Expected result**

**VIABILITY:** the expected viability by Trypan Blue should be $\geq 90\%$

**CELL YIELD:** $\pm 6 \times 10^6$ cells starting from 1 mL of Buffy Coat