

Version 3

Jul 24, 2020

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

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1 Works for me dx.doi.org/10.17504/protocols.io.biamkac6

Mattia Di Rocco

ABSTRACT

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

- Boydum A. Isolation of mononuclear cells and granulocytes from human blood. Scand.J.Clin.Lab. Invest. 21 (Suppl.97): 77-89, 1968

- Alex Mabou Tagne, Franca Marino, Massimiliano Legnaro, Alessandra Luini, Barbara Pacchetti and Marco Cosentino. A Novel Standardized Cannabis sativa L. Extract and Its Constituent Cannabidiol Inhibit Human Polymorphonuclear Leukocyte Functions. Int J Mol Sci 2019 Apr; 20(8): 1833. Published online 2019 Apr 13. doi: 10.3390/ijms20081833.

- Angela Scanzano, Laura Schembri, Emanuela Rasini, Alessandra Luini, Jessica Dallatorre, Massimiliano Legnaro, Raffaella Bombelli, Terenzio Congiu, Marco Cosentino, Franca Marino. Adrenergic Modulation of Migration, CD11b and CD18 Expression, ROS and interleukin-8 Production by Human Polymorphonuclear Leukocytes. Inflamm Res. 2015 Feb;64(2):127-35. doi: 10.1007/s00011-014-0791-8. Epub 2015 Jan 6.

DOI

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PROTOCOL CITATION

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MATERIALS

NAME	CATALOG #	VENDOR
Ethylenediaminetetraacetic acid disodium salt dihydrate	ED2SS	Sigma Aldrich
Ficoll Paque PLUS	17144003-500 ml	Ge Healthcare

NAME	CATALOG #	VENDOR
Fetal Bovine Serum (FBS)	ECS0180L-500 ml	EuroClone
RPMI 1640	ECM 0495L- 500 ml	EuroClone
NaCl	S9625	Sigma Aldrich
NH4Cl	1.01145.1000	Merck Serono GmbH
KHCO3	1.04854.500	Merck Serono GmbH
Acetic Acid 100%	A6283	Sigma Aldrich
Genitian violet 1%	not available	Marco Viti

MATERIALS TEXT


Optical Microscope (for manual cell count)

EQUIPMENT

NAME	CATALOG #	VENDOR
BD FACS Celesta	Milan Italy BD	
Allegra AVANTI 30	Beckman Italy	Beckman Coulter
Cellometer Auto T4	Euroclone	

BEFORE STARTING




All reagents used in this protocol must be at room temperature

- 1 Place 5 ml of venous 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





SOLUTION- 03 - Dextran solution 5%

by Farmacologia Medica

- 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.

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



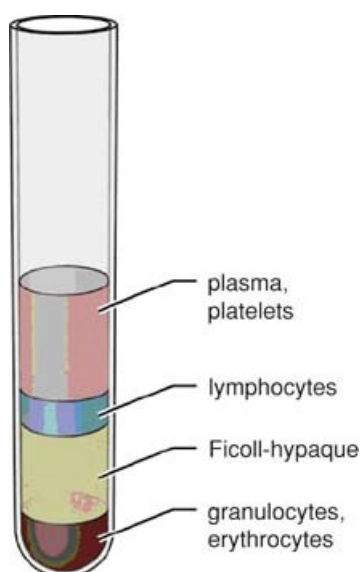
Important: when layering the sample, do not mix the Fycoll-HyPaque media solution and supernatant

6 Centrifuge at **400 x g, Room temperature 00:30:00 , no break**



Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy

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 [M]0.15 Molarity (M)** and centrifuge at **400 x g, Room temperature 00:05:00**



SOLUTION- 01 - Sodium Chloride (NaCl) solution
by Farmacologia Medica

9 Aspirate the supernatant with a plastic pipette Pasteur.

10 Lyse remaining red blood cells in  **5 mL** of **hypotonic Lysis Buffer** for  **00:05:00** .



SOLUTION- 06 - Lysis Buffer
by **Farmacologia Medica**



11 Centrifuge at  **400 x g, Room temperature 00:05:00**



Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy


12 Aspirate the supernatant with a plastic pipette Pasteur.

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



SOLUTION- 01 - Sodium Chloride (NaCl) solution
by **Farmacologia Medica**


14 Centrifuge at  **400 x g, Room temperature 00:05:00** .



Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy


15 Aspirate the supernatant with a plastic pipette Pasteur.

16 Resuspend the cell pellet in **5 mL NaCl [M]0.15 Molarity (M)** for cell counting.



SOLUTION- 01 - Sodium Chloride (NaCl) solution
by Farmacologia Medica

17 Mix **10 µl** of cell suspension with an equal amount of **Türk solution** (dilution factor=2) allow mixture
⌚ **00:03:00** at **Room temperature (RT)**.



SOLUTION- 08 - Türk solution
by Farmacologia Medica

Take **10 µl** of the mixture and place it inside a Bürker chamber and view under an optical microscope using 40x magnification

Count cell 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 cell per ml = mean number of cell x dilution factor x 10^4 (hemacitometer volume)

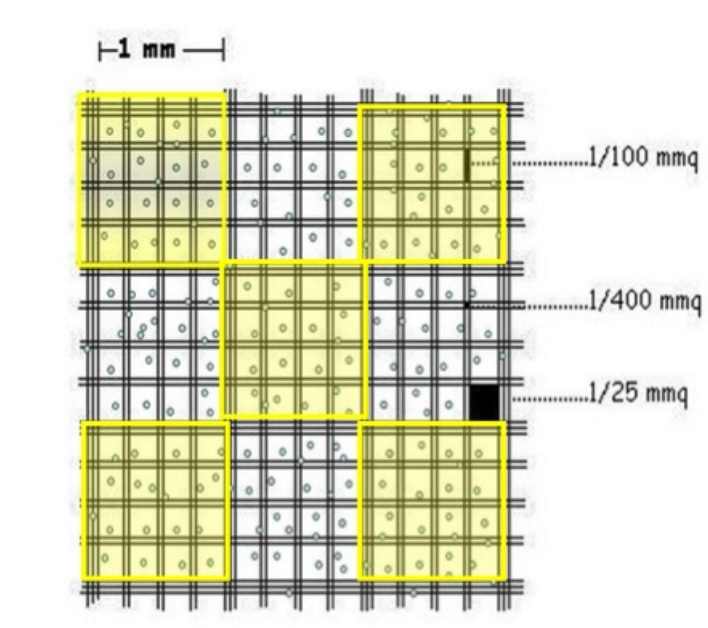


Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

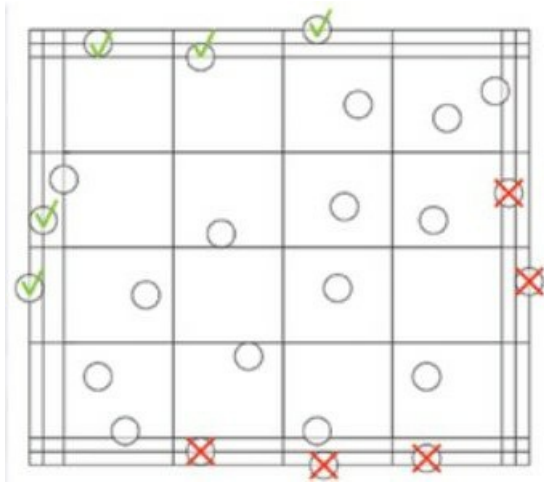




Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

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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.



Cellometer Auto T4
Automated Cell Counter
Nexcelom Bioscience Euroclone



SOLUTION- 09 - Trypan Blue solution
by Farmacologia Medica

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OPTIONAL STEP

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

For this test $0,5 \times 10^6$ PMN in  **500 µl** of PBS are enough.



BD FACS Celesta
Flow Cytometer
Becton Dickinson Milan Italy BD

20 EXPECTED RESULTS



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

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