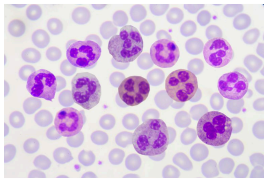
Jul 24, 2020 Version 3

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



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Protocol status: Working
We use this protocol and it's working

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Protocol Integer ID: 38957

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 Sci2019 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.

Materials

MATERIALS

- Ethylenediaminetetraacetic acid disodium salt dihydrate **Sigma Aldrich Catalog #**ED2SS
- 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

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

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NAME

SOLUTION- 03 - Dextran solution 5%

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PREVIEW

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.

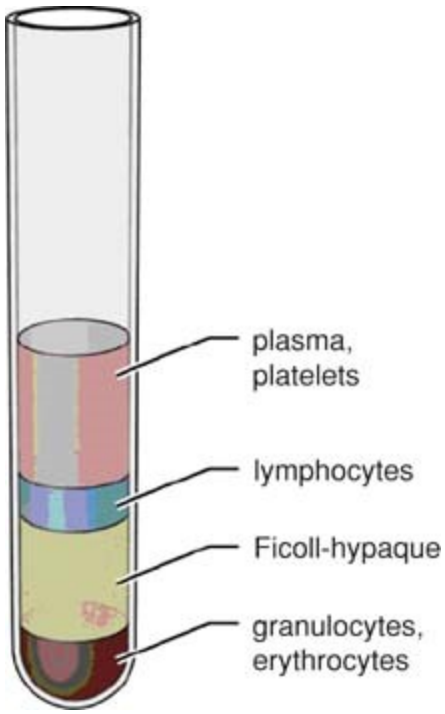
Note

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

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

Equipment	
Allegra AVANTI 30	NAME
Centrifuge	TYPE
Beckman Coulter	BRAND
Beckman Italy	SKU

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

Document

NAME

SOLUTION- 01 - Sodium Chloride (NaCl) solution

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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 .

Document

NAME

SOLUTION- 06 - Lysis Buffer

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Elisa Storelli

PREVIEW

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

Equipment

NAME	TYPE	BRAND	SKU
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** [M] 0.15 Molarity (M) .

Document

NAME

SOLUTION- 01 - Sodium Chloride (NaCl) solution

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PREVIEW

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

Equipment

NAME	TYPE	BRAND	SKU
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.

Document

NAME

SOLUTION- 01 - Sodium Chloride (NaCl) solution

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17 Mix 10 µL of cell suspention with an equal amount of **Türk solution** (diluition factor=2) allow mixture 00:03:00 at Room temperature (RT).

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NAME

SOLUTION- 08 - Türk solution

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

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 diluition factor x 10^4 (hemacitometer volume)

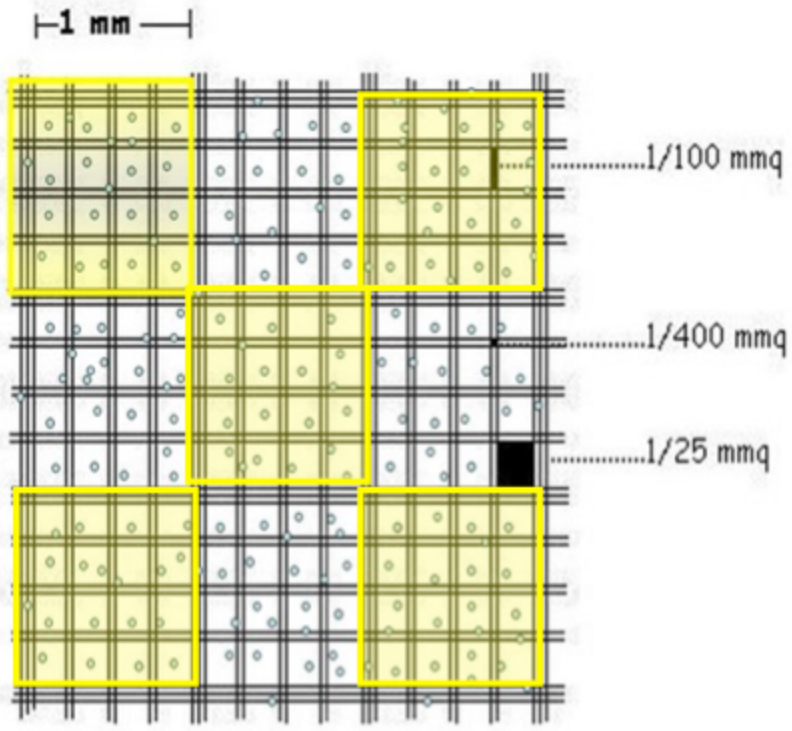

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

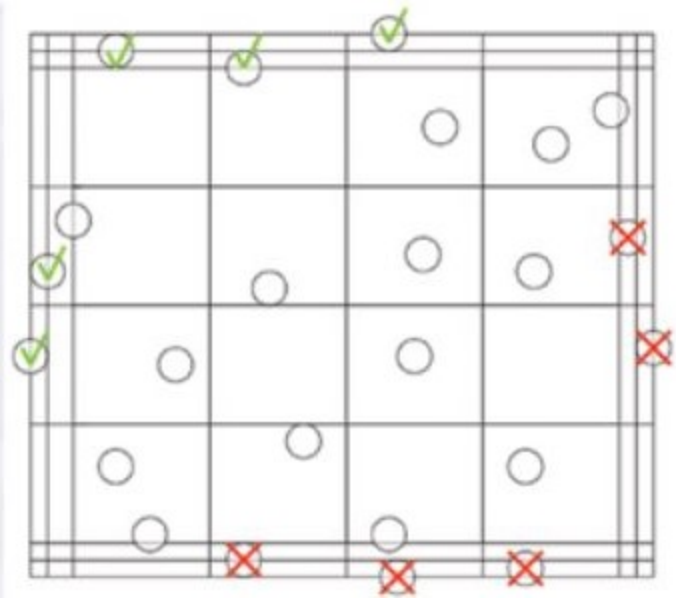

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

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.

Equipment	
Cellometer Auto T4	NAME
Automated Cell Counter	TYPE
Nexcelom Bioscience	BRAND
Euroclone	SKU

Document

NAME

SOLUTION- 09 - Trypan Blue solution

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PREVIEW

19 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.

Equipment

BD FACS Celesta	NAME
Flow Cytometer	TYPE
Becton Dickinson	BRAND
Milan Italy BD	SKU

20 **EXPECTED RESULTS**

Expected result

VIABILITY: the expeted viability by Tripan Blue shuold be ≥90%

CELL YIELD: ± 6 x10^6 cells starting from 1 mL of Buffy Coat