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PBMC- 01b Isolation of human PBMC from Whole Blood V.3

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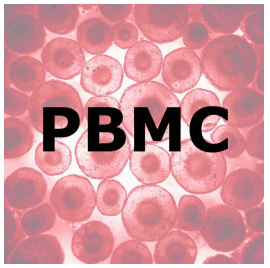

Marco Cosentino[1], Elisa Storelli[2], Alessandra Luini[1], Massimiliano LM Legnaro[3], Emanuela Rasini[3], Marco Ferrari[3], Franca Marino[3]

[1]Center for Research in Medical Pharmacology, University of Insubria (Varese, Italy);
[2]Center for Research in Medical Pharmacology, University of Insubria; [3]University of Insubria

Elisa Storelli
Center for Research in Medical Pharmacology, University of I...

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We use this protocol and it's working

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Abstract

Separation and purification of PBMC from FRESH BLOOD: list of published work using this protocol

Kustrimovic, N., Comi, C., Magistrelli, L., Rasini, E., Legnaro, M., Bombelli, R., Aleksic, I., Blandini, F., Minafra, B., Riboldazzi, G., Sturchio, A., Mauri, M., Bono, G., Marino, F., & Cosentino, M. (2018). Parkinson's disease patients have a complex phenotypic and functional Th1 bias: cross-sectional studies of CD4+ Th1/Th2/T17 and Treg in drug-naïve and drug-treated patients. Journal of neuroinflammation, 15(1), 205. https://doi.org/10.1186/s12974-018-1248-8

Kustrimovic, N., Rasini, E., Legnaro, M., Bombelli, R., Aleksic, I., Blandini, F., Comi, C., Mauri, M., Minafra, B., Riboldazzi, G., Sanchez-Guajardo, V., Marino, F., & Cosentino, M. (2016). Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson's Disease. Scientific reports, 6, 33738. https://doi.org/10.1038/srep33738

Cosentino M., Ferrari M., Kustrimovic N., Rasini E., Marino F. (2015). Influence of dopamine receptor gene polymorphisms on circulating T lymphocytes: A pilot study in healthy subjects. Human immunology, 76, 10, 747-752. https://doi.org/10.1016/j.humimm.2015.09.032

Materials

MATERIALS

- 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
- Trypan Blue solution 0.4% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8154- 100 ml
- NaCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S9625
- Na2HPO4*7H2O **Merck Catalog #**1.06574.1000
- NaH2PO4 **Merck Catalog #**1.06346.0500
- NH4Cl **Merck Catalog #**1.01145.1000
- KHCO3 **Merck Catalog #**1.04854.500
- EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**ED2SS
- Acetic Acid 100% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A6283
- Gentian violet 1% **Marco Viti Catalog #**not available

Instrumentation required:

- Laminar flow hood
- Autoclave

Troubleshooting

Before start

If you need to obtain **PBMC for cell culture**, make sure you are using **sterile PBS, culture medium, filtered Lysis Buffer and sterile plastic disposables as well.** Moreover, work under laminar flow hood when you are processing samples. Otherwise, use non-sterile solutions and plastic disposables, and process samples in cell isolation laboratory.

ALL REAGENTS USED IN THIS PROTOCOL MUST BE AT ROOM TEMPERATURE!

1 Put the needed amount of blood sampl into a 50 mL conical tube.

2 Add an equal volume of **PBS 1X** and mix well.

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SOLUTION- 02 - Phosphate Buffered Saline (PBS)

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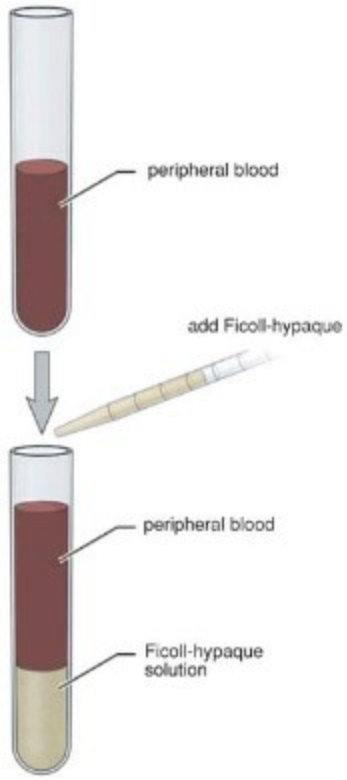
3 Place 3 mL of FICOLL in a 15 mL conical tube.

4 Carefully layer 12 mL of diluted blood on FICOLL with a glass Pasteur Pipette to a final volume of 15 ml as shown in the figure below.

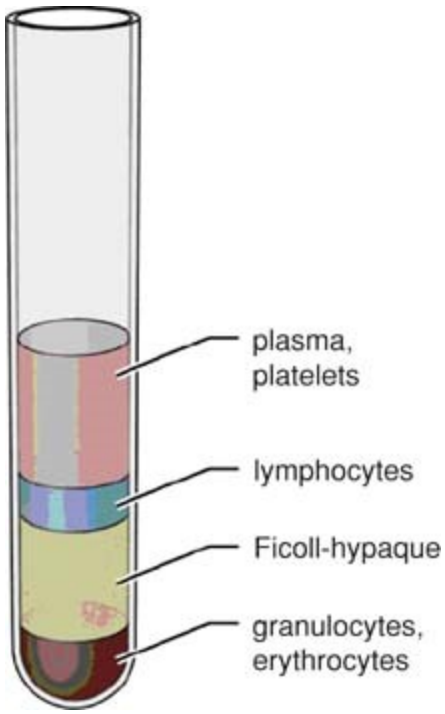


5 Centrifuge samples 400 x g, 00:40:00 at room temperature (RT) without break.

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

6 After centrifugation, take out the tubes carefully to not disturb the mononuclear cell layer that appears as a white, cloudy band between the plasma and FICOLL as shown in the

figure below.



7 Carefully with a glass Pasteur pipette transfer the mononuclear lymphocyte cell layer to another 15 ml conical tube.

8 Wash the isolated PBMC with **PBS/FBS 2%** to a final volume of 10 mL and centrifuge at 600 x g, 00:10:00 at RT.

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SOLUTION- 05 - Wash solution (PBS/FBS) for PBMC

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9 Remove supernatants, resuspend pellet in 1 mL of **Lysis Buffer** and add another 9 mL of **Lysis Buffer**. Immediately centrifuge tubes at 300 x g, 00:10:00 at RT.

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SOLUTION- 06 - Lysis Buffer

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10 Remove supernatant and resuspend pellet in 10 mL of **PBS/FBS 2%** and centrifuge at 600 x g, 00:10:00 at RT.

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SOLUTION- 05 - Wash solution (PBS/FBS) for PBMC

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11 Remove supernatant and resuspend the obtained pellet in 10 mL of **RPMI/FBS 10%** for cell counting.

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SOLUTION- 04 - Wash solution (RPMI/FBS) for PBMC

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12 **For manual cell count use Türk solution for checking purity.**

Mix 10 µL of cell suspension with an equal amount of Türk solution (dilution factor = 2), allow mixture 3 min at room temperature.

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

Count the cells in each square found in the four corners and in the central square (see 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 (hemacytometer volume).

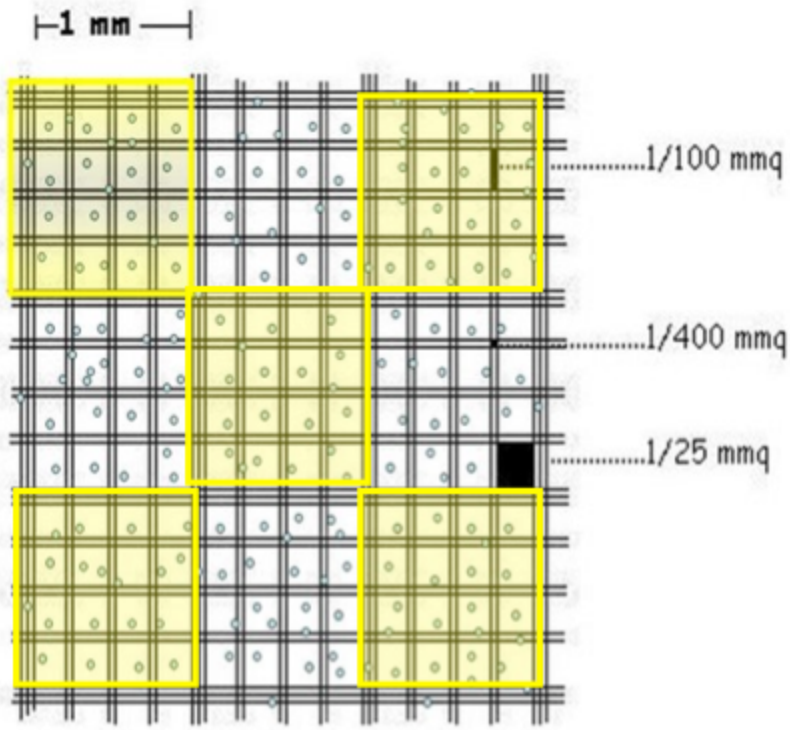

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

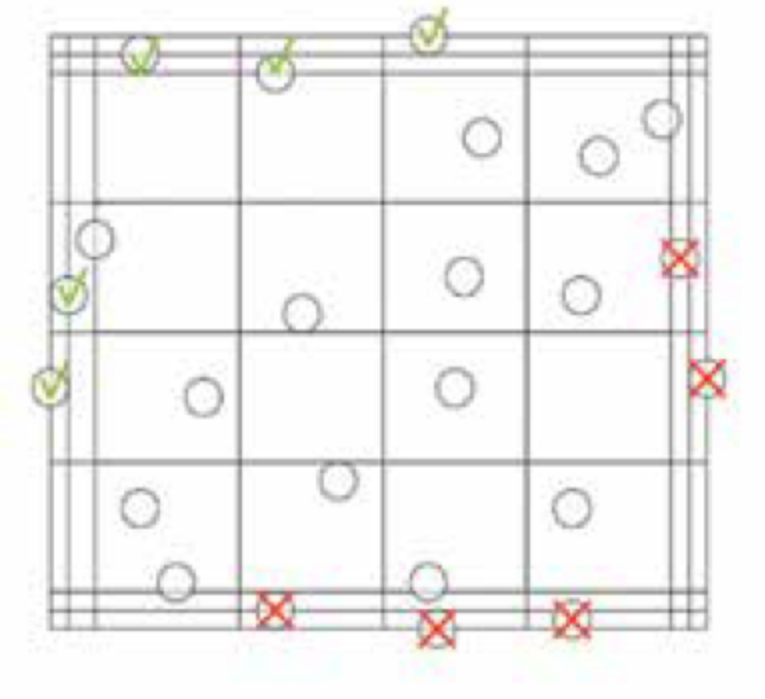

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

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SOLUTION- 08 - Türk solution

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13 **OPTIONAL STEP**

For automatic cell count with Cellometer machine use Trypan Blue. The machine will calculate the n° 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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14 If needed, check the purity of PBMC suspension by using morphological parameter of the flow cytometer.

For this test 0.5×10^6 PBMC in 500 µl of PBS are enough.

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

15 Expected results

Expected result

VIABILITY - The expected viability by Trypan Blue should be ≥90 %.

PURITY - The PBMC suspension obtained should contain at least 80% of lymphocytes, 10-15% of monocytes and few contaminant PMN cells (≤ 5%) as confirmed by flow cytometry.

YIELD - The expected amount of PBMCs should be ± 28,5x10^6 starting from 25 ml of fresh blood.