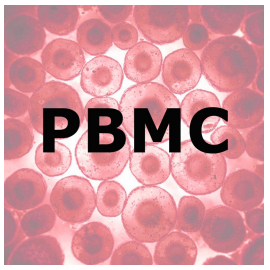
Jul 23, 2020 Version 1

PBMC- 01a - Isolation of Human PBMC from Buffy Coat V.1



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

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Protocol Integer ID: 39610

Keywords: PBMC, Buffy Coat, Neuroimmune-Pharmacology, Parkinson's Disease, Cell isolation, Primary cell culture,

Abstract

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

- Fetal bovine serum (FBS) **BioWest Catalog #**S181B-500
- Ficoll Paque PLUS **Ge Healthcare Catalog #**17144003-500 ml
- RPMI 1640 **EuroClone Catalog #**ECM 0495L- 500 ml
- Trypan Blue Solution 0.4% **Thermo Fisher Scientific Catalog #**15250061

Instrumentation required:

- Laminar flow hood
- Optical Microscope (manual cell count)

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 sample from buffy coat 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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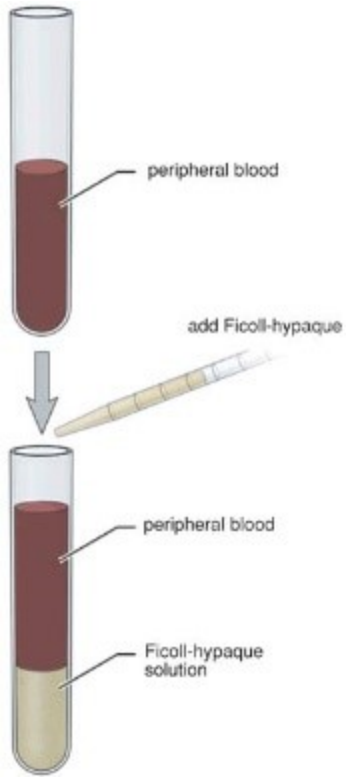
3 Place 3 mL of **FICOLL** in a 15 mL conical tube.

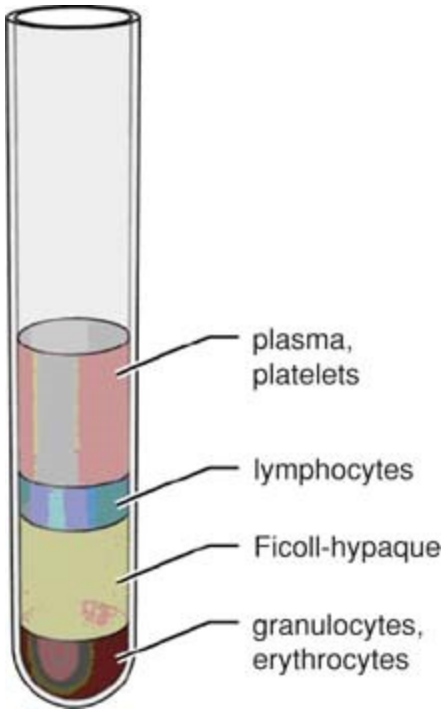
4 **CAREFULLY** layer 12 mL of diluted blood on the FICOLL with a glass Pasteur Pipette to a final volume of 15 ml as shown in the figure below.



5 Centrifuge samples 400 x g, 00:40:00 **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 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 300 x g, 00:10:00 at RT.

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

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Equipment

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

9 Remove supernatants, resuspend pellet in 1 mL of **Lysis Buffer** and add another 9 mL of **Lysis Buffer.** Immediately centrifuge the tubes at 100 x g, 00:10:00 at RT.

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NAME

SOLUTION- 06 - Lysis Buffer

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Equipment

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

10 Remove supernatant and resuspend pellet in 10 mL **PBS/FBS 2%** and centrifuge at 300 x g, 00:10:00 at RT.

Document

NAME

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

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Equipment

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

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

Document

NAME

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 suspention 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 104 (hemacytometer volume).

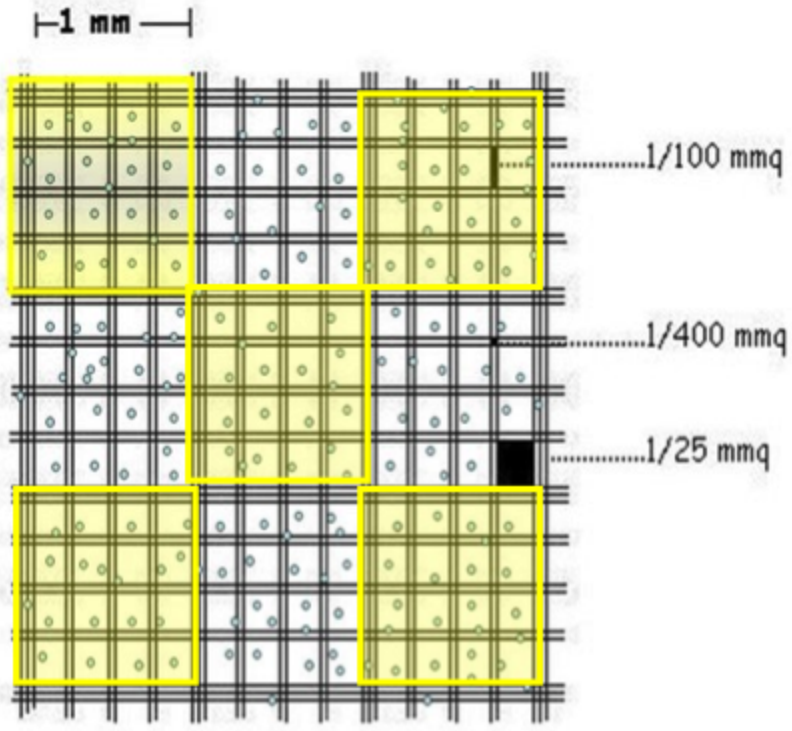

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

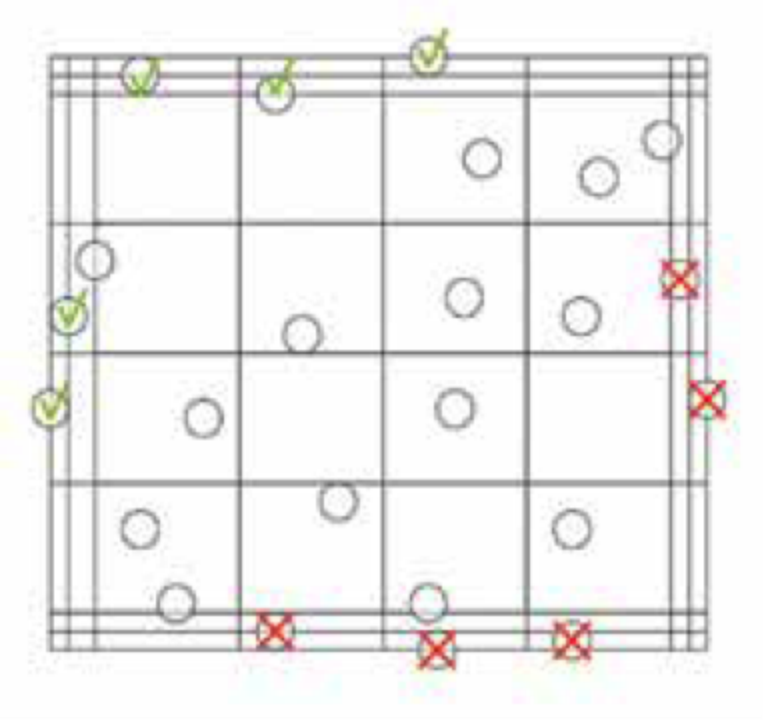

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

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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 suspention 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 $0.5x10^6$ 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 ± $100x10^6$ starting from 25 ml of buffy coat.