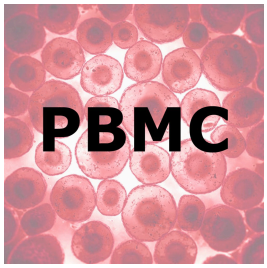
Jul 23, 2020 Version 1

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, isolation of human pbmc, human pbmc, patients with parkinson, parkinson, dopaminergic receptors on cd4, dopaminergic receptor, dopamine receptor gene polymorphism, pbmc, influence of dopamine receptor gene polymorphism, treg in drug, functional th1 bias, journal of neuroinflammation,

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.

Document

NAME

SOLUTION- 02 - Phosphate Buffered Saline (PBS)

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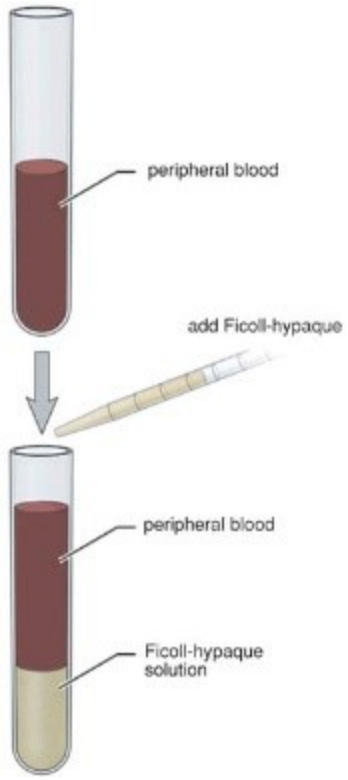
3 Place 3 mL of **FICOLL** in a 15 mL conical tube.

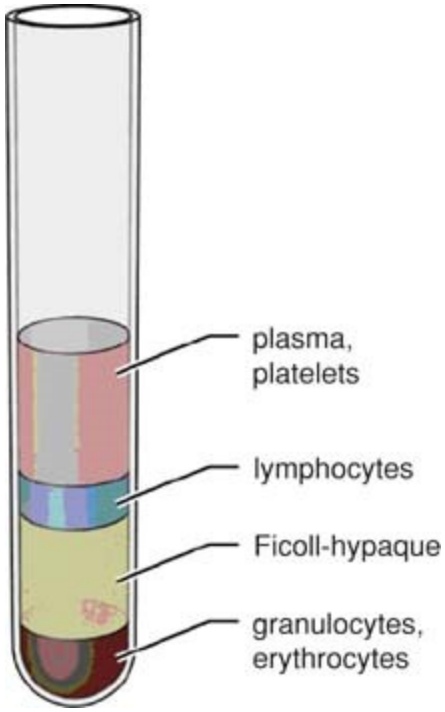
4 **CAREFULLY** layer 12 mL of diluted blood on the FICOLL with a glass Pasteur Pipette to a final volume of 15 ml as shown in the figure below.



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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NAME

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

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

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.

Document	
NAME	
SOLUTION- 06 - Lysis Buffer	
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Equipment

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

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

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

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).

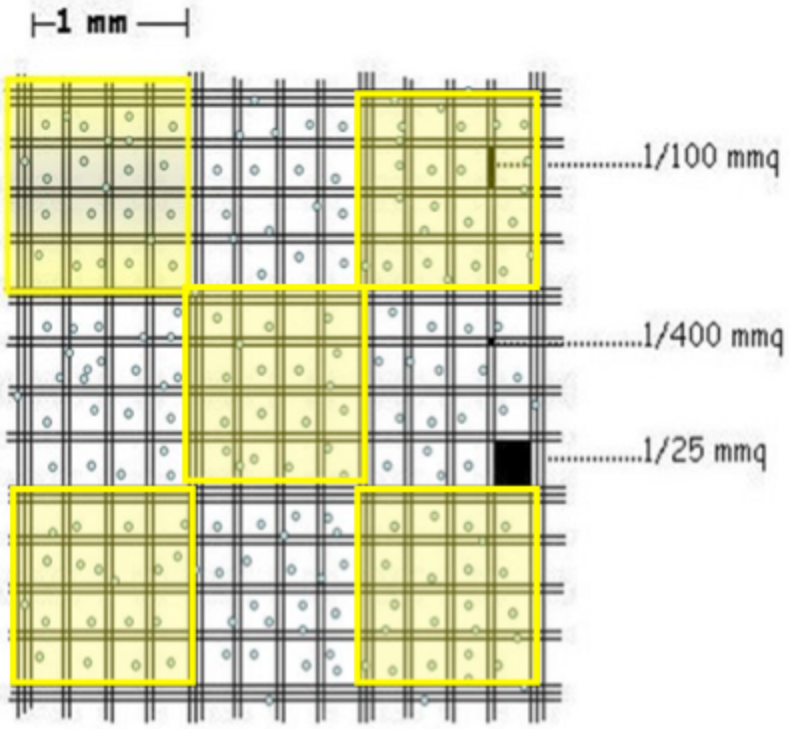

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

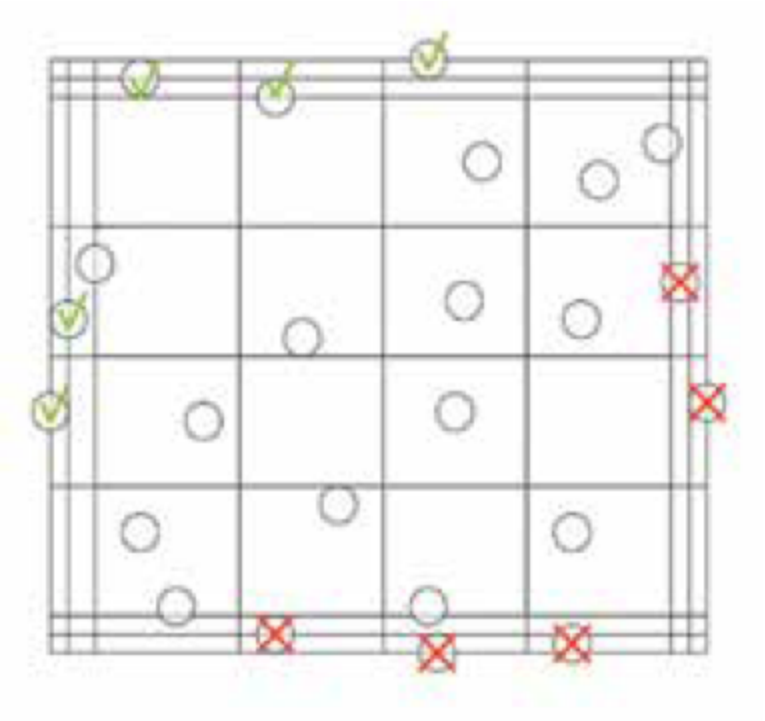

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

Document

NAME

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 0.5×10^6 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 ± $100x10^6$ starting from 25 ml of buffy coat.