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We use this protocol and it's working

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

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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>

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

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 INSTRUCTIONS

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.

Document

NAME

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

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Elisa Storelli

PREVIEW

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.

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NAME
	SOLUTION- 06 - Lysis Buffer
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Elisa Storelli	
	<u>PREVIEW</u>

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.

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

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PREVIEW

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.

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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 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 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 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

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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 μ l 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 $\geq 90\%$.

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

YIELD - The expected amount of PBMCs should be $\pm 100 \times 10^6$ starting from 25 ml of buffy coat.