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


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PROTOCOL CITATION

KEYWORDS
PBMC, Fresh Blood, Neuroimmune-Pharmacology, Parkinson’s Disease, Cell isolation, Primary cell culture

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MATERIALS TEXT
MATERIALS
- **Ficoll Paque PLUS**
- Healthcare Catalog #17144003-500 ml
- **Fetal Bovine Serum**
- EuroClone Catalog #ECS0180L-500 ml
- **RPMI 1640**
- EuroClone Catalog #ECM 0495L- 500 ml
- **Trypan Blue solution 0.4%**
- Sigma Aldrich Catalog #T8154- 100 ml

Instrumentation required:
- Laminar flow hood
- Centrifuge
- Cellometer (automated cell counter) or Optical Microscope (manual cell count)
- Flow Cytometer
- Autoclave

BEFORE STARTING
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 into a **50 mL** conical tube.

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

   **PBS 1X**
   by Marco Ferrari,
   University of Insubria

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.

Citation: Marco Cosentino, Elisa Storelli, Alessandra Luini, Massimiliano LM Legnaro, Emanuela Rasini, Marco Ferrari, Franca Marino (05/05/2020). Separation and purification of human PBMC from FRESH BLOOD. https://dx.doi.org/10.17504/protocols.io.bfxmjpk6

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

Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy

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

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.

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.

Remove supernatant and resuspend pellet in 10 mL of PBS/FBS 2% and centrifuge at 600 x g, 00:10:00 at RT.
11 Remove supernatant and resuspend the obtained pellet in **10 mL** of RPMI/FBS 10% for cell counting.

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).**
OPTIONAL STEP

Trypan blue and Turk solution
by Marco Ferrari,
University of Insubria

Figure 1

Figure 2
For automatic cell count with Cellometer machine use Trypan Blue. The machine will calculate the number of cells/ml and the percent 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.

Cellometer Auto T4
Automated Cell Counter
Nexcelom Bioscience Euroclone

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.

BD FACS Celesta
Flow Cytometer
Becton Dickinson Milan Italy BD

Expected results

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.