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PBMC- 03 - TEFF+TREG Isolation from PBMC with “Miltenyi CD4+CD25+ Regulatory T cell Isolation Kit” V.1

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

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Abstract

List of published work using this procedure:

- 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

Materials

MATERIALS

- MACS MultiStand **Miltenyi Biotec Catalog #**130-042-303
- EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**ED2SS
- BSA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A2153
- CD4 CD25 Regulatory T Cell Isolation Kit **Miltenyi Biotec Catalog #**130-091-301
- LD columns **Miltenyi Biotec Catalog #**130-042-901
- MS columns **Miltenyi Biotec Catalog #**130-042-201
- MidiMACS Separator **Miltenyi Biotec Catalog #**130-042-302
- MiniMACS Separator **Miltenyi Biotec Catalog #**130-042-102

INSTRUMENTATION REQUIRED

Laminar flow hood **(Room PS03)**

Before start

Make sure that the buffer is **cold (+4°C)** by putting it on **ice** for all the time needed to perform this protocol!

You need to obtaine **TEFF and TREG cells uncontaminated for the subsequent cell culture**, hence make sure you are using **sterile buffers** and **sterile plastic disposables** as well.

Moreover, work under laminar flow hood when you are processing samples (from the beginning to the end of the following procedure).

1 Isolate PBMCs according either to the standard protocol from fresh blood or from buffy coat (PBMC- 01a - Isolation of Human PBMC from Buffy Coat, PBMC- 01b - Isolation of Human PBMC from Whole Blood).

2 Determine the cell number and viability with the microscope by staining with either Türk or Trypan blue. You can use also Cellometer machine. **(PBMC purity should be ≥95% with few contaminant PMNs to prevent clogging of the column).**

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).

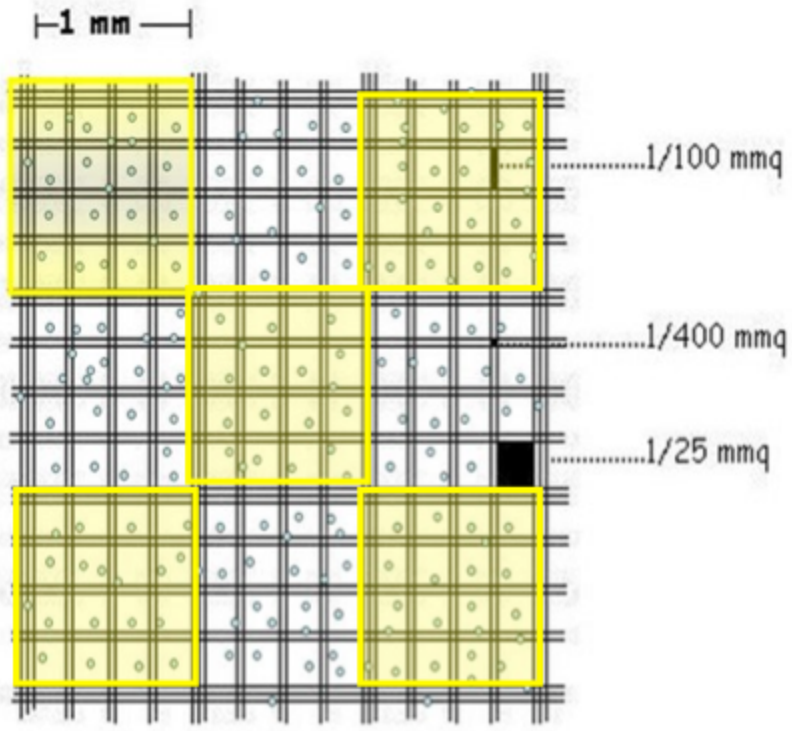

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

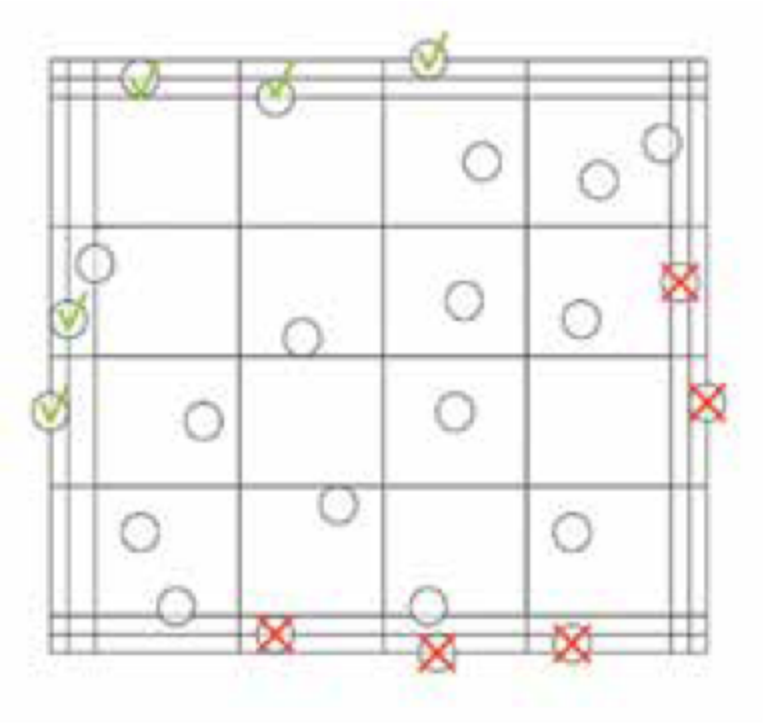

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

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.

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SOLUTION- 08 - Türk solution

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SOLUTION- 09 - Trypan Blue solution

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Equipment

Cellometer Auto T4	NAME
Automated cell counter	TYPE
Nexcelom Bioscience	BRAND
EuroClone	SKU

3 **OPTIONAL STEP**

Sorting of TREG is quite long procedure. Especially in clinical studies with whole blood of enrolled subject, it is possible to stop it after PBMC isolation and counting.

In this case, put cells in a flask with complete medium at a concentration of 1×10^6 cells/mL.

Put the flasks in an incubator (37°C, 5% CO2), and start sorting procedure the day after.

4 Centrifuge the obtained PBMCs at 1200 x g, Room temperature, 00:05:00 .

Aspirate supernatant completely. (Use 15 mL-conical tube)

Note

Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

Volumes for magnetic labeling indicated in this procedure are for up to $10x10^6$ total PBMCs. When working with higher than $10x10^6$ cells, scale up all reagent volumes and total volumes accordingly.

For optimal performance it is important to obtain a single-cell suspension before magnetic labeling.

Equipment

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

5 Resuspend the pellet in 100 µL of **cold SOLUTION- 10** (adjust volumes for $10×10^6$ cells).

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SOLUTION- 10 - TEFF/TREG isolation buffer

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6 Add 10 µL of **CD4+T Cell Biotin-Antibody Cocktail** (adjust volumes for 10×10^6 cells).

7 Mix well and incubate for 00:10:00 at 4 °C

8 Add 20 µL of **Anti-Biotin MicroBeads** (adjust volumes for 10×10^6 cells), mix well and incubate 00:15:00 at 4 °C

9 Add 5 µL of **cold SOLUTION- 10** and centrifuge at 1200 x g, Room temperature, 00:05:00

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SOLUTION- 10 - TEFF/TREG isolation buffer

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10 Discard the supernatant and resuspend the pellet in 500 µL of **cold SOLUTION- 10**.

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11 Place **LD column** in the magnetic field of suitable MACS Separator (violet, see figures below).

Separator must be attached to the MACS multistand (black) in order to work

12 Prepare column by rinsing it with 3 mL of **cold SOLUTION- 10** (**trash the effluent**).

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SOLUTION- 10 - TEFF/TREG isolation buffer

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13 Apply cell suspension onto the column.

14 Collect unlabeled cells that pass through column. Wait until the column reservoir is completely empty.

Wash again **2 times** with 3 mL of **cold SOLUTION- 10** and **1 last time** with 2 mL of of the same buffer.

Collect total effluent that is consisting of unlabeled pre-enriched CD4+ cell fraction.

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SOLUTION- 10 - TEFF/TREG isolation buffer

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15 Centrifuge the obtained effluent at 1200 x g, Room temperature, 00:05:00

Equipment

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

16 Remove supernatant and resuspend cell pellet in 100 µL of **cold SOLUTION- 10** (adjust the volumes for 10×10^6 cells).

Note

Use 15 mL-conical tubes.

Volumes for magnetic labeling indicated in this procedure are for an initial starting cell number of up to 10x106 total PBMCs. For higher initial cell numbers, scale up all reagent volumes accordingly.

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SOLUTION- 10 - TEFF/TREG isolation buffer

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17 Add 10 µL of **CD25 MicroBeads** (adjust volumes for 10×10^6 cells), mix well and incubate 00:15:00 at 4 °C in the dark.

18 Add 5 mL of **cold SOLUTION- 10** and centrifuge at 1200 x g, Room temperature, 00:05:00

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Equipment

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

19 Remove the supernatant and resuspend the cell pellet in 500 µL of **cold SOLUTION- 10**.

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SOLUTION- 10 - TEFF/TREG isolation buffer

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20 Place the **MS column** in the magnetic field of a suitable MACS Separator (green, see figures below).

Separator must be attached to the MACS multistand (black) in order to work

21 Prepare the column by rinsing with 500 µL of **cold SOLUTION- 10** and trash the effluent.

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SOLUTION- 10 - TEFF/TREG isolation buffer

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22 Apply cell suspension onto the column.

23 Collect the flow-through containing unlabeled negative fraction (T effector cells CD25-).

Wait until the column reservoir is completely empty, wash again **3 times** with 2 µL of **cold SOLUTION- 10**.

Note

Use 15 mL-conical tube

24 Remove the column from the magnet and place it on a suitable collection tube.

Note

Use 15 mL-conical tube

25 Pipette 1 mL of **cold SOLUTION- 10** onto the column.

Immediately flush out the magnetically labeled cells by **firmly pushing the plunger into the column**.

The cells that are flushed out are CD25 labeled cells positive fraction (T regulatory cells CD25+).

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SOLUTION- 10 - TEFF/TREG isolation buffer

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26 In order to make sure that collection of cells was complete, **repeat the last step TWO more times**.

27 Centrifuge isolated TEFF and TREG at 1200 x g, Room temperature, 00:05:00

Equipment

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

28 Resuspend TEFF cells in 1 mL of **SOLUTION- 4** and TREG cells in 0.2 mL of **SOLUTION- 4.**

Count them under microscope or Cellometer machine, according to the appropriate procedure (see step 2 of this protocol).

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

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29 **OPTIONAL STEPS**

If required, it is possible to check the purity of isolated TEFF and TREG.

Proceed as follows:

- Put PBMCs (0.5×10^6 cells), Teff (0.5×10^6 cells) and TREG (at least 0.3×10^6 cells) into 3 different BD Tubes;
- Centrifuge at 1200 x g, Room temperature, 00:05:00
- Remove the supernatant and resuspend the pellet in 50 µL **PBS 1X** ;
- Add the adequate antibodies such as: **CD4 APC-Cy7** (2.5 µL , BD cat. n. 557871), **CD25 PE** (10 µL , Miltenyi cat. n. 120-001-311) and **CD127 AF647** (10 µL , BD cat. n. 558598) or conjugated to other fluorochromes;
- Incubate for 00:20:00 , in the **dark at RT**;
- Wash with 1 mL of **PBS 1X** and centrifuge 1200 x g, Room temperature, 00:05:00 ;
- Resuspend the pellet in 350 µL **PBS 1X** and leave on ice until FACS acquisition with an appropriate protocol.