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

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1 Works for me dx.doi.org/10.17504/protocols.io.bjabkian

Farmacologia Medica

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

DOI

dx.doi.org/10.17504/protocols.io.bjabkian

PROTOCOL CITATION

Marco Cosentino, Elisa Storelli, Alessandra Luini, Massimiliano LM Legnaro, Emanuela Rasini, Marco Ferrari, Franca Marino 2020. PBMC- 03 - TEFF+TREG Isolation from PBMC with “Miltenyi CD4+CD25+ Regulatory T cell Isolation Kit”. **protocols.io** dx.doi.org/10.17504/protocols.io.bjabkian

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39971

MATERIALS

NAME	CATALOG #	VENDOR
MACS MultiStand	130-042-303	Miltenyi Biotec
EDTA	ED2SS	Sigma Aldrich
BSA	A2153	Sigma Aldrich
CD4 CD25 Regulatory T Cell Isolation Kit	130-091-301	Miltenyi Biotec
LD columns	130-042-901	Miltenyi Biotec
MS columns	130-042-201	Miltenyi Biotec

Citation: Marco Cosentino, Elisa Storelli, Alessandra Luini, Massimiliano LM Legnaro, Emanuela Rasini, Marco Ferrari, Franca Marino (08/03/2020). PBMC- 03 - TEFF+TREG Isolation from PBMC with Ã¢ÂÂMiltenyi CD4+CD25+ Regulatory T cell Isolation KitÃ¢ÂÂ. https://dx.doi.org/10.17504/protocols.io.bjabkian

NAME	CATALOG #	VENDOR
MidiMACS Separator	130-042-302	Miltenyi Biotec
MiniMACS Separator	130-042-102	Miltenyi Biotec

MATERIALS TEXT

INSTRUMENTATION REQUIRED

Laminar flow hood **(Room PS03)**

EQUIPMENT

NAME	CATALOG #	VENDOR
Cellometer Auto T4	EuroClone	
Allegra AVANTI 30	Beckman Italy	Beckman Coulter

BEFORE STARTING

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).

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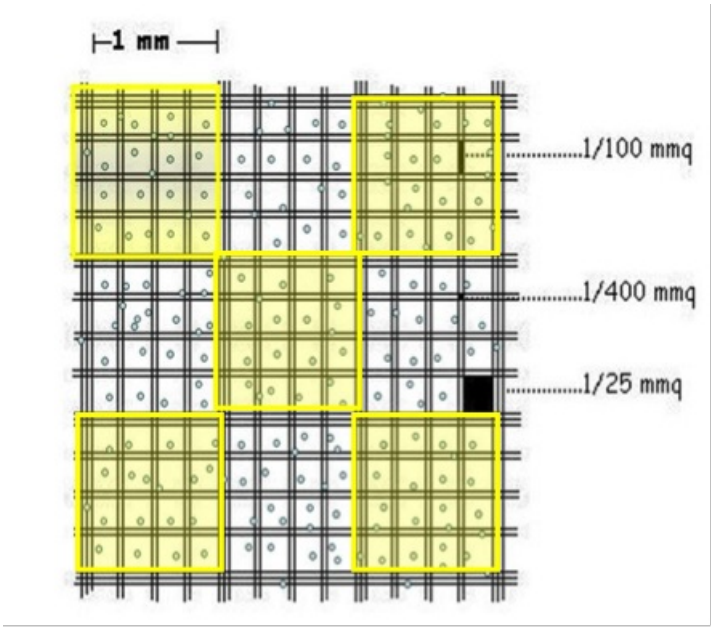

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

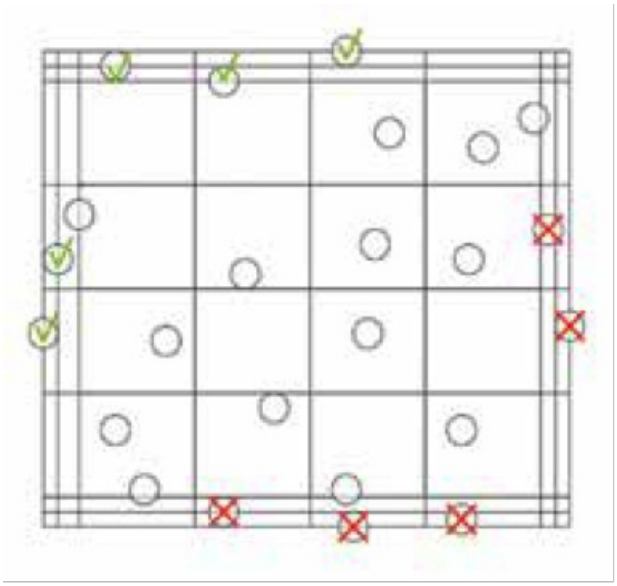

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

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.

SOLUTION- 08 - Türk solution
by Farmacologia Medica

Citation: Marco Cosentino, Elisa Storelli, Alessandra Luini, Massimiliano LM Legnaro, Emanuela Rasini, Marco Ferrari, Franca Marino (08/03/2020). PBMC- 03 - TEFF+TREG Isolation from PBMC with Ã¢ÂÂMiltenyi CD4+CD25+ Regulatory T cell Isolation KitÃ¢ÂÂ. https://dx.doi.org/10.17504/protocols.io.bjabkian

SOLUTION- 09 - Trypan Blue solution
by Farmacologia Medica

Cellometer Auto T4
Automated cell counter
Nexcelom Bioscience EuroClone

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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 $1x10^6$ 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)

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.

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Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy

5 Resuspend the pellet in **100 µl** of **cold SOLUTION- 10** (adjust volumes for 10x10^6 cells).

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

6 Add **10 µl** of **CD4+T Cell Biotin-Antibody Cocktail** (adjust volumes for 10x10^6 cells).

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

8 Add **20 µl** of **Anti-Biotin MicroBeads** (adjust volumes for 10x10^6 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**

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

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

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

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

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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**).

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

13 Apply cell suspension onto the column.

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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.

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

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

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 $10x10^6$ cells).

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.

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

17 Add **10 µl** of **CD25 MicroBeads** (adjust volumes for $10x10^6$ 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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SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy

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

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

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

Citation: Marco Cosentino, Elisa Storelli, Alessandra Luini, Massimiliano LM Legnaro, Emanuela Rasini, Marco Ferrari, Franca Marino (08/03/2020). PBMC- 03 - TEFF+TREG Isolation from PBMC with Ã¢ÂÂMiltenyi CD4+CD25+ Regulatory T cell Isolation KitÃ¢ÂÂ. https://dx.doi.org/10.17504/protocols.io.bjabkian

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.

SOLUTION- 10 - TEFF/TREG isolation buffer
by Farmacologia Medica

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**.

Use 15 mL-conical tube

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

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
by Farmacologia Medica

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**

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).

SOLUTION- 04 - Wash solution (RPMI/FBS) for PBMC
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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OPTIONAL STEPS

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

Proceed as follows:

- Put PBMCs ($0.5x10^6$ cells), Teff ($0.5x10^6$ cells) and TREG (at least $0.3x10^6$ cells) into 3 different BD Tubes;

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- 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.

Citation: Marco Cosentino, Elisa Storelli, Alessandra Luini, Massimiliano LM Legnaro, Emanuela Rasini, Marco Ferrari, Franca Marino (08/03/2020). PBMC- 03 - TEFF+TREG Isolation from PBMC with Ã¢ÂÂMiltenyi CD4+CD25+ Regulatory T cell Isolation KitÃ¢ÂÂ. https://dx.doi.org/10.17504/protocols.io.bjabkian