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

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🌐 PBMC- 03 - TEFF+TREG Isolation from PBMC with “Miltenyi CD4+CD25+ Regulatory T cell Isolation Kit” V.2

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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 **Sigma Aldrich Catalog #ED2SS**
- ☒ BSA **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 INSTRUCTIONS

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 $\geq 95\%$ with few contaminant PMNs to prevent clogging of the column).**

For manual cell count use Türk solution for checking purity.

Follow protocol CELL COUNT- 02.

For automatic cell count with Cellometer machine use Trypan Blue for checking viability.

Follow protocol CELL COUNT- 03

Document
NAME SOLUTION- 08 - Türk solution
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Document
NAME 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 **SOLUTION- 13** 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.

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SOLUTION- 13 - Complete culture medium

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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 10×10^6 total PBMCs. When working with higher than 10×10^6 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 10x10⁶ cells).

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NAME
	SOLUTION- 10 - TEFF/TREG isolation buffer
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- 6 Add 10 µL of **CD4+T Cell Biotin-Antibody Cocktail** (adjust volumes for 10x10⁶ cells).

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

- 8 Add 20 µL of **Anti-Biotin MicroBeads** (adjust volumes for 10x10⁶ 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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NAME 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 the same buffer.

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

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

- 16 Remove supernatant and resuspend cell pellet in 100 μ L 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 10×10^6 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 μ L 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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SOLUTION- 10 - TEFF/TREG isolation buffer

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

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

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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 μ L **PBS 1X** ;
- Add the adequate antibodies such as: **CD4 APC-Cy7** (2.5 μ L , BD cat. n. 557871), **CD25 PE** (10 μ L , Miltenyi cat. n. 120-001-311) and **CD127 AF647** (10 μ L , 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 μ L **PBS 1X** and leave on ice until FACS acquisition with an appropriate protocol.

Document
<div>NAME</div> <div>SOLUTION- 02 - Phosphate Buffered Saline (PBS)</div>
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