

Nov 21, 2020

Version 2

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

DOI

<https://dx.doi.org/10.17504/protocols.io.bpxumpnw>

Marco Cosentino¹, Elisa Storelli¹, Alessandra Luini¹, Massimiliano Legnaro¹, Emanuela Rasini¹, Marco Ferrari¹, Franca Marino¹

¹Center for Research in Medical Pharmacology, University of Insubria (Varese, Italy)



Farmacologia Medica

University of Insubria

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.bpxumpnw>

Protocol Citation: Marco Cosentino, Elisa Storelli, Alessandra Luini, Massimiliano 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 https://dx.doi.org/10.17504/protocols.io.bpxumpnw](https://dx.doi.org/10.17504/protocols.io.bpxumpnw) Version created by **Farmacologia Medica**

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: November 21, 2020

Last Modified: November 21, 2020

Protocol Integer ID: 44756

Keywords: treg in drug, parkinson, journal of neuroinflammation, functional th1 bias, treg, neuroinflammation, pbmc

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 $\geq 95\%$ 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

CREATED BY

Farmacologia Medica

Preview

Document

NAME

SOLUTION- 09 - Trypan Blue solution

CREATED BY

Farmacologia Medica

Preview



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.

Document

NAME

SOLUTION- 13 - Complete culture medium

CREATED BY

Farmacologia Medica

Preview

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 10×10^6 cells).



Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

[Preview](#)

- 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

Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

[Preview](#)



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

Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

Preview

- 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)**.



Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

[Preview](#)

- 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.

Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

[Preview](#)

- 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 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 10×10^6 total PBMCs. For higher initial cell numbers, scale up all reagent volumes accordingly.

Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

Preview



- 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

Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

Preview

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**.

Document

NAME

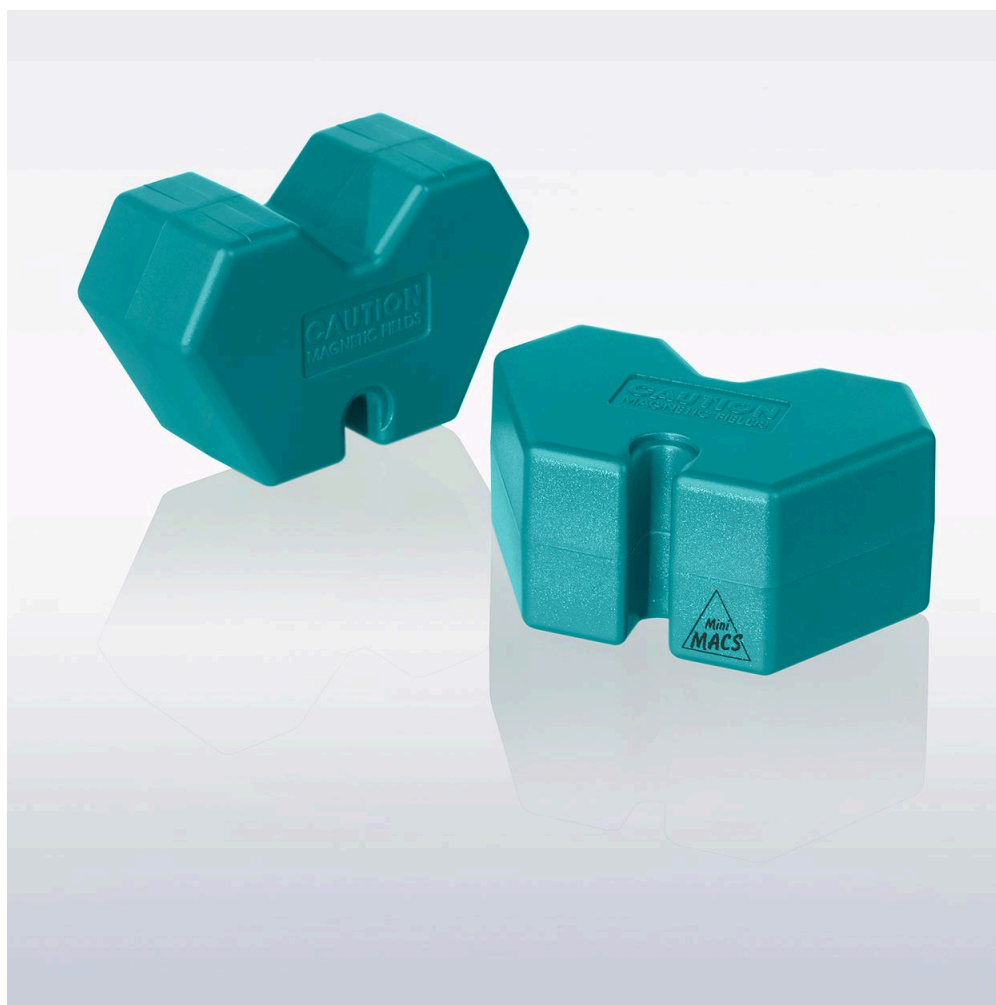
SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

[Preview](#)

- 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.

Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

Preview

- 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+).

Document

NAME

SOLUTION- 10 - TEFF/TREG isolation buffer

CREATED BY

Farmacologia Medica

Preview

- 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).

Document

NAME

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

CREATED BY

Farmacologia Medica

Preview

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 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

NAME

SOLUTION- 02 - Phosphate Buffered Saline (PBS)

CREATED BY

Farmacologia Medica

Preview