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

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ABSTRACT
List of published work using this procedure:


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PROTOCOL CITATION

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INSTRUMENTATION REQUIRED
Laminar flow hood (Room PS03)

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 obtain 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 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 104 (hemacytometer volume).

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 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.

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 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.
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 $1 \times 10^6$ 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 $1200 \times g$, 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 $10 \times 10^6$ total PBMCs. When working with higher than $10 \times 10^6$ 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.
5. Resuspend the pellet in **100 µl** of **cold SOLUTION- 10** (adjust volumes for 10x10^6 cells).

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**.

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

![SOLUTION-10 TEFF/TREG isolation buffer](https://www.farmacologia-medica.it)

**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.

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 10x10^6 total PBMCs. For higher initial cell numbers, scale up all reagent volumes accordingly.

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

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.

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+).
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 \(1200 \times g\), Room temperature, 00:05:00.

28 Resuspend TEFF cells in \(1 \text{ mL}\) of SOLUTION- 4 and TREG cells in \(0.2 \text{ mL}\) of SOLUTION- 4.

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

29 **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;
- 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.