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:

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 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).

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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.
For automatic cell count with Cellometer machine use Trypan Blue. The machine will calculate the number 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.

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

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<thead>
<tr>
<th>NAME</th>
<th>Type</th>
<th>Brand</th>
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<tbody>
<tr>
<td>Cellometer Auto T4</td>
<td>Automated cell counter</td>
<td>Nexclom Bioscience</td>
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<td></td>
<td></td>
<td>EuroClone</td>
<td>3</td>
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### 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)
### 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 $10 \times 10^6$ 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.**

### Equipment

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<tr>
<td>Allegra AVANTI 30</td>
<td>Centrifuge</td>
<td>Beckman Coulter</td>
<td>Beckman Italy</td>
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### 5

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

### Document

<table>
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<tr>
<td>SOLUTION- 10 - TEFF/TREG isolation buffer</td>
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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.

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).

13 Apply cell suspension onto the column.

14 Collect unlabeled cells that pass through the 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.
Centrifuge the obtained effluent at 1200 x g, Room temperature, 00:05:00.

Remove supernatant and resuspend cell pellet in 100 µL of cold SOLUTION- 10 (adjust the volumes for 10x10^6 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 10x10^6 total PBMCs. For higher initial cell numbers, scale up all reagent volumes accordingly.
Add 10 µL of CD25 MicroBeads (adjust volumes for 10x10^6 cells), mix well and incubate at 4 °C in the dark.

Add 5 mL of cold SOLUTION- 10 and centrifuge at 1200 x g, Room temperature, 00:05:00.
Remove the supernatant and resuspend the cell pellet in 500 µL of cold SOLUTION-10.

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.

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+).

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

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).

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

- Put PBMCs (0.5x10⁶ cells), Teff (0.5x10⁶ cells) and TREG (at least 0.3x10⁶ 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 20 minutes in the dark at RT;

- Wash with 1 mL of PBS 1X and centrifuge at 1200 x g, Room temperature, 5 minutes;

- Resuspend the pellet in 350 µL PBS 1X and leave on ice until FACS acquisition with an appropriate protocol.