Jun 08, 2020

Magnetic bead-based TREG-TEFF cell isolation from PBMC with Miltenyi CD4+CD25+ Regulatory T cell Isolation Kit

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

Magnetic bead-based TREG-TEFF cell isolation from PBMC with Miltenyi CD4+CD25+ Regulatory T cell Isolation Kit, 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

PROTOCOL CITATION

Marco Cosentino, Elisa Storelli, Alessandra Luini, Emanuela Rasini, Massimiliano LM Legnaro, Marco Ferrari, Franca Marino 2020. Magnetic bead-based TREG-TEFF cell isolation from PBMC with Miltenyi CD4+CD25+ Regulatory T cell Isolation Kit. **protocols.io** https://protocols.io/view/magnetic-bead-based-treg-teff-cell-isolation-from-bg4qjyvw

CREATED

Jun 03, 2020

LAST MODIFIED

Jun 08, 2020

PROTOCOL INTEGER ID

37744

GUIDELINES

Visit the link below to learn about the **MACS cell separation technology** and to download the **data sheet** of the kit (**catalogue number 130-091-301**):

http://www.miltenyibiotec.com/en/products-and-services/macs-cell-separation.aspx

MATERIALS

NAME	CATALOG #	VENDOR
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

NAME	CATALOG #	VENDOR
MidiMACS Separator	130-042-302	Miltenyi Biotec
MiniMACS Separator	130-042-102	Miltenyi Biotec
MACS Multistand	130-042-303	Miltenyi Biotec

MATERIALS TEXT

INSTRUMENTATION REQUIRED

Laminar flow hood **(Room PS03)**

EQUIPMENT

NAME	CATALOG #	VENDOR
Allegra AVANTI 30	Beckman Italy	Beckman Coulter
BD FACS Celesta	Milan Italy BD	
Cellometer Auto T4	EuroClone	

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 to the standard protocol "**Separation and purification of human PBMC from BUFFY COAT**" or "**Separation and purification of human PBMC from FRESH 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)**

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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).

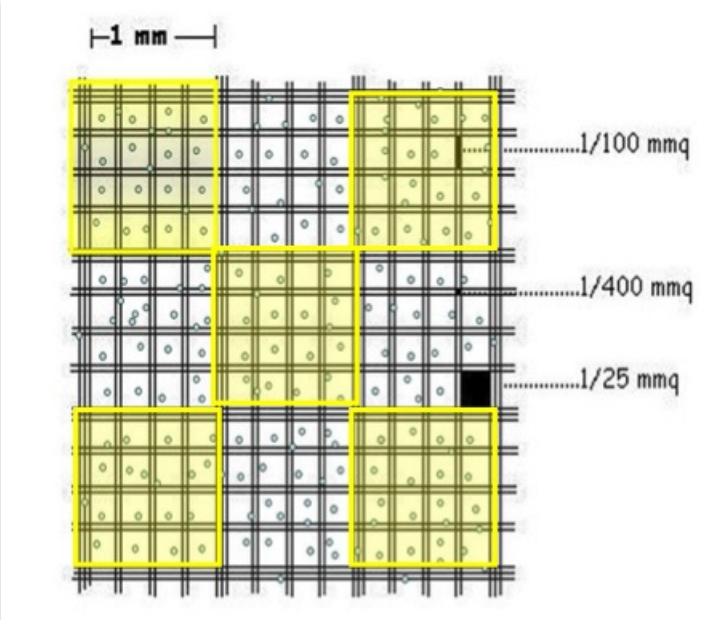

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

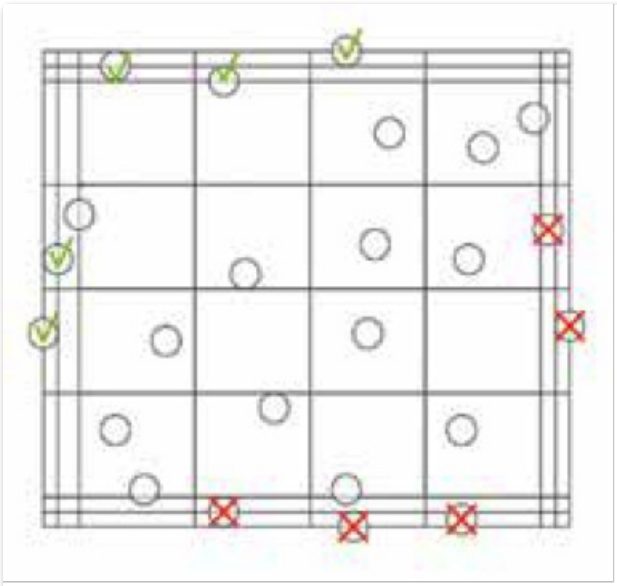

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

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.

Türk solution and Trypan Blue
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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.

Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy

5 Resuspend the pellet in **100 µl** of **cold** Miltenyi Buffer (for $10x10^6$ cells).

Miltenyi Buffer
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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

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

8 8. Add **20 µl** of **Anti-Biotin MicroBeads** (for 10x10^6 cells), mix well and incubate **00:15:00** at **4 °C**

9 Add **5 mL** of **cold** Miltenyi Buffer and centrifuge at **1200 x g, Room temperature 00:05:00**

Miltenyi Buffer
by Elisa Storelli,
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10 Discard the supernatant and resuspend the pellet in **500 µl** of **cold** Miltenyi Buffer.

Miltenyi Buffer
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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

Figure 3

Separator that goes with LD columns

Figure 4

Separator must be attached to the MACS multistand (black)

12 Prepare column by rinsing it with **3 mL** of Miltenyi Buffer (**trash the effluent**).

Miltenyi Buffer

by Elisa Storelli,

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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** Miltenyi Buffer and **1 last time** with **2 mL** of Buffer.

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

Miltenyi Buffer
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Center for Research in Medical Pharmacology, University of Insubria

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** Miltenyi Buffer (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.

Miltenyi Buffer
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

17 Add **10 µl** of CD25 MicroBeads (for $10x10^6$ cells), mix well and incubate **00:15:00** , at **4 °C** , **in the dark**.

18 Add **5 mL** of **cold** Miltenyi Buffer and centrifuge at **1200 x g, Room temperature 00:05:00**

Miltenyi Buffer
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy

19 Remove the supernatant and resuspend the cell pellet in **500 µl** of **cold** Miltenyi Buffer.

Miltenyi Buffer
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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

Magnetic separation: Positive selection of CD4+CD25+T regulatory cells.

Figure 5

Separator suitable for MS columns

Figure 6

Separator must be attached to the MACS multistand (black)

21 Prepare the column by rinsing with **500 µl** of **cold** Miltenyi Bufferand **trash the effluent.**

Miltenyi Buffer

by Elisa Storelli,

Center for Research in Medical Pharmacology, University of Insubria

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 mL** of **cold** Miltenyi Buffer.

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** Buffer 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+).

Miltenyi Buffer
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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 RPMI/10%FBS and TREG cells in **0.2 mL** of RPMI/10%FBS.

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

protocol).

(See this link for RPMI-FBS 10% recipe)

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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;
- 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.

PBS 1X
by Elisa Storelli,
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BD FACS Celesta
Flow Cytometer
Becton Dickinson Milan Italy BD

Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy