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PBMC- 02 - CD4+ T cell Isolation from PBMC with “Dynabeads CD4 Positive Isolation Kit” V.1

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

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Abstract

List of published works using this protocol:

- Kustrimovic N., Comi C., Magistrelli L., Rasini E., Legnaro M., Bombelli R., Aleksic I., Blandini F., Minafra B., Riboldazzi G., Struchio A., Mauri M., Bono G., Marino F., Cosentino M. 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 (2018). Journal of neuroinflammation, 15(1), 205. https://doi.org/10.1186/s12974-018-1248-8

- Kustrimovic, N., Rasini, E., Legnaro, M., Bombelli, R., Aleksic, I., Blandini, F., Comi, C., Mauri, M., Minafra, B., Riboldazzi, G., Sanchez-Guajardo, V., Marino, F., & Cosentino, M. (2016). Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson's Disease. Scientific reports, 6, 33738. https://doi.org/10.1038/srep33738

- Cosentino M., Ferrari M., Kustrimovic N., Rasini E., Marino F. (2015). Influence of dopamine receptor gene polymorphisms on circulating T lymphocytes: A pilot study in healthy subjects. Human immunology, 76, 10, 747-752. https://doi.org/10.1016/j.humimm.2015.09.032

Materials

MATERIALS

- Dynabeads™ CD4 Positive Isolation Kit **Thermo Fisher Catalog #**11331D
- Fetal Bovine Serum (FBS) **EuroClone Catalog #**ECS0180L-500 ml
- RPMI 1640 **EuroClone Catalog #**ECM 0495L- 500 ml
- BSA **Sigma Aldrich Catalog #**A2153
- BD tubes **Becton-Dickinson Catalog #**352054

Instrumentation required:

a.Magnet (DynaMag™)
b.Sample Mixer with rotation
c.Laminar flow hood

Before start

If you need to obtain **CD4+ T cell for subsequent cell culture**, 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). Otherwise, use non-sterile Buffers and disposables, and process samples in a cell isolation laboratory.

IMPORTANT NOTE: the isolation protocol is calibrated for using **25µL of beads for 10×10^6 PBMCs resuspended in 1mL.** For lower or higher cell number than 10×106, resize the volumes, accordingly. (See also Table 1on the data sheet of the kit).

ALL REAGENTS MUST BE AT ROOM TEMPERATURE WHEN USED!!!

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 Count the cells with Cellometer machine or by manual count, using either Trypan Blue or Türk solutions accordingly.

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 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 µlof 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).

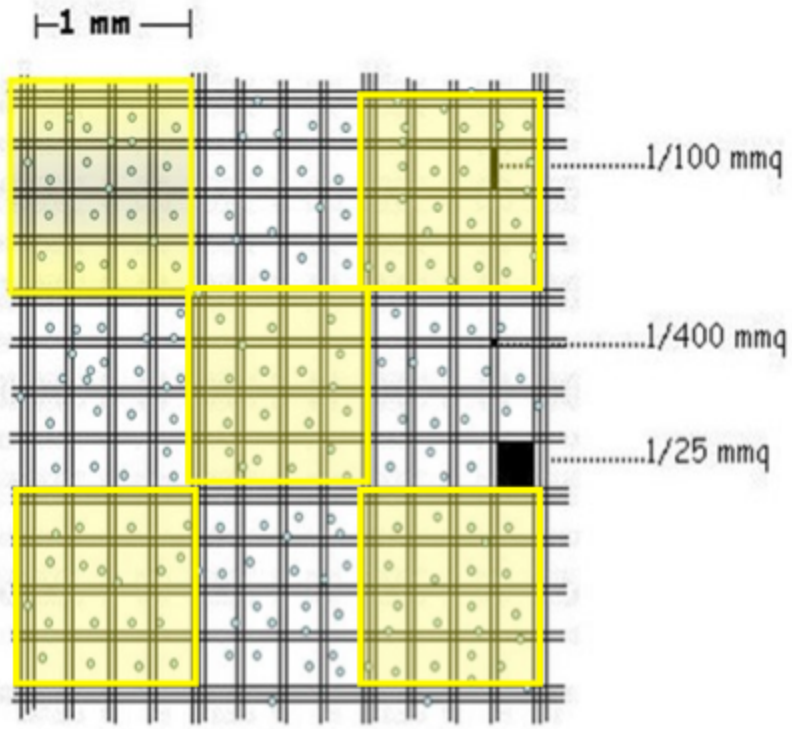

Figure 1
The gridded area of the chamber consists of nine 1 mmq squares. These squares are subdivided in three directions; 0.0625 mmq, 0.05 mmq and 0.04 mmq. The central square here in Figure 1 is further subdivided into 0.0025 mmq = 1/25 mmq squares. Count cells in 5 squares as shown.

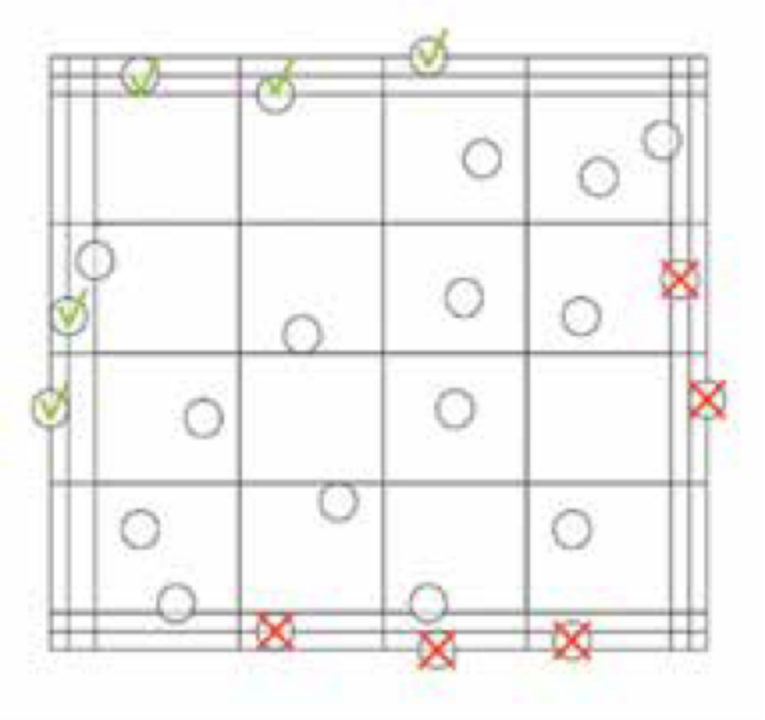

Figure 2
Concerning those cells that lay on the perimeter of the square, count following this scheme.

Document

NAME

SOLUTION- 09 - Trypan Blue solution

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NAME

SOLUTION- 08 - Türk solution

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Equipment

Cellometer Auto T4	NAME
Automated cell counter	TYPE
Nexcelom Bioscience	BRAND
EuroClone	SKU

3 Resuspend Dynabeads in the vial using a vortex for >30 sec.

4 Transfer the desired volume of Dynabeads to a 5mL-tube (use BD tubes cat. n. 352054) following this proportion: **25µL of beads for 10×10^{6}cells.**

5 Add 2 µL of **Solution- 11** (found in the kit materials as Buffer 1), resuspend and place the tube into the magnet: beads will attach to the magnet very quickly (few seconds).

Discard then the supernatant by using a glass Pasteur pipette.

Remove the tube from the magnet.

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SOLUTION- 11 - CD4+T cells isolation buffer

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6 Repeat the washing step 2 or 3 times to make sure that DMSO is all washed up.

7 After counting, centrifuge PBMCs sample at 1200 x g, 00:05:00 .

Equipment

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

8 Discard supernatant and resuspend pellet of 10×10^6 cells in 1 mL of **SOLUTION- 11**.

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SOLUTION- 11 - CD4+T cells isolation buffer

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9 Transfer cell suspension into the tube with beads, and resuspend vigorously.

10 Incubate the beads with cells for 00:20:00 at 4 °C with gentle rotation by putting the Sample Mixer in the fridge.

11 After incubation place the tube on the magnet and wait for 1-2 minutes, that is until the complex beads-cells is completely attached to the magnet.

12 While the tube is still in the magnet, carefully **remove** and **discard** the **supernatant** with a glass Pasteur pipette.

13 Remove the tube from the magnet, add 2 µL of **SOLUTION- 11** and resuspend the cells very vigorously because of aggregates.

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SOLUTION- 11 - CD4+T cells isolation buffer

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14 Repeat steps 11-13 twice (in total 3 times) to wash the bead-bound CD4+ T cells. These steps are critical to obtain a high purity of isolated cells.

15 Resuspend cell pellet in 100 µL of **SOLUTION- 07** (found in the kit materials as Buffer 2)
[The volume is calibrated for 10×10^6 cells, for lower or higher number of cell resize the volume accordingly].

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SOLUTION -07 - RPMI/FBS 1%

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16 Add 10 µL of **DETACHaBEAD® CD4** for each 10×10^6 PBMCs.
(Resize this volume if the number of starting cell is different)

17 Add another 500 µL of **SOLUTION- 07** to increase the volume and transfer everything in a 1.5 mL eppendorf.

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SOLUTION -07 - RPMI/FBS 1%

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18 Incubate 00:45:00 at Room temperature (RT) with gentle rotation by using a Sample Mixer.

19 Transfer the sample from eppendorf to BD tube, and place the tube on magnetand wait for 1-2 mins, that is until the complex beads-cells is completely attached to the magnet.

20 While the tube is still in the magnet, **transfer the supernatant** containing the released cells into a 15 mL conical tube.

To obtain residual cells, wash the beads 3 times with 500 µL of **SOLUTION- 07** and collect the supernatant each time.

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SOLUTION -07 - RPMI/FBS 1%

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21 Add to the detached cell suspension **SOLUTION- 07** to a final volume of 5 mL and centrifuge at 1200 x g, Room temperature, 00:05:00

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SOLUTION -07 - RPMI/FBS 1%

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Equipment

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

22 Resuspend the cells for cell counting in 1 mL : follow the appropriate protocol (see step 2 of this protocol).

Check the viability with Trypan blue.

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SOLUTION- 09 - Trypan Blue solution

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23 **OPTIONAL STEP**

Check the purity of the isolated CD4+ T cells by flow cytometry.

If needed, check the purity by labeling CD4 with the appropriate CD markers, such as CD3, CD4, CD8 and CD14 Ab and analyze samples with a flow cytometer to exclude the presence of undesired subsets.

Equipment

BD FACS Celesta	NAME
Flow Cytometer	TYPE
Becton Dickinson	BRAND
Milan Italy BD	SKU

24 **EXPECTED RESULTS**

Expected result

Cell Viability: ≥95 %

Cell Yield: ± 4,6 x10^6 cells starting from 25 mL of Fresh Blood
± 6 x10^6 cells starting from 25 mL of Buffy Coat

If checked, purity of the isolated CD4+ cells must be ≥95 %