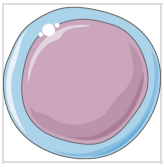

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Magnetic bead-based CD4+ T cell isolation from PBMCs with Dynabeads: CD4 Positive Isolation Kit

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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

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

MATERIALS TEXT

Instrumentation required:

a.Magnet (DynaMag™)

b.Sample Mixer with rotation

c.Laminar flow hood

EQUIPMENT

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

BEFORE STARTING

If you need to obtaine **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 10x10^{6}PBMCs resuspended in 1mL**. For lower or higher cell number than 10x10^6, resize the volumes, accordingly. (See also **Table 1** on 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.

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.

Trypan blue and Turk solution
by Marco Ferrari,
University of Insubria

Cellometer Auto T4
Automatic Cell Counter
Nexcelom Bioscience Euroclone

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 10x10^{6}cells.**

5 Add **2 ml** of **Buffer 1** (found in the kit materials), 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.

Buffer 1 and Buffer 2 for magnetic bead-based CD4+ T cell isolation with Dynabeads
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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** .

Allegra Avanti 30
Centrifuge
Beckman Coulter Beckman Italy

8 Discard supernatant and resuspend pellet of 10x106cells in **1 ml** of **Buffer 1**.

Buffer 1 and Buffer 2 for magnetic bead-based CD4+ T cell isolation with Dynabeads
by Elisa Storelli,
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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 ml** of **Buffer 1** and resuspend the cells very vigorously because of aggregates.

Buffer 1 and Buffer 2 for magnetic bead-based CD4+ T cell isolation with Dynabeads
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

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** **Buffer 2**.
[The volume is calibrated for $10x10^6$cells, for lower or higher number of cell resize the volume accordingly].

Buffer 1 and Buffer 2 for magnetic bead-based CD4+ T cell isolation with Dynabeads
by Elisa Storelli,
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16 Add **10 µl** of **DETACHaBEAD®** CD4 for each $10x10^6$ PBMCs.
(Resize this volume if the number of starting cell is different)

17 Add another **500 µl** of **Buffer 2** to increase the volume and transfer everything in a 1.5 mL eppendorf.

Buffer 1 and Buffer 2 for magnetic bead-based CD4+ T cell isolation with Dynabeads
by Elisa Storelli,
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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** Buffer 2 and **collect** the **supernatant** each time.

Buffer 1 and Buffer 2 for magnetic bead-based CD4+ T cell isolation with Dynabeads
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

21 Add to detached cell suspension **Buffer 2** to a final volume of 10 mL and centrifuge at **1200 x g, Room temperature 00:05:00** .

Buffer 1 and Buffer 2 for magnetic bead-based CD4+ T cell isolation with Dynabeads
by Elisa Storelli,
Center for Research in Medical Pharmacology, University of Insubria

Allegra AVANTI 30
Centrifuge
Beckman Coulter Beckman Italy

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.

Trypan blue and Turk solution
by Marco Ferrari,
University of Insubria

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.

BD FACS Celesta
Flow Cytometer
Becton Dickinson Milan Italy BD

24 EXPECTED RESULTS

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 %