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Version 2

◆ PBMC- 02 - CD4+ T cell Isolation from PBMC with "Dynabeads CD4 Positive Isolation Kit" V.2

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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 Merck MilliporeSigma (Sigma-Aldrich) Catalog #A2153
- BD tubes **Becton Dickinson (BD) Catalog** #352054

Instrumentation required:

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

Troubleshooting

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 10×10⁶ 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).

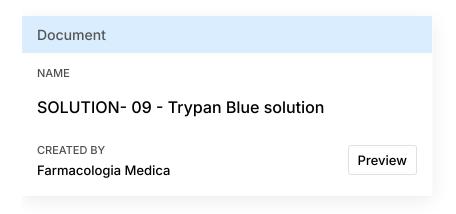
ALL REAGENTS MUST BE AT ROOM TEMPERATURE WHEN USED!!!



- 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.

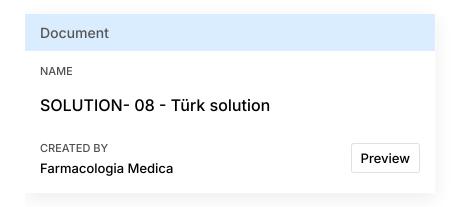
For automatic cell count with Cellometer machine use Trypan Blue.

Follow protocol **CELL COUNT- 03**



For manual cell count use Türk solution for checking purity.

Follow protocol **CELL COUNT- 02**





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 10×10⁶ cells.
- 5 Add \perp 2 μ L 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.





- Repeat the washing step 2 or 3 times to make sure that DMSO is all washed up.
- After counting, centrifuge PBMCs sample at 1200 x g, 00:05:00.



8 Discard supernatant and resuspend pellet of 10×10^6 cells in $\boxed{4 \text{ 1 mL}}$ of **SOLUTION-11**.



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



- Incubate the beads with cells for 00:20:00 at 4 °C with gentle rotation by putting the Sample Mixer in the fridge.
- 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.
- While the tube is still in the magnet, carefully **remove** and **discard** the **supernatant** with a glass Pasteur pipette.



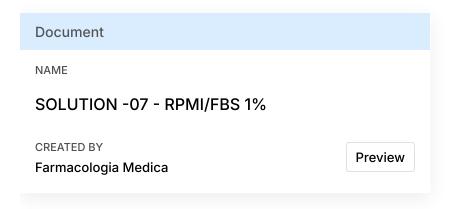
Remove the tube from the magnet, add $\underline{\underline{A}}$ 2 $\mu \underline{L}$ of **SOLUTION-11** and resuspend the cells very vigorously because of aggregates.



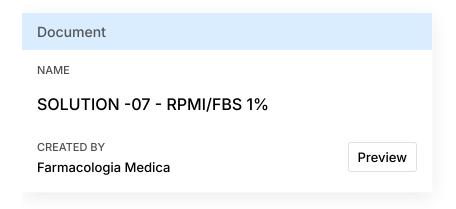
- Repeat steps 11-13 twice (in total 3 times) to wash the bead-bound CD4+ T cells. These steps are critical to obtain a high purity of isolated cells.

[The volume is calibrated for 10×10^6 cells, for lower or higher number of cell resize the volume accordingly].





- 16 Add Δ 10 μL of **DETACHaBEAD® CD4** for each 10×10⁶ PBMCs. (Resize this volume if the number of starting cell is different)
- 17 Add another Δ 500 μ L of **SOLUTION- 07** to increase the volume and transfer everything in a 1.5 mL eppendorf.

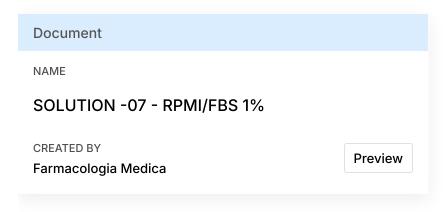


- Incubate 00:45:00 at Room temperature (RT) with gentle rotation by using a Sample Mixer.
- Transfer the sample from eppendorf to BD tube, and place the tube on magnetand wait for 1-2 mins, that is until the complex beads-cells is completely attached to the magnet.
- While the tube is still in the magnet, **transfer the supernatant** containing the released cells into a 15 mL conical tube.

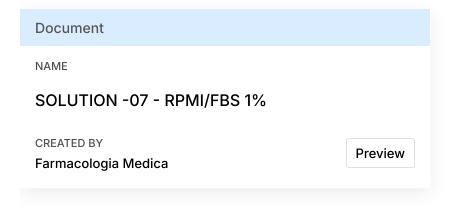




To obtain residual cells, wash the beads 3 times with Δ 500 μ L of **SOLUTION- 07** and collect the supernatant each time.



21 Add to the detached cell suspension **SOLUTION- 07** to a final volume of 4 5 mL and centrifuge at 1200 x g, Room temperature, 00:05:00

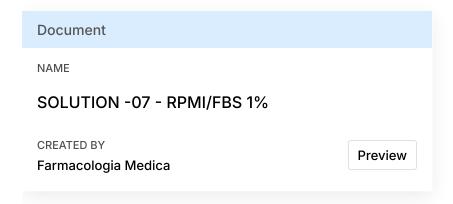






22 Resuspend the cells for cell counting in 4 1 mL of **SOLUTION- 07**: follow the appropriate protocol (see step 2 of this protocol).

Check the viability with Trypan blue.



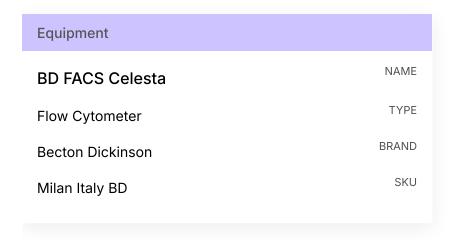


Document	
NAME SOLUTION- 09 - Trypan Blue solution	
CREATED BY Farmacologia Medica	Preview

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.



24 **EXPECTED RESULTS**



Expected result

Cell Viability: ≥95 %

Cell Yield: \pm 4,6 x10⁶ cells starting from 25 mL of Fresh Blood \pm 6 x10⁶ cells starting from 25 mL of Buffy Coat

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