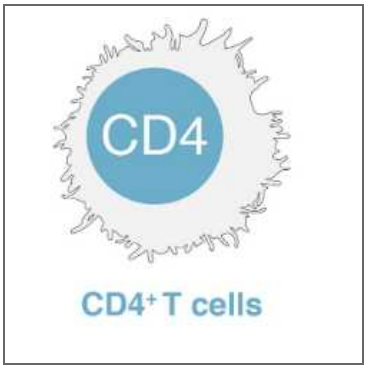

NOV 02, 2023

DOI:
dx.doi.org/10.17504/protocols.io.36wgq3mxolk5/v1

Protocol Citation: Anna Esman, Michael Vinokurov 2023. Two-step method for isolation of inactivated CD4+ T-cells from human blood mononuclear cells. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq3mxolk5/v1

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

Created: Oct 24, 2023

Two-step method for isolation of inactivated CD4+ T-cells from human blood mononuclear cells

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ABSTRACT

1. Obtaining human CD4+ T cells
2. Obtaining CD4+ inactivated cells

using

1. Dynabeads™ Untouched™ Human CD4 T Cells Kit **Thermofisher Catalog #11346D**
2. CELLection™ Biotin Binder Kit **Thermofisher Catalog #11533D**
3. CD71 (Transferrin Receptor) Monoclonal Antibody (OKT9 (OKT-9)) **eBioscience Catalog #14-0719-82**
4. CD25 Monoclonal Antibody (BC96), Biotin, eBioscience **eBioscience Catalog #13-0259-82**
5. HLA-DR Monoclonal Antibody (LN3), Biotin **eBioscience Catalog #13-9956-82**
6. CD69 Monoclonal Antibody (FN50), Biotin **eBioscience Catalog #13-0699-82**

and magnetic tube separator.

Last Modified: Nov 02, 2023

PROTOCOL integer ID: 89818

Keywords: CD4+ cells , magnetic separation , untouched human CD4+ T cells, CD4+ inactivated cells, CD4+ T-cells

Funders Acknowledgement:
Effect of DNA methylation in HIV-1 positive individuals on viral reservoir reactivation
Grant ID: 122053000056-2

MATERIALS

1. Dynabeads™ Untouched™ Human CD4 T Cells Kit **Thermofisher Catalog #11346D**
2. CELLection™ Biotin Binder Kit **Thermofisher Catalog #11533D**
3. CD71 (Transferrin Receptor) Monoclonal Antibody (OKT9 (OKT-9)) **eBioscience Catalog #14-0719-82**
4. CD25 Monoclonal Antibody (BC96), Biotin, eBioscience **eBioscience Catalog #13-0259-82**
5. HLA-DR Monoclonal Antibody (LN3), Biotin **eBioscience Catalog #13-9956-82**
6. CD69 Monoclonal Antibody (FN50), Biotin **eBioscience Catalog #13-0699-82**
7. Magnetic separator for 1.5 / 5 / 15 / 50 ml tubes
8. Mixer with tilt and rotation capabilities
9. PBS (Ca 2+ and Mg 2+ free)
   PBS, pH 7.4 **Thermo Fisher Catalog #10010023**
10. 0.1% BSA
11. [M] 2 millimolar (mM) EDTA
12. Fetal Bovine Serum **Gibco - Thermo Fischer Catalog #26140079**
13. RPMI-1640 **Pan-Eco Catalog #C310p**

PROTOCOL MATERIALS

PBS, pH 7.4 **Thermo Fisher Catalog #10010023** In Materials and 4 steps

Dynabeads™ Untouched™ Human CD4 T Cells Kit **Thermofisher Catalog #11346D**

In Materials, Abstract and 3 steps

CD25 Monoclonal Antibody (BC96), Biotin, eBioscience **eBioscience Catalog #13-0259-82**

In Materials, Abstract, Step 8.1

CD69 Monoclonal Antibody (FN50), Biotin **eBioscience Catalog #13-0699-82**

In Materials, Abstract, Step 8.1

Fetal Bovine Serum **Gibco - Thermo Fischer Catalog #26140079** Materials

CD71 (Transferrin Receptor) Monoclonal Antibody (OKT9 (OKT-9)) **eBioscience Catalog #14-0719-82**

In Materials, Abstract, Step 8.1

HLA-DR Monoclonal Antibody (LN3), Biotin **eBioscience Catalog #13-9956-82**

In Materials, Abstract, Step 8.1

CELLection™ Biotin Binder Kit **Thermofisher Catalog #11533D**

In Materials, Abstract

RPMI-1640 **Pan-Eco Catalog #C310p** Materials

BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube - Sodium Heparin **BD Biosciences Catalog #362753**

Before starting, Step 1.1

BEFORE START INSTRUCTIONS

Sample

BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube - Sodium Heparin **BD Biosciences Catalog #362753**

Negative depletion of CD4+ T-cells

1 **Preparation of PBMC** (peripheral blood mononuclear cells)

1.1 Collect at least 5 mL of human whole blood to the
BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube - Sodium Heparin **Pan-Eco Catalog #362753**

1.2 Store tube upright at Room temperature until centrifugation.

Note

To ensure proper barrier formation, blood samples should be centrifuged within 2 hours of blood collection. Centrifugation more than 2 hours after specimen collection may cause incomplete barrier formation.

1.3 1500-1800 rcf, 18-25°C in a horizontal rotor (swing-out head) for a minimum of 00:15:00 15m

1.4 Aspirate approximately half of the plasma without disturbing the cell layer.

Note

Mononuclear cells and platelets will be in a whitish layer just under the plasma layer

1.5 Collect cell layer with a Pasteur Pipette and transfer to a 15 mL size conical centrifuge tube
with cap.

Note

Collection of cells immediately following centrifugation will yield best results

Note

An alternative procedure for recovering the separated mononuclear cells is to resuspend the cells into the plasma by inverting the unopened BD Vacutainer® CPT™ Tube gently 5 to 10 times.
This is the preferred method for storing or transporting the separated sample for up to 24 hours after centrifugation. To collect the cells, open the BD Vacutainer® CPT™ Tube and
pipette the entire contents of the tube above the gel into a separate vessel.

Expected result

Separation of PBMC from whole blood.

2 Preparation of the *Isolation buffer*

PBS, pH 7.4 **Pan-Eco Catalog #10010023** supplemented with 0.1% BSA and [M] 2 millimolar (mM) EDTA.

3 Preparation of magnetic particles

3.1 Resuspend the 5m 30s
Dynabeads™ Untouched™ Human CD4 T Cells Kit **Pan-Eco Catalog #11346D** in the vial
(i.e. vortex for > 00:00:30 or tilt and rotate for 00:05:00)

3.2 Transfer the desired volume of
Dynabeads™ Untouched™ Human CD4 T Cells Kit **Pan-Eco Catalog #11346D** to a tube.

3.3 Add the same volume of *Isolation Buffer*, or at least 1 mL and resuspend.

3.4 Place the tube in a magnet for 00:01:00 and discard the supernatant. 1m

3.5 Remove the tube from the magnet and resuspend the washed Dynabeads™ Untouched™ Human CD4 T Cells Kit **Pan-Eco Catalog #11346D** in the same volume of Isolation Buffer as the initial volume of Dynabeads®.

4 Isolation Procedure

4.1 Transfer 100 µL (5×10^7) PBMC in *Isolation Buffer* to a tube.

4.2 Add 20 µL PBS, pH 7.4 **Pan-Eco Catalog #10010023**

4.3 Add 20 µL of Antibody Mix

> **Note**
>
> Contains mouse IgG antibodies towards human CD8, CD14, CD16 (specific for CD16a and
> CD16b), CD19, CD36, CD56, CDw123 and CD235a (Glycophorin A)

4.4 Mix well and incubate for 00:20:00 at 2-8 °C 20m

4.5 Wash the cells by adding 2 mL *Isolation Buffer*. Mix well by tilting the tube several times and 350 x g, 2-8°C, 00:08:00. Discard the supernatant. 8m

4.6 Resuspend the cells in 100 µL *Isolation Buffer*.

4.7 Add 100 µL pre-washed Dynabeads®.

4.8 Incubate for 00:15:00 at Room temperature with gentle tilting and rotation 15m

4.9 Add 1 mL *Isolation Buffer*.

> **Note**
>
> When working with lower cell volumes, never use less than 1 mL *Isolation Buffer*

4.10 Resuspend the bead-bound cells thoroughly by pipetting >10 times using a pipette with a narrow tip opening. Avoid foaming.

4.11 Place the tube in the magnet for 00:02:00. Transfer the supernatant containing the untouched human CD4+ T cells, to a new larger tube. 2m

4.12 Add 2 mL *Isolation Buffer* to the tube containing the Dynabeads® and resuspend the bead-bound cells by pipetting as described in step 4.10.

4.13 Place the tube in the magnet for 00:02:00 and then combine the two supernatants. 2m

Note

To remove residual beads; place the tube in the magnet for 00:02:00 and transfer cells to a new tube.

Expected result

The supernatant contains negatively isolated human CD4+ T-cells.

Negative depletion of inactivated CD4+ T-cells 46m 30s

5 Preparing buffers for operations

• *Buffer 1*: PBS, pH 7.4 **Pan-Eco Catalog #10010023** supplemented with 0.1% bovine serum albumin (BSA), pH 7.4

• *Buffer 2*: PBS, pH 7.4 **Pan-Eco Catalog #10010023** with 0.1% BSA and 0.6% sodium citrate or [M] 2 millimolar (mM) EDTA.

6 Prepare Release Buffer

1. For each vial of freeze-dried DNase I, transfer 300 µL from the Releasing Buffer Component
II to each tube of Releasing Buffer Component I (DNase I).

Note

Dissolve the enzyme gently. Vigorous mixing will destroy the enzyme.

2. Aliquot the reconstituted Release Buffer into suitable portions. Store at -20 °C. Thaw immediately before use and keep on ice once thawed. Thawed Release Buffer can be re-frozen once.

7 Wash Dynabeads®

7.1 Resuspend the Dynabeads® in the vial (i.e. vortex for > 00:00:30 or tilt and rotate for 00:05:00. Transfer the desired volume of Dynabeads® to a tube (25 µL for one sample). 5m 30s

7.2 Add the same volume of *Buffer 1*, or at least 1 mL and resuspend.

7.3 Place the tube in a magnet for 00:01:00 and discard the supernatant. 1m

7.4 Remove the tube from the magnet and resuspend the washed Dynabeads® in the same volume of *Buffer 1* as the initial volume of Dynabeads®.

8 Isolate Cells (Labeling Cells with Biotinylated Antibodies)

8.1 Add ~ 10 µg primary biotinylated antibody to 1 mL cell suspension and mix

- CD71 (Transferrin Receptor) Monoclonal Antibody (OKT9 (OKT-9)) **Pan-Eco Catalog #14-0719-82**
- CD25 Monoclonal Antibody (BC96), Biotin, eBioscience **Pan-Eco Catalog #13-0259-82**
- HLA-DR Monoclonal Antibody (LN3), Biotin **Pan-Eco Catalog #13-9956-82**
- CD69 Monoclonal Antibody (FN50), Biotin **Pan-Eco Catalog #13-0699-82**

8.2 Incubate for 00:10:00 at 2-8 °C 10m

8m

8.3 Wash the cells by adding 2 mL *Buffer 2* and 350 x g, 00:08:00. Discard the supernatant.

8.4 Suspend the cells in 4 mL *Buffer 2*.

8.5 Add 25 µL pre-washed and resuspended Dynabeads®

20m

8.6 Incubate for 00:20:00 at 2-8 °C with gentle tilting and rotation.

2m

8.7 Place the tube in the magnet for 00:02:00. Transfer the supernatant containing the inactivated human CD4+ T-cells to a new larger tube.

8.8 Add 4 mL *Buffer 2* to the tube containing the Dynabeads® and repeat step 8.7

8.9 Combine the two supernatants.

Expected result

The resulting supernatant contains the inactivated human CD4+ T cells.