Miltenyi Biotec

MACSxpress® Whole Blood Treg Isolation Kit
human

Order no. 130-109-557

Contents

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

1. Description

This product is for research use only.

Components	**3×1.5 mL MACSxpress® Whole Blood Treg Isolation Cocktail, human**: MACSxpress Beads conjugated to monoclonal antibodies and CD25 MicroBeads. **2×25 mL MACSxpress Buffer**
Capacity	For 3×30 mL whole blood.
Product format	MACSxpress Whole Blood Treg Isolation Cocktail is supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label. For information about reconstitution of the lyophilized cocktail refer to chapter 2.1.

1.1 Principle of the MACSxpress® Whole Blood Treg Isolation Kit

The MACSxpress® Whole Blood Treg Isolation Kit has been developed for the fast isolation of regulatory T (Treg) cells from freshly drawn anticoagulated whole blood without density gradient centrifugation. The isolation of $CD4^+CD25^+$ regulatory T cells is performed with only one labeling step and in a two-step separation procedure.

During the first MACSxpress isolation step erythrocytes are aggregated and sedimented, while non-$CD4^+$ and the majority of $CD127^{hi}$ cells are removed by immunomagnetic depletion with MACSxpress Beads. In a secondary enrichment step $CD25^+$ cells are magnetically sorted over a MACS® Column. The eluted cell fraction represents the $CD4^+CD25^+$ Treg cells, which can be immediately used for further downstream analysis.

▲ **Note:** As the majority of $CD127^{hi}$-expressing cells are removed during Treg cell isolation, the final flow-through fraction is not suited for use as responder cells in an *in vitro* suppression assay.

1.2 Background information

Regulatory $CD4^+$ T cells are suppressor cells that neutralize other immune cells by various mechanisms.[1] Their characteristic marker is the transcription factor FoxP3. $CD4^+CD25^+$ regulatory T cells were originally discovered in mice, but a population with identical phenotype has also been identified in humans.[2–6] CD25 is the interleukin-2–receptor α-chain which is not only expressed by regulatory T cells but also by activated effector T cells.

1.3 Applications

- Isolation of $CD4^+CD25^+$ regulatory T cells from whole blood without density gradient centrifugation for further phenotypical or functional characterization.

1.4 Reagent and instrument requirements

- Separation Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- MACS Columns and MACS Separators: The subsequent positive selection of $CD4^+CD25^+$ T cells can be performed with the use of LS Columns. Positive selection can also be performed by using the autoMACS Pro Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
autoMACS	2×10^8	4×10^9	autoMACS Pro

- 5 mL polystyrene round-bottom test tube or 15 mL or 50 mL tubes

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- LS Columns (# 130-042-401)
- MACSxpress® Separator (# 130-098-308)
- MidiMACS™ Separator (# 130-042-302) or QuadroMACS™ Separator (# 130-090-976)
- MACSmix™ Tube Rotator (# 130-090-753)
- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD45-VioBlue®, CD25-PE, CD4-FITC, and CD127-APC. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.

  ▲ **Note:** It is strongly recommended to include a CD45 staining in the analysis to distinguish between white blood cells and residual erythrocytes.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells.

2. Protocol

▲ EDTA as anticoagulant is recommended. Use of other anticoagulants, e.g., heparin or sodium citrate may decrease the yield of target cells.

▲ Bring all reagents and materials to room temperature (19–25 °C) before use.

▲ Pipette gently to avoid foam formation.

▲ (Optional) For the evaluation of purity and recovery of the target cell fraction, take aliquots where indicated in the protocol.

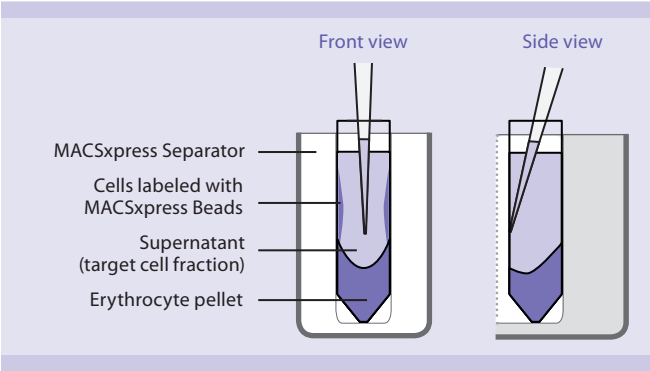


Figure 1: Front and side view of the MACSxpress Separator containing a separated blood sample in a 50 mL tube.

2.1 Protocol overview

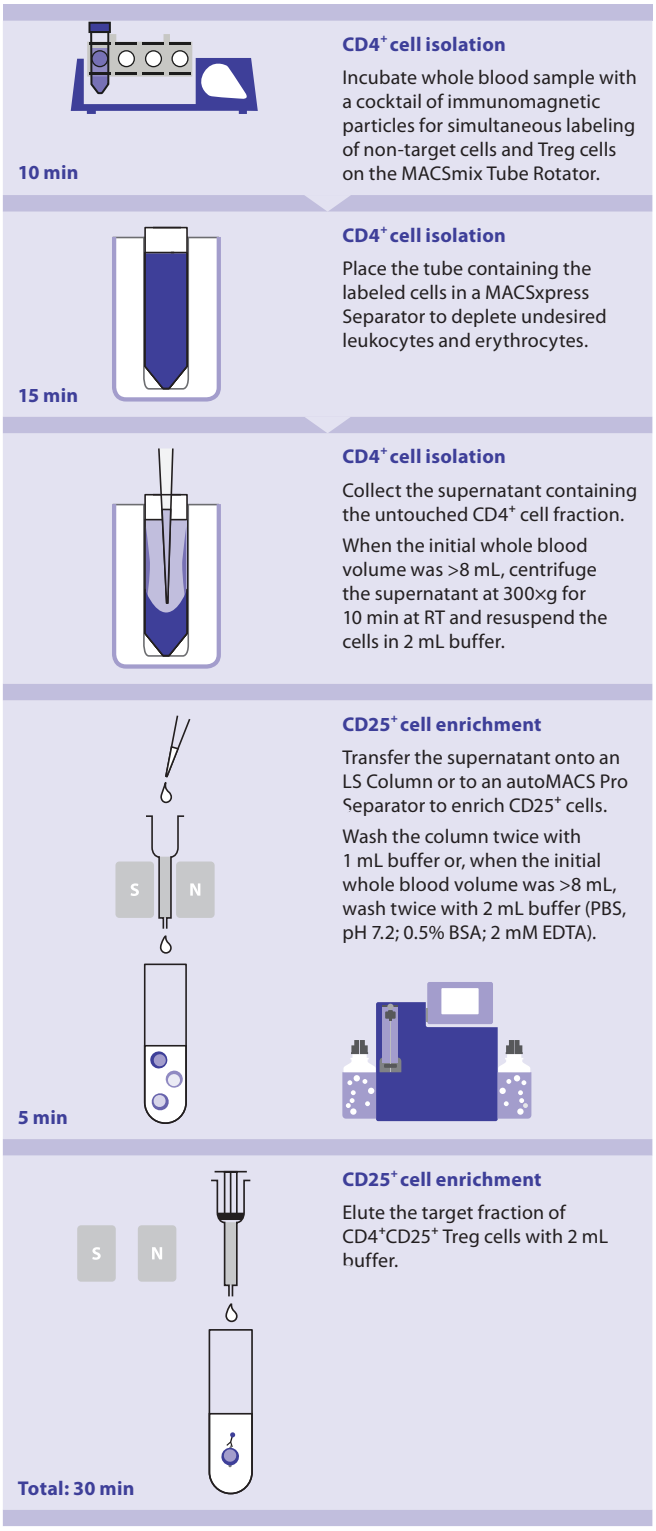


Figure 2: Isolation of CD4$^+$CD25$^+$ Treg cells from whole blood.

2.2 Magnetic separation

▲ Reagent volumes for magnetic labeling given below are for 8 or 30 mL of whole blood. When working with other volumes, scale the reagent volumes accordingly, e.g., use **500 µL** of MACSxpress® Buffer and **50 µL** of MACSxpress Whole Blood Treg Isolation Cocktail for each mL of whole blood, and consult the table below for the appropriate tube size.

Whole blood sample volume	Tube size
1–3 mL	5 mL tube
>3–8 mL	15 mL tube
>8–30 mL	50 mL tube

2.2.1 Sample volume 1–8 mL

1. (Optional) Take an aliquot of whole blood for cell counting and staining, to determine target cell frequency in the starting material (refer to section 2.4).
2. Pipette 8 mL of anticoagulated whole blood into a 15 mL tube.
3. Add 4 mL of MACSxpress Buffer to the whole blood.

   ▲ **Note:** Use 500 µL of MACSxpress Buffer and 50 µL of MACSxpress Whole Blood Treg Isolation Cocktail per mL whole blood.
4. Resuspend MACSxpress Whole Blood Treg Isolation Cocktail thoroughly before use to obtain a homogeneous dispersion of particles in solution.
5. Add 400 µL of MACSxpress Whole Blood Treg Isolation Cocktail to the whole blood.
6. Close the tube tightly and invert gently three times. Incubate sample for 10 minutes at room temperature using the MACSmix™ Tube Rotator on permanent run speed of 12 rpm.

   ▲ **Note:** If another rotator is used, make sure it supports overhead mixing of tubes and adjust rotation speed.
7. Remove the tube containing the sample from the MACSmix Tube Rotator and carefully open the cap.
8. Place the open tube in the magnetic field of the MACSxpress Separator for 15 minutes. The magnetically labeled cells will adhere to the wall of the tube while the aggregated erythrocytes sediment to the bottom.

   ▲ **Note:** Do not move the tube during the separation process.
9. Prepare LS Column by rinsing with 2 mL of Separation Buffer. Discard effluent and change collection tube. For details see the LS Column data sheet.
10. While the tube is still inside the MACSxpress Separator, carefully collect the supernatant and apply instantly onto the prepared LS Column or to the autoMACS® Pro Separator (refer to 2.3). When using LS Column: Collect flow-through containing unlabeled cells.

    ▲ **Note:** For optimal recoveries, collect supernatant by moving the pipette tip top-to-bottom down the front wall of the tube (fig. 1). The supernatant contains the $CD4^+$ cell fraction.

    ▲ **Note:** Leave a residual volume of supernatant (approximately 1–2 mm above erythrocyte pellet) to avoid unintended aspiration of erythrocytes.
11. Wash column with 2×1 mL of Separation Buffer. Collect unlabeled cells that pass through and combine with the effluent from step 10.

    ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
12. Remove column from the separator and place it on a suitable collection tube. Pipette 2 mL of Separation Buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. The eluted fraction represents $CD4^+CD25^+$ Treg cells.

2.2.2 Sample volume >8–30 mL

1. (Optional) Take an aliquot of whole blood for cell counting and staining, to determine target cell frequency in the starting material.
2. Pipette 30 mL of anticoagulated whole blood into a 50 mL tube.

   ▲ **Note:** Use 500 µL of MACSxpress Buffer and 50 µL of MACSxpress Whole Blood Treg Isolation Cocktail per mL whole blood.
3. Add 15 mL of MACSxpress Buffer to the whole blood.
4. Resuspend MACSxpress Whole Blood Treg Isolation Cocktail thoroughly before use to obtain a homogeneous dispersion of particles in solution.
5. Add 1500 µL of MACSxpress Whole Blood Treg Isolation Cocktail to the whole blood.
6. Close the tube tightly and invert gently three times. Incubate sample for 10 minutes at room temperature using the MACSmix Tube Rotator on permanent run speed of 12 rpm.

   ▲ **Note:** If another rotator is used, make sure it supports overhead mixing of tubes and adjust rotation speed.
7. Remove the tube containing the sample from the MACSmix Tube Rotator and carefully open the cap.
8. Place the open tube in the magnetic field of the MACSxpress Separator for 15 minutes. The magnetically labeled cells will adhere to the wall of the tube while the aggregated erythrocytes sediment to the bottom.

   ▲ **Note:** Do not move the tube during the separation process.
9. While the tube is still inside the MACSxpress Separator, carefully collect the supernatant into a new tube.

   ▲ **Note:** For optimal recoveries, collect supernatant by moving the pipette tip top-to-bottom down the front wall of the tube (fig. 1). The supernatant contains the $CD4^+$ cell fraction.

   ▲ **Note:** Leave a residual volume of supernatant (approximately 1–2 mm above erythrocyte pellet) to avoid unintended aspiration of erythrocytes.
10. Centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
11. Prepare LS Column by rinsing with 2 mL of Separation Buffer. Discard effluent and change collection tube. For details refer to the LS Column data sheet.
12. Resuspend cell pellet in 2 mL of Separation Buffer.
13. Apply resuspended cells onto the prepared LS Column or to the autoMACS® Pro Separator (refer to 2.3). When using LS Column: Collect flow-through containing unlabeled cells.
14. Wash column with 2×2 mL of Separation Buffer. Collect unlabeled cells that pass through and combine with the effluent from step 13.

    ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
15. Remove column from the separator and place it on a suitable collection tube. Pipette 2 mL of Separation Buffer onto the column. Immediately flush out the magnetically labeled cells

by firmly pushing the plunger into the column. The eluted fraction represents $CD4^+CD25^+$ Treg cells.

2.3 Separation with the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

1. Prepare and prime the instrument.
2. Apply tube containing the supernatant (from 2.2.1., step 10 or 2.2.2, step 13) and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:

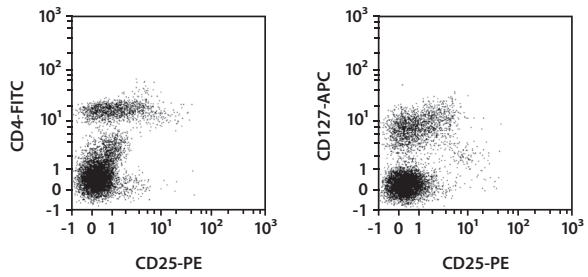
   Positive selection: possels
   Collect positive fraction in row C of the tube rack, representing $CD4^+CD25^+$ Treg cells.

3. Example of a separation using the MACSxpress® Whole Blood Treg Isolation Kit

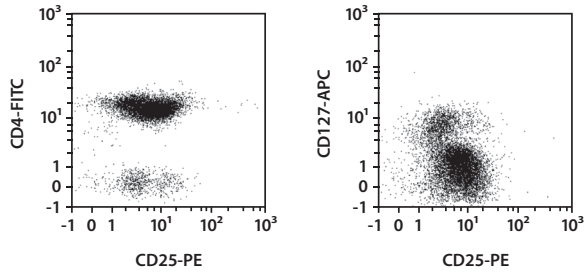
$CD4^+CD25^+$ regulatory T cells were isolated from 8 mL of human EDTA-anticoagulated whole blood using the MACSxpress® Whole Blood Treg Isolation Kit, an LS Column, an overhead rotator, a MidiMACS™ Separator, and a MACSxpress Separator.
An aliquot of the original whole blood was taken and red blood cells were lysed. Cells taken before and after separation were fluorescently stained with CD45-VioBlue®, CD4-FITC, CD25-PE, and CD127-APC and were analyzed by flow cytometry using the MACSQuant® Analyzer 10. Cell debris, non-leukocytes, and dead cells were excluded from the analysis based on CD45 expression, scatter signals, and propidium iodide fluorescence.

▲ **Note:** For optimal staining intensity of magnetically labeled $CD25^+$ cells the recommended antibody dilution is 1:6 for up to 10^7 cells/100 µL of buffer.

Before separation



After separation



4. References

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Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.

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