Miltenyi Biotec

Treg Expansion Kit
mouse

Order no. 130-095-925

Contents

1. Description

Components	2 mL CD3/CD28 MACSiBead™ Particles, cell culture grade, corresponding to 1.2×10^8 MACSiBead Particles pre-loaded with CD3 and CD28 antibodies.
Product format	All components are supplied in azide-free buffer; MACSiBead Particles contain stabilizer.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Principle of the Treg Expansion Kit

The Treg Expansion Kit, mouse is based on MACSiBead Particles pre-loaded with CD3 and CD28 antibodies. Best expansion of Treg cells is accomplished by using pre-loaded MACSiBead Particles and Treg cells at a bead-to-cell ratio of 3:1 and recombinant interleukin 2 (rIL-2) with a concentration of 2000 U/mL.

1.2 Background information

Regulatory T cells (Treg cells) have been described to be hypoproliferative in reponse to polyclonal stimulation and interleukin 2 (IL-2) *in vitro*. Thus, *in vitro* expansion of Treg cells often results in low expansion rates or low frequencies of $FoxP3^+$ Treg cells due to overgrowth by conventional T cells or loss of FoxP3 expression.

The kit is designed to efficiently expand Treg cells and to maintain FoxP3 expression after isolation with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit from single-cell suspensions of mouse spleen.

Expanded Treg cells can be used for any downstream application, such as adoptive Treg cell transfer, cytokine analysis, gene expression analysis, and suppression assays.

MACSiBead Particles show no autofluorescence and normally do not need to be removed prior to flow cytometric analysis. However, if desired, removal of MACSiBead Particles is easily achieved by using the MACSiMAG™ Separator (refer to 2.5)

1.3 Applications

- Expansion of mouse Treg cells after isolation with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse.

1.4 Reagent and instrument requirements

- Medium: RPMI 1640 with stable glutamine (# 130-091-439) supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 10 mM HEPES, and 2000 U/mL rIL-2.

  ▲ **Note:** 2-Mercaptoethanol (0.01 mM) can be added to preserve cell viability in case of rapid cell growth.

- (Optional) $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse (# 130-091-041).
- Recombinant interleukin 2 (rIL-2), e.g., Mouse IL-2 (5 µg, # 130-094-054).
- Humidified incubator.
- 96-well flat bottom plates.
- (Optional) MACSiMAG Separator (# 130-092-168) for removal of MACSiBead Particles after Treg cell expansion prior to downstream experiments.

  ▲ **Note:** Do not remove MACSiBead Particles by using MACS® Columns and MiniMACS™, MidiMACS™, VarioMACS™, SuperMACS™, autoMACS® or autoMACS Pro Separators.

- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis of Treg cells, for example, the Treg Detection Kit (CD4/CD25/FoxP3) (APC) (# 130-094-164) including CD4 and CD25 antibodies, FoxP3 antibodies, FoxP3 Staining Buffer Set, and an optimized protocol. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.

2. Protocol

▲ All steps in the protocol have to be performed under sterile conditions.

▲ The protocol is intended for the stimulation of 1×10^6 Treg cells.

2.1 Sample preparation

When working with lymphoid organs, non-lymphoid tissues, or peripheral blood, prepare a single-cell suspension using manual methods or the gentleMACS™ Dissociator.

For details refer to the protocols section at www.miltenyibiotec.com/protocols.

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2.2 Preparation of CD3/CD28 MACSiBead™ Particles

1. Resuspend CD3/CD28 MACSiBead™ Particles thoroughly and transfer 50 µL to a suitable tube.

   ▲ **Note:** The concentration of the Treg Expansion Kit is 6×10^7 MACSiBead Particles per mL.

2. Add 300–600 µL of culture medium and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
3. Resuspend CD3/CD28 MACSiBead Particles in 500 µL medium (concentration of 6×10^6 beads/mL). The reagent is ready to use.

2.3 Preparation of cells and expansion

▲ Start with Treg cells isolated with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse. For details concerning Treg isolation refer to the respective data sheet.

1. Determine the concentration and the total number of Treg cells. 1×10^5 cells per well are needed.
2. Transfer required volumes of cell suspension to suitable tubes.
3. Add 5–10 volumes culture medium to the cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
4. Resuspend cells at a concentration of 2×10^6 cells/mL of medium including 2000 U/mL rIL-2. Pipette 50 µL in a well of a 96-well flat bottom plate (day 0).
5. Add 50 µL of CD3/CD28 MACSiBead Particles in every well.
6. At day 1, add 100 µL media including 2000 U/mL rIL-2.
7. At day 3–5, according to media usage either split cells or aspirate 100 µL medium and add 100 µL medium including 2000 U/mL rIL-2.
8. For restimulation proceed to 2.4.

2.4 Restimulation of Treg cells

▲ For restimulation of Treg cells after 6–7 days of culture, CD3/CD28 MACSiBead Particles may be removed. Please refer to 2.5 Removal of MACSiBead Particles.

▲ **Note:** Restimulation is performed with a bead-to-cell ratio of 1:1.

Please follow steps 1–8 in section 2.3 Preparation of cells and expansion. Restimulation may also be performed in 96-well plates with a ratio of one CD3/CD28 MACSiBead Particle per Treg cell.

2.5 Removal of MACSiBead™ Particles

▲ Removal of MACSiBead™ Particles may be required before magnetic separation of cells with MACS MicroBeads or before restimulation with different agents or antigens.

1. Harvest cells and transfer to a 5 mL, 15 mL, or 50 mL tube and wash once with buffer.
2. Resuspend cells in buffer at a density of up to 2×10^7 cells per 1 mL and vortex thoroughly.
3. Place the tube in the magnetic field of the MACSiMAG Separator.

   ▲ **Note:** Use tube rack to insert 5 mL tube into the magnetic field of the separator. For details see MACSiMAG Separator data sheet.

4. Allow the MACSiBead Particles to adhere to the wall of the tube:

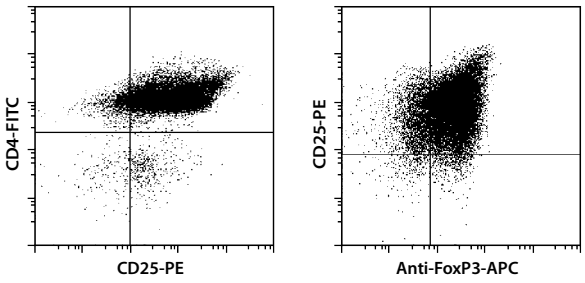
5 mL tubes:	2 minutes
15 mL or 50 mL tubes:	4 minutes

5. Retaining the tube in the magnet, carefully remove the supernatant containing the MACSiBead-depleted cells and place in a new tube.
6. Remove the tube from the separator and add buffer to the same volume as before.
7. Vortex sample, replace tube in the MACSiMAG Separator and repeat steps 4–5.
8. Collected cells can now be further processed as required.

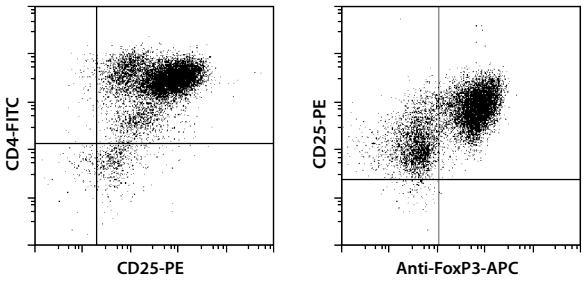
3. Examples of Treg cell expansion using the Treg Expansion Kit

A single-cell suspension from mouse spleen was prepared using the program m_spleen_01 on the gentleMACS Dissociator.
Mouse Treg cells were isolated from this single-cell suspension using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit and expanded for 13 days with the Treg Expansion Kit according to the protocol. Treg cells before (A) and after expansion (B) were fluorescently stained with the Treg Detection Kit (CD4/CD25/FoxP3) (APC) (# 130-094-164) and analyzed by flow cytometry using the MACSQuant® Analyzer. Expansion of Treg cells (C) was calculated from FoxP3-expressing cells on day 0, 7, and day 13, respectively.

A) Treg cells before expansion



B) Expanded Treg cells



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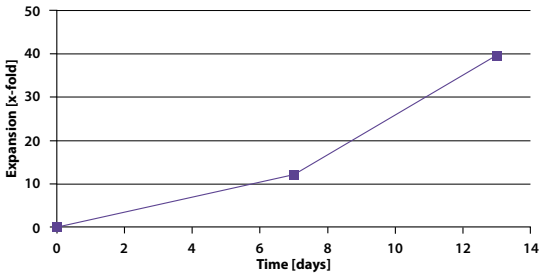
C) Expansion of Treg cells



All protocols and data sheets are available at **www.miltenyibiotec.com**.

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