Miltenyi Biotec

Treg Expansion Kit
human

2 mL	130-095-345
2×2 mL	130-095-353

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1. Description

This product is for research use only.

Components	2 mL CD3/CD28 MACSiBead™ Particles, cell culture grade, corresponding to 4×10^7 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 MACS® Separation

The Treg Expansion Kit 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 4:1 and recombinant interleukin 2 (rIL-2) with a concentration of 500 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 $CD4^+CD25^+CD127^{dim/-}$ or $CD4^+CD25^+CD45RA^+$ Regulatory T Cell Isolation Kits from human blood or peripheral blood mononuclear cells (PBMCs).[1] The kit is also suitable for usage with Rapamycin.[2]

Expanded Treg cells can be used for any downstream application such as cytokine analysis, gene expression, 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 human Treg cells after isolation with the $CD4^+CD25^+CD127^{dim/-}$ or $CD4^+CD25^+CD45RA^+$ Regulatory T Cell Isolation Kits from human blood or PBMCs.

1.4 Reagent and instrument requirements

- Culture medium: TexMACS™ Medium (# 130-097-196) supplemented with 500 U/mL rIL-2 and 5% AB serum.

  ▲ **Note:** 2-Mercaptoethanol (0.01 mM) can be added to preserve cell viability in case of rapid cell growth.
- (Optional) $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, human (# 130-094-775) or $CD4^+CD25^+CD45RA^+$ (# 130-093-631) Regulatory T Cell Isolation Kit, human.
- Recombinant interleukin 2 (rIL-2), e.g., Human IL-2 IS, premium grade (# 130-097-744).
- Human AB serum.
- Humidified incubator.
- 96-well round-bottom plates.
- 24-well plate.
- (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, CD4 (VIT4)-FITC, CD25-APC, CD127-PE, or the Treg Detection Kit (PE) (# 130-094-163) including CD4, CD25, and FoxP3 antibodies and FoxP3 Staining Buffer Set, FcR Blocking Reagent, and an optimized protocol. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Treg Suppression Inspector, human (# 130-092-909).
- (Optional) Rapamycin.

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2. Protocol

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

2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues or lysed blood, prepare a single-cell suspension using standard methods.

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

▲ Dead cells may bind non-specifically to MACS® MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).

2.2 Preparation of CD3/CD28 MACSiBead™ Particles

1. Resuspend CD3/CD28 MACSiBead Particles thoroughly and transfer 200 µL to a suitable tube.
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 200 µL medium (concentration of 2×10^7 beads/mL). The reagent is ready to use.

   ▲ **Note:** The concentration of the Treg Expansion Kit is 2×10^7 MACSiBead Particles per mL.

2.3 Preparation of cells and expansion

▲ Start with Treg cells isolated either with the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II or the $CD4^+CD25^+CD45RA^+$ Regulatory T Cell Isolation Kit. 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 1×10^6 cells/mL of medium including 500 U/mL rIL-2. Pipette 100 µL in a well of a 96-well round-bottom plate (day 0).
5. Add 20 µL of CD3/CD28 MACSiBead Particles in every well.
6. At day 1, add 100 µL media including 500 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 500 U/mL rIL-2.
8. At day 5–8, transfer Treg cells into a 24-well plate or split accordingly in a 96-well plate.
9. At day 14, count cells and stain cells using the Treg Detection Kit.
10. For restimulation proceed to 2.4.

2.4 Restimulation of Treg cells

▲ For restimulation of Treg cells after 14 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 4:1.

Please follow steps 1–9 in section 2.3 Preparation of cells and expansion. Restimulation may also be performed in 24-well plates in higher volumes, for example, 5×10^5 Treg cells in 500 µL medium and 100 µL CD3/CD28 MACSiBead Particles.

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.

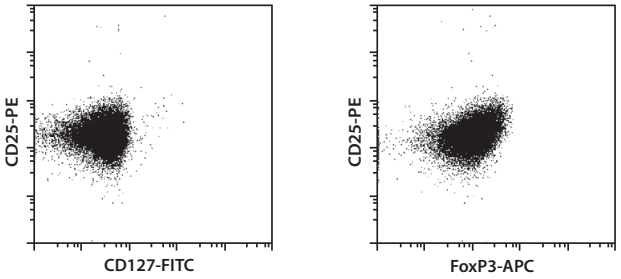
   ▲ **Note:** Use tube rack to insert 5 mL tube into the magnetic field of the separator. For details see MACSiMAG Separator data sheet.
4. Allow the MACSiBead Particles to adhere to the wall of the tube:

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.

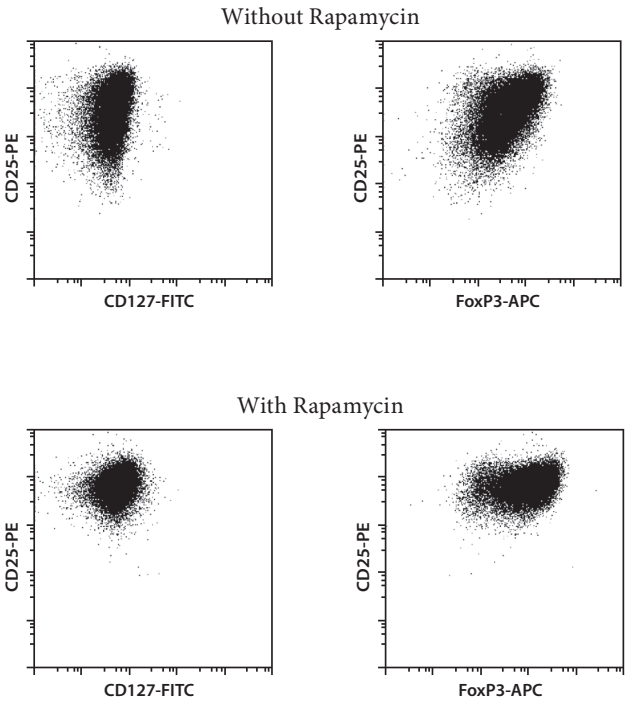
3. Examples of Treg cell expansion using the Treg Expansion Kit

Treg cells were isolated from human peripheral blood mononuclear cells (PBMCs) by using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II and expanded with the Treg Expansion Kit according to the protocol. Treg cells before (A) and after expansion (B) were fluorescently stained with CD4 (VIT4)-VioBlue®, CD25-PE, CD127-FITC, and Anti-FoxP3-APC and analyzed by flow cytometry by using the MACSQuant® Analyzer. Gating was performed according to CD4-expression. Expansion rate (C) of Treg cells were calculated from FoxP3-expressing cells before and after expansion.

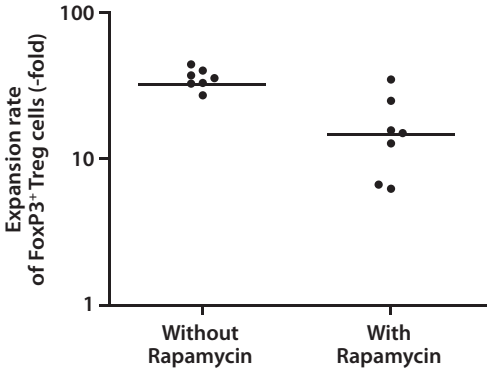
A) Isolated Treg cells



B) Expanded Treg cells



C) Treg cell expansion rates



4. References

1. Hoffmann, P. *et al.* (2006) Only the $CD45RA^+$ subpopulation of $CD4^+CD25^{high}$ T cells gives rise to homogeneous regulatory T-cell lines upon *in vitro* expansion. Blood 108: 4260–4267.
2. Battaglia, M. *et al.* (2005) Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. Blood 105: 4743–4748.

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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