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1. Description

Components	1 mL monoclonal Anti-LAP (TGF- β 1) antibodies, human conjugated to various dyes.
	PE 130-096-666
	APC 130-096-573
	Biotin 130-096-735
Clone	CH6-17E5.1 (isotype: mouse IgG1).
Capacity	100 tests or up to 10 ⁹ total cells.
Product format	Antibodies are 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.

1.1 Background information

Transforming growth factor β 1 (TGF- β 1) belongs to a family of homologous, disulfide-linked, homodimeric proteins. These highly pleiotropic cytokines inhibit proliferation of most cells, but can promote the growth of mesenchymal cells and enhance extracellular matrix formation. The pivotal function of TGF- β 1 in the immune system is to mediate immunosuppression and maintain tolerance by regulating lymphocyte proliferation, differentiation, and survival. TGF- β 1 is produced by many cell types, but is reported to be most abundant in mammalian platelets and bone. It is secreted predominantly as an inactive latent complex. After proteolytical processing of the TGF- β 1 precursor, the resulting N-terminal latency-associated peptide (LAP) remains non-covalently associated with the TGF- β 1 dimer. Mature and biologically active TGF- β 1 can be released from the complex by action of proteases and/or conformational changes. LAP-TGF- β 1 was recently described to be expressed on the cell

surface of activated human regulatory T cells.^{1,2} Clone CH6-17E5.1 reacts with LAP.

1.2 Applications

- Identification and enumeration of LAP (TGF- β 1)⁺ cells by flow cytometry.

1.3 Recommended antibody dilution

The recommended antibody dilution for all Anti-LAP (TGF- β 1) conjugates is **1:11 for up to 10⁷ cells/100 μ L** of buffer for labeling of cells and analysis by flow cytometry.

Cells should be stained prior to fixation, if formaldehyde is used as a fixative.

1.4 Reagent requirements

- Medium: X-VIVO® 15 supplemented with 5% human AB serum.
 - ▲ **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, human (# 130-091-301), or CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II, human (# 130-094-775).
- Treg Expansion Kit, human (# 130-095-345) for *in vitro* stimulation of regulatory T cells with CD3/CD28 MACSiBead™ Particles.
- Humidified incubator.
- 48-well plate.
- (Optional) MACSiMAG™ Separator (# 130-092-168) for removal of MACSiBead Particles after cell stimulation prior to downstream experiments.
 - ▲ **Note:** Do not remove MACSiBead Particles by using MACS® Columns and MiniMACS™, MidiMACS™, VarioMACS™, SuperMACS™, autoMACS® or autoMACS Pro Separators.
- 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).
 - ▲ **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 Ca²⁺ or Mg²⁺ are not recommended for use.
- FcR Blocking Reagent, human (# 130-059-901) to avoid Fc receptor-mediated antibody labeling.
- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis of the cells, for example, CD4 (VIT4)-FITC, human (# 130-092-358), Anti-FoxP3-PE, human and mouse (# 130-093-014), Anti-FoxP3-APC, human and mouse (# 130-093-013), and FoxP3 Staining Buffer Set (# 130-093-142). For more information about antibodies refer to www.miltenyibiotec.com/antibodies.

- (Optional) Anti-Biotin antibodies conjugated to, e.g., PE (# 130-090-756) as secondary antibody reagent in combination with Anti-LAP (TGF- β 1)-Biotin.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells without fixation.

2. Protocols

2.1 Sample preparation

▲ All steps have to be performed under sterile conditions.

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

▲ All steps have to be performed under sterile conditions.

1. Resuspend CD3/CD28 MACSiBead™ Particles thoroughly and transfer 200 μ L to a suitable tube.
2. Add 300–600 μ L of culture medium and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
3. Resuspend CD3/CD28 MACSiBead Particles in 200 μ L 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 stimulation

▲ All steps have to be performed under sterile conditions.

▲ Start with PBMC or Treg cells isolated with the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit or the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II. For details concerning Treg isolation refer to the respective data sheet.

▲ Include a negative control in the experiment. The sample should be treated exactly the same as the stimulated sample, except for the addition of CD3/CD28 MACSiBead Particles.

1. Determine the concentration and the total number of cells. 1×10^6 cells per cm^2 and 300 μ L are needed for the stimulation.
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 1×10^7 cells/mL of culture medium and pipette cells into an appropriate culture vessel, e.g. for stimulation of 1×10^6 cells pipette 100 μ L in a well of a 48-well-plate (day 0).

5. Add 200 μ L of CD3/CD28 MACSiBead Particles in every well.
6. Incubate at 37 °C, 5–10% CO₂ for 2 days.
▲ **Note:** Inspect cultures daily and add fresh medium if required.
7. (Optional) For removal of the MACSiBead Particles, proceed to 2.4. MACSiBead Particles show no autofluorescence and normally do not need to be removed prior to flow cytometric analysis (refer to 2.4). For immunofluorescent staining proceed to 2.5.

2.4 (Optional) Removal of MACSiBead™ Particles

▲ Removal of MACSiBead™ Particles may be required before magnetic separation of cells with MACS MicroBeads.

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 refer to the 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.

2.5 General protocol for immunofluorescent staining

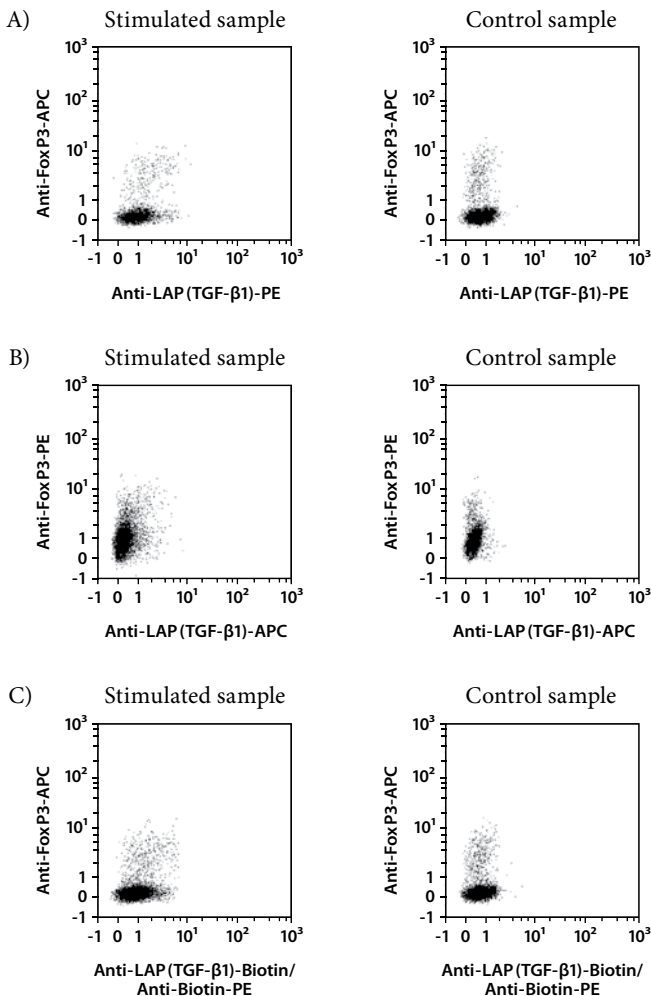
▲ It is recommended to stain 10^6 cells per sample. When working with up to 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^7 nucleated cells per 70 μ L of buffer.
4. Add 20 μ L FcR Blocking Reagent, human.
5. Add 10 μ L of the Anti-LAP (TGF- β 1) antibody.
6. (Optional) Add additional staining antibodies to the solution, for example CD4-FITC (# 130-080-501).
7. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
▲ **Note:** Higher temperatures and/or longer incubation times may lead to nonspecific cell labeling. Working on ice requires increased incubation times.
8. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

9. (Optional) If Anti-LAP (TGF- β 1)-Biotin was used, resuspend the cell pellet in 100 μ L of buffer, add 10 μ L of anti-biotin antibody, and continue as described in steps 7 and 8.
10. (Optional) For staining of FoxP3 refer to the respective data sheet.
11. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.

3. Examples of immunofluorescent staining with Anti-LAP (TGF- β 1) antibodies

Human PBMCs were incubated with and without Treg Expansion CD3/CD28 MACSiBead Particles for 48 hours. The cells were stained with Anti-LAP (TGF- β 1) antibodies conjugated to PE (A) and APC (B) as well as with CD4 (VIT4)-FITC (# 130-092-358). Cells labeled with Anti-LAP (TGF- β 1)-Biotin (C) were stained with Anti-Biotin-PE (# 130-090-756) as well as with CD4 (VIT4)-FITC. LAP-TGF- β 1-staining was controlled by incubating cells with unconjugated Anti-LAP (TGF- β 1) antibodies prior to labeling with fluorochrome-conjugated antibodies. After that the cells were fixed, permeabilized, and intracellularly stained with Anti-FoxP3-APC (# 130-093-013) or Anti-FoxP3-PE (# 130-093-014) using the FoxP3 Staining Buffer Set and analyzed by flow cytometry using the MACSQuant[®] Analyzer. Gating was performed according to the CD4 expression and side scatter properties of the cells. Autofluorescent cell debris was excluded from the analysis in a fluorescence 3 versus fluorescence 4 dot plot.



4. References

1. Tran, D.Q. *et al.* (2009) Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3⁺ regulatory T cells allows for their purification from expansion cultures. *Blood* 113: 5125–5133.
2. Tran, D.Q. *et al.* (2009) GARP (LRRC32) is essential for the surface expression of latent TGF- β on platelets and activated FOXP3⁺ regulatory T cells. *Proc. Nat. Acad. Sci.* 106: 13445–13450.

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

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.

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