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1. Description

Components	<p>1 mL Anti-IL-17A antibodies, mouse conjugated to various dyes.</p> <table border="0"> <tr> <td>FITC</td> <td>130-094-298</td> </tr> <tr> <td>PE</td> <td>130-094-296</td> </tr> <tr> <td>APC</td> <td>130-094-294</td> </tr> </table> <p>or</p> <p>0.5 mL Anti-IL-17A antibodies, mouse pure – functional grade 130-095-732</p>	FITC	130-094-298	PE	130-094-296	APC	130-094-294
FITC	130-094-298						
PE	130-094-296						
APC	130-094-294						
Clone	TC11-18H10 (isotype: rat IgG1).						
Capacity	<p>100 tests or up to 10⁹ total cells.</p> <p>The functional grade antibody is supplied at a concentration of 1 mg/mL.</p>						
Product format	<p>Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.</p> <p>Functional grade antibodies are supplied in phosphate-buffered saline (PBS), pH 7.2. Endotoxin levels have been tested and do not exceed 0.01 ng/μg of protein.</p> <p><i>The functional grade product contains no preservative and is sterile filtered; always handle under aseptic conditions.</i></p>						
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

The Anti-IL-17A antibodies are designed for intracellular staining of IL-17A-producing mouse cells.

Interleukin 17 (IL-17A–E) is a family of cytokines that play a central role in adaptive immunity as well as autoinflammatory disorders.¹ Data from several mouse models suggest that IL-17A plays a key role in the host defense against certain extracellular bacterial infections.

A mouse T helper cell subset has been identified, which is distinct from Th1 and Th2 cells: IL-17A-producing CD4⁺ T helper (Th17) cells. Th17 cells preferentially produce IL-17A, IL-17F, IL-21, and IL-22. Receptors for IL-17A and IL-22 are expressed on various epithelial tissues, thus Th17 cells are crucial for the cross-talk between immune system and tissues.² Th17 cells are involved in autoimmune inflammation.

IL-17A is not only secreted by CD4⁺ T cells, but also by CD8⁺ T cells, γδ T cells, NK cells, and granulocytes.¹

1.2 Applications

- Identification and enumeration of IL-17A-producing cells by flow cytometry or fluorescence microscopy.
- Identification and enumeration of IL-17A-producing antigen-specific T cells upon restimulation with the respective antigen or detection of IL-17A-producing T cells upon polyclonal restimulation.
- Analysis of cytokine production of rare cells by solid-phase staining technology, in combination with magnetic enrichment of cells (see protocol 2.3.2)
- The Anti-IL-17A antibody pure - functional grade is suited for functional assays, e.g. neutralization of IL-17A activity.

1.3 Recommended antibody dilution

- Anti-IL-17A antibodies conjugated to FITC, PE, or APC should be used at a dilution of 1:10.

1.4 Reagent requirements

- 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 mouse serum albumin, mouse serum, or fetal bovine serum. Buffers or media containing Ca²⁺ or Mg²⁺ are not recommended for use.
- Culture medium, e.g., RPMI 1640 (# 130-091-440) containing 5% mouse serum (do not use BSA or FCS because of non-specific stimulation!).
- Reagents for T cell stimulation, such as staphylococcal enterotoxin B (SEB), phorbol myristate acetate (PMA)/ionomycin, antigenic peptide, or protein.

- Secretion inhibitor, e.g., brefeldin A.
- Inside Stain Kit (#130-090-477) for fixation and permeabilization of cells.
- (Optional) Fluorochrome-conjugated antibodies for cell surface staining, e.g., CD4-FITC (# 130-091-608), CD4-PE (# 130-091-607), CD4-APC (# 130-091-611), CD8a-FITC (# 130-091-605), CD8a-PE (# 130-091-603), CD8a-APC (# 130-091-606), or CD45R (B220)-PerCP (# 130-094-966).
- Fluorochrome-conjugated antibodies for intracellular staining of activation markers, e.g., CD154-PE (# 130-092-106), CD154-APC (# 130-092-105).

Additional requirements for intracellular cytokine staining in combination with magnetic cell separation (see protocol 2.3.2)

- MACS MicroBeads of choice, e.g., CD4 (L3T4) MicroBeads (# 130-049-201).
- MS Columns and suitable MACS Separator (MiniMACS™, OctoMACS™, VarioMACS™, or SuperMACS™ Separator).
 - ▲ **Note:** Column adapters are required to insert certain columns into VarioMACS or SuperMACS Separators. For details see the respective MACS Separator data sheet.
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.

2. Protocols

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.

2.2 *In vitro* stimulation of T cells

▲ Always include a negative control in the experiment. The sample should be treated exactly the same as the stimulated sample, except for the addition of the stimulus.

▲ A positive control may also be included in the experiment.

▲ Do **not** use media containing any non-mouse proteins.

1. Wash cells by adding medium and centrifuge at 300×g for 10 minutes.
2. Resuspend cells at a density of 10⁷ per mL in culture medium, containing 5% mouse serum. Plate cells in dishes at a density of 5×10⁶ cells/cm². For details see section 4. Appendix: Flask and dish sizes for stimulation.
3. Add stimulus or control reagent: 10 ng/mL PMA and 1 µg/mL ionomycin
4. Incubate cells for 2 hours at 37 °C and 5% CO₂.
5. Add 1 µg/mL brefeldin A and incubate for an additional 4 hours at 37 °C and 5% CO₂.
6. Collect cells carefully by pipetting up and down when working with smaller volumes or by using a cell scraper. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

2.3 Intracellular immunofluorescent cytokine staining protocols

2.3.1 Intracellular staining in suspension

▲ Volumes for magnetic labeling given below are for up to 10⁷ total cells. When working with fewer than 10⁷ 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 2×10⁷ total cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Wash up to 10⁷ nucleated cells by adding 1–2 mL of buffer. Centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. (Optional) Stain for cell surface antigens, which are sensitive to fixation, according to the manufacturer's recommendations. Subsequently, wash cells by adding 1–2 mL of buffer. Centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend 10⁷ cells in 500 µL of buffer.
4. Add 500 µL of Inside Fix. Mix well and incubate for 20 minutes at room temperature.
5. Centrifuge for 5 minutes at 300×g. Aspirate supernatant carefully.
6. Wash cells by adding 1 mL of buffer. Centrifuge for 5 minutes at 300×g and aspirate supernatant carefully.
 - ▲ **Note:** Fixed cells may be stored in azide-containing buffer at 2–8 °C for up to three days.
7. (Optional) Stain for cell surface antigens which are sensitive to permeabilization, according to manufacturer's recommendation. Subsequently, wash cells by adding 1–2 mL of buffer. Centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
8. Wash cells by adding 1 mL of Inside Perm and centrifuge for 5 minutes at 300×g. Aspirate supernatant carefully.
9. Resuspend cells in 90 µL of Inside Perm. Add 10 µL of Anti-IL-17A antibodies.
10. (Optional) Add additional staining antibodies to the solution, e.g., 10 µL of CD4-PE (# 130-091-607) and 10 µL of CD154 antibodies or CD45R (B220)-PerCP (# 130-094-966).
 - ▲ **Note:** For efficient permeabilization upon intracellular staining the volume of Inside Perm should be at least 5× the volume of staining antibodies.
11. Mix well and incubate for 10 minutes at room temperature.
12. Wash cells by adding 1 mL of Inside Perm. Centrifuge at 300×g for 5 minutes and aspirate supernatant carefully.
13. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy. Store cells at 2–8 °C in the dark until analyzed. Mix well before flow cytometric acquisition.
 - ▲ **Note:** Samples may be stored at 2–8 °C in the dark for up to 24 hours.
 - ▲ **Note:** Do not use propidium iodide (PI) or 7-AAD staining.

2.3.2 Intracellular staining in combination with magnetic cell separation (solid phase intracellular staining)

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for up to 10⁷ total cells. When working with fewer than 10⁷ 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 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic separation. Pass cells through a 30 μm nylon mesh (Pre-Separation Filters, 30 μm # 130-041-407) to remove cell clumps which may clog the column. Wet filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

1. Wash up to 10^7 nucleated cells by adding 1–2 mL of buffer. Centrifuge at $300 \times g$ for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 90 μL of buffer per 10^7 total cells.
3. Add 10 μL of MACS® MicroBeads of choice, e.g. CD4 MicroBeads (# 130-049-201) per 10^7 total cells.
▲ Note: For details on the procedure refer to the respective data sheet of MACS MicroBeads.
4. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
5. (Optional) Add surface staining antibodies, which are sensitive to fixation, according to the manufacturer's recommendations, and incubate for an additional 5 minutes in the refrigerator (2–8 °C).
6. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at $300 \times g$ for 10 minutes. Aspirate supernatant completely.
7. Resuspend cells in 500 μL of buffer.
8. Place MS Column in the magnetic field of a suitable MACS Separator.
9. Prepare column by rinsing with 500 μL of buffer.
10. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
11. Wash column with $3 \times 500 \mu\text{L}$ of buffer. Collect unlabeled cells that pass through and combine with effluent from step 10.
12. Remove column from the separator and place it on a suitable collection tube.
13. Pipette 500 μL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
14. Add 500 μL of Inside Fix to the eluted cell fraction and incubate for 20 minutes at room temperature.
15. Place a second MS Column in the magnetic field of a suitable MACS Separator and prepare column by rinsing with 500 μL of buffer.
16. Apply the fixed cell suspension onto the column.
17. Wash cells by rinsing the column with $1 \times 500 \mu\text{L}$ of buffer, followed by $2 \times 500 \mu\text{L}$ of Inside Perm.
18. Prepare a solution of 10 μL of Anti-IL-17A antibodies and 90 μL of Inside Perm.

19. (Optional) Add additional staining antibodies to the solution, e.g., 10 μL of CD4-PE (# 130-091-607) and 10 μL of CD154 antibodies.

▲ Note: Do not exceed a total volume of 150 μL .

20. Apply the solution onto the column and incubate for 10 minutes at room temperature.

▲ Note: The MACS Column has a flow-stop mechanism that will retain the solution in the column.

21. Wash cells by rinsing the column with $2 \times 500 \mu\text{L}$ of Inside Perm followed by $1 \times 500 \mu\text{L}$ of buffer.

22. Remove column from the separator and place it on a suitable collection tube.

23. Pipette 500 μL of buffer onto the column. Immediately flush out fraction with magnetically labeled cells by firmly applying the plunger supplied with the column.

24. Cells are now ready for analysis. Store cells at 2–8 °C in the dark until analyzed. Mix well before flow cytometric acquisition.

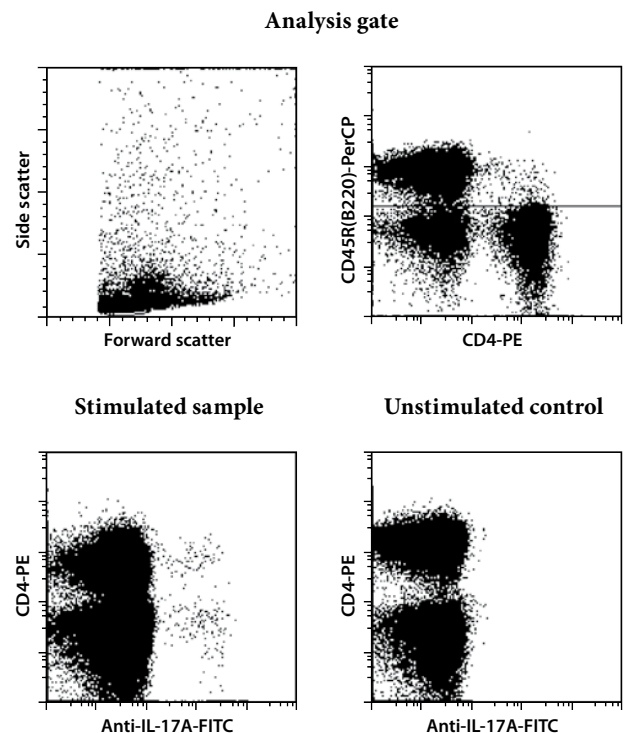
▲ Note: Samples may be stored at 2–8 °C in the dark for up to 24 hours.

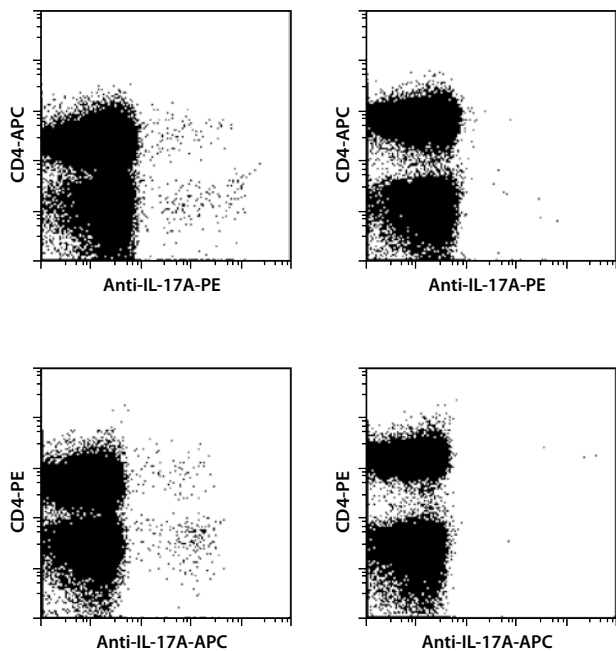
▲ Note: Do not use propidium iodide (PI) or 7-AAD staining.

3. Examples of immunofluorescent staining with Anti-IL-17A antibodies

Mouse spleen cells were incubated with or without PMA/ionomycin for 6 hours. After 2 hours brefeldin A was added.

The cells were harvested, fixed, permeabilized, and intracellularly stained with Anti-IL-17A conjugated with FITC, PE, or APC. Cell surface staining was performed with CD4-PE (# 130-091-607) or CD4-APC (# 130-091-611) and CD45R (B220)-PerCP (# 130-094-966). Cells were analyzed by flow cytometry. Gating was performed according to forward scatter and side scatter properties of the cells. B cells and cell debris were excluded from the analysis in a fluorescence 2 versus fluorescence 3 dot plot.





4. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

For *in vitro* stimulation of T cells (refer to 2.2) the cells should be resuspended in culture medium, containing 5% of mouse serum, at a dilution of 10^7 cells/mL. The cells should be plated at a density of 5×10^6 cells/cm². Both the dilution and the cell density are important to assure optimum stimulation.

The following table lists culture plate, dish and flask sizes suitable for different cell numbers. It also indicates the appropriate amount of medium to add.

Total cell number	Medium volume to add	Culture plate	Well diameter
0.15×10^7	0.15 mL	96 well	0.64 cm
0.50×10^7	0.50 mL	48 well	1.13 cm
1.00×10^7	1.00 mL	24 well	1.60 cm
2.00×10^7	2.00 mL	12 well	2.26 cm
5.00×10^7	5.00 mL	6 well	3.50 cm
Total cell number	Medium volume to add	Culture dish	Dish diameter
4.5×10^7	4.5 mL	small	3.5 cm
10.0×10^7	10.0 mL	medium	6 cm
25.0×10^7	25.0 mL	large	10 cm
50.0×10^7	50.0 mL	extra large	15 cm
Total cell number	Medium volume to add	Culture flask	Growth area
12×10^7	12 mL	50 mL	25 cm ²
40×10^7	40 mL	250 mL	75 cm ²
80×10^7	80 mL	720 mL	162 cm ²
120×10^7	120 mL	900 mL	225 cm ²

5. References

- Weaver, C. T. *et al.* (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25: 821–852.
- Ouyang, W. *et al.* (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28: 454–467.

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.

Warranty

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