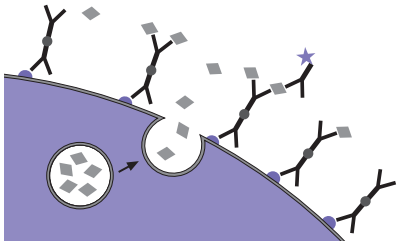
Miltenyi Biotec

IL-17 Secretion Assay – Detection Kit (PE)

human

IL-17 Secretion Assay – Detection Kit (PE)	130-094-537
IL-17 Secretion Assay – Detection Kit (APC)	130-094-536

For 100 tests with 10^6 cells



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140-002-700.03

Contents

1. Description

Components	**1 mL IL-17 Catch Reagent:** anti-IL-17A monoclonal antibody (mouse IgG1) conjugated to CD45-specific monoclonal antibody (mouse IgG2a). **1 mL IL-17 Detection Antibody:** anti-IL-17A monoclonal antibody (mouse IgG1) conjugated to R-phycoerythrin (PE) or allophycocyanin (APC).
Size	For 100 tests with 10^6 cells.
Product format	All components 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 dates are indicated on the vial labels.

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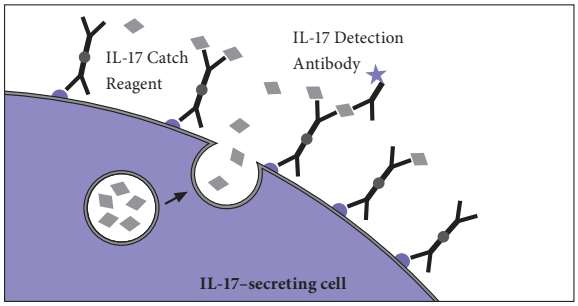


1.1 Principle of the IL-17 Secretion Assay

Antigen-specific T cells are analyzed using the IL-17 Secretion Assay starting from whole blood, peripheral blood mononuclear cells (PBMCs), or other leukocyte containing single-cell preparations. The cells are restimulated for a short period of time with a polyclonal stimulus such as CytoStim™ or specific peptide, protein, or other antigen preparations, e.g., from *Candida albicans* (*C. albicans*).
Subsequently, an IL-17–specific **Catch Reagent** is attached to the cell surface of all leukocytes. The cells are then incubated for a short time at 37 °C to allow cytokine secretion. The secreted IL-17 binds to the IL-17 Catch Reagent on the positive, secreting cells. These cells are subsequently labeled with a second IL-17–specific antibody, the **IL-17 Detection Antibody** conjugated to fluorochrome for sensitive detection by flow cytometry. Since viable cells are analyzed, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background information

Interleukin 17 (IL-17, IL-17A, CTLA8), a member of the IL-17 family (IL-17A–F), is a disulfide-linked homodimeric glycoprotein. Human IL-17 consists of 155 amino acids with a molecular weight of around 35 kDa.[1] IL-17 is produced by $CD4^+$ T helper cells, a third T cell subset termed Th17, which secrete also cytokines such as IL-17F and IL-22 and express the NK cell marker CD161.[2] IL-17 secretion has also been described for other cell types, such as $CD8^+$ memory T cells.[3] Furthermore, intracellular IL-17 has also been detected in eosinophils, neutrophils, and blood monocytes. Emerging data about Th17 cells suggest that these cells are involved in the recruitment of neutrophils to control early stages of infections to a number of pathogens, such as extracellular bacteria and fungi. IL-17 and Th17 cells have been shown to play an important role in immune-mediated diseases, such as rheumatoid arthritis, psoriasis, multiple sclerosis, asthma, inflammatory bowel diseases, and other immune-mediated inflammatory conditions.[4]
Depending on the cytokine milieu present at time of the initial engagement, $CD4^+$ naive T cells can differentiate into various subsets (Th1, Th2, and Th17). For the differentiation into Th17 cells several cytokines have been described, including TGF-β, IL-1β, IL-6, IL-21, and IL-23.[5,6,7] RORγt was identified as a master regulator gene for Th17 cells.[8]

1.3 Applications

- Detection of viable IL-17–secreting leukocytes.
- Detection of IL-17–secreting, antigen-specific T cells for enumeration and phenotypic characterization.
- Monitoring and analysis of antigen-specific T cell immunity, e.g., bacterial and fungal immunity, autoimmunity, and other inflammatory disorders.
- Analysis of IL-17–secreting cells for determination of functional antigens in disease and for T cell receptor (TCR) epitope mapping.
- Analysis of TCR repertoire of antigen-specific T cells.

1.4 Reagent and instrument requirements

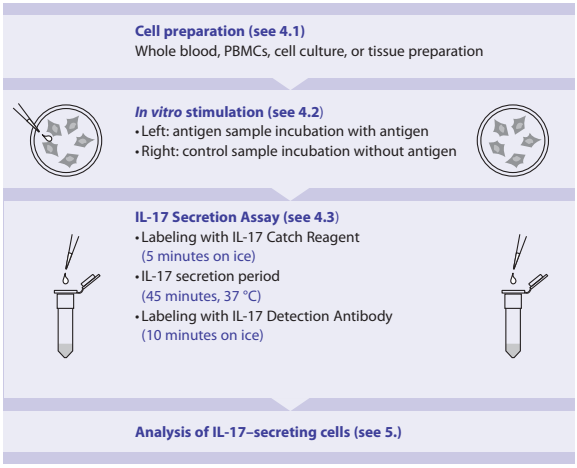
- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles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

- Culture medium, e.g., RPMI 1640 (# 130-091-440), containing 5% human serum, like autologous or AB serum (do not use BSA or FBS because of non-specific stimulation!).
- (Optional) Cell stimulation reagents, e.g., CytoStim (# 130-092-172, # 130-092-173). For details see the respective data sheet. For more information about other stimulation reagents see www.miltenyibiotec.com.
- (Optional) For detection of activated T cells with CD154, the incubation with CD40 pure – functional grade (# 130-094-133) is recommended to avoid downregulation of CD154 expression.
- Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells without fixation. For cell fixation and flow cytometric exclusion of dead cells, the Fixation and Dead Cell Discrimination Kit (# 130-091-163) is recommended.

- (Optional) Staining reagents such as CD154-APC, CD4-FITC, CD8-FITC, CD161-PE, or CD161-APC.
- Refrigerated centrifuge (2–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.

2. Protocol overview



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3. Experimental setup

3.1 Controls

Negative control
For accurate detection of IL-17–secreting cells, a negative control sample should always be included. This will provide information about IL-17 secretion unrelated to the specific antigen-stimulation, but, e.g., due to ongoing *in vivo* immune responses. The control sample should be treated exactly the same as the antigen-stimulated sample except for the addition of antigen, or by using a control antigen.

Positive control
When setting up a new experiment, it is recommended to include a positive control. As a positive control, a sample stimulated with CytoStim (# 130-092-172, # 130-092-173) 20 µL/mL for 4–6 hours, may be included in the experiment.

3.2 Kinetics of restimulation and proposed time schedule

Peptides
Upon stimulation with peptide, the cells can be analyzed for IL-17 secretion 3–6 hours later.
Proteins
Upon stimulation with protein antigen preparations, e.g., from *C. albicans*, the cells can be analyzed for IL-17 secretion 8–16 hours later.
It is possible to start the stimulation of the cells late in the afternoon and perform the IL-17 Secretion Assay the following morning.

3.3 Counterstaining of cytokine-secreting cells

The IL-17–secreting cells are stained with fluorochrome-conjugated IL-17 Detection Antibodies. To identify cells of interest, counterstaining for T cells with, e.g., CD4-FITC (# 130-080-501) and CD154-APC (# 130-092-290) is important.

▲ Upon activation of T cells, TCR, and some associated molecules, like CD3, might be down-regulated.

▲ The samples should be stained with propidium iodide (PI) or 7-AAD prior to acquisition, to exclude dead cells from analysis. This will reduce non-specific background staining and increase sensitivity.

▲ For optimal sensitivity, we recommend labeling of undesired non–T cells such as monocytes with antibodies conjugated to Peridinin-chlorophyll-protein (PerCP), e.g., CD14-PerCP. These cells together with PI stained dead cells can then be excluded by gating.

3.4 Two-color cytokine analysis

The IL-17 Secretion Assay – Detection Kit can be combined with other Secretion Assay – Detection Kits for two-color cytokine analysis, e.g., IFN-γ, IL-4, or IL-10. For more information about Cytokine Secretion Assay – Detection Kits see www.miltenyibiotec.com/protocols.

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3.5 Combination with peptide-MHC tetramer staining

IL-17–secreting cells can be analyzed simultaneously for peptide-MHC tetramers combining the IL-17 Secretion Assay –Detection Kit (PE) with APC-conjugated peptide-MHC tetramers or the IL-17 Secretion Assay – Detection Kit (APC) with PE-conjugated peptide-MHC tetramers. A special protocol is available at www.miltenyibiotec.com/protocols.

3.6 Detection of very low frequencies

(Optional, reagents not included) If the sample contains less than 0.01–0.1% of IL-17–secreting cells, it is possible to enrich these cells magnetically using the IL-17 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-094-542). Thereby it is possible to detect antigen-specific T cells down to frequencies as low as 0.0001% (1 in 10^6).

4. Protocol for the IL-17 Secretion Assay

4.1 Cell preparation

To detect and isolate cytokine-secreting cells, best results are achieved by starting the assay with fresh PBMCs, or with other leukocyte containing single-cell preparations from tissues or cell lines. Alternatively, frozen cell preparations can be used.

▲ **Note:** PBMCs may be stored overnight. The cells should be resuspended and incubated in culture medium as described in 4.2 step 2.

▲ **Note:** Remove platelets after density gradient separation. Resuspend cell pellet, fill tube with buffer, and mix. Centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully remove supernatant.

Special protocols for whole blood: You can start the IL-17 Secretion Assay directly from whole blood. For details on the procedure refer to www.miltenyibiotec.com/protocols.

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4.2 *In vitro* stimulation

▲ Always include a negative control in the experiment. A positive control may also be included (see 3.1).

▲ Do not use media containing any non-human proteins, such as BSA or FBS, as they lead to non-specific stimulation.

Protocol for *in vitro* stimulation

1. Wash cells by adding medium, centrifuge at 300×g for 10 minutes.
2. Resuspend cells in culture medium, containing 5% human serum, adjust to 10^7 cells/mL and 5×10^6 cells/cm² (see 7. Appendix: Flask and dish sizes for stimulation).
3. Add antigen or control reagent:

   CytoStim: 4–6 hours at 37 °C, 5–7% CO_2, e.g., 20 µL/mL
   Peptide: 3–6 hours at 37 °C, 5–7% CO_2, e.g., 1–10 µg/mL
   Protein preparation: 8–16 hours at 37 °C, 5–7% CO_2, e.g., 20 µg/mL

   For comparison of different experiments, the stimulation time should always be the same (see 3.2).

   ▲ **Note:** If CD154 antibodies are used in the labeling step of the cytokine secretion assay to stain activated $CD4^+$ T cells, a CD40-blocking antibody has to be added during the in vitro stimulation step to prevent CD154 down-regulation.

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4. Collect cells carefully by using a cell scraper or by pipetting up and down when working with smaller volumes. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

4.3 Cytokine Secretion Assay

General considerations

▲ The assay is optimized for cell samples containing <1% of total IL-17–secreting cells. If ≥1% of IL-17–secreting cells are expected, it is necessary to dilute the cells further during the cytokine secretion period, and therefore a larger test tube will be needed (see table below). The dilution prevents non-specific staining of cells not secreting IL-17 during this period.

▲ For each test with 10^6 total cells, prepare:
50 mL of **cold buffer** (2–8 °C)
100 µL of **cold medium** (2–8 °C)
1 mL (or 10 mL; see table below) of **warm medium** (37 °C).

▲ 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 (exception: warm medium during secretion period).

▲ Volumes given below are for up to 10^6 total cells. When working with fewer than 10^6 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 2×10^6 total cells, use twice the volume of all indicated reagent volumes and total volumes).

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▲ Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes. Aspirate supernatant.

▲ Dead cells may bind non-specifically to antibodies. Therefore, when working with cell preparations containing large amounts of dead cells, they should be removed before starting the IL-17 Secretion Assay, e.g., by density gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with IL-17 Catch Reagent

1. Use 10^6 total cells in a 2 mL closable tube per sample.
2. Wash cells by adding 1–2 mL of **cold buffer**, centrifuge at 300×g for 5 minutes at 2–8 °C, aspirate supernatant completely.
3. Resuspend cell pellet in 90 µL of **cold medium** per 10^6 total cells.
4. Add 10 µL of **IL-17 Catch Reagent** per 10^6 total cells, mix well and incubate for 5 minutes on ice.

IL-17 secretion period

1. Add **warm** (37 °C) medium to dilute the cells according to the following table:

Expected number of IL-17–secreting cells	Dilution	Amount of medium to add per 10^6 total cells
<1%	10^6 cells/mL	1 mL
≥1%	≤10^5 cells/mL	10 mL

▲ **Note:** For frequencies of cytokine-secreting cells >20% the cells need to be further diluted, e.g., by a factor of 5.

2. Incubate cells in closed tube for 45 minutes at 37 °C under slow continuous rotation using the MACSmix Tube Rotator (# 130-090-753), or turn tube every 5 minutes to resuspend settled cells.

▲ **Note:** During this step it is crucial to prevent contact of cells to avoid cross contamination with cytokines.

Labeling cells with IL-17 Detection Antibody

1. Put the tube on ice.
2. Wash the cells by filling up the tube with **cold buffer** and centrifuge at 300×g for 5 minutes at 2–8 °C. Aspirate supernatant completely.

   ▲ **Note:** If the volume of the cell suspension was higher than the volume of the added buffer, then repeat the wash step.
3. Resuspend cell pellet in 90 µL of **cold buffer** per 10^6 total cells.
4. Add 10 µL of **IL-17 Detection Antibody** per 10^6 total cells.
5. (Optional) Add staining antibodies, e.g., 10 µL of CD4-FITC and 10 µL of CD154-APC.
6. Mix well and incubate for 10 minutes on ice.
7. Add 1 mL of cold buffer and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.
8. Proceed to analysis (5.).

5. Detection and analysis of IL-17–secreting T cells

▲ Add propidium iodide (PI) or 7-AAD to a final concentration of 0.5 µg/mL just prior to acquisition to exclude dead cells from flow cytometric analysis. Incubating with PI for longer periods will affect the viability of the cells.
Do not fix the cells when using PI or 7-AAD.

▲ For optimized sensitivity, an appropriate number of viable cells has to be acquired from the antigen stimulated sample as well as from the control sample.

▲ **Note:** Acquire 2×10^5 viable cells from each sample.

To illustrate the analysis, we describe the detection of IL-17–secreting T cells using the IL-17 Secretion Assay. The detailed description, including how to set gates, should serve as a model for the analysis of your own sample.

1. 10^6 human PBMCs have been incubated for 4 hours with and without CytoStim (20 µL/mL) and CD40 pure – functional grade (1µg/mL).
2. The IL-17 Secretion Assay was performed on the stimulated and the unstimulated sample.
3. Counterstaining of T cells was performed using CD4-FITC and CD154-APC.

4. Dead cells were stained with propidium iodide (PI), which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.
5. 200,000 viable cells were acquired by flow cytometry, from the stimulated as well as from the unstimulated sample.
6. A lymphocyte gate based on $CD4^+$ staining and side scatter properties was activated prior to further gating to exclude debris (A).
7. Dead cells were excluded according to PI-staining (B).

   ▲ **Note:** The dead cell exclusion is crucial for the analysis of rare antigen-specific T cells, as dead cells may bind non-specifically to antibodies. This could lead to false positive events.

   ▲ **Note:** The sensitivity of detection is further enhanced by exclusion of undesired non–T cells, like monocytes which may cause non-specific background staining.
8. Analysis of secreted IL-17 (PE) versus CD154-APC staining of $CD4^+$ viable lymphocytes is displayed (C).

   Analysis of secreted IL-17 (APC) versus CD154-PE staining of $CD4^+$ viable lymphocytes is displayed (D).

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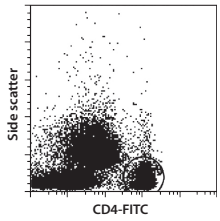
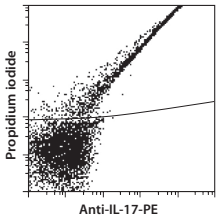
A) $CD4^+$ lymphocyte gate



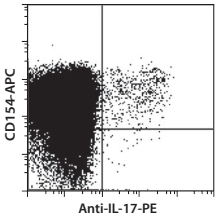
B) Dead cell exclusion



5. Detection and analysis of IL-17-secreting T cells

C) Antigen-specific $CD4^+$ T cells stained for secreted IL-17 (PE)

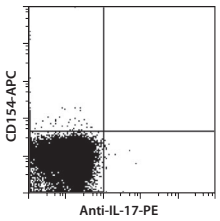
Sample stimulated with CytoStim



0.44% of the total $CD4^+CD154^+$ T cell population secrete IL-17 (see formula below).

$$\%\ \text{IL-17}^+\ \text{cells among CD4}^+ = \frac{\#\ \text{of IL-17}^+\text{CD4}^+\ \text{cells in the analyzed sample}}{\#\ \text{of total CD4}^+\ \text{cells in the analyzed sample}} \times 100$$

Unstimulated control sample



0.00% of the total $CD4^+$ $CD154^+$ T cell population secrete IL-17.

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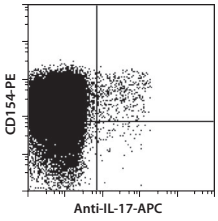
D) Antigen-specific $CD4^+$ T cells stained for secreted IL-17 (APC)

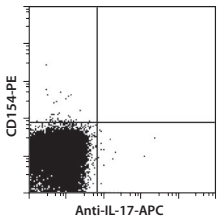
Sample stimulated with CytoStim



0.39% of the total $CD4^+CD154^+$ T cell population secrete IL-17 (see formula below).

$$\%\ \text{IL-17}^+\ \text{cells among CD4}^+ = \frac{\#\ \text{of IL-17}^+\text{CD4}^+\ \text{cells in the analyzed sample}}{\#\ \text{of total CD4}^+\ \text{cells in the analyzed sample}} \times 100$$

Unstimulated control sample



0.03% of the total $CD4^+CD154^+$ T cell population secrete IL-17.

6. References

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7. Appendix: Flask and dish sizes for in vitro stimulation

For *in vitro* stimulation (see 4.2) the cells should be resuspended in culture medium, containing 5% of human serum, at a dilution of 10^7 cells/mL. The cells should be plated at a density of 5×10^6 cells/cm^2. 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

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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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