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140-002-556.01

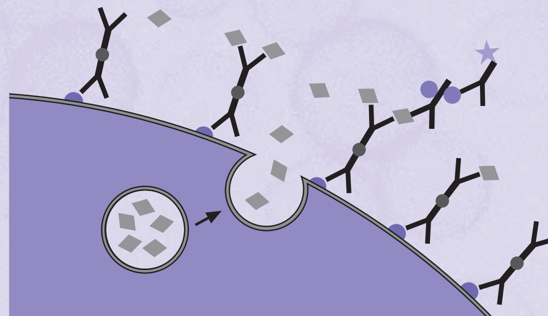
Miltenyi Biotec

Mouse IL-17 Secretion Assay

Detection Kit (PE)

For 100 tests with 10^6 cells

Order no. 130-094-205



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1. Description

Components

1 mL **Mouse IL-17 Catch Reagent**: anti-IL-17 monoclonal antibody (rat IgG1) conjugated to cell surface-specific monoclonal antibody (rat IgG2b).

1 mL **Mouse IL-17 Detection Antibody (Biotin)**: anti-IL-17 monoclonal antibody (rat IgG1) conjugated to biotin.

1 mL **Anti-Biotin-PE**: monoclonal anti-biotin antibody (mouse IgG1) conjugated to R-phycoerythrin (PE).

Capacity For 100 tests with 10^6 cells.

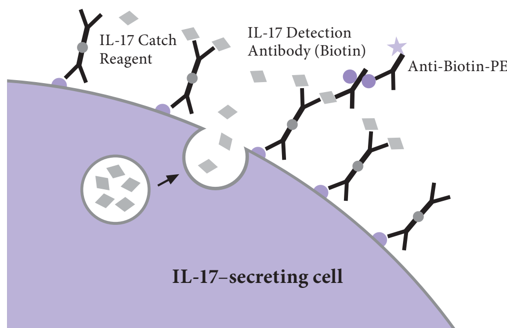
Product format All components are supplied in buffer containing stabilizer and 0.05% sodium azide.

Storage Store protected from light at 2–8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Principle of the Mouse IL-17 Secretion Assay

For analysis of mouse IL-17–secreting leukocytes using the Mouse IL-17 Secretion Assay, mouse spleen cells or other leukocyte-containing single-cell preparations are restimulated for a certain period of time with the desired stimulus.

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 **Mouse IL-17 Detection Antibody** conjugated to biotin and Anti-Biotin-PE 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

The Mouse IL-17 Secretion Assay is designed for the detection and analysis of viable IL-17A–secreting mouse leukocytes.

Interleukin 17 (IL-17) 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-17 plays a key role in the host defense against certain extracellular bacterial infections.
Recently, a new mouse T helper cell subset has been identified, which is dinstinct from TH1 and TH2 cells: IL-17–producing CD4$^+$ T helper (TH17) cells. TH17 cells preferentially produce IL-17A, IL-17F, IL-21, and IL-22. Receptors for IL-17 and IL-22 are expressed on various epithelial tissues, thus TH17 cells are crucial for the cross-talk between immune system and tissues.[2] It is now established that TH17 cells are responsible for driving autoimmune inflammation.
IL-17 is not only secreted by CD4$^+$ T cells, but also by CD8$^+$ T cells, γδ T cells, NK cells, and granulocytes.[1]

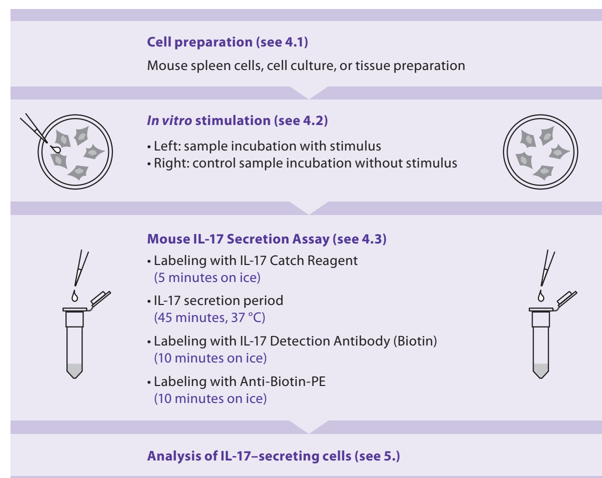
1.3 Applications

- Detection of viable IL-17–secreting mouse leukocytes.
- Detection of IL-17–secreting antigen-specific T cells for enumeration and phenotypic characterization.

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.
- Culture medium, e.g., RPMI 1640 (# 130-091-440), containing 5% mouse serum. Do not use BSA or FCS because of non-specific stimulation.
- Propidium Iodide Solution (# 130-093-233) or 7-AAD to exclude dead cells from analysis.
- Refrigerated centrifuge (2−8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Staining reagents such as CD4-APC (# 130-091-611), CD8a-FITC (# 130-091-605), and CD45R/B220-PerCP™. For more information about other fluorochrome conjugates see www.miltenyibiotec.com.
- (Optional) FcR Blocking Reagent, mouse (# 130-092-575).
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.

2. Protocol overview



3. Experimental set-up

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 *in vitro* stimulation but, for example, due to ongoing *in vivo* immune responses. The control sample should be treated in exactly the same way as the stimulated sample, with the exception of not adding the stimulus.

When working with immunized mice, it could be relevant to include an experiment analyzing cells of a non-immunized mouse.

Positive control
When setting up a new experiment, it is recommended to include a positive control.

3.2 Kinetics of stimulation and proposed time schedule

PMA/ionomycin
Upon stimulation with PMA/ionomycin, the cells can be analyzed for IL-17 secretion 1–4 hours after onset of stimulation.

Costimulation
The addition of costimulatory agents like CD28 antibody may enhance the response to peptide or protein antigens. If costimulatory agents are added to the antigen sample, they also have to be included in the control sample.

3.3 Counterstaining of cytokine-secreting cells

The IL-17-secreting cells are stained with biotin-conjugated IL-17 Detection Antibodies and Anti-Biotin-PE. To identify cells of interest, counterstaining for T cells with, for example, CD4-APC or CD8-FITC 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 B cells with antibodies conjugated to PerCP™, e.g. CD45R/B220-PerCP™. These cells can then be excluded together with PI stained dead cells by gating.

3.4 Two-color cytokine analysis

IL-17–secreting cells can be analyzed simultaneously for, e.g., IFN-γ production by two-color cytokine analysis combining the Mouse IL-17 Secretion Assay – Detection Kit (PE) with the Mouse IFN-γ Secretion Assay – Detection Kit (APC) (# 130-090-984). Detailed protocols are included in the data sheets of the Cytokine Secretion Assay – Detection Kits (APC) and are available from our website www.miltenyibiotec.com/protocols.

3.5 Detection with 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 Mouse IL-17 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-094-213). Thereby it is possible to detect IL-17–secreting T cells down to frequencies as low as 0.0001% (1 in 10^6). A detailed protocol is included in the data sheet of the Mouse IL-17 Secretion Assay – Enrichment and Detection Kit and is available from our website www.miltenyibiotec.com/protocols.

4. Protocol for the Mouse IL-17 Secretion Assay

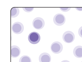
4.1 Cell preparation

Mouse spleen preparation
Prepare fresh mouse spleen cells or other leukocyte containing single-cell preparations under sterile conditions according to standard protocols. Avoid excess of dead cells.

4.2 *In vitro* stimulation

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

▲ Do **not use** media containing any non-murine proteins, like BSA or FCS, because of non-specific stimulation.



Protocol for *in vitro* stimulation

1. Wash cells by adding medium, centrifuge at 200×g for 10 minutes. Aspirate supernatant.
2. Resuspend cells in culture medium at 10^7 cells/mL and 5×10^6 cells/cm² (see 7. Appendix: Flask and dish sizes for stimulation).
3. Add ionomycin (1 µg/mL) and PMA (10 ng/mL). Incubate for 3 hours at 37 °C.

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

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 <2% of total IL-17-secreting cells. If higher percentages of IL-17–secreting cells are expected, it is necessary to dilute the cells further during the cytokine secretion period. A larger test tube will then 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).

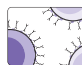
▲ Work fast, keep the cells cold, use pre-cooled solutions which will prevent capping of antibodies on the cell surface and a non-specific cell labeling (exception: warm medium during secretion period).

▲ Volumes shown below are for 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).

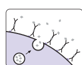
▲ Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes. Aspirate supernatant.

▲ Dead cells may bind non-specifically to MACS MicroBeads or 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 Mouse IL-17 Catch Reagent

1. Use 10^6 total cells in a 2 mL closable tube per sample.
   ▲ **Note:** For larger cell numbers, scale up all volumes accordingly. For fewer than 10^6 cells, use same volumes.
2. Wash cells by adding 2 mL of cold buffer and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.
   ▲ **Note:** Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes.
3. Repeat wash step. Aspirate supernatant completely.
4. Resuspend cell pellet in 90 µL of **cold medium** per 10^6 total cells.
5. Add 10 µL of **Mouse IL-17 Catch Reagent** per 10^6 total cells, mix well and incubate for 5 minutes **on ice**.



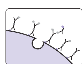
IL-17 secretion period

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
< 2 %	10^6 cells/mL	1 mL
2–20 %	≤ 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 Mouse IL-17 Detection Antibody (Biotin) and Anti-Biotin-PE

1. Put the tube **on ice**.
2. Wash the cells by filling up the tube with **cold buffer**, and centrifuge at 300×g for 10 minutes at **2–8 °C**. Aspirate supernatant completely.
3. Repeat wash step, aspirate supernatant completely.

4. Resuspend cell pellet in 90 µL of **cold buffer** per 10^6 total cells.
5. Add 10 µL of **Mouse IL-17 Detection Antibody (Biotin)** per 10^6 total cells.
   ▲ **Note:** To avoid non-specific binding the use of FcR Blocking Reagent is recommended.
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 2 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant.
8. Resuspend cell pellet in 90 uL of cold buffer per 10^6 total cells.
9. Add 10 µL of Anti-Biotin-PE per 10^6 total cells.
10. (Optional) Add additional staining reagents, e.g., CD4-APC and CD45R/B220-PerCP™.
11. Mix well and incubate for 10 minutes on ice.
12. Wash cells by adding 2 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant.
13. Resuspend cells in 500 µL of **cold buffer** and proceed to analysis (see section 5).

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

5. Detection and analysis of IL-17-secreting 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 stimulated sample as well as from the control sample.

- Acquire 2×10^5 viable cells from each fraction.

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

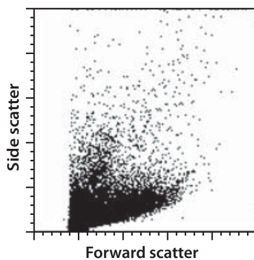
1. Mouse splenocytes were stimulated for 3 hours with ionomycin (1 µg/mL) and PMA (10 ng/mL) or left untreated.
2. The Mouse IL-17 Secretion Assay was performed on the stimulated and the unstimulated sample.
3. **Counterstaining of T cells** was performed using CD4-APC.
4. **B lymphocytes** were stained with CD45R (B220)-PerCP.
5. **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.
6. 200,000 viable cells were acquired by flow cytometry, from the stimulated and the unstimulated samples.
7. A **lymphocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude B cells and debris (see A.).
8. Dead cells and B cells were excluded according to PI- and CD45R (B220)-PerCP staining in a fluorescence 2 (PE) versus fluorescence 3 plot (PI) (see B.).

   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.

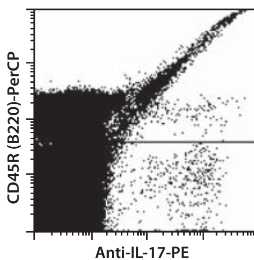
   The sensitivity of the detection will further be enhanced by exclusion of undesired non-T cells, which may cause non-specific background staining.
9. For analysis IL-17 (PE) versus CD4-APC staining of viable lymphocytes is displayed (see C.).

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

A. Lymphocyte gate in the forward versus side scatter plot

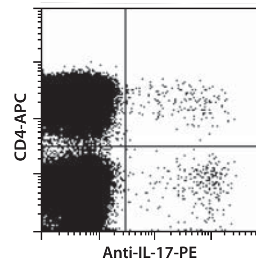


B. Dead cell and B cell exclusion



C. IL-17–secreting CD4⁺ T cells

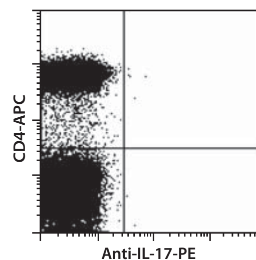
Splenocytes stimulated with ionomycin and PMA



0.259% of the total CD4⁺ 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 splenocytes



0.016% of the total CD4⁺ T cell population secrete IL-17.

6. References

1. 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.
2. Ouyang, W. *et al.* (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 28: 454–467.

For further references visit our website **www.miltenyibiotec.com**.

7. Appendix: Flask and dish sizes for stimulation

For (antigen-specific) stimulation (see 4.2) 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 cm²
40×10^7	40 mL	250 mL	75 cm²
80×10^7	80 mL	720 mL	162 cm²
120×10^7	120 mL	900 mL	225 cm²

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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