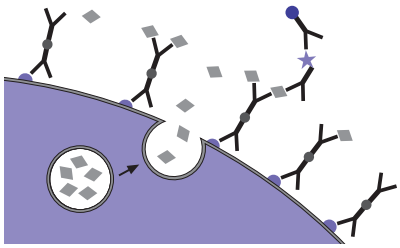
Miltenyi Biotec

Mouse IL-17 Secretion Assay – Cell Enrichment and Detection Kit (PE)

For 50 tests with 10^7 cells

Order no. 130-094-213



Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macsde@miltenyi.com, **www.miltenyibiotec.com**

140-002-557.06

Contents

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

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

1 mL Anti-PE MicroBeads UltraPure: colloidal superparamagnetic MicroBeads UltraPure conjugated to monoclonal mouse anti-PE antibody (mouse IgG1).

Capacity

For 50 tests with 10^7 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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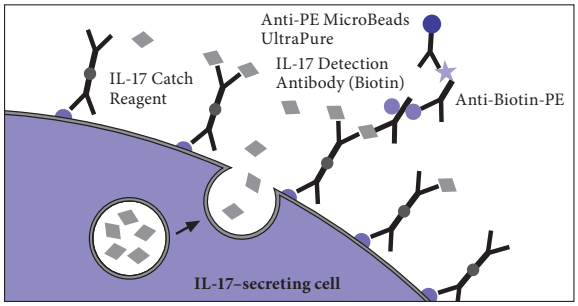


1.1 Principle of the Mouse IL-17 Secretion Assay

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.

The IL-17–secreting cells can now be magnetically labeled with

Anti-PE MicroBeads UltraPure and enriched over a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled cells are retained in the MACS Column while the unlabeled cells run through. After the column has been removed from the magnetic field, the magnetically retained cells can be eluted as positively selected cell fraction, enriched for cytokine-secreting cells. The cells can now be used for cell culture or analysis. 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 isolation, 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]

Enrichment of the IL-17–secreting leukocytes using the MACS Technology increases the sensitivity of analysis and also enables further functional characterization, culturing of the cells, as well as downstream applications.

1.3 Applications

- Detection and enrichment of viable IL-17–secreting mouse leukocytes.
- Detection and enrichment of IL-17–secreting antigen-specific T cells for enumeration and phenotypic analysis as well as for culturing and functional 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.

  ▲ **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 (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.
- Culture medium, e.g., TexMACS Medium, research grade (# 130-097-196), containing 5% mouse serum. Do not use BSA or FBS because of non-specific stimulation!
- Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells without fixation.
- (Optional) FcR Blocking Reagent, mouse (# 130-092-575).
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Staining reagents such as CD4-APC, Anti-IL-17A-PE, and CD45R (B220)-VioBlue®. For more information about other fluorochrome conjugates refer to www.miltenyibiotec.com/antibodies.

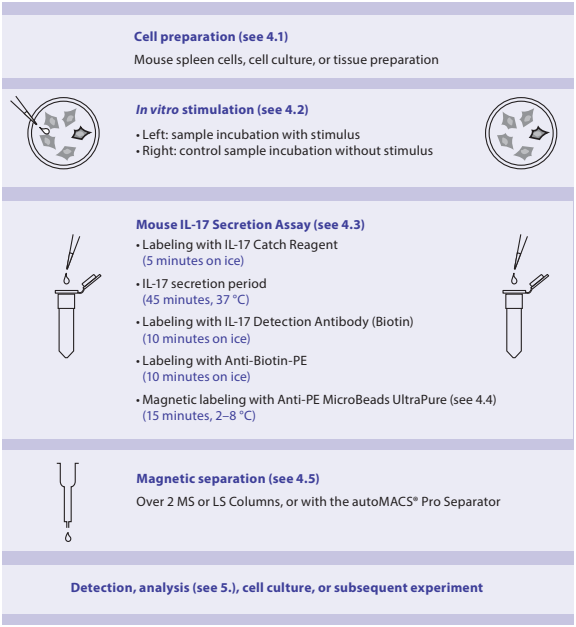
- MACS Columns and MACS Separators:

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
autoMACS	2×10^8	4×10^9	autoMACS Pro

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ II Separators. For details refer to the respective MACS Separator data sheet.

- Refrigerated centrifuge (2–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.

2. Protocol overview



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 *in vitro* stimulation but, for example, due to ongoing *in vivo* immune responses. The control sample should be treated exactly the same as the stimulated sample exept for the addition of stimulus or by using a control 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-VioBright™ FITC or CD8a-PE is important.

▲ Do not use tandem conjugates of phycoerythrin, such as Cy™-Chrome, PE-Cy5, PE-Vio® 770, ECD, or PC5. They may also be recognized by the Anti-PE MicroBeads UltraPure.

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

▲ The samples should be stained with Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) 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 VioBlue®, e.g., CD45R (B220)-VioBlue . These cells can then be excluded together with propidium iodide (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 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.

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 – Cell Enrichment and Detection Kit (PE) with APC-conjugated peptide-MHC tetramers. A special protocol with detailed recommendations for the experimental setup and the procedure is available at www.miltenyibiotec.com.

3.6 Detection without prior enrichment

(Optional) If the sample contains more than 0.01–0.1% of IL-17–secreting cells, you may be able to analyze IL-17–secreting cells without prior enrichment (see also: Mouse IL-17 Secretion Assay – Detection Kit (PE), # 130-094-205, and Mouse IL-17 Secretion Assay – Detection Kit (APC), # 130-094-207). A detailed protocol is included in the data sheet of the Mouse IL-17 Secretion Assay – Detection Kits and is available at www.miltenyibiotec.com.

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. A positive control may also be included (see 3.1).

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

Protocol for *in vitro* stimulation

1. Wash cells by adding medium and 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 (refer to 7. Appendix: Flask and dish sizes for *in vitro* 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 (refer to 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, and therefore 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 10^7 total cells, prepare:
100 mL of **cold buffer** (2–8 °C)
100 µL (or 500 µL, refer to table below) of **cold medium** (2–8 °C)
10 mL (or 100 mL, refer to 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 10^7 total cells. When working with fewer than 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 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 densitiy gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with Mouse IL-17 Catch Reagent

1. Use 10^7 total cells in a 15 mL closable tube per sample.

   ▲ Note: For larger cell numbers, scale up all volumes accordingly. For fewer than 10^7 cells, use same volumes

2. Wash cells by adding 10 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 **cold medium** per 10^7 total cells and add **Mouse IL-17 Catch Reagent** per 10^7 total cells, according to the following table:

Expected number of IL-17–secreting cells	Cold medium	Mouse IL-17 Catch Reagent
<2%	80 µL	20 µL
2–20%	450 µL	50 µL

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

5. 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 10^7 total cells
<2%	10^6 cells/mL	10 mL
2–20%	10^5 cells/mL	100 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 (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 **cold buffer** per 10^7 total cells and add **Mouse IL-17 Detection Antibody (Biotin)** per 10^7 total cells, according to the following table:

Expected number of IL-17–secreting cells	Cold buffer	Mouse IL-17 Detection Reagent
<2%	80 µL	20 µL
2–20%	450 µL	50 µL

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

▲ Note: To avoid non-specific binding the use of FcR Blocking Reagent is recommended.

5. Mix well and incubate for 10 minutes on ice.
6. Wash cells by adding 10 mL of **cold buffer** and centrifuge at 300 × g for 10 minutes at 2–8 °C. Aspirate supernatant.

7. Resuspend cell pellet in **cold buffer** per 10^7 total cells and add **Anti-Biotin-PE** per 10^7 total cells, according to the following table:

Expected number of IL-17–secreting cells	Cold buffer	Anti-Biotin-PE
<2%	96 µL	4 µL
2–20%	490 µL	10 µL

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

8. (Optional) Add additional staining reagents, e.g., CD4-APC and CD45R (B220)-VioBlue®.
9. Mix well and incubate for 10 minutes on ice.
10. Wash cells by adding 10 mL of **cold buffer** and centrifuge at 300 × g for 10 minutes at 2–8 °C. Aspirate supernatant.

4.4 Magnetic labeling

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

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

Magnetic labeling with Anti-PE MicroBeads UltraPure

1. Resuspend cell pellet in **cold buffer** per 10^7 total cells and add **Anti-PE MicroBeads UltraPure** per 10^7 total cells, according to the following table:

Expected number of IL-17–secreting cells	Cold buffer	Anti-PE MicroBeads UltraPure
<2%	80 µL	20 µL
2–20%	450 µL	50 µL

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

2. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
   ▲ Note: Do not work on ice during this step
3. Wash cells by adding 10 mL of **cold buffer** and centrifuge at 300 × g for 10 minutes at 2–8 °C. Aspirate supernatant.
4. Resuspend cell pellet in 500 µL of **cold buffer**. For higher cell numbers use a dilution of 10^8 cells/mL.
5. (Optional) Take an aliquot of the fraction before enrichment for flow cytometric analysis and cell count.
6. Proceed to magnetic separation (see 4.5).

4.5 Magnetic separation

Magnetic separation with MS or LS Columns

▲ Choose an appropriate MACS® Column and MACS Separator according to the number of total cells. For details refer to the table in 1.4.

▲ When enriching IL-17–secreting cells, **always perform two consecutive column runs** to achieve best results.

1. Place column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
2. Prepare column by rinsing with the appropriate amount of buffer:
   MS: 500 µL LS: 3 mL
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with the appropriate amount of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
   MS: 3×500 µL LS: 3×3 mL
5. Remove column from the separator and place it on a suitable collection tube.
   ▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.

6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
   MS: 1 mL LS: 5 mL
7. (Optional) To increase the purity of IL-17–secreting cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.
   ▲ **Note:** For subsequent cell culture, the cells can also be eluted with medium. If part of the cells are analyzed by flow cytometry, the medium should not contain phenol red.
8. Proceed to analysis (see section 5.), cell culture, or other subsequent experiment.

Magnetic separation using the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥ 10 °C.

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual.

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:
   Positive selection: Posseld2
   Collect positive fraction in row C of the tube rack.
4. Proceed to analysis (section 5.), cell culture, or other subsequent experiment.

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.

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

▲ **Note:** For enumeration of low frequent IL-17–secreting cells, acquire all of the positive fraction. For preparative purposes, acquire an aliquot of the positive fraction to determine the performance of the cell enrichment.

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 µg/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)-VioBlue®.

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 of the fractions before enrichment and the complete enriched fractions were acquired by flow cytometry using the MACSQuant® Analyzer, 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 monocytes and debris (A).

8. Dead cells were excluded according to PI-staining in channel B2 (PE) versus channel R1 (PerCP) plot (B).

9. B cells were excluded according to CD45R (B220)-VioBlue®–staining in channel B2 (PE) versus channel V1 (VioBlue) plot.

   The dead cell exclusion is crucial for the analysis of rare antigen-specific T cells, as dead cells may bind non-specifically to antibodies or MicroBeads. 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 (C).

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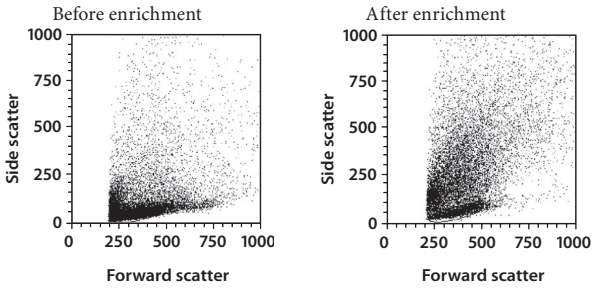
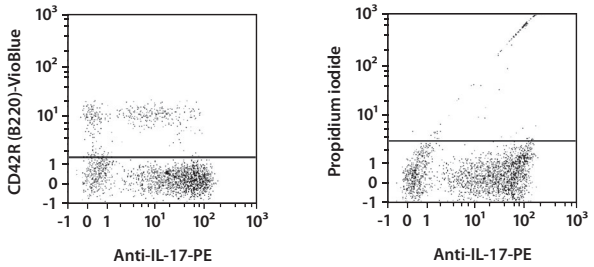
5. Detection and analysis of IL-17–secreting cells

A. Lymphocyte gate in the forward versus side scatter plot



B. Dead cell exclusion

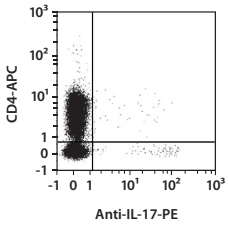


5. Detection and analysis of IL-17–secreting cells

C. IL-17–secreting CD4⁺ T cells

Splenocytes stimulated with ionomycin and PMA

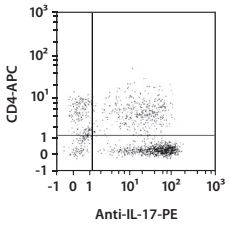
Before enrichment



0.16% of the total CD4⁺ T cell population secrete IL-17 (see formula below).

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

After enrichment



The IL-17–secreting cells have been enriched to 66.43%, within the total CD4⁺ T cell population.

920 IL-17⁺CD4⁺ T cells were enriched from 10^6 CD4⁺ cells (= 0.0.092%; see formula below).

$$\%\ \text{IL-17}^+\ \text{cells among CD4}^+ = \frac{\text{abs. \# of IL-17}^+\text{CD4}^+\ \text{cells in the enriched fraction}}{\text{abs. \# of total CD4}^+\ \text{cells before enrichment}} \times 100$$

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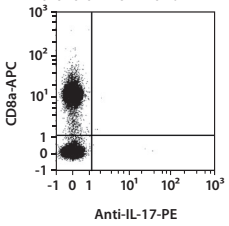
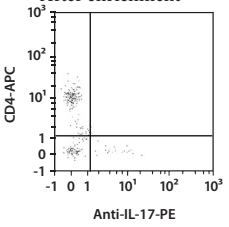
Unstimulated splenocytes

Before enrichment



0.01% of the total CD4⁺ T cell population secrete IL-17.

After enrichment



1 IL-17⁺CD4⁺ T cells were enriched from 10^6 CD4⁺ cells (= 0.01%).

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.

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

For *in vitro* stimulation (refer to 4.2) the cells should be resuspended in culture medium, containing 5% of mouse serum, at 10^7 cells/mL and 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

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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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