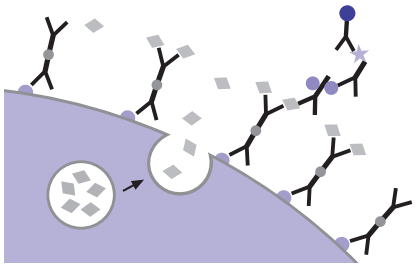
Miltenyi Biotec

IL-13 Secretion Assay – Cell Enrichment and Detection Kit (PE)

human

For 50 tests with 10^7 cells

Order no. 130-093-480



Miltenyi Biotec

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140-002-214.03

Contents

1. Description

Components

1 mL IL-13 Catch Reagent:
anti-IL-13 monoclonal antibody (rat IgG1) conjugated to CD45-specific monoclonal antibody (mouse IgG2a).

1 mL IL-13 Detection Antibody (Biotin):
anti-IL-13 monoclonal antibody (mouse IgG1) conjugated to biotin.

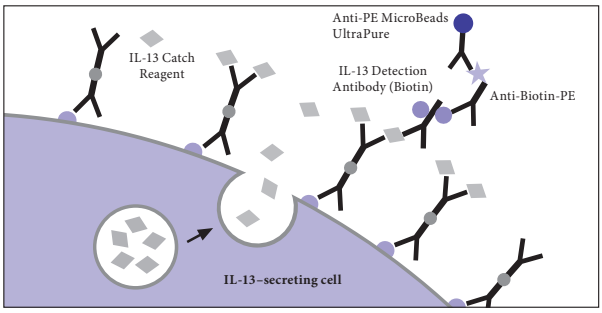
0.2 mL Anti-Biotin-PE:
monoclonal anti-biotin antibody conjugated to R-phycoerythrin (PE).

1 mL Anti-PE MicroBeads UltraPure:
MicroBeads conjugated to monoclonal human anti-PE antibodies (isotype: 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 date is indicated on the vial label.



1.1 Principle of the IL-13 Secretion Assay

Antigen-specific T cells are analyzed using the IL-13 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 or specific peptide, protein, or other protein antigen preparations.
Subsequently, an IL-13–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-13 binds to the IL-13 Catch Reagent on the positive, secreting cells. These cells are subsequently labeled with a second IL-13–specific antibody, the **IL-13 Detection Antibody** conjugated to biotin and Anti-Biotin-PE for sensitive detection by flow cytometry.

The IL-13–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 within the column. The unlabeled cells run through. After removing the column from the magnetic field, the magnetically retained IL-13–secreting cells can be eluted as positively selected cell fraction. 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

Interleukin-13 (IL-13) is produced primarily by activated Th2 cells, but also by mast cells, basophils, NK cells, and dendritic cells.[1] IL-13 plays an important role in resistance to gastrointestinal nematodes. Moreover, it is a central mediator of allergic asthma. IL-13 has also been shown to enhance B cell proliferation and to induce isotype switching, resulting in an increased production of IgE.
IL-13, in contrast to IL-4, fails to induce Th2 cell differentiation, one of the hallmarks of the allergic response.

1.3 Applications

- Detection and enrichment of viable IL-13-secreting leukocytes.
- Detection and enrichment of viable IL-13-secreting leukocytes from whole blood. You can start the IL-13 Secretion Assay directly from whole blood. For details on the procedure refer to www.miltenyibiotec.com/protocols.
- Detection and enrichment of IL-13-secreting, antigen-specific T cells for enumeration and phenotypic characterization as well as for expansion and functional characterization.
- Monitoring and analysis of antigen-specific T cell immunity, for example, in infection, autoimmunity, cancer, allergy, or alloreactivity.
- Isolation and expansion of antigen-specific T cells.
- Enrichment and analysis of IL-13-secreting cells for determination of functional antigens in disease and for T cell receptor (TCR) epitope mapping.
- Analysis or cloning 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 with stable glutamin, containing 5% human serum, like autologous or AB serum (do not use BSA or FBS because of non-specific stimulation!).
- (Optional) Cell stimulation reagents, for example, CytoStim (# 130-092-172, # 130-092-173), CMV pp65 – Recombinant Protein (# 130-091-823, # 130-091-824), or PepTivator® – CMV pp65 (# 130-093-435, # 130-093-438) for restimulation of human T cells. For more information refer to www.miltenyibiotec.com/peptivators.
- (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 Staining Solution (# 130-111-568) 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.

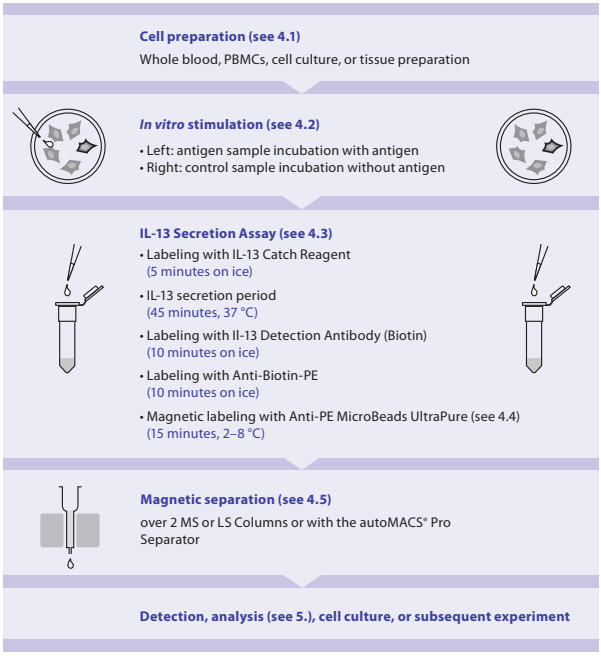
- (Optional) Staining reagents such as CD154-APC, CD4-FITC, and CD14-PerCP-Vio® 700.
- MACS Columns and MACS Separators:

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

▲ **Note:** Column adapters are required to insert certain columns into 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-13–secreting cells, a negative control sample should always be included. This will provide information about IL-13 secretion unrelated to the specific antigen-stimulation but, for example, 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 1–4 hours or with the superantigen Staphylococcal Enterotoxin B (Sigma) 1 µg/mL for 3–16 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-13 secretion 3–6 hours later.

It is possible to prepare the cells first and take them into culture overnight, but without adding the antigen (see 4.2 step 2.). Peptide is then added the next morning for 3 hours of stimulation, directly followed by the IL-13 Secretion Assay.

Proteins
Upon stimulation with protein, the cells can be analyzed for IL-13 secretion 6–16 hours later.
It is possible to start the stimulation of the cells late in the afternoon, and to perform the IL-13 Secretion Assay the following morning.

Costimulation
The addition of costimulatory agents like CD28 or CD49d antibody may enhance the response to the antigen. 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-13-secreting cells are stained with biotin-conjugated IL-13 Detection Antibodies and Anti-Biotin-PE. To identify cells of interest, counterstaining for T cells with, for example, CD4-FITC or CD154-APC is important.

▲ Do not use tandem conjugates of phycoerythrin, like Cy-Chrome, PE-Cy5, ECD, or PC5. They may also be recognized by the Anti-PE MicroBeads.

▲ 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 appropriate fluorochrome-

conjugated antibodies, e.g., CD14-PerCP-Vio® 700. These cells together with PI stained dead cells can then be excluded by gating.

3.4 Two-color cytokine analysis

IL-13-secreting cells can be analyzed simultaneously for IFN-γ, IL-2, IL-10, or TNF-α production by two-color cytokine analysis combining the IL-13 Secretion Assay with the IFN-γ Secretion Assay – Detection Kit (APC) (# 130-090-762) or IFN-γ Secretion Assay – Detection Kit (FITC) (# 130-090-433), IL-2 Secretion Assay – Detection Kit (APC) (# 130-090-763), IL-10 Secretion Assay – Detection Kit (APC) (# 130-090-761), or TNF-α Secretion Assay – Detection Kit (APC) (# 130-091-267). Detailed protocols are included in the data sheets of the Cytokine Secretion Assay – Detection Kits (APC) and are also available at www.miltenyibiotec.com.

3.5 Combination with peptide-MHC tetramer staining

IL-13-secreting cells can be analyzed simultaneously for peptide-MHC tetramers combining the IL-13 Secretion Assay – Detection Kit (PE) with APC-conjugated peptide-MHC tetramers. A special protocol 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-13-secreting cells, you may be able to analyze IL-13-secreting cells without prior enrichment (see also: IL-13 Secretion Assay – Detection Kit (PE), # 130-093-479). The assay can also be performed directly starting from

whole blood. For details on the procedure refer to www.miltenyibiotec.com.

4. Protocol for the IL-13 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-13 Secretion Assay directly from whole blood. For details on the procedure refer to www.miltenyibiotec.com.

4.2 *In vitro* stimulation

▲ Always include a negative control in the experiment. A positive control may also be included (refer to 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 (refer to 6. Appendix: Flask and dish sizes for *in vitro* stimulation).
3. Add antigen or control reagent:

peptide:	3–6 hours at 37 °C, 5–7% CO_2, e.g. 1–10 µg/mL
protein:	6–16 hours at 37 °C, 5–7% CO_2, e.g. 10 µg/mL
CytoStim:	1–4 hours at 37 °C, 5–7% CO_2, e.g. 20 µL/mL
SEB:	3–16 hours at 37 °C, 5–7% CO_2, e.g. 1 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. Stimulation with CytoStim should be performed for 4 hours.

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 <5% of total IL-13-secreting cells. If ≥5% of IL-13-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-13 during this period.

▲ For each test with 10^7 total cells, prepare:
50 mL of **cold buffer** (2–8 °C)
100 µL 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 up to 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 antibodies. Therefore, when working with cell preparations containing large amounts of dead cells,

they should be removed before starting the IL-13 Secretion Assay, for example, by densitiy gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with IL-13 Catch Reagent

1. Use 10^7 total cells in a 15 mL closable tube per sample.
2. Wash cells by adding 10 mL of cold buffer, centrifuge at 300×g for 10 minutes at 2–8 °C, aspirate supernatant completely.
3. Resuspend cell pellet in 80 µL of cold medium per 10^7 total cells.
4. Add 20 µL of IL-13 Catch Reagent per 10^7 total cells, mix well and incubate for 5 minutes on ice.

IL-13 secretion period

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

Expected number of IL-13–secreting cells	Dilution	Amount of medium to add per 10^7 total cells
<5%	10^6 cells/mL	10 mL
≥5%	≤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-13 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.

▲ **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 80 µL of **cold buffer** per 10^7 total cells.
4. Add 20 µL of **IL-13 Detection Antibody (Biotin)** per 10^7 total cells.
5. (Optional) Add staining antibodies, e.g., CD4-FITC and CD154-APC according to manufacturer's recommendation.
6. Mix well and incubate for 10 minutes on ice.
7. Add 10 mL of **cold buffer** and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.

8. Resuspend cell pellet in 96 µL of cold buffer per 10^7 total cells.
9. Add 4 µL of **Anti-Biotin-PE** and incubate for 10 minutes on ice.
10. Add 10 mL of **cold buffer** and centrifuge at 300×g for 10 minutes at 2–8° C. Aspirate supernatant.
11. Proceed to magnetic labeling (4.4).

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 80 µL of **cold buffer** per 10^7 total cells.
2. Add 20 µL of **Anti-PE MicroBeads UltraPure** per 10^7 total cells.
3. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
4. Wash cells by adding 10 mL of **cold buffer** per 10^7 total cells and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.
5. Resuspend cell pellet in 500 µL of **cold buffer**. For higher cell numbers than 5×10^7 use a dilution of 10^8 cells/mL.
6. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
7. Proceed to magnetic separation (4.5).

4.5 Magnetic separation

Magnetic separation using MS or LS Columns

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of IL-13–secreting cells. For details refer to the table in section 1.4.

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

▲ When enriching antigen-specific T 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

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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. To increase the purity of IL-13–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 (refer to section 5), cell culture, or other subsequent experiment.

Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the 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 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: Posseld
   Collect positive fraction in row C of the tube rack.
4. Proceed to analysis (refer to section 5), cell culture, or other subsequent experiment.

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5. Detection and analysis of IL-13–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-13–secreting T cells using the IL-13 Secretion Assay. The detailed description, including how to set gates, should serve as a model for the analysis of your own sample.

1. 10^7 human PBMCs have been restimulated for 4 hours with and without CytoStim (20 µL/mL).
2. The IL-13 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 PI, which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.
5. 100,000 viable cells of the fractions before enrichment and the complete enriched fractions were acquired by flow cytometry, from the stimulated as well as from the unstimulated samples.
6. A lymphocyte gate based on $CD4^+$ staining and side scatter properties was activated prior to further gating to exclude debris (see A.).
7. Dead cells were excluded according to PI-staining in a fluorescence 2 (PE) versus fluorescence 3 (PI) plot (see 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 MicroBeads or 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-13 (PE) versus CD154-APC staining of $CD4^+$ viable lymphocytes is displayed (see C.).

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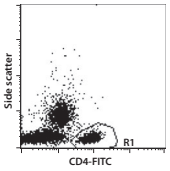
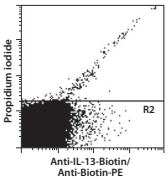
A) $CD4^+$ lymphocyte gate



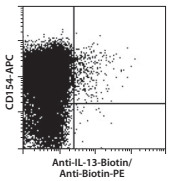
B) Dead cell exclusion



C) IL-13–secreting $CD4^+$ T cells after stimulation with CytoStim

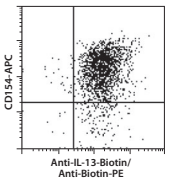
Sample stimulated with CytoStim

Before enrichment



1.25% of the total $CD4^+$ T cell population secrete IL-13 (see formula below).

After enrichment



The IL-13-secreting $CD4^+$ T cells have been enriched to 90.17%.

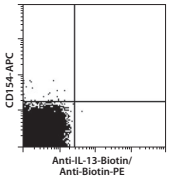
1529 $IL\text{-}13^+CD4^+$ T cells were enriched from 10^6 $CD4^+$ cells (= 0.15%; see formula below).

$$\%\ IL\text{-}13^+\ \text{cells among}\ CD4^+ = \frac{\#\ \text{of}\ IL\text{-}13^+CD4^+\ \text{cells in the analyzed sample}}{\#\ \text{of total}\ CD4^+\ \text{cells in the analyzed sample}} \times 100$$

$$\%\ IL\text{-}13^+\ \text{cells among}\ CD4^+ = \frac{\text{abs.}\ \#\ \text{of}\ IL\text{-}13^+CD4^+\ \text{cells in the enriched fraction}}{\text{abs.}\ \#\ \text{of total}\ CD4^+\ \text{cells before enrichment}} \times 100$$

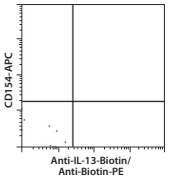
Unstimulated control sample

Before enrichment



0.00% of the total $CD4^+$ T cell population secrete IL-13.

After enrichment



No $IL\text{-}13^+CD4^+$ T cell was enriched from 10^6 $CD4^+$ cells (0.00%).

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6. Reference

1. Wynn, T. (2003) IL-13 effector functions. Annu. Rev. Immunol. 21: 425–456.

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 human 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

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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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