MACS

Cytokine Secretion Assays for murine cells

Mouse IL-10 Secretion Assay

Cell Enrichment and Detection Kit

1 ml Mouse IL-10 Catch Reagent
1 ml Mouse IL-10 Detection Antibody (PE*)
1 ml Anti-PE MicroBeads
For 50 tests with 10^7 cells Order No. 130-090-490

Contents

1. Description

Components

Mouse IL-10 Catch Reagent: anti-IL-10 monoclonal antibody (rat IgG2b) conjugated to cell surface specific monoclonal antibody (rat IgG2b).

Mouse IL-10 Detection Antibody: anti-IL-10 monoclonal antibody (rat IgG1) conjugated to PE* (R-phycoerythrin).

Anti-PE MicroBeads: colloidal super-paramagnetic MicroBeads conjugated to monoclonal mouse anti-PE antibody (mouse IgG1).

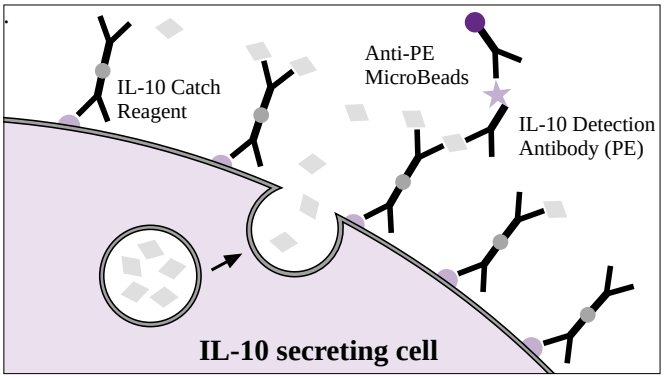
Product format All components are supplied as suspension containing 0.1% gelatine and 0.05% sodium azide.

Storage Store protected from light at 4°C. Do not freeze.

1.1 Principle of the Mouse IL-10 Secretion Assay

For analysis of murine antigen-specific T cells using the Mouse IL-10 Secretion Assay, mouse spleen cells or other leukocyte containing single cell preparations are restimulated for a short period of time with specific peptide, protein or other antigen preparations.

Subsequently, an IL 10–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-10 binds to the IL-10 Catch Reagent on the positive cells. These cells can subsequently be labeled with a second IL-10 specific antibody, the **Mouse IL-10 Detection Antibody** conjugated to phycoerythrin (PE)



for sensitive detection by flow cytometry.

The IL-10 secreting cells can now be magnetically labeled with **Anti-PE MicroBeads** 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 IL-10 secreting cells can be eluted as positively selected cell fraction, enriched for cytokine secreting cells. The cells can now be used for cell culture or for analysis. Since viable cells are analyzed, nonspecific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Instrument and reagent requirements

- Columns and separators:

Column	max. number of labeled cells	max. number of total cells	Separator
MS	10^7	2×10^8	MiniMACS, OctoMACS; with Column Adapter: VarioMACS, SuperMACS
LS	10^8	2×10^9	MidiMACS with Column Adapter: VarioMACS, SuperMACS
autoMACS	2×10^8	4×10^9	autoMACS

- **Buffer** (degassed): phosphate buffered saline pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA (e.g. 4 ml of a 0.5 M EDTA stock solution per 1 liter of buffer).
- (Optional) 0.5 M EDTA stock solution: dissolve 56 g sodium hydroxide (NaOH) in 900 ml distilled water. Add 146.2 g ethylene-diamine-tetraacetic acid, adjust pH to 7.5, fill up to 1000 ml.
- **Culture medium**, e.g. RPMI 1640 containing 5% of murine serum (do **not** use **BSA** or **FCS** because of nonspecific stimulation!).
- **Propidium iodide (PI)** or **7-AAD** to exclude dead cells from the analysis.
- (Optional) Staining reagents such as CD4-FITC or CD8-FITC and CD45R/B220-PerCP.
- Rotation device for tubes: MACSmix (# 130-090-753).
- (Optional) Pre-Separation Filter (# 130-041-407) or 30 µm nylon mesh.

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1.3 Background and applications (see also 6.)

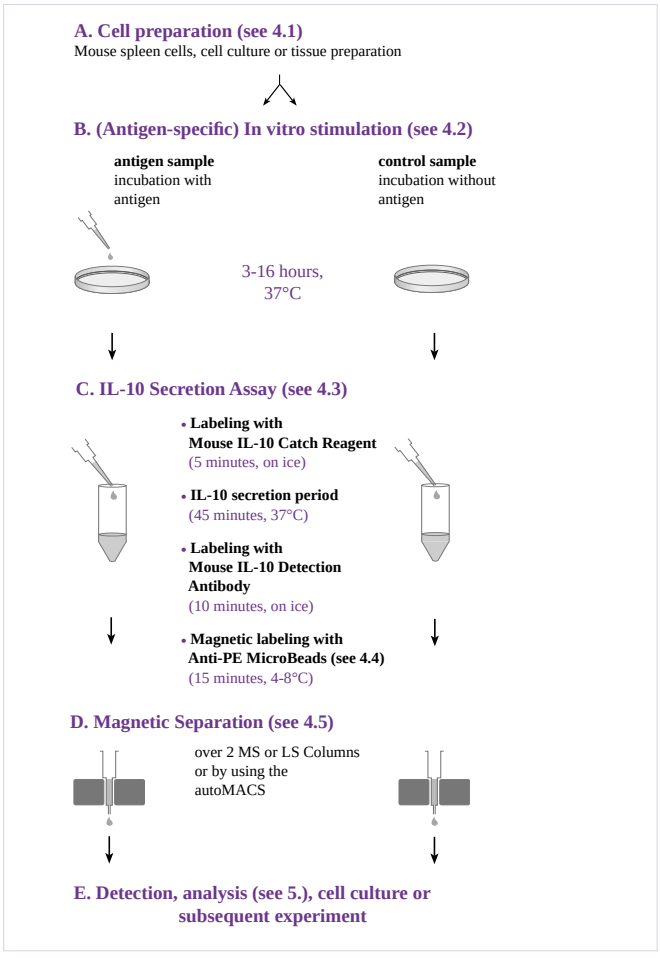
The Mouse IL-10 Secretion Assay is designed for the isolation, detection and analysis of viable IL-10 secreting murine leukocytes. It is especially useful for the detection and isolation of antigen-specific T cells after in vitro restimulation with specific antigen to induce secretion of IL-10.

IL-10 is predominantly secreted by human $CD4^+$ memory and effector T cells, and antigen-presenting cells, e.g. monocytes/macrophages. It is believed to have important suppressive functions on immune responses and may, for example, be involved in the maintenance of tolerance.

Quantitative analysis of antigen-specific T cell populations can provide important information on the natural course of immune responses. MACS enrichment of the antigen-specific T cells increases the sensitivity of analysis, allowing detection of frequencies as low as one in a million cells.
The MACS enrichment also enables further functional characterization of the antigen-specific cells and downstream experiments, as well as the expansion of antigen-specific cells allowing research on potential future immuno-therapeutical applications.

- ▲ Detection and enrichment of viable IL-10 secreting mouse leukocytes for phenotypic and functional characterization.
- ▲ Detection and enrichment of IL-10 secreting antigen-specific T cells for enumeration, expansion and phenotypic as well as functional characterization.
- ▲ Isolation and expansion of antigen-specific T cells for research in immunotherapy, for example for adoptive transfer experiments.

2. Protocol overview



3. Experimental set-up

3.1 Controls

Negative control

For accurate detection of antigen-specific cells secreting IL-10, a negative control sample should always be included. This will provide information about IL-10 secretion not related to the in vitro stimulation with the specific antigen, e.g. ongoing in vivo immune response. 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.
When working with immunized mice, it could be relevant to include an experiment analyzing cells of a nonimmunized mouse.

Positive control

When setting up a new experiment, it is recommended to include a positive control. As a positive control, a sample stimulated with the superantigen Staphylococcal Enterotoxin B (Sigma, St. Louis, USA) 1 µg/ml for 3-16 hours, may be included in the experiment.

▲ **Note:** Mitogens like PHA or PMA/Ionomycin are not recommended for stimulation of a positive control, as the resulting high frequencies of IL-10 secreting cells do not allow conclusions on the performance (e.g. sensitivity) of the Mouse IL-10 Secretion Assay.

3.2 Kinetics of restimulation and proposed time schedule

Restimulation with peptide

Upon stimulation with peptide, the cells can be analyzed for IL-10 secretion 3-6 hours after onset of stimulation.

Restimulation with protein

Upon stimulation with protein, the cells can be analyzed for IL-10 secretion 6-16 hours after onset of stimulation. Therefore you can start the stimulation of the cells late in the afternoon, and perform the IL-10 Secretion Assay the following morning.

Costimulation

The addition of costimulatory agents like CD28 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-10 secreting cells are stained with PE-conjugated IL-10 Detection Antibodies. To identify cells of interest, counterstaining for T cells with e.g. CD4-FITC or CD8-FITC is important.

▲ Do **not use** tandem conjugates of R-phycoerythrin and other fluorescent dyes that are often used in flow cytometry. They may also be recognized by the Anti-PE MicroBeads, e.g. Cy-Chrome (PharMingen), PE-Cy5 (Serotec), ECD, PC5 (Coulter-Immunotech).

▲ 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 nonspecific 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.

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3.4 Detection without prior enrichment

(Optional) If the sample contains more than 0.01-0.1% of IL-10 secreting cells, the analysis can also be performed without prior magnetic separation (see also: Mouse IL-10 Secretion Assay - Detection Kit (PE) # 130-090-489).

4. Protocol for the Mouse IL-10 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

▲ A **negative control** should **always,** and a **positive control** may be included in the experiment (see 3.1).

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

▲ Dead cells may bind nonspecifically to MACS MicroBeads. Therefore, dead cells should be removed before starting the Mouse IL-10 Secretion Assay, e.g. by density gradient centrifugation using Ficoll-Paque® or by using the Dead Cell Removal Kit (# 130-090-101).

Protocol for in vitro stimulation

1. Wash cells by adding medium, centrifuge at 200 x g for 10 minutes. Pipet off supernatant.
2. Resuspend cells in culture medium at 1×10^7 cells/ml and 5×10^6 cells/cm^2 (see 7. Appendix: Flask/dish sizes for stimulation) and incubate with antigen:

   peptide: 3-6 hours at 37°C, 7% CO_2, e.g. 1-10 µg/ml

   protein: 6-16 hours at 37°C, 7% CO_2, e.g. 10 µg/ml

   For comparison of different experiments, the stimulation time should be kept constant (see 3.).
3. 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. Control dish microscopically to check whether cells are left. 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-10 secreting cells. If ≥ 5 % of IL-10 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 avoids nonspecific staining of cells not secreting IL-10 during this period.

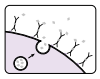
▲ For each test with 10^7 total cells, prepare 100 ml of **cold buffer** (4-8°C), 100 µl of **cold medium** (4-8°C) and 10 ml (or 100 ml; see table below) of **warm medium** (37°C).

▲ Avoid capping of antibodies on the cell surface during staining. **Work fast, keep cells cold, use cold solutions only** (exception: **warm medium** during secretion period).

▲ Higher temperatures and longer incubation times for staining and magnetic labeling should be avoided. This will lead to nonspecific cell labeling.

Labeling cells with Mouse IL-10 Catch Reagent

1. Use 10^7 total cells in a 15 ml closable test tube per sample. For larger cell numbers, scale up **all** volumes accordingly. For less than 10^7 cells, use same volumes.
2. Wash cells once by adding 10 ml of **cold buffer**, centrifuge at 300 x g for 10 minutes at **4-8°C**, pipet off supernatant.
3. Repeat wash step, pipet off supernatant **completely**.
4. Resuspend cell pellet in 80 µl of **cold medium** per 10^7 total cells. For fewer cells, use same volume.
5. Add 20 µl of **Mouse IL-10 Catch Reagent** per 10^7 total cells, mix well and incubate for 5 minutes **on ice**. For less than 10^7 total cells, use same volume.



IL-10 secretion period

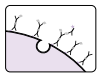
1. Add **warm** (37°C) medium to dilute the cells according to the following table:

Expected number of IL-10 secreting cells	Dilution	Amount of medium to add per 10^7 total cells
< 5 %	1×10^6 cells/ml	10 ml
≥ 5 %	≤ 1×10^5 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 by using the MACSmix (# 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 with Mouse IL-10 Detection Antibody

1. Put the tube **on ice.**
2. Wash the cell by filling up the tube with **cold buffer**, centrifuge at 300 x g for 10 minutes at **4-8°C**. Pipet off supernatant **completely**.
3. Repeat wash step, pipet off supernatant **completely**.
4. Resuspend cell pellet in 80 µl of **cold buffer** per 10^7 total cells. For fewer cells, use same volume.
5. Add 20 µl of **Mouse IL-10 Detection Antibody (PE)** per 10^7 total cells. For less than 10^7 total cells, use same volume.
6. (Optional) Add additional staining antibodies, e.g. CD4-FITC or CD8-FITC and CD45R/B220-PerCP.
7. Mix well and incubate for 10 minutes **on ice**.
8. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300 x g for 10 minutes at **4-8°C**. Pipet off supernatant.
9. Proceed to magnetic labeling (4.4).

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4.4 Magnetic labeling

Magnetic labeling with Anti-PE MicroBeads

1. Resuspend cell pellet in 80 µl of **cold buffer** per 10^7 total cells. For fewer cells, use same volume.
2. Add 20 µl of **Anti-PE MicroBeads** per 10^7 total cells, mix well and incubate for 15 minutes at **4-8° C**. For less than 10^7 total cells, use same volume.

   ▲ **Note:** Incubate in refrigerator at 4°–8°C, do not work on ice during this step.
3. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300 x g for 10 minutes at 4-8°C, pipet off supernatant.
4. Resuspend cell pellet in 500 µl of **cold buffer** per 10^7 cells, for higher cell numbers use a dilution of 10^8 cells/ml.
5. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
6. Proceed to magnetic separation (4.5).

4.5 Magnetic separation

Magnetic separation using MS or LS Columns

▲ **Note:** To choose suitable MACS Columns and MACS Separator, refer to table in 1.2.

▲ **Note:**When enriching antigen-specific T cells, **always perform two consecutive column runs** to achieve best results.

1. Prepare **two columns** per sample by rinsing with **cold buffer**:

   MS: 500 µl LS Column: 3 ml

   discard effluent.
2. Place the first column into the magnetic field of a MACS Separator (use column adapter with VarioMACS or SuperMACS).
3. (Optional) Pass the cells through 30 µm nylon mesh (Pre-Separation Filter # 130-041-407) to remove clumps.
4. Apply the magnetically labeled cells to the column, allow the cells to pass through the column. Wash with **cold buffer**:

   MS: 3 x 500 µl LS: 3 x 3 ml

   Collect effluent as unlabeled fraction.
5. Remove the first column from separator, place the second column into the separator, and put the first column on top of the second one. Pipet **cold buffer** on top of the first column:

   MS: 1 ml LS: 5 ml
6. **Firmly** flush out the retained cells from the first column, by using the plunger supplied with the column, directly onto the second column. Allow the cells to pass through the column.
7. Wash with **cold buffer:**

   MS: 3 x 500 µl LS: 3 x 3 ml
8. Remove the second column from separator, place the column on a suitable collection tube. Pipet **cold buffer** on top of the column:

   MS: 500 µl LS: 5 ml
9. **Firmly** flush out the retained cells by using the plunger supplied with the column.

   ▲ **Note**: For subsequent cell culture, the cells can also be eluted with medium. If part of the cells are analysed by flow cytometry, the medium should **not contain** phenol red.
10. Proceed to analysis (see 5.), cell culture or other subsequent experiment.

Magnetic separation using the autoMACS

1. Prepare and prime the autoMACS instrument according to the autoMACS User Manual.
2. (Optional) Pass cells through 30 µm nylon mesh (Pre-Separation Filter # 130-041-407) to remove clumps.
3. Apply magnetically labeled cells to the autoMACS. Choose the separation program POSSELD. Collect the separated fractions and proceed to analysis (see 5.), cell culture or other subsequent experiment.

5. Detection and analysis of IL-10 secreting T cells

▲ Add **propidium iodide** (PI) or 7-AAD to a final concentration of **0.5 µg/ml just prior** to acquisition for exclusion of 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**.

- Acquire 2×10^5 events from the fraction before enrichment (see 4.4 step 5.).
- For **enumeration** of low frequent IL-10 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-10 secreting T cells by using the Mouse IL-10 Secretion Assay. The detailed description, including how to set gates, may serve as a model for the analysis of your own sample.

1. BALB/c mice were intraperitoneally (i.p.) immunized with 100 µg Henn eggwhite lysozyme (HEL) in incomplete Freund´s adjuvant with 200 ng Pertussis Toxin. 200 ng Pertussis Toxin in PBS were i.p. injected again 24 hours later.
2. After 2 weeks 10^7 mouse spleen cells of the immunized mouse were incubated in vitro for 16 hours with or without 100 µg/ml HEL.
3. The Mouse IL-10 Secretion Assay was performed on the stimulated and the unstimulated sample.
4. **Counterstaining of T cells** was performed by using CD4-FITC.
5. **B lymphocytes** were stained with CD45R/B220-PerCP.
6. **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**.
7. 200,000 viable cells of the original fractions and the complete enriched fractions were acquired by flow cytometry, from the stimulated and the unstimulated samples.

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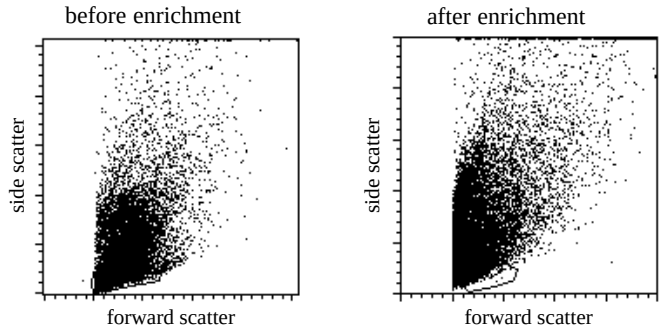
8. A **lymphocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude monocytes and debris.
9. Dead cells and B cells were excluded according to PI- and CD45R/B220-PerCP-staining in a fluorescence 2 versus fluorescence 3 plot.

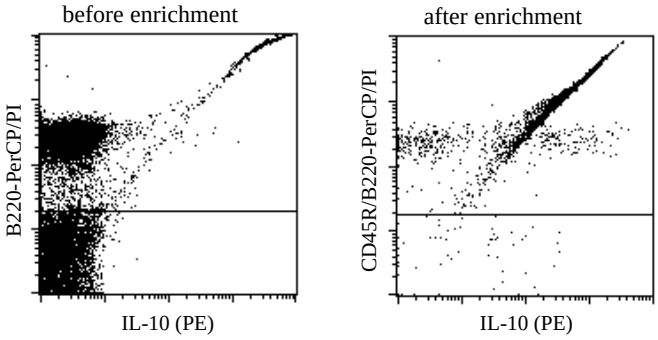
   The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as immunoglobulins or MicroBeads may bind nonspecifically to dead cells. This could lead to false positive events.

   The sensitivity of the detection will further be enhanced by exclusion of undesired non-T cells which may cause nonspecific background staining.
10. For analysis IL-10 (PE) versus CD4-FITC staining of viable lymphocytes is displayed.

1. Lymphocyte gate in the forward versus side scatter plot

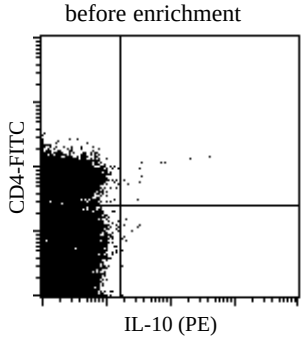


2. Dead cell gate and B cell exclusion in FL-2 versus FL-3 plot

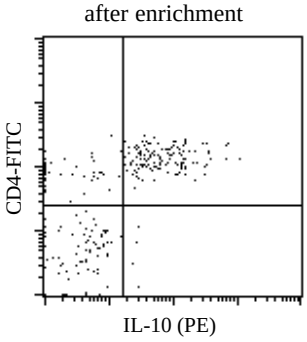


3. IL-10 secreting CD4$^+$ T cells

Splenocytes **restimulated with HEL** from a mouse **immunized with HEL**



0.041% of the total CD4$^+$ T cells population secrete IL-10 (see formula below).



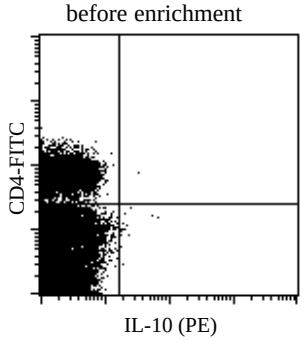
The IL-10 secreting CD4$^+$ T cells have been enriched to 51.0% in this sample.

144 IL-10$^+$CD4$^+$ T cells were enriched from 10^6 CD4$^+$ cells (= 0.0144%; see formula below).

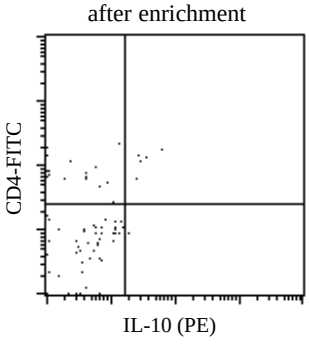
$$\%\ \text{IL-10}^+\ \text{cells}_{\text{among CD4}^+} = \frac{\#\ \text{of IL-10}^+\text{CD4}^+\ \text{cells in the analyzed sample}}{\#\ \text{of total CD4}^+\ \text{cells in the analyzed sample}} \times 100$$

$$\%\ \text{IL-10}^+\ \text{cells}_{\text{among CD4}^+} = \frac{\text{abs.}\ \#\ \text{of IL-10}^+\text{CD4}^+\ \text{cells in the enriched fraction}}{\text{abs.}\ \#\ \text{of total CD4}^+\ \text{cells before enrichment}} \times 100$$

Unstimulated splenocytes from a mouse **immunized with HEL**



0.003% of the total CD4$^+$ T cells population secrete IL-10.



7 IL-10$^+$ CD4$^+$ T cells were enriched from 10^6 CD4$^+$ cells (= 0.0007%).

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6. References

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

7. Appendix: Flask/dish sizes for stimulation

For (antigen-specific) stimulation (see 4.2), the cells should be resuspended in culture medium at 1×10^7 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 amounts of medium to add.

total cell number	buffer volume to add	culture plate	well diameter
0.15×10^7	0.15 ml	96 well	0.64 cm
0.5×10^7	0.5 ml	48 well	1.13 cm
1×10^7	1 ml	24 well	1.6 cm
2×10^7	2 ml	12 well	2.26 cm
5×10^7	5 ml	6 well	3.5 cm

total cell number	buffer volume to add	culture dish	dish diameter
4.5×10^7	4.5 ml	small	3.5 cm
10×10^7	10 ml	medium	6 cm
25×10^7	25 ml	large	10 cm
50×10^7	50 ml	extra large	15 cm

total cell number	buffer 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

Warranty

Products sold hereunder are warranted only to be free from defects in workmanship and material at the time of delivery to the customer. MILTENYI BIOTEC GmbH makes no warranty or representation, either expressed or implied, with respect to the fitness of a product for a particular purpose. There are no warranties, expressed or implied, which extend beyond the Technical Specifications of the products. MILTENYI BIOTEC GmbH´s liability is limited to either replacement of the products or refund of the purchase price. MILTENYI BIOTEC GmbH is not liable for any property damage, personal injury or economic loss caused by the product.

Warning

Reagents contain sodium azide. Sodium azide yields hydrazoic acid under acid conditions, which is extremely toxic. Azide compounds should be diluted with running water before being discarded. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

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