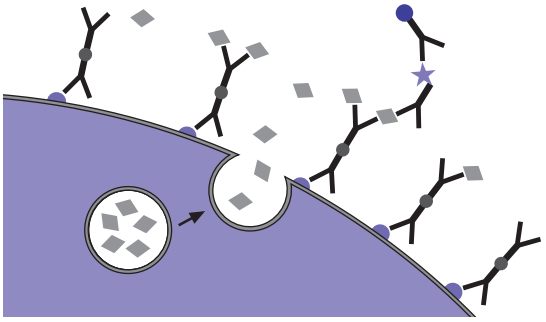
Miltenyi Biotec

Mouse IL-4 Secretion Assay – Cell Enrichment and Detection Kit (PE)

For 50 tests with 10^7 cells

Order no. 130-090-515



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**

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1. Description

Components

1 mL **Mouse IL-4 Catch Reagent**: anti-IL-4 monoclonal antibody (rat IgG1) conjugated to cell surface specific monoclonal antibody (rat IgG2b).

1 mL **Mouse IL-4 Detection Antibody**: anti-IL-4 monoclonal antibody (rat IgG2b) conjugated to PE (R-phycoerythrin).

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

Size

For 50 tests with 10^7 cells

Product format

Mouse IL-4 Catch Reagent and Mouse IL-4 Detection Antibody are supplied in a solution containing 0.1% gelatine and 0.05% sodium azide.

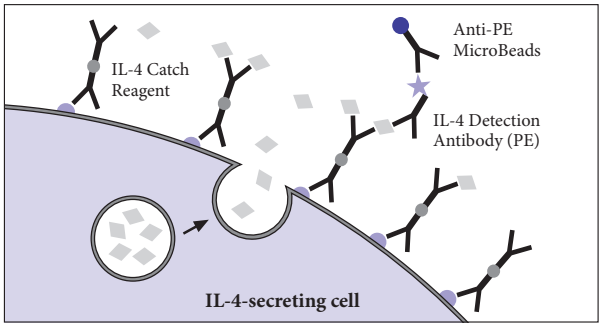
Storage

Store protected from light at 4–8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Principle of the Mouse IL-4 Secretion Assay

For analysis of murine antigen-specific T cells using the Mouse IL-4 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-4-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-4 binds to the IL-4 Catch Reagent



on the positive cells. These cells can subsequently be labeled with a second IL-4 specific antibody, the **Mouse IL-4 Detection Antibody** conjugated to phycoerythrin (PE) for sensitive detection by flow cytometry.

The IL-4-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 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, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background and product applications

The Mouse IL-4 Secretion Assay is designed for the isolation, detection and analysis of viable IL-4-secreting murine leukocytes. It is specially developed for the detection and isolation of antigen-specific T cells after *in vitro* restimulation with specific antigen to induce secretion of IL-4.

IL-4 is predominantly secreted by $CD4^+$ memory and effector T cells, basophils and mast cells. IL-4 especially induces and supports humoral responses, e.g. by its effects on activation, proliferation and antibody production by B cells.

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 immunotherapeutical applications.

Examples of applications

- Detection and enrichment of viable IL-4-secreting mouse leukocytes for phenotypic and functional characterization.
- Detection and enrichment of IL-4-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, e.g. for adoptive transfer experiments.

1.3 Reagent and instrument requirements

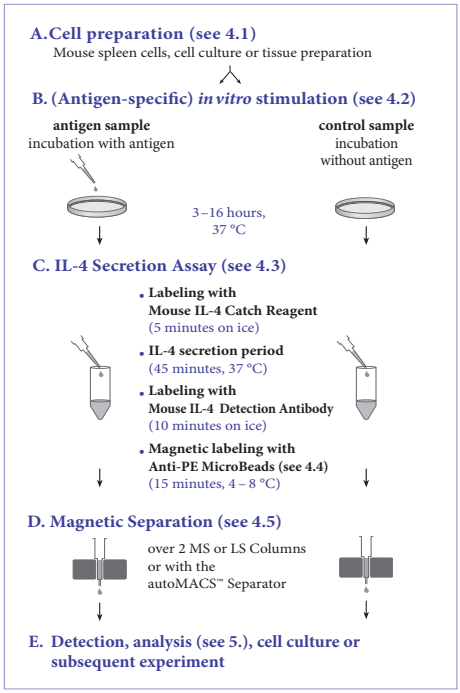
- **Buffer** (degassed): Prepare a solution containing PBS (phosphate buffered saline) pH 7.2, 0.5% BSA and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 in autoMACS™ Rinsing Solution (# 130-091-222).
- **Culture medium**, e.g., RPMI 1640 (# 130-091-440) containing 5% murine serum (do **not** use **BSA** or **FCS** because of non-specific 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.
- MACS Columns and MACS Separators:

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

▲ **Note:** Column adapters are required to insert certain columns into SuperMACS Separators. For details, see MACS Separator data sheets.

- Refrigerated centrifuge (4 – 8 °C).
- Rotation device for tubes: MACSmix™ tube rotator (# 130-090-753).
- (Optional) Pre-Separation Filter (# 130-041-407).

2. Protocol overview



3. Experimental set-up

3.1 Controls

Negative control

For accurate detection of antigen-specific cells secreting IL-4, a negative control sample should always be included. This will provide information about IL-4 secretion unrelated to the in vitro stimulation with the specific antigen, e.g. due to 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 non-immunized 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) 10 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-4 secretion 3–6 hours after onset of stimulation.

Proteins

Upon stimulation with protein, the cells can be analyzed for IL-4 secretion 6–16 hours later.

It is possible to start the stimulation of the cells late in the afternoon, and to perform the IL-4 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-4-secreting cells are stained with PE-conjugated Mouse IL-4 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 phycoerythrin, like Cy-Chrome® (PharMingen), PE-Cy5 (Serotec), ECD, PC5 (Coulter-Immunotech) etc., 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 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 Detection without prior enrichment

(Optional) If the sample contains more than 0.01– 0.1% of IL-4-secreting cells, the analysis can also be performed without prior magnetic enrichment (see also: Mouse IL-4 Secretion Assay - Detection Kit (PE) # 130-090-479).

4. Protocol for the Mouse IL-4 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, 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. Pipette off supernatant.
2. Resuspend cells in culture medium at 10^7 cells/mL and 5×10^6 cells/cm^2 (see 7. Appendix: Flask and dish sizes for stimulation).
3. Add antigen or control reagent:
   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
   SEB: 3 – 16 hours at 37 °C, 5–7% CO_2, e.g. 10 µg/mL
   For comparison of different experiments, the stimulation time should be kept constant (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 < 5% of total IL-4-secreting cells. If ≥ 5% of IL-4-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 non-specific staining of cells not secreting IL-4 during this period.

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▲ 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)
10 mL (or 100 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 10^7 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. Pipette off or 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-4 Secretion Assay, e.g. by density gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with Mouse IL-4 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**, centrifuge at 300×g for 10 minutes at **4 – 8 °C**, pipette off supernatant completely.
   ▲ **Note:** Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes.
3. Repeat wash step, pipette off supernatant completely.
4. Resuspend cell pellet in 80 µL of **cold medium** per 10^7 total cells.
5. Add 20 µL of **Mouse IL-4 Catch Reagent** per 10^7 total cells and incubate for 5 minutes **on ice**.

IL-4 secretion period

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

Expected number of IL-4 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 by 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.

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Labeling cells with Mouse IL-4 Detection Antibody

1. Put the tube **on ice**.
2. Wash the cell by filling up the tube with **cold buffer**, centrifuge at 300×g for 10 minutes at **4 – 8 °C**. Pipette off supernatant completely.
3. Repeat wash step, pipette off supernatant completely.
4. Resuspend cell pellet in 80 µL of **cold buffer** per 10^7 total cells.
5. Add 20 µL of **Mouse IL-4 Detection Antibody (PE)** per 10^7 total cells.
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×g for 10 minutes at **4 – 8 °C**. Pipette of supernatant.

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.
2. Add 20 µL of **Anti-PE MicroBeads** per 10^7 total cells, mix well and incubate for 15 minutes at **4 – 8 °C**.

   ▲ **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×g for 10 minutes at **4 – 8 °C**, pipette 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 (see 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 (see table in 1.3).

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

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1. Prepare **two columns** per sample by rinsing with **cold buffer**:
   MS: 500 µL LS Column: 3 mL
   and discard effluent.
2. Place the first column into the magnetic field of a MACS Separator (use column adapter with VarioMACS™ Separator or SuperMACS™ Separator).
3. (Optional) Pass the cells through Pre-Separation Filter (# 130-041-407) to remove clumps.
4. Apply cell suspension onto the column.
5. Collect unlabeled cells which pass through and wash with appropriate amount of cold buffer. Perform washing steps by adding buffer successively once the column reservoir is empty.
   MS: 3×500 µL LS: 3×3 mL
   Collect total effluent. This is the unlabeled cell fraction.
6. Remove the first column from separator, place the second column into the separator, and put the first column on top of the second one.
7. Pipette appropriate amount of cold buffer onto the first column. Immediately and firmly flush out fraction with the magnetically labeled cells using the plunger supplied with the column, directly onto the second column.
   MS: 1 mL LS: 5 mL
8. Collect unlabeled cells that pass through and wash with appropriate amount of cold buffer. Perform washing steps by adding buffer successively once the column reservoir is empty.

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MS: 3×500 µL LS: 3×3 mL

9. Remove the second column from separator, place the column on a suitable collection tube.
10. Pipette appropriate amount of cold buffer onto the column. Immediately flush out the fraction with the magnetically labeled cells by firmly applying the plunger supplied with the column.
    MS: 500 µL LS: 5 mL

    ▲ **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.
11. Proceed to analysis (see section 5.), cell culture or other subsequent experiment.

Magnetic separation using the autoMACS™ Separator

▲ Refer to the autoMACS™ User Manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime autoMACS Separator.
2. (Optional) Pass cells through Pre-Separation Filter (# 130-041-407) to remove clumps.
3. Place tube containing magnetically labeled cells in autoMACS Separator. Choose separation program "Posseld". Collect the separated fractions from outlet port "pos2".
4. Proceed to analysis (see section 5.), cell culture or other subsequent experiment.

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5. Detection and analysis of IL-4-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-4-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-4-secreting T cells by using the Mouse IL-4 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 was i.p. injected again 24 hours later.

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2. After 3 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-4 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.
8. 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.).
9. Dead cells and B cells were excluded according to PI- and CD45R/B220-PerCP™-staining in a fluorescence 2 versus fluorescence 3 plot (see B.).

   The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as immunoglobulins or MicroBeads may bind non-specifically 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 non-specific background staining.

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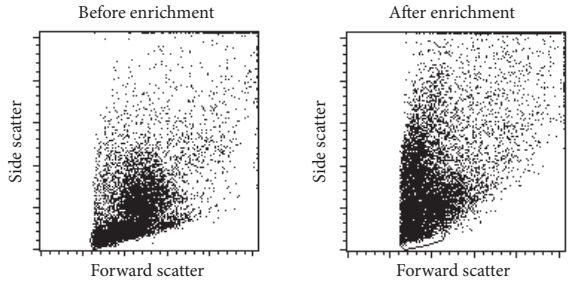
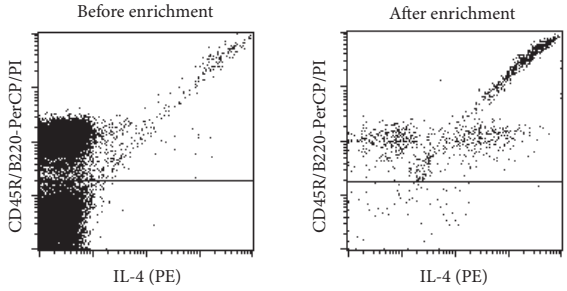
10. For analysis IL-4 (PE) versus CD4-FITC staining of viable lymphocytes is displayed (see C.).

A. Lymphocyte gate in the forward versus side scatter plot



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



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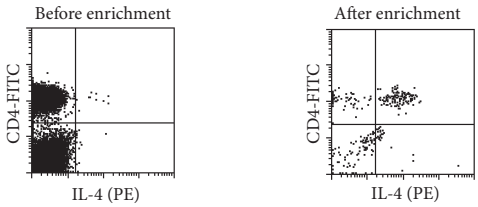
C. IL-4-secreting CD4⁺ T cells

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



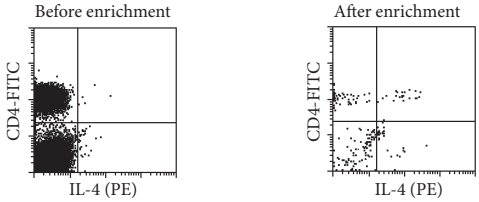
0.035% of the total $CD4^+$ T cell population secrete IL-4 (see formula below).

236 $IL\text{-}4^+CD4^+$ T cells were enriched from 10^6 $CD4^+$ cells (= 0.024%; see formula below). The IL-4-secreting $CD4^+$ T cells have been enriched to 40.6% in this sample.

$$\%\ IL\text{-}4^+\ \text{cells among}\ CD4^+ = \frac{\#\ \text{of}\ IL\text{-}4^+CD4^+\ \text{cells in the analyzed sample}}{\#\ \text{of total}\ CD4^+\ \text{cells in the analyzed sample}} \times 100$$

$$\%\ IL\text{-}4^+\ \text{cells among}\ CD4^+ = \frac{\text{abs.}\ \#\ \text{of}\ IL\text{-}4^+CD4^+\ \text{cells in the enriched fraction}}{\text{abs.}\ \#\ \text{of total}\ CD4^+\ \text{cells before enrichment}} \times 100$$

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



0.018% of the total $CD4^+$ T cell population secrete IL-4.

48 $IL\text{-}4^+CD4^+$ T cells were enriched from 10^6 $CD4^+$ cells (= 0.005%).

6. References

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

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7. Appendix: Flask and dish sizes for stimulation

For (antigen-specific) stimulation (see 4.2 step 2.) the cells should be resuspended in culture medium at 10^7 cells/mL and 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
0.5×10^7	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	medium 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	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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Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

Warnings

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

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