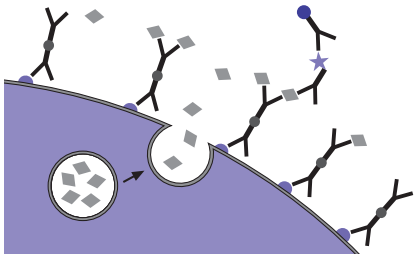
Miltenyi Biotec

Mouse IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE)

For 50 tests with 10^7 cells

Order no. 130-090-517



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140-000-474.06

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1. Description

Components

1 ml **Mouse IFN-γ Catch Reagent**: anti-IFN-γ monoclonal antibody (rat IgG1) conjugated to cell surface specific monoclonal antibody (rat IgG2b).

1 ml **Mouse IFN-γ Detection Antibody**: anti-IFN-γ monoclonal antibody (rat IgG1) conjugated to PE* (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

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

Storage

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

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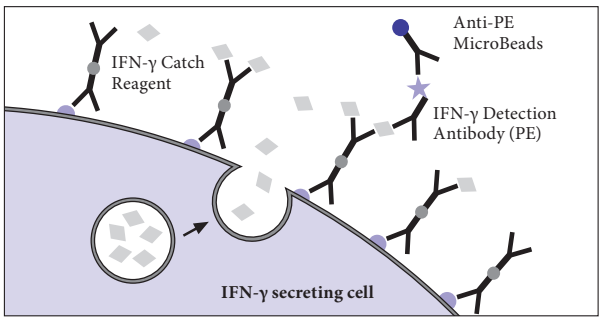


1.1 Principle of the Mouse IFN-γ Secretion Assay

For analysis of murine antigen-specific T cells using the Mouse IFN-γ 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 IFN-γ-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 IFN-γ binds to the IFN-γ Catch Reagent on the positive cells. These cells can subsequently be labeled with a second IFN-γ specific antibody, the **Mouse IFN-γ Detection Antibody** conjugated to phycoerythrin (PE) for sensitive detection by flow cytometry.

The IFN-γ 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 IFN-γ Secretion Assay is designed for the isolation, detection and analysis of viable IFN-γ 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 IFN-γ.

IFN-γ is predominantly secreted by activated $CD4^+$ and $CD8^+$ memory and effector T cells and by NK cells upon activation.

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 IFN-γ secreting mouse leukocytes for phenotypic and functional characterization.
- Detection and enrichment of IFN-γ 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): 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 dd H_2O. Add 146.2 g ethylene-diamine-tetraacetic acid, adjust pH to 7.5, fill up to 1000 ml with dd H_2O.

- **Culture medium**, e.g. RPMI 1640 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.

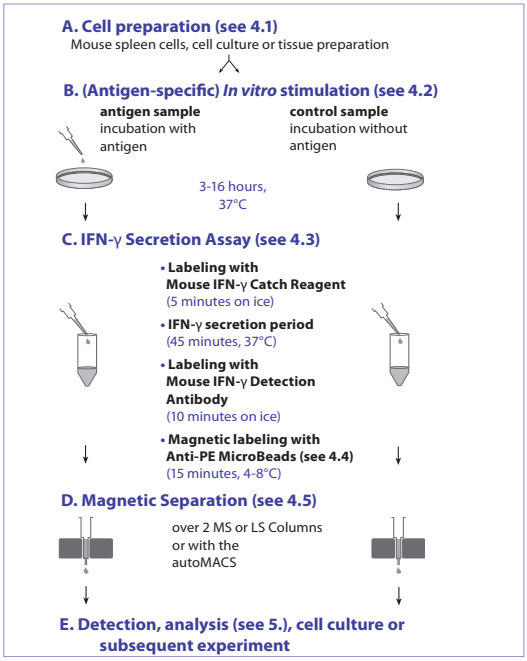
- MACS Columns and MACS Separators:

Column	max. number of labeled cells	max. number of total cells	Separator
MS	10^7	$2x10^8$	MiniMACS, OctoMACS; with Column Adapter: VarioMACS, SuperMACS
LS	10^8	$2x10^9$	MidiMACS; with Column Adapter: VarioMACS, SuperMACS
autoMACS	$2x10^8$	$4x10^9$	autoMACS

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

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

2. Protocol overview



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3. Experimental set-up

3.1 Controls

Negative control

For accurate detection of antigen-specific cells secreting IFN-γ, a negative control sample should always be included. This will provide information about IFN-γ secretion unrelated to the in vitro stimulation with the specific antigen, e.g. due to ongoing in vivo immune response (see section 5.). 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 (see section 5.).

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.

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

3.2 Kinetics of restimulation and proposed time schedule

Peptides

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

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Proteins

Upon stimulation with protein, the cells can be analyzed for IFN-γ secretion 6-16 hours later.

It is possible to start the stimulation of the cells late in the afternoon, and to perform the IFN-γ 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 IFN-γ secreting cells are stained with PE-conjugated Mouse IFN-γ 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.

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▲ 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 IFN-γ secreting cells, the analysis can also be performed without prior magnetic enrichment (see also: Mouse IFN-γ Secretion Assay - Detection Kit (PE) # 130-090-516).

4. Protocol for the Mouse IFN-γ 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.

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Protocol for *in vitro* stimulation

1. Wash cells by adding medium, centrifuge at 200xg for 10 minutes. Pipette off supernatant.
2. Resuspend cells in culture medium at 10^7 cells/ml and $5x10^6$ 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 < 2% of total IFN-γ secreting cells. If ≥ 2% of IFN-γ 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 IFN-γ 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)

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 less 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 $2x10^7$ 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 IFN-γ Secretion Assay, e.g. by density gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with Mouse IFN-γ 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 less than 10^7 cells, use same volumes.
2. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300xg 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 **cold medium** per 10^7 total cells and add **Mouse IFN-γ Catch Reagent (PE)** per 10^7 total cells, according to the following table:

Expected number of IFN-γ secreting cells	cold medium	Mouse IFN-γ 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**.

IFN-γ secretion period

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

Expected number of IFN-γ 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 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.

Labeling cells with Mouse IFN-γ Detection Antibody

1. Put the tube **on ice**.
2. Wash the cell by filling up the tube with **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**. Pipette off supernatant completely.
3. Repeat wash step, pipette off supernatant completely.

4. Resuspend cell pellet in **cold buffer** per 10^7 total cells and add **Mouse IFN-γ Detection Antibody (PE)** per 10^7 total cells, according to the following table:

Expected number of IFN-γ secreting cells	cold buffer	Mouse IFN-γ Detection Antibody
< 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. (Optional) Add additional staining antibodies, e.g. CD4-FITC or CD8-FITC and CD45R/B220-PerCP™.
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**. Pipette of supernatant completely.

4.4 Magnetic labeling

Magnetic labeling with Anti-PE MicroBeads

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

Expected number of IFN-γ secreting cells	cold buffer	Anti-PE MicroBeads
< 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.

3. 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.

4. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**, pipette off supernatant.
5. 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.
6. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
7. 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.

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 or SuperMACS).
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: 3x500 µl LS: 3x3 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.
   MS: 3x500 µl LS: 3x3 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

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

1. Prepare and prime autoMACS.
2. (Optional) Pass cells through Pre-Separation Filter (# 130-041-407) to remove clumps.
3. Place tube containing magnetically labeled cells in autoMACS. 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.

5. Detection and analysis of IFN-γ 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.

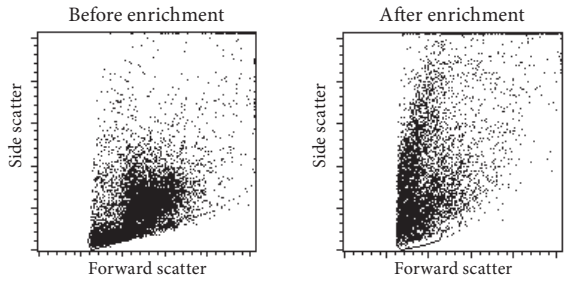
- Acquire $2x10^5$ events from the fraction before enrichment (see 4.4 step 5.).
- For **enumeration** of low frequent IFN-γ secreting cells, acquire all of the positive fraction. For **preparative purposes**, acquire an aliquot of the positive fraction to determine the performance of the cell enrichment.

To illustrate the analysis, we describe the detection of IFN-γ secreting T cells by using the Mouse IFN-γ Secretion Assay. The detailed description, including how to set gates, may serve as a model for the analysis of your own sample.

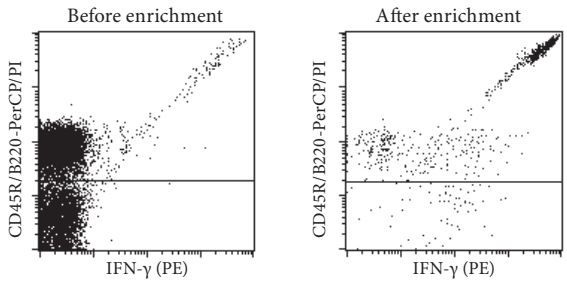
1. BALB/c mice were intraperitoneally (i.p.) immunized with 100 µg Henn eggwhite lysozyme (HEL) in incomplete Freund´s adjuvant with 200 ng Pertussis Toxin. 200 ng Pertussis Toxin in PBS was i.p. injected again 24 hours later.
2. After 3 weeks 10^7 mouse spleen cells of the immunized mouse and from an non-immunized control mouse were incubated in vitro for 16 hours with or without 100 µg/ml HEL.

3. The Mouse IFN-γ Secretion Assay was performed on the stimulated and the unstimulated sample from the HEL-immunized mouse, and on the stimulated sample from the non-immunized mouse.
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.
10. For analysis IFN-γ (PE) versus CD4-FITC staining of viable lymphocytes is displayed (see C.).

A. Lymphocyte gate in the forward versus side scatter plot

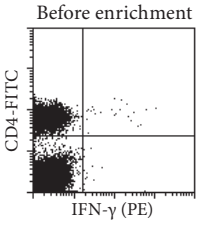


B. Dead cell and B cell exclusion in FL-2 versus FL-3 plot



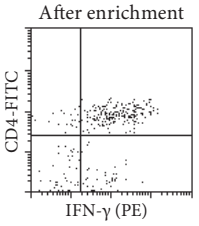
C. IFN-γ secreting $CD4^+$ T cells

Splenocytes restimulated with HEL from a mouse immunized with HEL



0.167% of the total $CD4^+$ T cell population secrete IFN-γ (see formula below).

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



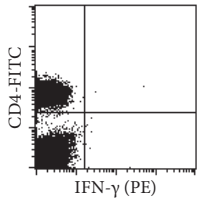
693 IFN-γ^+CD4$^+$ T cells were enriched from 10^6 CD4$^+$ cells (= 0.069%; see formula below).

The IFN-γ secreting $CD4^+$ T cells have been enriched to 67.0% in this sample.

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

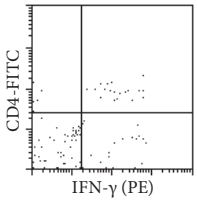
Unstimulated splenocytes from a mouse immunized with HEL

Before enrichment



0.018% of the total CD4⁺ T cell population secrete IFN-γ.

After enrichment

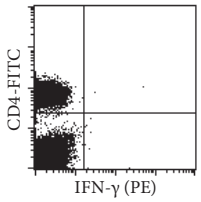


67 IFN-γ⁺CD4⁺ T cells were enriched from 10^6 CD4⁺ cells (= 0.007%).

This control sample indicates that there was still an ongoing in vivo immune reaction three weeks after immunization. This was further supported by samples of HEL-stimulated splenocytes (see below) and unstimulated splenocytes (data not shown) from non-immunized mice.

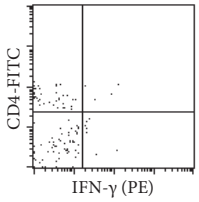
Splenocytes restimulated with HEL from a non-immunized mouse

Before enrichment



0.011% of the total CD4⁺ T cell population secrete IFN-γ.

After enrichment



8 IFN-γ⁺CD4⁺ T cells were enriched from 10^6 CD4⁺ cells (= 0.001%)

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6. References

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

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 $5x10^6$ 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 x 10^7	0.15 ml	96 well	0.64 cm
0.5 x 10^7	0.5 ml	48 well	1.13 cm
1 x 10^7	1 ml	24 well	1.6 cm
2 x 10^7	2 ml	12 well	2.26 cm
5 x 10^7	5 ml	6 well	3.5 cm

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

total cell number	medium volume to add	culture flask	growth area
12 x 10^7	12 ml	50 ml	25 cm²
40 x 10^7	40 ml	250 ml	75 cm²
80 x 10^7	80 ml	720 ml	162 cm²
120 x 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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