

Contents

1. Description
 - 1.1 Background information
 - 1.2 Applications
 - 1.3 Recommended antibody dilution
 - 1.4 Reagent requirements
2. Protocols
 - 2.1 Sample preparation
 - 2.2 *In vitro* stimulation of T cells
 - 2.3 Intracellular immunofluorescent cytokine staining protocols
 - 2.3.1 Intracellular staining of cells in suspension
 - 2.3.2 Intracellular staining in combination with magnetic cell separation (solid phase intracellular staining)
3. Examples of immunofluorescent staining with Anti-IFN- γ antibodies
4. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

1. Description

Components	1 mL Anti-IFN-γ antibodies, mouse conjugated to various dyes.
	PE 130-092-346
	APC 130-092-347
	or
	0.5 mL Anti-IFN-γ antibodies, mouse pure – functional grade 130-095-729
Clone	AN18.17.24 (isotype: rat IgG1).
Capacity	100 tests or up to 10^9 total cells. The functional grade antibody is supplied at a concentration of 1 mg/mL.
Product format	Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide. Functional grade antibodies are supplied in phosphate-buffered saline (PBS), pH 7.2. Endotoxin levels have been tested and do not exceed 0.01 ng/ μ g of protein. <i>The functional grade product contains no preservative and is sterile filtered; always handle under aseptic conditions.</i>
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

Interferon- γ (IFN- γ) is a cytokine predominantly produced by activated CD4⁺ and CD8⁺ memory and effector T cells and by NK cells upon activation. Quantitative analysis of IFN- γ -producing cells can provide important information on the course of immune responses.

Anti-IFN- γ antibodies are designed for intracellular staining of IFN- γ -producing cells. Cells can be stimulated for IFN- γ -production, for example, by polyclonal stimulation with mitogens. For induction of IFN- γ production by antigen-specific T cells, cells are restimulated with the respective antigen. IFN- γ can be accumulated in the cells by addition of secretion inhibitors like brefeldin A. After fixation and permeabilization of the cell sample, IFN- γ -producing cells can be stained intracellularly with Anti-IFN- γ antibodies. Staining of surface and activation markers allows simultaneous flow cytometric analysis of subsets and activation status of the IFN- γ -producing cells.

Magnetically enriched cells can be stained intracellularly for IFN- γ production directly on the MACS[®] Column. This procedure ensures higher sensitivity of detection and minimizes loss of cells during washing procedures. The protocol is very useful for cytokine analysis of rare cells (refer to protocol 2.3.2).

1.2 Applications

- Identification and enumeration of IFN- γ -producing cells upon polyclonal stimulation with mitogens by flow cytometry.
- Identification and enumeration of IFN- γ -producing antigen-specific T cells upon restimulation with the respective antigen.
- Monitoring of specificity of antigen-specific T cell lines.
- Analysis of cytokine production of rare cells by solid-phase staining technology, in combination with magnetic enrichment of cells (refer to protocol 2.3.2).
- The Anti-IFN- γ pure – functional grade antibody is suited for functional assays, for example, neutralization of IFN- γ activity.

1.3 Recommended antibody dilution

- Anti-IFN- γ antibodies should be used at a dilution of 1:10.

1.4 Reagent 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).

▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as mouse serum albumin, mouse serum, or fetal bovine serum (FBS). Buffers or media containing Ca²⁺ or Mg²⁺ are not recommended for use.

- Culture medium, e.g., RPMI 1640 (# 130-091-440) containing 5% mouse serum (do not use BSA or FBS because of non-specific stimulation!).
- Reagents for cell culture and stimulation, e.g., staphylococcal enterotoxin B (SEB), phorbol myristate acetate (PMA)/ionomycin, antigenic peptide, or protein for the stimulation of mouse T cells. For details refer to the respective data sheet. For more information about antigens refer to www.miltenyibiotec.com.
- Secretion inhibitor, e.g., brefeldin A.
- (Optional) Inside Stain Kit (# 130-090-477) for the fixation and permeabilization of cells.
- (Optional) Fluorochrome-conjugated antibodies for cell surface staining, e.g., CD4-FITC (# 130-091-608), CD4-PE (# 130-091-607), CD4-APC (# 130-091-611), CD8a-FITC (# 130-091-605), CD8a-PE (# 130-091-603), or CD8a-APC (# 130-091-606). For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Fluorochrome-conjugated antibodies for intracellular staining of activation markers, e.g., CD154-PE (# 130-092-106) or CD154-APC (# 130-092-105).

Additional requirements for intracellular cytokine staining in combination with magnetic cell separation (refer to protocol 2.3.2)

- MACS MicroBeads of choice, e.g., CD4 (L3T4) MicroBeads (# 130-049-201).
- MS Columns and suitable MACS Separator (MiniMACS™, OctoMACS™, VarioMACS™, or SuperMACS™ II Separator).
▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS or SuperMACS Separators. For details refer to the respective MACS Separator data sheet.
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.

2. Protocols

2.1 Sample preparation

When working with lymphoid organs, prepare a single-cell suspension using manual methods or the gentleMACS™ Dissociator.

For details refer to the protocols section at www.miltenyibiotec.com/protocols.

2.2 *In vitro* stimulation of T cells

▲ Always include a negative control in the experiment. The sample should be treated in exactly the same manner as the stimulated sample, except for the addition of the stimulus.

▲ A positive control should also be included in the experiment, for example, a sample stimulated with SEB or PMA/ionomycin.

▲ Do not use media containing any non-mouse proteins, such as BSA or FBS, because of non-specific stimulation.

1. Wash cells by adding medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant.
2. Resuspend cells at a density of 10⁷ per mL in culture medium containing 5% mouse serum. Plate cells in dishes at a density of 5×10⁶ cells/cm². For details refer to section 4. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells.
3. Add an antigen or control reagent:
 - 1–10 µg/mL peptide
 - 10–100 µg/mL protein
 - 10 µg/mL SEB
 - 20 ng/mL PMA and 1 µg/mL ionomycin
4. Incubate cells for 2 hours at 37 °C and 5% CO₂.
5. Add 1 µg/mL brefeldin A and incubate for an additional 4 hours at 37 °C and 5% CO₂.
6. Collect cells carefully by pipetting up and down when working with smaller volumes or by using a cell scraper. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

2.3 Intracellular immunofluorescent cytokine staining protocols

2.3.1 Intracellular staining of cells in suspension

▲ It is recommended to stain 10⁶ cells per sample. When working with up to 10⁷ 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 2×10⁷ nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Wash up to 10⁷ cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. (Optional) Stain cell surface antigens that are sensitive to fixation with appropriate antibodies according to the manufacturer's recommendations. Then wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10⁷ cells in 500 µL of buffer.
4. Add 500 µL of Inside Fix (Inside Stain Kit). Mix well and incubate for 20 minutes in the dark at room temperature.
5. Centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
6. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
▲ **Note:** Fixed cells may be stored in azide-containing buffer at 2–8 °C for up to 1 week.
7. (Optional) Stain cell surface antigens that are sensitive to permeabilization with appropriate antibodies according to the manufacturer's recommendations. Then wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
8. Wash cells by adding 1 mL of Inside Perm (Inside Stain Kit) and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
9. Resuspend cells in 90 µL of Inside Perm. Add 10 µL of the Anti-IFN-γ antibody.

10. (Optional) Add additional staining antibodies to the solution, for example, 10 μ L of CD4-FITC (# 130-091-608) and 10 μ L of CD154 antibodies.
 - ▲ **Note:** For efficient permeabilization upon intracellular staining the volume of Inside Perm should be at least 5 \times the volume of staining antibodies.
11. Mix well and incubate for 10 minutes in the dark at room temperature.
12. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300 \times g for 5 minutes. Aspirate supernatant carefully.
13. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy. Store cells at 2–8 $^{\circ}$ C in the dark until analysis. Mix well before flow cytometric acquisition.
 - ▲ **Note:** Samples may be stored at 2–8 $^{\circ}$ C in the dark for up to 24 hours.
 - ▲ **Note:** Do not use propidium iodide (PI) or 7-AAD staining.
6. Wash cells by adding 1–2 mL of buffer per 10⁷ cells and centrifuge at 300 \times g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cells in 500 μ L of buffer.
8. Place MS Column in the magnetic field of a suitable MACS Separator.
9. Prepare column by rinsing with 500 μ L of buffer.
10. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
11. Wash column with 3 \times 500 μ L of buffer. Collect unlabeled cells that pass through and combine with effluent from step 10.
12. Remove column from the separator and place it on a suitable collection tube.

2.3.2 Intracellular staining in combination with magnetic cell separation (solid phase intracellular staining)

▲ 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.

▲ Volumes for magnetic labeling given below are for up to 10⁷ total cells. When working with fewer than 10⁷ 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 \times 10⁷ total cells, use twice the volume of all indicated reagent volumes and total volumes).

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

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

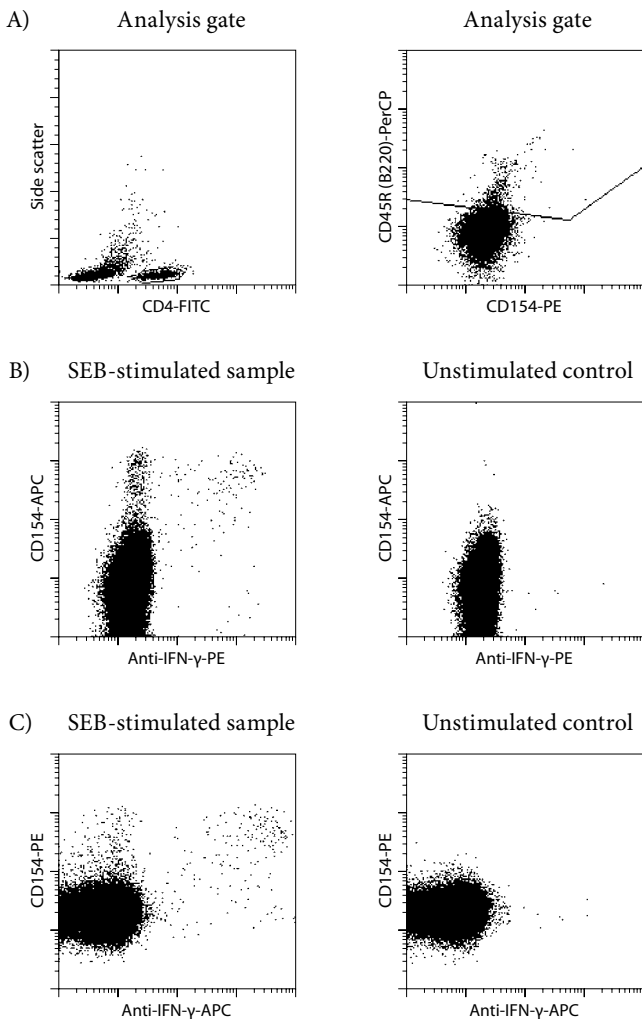
▲ Always wait until the column reservoir is empty before proceeding to the next step.

1. Wash cells by adding 1–2 mL of buffer per 10⁷ cells and centrifuge cell suspension at 300 \times g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 90 μ L of buffer per 10⁷ total cells.
3. Add 10 μ L of MACS MicroBeads, , e.g., CD4 MicroBeads (# 130-049-201,) per 10⁷ total cells.
 - ▲ **Note:** For details on the procedure refer to the respective MACS MicroBeads data sheet.
4. Mix well and incubate for 15 minutes in the refrigerator (2–8 $^{\circ}$ C).
5. (Optional) Counterstain cell surface antigens with antibodies that are sensitive to fixation according to the manufacturer's recommendations.

13. Pipette 500 μ L of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
14. Add 500 μ L of Inside Fix to the eluted cell fraction and incubate for 20 minutes at room temperature.
15. Place a second MS Column in the magnetic field of a suitable MACS Separator and prepare column by rinsing with 500 μ L of buffer.
16. Apply the fixed cell suspension onto the column.
17. Wash cells by rinsing the column with 1 \times 500 μ L of buffer, followed by 2 \times 500 μ L of Inside Perm.
18. Prepare a solution of 10 μ L of Anti-IFN- γ antibodies and 90 μ L of Inside Perm.
19. (Optional) Add additional staining antibodies to the solution, e.g., 10 μ L of CD4-FITC (# 130-091-608) and 10 μ L of CD154 antibodies.
 - ▲ **Note:** Do not exceed the total solution volume of 150 μ L.
20. Apply the solution onto the column and incubate for 10 minutes at room temperature.
 - ▲ **Note:** The MACS Column has a flow-stop mechanism that will retain the solution in the column.
21. Wash cells by rinsing the column with 2 \times 500 μ L of Inside Perm followed by 1 \times 500 μ L of buffer.
22. Remove column from the separator and place it on a suitable collection tube.
23. Pipette 500 μ L of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
24. Cells are now ready for analysis. Store cells at 2–8 $^{\circ}$ C in the dark until analysis. Mix well before flow cytometric acquisition.
 - ▲ **Note:** Samples may be stored at 2–8 $^{\circ}$ C in the dark for up to 24 hours.
 - ▲ **Note:** Do not use propidium iodide (PI) or 7-AAD staining.

3. Examples of immunofluorescent staining with Anti-IFN- γ antibodies

Mouse spleen cells were incubated with or without SEB for 6 hours. After 2 hours, brefeldin A was added. Cells were then fixed, permeabilized, and intracellularly stained with Anti-IFN- γ antibodies conjugated to PE (B) or APC (C) as well as with CD154-APC (# 130-092-105) or CD154-PE (# 130-092-106) and analyzed by flow cytometry. Cell surface staining was performed with CD4-FITC (# 130-091-608). Gating was performed according to CD4 expression and side scatter properties of the cells. Cell debris were excluded from the analysis in a FL-2 versus FL-3 dot plot.



5. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

For *in vitro* stimulation of T cells (refer to 2.2) the cells should be resuspended in culture medium, containing 5% of mouse 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 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

All protocols and data sheets are available at www.miltenyibiotec.com.

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.

Warranty

The 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.

autoMACS and MACS are registered trademarks and gentleMACS, MiniMACS, OctoMACS, SuperMACS, and VarioMACS are trademarks of Miltenyi Biotec GmbH.

Copyright © 2010 Miltenyi Biotec GmbH. All rights reserved.