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1. Description

Components	1 mL monoclonal Anti-IL-9 antibodies, mouse conjugated to various dyes.
	PE 130-096-721
	APC 130-096-723
Clone	RM9A4 (isotype: rat IgG1).
Capacity	100 tests or up to 10 ⁹ total cells.
Product format	Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

Interleukin-9 (IL-9) is secreted by mast cells and T-helper (TH) cells. First IL-9 production was associated to TH2 cells, but it has also been found in other TH subsets. Recently, evidence for the existence of a specialized lineage of TH cells producing IL-9, named TH9, came up. IL-9 has pleiotropic effects, e.g. it promotes mast cell growth and function, is involved in CD4⁺ T cell subset differentiation and growth, enhances IgE production, supports hematopoiesis, and acts on airway and gut epithelial cells.

1.2 Applications

- Identification and enumeration of IL-9-producing cells by flow cytometry or fluorescence microscopy.
- Identification and enumeration of IL-9-producing antigen-specific T cells upon stimulation with the respective antigen or detection of IL-9-producing T cells upon polyclonal stimulation.

- Analysis of cytokine production of rare cells by solid-phase staining technology, in combination with magnetic enrichment of cells. For details see section 2.3.2.

1.3 Recommended antibody dilution

- Anti-IL-9 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, for example, autologous AB serum (do not use BSA or FBS because of non-specific stimulation!).
- Secretion inhibitor, e.g., brefeldin A.
- (Optional) FcR Blocking Reagent, mouse (# 130-092-575) to avoid Fc receptor-mediated antibody labeling.
- (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), or CD4-VioBlue® (# 130-096-056). 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).
- (Optional) Fixation and Dead Cell Discrimination Kit (# 130-091-163) for cell fixation and flow cytometric exclusion of dead cells.

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 II 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, non-lymphoid tissues, or peripheral blood, prepare a single-cell suspension using manual methods or the gentleMACS™ Dissociator.

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

▲ Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).

2.2 Intracellular immunofluorescent cytokine staining protocols

2.2.1 Intracellular staining of cells in suspension

▲ It is recommended to stain 10^6 cells per sample. When working with up to 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 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Wash up to 10^7 cells by adding 1–2 mL of buffer and centrifuge at $300 \times g$ 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 $300 \times g$ for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^7 cells in 500 μ L of buffer.
4. Add 500 μ L of Inside Fix (Inside Stain Kit). Mix well and incubate for 20 minutes in the dark at room temperature.
5. Centrifuge at $300 \times g$ for 5 minutes. Aspirate supernatant carefully.
6. Wash cells by adding 1 mL of buffer and centrifuge at $300 \times g$ 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 $300 \times g$ for 10 minutes. Aspirate supernatant completely.
8. Wash cells by adding 1 mL of Inside Perm (Inside Stain Kit) and centrifuge at $300 \times g$ for 5 minutes. Aspirate supernatant carefully.
9. Resuspend cells in 90 μ L of Inside Perm. Add 10 μ L of the Anti-IL-9 antibody.
10. (Optional) Add additional staining antibodies to the solution, for example, 10 μ L of CD4-FITC (# 130-091-608).
▲ 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 °C in the dark until analysis. Mix well before flow cytometric acquisition.

▲ Note: Samples may be stored at 2–8 °C in the dark for up to 24 hours.

▲ Note: Do not use propidium iodide (PI) or 7-AAD staining.

2.2.2 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 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).

▲ 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 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

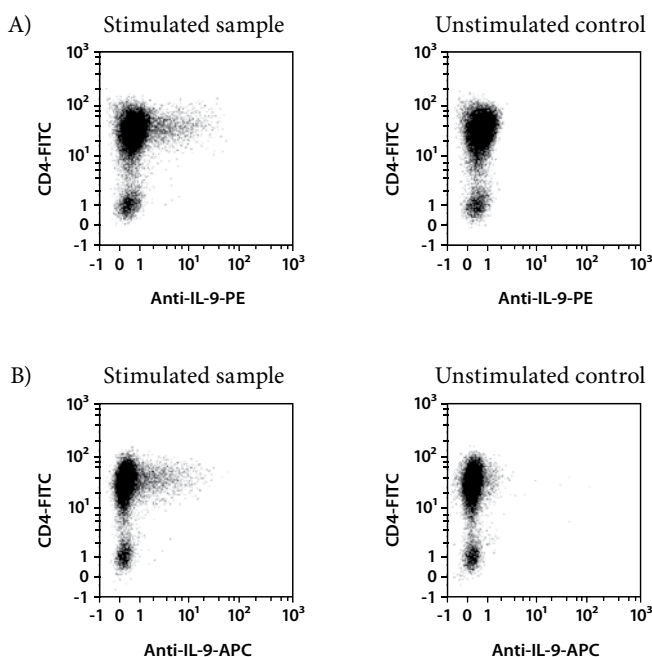
▲ Always wait until the column reservoir is empty before proceeding to the next step.

1. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge cell suspension at $300 \times g$ for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 80 μ L of buffer per 10^7 total cells.
3. Add 20 μ L of MACS MicroBeads, e.g., CD4 (L3T4) MicroBeads (# 130-049-201), per 10^7 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 °C).
5. (Optional) Counterstain cell surface antigens with antibodies that are sensitive to fixation according to the manufacturer's recommendations.
6. Wash cells by adding 1–2 mL of buffer per 10^7 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 3×500 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.

13. Pipette 500 μL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
14. Add 500 μL 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 μL of buffer.
16. Apply the fixed cell suspension onto the column.
17. Wash cells by rinsing the column with 1 \times 500 μL of buffer, followed by 2 \times 500 μL of Inside Perm.
18. Prepare a solution of 10 μL of Anti-IL-9 antibodies and 90 μL of Inside Perm.
19. (Optional) Add additional staining antibodies to the solution.
 - ▲ **Note:** Do not exceed the total solution volume of 150 μL .
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 μL of Inside Perm followed by 1 \times 500 μL of buffer.
22. Remove column from the separator and place it on a suitable collection tube.
23. Pipette 500 μL 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 °C in the dark until analysis. Mix well before flow cytometric acquisition.
 - ▲ **Note:** Samples may be stored at 2–8 °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-IL-9 antibodies

Naive CD4⁺ T cells were enriched (CD4⁺CD62L⁺ T Cell Isolation Kit II, mouse) from spleen cells of a BALB/c mouse. Cells were stimulated with plate-bound CD3 and soluble CD28 antibodies in the presence of recombinant TGF- β , recombinant IL-4, recombinant IL-2, and neutralizing Anti-IL-6 antibody, Anti-IFN- γ antibody and Anti-IL-12 antibody. On day 5 brefeldin A was added for 4 hours. The cells were harvested, fixed, permeabilized, and intracellularly stained with Anti-IL-9 antibodies conjugated to PE (A), or APC (B), as well as with CD4-FITC. Cells were analyzed by flow cytometry using the MACSQuant Analyzer. Gating was performed according to forward scatter and side scatter properties of the cells. Cell debris was excluded from the analysis in a FL-3 versus FL-4 dot plot.



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.

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