Miltenyi Biotec

Treg Analysis Kits, anti-mouse, REAfinity™

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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.

1. Description

This product is for research use only.

Products

Treg Analysis Kits, anti-mouse, REAfinity

Product	Order no.
Treg Analysis Kit, with FoxP3-PE, anti-mouse, REAfinity	130-120-674
Treg Analysis Kit, with FoxP3-Vio® R667, anti-mouse, REAfinity	130-120-671

Components

25 mL Fixation/Permeabilization Solution 1

2× 40 mL Fixation/Permeabilization Solution 2

40 mL 10× Permeabilization Buffer

200 µL CD4 Antibody, anti-mouse, VioBlue®, REAfinity (clone: REA604, isotype: recombinant human IgG1)

Additionally for Treg Analysis Kit, with FoxP3-PE, anti-mouse, REAfinity:

200 µL FoxP3 Antibody, anti-mouse, PE, REAfinity (clone: REA788, isotype: recombinant human IgG1).

200 µL CD25 Antibody, anti-mouse, APC, REAfinity (clone: REA568, isotype: recombinant human IgG1)

Additionally for Treg Analysis Kit, with FoxP3-Vio R667, anti-mouse, REAfinity:

200 µL FoxP3 Antibody, anti-mouse, Vio R667, REAfinity (clone: REA788, isotype: recombinant human IgG1).

200 µL CD25 Antibody, anti-mouse, PE, REAfinity (clone: REA568, isotype: recombinant human IgG1)

Capacity

100 tests or up to 10^8 total cells.

Product format

Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.

Storage

Store 10× Permeabilization Buffer at room temperature. Store all other reagents protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

The Treg Analysis Kits, anti-mouse, REAfinity have been developed for the convenient detection of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) by flow cytometry. Tregs are a subset of T cells with the ability to suppress harmful immunological reactions to self and foreign antigens. Due to the lack of a specific marker for Tregs, a combination of CD4, CD25, and FoxP3 antibodies is commonly used for their identification.

The Treg Analysis Kits, anti-mouse, REAfinity include all reagents necessary for cell surface staining of CD4 and CD25 and intracellular staining of FoxP3 together with a special staining buffer set and an optimized protocol.

The kit applies recombinantly engineered REAfinity Antibodies. REAfinity Antibodies are recombinant antibodies that provide superior lot-to-lot consistency and purity compared to mouse or rat hybridoma-derived, monoclonal antibodies. They have been recombinantly engineered to produce highly specific antibodies that require no FcR blocking step. Additionally, they all have the same IgG1 isotype, requiring less isotype controls.

1.2 Applications

- Identification and enumeration of $CD4^+CD25^+FoxP3^+$ Tregs by flow cytometry.
- Analysis of Tregs separated using MACS Technology by flow cytometry. Mouse $CD4^+CD25^+$ Tregs can be isolated using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse (# 130-091-041).

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1.3 Reagent and instrument 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 human serum albumin, human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- Flow cytometer equipped with a red (640 nm), a blue (488 nm), and a violet (405 nm) laser, e.g., MACSQuant® Analyzer 10 (# 130-096-343) or MACSQuant Analyzer 16 (# 130-109-803).
- Deionized or distilled water.
- (Optional) MACS Comp Bead Kit, anti-REA (# 130-104-693) for optimal compensation of the fluorescence spillover from fluorochrome-conjugated antibodies.

2. Protocol

2.1 Reagent preparation

▲ Always prepare reagents freshly. Failure to do so may lead to suboptimal results.

▲ The required total buffer volumes should be calculated beforehand; volumes will depend on the number of cells to be analyzed as well as the number of tests to be performed.

Fixation/Permeabilization Solution
To achieve the appropriate working concentration for safe fixation and permeabilization of cells, the Fixation/Permeabilization Solution 1 must be diluted 1:4 with the Fixation/Permeabilization Solution 2 (i.e. for 10^6 cells use 0.25 mL of Fixation/Permeabilization Solution 1 plus 0.75 mL of Fixation/Permeabilization Solution 2).

Permeabilization Buffer
To achieve the appropriate working concentration for safe permabilization of cells, the 10× Permeabilization Buffer must be diluted 1:10 with deionized or distilled water before use (i.e. 1 mL of 10× Permeabilization Buffer plus 9 mL of deionized or distilled water).

▲ **Note:** Before preparing the dilution make sure that buffer does not contain any precipitates.

2.2 Immunofluorescent staining of Tregs

▲ Volumes given below are for **up to 10^6** nucleated cells. When working with fewer than 10^6 cells, use the same volumes as indicated except for the fixation step due to the impact on cell morphology. When working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly (e.g. for 2×10^6 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ 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.

2.2.1 Surface staining of CD4 and CD25

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^6 nucleated cells per 96 µL of buffer.
4. Add 2 µL of CD4-VioBlue and 2 µL of the CD25-APC or CD25-PE, respectively.
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).

   ▲ **Note:** Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.
6. Wash cells by adding 1–2 mL of buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Aspirate supernatant completely.
7. Proceed immediately to 2.2.2.

2.2.2 Intracellular staining of FoxP3

1. Resuspend 10^6 nucleated cells in 1 mL of cold, freshly prepared Fixation/Permeabilization Solution.
2. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).
3. Wash cells by adding 1–2 mL of cold buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Aspirate supernatant completely.
4. Wash cells by adding 1–2 mL of cold 1× Permeabilization Buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Aspirate supernatant completely.
5. Resuspend up to 10^6 nucleated cells in 98 µL of cold 1× Permeabilization Buffer.
6. Add 2 µL of FoxP3 Antibody-PE or FoxP3-Vio R667, respectively.
7. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).
8. Wash cells by adding 1–2 mL of cold 1× Permeabilization Buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Aspirate supernatant completely.
9. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry.

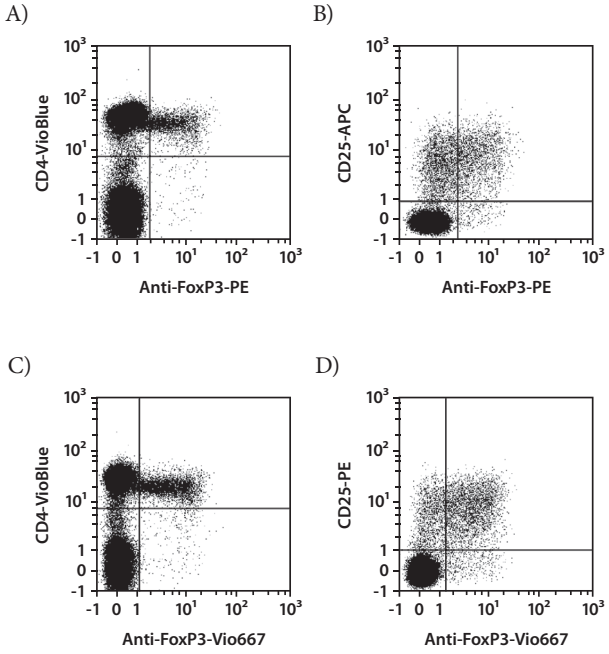
   ▲ **Note:** Due to fixation and permeabilization, cells can be smaller than viable cells. Thus, FSC/SSC settings of the flow cytometer might need to be adjusted.

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3. Example of immunofluorescent staining with the Treg Analysis Kits, anti-mouse, REAfinity

Mouse lymph nodes were prepared and stained with either the Treg Analysis Kit, with FoxP3-PE, anti-mouse, REAfinity (A, B) or with the Treg Analysis Kit, with FoxP3-Vio® R667, anti-mouse, REAfinity (C, D). Cells were stained with CD4-VioBlue and the respective CD25 antibody. Cells were then fixed, permeabilized, and stained with the respective FoxP3 antibody. Cells were analyzed by flow cytometry. Autofluorescent cell debris was excluded in an FL-2 versus FL-3 dot plot (data not shown). Gating was performed according to CD4 expression (except for the CD4/FoxP3 dot plot).



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