Miltenyi Biotec

Rapid Cytokine Inspector (CD4/CD8 T Cell) Kit
human

Order no. 130-097-343

Contents

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.

Components **1 mL CD4/CD8 T Cell Detection Cocktail, human:** Cocktail of fluorochrome-conjugated monoclonal anti-human antibodies containing CD3-VioBlue® (clone: BW264-56, isotype: mouse IgG2a), CD4-APC (clone: VIT4, isotype: mouse IgG2a), CD8-FITC (clone: BW135/80, isotype: mouse IgG2a), CD14-PerCP (clone: TÜK4, isotype: mouse IgG2a), CD20-PerCP (clone: LT20, isotype: mouse IgG1), and FcR Blocking Reagent.

2× 200 µL brefeldin A (100 µg/mL)

2.5 mL Inside Fix

2.5 mL Inside Perm

Capacity 100 tests or up to 10^8 total cells.

Product format Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide. Brefeldin A is supplied in buffer containing 10% DMSO. Inside Fix contains 3.7% formaldehyde. Inside Perm contains a detergent.

Storage Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Protocol overview

Stimulation	Plate 10^6 cells (100 µL) per well. Add antigen or control. After 2 hours of cultivation, add brefeldin A and continue cultivation for additional 4 hours.	
Cell surface staining	Pipette off 80 µL cell culture supernatant. Add 50 µL staining mix containing the CD4/CD8 T Cell Detection Cocktail and Rapid Cytokine Inspector Anti-Cytokine antibodies. Shake plate for 2 minutes and incubate for 8 minutes at room temperature.	45 minutes
Fixation	Add 25 µL Inside Fix. Shake plate for 2 minutes and incubate for 18 minutes at room temperature.	
Permeabilization and intracellular staining	Add 25 µL Inside Perm. Shake plate for 2 minutes and incubate for 8 minutes at room temperature.	
Washing step	Add 100 µL of buffer. Centrifuge plate for 5 minutes at 300×g. Remove supernatant and resuspend cells in 200 µL of buffer.	
Flow cytometric analysis	Proceed to sample aquisition and analysis.	

1.2 Principle of the Rapid Cytokine Inspector (CD4/CD8 T Cell) Kit

The Rapid Cytokine Inspector (CD4/CD8 T Cell) Kit, human, has been developed for the fast and easy evaluation of cytokine expression in activated T cells by intracellular staining. The use of 96-well cell culture plates enables the analysis on multiple samples in parallel. Washing steps after T cell stimulation, and before immunofluorescent staining, fixation, and permeabilization are not required.

The kit contains an antibody cocktail for the identification of $CD4^+$ and $CD8^+$ T cells and the exclusion of monocytes and B cells as well as brefeldin A and reagents for the fixation and permeabilization of cells after T cell stimulation. The kit is optimized for use in combination with Rapid Cytokine Inspector Anti-Cytokine antibodies of interest (not included in the kit).

Peripheral blood mononuclear cells (PBMCs) are cultured with or without antigen for a total of 6 hours. Upon activation, the cells start to secrete cytokines. After 2 hours of cultivation, brefeldin A is added to the cells to inhibit transport of proteins to the cellular membrane. After stimulation, the culture supernatant is removed, and the CD4/CD8 T Cell Detection Cocktail is added together with Rapid Cytokine Inspector Anti-Cytokine antibodies of interest. Cell surface markers are stained during a short incubation step.

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Fixation and permeabilization of the cells is performed and enables subsequent intracellular staining of cytokines. The cells are washed and analyzed by flow cytometry.

1.3 Background information

T lymphocytes execute and control immunological reactions with a repertoire of cytokines, cytotoxic substances, and other mediators. The quantitative and qualitative analysis of $CD4^+$ T cells and $CD8^+$ T cells specifically recognizing and reacting towards a defined antigen provide important information to understand their function in various immunological situations. Antigen-specific T cells can be identified and characterized by analyzing their effector function, e.g., production of cytokines.

1.4 Applications

- High-throughput multiparameter analysis of antigen-specific T cells.
- Rapid identification and enumeration of cytokine-producing activated antigen-specific $CD4^+$ and $CD8^+$ T cells upon *in vitro* stimulation with the respective antigen or upon polyclonal restimulation.
- Immunomonitoring of antigen-specific T cells.

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

- Anti-cytokine antibodies for intracellular staining: Rapid Cytokine Inspector Anti-IFN-γ-PE (# 130-097-600), Rapid Cytokine Inspector Anti-IL-2-PE (# 130-099-450), or Rapid Cytokine Inspector Anti-IL-17A-PE (# 130-097-599).

  ▲ **Note:** Use only Rapid Cytokine Inspector antibodies.

- Culture medium, e.g., RPMI 1640 containing 5% human serum, e.g., autologous or AB serum.

  ▲ **Note:** Do not use bovine serum albumin (BSA) or fetal bovine serum (FBS)

- Reagents for T cell stimulation, e.g., antigenic peptide, peptide pool, such as PepTivator® CMV pp65, human (# 130-093-438, # 130-093-435) or PepTivator CMV pp65 (HT), human (# 130-097-727), staphylococcal enterotoxin B (SEB), or protein. For more information about antigens refer to www.miltenyibiotec.com.
- 96-well flat-bottom cell culture plates for *in vitro* stimulation of T cells.
- Orbital shaker
- Flow cytometer, e.g., MACSQuant Analyzer 10 (# 130-096-343) or MACSQuant VYB (# 130-096-116) for flow cytometric analysis.

2. Protocols

2.1 Sample preparation

For optimal analysis of activated cytokine-producing T cells, perform assay with freshly prepared PBMCs or cell lines. Alternatively, frozen cell preparations can be used.

When working with anticoagulated peripheral blood or buffy coat, PBMCs should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

▲ **Note:** To remove platelets after density gradient centrifugation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

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

2.2 *In vitro* stimulation and immunofluorescent staining 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.

▲ **Note:** Do not use CytoStim for stimulation.

▲ Do not use media containing any non-human proteins, such as BSA or FBS to avoid non-specific stimulation.

▲ Do not use propidium iodide or 7-AAD staining.

▲ Avoid formation of air bubbles during the procedure, i.e., avoid extensive pipetting up and down of the samples.

1. Wash cells by adding culture medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant.
2. Resuspend cells at a density of 10^7 per mL in culture medium containing 5% human serum. Plate 1×10^6 cells per well in a 96-well plate.
3. Add antigen, for example, PepTivator CMV pp65, or control reagent in the appropriate concentration.

   ▲ **Note:** When using PepTivator – HT products, pipette 1×10^6 cells into each well. For details, refer to the respective data sheet.
4. Incubate cells for 2 hours at 37 °C and 5% CO_2.
5. Prepare 10 µL of a brefeldin A dilution for each well: Add 2 µL of brefeldin A to 8 µL of medium. Add 10 µL of the dilution to each well and incubate for an additional 4 hours at 37 °C and 5% CO_2.
6. Very carefully aspirate 80 µL of cell culture supernatant. Avoid resuspension of the cells.

   ▲ **Note:** The volume per well may vary, depending on the volume of the antigen added in step 3. The remaining volume per well should not exceed 40 µL.
7. Prepare 50 µL of staining mix for each well: Add 10 µL of the CD4/CD8 T Cell Detection Cocktail and 10 µL of fluorochrome-conjugated Rapid Cytokine Inspector Anti-Cytokine antibody to 30 µL of buffer.

   ▲ **Note:** When using more than one Rapid Cytokine Inspector Anti-Cytokine antibody conjugate, reduce amount of buffer accordingly.
8. Add 50 µL of staining mix to each well. Mix well for 2 minutes using an orbital shaker and incubate for 8 minutes in the dark at room temperature.

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9. Add 25 µL Inside Fix to each well. Mix well for 2 minutes using an orbital shaker and incubate for 18 minutes in the dark at room temperature.
10. Add 25 µL Inside Perm to each well. Mix well for 2 minutes using an orbital shaker and incubate for 8 minutes in the dark at room temperature.
11. Add 100 µL buffer to each well. Centrifuge cell culture plate at 300×g for 5 minutes. Aspirate supernatant very carefully.
12. (Optional) Add 200 µL buffer to each well. Centrifuge at 300×g for 5 minutes. Aspirate supernatant very carefully.
13. Add 200 µL of buffer to each well, resuspend the cells and perform flow cytometric analysis.

▲ **Note:** Cell culture plates can be stored over night at 2–8 °C in the dark until analysis. Mix well before flow cytometric analysis.

2.3 Fully automated flow analysis with the MACSQuant Analyzer 10 using the Express Mode

For fully automated flow cytometric analysis with the MACSQuant Analyzer 10, use the Express Mode **RCI_CD4CD8_h**. For details of how to use the Express Modes, please refer to the MACSQuant Instrument user manual, the MACSQuantify™ Software guide, or visit www.macsquant.com.

By selecting the Express Mode all experiment settings are automatically loaded. The loaded values are shown in the respective fields in the Experiment tab. These can be adapted manually if needed, for example, uptake volume.

For data analysis using other flow cytometers, follow the gating strategy provided in the example.

3. Examples of immunofluorescent staining with Rapid Cytokine Inspector (CD4/CD8 T Cell) Kit

Human PBMCs were incubated with or without PepTivator CMV pp65 for 6 hours; brefeldin A was added after 2 hours. The cells were stained with the CD4/CD8 T Cell Detection Cocktail and Rapid Cytokine Inspector Anti-IFN-γ-PE as described and analyzed by flow cytometry using the MACSQuant Analyzer.

Gating was performed with the following strategy. Gate P1: single cells were selected within a FSC-A/FSC-H dot plot (A). Gate P2: Cell debris, monocytes, and B cells were excluded from the single cells with a CD14- and CD20-PerCP/Rapid Cytokine Inspector Anti-IFN-γ-PE dot plot (B3/B2 channels) (B). Gate P3: Lymphocytes were selected with a FSC-A/SSC-A dot plot (C). Gate P4: $CD3^+$ T cells were gated in a CD3-VioBlue/Rapid Cytokine Inspector Anti-IFN-γ-PE dot plot (V1/B2 channels) (D). Quadrant gate 5: $CD8^+$ T cells and $CD4^+$ T cells were gated in the UL and LR quadrants, respectively (E).

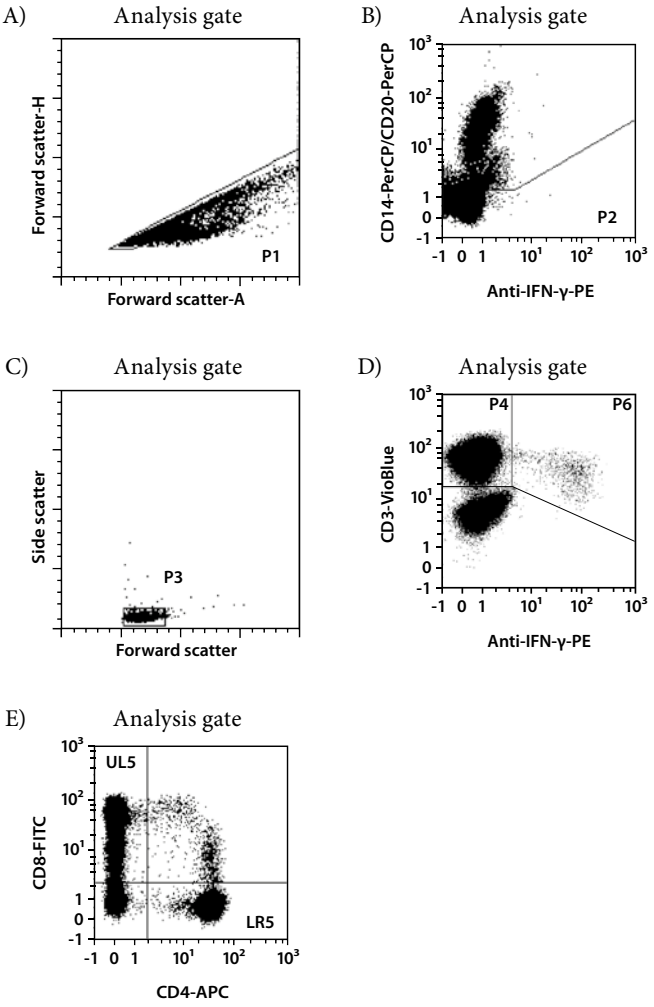
IFN-γ expression was analyzed for $CD4^+$ and $CD8^+$ T cells in stimulated and unstimulated samples. Dot plots of CD4-APC/Anti-IFN-γ-PE (R1/B2 channels) (F and G) or CD8-FITC/Anti-IFN-γ-PE (B1/B2 channels) (H and I) were used for analysis of IFN-γ-PE expression using quadrant gates 6 and 7, respectively.

Gating strategy

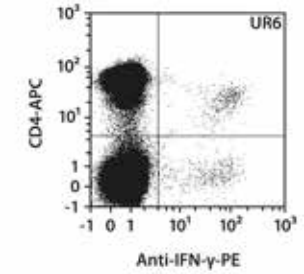
Population	Parameter/Label	Definition
Single cells	FSC-H/FSC-A	P1
$CD14^-CD20^-$ cells	CD14- and CD20-PerCP/ RCI Anti-IFN-γ-PE	P1/P2
Lymphocytes	FSC-A/SSC-A	P1/P2/P3
$CD3^+$ T cells	CD3-VioBlue/ RCI Anti-IFN-γ-PE	P1/P2/P3/P4/P6
$CD8^+$ T cells	CD8-FITC/CD4-APC	UL5
$CD4^+$ T cells	CD8-FITC/CD4-APC	LR5

Analysis

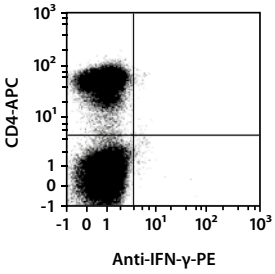
Population	Parameter/Label	Definition
IFN-γ^+ cells among $CD4^+$ T cells	CD4-APC/ RCI Anti-IFN-γ-PE	UR6
IFN-γ^+ cells among $CD8^+$ T cells	CD8-FITC/ RCI Anti-IFN-γ-PE	UR7



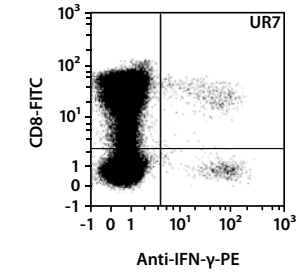
F) Stimulated sample



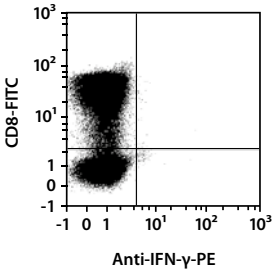
G) Unstimulated control



H) Stimulated sample



I) Unstimulated control



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.

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