

Contents

1. Description
 - 1.1 Protocol overview
 - 1.2 Principle of the Rapid Cytokine Inspector (CD8 T Cell) Kit
 - 1.3 Background information
 - 1.4 Applications
 - 1.5 Reagent requirements
2. Protocols
 - 2.1 Sample preparation
 - 2.2 *In vitro* stimulation and immunofluorescent staining of T cells
 - 2.3 Fully automated flow analysis with the MACSQuant® Analyzer 10 using the Express Mode
3. Examples of immunofluorescent staining with the Rapid Cytokine Inspector (CD8 T Cell) Kit

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 CD8 T Cell Detection Cocktail, human: Cocktail of fluorochrome-conjugated monoclonal anti-human antibodies containing CD3-VioBlue® (clone: BW264-56, isotype: mouse IgG2a), CD8-FITC (clone: BW135/80, isotype: mouse IgG2a), CD14-PerCP (clone: TÛK4, 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.	45 minutes
Cell surface staining	Pipette off 80 µL cell culture supernatant. Add 50 µL staining mix containing the 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.	
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 acquisition and analysis.	

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

The Rapid Cytokine Inspector (CD8 T Cell) Kit, human has been developed for the fast and easy evaluation of cytokine expression in activated CD8⁺ 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 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, CD8⁺ T cells start to produce 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 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. 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 CD8⁺ T cells specifically recognizing and reacting towards a defined antigen provide important information to understand their function in various immunological situations. Antigen-specific CD8⁺ 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 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 Ca²⁺ or Mg²⁺ are not recommended for use.
- Anti-cytokine antibodies for intracellular staining: 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 10⁷ per mL in culture medium containing 5% human serum. Plate 1×10⁶ 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 1×10⁶ cells into each well. For details, refer to the respective data sheet.
4. Incubate cells for 2 hours at 37 °C and 5% CO₂.
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% CO₂.
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 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.

9. Add 25 μ L 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 μ L 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 μ L buffer to each well. Centrifuge cell culture plate at 300 \times g for 5 minutes. Aspirate supernatant very carefully.
12. (Optional) Add 200 μ L buffer to each well. Centrifuge at 300 \times g for 5 minutes. Aspirate supernatant very carefully.
13. Add 200 μ L 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_CD8_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 (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 CD8 T Cell Detection Cocktail, Rapid Cytokine Inspector Anti-IFN- γ -APC, and Rapid Cytokine Inspector Anti-IL-2-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/RCI Anti-IL-2-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-IL-2-PE dot plot (V1/B2 channels) (D). Gate P5: CD8⁺ T cells were gated in a CD8-FITC/RCI Anti-IL-2-PE dot plot (B1/B2) (E).

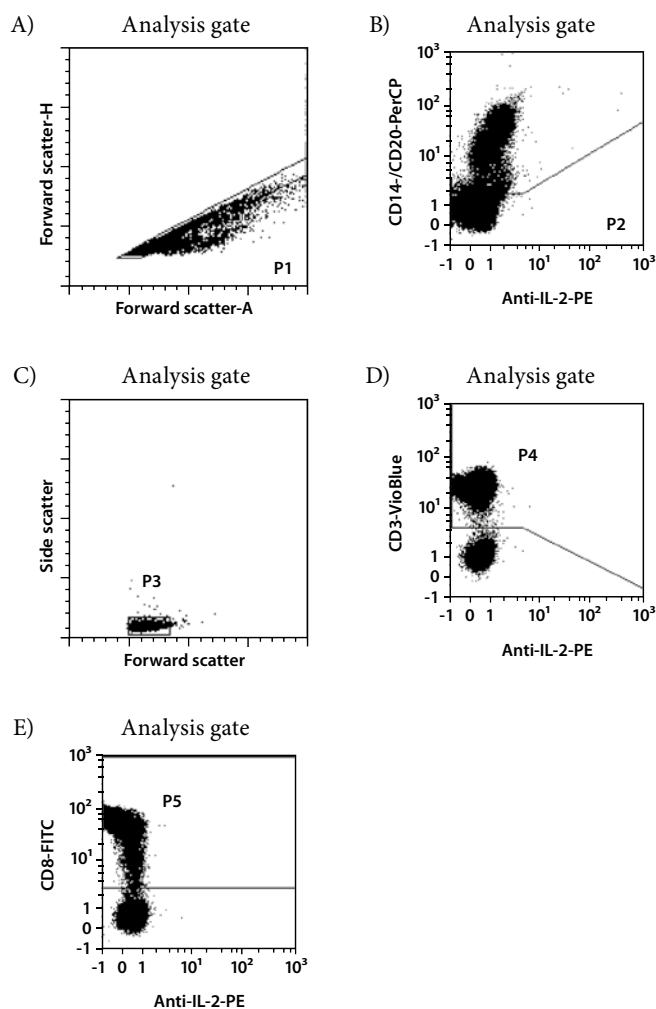
IFN- γ expression was analyzed for CD8⁺ T cells in stimulated and unstimulated samples. Dot plots of RCI Anti-IFN- γ -APC/RCI Anti-IL-2-PE (R1/B2 channels) (F and G) were used for analysis of IFN- γ and IL-2 expression using quadrant gate 6.

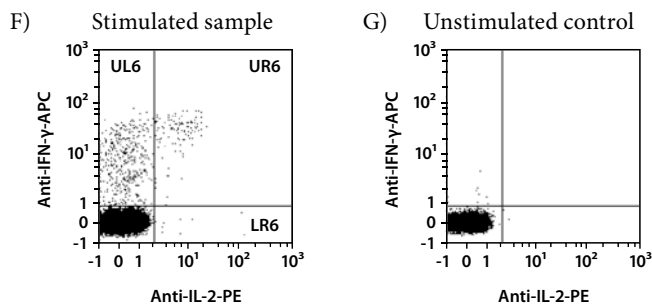
Gating strategy

Population	Parameter/Label	Definition
Single cells	FSC-H/FSC-A	P1
CD14 ⁻ CD20 ⁻ cells	CD14- and CD20-PerCP/ RCI Anti-IL-2-PE	P1/P2
Lymphocytes	FSC-A/SSC-A	P1/P2/P3
CD3 ⁺ T cells	CD3-VioBlue/ RCI Anti-IL-2-PE	P1/P2/P3/P4
CD8 ⁺ T cells	CD8-FITC/ RCI Anti-IL-2-PE	P1/P2/P3/P4/P5

Analysis

Population	Parameter/Label	Definition
IFN- γ ⁺ cells among CD8 ⁺ T cells	RCI Anti-IFN- γ -APC/ RCI Anti-IL-2-PE	UR6 + UL6
IL-2 ⁺ among CD8 ⁺ T cells	RCI Anti-IFN- γ -APC/ RCI Anti-IL-2-PE	UR6 + LR6
IL-2 ⁺ /IFN- γ ⁺ cells among CD8 ⁺ T cells	RCI Anti-IFN- γ -APC/ RCI Anti-IL-2-PE	UR6
IL-2 ⁻ /IFN- γ ⁻ cells among CD8 ⁺ T cells	RCI Anti-IFN- γ -APC/ RCI Anti-IL-2-PE	LR6
IL-2 ⁻ /IFN- γ ⁺ cells among CD8 ⁺ T cells	RCI Anti-IFN- γ -APC/ RCI Anti-IL-2-PE	UL6





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.

Legal notices

Limited product warranty

Miltenyi Biotec B.V. & Co. KG and/or its affiliate(s) warrant this product to be free from material defects in workmanship and materials and to conform substantially with Miltenyi Biotec's published specifications for the product at the time of order, under normal use and conditions in accordance with its applicable documentation, for a period beginning on the date of delivery of the product by Miltenyi Biotec or its authorized distributor and ending on the expiration date of the product's applicable shelf life stated on the product label, packaging or documentation (as applicable) or, in the absence thereof, ONE (1) YEAR from date of delivery ("Product Warranty"). Miltenyi Biotec's Product Warranty is provided subject to the warranty terms as set forth in Miltenyi Biotec's General Terms and Conditions for the Sale of Products and Services available on Miltenyi Biotec's website at www.miltenyibiotec.com, as in effect at the time of order ("Product Warranty"). Additional terms may apply. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.

THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING IF A PRODUCT IS SUITABLE FOR CUSTOMER'S PARTICULAR PURPOSE AND APPLICATION METHODS.

Technical information

The technical information, data, protocols, and other statements provided by Miltenyi Biotec in this document are based on information, tests, or experience which Miltenyi Biotec believes to be reliable, but the accuracy or completeness of such information is not guaranteed. Such technical information and data are intended for persons with knowledge and technical skills sufficient to assess and apply their own informed judgment to the information. Miltenyi Biotec shall not be liable for any technical or editorial errors or omissions contained herein.

All information and specifications are subject to change without prior notice. Please contact Miltenyi Biotec Technical Support or visit www.miltenyibiotec.com for the most up-to-date information on Miltenyi Biotec products.

Licenses

This product and/or its use may be covered by one or more pending or issued patents and/or may have certain limitations. Certain uses may be excluded by separate terms and conditions. Please contact your local Miltenyi Biotec representative or visit Miltenyi Biotec's website at www.miltenyibiotec.com for more information.

The purchase of this product conveys to the customer the non-transferable right to use the purchased amount of the product in research conducted by the customer (whether the customer is an academic or for-profit entity). This product may not be further sold. Additional terms and conditions (including the terms of a Limited Use Label License) may apply.

CUSTOMER'S USE OF THIS PRODUCT MAY REQUIRE ADDITIONAL LICENSES DEPENDING ON THE SPECIFIC APPLICATION. THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING FOR ITSELF WHETHER IT HAS ALL APPROPRIATE LICENSES IN PLACE. Miltenyi Biotec provides no warranty that customer's use of this product does not and will not infringe intellectual property rights owned by a third party. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.

Trademarks

autoMACS, MACS, MACSQuant, MACSQuantify, the Miltenyi logo, PepTivator, and VioBlue are registered trademarks or trademarks of Miltenyi Biotec and/or its affiliates in various countries worldwide.

Ficoll-Paque is a trademark of GE Healthcare companies.

Copyright © 2021 Miltenyi Biotec and/or its affiliates. All rights reserved.