Miltenyi Biotec

Antigen-specific T Cell Analysis Kit (Whole Blood), anti-human, REAfinity®

Order no. 130-138-372

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1. Description

This product is for research use only.

Components

200 µL CD3 Antibody, anti-human, APC, REAfinity (clone: REA613, isotype: recombinant human IgG1)

200 µL CD4 Antibody, anti-human, Vio® Bright B515, REAfinity (clone: REA623, isotype: recombinant human IgG1)

200 µL CD8 Antibody, anti-human, VioGreen™, REAfinity (clone: REA734, isotype: recombinant human IgG1)

200 µL IFN-γ Antibody, anti-human, PE, REAfinity (clone: REA600, isotype: recombinant human IgG1)

200 µL TNF-α Antibody, anti-human, PE-Vio 770, REAfinity (clone: REA656, isotype: recombinant human IgG1)

200 µL CD14 Antibody, anti-human, VioBlue®, REAfinity (clone: REA599, isotype: recombinant human IgG1)

200 µL CD20 Antibody, anti-human, VioBlue, REAfinity (clone: REA780, isotype: recombinant human IgG1)

200 µL CD154 Antibody, anti-human, APC-Vio 770, REAfinity (clone: REA238, isotype: recombinant human IgG1)

200 µL IL-2 Antibody, anti-human, PE-Vio 615, REAfinity (clone: REA689, isotype: recombinant human IgG1)

2×200 µL CytoStim™, human

25 mL Inside Fix

2×105 mL Inside Perm

2 mL Brefeldin A (100 µg/mL)

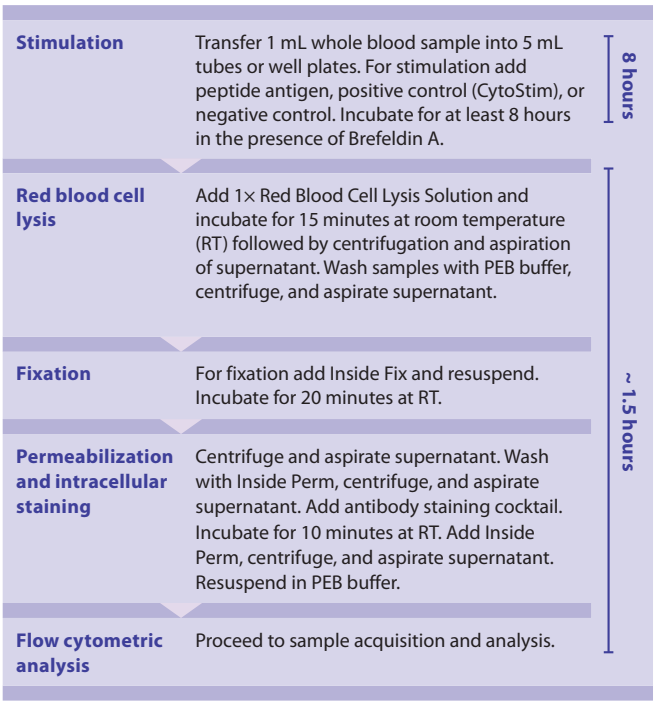
50 mL Red Blood Cell Lysis Solution (10×)

Capacity 100 tests for up to 100 mL whole blood.

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. CytoStim is supplied in buffer containing stabilizer.

Storage Store protected from light at +2 to +8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Protocol overview



Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macsde@miltenyi.com
www.miltenyibiotec.com

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1.2 Principle

The Antigen-specific T Cell Analysis Kit (Whole Blood), anti-human, REAfinity has been developed for the fast and easy detection of antigen-specific T cells by intra- and extracellular staining of activation markers and cytokines directly from whole blood samples. The kit contains antibodies for the identification of $CD4^+$ and $CD8^+$ T cells, the exclusion of monocytes and B cells as well as the staining of activation markers and cytokines. Furthermore, a positive control (CytoStim), Brefeldin A, Red Blood Cell Lysis Solution for removal of erythrocytes, and reagents for the fixation and permeabilization of cells after stimulation (Inside Fix and Inside Perm) are included. The optimized antibody panel and protocol ensure a comprehensive and efficient analysis of antigen-reactive T cells in minimal amount of whole blood.
The kit contains recombinantly engineered REAfinity Antibodies. REAfinity Antibodies provide superior lot-to-lot consistency and purity compared to mouse or rat hybridoma-derived monoclonal antibodies. They have been designed to carry a mutated Fc part and do not require an FcR blocking step. Furthermore, REAfinity Antibodies are all based on human IgG1 and require only one common isotype control.

1.3 Background information

T lymphocytes execute and control immunological reactions with a repertoire of specific T cell receptors, 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 provides important information to understand their function in various immunological responses. The presence and function of antigen-specific T cells allows conclusions about disease progression, severity, and immune status, e.g., in cancer, infection, and autoimmunity. Antigen-reactive T cells can thereby be identified and characterized by analyzing their effector function, e.g., upregulation of activation markers and production of cytokines.

1.4 Applications

- Rapid detection of $CD4^+$ and $CD8^+$ T cells reactive towards any antigen of interest
- Phenotypical characterization of activated T cells after stimulation with PepTivator® Peptide Pools or MACSpep Single Peptides by flow cytometry
- Immune monitoring of antigen-specific T cells
- Research and monitoring of infection- and vaccine-specific T cell responses in individuals

1.5 Reagent and instrument requirements

- Antigenic peptide pool (PepTivator) or single epitope peptides (MACSpep Single Peptides) of interest for peptide-based stimulation of T cells
- Phosphate-buffered saline (PBS), pH 7.2, without azide, protein, or other amine-containing compounds
- PEB 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 to +8 °C). Alternatively, use autoMACS Running Buffer (# 130-091-221).

  ▲ **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.
- Double-distilled water (ddH_2O)
- Cell culture–grade DMSO
- 5 mL tubes or a polypropylene deep well plate with round bottom with a minimal volume of 3.5 mL per well
- 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)
- (Optional) MACS Comp Bead Kit, anti-REA (# 130-104-693) for optimal compensation of the fluorescence spillover from fluorochrome-conjugated antibodies.
- (Optional) StemMACS™ Cryo-Brew (# 130-109-558)
- (Optional) PepTivator CEF MHC Class I Plus – premium grade (# 130-098-426) as a peptide-specific positive control.
- (Optional) PepTivator Negative Control – premium grade (# 130-131-610, # 130-131-612) as a matrix-specific control reagent.

2. Protocol

▲ For a detailed gating strategy refer to the product page at **www.miltenyibiotec.com/130-138-372**.

2.1 Reagent preparation

Preparation of stimulant

For peptide-based stimulation of antigen-specific T cells, it is recommended using MACSpep single peptides or PepTivator peptide pools. For appropriate preparation of the peptide stimulant please follow the instructions below.

▲ For dissolving PepTivator peptide pools please refer to the respective data sheet of each product. It is recommended to use for the first experiments a concentration of 30 nmol/mL for the working solution, resulting in a 0.6 nmol/mL assay concentration.

▲ **Note:** For higher or lower peptide concentration in the assay, either the initial dissolving volume or the pipetted volume of working solution can be adopted accordingly.

▲ For details on how to correctly dissolve MACSpep single peptides please refer to the application protocol "Preparation of MACSpep single peptides for stimulation and expansion of antigen-specific T cells".

Preparation of antibody staining cocktail

For each staining of 1 mL whole blood add 2 µL of each of the nine antibodies to 82 µL of Inside Perm to a total volume of 100 µL per staining.

▲ **Note:** Prepare staining master mix freshly before use. Store staining master mix at +2 to +8 °C in the dark. Discard unused solution at the end of the day.

Preparation 1×Red Blood Cell (RBC) Lysis Solution

Dilute Red Blood Cell Lysis Solution (10×) 1:10 with double-distilled water (ddH_2O). For example, dilute 2 mL of Red Blood Cell Lysis Solution (10×) with 18 mL of ddH_2O.

▲ **Note:** Do not dilute with deionized water. Store prepared 1× Red Blood Cell Lysis Solution at room temperature. Discard unused solution at the end of the day.

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Preparation of fixation mix

Prepare for each sample of 1 mL whole blood:

1. Mix 250 µL PEB buffer with 250 µL Inside Fix.
2. Mix the solution by 3 seconds of vortexing.

   ▲ **Note:** Prepare fixation mix freshly before use. Store fixation mix at room temperature. Discard unused solution at the end of the day.

2.2 Sample preparation and cell stimulation

▲ For optimal stimulation, use only blood collection tubes containing heparin. EDTA and citrate as anticoagulants impair T cell stimulation.

▲ For optimal results use freshly drawn whole blood stored at room temperature not longer than 24 hours.

▲ Volumes given below are for 1 mL whole blood. Perform three tests for one donor. Use 1 mL for each antigen stimulation, positive control, and negative control.

1. Collect at least 3 mL of venous blood in a collection tube containing an appropriate anticoagulant.
2. Mix whole blood collection tubes by inverting five times.
3. Transfer 1 mL of whole blood into a 5 mL tube or well per sample.
4. For antigen stimulation, add 20 µL working solution containing PepTivator Peptide Pools or MACSpep Single Peptides in sterile solvent. Mix by pipetting up and down.
5. For the positive control: Add 10 µL CytoStim, human to the respective 5 mL tubes or wells. Mix by pipetting up and down.
6. For the negative control: Add sterile solvent (composition and volume identical to the solvent used for the antigen). Mix by pipetting up and down.

   ▲ **Note:** It is recommended to include at least one positive and negative control for each donor.
7. Add 20 µL of Brefeldin A to each 5 mL tube or well. Mix the samples by pipetting up and down.

   ▲ **Note:** Thorough mixing is required for a homogeneous stimulation.
8. Incubate samples at +37 °C, 5% CO_2, and approx. 95% humidity for 6–12 hours.

   ▲ **Note:** Stimulation results may vary between donors and peptide used.

2.3 Staining protocol

▲ Prepare a sufficient amount of 1× RBC Lysis Solution, fixation mix, and antibody staining cocktail (refer to chapter 2.1).

▲ If the cell pellet is still red from non-lysed erythrocytes after aspiration of supernatant, the pellet might be "fluffy". Do not disturb the pellet when aspirating the supernatant.

1. Remove the samples from the incubator and open the lids.
2. Add 2 mL of 1× RBC Lysis Solution to each sample.
3. Mix by pipetting up and down.
4. Incubate for 15 minutes at room temperature (+19 to +25 °C).
5. Centrifuge at 300×g for 5 minutes.
6. Carefully aspirate supernatant by using a vacuum pump or by pipetting. Do not disturb the pellet.
7. Add 1 mL PEB buffer to each sample.
8. Mix by pipetting up and down.
9. Centrifuge at 300×g for 5 minutes.
10. Carefully aspirate supernatant by using a vacuum pump or by pipetting.
11. Add 500 µL fixation mix to each sample.
12. Mix by pipetting up and down.
13. Incubate for 20 minutes at room temperature (+19 to +25 °C).
14. Centrifuge at 300×g for 5 minutes.
15. Carefully aspirate supernatant by using a vacuum pump or by pipetting.
16. (Optional) Freeze and store the cells (refer to chapter 2.4).
17. Add 1 mL Inside Perm to each sample.
18. Mix by pipetting up and down.
19. Centrifuge at 300×g for 5 minutes.
20. Carefully aspirate supernatant by using a vacuum pump or by pipetting.
21. Add 100 µL antibody staining cocktail to each sample.
22. Mix by pipetting up and down.
23. Incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).
24. Add 1 mL Inside Perm to each sample.
25. Mix by pipetting up and down.
26. Centrifuge at 300×g for 5 minutes.
27. Carefully aspirate supernatant completely by using a vacuum pump or by pipetting.

    ▲ **Note:** The cell pellet should be white and more "solid". Do not disturb the pellet when aspirating the supernatant.
28. Resuspend the cell pellet in 250 µL PEB buffer.
29. Analyze cell suspension on a suited flow cytometer, e.g., the MACSQuant Analyzer 16 or store cells for up to 24 hours at +2 to +8 °C in the dark.

2.4 (Optional) Freeze and thawing protocol

Freeze protocol

1. Add 1 mL PEB buffer to each sample.
2. Mix by pipetting up and down.
3. Centrifuge at 300×g for 5 minutes.
4. Carefully aspirate supernatant.
5. Add 1 mL StemMACS CryoBrew to each sample.
6. Mix by pipetting up and down.
7. Transfer the cells of each sample into a cryotube and store tubes at –80 °C.

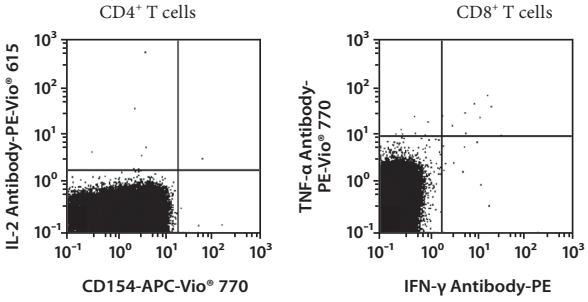
Thawing protocol

1. Carefully thaw cells at +37 °C.
2. Transfer cell suspension into a 5 mL tube or well.
3. Add 3 mL PEB buffer to each sample.
4. Mix by pipetting up and down.
5. Centrifuge at 300×g for 5 minutes.
6. Carefully aspirate supernatant.
7. Add 3 mL PEB buffer to each sample.
8. Mix by pipetting up and down.
9. Centrifuge at 300×g for 5 minutes.
10. Carefully aspirate supernatant.
11. Proceed with step 17 in chapter 2.3.

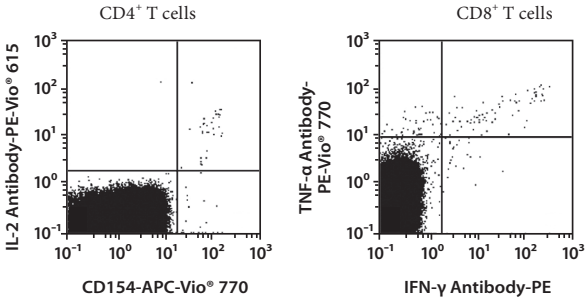
3. Example of immunofluorescent staining using the Antigen-specific T Cell Analysis Kit (Whole Blood), anti-human, REAfinity in combination with SARS-CoV-2 PepTivator Peptide Pools

Human whole blood (1 mL) from SARS-CoV-2–reactive donors were incubated for 8 hours with Brefeldin A. Simultaneously, cells were either left unstimulated (A) or incubated with a selection of SARS-CoV-2 PepTivators Prot_N (B) or Prot_S1 (C). Blood samples were lysed, fixed, and permeabilized afterwards. Subsequently, cells were stained with the antibody panel included in this kit. Cells were analyzed using a MACSQuant Analyzer 16. Doublets, debris, and dead cells as well as $CD14^+$ and $CD20^+$ cells were excluded. After pregating on CD3 as well as CD4 and CD8, respectively, activation marker and cytokine expression were assessed, e.g., CD154 and IL-2 for $CD4^+$ T cells and TNF-α and IFN-γ for $CD8^+$ T cells.

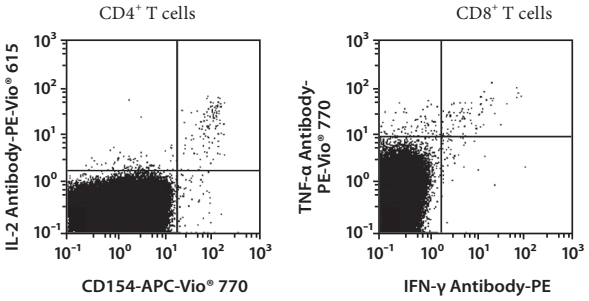
A) Unstimulated cells



B) Cells stimulated with PepTivator SARS-CoV-2_Prot_N



C) Cells stimulated with PepTivator SARS-CoV-2_Prot_S1



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