Miltenyi Biotec

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

Order no. 130-138-375

Contents

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)

100 µL Viobility™ 405/452 Fixable Dye

2×200 µL CytoStim™, human

25 mL Inside Fix

55 mL Inside Perm

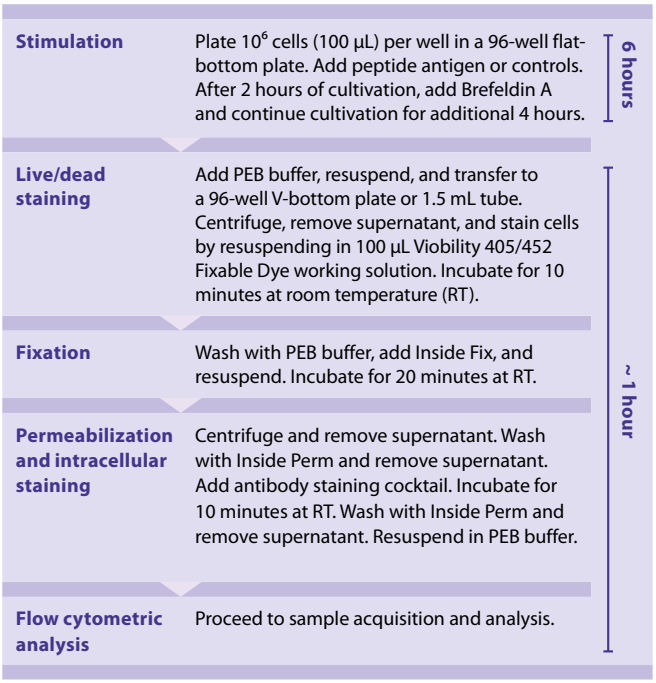
200 µL Brefeldin A (100 µg/mL)

Capacity 100 tests for up to 10^6 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. CytoStim is supplied in buffer containing stabilizer. Lyophilized peptides contain stabilizer. Viobility 405/452 Fixable Dye is supplied dissolved in anhydrous DMSO.

Storage Upon arrival store Viobility 405/452 Fixable Dye dry and protected from light at –20 °C. For information about reconstitution and storage after reconstitution refer to chapter 2.1. Store all other components protected from light at +2 to +8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Protocol overview



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1.2 Principle

The Antigen-specific T Cell Analysis Kit (PBMC), anti-human, REAfinity has been developed for the fast and easy detection of antigen–reactive T cells by intra- and extracellular staining of activation markers and cytokines. The use of 96-well cell culture plates enables the processing and analysis of multiple samples in parallel. 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), a live/dead marker (Viobility 405/452 Fixable Dye), Brefeldin A, 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.
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 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).
- Culture medium, e.g., RPMI 1640 containing 5% AB serum

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

  ▲ **Note:** AB serum needs to be tested in advance for low background activation of T cells.
- Cell culture–grade DMSO
- 96-well flat-bottom cell culture plates for *in vitro* stimulation of T cells
- 96-well V-bottom cell culture plates for immunofluorescent staining of T cells
- Centrifuge suited for cell culture plates and 1.5 mL microcentrifuge tubes
- 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) MACSprep™ PBMC Isolation Kit, human (# 130-115-169)
- (Optional) 8-Color Immunophenotyping Kit, anti-human, REAfinity (# 130-120-640)
- (Optional) 24- or 12-well cell culture plates
- (Optional) 7-AAD Staining Solution (# 130-111-568)
- (Optional) Tandem Signal Enhancer, human (# 130-099-888)
- (Optional) PE-Vio 615–conjugated REAfinity Antibodies for flow cytometric analysis in the free PE-Vio 615 channel (refer to chapter 4. Appendix), e.g., IL-2 Antibody, anti-human, CD69 Antibody, anti-human, CD137 Antibody, anti-human, or TIGIT Antibody, anti-human. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) MACS Comp Bead Kit, anti-REA (# 130-104-693) for optimal compensation of the fluorescence spillover from fluorochrome-conjugated antibodies.

2. Protocol

▲ For a detailed gating strategy refer to the product page at **www.miltenyibiotec.com/130-128-375**.

2.1 Reagent preparation

Preparation of antibody staining cocktail

For each staining add 2 µL of each of the eight antibodies to 84 µL of Inside Perm to a total volume of 100 µL per staining.

▲ **Note:** Prepare freshly before staining.

▲ **Note:** If less or more antibodies are used, please adjust the volume of Inside Perm accordingly to a final volume of 100 µL.

▲ **Note:** (Optional) To increase signal specificity of tandem-dye–conjugated antibodies, Tandem Signal Enhancer, human can be added to the antibody staining cocktail (1:11). Reduce volume of Inside Perm accordingly to reach a total volume of 100 µL.

Preparation of Viobility 405/452 Fixable Dye working solution

The recommended dye dilution is 1 µL for up to 10^7 cells/100 µL cell suspension. Prepare a working solution, e.g., prepare 10 µL Viobility 405/452 Fixable Dye in 990 µL PBS according to the required sample number.

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2.2 Sample preparation

▲ For optimal analysis of activated cytokine-producing T cells, perform assay with freshly prepared peripheral blood mononuclear cells (PBMCs). Alternatively, frozen cell preparations can be used.

▲ **Note:** It is strongly recommended to work with fresh PBMCs for best results.

▲ When working with frozen PBMCs let cells rest overnight. For resting, determine number of living cells after thawing (e.g. by using the 7-AAD Staining Solution) and plate cells at a density of 5×10^6/mL in fresh cell culture medium in an appropriated cell culture dish (e.g. 1 mL or 2 mL cell suspension per well in a 24-or 12-well cell culture plate, respectively). Incubate at +37 °C and 5% CO_2 overnight.

▲ When working with anticoagulated peripheral blood, buffy coat or other blood products, PBMCs should be isolated by density gradient centrifugation or using the MACSprep PBMC Isolation Kit, human (# 130-115-169).

▲ **Note:** To remove platelets after density gradient separation, 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.

2.3 Cell stimulation

1. On the day of stimulation harvest PBMCs and determine number of viable cells (e.g. by using the 7-AAD Staining Solution). Centrifuge cells at 300×g for 5 minutes and remove supernatant. Resuspend cells in culture medium at a density of 1×10^7 viable cells per mL.
2. Plate 100 µL cell suspension per well in a flat-bottom 96-well plate reaching a total number of 1×10^6 cells per well.
3. For antigen stimulation, add PepTivator Peptide Pools or MACSpep Single Peptides in an appropriate concentration in 2 µL volume. Mix by pipetting up and down.
4. For the positive control: Add 2 µL CytoStim, human to the respective wells. Mix by pipetting up and down.
5. For the negative control: Add 2 µL sterile water/10% DMSO solution to the respective wells. Mix by pipetting up and down.

   ▲ **Note:** It is recommended to include at least one positive and negative control for each donor.

5. Incubate at incubate at +37 °C and 5% CO_2 for 2 hours.
6. Add 2 µL of Brefeldin A to each well. Mix by pipetting up and down.
7. Incubate at +37 °C and 5% CO_2 for 4 additional hours.

   ▲ **Note:** Stimulation results may vary between donors and individual PepTivator specificities.

2.4 Cell harvest and staining of dead cells

1. Add 100 µL of PEB buffer to each well and mix by pipetting up and down to detach all adherent cells.
2. Transfer each setup into a 96-well V-bottom plate.

   ▲ **Note:** Alternatively, a 1.5 mL microcentrifuge tube can be used.

3. Centrifuge at 300×g for 5 minutes and aspirate supernatant.
4. (Optional) Resuspend cells in 100 µL Viobility 405/452 Fixable Dye working solution.
5. (Optional) Incubate for 10 minutes at room temperature (+20 to +25 °C).
6. Add 100 µL of PEB buffer to each well and centrifuge at 300×g for 5 minutes.
7. Resuspend cells in 100 µL of PEB buffer.

2.5 Fixation

1. Add 100 µL Inside Fix to each well. Mix by pipetting up and down.
2. Incubate for 20 minutes at at room temperature (+20 to +25 °C).
3. Centrifuge at 300×g for 5 minutes and aspirate supernatant.

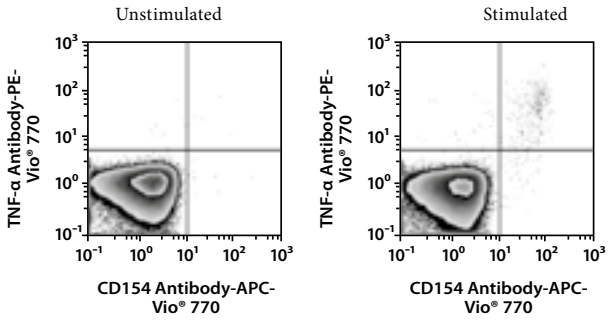
2.6 Permeabilization and intracellular staining

1. Add 100 µL Inside Perm to each well and resuspend by carefully pipetting up and down.
2. Centrifuge at 300×g for 5 minutes and aspirate supernatant.
3. Add 100 µL antibody staining cocktail (refer to 2.1 Reagent preparation) to each well.
4. Incubate for 10 minutes at room temperature.
5. Add 100 µL Inside Perm to each well.
6. Centrifuge at 300×g for 5 minutes and aspirate supernatant.
7. Add 200 µL of PEB buffer to each well and mix by pipetting up and down.
8. Analyze 180 µL 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.

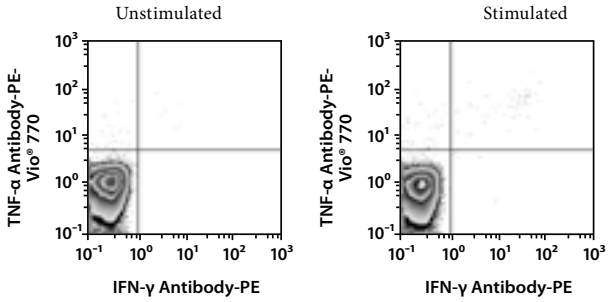
3. Example of immunofluorescent staining using the Antigen-specific T Cell Analysis Kit (PBMC), human in combination with SARS-CoV-2 PepTivator Peptide Pools

Human PBMCs from a SARS-CoV-2–reactive donor were incubated for 6 hours with PepTivator SARS-CoV-2 Prot_S Complete or left unstimulated (negative control). Brefeldin A was added after 2 hours. Cells were then stained with the live/dead marker Viobility 405/452 Fixable Dye, 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 TNF-α for $CD4^+$ T cells (A) and TNF-α and IFN-γ for $CD8^+$ T cells (B).

A) $CD4^+$ T cells



B) $CD8^+$ T cells



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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