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1. Description

This product is for research use only.

Components	<p>200 µL CD3 Antibody, anti-human, APC, REAfinity (clone: REA613, isotype: recombinant human IgG1)</p> <p>200 µL CD4 Antibody, anti-human, Vio® Bright B515, REAfinity (clone: REA623, isotype: recombinant human IgG1)</p> <p>200 µL CD8 Antibody, anti-human, VioGreen™, REAfinity (clone: REA734, isotype: recombinant human IgG1)</p> <p>200 µL IFN-γ Antibody, anti-human, PE, REAfinity (clone: REA600, isotype: recombinant human IgG1)</p> <p>200 µL TNF-α Antibody, anti-human, PE-Vio 770, REAfinity (clone: REA656, isotype: recombinant human IgG1)</p> <p>200 µL CD14 Antibody, anti-human, VioBlue®, REAfinity (clone: REA599, isotype: recombinant human IgG1)</p> <p>200 µL CD20 Antibody, anti-human, VioBlue, REAfinity (clone: REA780, isotype: recombinant human IgG1)</p> <p>200 µL CD154 Antibody, anti-human, APC-Vio 770, REAfinity (clone: REA238, isotype: recombinant human IgG1)</p>
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- 100 µL Viability™ 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. Viability 405/452 Fixable Dye is supplied dissolved in anhydrous DMSO.
Storage	Upon arrival store Viability 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

Stimulation	Plate 10 ⁶ cells (100 µL) per well in a 96-well flat-bottom plate. Add peptide antigen or controls. After 2 hours of cultivation, add Brefeldin A and continue cultivation for additional 4 hours.	6 hours
Live/dead staining	Add PEB buffer, resuspend, and transfer to a 96-well V-bottom plate or 1.5 mL tube. Centrifuge, remove supernatant, and stain cells by resuspending in 100 µL Viability 405/452 Fixable Dye working solution. Incubate for 10 minutes at room temperature (RT).	~ 1 hour
Fixation	Wash with PEB buffer, add Inside Fix, and resuspend. Incubate for 20 minutes at RT.	
Permeabilization and intracellular staining	Centrifuge and remove supernatant. Wash with Inside Perm and remove supernatant. Add antibody staining cocktail. Incubate for 10 minutes at RT. Wash with Inside Perm and remove supernatant. Resuspend in PEB buffer.	
Flow cytometric analysis	Proceed to sample acquisition and analysis.	

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 (Viability 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 MACSprep 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 ([MACSprep 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.
- (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/130-138-375.

2.1 Reagent preparation

Preparation of stimulant

For peptide-based stimulation of antigen-specific T cells, it is recommended using [MACSprep 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. For the first experiments we recommend 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 MACSprep single peptides please refer to the application protocol “[Preparation of MACSprep single peptides for stimulation and expansion of antigen-specific T cells](#)”.

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 Viability 405/452 Fixable Dye working solution

The recommended dye dilution is 1 µL for up to 10⁷ cells/100 µL cell suspension. Prepare a working solution, e.g., prepare 10 µL Viability 405/452 Fixable Dye in 990 µL PBS according to the required sample number.

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 5×10⁶/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% CO₂ 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 1×10⁷ viable cells per mL.
2. Plate 100 µL cell suspension per well in a flat-bottom 96-well plate reaching a total number of 1×10⁶ cells per well.
3. For antigen stimulation, add PepTivator Peptide Pools or MACSprep 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 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.

6. Incubate at incubate at +37 °C and 5% CO₂ for 2 hours.
7. Add 2 µL of Brefeldin A to each well. Mix by pipetting up and down.
7. Incubate at +37 °C and 5% CO₂ 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 Viability 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 room temperature (+20 to +25 °C).
3. Centrifuge at 300×g for 5 minutes and aspirate supernatant.

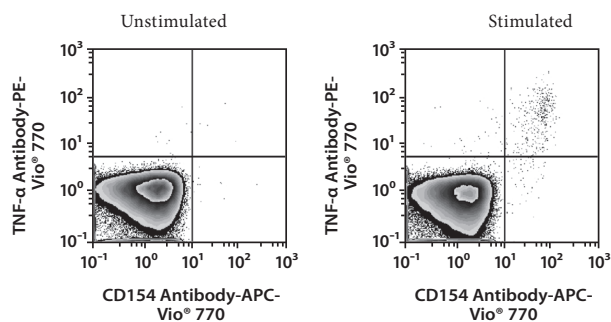
2.6 Permeabilization and intracellular staining

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.

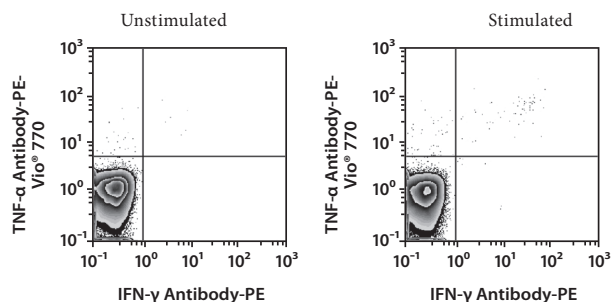
3. Example of immunofluorescent staining using the Antigen-specific T Cell Analysis Kit (PBMC), anti-human, REAfinity in combination with SARS-CoV-2 PepTivator Peptide Pools

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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