CD19 CAR (FMC63 scFv) Cellular Immunogenicity Flow Analysis Kit, anti-human, REAfinity®

Order no. 130-140-833

Contents

1. Description
   - 1.1 Protocol overview
   - 1.2 Principle
   - 1.3 Background information
   - 1.4 Applications
   - 1.5 Reagent and instrument requirements
2. Protocol
   - 2.1 Reagent preparation
   - 2.2 Sample preparation
   - 2.3 Cell stimulation
   - 2.4 Cell harvest and staining of dead cells
   - 2.5 Fixation
   - 2.6 Permeabilization and intracellular staining
3. Example of immunofluorescent staining using the CD19 CAR (FMC63 scFv) Cellular Immunogenicity Flow Analysis Kit, anti-human, REAfinity

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

1× 200 µL CytoStim™, human

6 nmol/peptide PepTivator FMC63 scFv (CD19 CAR) Immunogenicity – research grade Pool of lyophilized peptides consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, covering the complete FMC63 scFv protein.

25 mL Inside Fix

55 mL Inside Perm

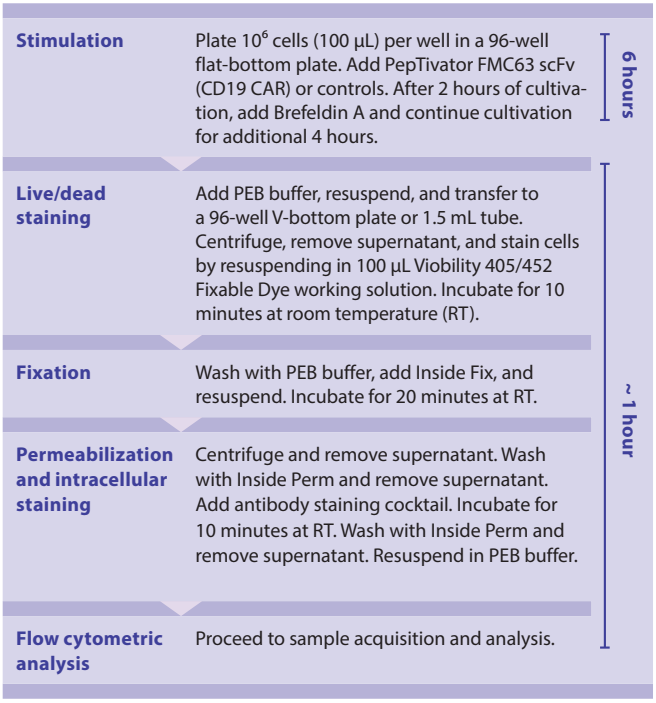
200 µL Brefeldin A (100 µg/mL)

Capacity 100 tests for up to 10^6 total cells per well.

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 Store all 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 CD19 CAR (FMC63 scFv) Cellular Immunogenicity Flow Analysis Kit, 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. Moreover, it also allows pooling of multiple samples to increase assay sensitivity. 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

The CD19 CAR (FMC63 ScFv) Cellular Immunogenicity Flow Analysis Kit, anti-human, REAfinity is designed to evaluate whether the FMC63 single-chain variable fragment (scFv) used in CD19 CAR-T cells induces T-cell activation, thereby indicating the potential for anti- CD19 CAR (FMC63 ScFv) immune responses. The FMC63 scFv is an ectodomain commonly incorporated into CAR constructs that target B cells via CD19. Because FMC63 scFv possesses immunogenic potential, it has been widely used to assess immune responses directed against CAR-T cells.
In this assay, both $CD4^+$ and $CD8^+$ T cells reactive to the FMC63 scFv can be detected. This is accomplished by incubating peripheral blood mononuclear cells (PBMCs) with a pool of 15-mer peptides overlapping by 11 amino acids, which together span the entire FMC63 scFv sequence. The peptides are taken up, processed, and presented as antigenic peptides to T cells via major histocompatibility complex (MHC) molecules. T cells that recognize their cognate peptides become activated, and this activation is detected by measuring the expression of CD154, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α).

1.4 Applications

- Rapid detection and immune monitoring of $CD4^+$ and $CD8^+$ T cells reactive towards CD19 CAR (FMC63 Fv containing) T cells
- Phenotypical characterization of activated T cells after stimulation with PepTivator Peptide Pools by flow cytometry

1.5 Reagent and instrument requirements

- 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 SARS-CoV-2 Prot_S Complete (#130-127-951) as a positive control or 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

2.1 Reagent preparation

Preparation of stimulant

For appropriate preparation of the stimulant please follow the instructions below.

▲ For dissolving the PepTivator peptide pool 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 adapted accordingly.

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.

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▲ **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.

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 allow cells to 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 appropriate 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.

   ▲ **Note:** Assay sensitivity can be improved by increasing the total number of cells analyzed. For enhanced sensitivity, pool multiple wells prior to downstream analysis.
3. For antigen stimulation, add PepTivator Peptide Pools 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. (Optional) Use one of the indicated positive control PepTivator mentioned in section 1.5, alternatively.
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.

   ▲ **Note:** If multiple wells are seeded to increase assay sensitivity, pool the same number of corresponding negative control wells prior to analysis.
6. Incubate at +37 °C and 5% CO_2 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% 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

▲ For increased sensitivity and improved detection, it is recommended to pool cells from five stimulated conditions and five negative controls by combining 200 µL of cell suspension per well before analysis.

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 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 suitable flow cytometer, e.g., the MACSQuant Analyzer 16 or store cells for up to 24 hours at +2 to +8 °C in the dark.

   ▲ **Note:** If multiple wells were seeded (see Section 2.3.2), pool 200 µL of cell suspension from each well into a suitable tube or deep-well plate. Proceed to acquire and analyze the leftover pooled sample.

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3. Example of immunofluorescent staining using PepTivator® SARS-CoV-2 (positive control) with the CD19 CAR (FMC63 scFv) Cellular Immunogenicity Flow Analysis Kit, anti-human, REAfinity

▲ Antigen-specific T cell responses may vary due to prior treatments, disease status, infection or inflammation, and individual HLA haplotypes. These factors can influence responding T cell subsets, activation marker expression, and response magnitude. The suggested gating strategy is intended for initial screening and may require application-specific optimization.

Hierarchical gating strategy

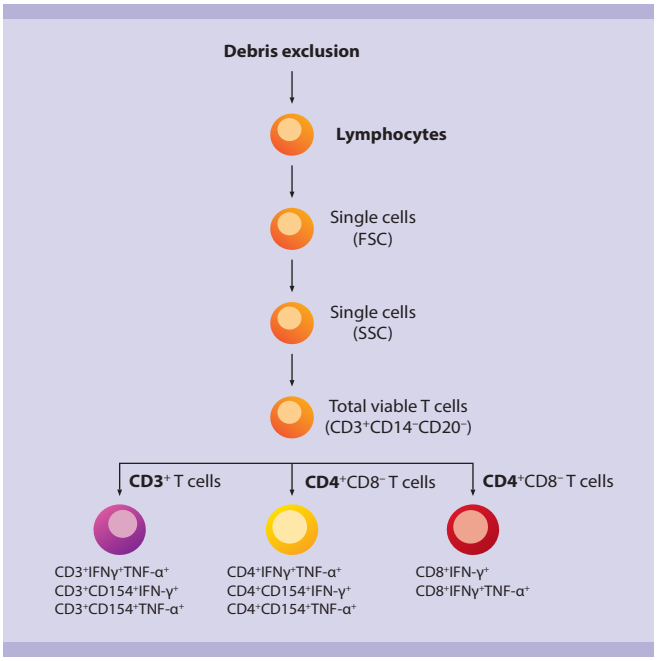


Figure 1: Hierarchical gating strategy to identify total CD3⁺ T cells, CD4⁺ T cells, and CD8⁺ T cells.

Example of pre-gating strategy to identify total CD3⁺ T cells, CD4⁺ T cells, and CD8⁺ T cells.

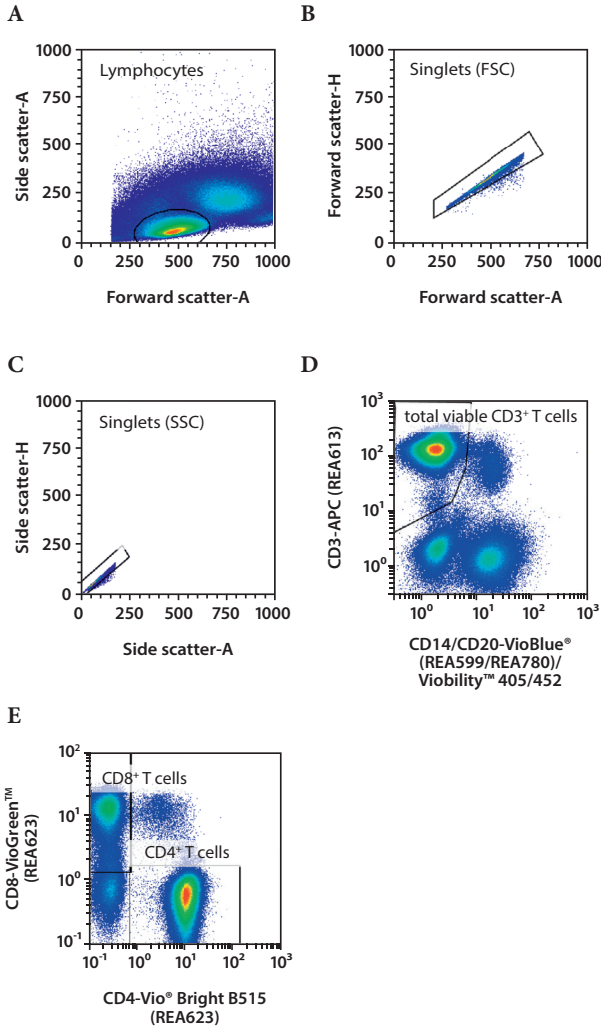


Figure 2: Example of pre-gating strategy to identify total CD3⁺ T cells, CD4⁺ T cells, and CD8⁺ T cells. Gating on total viable CD3⁺ T cells (**Figure 3D**) allows to determine cell counts and frequencies of total CD3⁺ reactive T cells. Gating on total viable CD4⁺ T cells (**Figure 3E**) allows to determine cell counts and frequencies of total CD4⁺ reactive T cells. Gating on total viable CD8⁺ T cells (**Figure 3E**) allows to determine cell counts and frequencies of total CD8⁺ reactive T cells.

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Identification of total reactive CD4$^+$ and CD8$^+$ T cells stimulated with positive control (PepTivator® SARS-CoV-2 Prot_S Complete)

▲ **Note:** Due to the limited availability of target-specific positive samples, representative data were obtained using a SARS-CoV-2-specific immune response as a surrogate positive control to illustrate assay functionality and expected signal characteristics

Human PBMCs were incubated for 6 hours with the PepTivator® SARS-CoV-2 Prot_S Complete as positive control (as indicated in section 1.5) 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 (**Figure 3A**) and TNF-α and IFN-γ for CD8$^+$ T cells (**Figure 3B**).

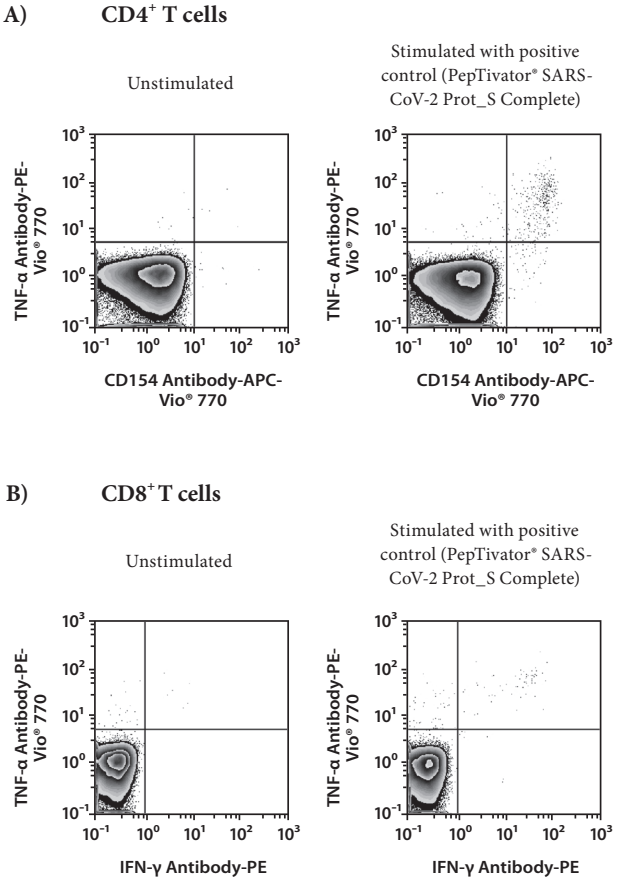


Figure 3: Identification of SARS-CoV-2–reactive CD4$^+$ T cells (A). Identification of SARS-CoV-2–reactive CD8$^+$ T cells (B).

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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