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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	0.5 mL 8-Color Immunophenotyping Cocktail, anti-human, REAfinity containing:
	CD3 Antibody, anti-human, PE, REAfinity (clone: REA613, isotype: recombinant human IgG1)
	CD4 Antibody, anti-human, Vio® Bright 667, REAfinity (clone: REA623, isotype: recombinant human IgG1)
	CD8 Antibody, anti-human, APC-Vio 770, REAfinity (clone: REA734, isotype: recombinant human IgG1)
	CD14 Antibody, anti-human, VioBlue®, REAfinity (clone: REA599, isotype: recombinant human IgG1)
	CD16 Antibody, anti-human, Vio Bright 515, REAfinity (clone: REA423, isotype: recombinant human IgG1)

CD19 Antibody, anti-human, PE-Vio 770, REAfinity (clone: REA675, isotype: recombinant human IgG1)

CD45 Antibody, anti-human, VioGreen™, REAfinity (clone: REA747, isotype: recombinant human IgG1)

CD56 Antibody, anti-human, Vio Bright 515, REAfinity (clone: REA196, isotype: recombinant human IgG1)

0.1 mL CD4 Antibody, anti-human, Vio Bright 667, REAfinity (clone: REA623, isotype: recombinant human IgG1)

0.1 mL CD8 Antibody, anti-human, APC-Vio 770, REAfinity (clone: REA734, isotype: recombinant human IgG1)

0.1 mL CD19 Antibody, anti-human, PE-Vio 770, REAfinity (clone: REA675, isotype: recombinant human IgG1)

0.1 mL CD56 Antibody, anti-human, Vio Bright 515, REAfinity (clone: REA196, isotype: recombinant human IgG1)

0.5 mL 7-AAD Staining Solution

10 mL Red Blood Cell Lysis Solution (10×)

Capacity

50 tests or up to 5×10^8 total cells.

Product format

Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.

Storage

Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

The 8-Color Immunophenotyping Kit, anti-human, REAfinity simplifies the flow cytometric evaluation of cell fractions for immunofluorescent staining of whole blood, peripheral mononuclear cells (PBMCs), lysed whole blood samples, or other single-cell suspensions from human tissue.

The 8-Color Immunophenotyping Cocktail, anti-human, REAfinity has been designed for the reliable identification of human monocytes, neutrophils, eosinophils, and T, B, and NK lymphocyte populations as well as CD4⁺, CD8⁺, and CD56⁺CD3⁺ T cell subsets in human blood.

The kit applies recombinantly engineered REAfinity Antibodies. REAfinity Antibodies are recombinant antibodies that provide superior lot-to-lot consistency and purity compared to mouse or rat hybridoma-derived, monoclonal antibodies. They have been recombinantly engineered to produce highly specific antibodies that require no FcR blocking step. Additionally, they all have the same IgG1 isotype, requiring less isotype controls.

1.2 Applications

- Evaluation of leukocyte subsets in whole blood, PBMCs, lysed whole blood, or other single-cell suspensions from human tissue.

1.3 Reagent and instrument 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 Ca^{2+} or Mg^{2+} are not recommended for use.
- 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.

2. Protocol

▲ For a detailed gating strategy refer to the application protocol “Immunophenotyping of tumor-infiltrating lymphocytes from human ovarian carcinoma using flow cytometry” at www.miltenyibiotec.com/applications.

2.1 Immunofluorescent staining of nucleated cells, e.g., PBMCs

▲ Volumes given below are for up to 10^7 nucleated cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Determine cell number.
2. Centrifuge cell suspension at $300 \times g$ for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^7 nucleated cells per 100 μL of buffer.
4. Add 10 μL 8-Color Immunophenotyping Cocktail, anti-human, REAfinity.
5. (Optional) Add 1 μL of 7-AAD Staining Solution.
6. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
▲ Note: Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.
7. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at $300 \times g$ for 10 minutes. Aspirate supernatant completely.
8. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry.
▲ Note: Store samples at 2–8 °C protected from light until analysis.
9. Proceed to flow cytometric analysis.

2.2 Immunofluorescent staining and lysis of whole blood (lyse/no wash)

1. Dilute 10 \times Red Blood Cell Lysis Solution 1:10 with double-distilled water (ddH_2O), for example, dilute 1 mL of 10 \times Red Blood Cell Lysis Solution with 9 mL of ddH_2O .
▲ Note: Do not dilute with deionized water. Store prepared 1 \times Red Blood Cell Lysis Solution at room temperature. Discard unused solution at the end of the day.
2. Add 10 μL 8-Color Immunophenotyping Cocktail, anti-human, REAfinity per 100 μL of whole blood.
3. (Optional) Add 10 μL of 7-AAD Staining Solution.
4. Mix well and incubate for 10 minutes in the dark at room temperature.
▲ Note: Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.
5. Add 2 mL of 1 \times Red Blood Cell Lysis Solution and immediately vortex thoroughly for 3 seconds. Incubate for 15 minutes in the dark at room temperature.
6. Proceed immediately to flow cytometric analysis.

2.3 Immunofluorescent staining and lysis of whole blood (lyse/wash)

1. Dilute Red Blood Cell Lysis Solution (10 \times) 1:10 with double-distilled water (ddH_2O), for example, dilute 1 mL of Red Blood Cell Lysis Solution (10 \times) with 9 mL of ddH_2O .
▲ Note: Do not dilute with deionized water. Store prepared 1 \times Red Blood Cell Lysis Solution at room temperature. Discard unused solution at the end of the day.
2. Add 10 μL 8-Color Immunophenotyping Cocktail, anti-human, REAfinity per 100 μL of whole blood.
3. (Optional) Add 10 μL of 7-AAD Staining Solution.
4. Mix well and incubate for 10 minutes in the dark at room temperature.
▲ Note: Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.
5. Add 2 mL of 1 \times Red Blood Cell Lysis Solution and immediately vortex thoroughly for 3 seconds. Incubate for 15 minutes in the dark at room temperature.
6. Centrifuge at $300 \times g$ for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry.
▲ Note: Store samples at 2–8 °C protected from light until analysis.
8. Proceed immediately to flow cytometric analysis.

2.4 Immunofluorescent staining of whole blood (no lyse/no wash)

1. Add 100 μL of buffer to 100 μL whole blood.
2. Add 10 μL 8-Color Immunophenotyping Cocktail, anti-human, REAfinity per 200 μL total volume.
3. (Optional) Add 10 μL of 7-AAD Staining Solution.
4. Mix well and incubate for 10 minutes in the dark at room temperature.
▲ Note: Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.

5. Add 3.8 mL of buffer and mix well.
6. Proceed immediately to flow cytometric analysis.
Use the flow rate “low” for acquisition and an uptake volume of at least 200 μL to ensure a sufficient number of cells for data analysis.

2.5 Flow cytometric data acquisition with the MACSQuant Analyzer 10 using the analysis template

▲ Refer to the MACSQuant Instrument user manual and software guide for detailed information on using the MACSQuant Analyzer 10.

▲ For automated flow cytometric analysis using the MACSQuant Analyzer flow cytometers, the Express Mode **Immunophenotyping_8_Color_Kit_h** can be used. Express Modes are unique add-on features for the MACSQuantify™ Software. They are standardized data analysis tools that are optimized to automate flow cytometric measurements and analyses via predefined experiment settings, acquisition, and automated gating. Derived from mathematical algorithms, they reduce human error and therefore increase experimental reproducibility. For details refer to the MACSQuant user manual, the MACSQuantify Software guide, or visit www.macsquant.com. For more information on the usage of Express Modes refer to the application note “How to use a MACSQuant® Instrument Express Mode in Custom Login” in the Resources section at www.miltenyibiotec.com.

▲ The analysis template is available on the product page of the 8-Color Immunophenotyping Kit, anti-human, REAfinity at www.miltenyibiotec.com/130-120-640.

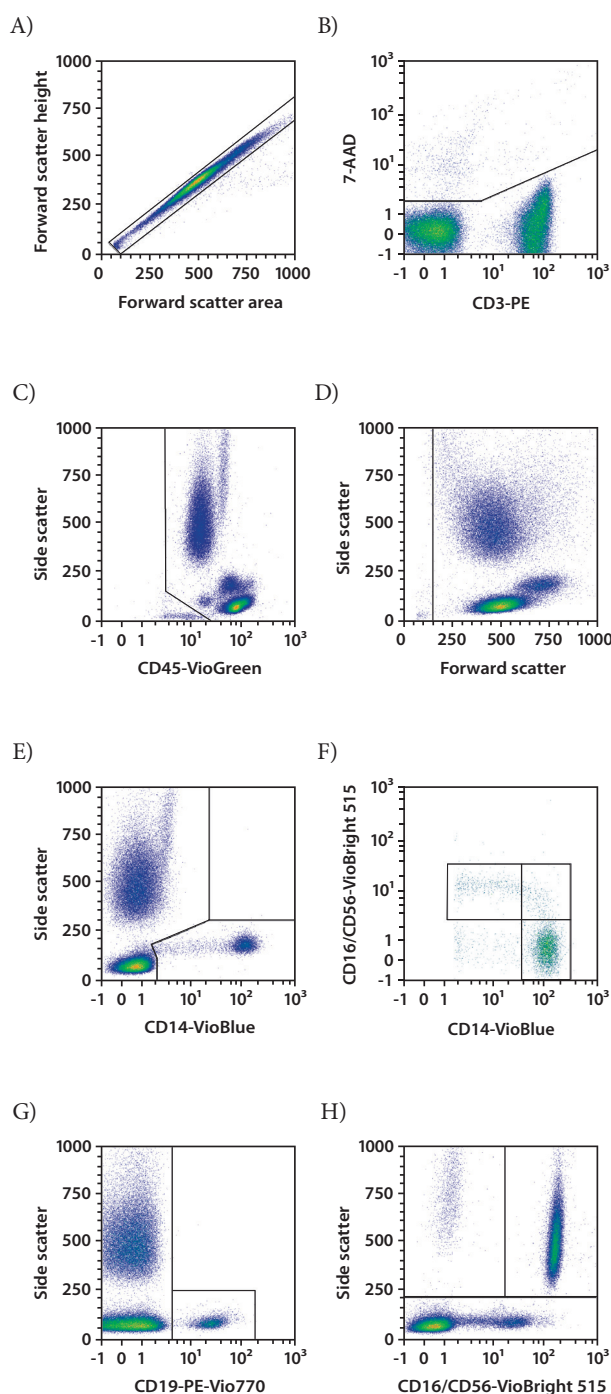
1. Prepare and prime the MACSQuant Analyzer. Make sure the calibration and instrument settings of the instrument have been optimized for acquisition of the 8-Color Immunophenotyping Cocktail, anti-human, REAfinity.
For Vio Bright 515, Vio Bright 667, PE-Vio 770, and APC-Vio 770 compensation use CD56-Vio Bright 515, CD4-Vio Bright 667, CD19-PE-Vio 770, and CD8-APC-Vio 770 antibodies provided with the kit.
2. Import and load the respective analysis template.
3. For doublets discrimination choose **Height**. Therefore click the **Advanced** button located in the **Channels** tab and click on the **Height** button.
4. Define an appropriate threshold, based on CD45-VioGreen versus side scatter (SSC), for the exclusion of debris and erythrocytes from the data acquisition
5. Start flow cytometric data acquisition.

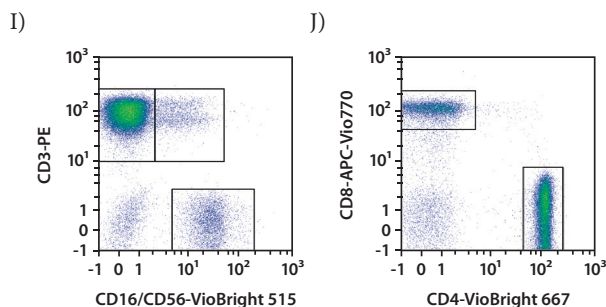
3. Example of immunofluorescent staining with the 8-Color Immunophenotyping Kit, anti-human, REAfinity

Whole blood from a healthy donor was stained with the 8-Color Immunophenotyping Kit, anti-human, REAfinity. Staining was carried out at room temperature for 10 minutes. Subsequently, red blood cells were lysed by incubation using 1 \times Red Blood Cell Lysis Solution at room temperature for 15 minutes. Cells were analyzed by flow cytometry using the MACSQuant Analyzer 10.

As a preliminary step for elimination of doublets a gate around single cells in forward scatter area (FSC-A) versus forward scatter

height (FSC-H) (A) as well as a gate around viable cells was set (B). To identify the major circulating blood cell types CD45 was used to target all leukocytes (C). These cells were further separated from debris via forward scatter (FSC) and side scatter (SSC) (D). Monocytes were discriminated based on their CD14 expression (E) and then further divided into classical, intermediate, and non-classical monocytes via CD16 (F). Among the non-monocyte population, B cells were defined as CD19⁺ (G). The remaining cells were separated into CD16⁺/SSC^{high} neutrophils, CD16⁻/SSC^{high} eosinophils as well as a CD16^{-dim}/SSC^{low} population (H). CD3 and CD56 were used to distinguish CD56⁺ NK cells, CD3⁺ T cells, and a CD3⁺CD56⁺ T cell population (I). CD3⁺ T cells were divided into CD4⁺ and CD8⁺ T cells (J).





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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