Miltenyi Biotec

StainExpress™ T Cell Subsets Cocktail

human

Order no. 130-135-459

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1. Description

This product is for research use only.

Components

24 tubes StainExpress T Cell Subsets Cocktail, human:

Dried cocktail of fluorochrome-conjugated recombinant engineered REAfinity® Antibodies (isotype: recombinant human IgG1) containing:

CD45-VioGreen™ (clone: REA747),

CD4-APC-Vio® 770 (clone: REA623),

CD3-FITC (clone: REA613),

CD8-VioBlue® (clone: REA734),

7-AAD Staining Solution.

2 StainExpress T Cell Subsets Compensation Sets, human:

Each set consists of four tubes containing dried single antibody fluorochrome-conjugated recombinant engineered REAfinity Antibodies (isotype: recombinant human IgG1) for compensation controls:

CD45-VioGreen (clone: REA747),

CD4-APC-Vio 770 (clone: REA623),

CD3-FITC (clone: REA613),

CD8-VioBlue (clone: REA734).

Capacity 24 tests, one test for up to 10^6 total cells.

Product format Antibodies are supplied in a dry format containing stabilizer.

Storage Store at dry conditions in a closed pouch. Store protected from light at +19 to +25 °C. The expiration date is indicated on the pouch label.

1.1 Background information

The StainExpress T Cell Subsets Cocktail, human provides a pre-formulated antibody backbone panel to simplify and standardize the characterization of T cell subsets using flow cytometry. The unified panel includes a free PE or APC channel for the addition of a CAR detection reagent of choice. The Miltenyi Biotec CAR Detection Reagents have been developed for the detection of transduced T cells that are engineered to express specific CARs (Chimeric Antigen Receptors) on the cell surface. In combination with these reagents, the cocktail is optimized to monitor engineered CAR T cells, e.g. to evaluate their expansion and persistence in human peripheral blood.

For flow cytometric analysis use a flow cytometer equipped with a red (638 nm), a blue (488 nm), and a violet (405 nm) laser, for example, the MACSQuant Analyzer 16.

1.2 Applications

- Identification and analysis of CAR^+ T cells by flow cytometry.
- Evaluation of the expansion and persistence of CAR^+ T cells in patient monitoring research.

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 to +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, e.g., MACSQuant Analyzer 16 (# 130-109-803) or MACSQuant Analyzer 10 (# 130-096-343).

  ▲ **Note:** The MACSQuant VYB cannot be used.

- MACSQuant Running Buffer (# 130-092-747)
- CAR Detection Reagent of choice detectable in the PE channel, e.g., CD19 CAR Detection Reagent, human, Biotin (# 130-129-550), CD19 CAR FMC63 Idiotype Antibody, PE, REAfinity (# 130-127-342), CD22 CAR Detection Reagent, human, Biotin (# 130-126-727), or BCMA CAR Detection Reagent, human, PE (# 130-133-888).
- (Optional) Biotin Antibody, PE, REAfinity (# 130-110-951).
- (Optional) MACS Comp Bead Kit, anti-REA (# 130-104-693), for compensation of the fluorescence spillover from fluorochrome-conjugated antibodies.

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- (Optional) MACS MiniSampler Plus (# 130-105-745).
- (Optional) Chill 5 Rack (# 130-092-951).
- (Optional) Red Blood Cell Lysis Solution (10×) (# 130-094-183)
- (Optional) StainExpress Immune Cell Composition Cocktail, human (# 130-127-637)
- (Optional) Markers for customization to expand the StainExpress T Cell Subsets Cocktail by incorporating liquid antibodies into available channels. Recommended antibodies include:

Antibody	Clone
CD14	REA599
CD19	REA675
CD45RO	REA611
CD197 (CCR7)	REA546

2. Protocols

2.1 Immunofluorescent staining of nucleated cells, e.g., T cells, using the StainExpress T Cell Subsets Cocktail, optional in combination with CAR Detection Reagents and additional antibodies

▲ Volumes given below are for up to 10^6 nucleated cells. When working with fewer than 10^6 cells, use the same volumes as indicated. When working with higher cell numbers, use multiple tubes accordingly (e.g. for 2×10^6 nucleated cells, use two tubes).

1. Determine cell number.
2. Adjust cell concentration to up to 10^6 nucleated cells per 100 µL using buffer.

   ▲ **Note:** If necessary, centrifuge cell suspension at 300×g for 5 minutes, aspirate supernatant completely, and resuspend up to 10^6 nucleated cells per 100 µL of buffer.
3. Add 100 µL of cell suspension to one tube of StainExpress T Cell Subsets Cocktail.
4. (Optional) Add suitable amounts of the CAR Detection Reagent of choice and other liquid antibodies for available channels (refer to table in chapter 1.3) according to manufacturer's recommendation.
5. Incubate for 10–20 seconds, then vortex at high speed for 5 seconds and incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).

   ▲ **Note:** Working at lower temperatures requires increased incubation times.
6. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
7. (Optional) If using a biotin-conjugated CAR Detection Reagent follow steps 7a–d:

   a. Repeat step 6.

   b. Resuspend cells in 98 µL buffer and add 2 µL of Biotin Antibody, PE, REAfinity.

   c. Vortex shortly at high speed and incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).

   d. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
8. (Optional for fixation) Add 250 µL of buffer and 250 µL of Inside Fix to the cells and incubate for 20 minutes in the dark at room temperature (+19 to +25 °C).
9. (Optional for fixation) Add up to 2 mL of buffer.
10. (Optional for fixation) Centrifuge cells at 300×g for 5 minutes at room temperature (+19 to +25 °C). Aspirate supernatant completely.
11. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry.

    ▲ **Note:** Store samples at +2 to +8 °C protected from light until analysis. Acquire samples within 1 hour after staining.
12. (Optional) As a negative control, fluorescence-minus-one (FMO) controls can be prepared by adding a sample into a StainExpress T Cell Subsets tube without adding a CAR Detection Reagent. Apart from that, these controls should be treated exactly the same as the fully stained samples to ensure accurate gating of CAR^+ T cell populations.
13. Proceed to flow cytometric analysis.

2.2 Immunofluorescent staining and lysis of whole blood or leukapheresis products using the StainExpress T Cell Subsets Cocktail, optional in combination with CAR Detection Reagents and additional antibodies

▲ (Optional) To determine cell counts, prepare a replicate sample using a no wash protocol. For optimal convenience and comprehensive cellular composition information, run the StainExpress Immune Cell Composition Cocktail on a MACSQuant Analyzer with precise volumetric counting. Calculate cell count/mL by multiplying the number of cells/mL obtained with the StainExpress Cocktail by the dilution factor (e.g. ×20). To determine the number of PE-positive cells, such as CAR^+ T cells, use the T Cell Subsets Cocktail. For example, if 50% of the $CD3^+$ T cells are PE-positive and the measure is 1×10^6 $CD3^+$ T cells/mL, then 5×10^5 CAR^+ T cells/mL are in the sample.

1. Dilute 10× Red Blood Cell Lysis Solution 1:10 with double-distilled water (ddH_2O), for example, dilute 1 mL of 10× Red Blood Cell Lysis Solution with 9 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.
2. Add 100 µL of whole blood or leukapheresis product to one StainExpress T Cell Subsets Cocktail tube.
3. (Optional) Add suitable amounts of the CAR Detection Reagent of choice and other liquid antibodies for available channels (refer to table in chapter 1.3) according to manufacturer's recommendation.
4. Incubate for 10–20 seconds, then vortex at high speed for 5 seconds and incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).

   ▲ **Note:** Working at lower temperatures requires increased incubation times.
5. Add 1900 µL of 1× Red Blood Cell Lysis Solution and immediately vortex thoroughly for 3 seconds. Incubate for 15 minutes in the dark at room temperature (+19 to +25 °C).
6. Centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.

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7. (Optional) If using a biotin-conjugated CAR Detection Reagent follow steps 7a–d:

   a. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.

   b. Resuspend cells in 98 µL buffer and add 2 µL of Biotin Antibody, PE, REAfinity.

   c. Vortex shortly at high speed and incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).

   d. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.

8. (Optional for fixation) Add 250 µL of buffer and 250 µL of Inside Fix to the cells and incubate for 20 minutes in the dark at room temperature (+19 to +25 °C).

9. (Optional for fixation) Add up to 2 mL of buffer.

10. (Optional for fixation) Centrifuge cells at 300×g for 5 minutes at room temperature (+19 to +25 °C). Aspirate supernatant completely.

11. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry.

    ▲ **Note:** Store samples at +2 to +8 °C protected from light until analysis. Acquire samples within 1 hour after staining.

12. (Optional) As a negative control, fluorescence-minus-one (FMO) controls can be prepared by adding a sample into a StainExpress T Cell Subsets tube without adding a CAR Detection Reagent. Apart from that, these controls should be treated exactly the same as the fully stained samples to ensure accurate gating of CAR^+ T cell populations.

13. Proceed to flow cytometric analysis.

2.3 Flow cytometric data acquisition with the MACSQuant Analyzer 16

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

▲ Please refer to the data sheet of the MACS Comp Bead Kit, anti-REA, when using beads for compensation.

1. Prepare and prime the MACSQuant Analyzer. Make sure the calibration and instrument settings of the instrument have been optimized for acquisition of the StainExpress T Cell Subsets Cocktail.

2. For optimal compensation, prepare single stainings of suitable beads or cells with all single antibody tubes from one pouch of the matching StainExpress Compensation Set. If using beads, add one full drop of MACS Comp Beads – anti-REA and one full drop of MACS Comp Beads – blank directly to each compensation tube.
   (Optional) If using a CAR Detection Reagent in the free PE channel, or if additional liquid markers have been added in available channels, prepare an additional tube for optimal compensation of the used reagent.
   (Optional) Prepare an additional tube for optimal compensation of 7-AAD. If using beads, add 500 µL of MACSQuant Running Buffer and one drop of MACS Comp Beads - blank. Use this sample to set the compensation values for 7-AAD, ensuring precise correction for any signal spillover into the 7-AAD channel.

   ▲ **Note:** For optimal compensation always use the provided StainExpress Compensation Set matching the StainExpress Cocktails used. To ensure they match, check for identical order number-related lot numbers indicated on the pouches next to the expiration date.

   ▲ **Note:** One full drop of beads is approximately 50 µL.

   ▲ **Note:** For compensation on cells add 100 µL cell suspension instead of beads directly to each compensation tube.

3. Incubate for 10–20 seconds, then vortex at high speed for 5 seconds and incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).

   ▲ **Note:** Working at lower temperatures requires increased incubation times.

4. Dilute each sample by adding 1 mL of MACSQuant Running Buffer. Mix well.

5. If using the MACS Comp Bead Kit, anti-REA, follow the protocol for compensation set up of the MACSQuant Analyzer.

   ▲ **Note:** For automated compensation choose the "CompensationMultiColor" Express Mode.

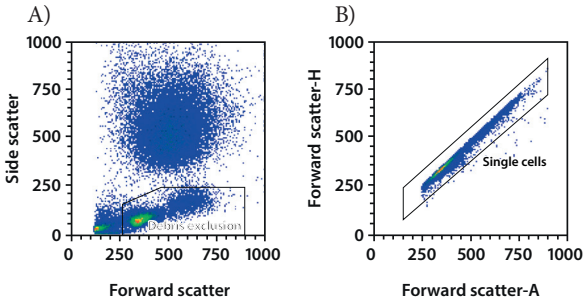
6. Choose appropriate voltage settings for forward scatter (FSC) and side scatter (SSC).

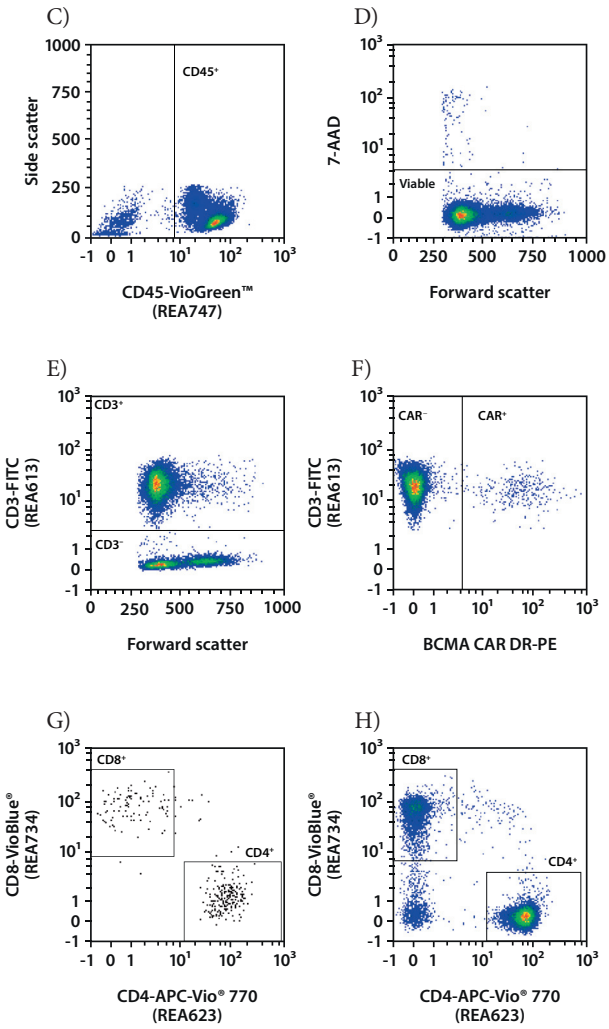
7. Define an appropriate threshold, based on FSC versus SSC, for the exclusion of debris from the data acquisition.

8. Start flow cytometric data acquisition.

3. Examples of immunofluorescent staining with the StainExpress T Cell Subsets Cocktail

CAR T cells, generated within 12 days using the CliniMACS Prodigy® T Cell Transduction process, were spiked into healthy donor blood samples and were stained with the StainExpress T Cell Subsets Cocktail and BCMA CAR Detection Reagent, human, PE. Staining was carried out at +19 to +25 °C for 10 minutes. Cells were analyzed by flow cytometry using the MACSQuant Analyzer 16.
To exclude debris, a gate was set on FSC versus SSC encompassing lymphocytes and monocytes (A). Single cells were identified and doublets excluded by gating on the FSC-A and FSC-H plot, selecting the population with uniform scatter characteristics (B). To exclude residual erythrocytes and to identify leukocytes, CD45 was used to gate on $CD45^+$ leukocytes (C). Dead cells were excluded by gating on 7-AAD^- cells (D). $CD3^+$ cells were identified (E) and discriminated in CAR^+ and CAR^- cells (F). CAR^+ cells were further divided into $CD4^+$ and $CD8^+$ T cells (G), as were CAR^- cells (H).





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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