Miltenyi Biotec

CAR Antibody (G4S Linker), REAfinity®

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1. Description

This product is for research use only.

Components 60 µL CAR Antibody (G4S Linker), REAfinity

Product	Order no.
CAR Antibody (G4S Linker), PE, REAfinity (clone: REA1410)	130-139-897
CAR Antibody (G4S Linker), APC, REAfinity (clone: REA1410)	130-139-898
CAR Antibody (G4S Linker), Biotin, REAfinity (clone: REA1410)	130-140-940

Capacity For 30 tests or up to 3×10^7 total cells..

Product format The reagent is supplied in buffer containing stabilizer and 0.05% sodium azide.

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

1.1 Background information

The CAR Antibody (G4S Linker), REAfinity, has been developed for the detection of the G4S linker sequence. This linker sequence is frequently used in chimeric antigen receptors (CARs) to connect the variable heavy (VH) and variable light (VL) domains within the antigen-recognizing single-chain variable fragment (scFv), improving their stability and resistance to proteolysis and aggregation. The CAR Antibody (G4S Linker), REAfinity (clone: REA1410), specifically recognizes the G4S linker region within CAR T cells. This antibody is designed for use in flow cytometry to identify and analyze CAR-expressing T cells. The mutated human IgG1 Fc region of the CAR Antibody (G4S Linker), REAfinity abolishes its binding to Fcγ receptors. This allows for background-free analysis and eliminates the need for additional blocking steps, such as using an FcR blocking reagent.

1.2 Applications

- Identification and enumeration of G4S Linker CAR$^+$ T cells by flow cytometry.

1.3 Recommended antibody dilution

The recommended dilution for CAR Antibody (G4S Linker), REAfinity is **1:50 for up to 10^6 cells/100 µL**, e.g., 2 µL in a final staining volume of 100 µL for labeling of up to 10^6 cells and subsequent analysis by flow cytometry.

Cells should be stained prior to fixation, if formaldehyde is used as a fixative.

1.4 Reagent and instrument requirements

- PEB buffer: autoMACS® Running Buffer (# 130-091-221). Alternatively, 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).
- (Optional) Red Blood Cell Lysis Solution (10×) (# 130-094-183)
- (Optional) Double-distilled water (ddH_2O).
- (Optional) 7-AAD Staining Solution (# 130-111-568 or # 170-100-042, CE-IVD).
- (Optional) Inside Fix, a component of the Inside Stain Kit (# 130-090-477).
- (Optional) If staining is done with a Biotin conjugate, additionally use Biotin Antibody, REAfinity (clone: REA746). It is recommended to use Biotin Antibody, PE, REAfinity (# 130-110-951). The total volume of the staining cocktail is 100 µL (including Biotin Antibody, REAfinity, 7-AAD Staining Solution, and additional flourochrome-conjugated antibodies). See table 1 for a recommendation of antibodies for the staining cocktail.

Product name	Clone
CD3 Antibody, anti-human, REAfinity	REA613
CD4 Antibody, anti-human, REAfinity	REA623
CD8 Antibody, anti-human, REAfinity	REA734
CD14 Antibody, anti-human, REAfinity	REA599
CD45 Antibody, anti-human, REAfinity	REA747

Table 1: Recommended antibodies to include in the staining panel.

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2. Protocol

2.1 (Optional) Lysis of whole blood

1. Dilute 10× Red Blood Cell Lysis Solution 1:10 with double-distilled water (ddH_2O). For example, dilute 2 mL of 10× Red Blood Cell Lysis Solution with 18 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 an appropriate amount of whole blood to a suitable tube, e.g., add 1 mL of whole blood to a tube with 50 mL capacity.
3. Add 1× Red Blood Cell Lysis Solution in 20-fold excess to the whole blood. For example, add 20 mL of 1× Red Blood Cell Lysis Solution to 1 mL of whole blood.
4. Vortex immediately and thoroughly for 3 seconds and incubate for 10–20 minutes in the dark at room temperature (+19 to +25 °C).
5. Centrifuge at 300 × g for 10 minutes. Remove supernatant.
6. (Optional) Add more than 20× volume of PEB buffer of the initial cell sample volume, e.g., add 20 mL buffer when using 1 mL blood. Centrifuge at 300 × g for 10 minutes. Remove supernatant.

   ▲ **Note:** An additional washing step reduces staining background.

7. Resuspend cell pellet in a suitable amount of buffer and proceed to immunofluorescent staining (chapter 2.2, step 4).

2.2 General protocol for immunofluorescent staining

▲ 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, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^6 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

Immunofluorescent staining with Biotin conjugates

▲ Prepare a staining cocktail containing Biotin Antibody, REAfinity, 7-AAD Staining Solution for dead cell exclusion, and additional fluorochrome-conjugated antibodies. For examples refer to table 1. For details refer to the respective data sheets.

1. Determine cell number.
2. Centrifuge cell suspension at 300 × g for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^6 nucleated cells per 98 µL of buffer.
4. Add 2 µL of the CAR Antibody (G4S Linker), REAfinity.
5. Mix well and incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).
6. Wash cells by adding 1 mL of buffer per 10^6 cells.
7. Mix well and centrifuge at 300 × g for 5 minutes at room temperature (+19 to +25 °C). Aspirate supernatant completely.
8. Repeat steps 6 and 7.
9. Add 100 µL of staining cocktail containing Biotin Antibody, REAfinity, 7-AAD Staining Solution, and additional fluorochrome-conjugated antibodies. Mix cells by pipetting up and down.
10. Incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).
11. Wash cells by adding 1 mL of buffer per 10^6 cells.
12. Mix well and centrifuge at 300 × g for 5 minutes at room temperature (+19 to +25 °C). Aspirate supernatant completely.
13. (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).
14. (Optional for fixation) Add up to 2 mL of buffer.
15. (Optional for fixation) Centrifuge cells at 300 × g for 5 minutes at room temperature (+19 to +25 °C). Aspirate supernatant completely.
16. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry.

    ▲ **Note:** Acquire the samples within 1 hour after staining.

Immunofluorescent staining with PE or APC conjugates

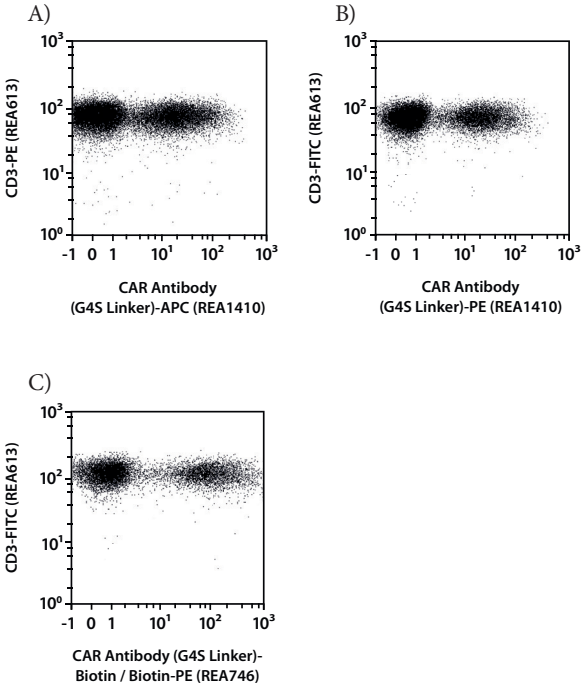
▲ Prepare a staining cocktail containing CAR Antibody (G4S Linker), REAfinity, 7-AAD Staining Solution for dead cell exclusion, and additional fluorochrome-conjugated antibodies. For examples refer to table 1. For details refer to the respective data sheets.

1. Determine cell number.
2. Centrifuge cell suspension at 300 × g for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^6 nucleated cells per 50 µL of buffer.
4. Add 50 µL staining cocktail containing 2 µL CAR Antibody (G4S Linker), REAfinity, 7-AAD Staining Solution, and additional fluorochrome-conjugated antibodies.
5. Mix well by pipetting up and down and incubate for 10 minutes in the dark at room temperature (+19 to +25 °C).
6. Wash cells by adding 1 mL of buffer per 10^6 cells.
7. Mix well and centrifuge at 300 × g for 5 minutes at room temperature (+19 to +25 °C). 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:** Acquire the samples within 1 hour after staining.

3. Example of immunofluorescent staining with CAR Antibody (G4S Linker), REAfinity

T cells expressing a chimeric antigen receptor (CAR) designed with the G4S linker were stained with CAR Antibody (G4S Linker), REAfinity, CD3-PE / CD3-FITC, and 7-AAD Staining Solution. Flow cytometry analysis was performed using the MACSQuant®Analyzer 10. Cell debris and dead cells were excluded based on scatter signals and 7-AAD fluorescence. The data presented show viable $CD3^+$ CAR 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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