Miltenyi Biotec

Protocol for labeling of cells with MACS® GMP Fluorescent Antibodies

Contents

Important note

The present protocols are research protocols, based on laboratory experience. Miltenyi Biotec GmbH cannot and will not accept any liability as to the outcome of procedures. The procedures are for research use only, not for diagnostic or therapeutic purposes.

1. Description

1.1 Purpose

This protocol describes the process for labeling of cells with MACS® GMP Fluorescent Antibodies. MACS GMP Fluorescent Antibodies have been developed for the flow cytometric analysis followed by flow cytometric sorting of cell populations from human heterogeneous blood products in the clinical setting. They are intended for *in vitro* use only and not to be used for therapeutic application or direct infusion into patients.

1.2 Reagent and instrument requirements

- MACS GMP Fluorescent Antibodies, e.g., MACS GMP CD4-VioBlue® (# 170-076-504), MACS GMP CD25-PE (# 170-076-503), MACS GMP CD45RA-FITC (# 170-076-502), or MACS GMP CD127-APC (# 170-076-501)
- CliniMACS® PBS/EDTA Buffer (# 700-25 or # 700-29)
- Human serum albumin (HSA) as supplement to CliniMACS PBS/EDTA Buffer, final concentration 0.5%
- Suitable buffer for flow sorting, e.g., CliniMACS PBS/EDTA buffer with 0.5% HSA
- Transfer Bags 600 mL (# 130-019-001)
- Luer/Spike Interconnectors (# 130-018-701)
- Sterile tubing welder, e.g., Terumo® Sterile Connection Device TSCD® SC-201A
- Flow cytometer, e.g., MACSQuant Analyzer 10 (# 130-096-343) for analysis
- Centrifuge, suitable for bag processing
- Plasma extractor
- Digital balance
- Tubing slide clamps or scissor clamps
- (Optional, for analysis only) Fluorochrome-conjugated antibodies for flow cytrometric analysis, e.g., CD45-VioGreen™ (# 130-096-906)
- (Optional, for analysis only) Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells without fixation

Disclaimer

MACS GMP Products are for research use and *ex vivo* cell culture processing only, and are not intended for human *in vivo* applications. For regulatory status in the USA, please contact your local representative.

Quality statement

MACS GMP Products are manufactured and tested under a quality management system (ISO 13485) and are in compliance with relevant GMP guidelines. They are designed following the recommendations of USP <1043> on ancillary materials.

2. Protocol

2.1 Labeling of cells in bags

▲ Volumes given below are for up to 1×10^9 total cells.

▲ The process requires 2×1L CliniMACS PBS/EDTA buffer supplemented with HSA to a final concentration of 0,5%.

▲ The cell number (and optionally viability) of the starting material prior to labeling has to be determined.

1. Spike the original starting material bag with a Luer/Spike Interconnector and remove a small sample (0.5 mL) using a syringe. Perform cell counts and viability assessment on this sample e.g., by using the MACSQuant Analyzer 10.
2. Calculate the volume to be used in the experiment, with a maximum of 1×10^9 total cells.
3. Weigh an empty Transfer Bag 600 mL including a connected Luer/Spike Interconnector and clamp and label it as 'Cell Preparation Bag'.
4. Transfer correct volume from starting material to the Cell Preparation Bag by either syringe transfer or weight via sterile welding of the leukapheresis bag.
5. Fill Cell Preparation Bag up to 500 mL with CliniMACS PBS/EDTA Buffer containing 0.5% HSA by sterile welding a buffer bag.
6. Connect an empty 600 mL Transfer Bag via sterile welding to the Cell Preparation Bag prior to centrifugation (to be used as waste bag).

33861/02 | Issued: 2018-10

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7. Centrifuge cells at 300×g for 15 minutes at room temperature (19–25 °C) without brake.
8. Remove supernatant using the plasma extractor (taking care not to disturb the pellet), disconnect waste bag, and thereafter resuspend the cell pellet completely.
9. Weigh the Cell Preparation Bag with the cells and calculate weight of cell pellet. Add CliniMACS PBS/EDTA Buffer containing 0.5% HSA to a final volume (weight) as described below, depending on the number of MACS GMP Fluorescent Antibodies to be used (recommended antibody dilution of 1:11 in a total staining volume of 55 mL should be used).

   ▲ **Note:** Depending on the number of MACS GMP Fluorescent Antibodies to be applied to one sample, the added volume of buffer should be adjusted in order to achieve a final dilution of 1:11. For example, if only one MACS GMP Fluorescent Antibody is used, cells are resuspended in 50 mL buffer with 5 mL MACS GMP Fluorescent Antibody. When four MACS GMP Fluorescent Antibodies are used together, cells are resuspended in 35 mL buffer and 5 mL of each MACS GMP Fluorescent Antibody is added to end up with a total staining volume of 55 mL.
10. Add 5 mL of each MACS GMP Fluorescent Antibody by using a syringe. The total staining volume should now be 55 mL.
11. Mix the bag thoroughly and incubate for 10 minutes in the dark at 2–8 °C, e.g., in the refrigerator.
12. Wash the cells by adding a suitable buffer for flow sorting, e.g., CliniMACS PBS/EDTA buffer containing 0.5% HSA to a final volume of 500 mL.
13. Connect a 600 mL Transfer Bag via sterile welding to be used as waste bag after centrifugation.
14. Centrifuge at 300×g for 15 minutes at room temperature (19–25 °C) without brake.
15. Remove as much supernatant as possible using the plasma extractor (taking care not to disturb the pellet), remove waste bag, and resuspend the pellet completely.
16. Resuspend the cells in an appropriate volume of a suitable buffer for flow sorting, e.g., CliniMACS PBS/EDTA buffer containing 0.5% HSA for further processing/sorting of cells.

    ▲ **Note:** When small volumes such as 10–20 mL are required, an additional transfer and wash in a 150 mL bag or a 50 mL tube might be beneficial for sufficient supernatant removal without concomitant loss of cells.

2.2 Evaluation and analysis of labeling performance

The labeling performance of MACS GMP Fluorescent Antibodies can be determined by flow cytometry. Propididum Iodide (PI) Solution (# 130-093-233) can be used for flow cytometric exclusion of dead cells.

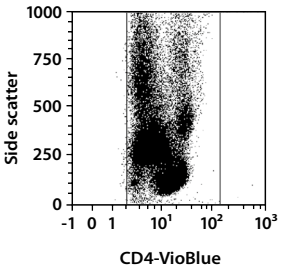
1. Transfer a small sample (e.g. 0.2 mL) to a tube for flow cytometric analysis. It is recommended to determine at least cell concentration, viability, and frequency/number of target cells.
2. Perform additional staining if needed (e.g., CD45-VioGreen™ or propidium iodide).
3. Analyze the sample using, e.g., the MACSQuant Analyzer 10.

3. Example of immunofluorescent labeling of cells with MACS® GMP Fluorescent Antibodies

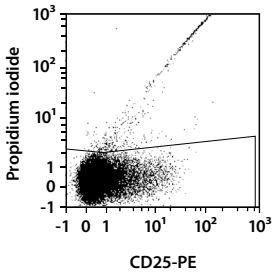
A leukapheresis sample was labeled with MACS® GMP CD25-PE, MACS GMP CD45RA-FITC, MACS GMP CD4-VioBlue, and MACS GMP CD127-APC. Cells were analysed by flow cytometry using the MACSQuant Analyzer 10. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

3.1 Description of the detailed gating strategy

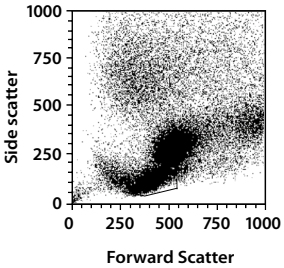
A) CD4-VioBlue (trigger) versus side scatter (SSC)
Activated gate: no gate
Set trigger/R1 to include $CD4^+$ cells.



B) PE versus PI – exclusion of dead cells
Activated gate: R1 = $CD4^+$ cells
Set R2 to exclude dead (PI positive) cells.



C) Forward scatter (FSC) versus side scatter (SSC)
Activated gate: R2 = R1*R2 = viable $CD4^+$ cells
Set R3 to include lymphocytes only.



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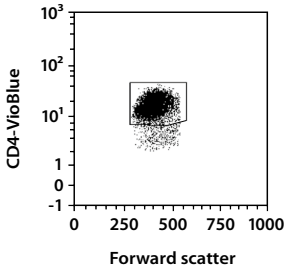
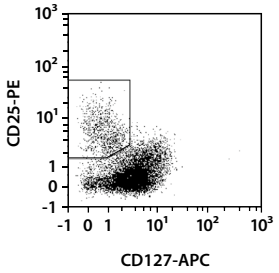
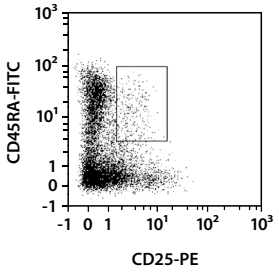
D) FSC versus CD4-VioBlue
Activated gate: G3 = R1*R2*R3 = viable $CD4^+$ lymphocytes
Set gate R4 including all $CD4^+$ cells.



E) CD127-APC versus CD25-PE for regulatory T cells
Activated gate: G4 = R1*R2*R3*R4 = viable $CD4^+$ lymphocytes
Set gate R5 including $CD4^+CD25^+CD127^-$ to distinguish regulatory T cells.



F) CD25-PE versus CD45RA-FITC for naive regulatory T cells
Activated gate: G4 = R1*R2*R3*R4 = viable $CD4^+$ lymphocytes
Set gate R6 including $CD4^+CD25^+CD45RA^+$ to distinguish naive regulatory T cells.



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