Miltenyi Biotec

Protocol for the removal of MACS® GMP ExpAct Treg Beads

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 clinical use.

1. Description

1.1 Purpose

This protocol describes the process for removal of MACS GMP ExpAct Treg Beads. MACS GMP ExpAct Treg Beads are antibody coated particles (3.5 µm) that can be used for the clinical grade expansion of T cell populations. This protocol describes the process how to remove the particles by using the CliniMACS® Plus Instrument. The protocol also describes how the number of residual particles within the cell product can be determined by flow cytometry.

1.2 Required Materials

- CliniMACS Plus Instrument (# 151-01)
- Two-laser flow cytometer (equipped with lasers 405 nm, and 488 nm to allow simultaneous analysis of FITC, PE, and APC), e.g. MACSQuant Analyzer (# 130-092-197) and appropriate software
- CliniMACS Tubing Set LS (# 162-01)
- Propidium Iodide Solution (# 130-093-233) or 7-AAD
- CliniMACS PBS/EDTA Buffer (# 700-25)
- Human serum albumin (HSA) or bovine serum albumin (BSA) as supplement to CliniMACS PBS/EDTA Buffer, final concentration 0.5%
- DNase solution (1000 U/mL) (e.g. DNase 1 from Worthington # LS006333)
- MACSQuant® Washing Solution (# 130-092-749)
- Labeling Check Reagent-PE (# 130-095-228)
- Labeling Check Reagent-APC (# 130-095-237)
- (Optional) MACSmix™ Tube Rotator (# 130-090-753)

2. Protocol

T cells or Treg cells are expanded for 10-14 days using standard condition with, e.g., MACS GMP ExpAct Treg Kit in TexMACS™ GMP Medium (# 170-076-306).

Cell count and viability have to be determined. It is recommended to determine the frequency of the T cells respectively Tregs:

1. Use a small aliquot of the cells for determination of the cell number, viability and T cell analysis, e.g., by using the MACSQuant® Analyzer. For Treg cell analysis please refer to the special protocol "Proposal for $CD4^+CD25^{hi}$ cell determination by flow cytometry".
2. Wash the residual cell suspension by adding at least two fold volume of CliniMACS PBS/EDTA Buffer containing 0.5% HSA.
3. Centrifuge at 300×g for 10 minutes at room temperature. Aspirate supernatant.
4. Resuspend cells at a concentration of 4×10^7 cells/mL including MACS GMP ExpAct Treg Beads in CliniMACS PBS/EDTA Buffer containing 0.5% HSA. The final volume should not exceed 300 mL.
5. Determine the weight of the empty Cell Collection Bag.
6. Transfer volume in a 600 mL bag. For analysis purpose take an aliquot of 0.5 mL (= original fraction).

2.2 Automated separation using the CliniMACS® Plus Instrument

The depletion of MACS GMP ExpAct Treg Beads from the original fraction is performed by automatic cell separation using the CliniMACS® Plus Instrument in combination with CliniMACS PBS/EDTA Buffer, the CliniMACS Tubing Set LS and software sequence DEPLETION 2.1. The depleted fraction (cells without MACS GMP ExpAct Treg Beads) is collected in the Cell Collection Bag.

1. Switch on the CliniMACS® Plus Instrument and select DEPLETION 2.1. for depletion of MACS GMP ExpAct Treg Beads.
2. Confirm your choice by pressing "ENT" and select a tubing set. Enter the order number of the selected tubing set. The order number (Ref. no.) can be found on the product label. Be aware that the separation program DEPLETION 2.1 is a "staged loading" program. The program includes a query for the following parameters in order to adjust the separation sequence for each individual sample and to provide important information on the required buffer and bag volumes:

   - WBC concentration (in this application: Cell number plus MACS GMP ExpAct Treg Beads number/mL)

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▲ **Note:** If cell number plus MACS GMP ExpAct Treg Beads number/mL is lower than minimal concentration choose 20×10^6 cells/mL.

- Percentage of labeled cells (in this application: Percentage of MACS GMP ExpAct Treg Beads among total cells (cells + MACS GMP ExpAct Treg Beads))

- Total volume of the sample ready for loading on the CliniMACS Tubing Set.

▲ **Note:** If the instrument screen refers to output of 1200 mL of target cells please connect a second 600 mL Transfer Bag to the pre-connected Cell Collection Bag.

3. Follow the instructions given on the CliniMACS Plus Instrument screen and connect appropriate bags to the tubing set using Luer/Spike Interconnectors. Ensure that the slide clamps of the Luer/Spike Interconnectors are open.
4. If more than 1 L of buffer is needed, connect two buffer bags using a Plasma Transfer Set with two couplers. Use the second port of one of the buffer bags for the connection to the tubing set.
5. Follow the instructions on the instrument screen for the installation of the tubing set and start the automated separation program.
6. After the separation has been finished, determine the weight of the Cell Collection Bag and take a sample of the Cell Collection Bag for flow cytometric analysis.

2.2 Analysis of MACS® GMP ExpAct Treg Beads removal

An effective depletion of MACS® GMP ExpAct Treg Beads requires an optimized staining protocol for flow cytometric evaluation of the separation performance. Before (original fraction) and after the separation (target cell fraction) aliquots are taken for flow cytometric analysis. The total number of cells in the target cell fraction can be analyzed by flow cytomtery. After lysis of the cells MACS GMP ExpAct Treg Beads are stained with Labeling Check Reagent-APC and Labeling Check Reagent-PE.
A detailed step-by-step description of the cell lyses and staining procedure is given below:
Controls:

- Negative control: Cells without MACS GMP ExpAct Treg Beads; lysed and stained with Labeling Check Reagent

1. Use an aliquot of the Target cell fraction for determination of cell number and viability.
2. Transfer 10^8 cells from the Target cell fraction and an adequate volume of the original fraction ($5\text{-}20\times10^4$ MACS GMP ExpAct Treg Beads) into 15 mL tubes; each fraction is analyzed as triplicate.
3. Fill up to 15 mL with CliniMACS PBS/EDTA Buffer containing 0.5% HSA and centrifuge at 300×g for 10 minutes at room temperature.
4. Remove supernatant completely and fill up to 2 mL with double distilled water. Incubate for 1 hour at 37 °C.
5. Add 200 µL of DNase solution (with a concentration of 1000 U/mL DNase).
6. Incubate sample for 10 minutes at 37 °C under slow, continuous rotation, for example, using the MACSmix™ Tube Rotator.
7. For cell lysis add 10 mL of MACSQuant Washing Solution and incubate for 5–10 minutes at room temperature with occasional mixing to generate a clear solution.
8. Centrifugation at 1000×g for 15 minutes at room temperature.
9. Remove the supernatant and leave 0.1 mL in the tube.
10. Add 75 µL of Labeling Check Reagent-APC and 75 µL of Labeling Check Reagent-PE.
11. Incubate for 15 minutes at RT under slow continuous rotation, for example, using the MACSmix Tube Rotator.
12. Fill up to 5 mL with CliniMACS PBS-EDTA Buffer containing 0.5% HSA and centrifuge at 300×g for 10 minutes at room temperature.
13. Remove the supernatant and leave a residue of 100 µL in the tube. Fill up the pellet to 250 µL with CliniMACS PBS/EDTA Buffer containing 0.5% HSA.
14. Analyze 100 µL of each sample using a flow cytometer (acquisition volume).

3. Example of a removal of MACS® GMP ExpAct Treg Beads

Analysis of residual MACS GMP ExpAct Treg Beads in the final fraction is performed by flow cytometric analysis, e.g. by using a MACSQuant Analyzer. Set the instrument to a standard 4-color data acquisition protocol. Make sure the calibration and compensation settings have been optimized. Due to the MACS GMP ExpAct Treg Beads, which are smaller than cells, FSC/SSC settings of the flow cytometer might need to be adjusted.

At least 100 µL of each sample are measured. Start with the target fraction, clean the flow cytometer carefully and go on with the original fraction, clean the flow cytometer carefully and go on with the control. It is important to measure the samples in this order and to clean the instrument after each sample to avoid carry over. The following protocol is designed for data acquisition and for analysis of all samples.

3.1 Description of the detailed gating strategy

Labeling Check Reagent is developed to stain MACS GMP ExpAct Treg Beads. Particles or cell debris are unspecifically stained with Labeling Check Reagent. Therefore, a stringent gating strategy and a negative control are used. The analysis of the MACS GMP ExpAct Treg Bead removal procedure (original fraction, target fraction and negative control) is performed by using Labeling Check Reagents. The negative control is used to determine the non-specific staining of cell debris and particles. The original fraction is used to determine the MACS GMP ExpAct Treg Beads.

Negative control: Cells without MACS GMP ExpAct Treg Beads (lysed and stained with Labeling Check Reagents).

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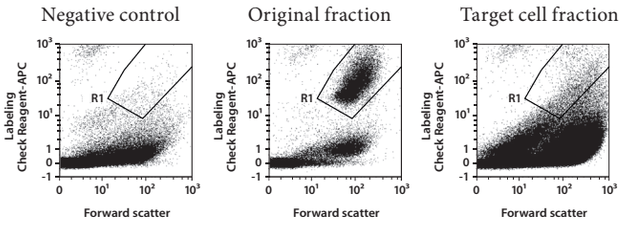
A Plot: Forward scatter (FSC) versus Labeling Check-Reagent-APC

Activated gate: No gate.

Set R1 to exclude non-specifically stained cell debris or particles .

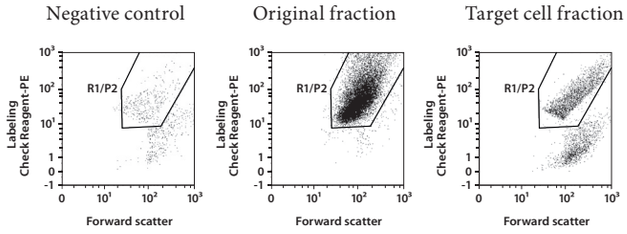


Define G1 = R1 = Labeling Check Reagent-APC $^{+}$

B Plot: Forward scatter (FSC) versus Labeling Check-Reagent-PE

Activated gate: G1 = R1 = Labeling Check Reagent APC^{+}.

Set R2 to exclude non-specifically stained cell debris or particles.



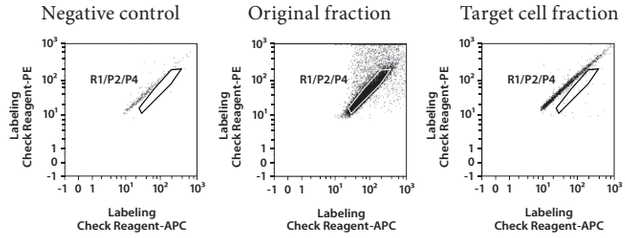
Define G2 = R1*R2 = Labeling Check Reagent-PE^{+}/Labeling Check Reagent-APC^{+}

C Plot: Labeling Check Reagent-APC versus Labeling Check Reagent-PE

Activated gate: G2 = R1*R2 = Labeling Check Reagent PE^{+}/APC^{+}.

Set R3 thereby including MACS GMP ExpAct Treg Beads.

▲ **Note:** Exclude non-specifically stained cell debris or particles.



Define G3 = R1*R2*R3 = MACS GMP ExpAct Treg Beads
Generate a gate statistic of the dot plot for later calculation.

3.2 Determination of absolute numbers of not removed MACS GMP ExpAct Treg Beads

For calculation of the MACS GMP ExpAct Treg Bead removal process the events of the target cell fraction in gate 3 have to be shown.

Calculate mean value of triplicate of gate 3 (for target cell fraction).

$$\textbf{Mean value triplicate} = \frac{(\text{events triplicate 1} + \text{events triplicate 2} + \text{events triplicate 3})}{3}$$

To calculate the total events of MACSiBeads in the aliquot of the target cell fraction, the mean value triplicate is multiplied with the dilution factor 2.5 (acquisition volume is 100 µL, sample volume is 250 µL).

Total events/aliquot = Mean value triplicate × 2.5

The mean of the recovery is 20%. Concluding the recovery factor is 5.

Total MACSiBeads per 10^8 target cells = 5× Total events/aliquots

The total amount of MACS GMP ExpAct Treg Beads in the target cell fraction should be <1000 Beads per 10^8 cells.

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