Miltenyi Biotec

MACS® GMP labeling of cells and preparation for cell sort on MACSQuant® Tyto® Cell Sorter using MACS GMP Tytonase EDTAble

Contents

1. Description
   - 1.1 Purpose
   - 1.2 Reagent and instrument requirements
2. Protocol
   - 2.1 Labeling of cells in bags and rebuffering of cells
   - 2.2 Automated priming of the MACS GMP Tyto Cartridge
   - 2.3 Loading of cells in MACS GMP Tyto Cartridge
   - 2.4 Loading the MACS GMP Tyto Cartridge into the MACSQuant Tyto Cell Sorter
   - 2.5 Retrieval of cells from the MACS GMP Tyto Cartridge
   - 2.6 Evaluation and analysis of labeling and sorting performance
   - 2.7 Optional release of label (MACS GMP REAlease Kits only)

Important note

The present protocols are research protocols, based on laboratory experience. Miltenyi Biotec B.V. & Co. KG 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. The protocol is valid for the MACS GMP Tyto Cartridge and the MACS GMP Tyto Cartridge HS.

It is recommended that isolated cells will be analyzed for quantity and quality before use (see 2.6).

1. Description

1.1 Purpose

This protocol describes the process for labeling of cells with MACS GMP Fluorescent Antibodies or MACS GMP REAlease component A, and the preparation for cell sorting on the MACSQuant Tyto Cell Sorter. MACS GMP Fluorescent Antibodies and MACS GMP REAlease Kits have been developed for the flow cytometric analysis followed by cell 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 and MACS GMP REAlease Kits
- CliniMACS® PBS/EDTA Buffer CR/GMP (# 220-001-827)
- Human serum albumin (HSA) as supplement to CliniMACS PBS/EDTA Buffer CR/GMP in a final concentration 0.5% (w/v)
- MACS GMP Tytonase EDTAble (# 170-100-122)
- Transfer Bags 600 mL
- Luer/Spike Interconnectors
- 20 µm filter, e.g., Pre-Separation Filter 20 µm (# 130-101-812)
- MACS GMP Tyto Cartridge (# 170-076-011)
- MACS GMP Tyto Cartridge HS (# 170-084-001)
- 10 mL Syringe (with Luer-Lock tip)
- (Optionally) 50 mL centrifuge tubes / Serological pipets / Capillary pipet tips
- Sterile tubing welder, e.g., Terumo® Sterile Connection Device TSCD® SC-201A
- Flow cytometer, e.g., MACSQuant Analyzer 10 (# 130-096-343) for analysis
- MACSQuant Tyto Cell Sorter family (# 130-103-931, # 130-133-903)
- 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™ (# 130-113-124)
- (Optional, for analysis only) Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells without fixation

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Disclaimer

MACS GMP Products are for *ex vivo* cell 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 designed, manufactured and tested under an ISO 13485 quality management system and are in compliance with relevant GMP guidelines. They are designed following the recommendations of USP <1043> on ancillary materials. The manufacturing and testing of this product is in compliance with EP chapter 5.2.12 for "Raw materials of biological origin for the production of cell-based and gene therapy medicinal products". The declaration of animal- or human-derived materials is given on the Certificate of Origin.

2. Protocol

2.1 Labeling of cells in bags and rebuffering of cells

- Volumes given below are for up to 1×10^9 total cells.
- The process requires 2×1 L CliniMACS PBS/EDTA Buffer CR/GMP supplemented with HSA to a final concentration of 0.5% (w/v). This buffer is called process buffer.
- The cell number (and optionally the viability) of the starting material prior to labeling must be determined.
- To prepare buffer for cell sorting with the MACSQuant Tyto Cell Sorter, mix 95 mL process buffer with 5 mL MACS GMP Tytonase EDTAble (20× stock solution). This buffer is called sorting buffer.

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 the calculated volume from the starting material to the Cell Preparation Bag by either syringe transfer or weight via sterile welding of the starting material bag.
5. Fill the Cell Preparation Bag up to 500 mL with process buffer 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).
7. Centrifuge cells at 300×g for 15 minutes at room temperature (+19°C to +25 °C [+66 °F to +77 °F]) without brake.
8. Remove the supernatant using the plasma extractor and take care not to disturb the pellet. Disconnect the waste bag.
9. Weigh the Cell Preparation Bag with the cells and calculate weight of cell pellet. Add process buffer to a final volume (weight) as described below, depending on the number of MACS GMP Fluorescent Antibodies or MACS GMP REAlease component A to be used (recommended antibody dilution of 1:11 in a total staining volume of 55 mL should be used). Resuspend the pellet completely.

   Note: Depending on the number of MACS GMP Fluorescent Antibodies or MACS GMP REAlease component A 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 or MACS GMP REAlease component A is used, cells are resuspended in 50 mL buffer with 5 mL MACS GMP Fluorescent Antibody or MACS GMP REAlease component A. When four MACS GMP Fluorescent Antibodies or MACS GMP REAlease component A are used together, cells are resuspended in 35 mL buffer and 5 mL of each MACS GMP Fluorescent Antibody or MACS GMP REAlease component A is added to end up with a total staining volume of 55 mL.
10. Add 5 mL of each MACS GMP Fluorescent Antibody or MACS GMP REAlease component A 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 °C to +8 °C (+36 °F to +46 °F), e.g., in the refrigerator.
12. Wash the cells by adding process buffer 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°C to +25 °C [+66 °F to +77 °F]) without brake.
15. Remove as much supernatant as possible using the plasma extractor, taking care not to disturb the pellet. Remove the waste bag and completely resuspend the pellet in the sorting buffer (See preparation above).

    Note: MACS GMP Tyto Cartridge can be filled with a maximal volume of 10 mL; take maximal concentration of cells into consideration

2.2 Automated priming of the MACS GMP Tyto Cartridge

- For manual priming of cartridge please refer to MACSQuant® Tyto® Instrument short instructions: Cartridge priming and sample loading.
- The automated priming feature requires MACSQuantify™ Tyto Software 3.2 or higher.
- The automated priming feature enables automated cartridge priming on the instrument. After priming, the instrument

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automatically proceeds with sample sorting. Cells, which are sorted to the positive collection chamber during the priming process, are taken into account in the sort statistics.

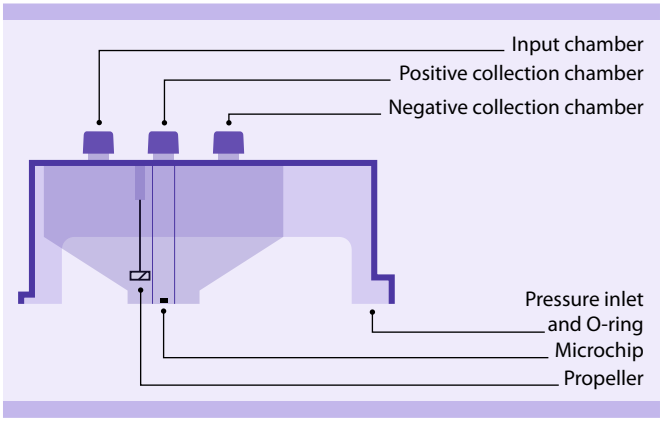
1. Open or create a suitable workspace for the desired sort. Refer to the MACSQuant Tyto instrument short instructions Setting up a sort workspace.
2. Load the sample into the cartridge. See 2.3 Loading of cells in the MACS GMP Tyto Cartridge.
3. To activate automated priming, select the checkbox Automated Priming in the Misc Controls tab of the Cartridge tab on the touchscreen.
4. Insert the cartridge and click the Start measurement button in the progress bar.
5. To start the priming process, click the Sort button. The priming process takes approximately one minute and is indicated in the instrument mode section of the progress bar.
6. After priming, the instrument automatically proceeds with sample sorting.

2.3 Loading of cells in the MACS GMP Tyto Cartridge

▲ Refer to the MACSQuant Tyto Cell Sorter User Manual for detailed information on using the instrument.

▲ All open cartridge steps must be performed in a biosafety cabinet

▲ Do not touch the microchip.



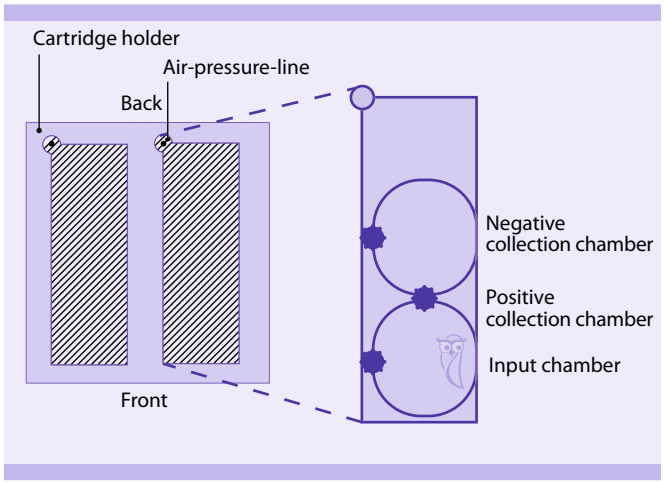
1. Remove plunger from a 10 mL syringe with Luer-Lock tip. Store plunger upside down.
2. Remove cap from the input chamber and attach syringe. Store cap upside down.

   Note: It has to be ensured that the propeller within the input chamber of the cartridge is located to the lower end of the shaft.
3. Place a 20 µm nylon mesh filter (Pre-Separation Filters 20 µm), on top of syringe.
4. Apply cell suspension onto the filter to remove cell clumps.
5. Discard the filter. Save an aliquot (e.g., 500 µL) as original fraction.
6. Carefully place plunger on top of syringe.
7. Flush out the sample by firmly applying the plunger at a rate of 0.5 mL/s.

   Note: Do not load the cartridge with higher rate as cells might be lost in the negative collection chamber.
8. Remove the syringe and reconnect the cap to the input chamber.
9. Proceed with loading into the MACSQuant Tyto Cell Sorter (2.4).

2.4 Loading the MACS GMP Tyto Cartridge into the MACSQuant Tyto Cell Sorter

1. Turn on the MACSQuant Tyto Cell Sorter for automatic initialization. Scan the 2D barcode on the MACS GMP Tyto Cartridge with the barcode scanner of the instrument.
2. Open the lid on top of the MACSQuant Tyto Cell Sorter and insert the MACS GMP Tyto Cartridge with the owl icon in the right direction. Make sure that the O-ring is placed on the air-pressure-line of the instrument. The MACS GMP Tyto Cartridge is locked automatically.



3. After the sort has been completed, the MACS GMP Tyto Cartridge can be removed from the instrument and fractions can be retrieved (2.5).

2.5 Retrieval of cells from the MACS GMP Tyto Cartridge

For calculation of recovery and yield, the absolute cell number of each fraction (original, positive, and negative) has to be determined. Assess the cell concentration either using a cell counter or e.g. the MACSQuant Analyzer 10.

Always start with the positive collection chamber.

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2.5.1 Positive collection chamber

The sorted cells are contained in the positive collection chamber.

1. (Optional) When volume is <100 µL, add process buffer or infusion fluid of choice into the positive collection chamber by using a syringe or an capillary pipet tipp.
2. Resuspend the sedimented cells by flushing/pipetting up and down.
3. Retrieve the full volume of cell suspension by using the syringe or pipet.
4. (Optional) For optimal retrieval wash the chamber with appropriate volume of MACS GMP PBS/$MgCl_2$ Buffer or infusion fluid of choice.

2.5.2 Negative collection chamber

The unsorted cells are contained in the negative collection chamber.

1. Invert or vortex the MACS GMP Tyto Cartridge to resuspend the sedimented cells.
2. Retrieve the needed volume (or complete sample) of unsorted cells from the negative collection chamber by using a syringe or or a capillary pipet tip.

2.6 Evaluation and analysis of labeling and sorting performance

▲ It is recommended to analyze the isolated cells for quantity and quality before use.

The labeling performance of MACS GMP Fluorescent Antibodies or MACS GMP REAlease component A and sorting performance of MACSQuant Tyto Cell Sorter can be determined by flow cytometry. Propididum Iodide Solution (# 130-093-233) can be used for flow cytometric exclusion of dead cells.

1. Transfer a small sample of the sorted fraction (e.g. 0.2 mL) to a tube for flow cytometric analysis; include also a pre-sort and non-sort sample to evaluate sorting performance. 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 samples using e.g., the MACSQuant Analyzer 10.

2.7 Optional release of label (MACS GMP REAlease Kits only)

▲ The process requires 2×1 L process buffer.

1. Weigh an empty 600 mL Transfer Bag, including a connected Luer/Spike Interconnector and clamp, and label it as 'Cell Release Bag'.
2. Transfer the labelled cells to the "Cell Release Bag" by either syringe transfer or weight via sterile welding the starting material bag.
3. Weigh the bag with the cells and add process buffer to a final volume (= weight) of 95 mL.
4. Add 5 mL (eg. by using a syringe) of the MACS GMP REAlease Release Reagent (component B in the MACS GMP REAlease Kit), the total staining volume should now be 100 mL.
5. Mix the bag thoroughly and incubate for 10 minutes at room temperature.
6. Wash the cells by adding process buffer to a final volume of 500 mL.
7. Prior to centrifugation, connect a 600 mL transfer bag via sterile welding to be used as waste bag after centrifugation.
8. Centrifuge at 300 × g for 15 minutes at room temperature without brake.
9. Resuspend the cells in an appropriate volume of a suitable buffer for downstream processing and take an adequate aliquot for analysis.

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