Miltenyi Biotec

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

Contents

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 2.7).

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 MACSQuant Tyto Cell Sorter. MACS GMP Fluorescent Antibodies and MACS GMP REAlease Kits 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 and MACS GMP REAlease Kits
- CliniMACS® PBS/EDTA Buffer
- MACS GMP PBS/$MgCl_2$ Buffer (# 170-076-155)
- Human serum albumin (HSA) as supplement to CliniMACS PBS/EDTA Buffer and MACS GMP PBS/$MgCl_2$ Buffer, final concentration 0.5%
- MACS GMP Tytonase (# 170-076-210)
- Transfer Bags 150 mL
- 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 Falcon tubes / Serological pipets / Extra long 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 (# 130-103-931)
- 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

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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 2×1 L CliniMACS PBS/EDTA buffer supplemented with HSA to a final concentration of 0.5% (w/v).
- 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% (w/v) 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).
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 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% (w/v) HSA 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).

- **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 CliniMACS PBS/EDTA Buffer containing 0.5% (w/v) 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°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 waste bag, and resuspend the pellet completely.

2.2 Preparation of MACS GMP Tyto Running Buffer and Rebuffering of the cells

- The process requires 1×1 L MACS GMP PBS/$MgCl_2$ Buffer supplemented with HSA to a final concentration of 0.5% (w/v) HSA and 1×5 mL MACS GMP Tytonase (20× Stock solution).

1. For removal of EDTA, fill the bag up to 500 mL with MACS GMP PBS/$MgCl_2$ Buffer containing 0.5% (w/v) HSA by connecting a MACS GMP PBS/$MgCl_2$ Buffer bag via sterile welding.
2. Connect a 600 mL Transfer Bag via sterile welding to be used as waste bag after centrifugation.
3. Centrifuge at 300×g for 15 minutes at room temperature (+19°C to +25 °C [+66 °F to +77 °F]) without brake.
4. 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.
5. Prepare MACS GMP Tyto Running Buffer for MACSQuant Tyto Cell Sorter: connect the MACS GMP PBS/$MgCl_2$ Buffer

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with 0.5% HSA to a 150 mL transfer bag via sterile welding and transfer 95 mL. Remove 5 mL MACS GMP Tytonase from the vial using a syringe and transfer to the 150 mL bag; mix well (final dilution 1:20).

6. Resuspend the cells in 50 mL of MACS GMP Tyto Running Buffer and transfer the cells either in a 150 mL bag or in a 50 mL Falcon for further processing.
7. If transferred in a 150 mL bag, connect a 150 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 (+19°C to +25 °C [+66 °F to +77 °F]) without brake.
9. Remove as much supernatant as possible.
10. Resuspend the cells in an appropriate volume of MACS GMP Tyto Running Buffer for MACSQuant Tyto Cell Sorter.

▲ **Note:** MACS GMP Tyto Cartridge can be filled with a maximal volume of 10 mL; take maximal cell sorting concentration in consideration.

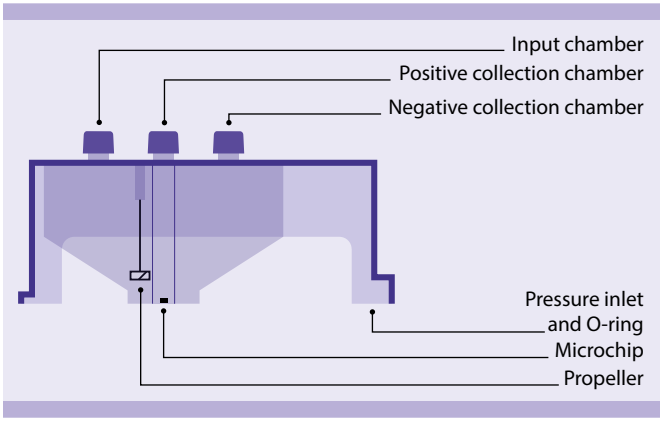
2.3 Priming of the MACS GMP Tyto Cartridge

1. Orient the cartridge so the owl icon is in the upper right corner and the caps appear in a shape.
2. Place the cartridge into the MACSQuant Tyto Priming Fixture by sliding the feet of the cartridge into the corresponding slots of the base. Start with the two slots on the right and lower into the two slots on the left. Verify that the cartridge is leveled out.
3. Fill 3 mL of the MACS GMP Tyto Running Buffer into a 10 mL syringe with a male Luer-Lock connection fitting.
4. Attach an 0.2 µm inline disk filter to the syringe. Discard 2 mL of buffer to flush the filter.
5. Remove cap from the input chamber of the cartridge and screw the filter with syringe. Store cap upside down.
6. Fill MACS GMP Tyto Running Buffer into the input chamber until the buffer level reaches the propeller (~500 µL).
7. Unscrew the filter with syringe from the input chamber. Discard the filter and remaining buffer from the syringe.
8. Pull the syringe plunger out to its stop and screw the syringe back to the input chamber.
9. Push the plunger completely while pressing on the left side of the cartridge to flow the MACS GMP Tyto Running Buffer to the positive collection chamber. Hold the plunger down until approximately half of the buffer has been pushed through (~250 µL).
10. Remove the cartridge from the MACSQuant Tyto Priming Fixture.
11. Unscrew the syringe from the cartridge and pull the plunger out to its stop. Screw the syringe to the input chamber again.
12. Close the pressure inlet with O-ring with your finger. Push the plunger of the syringe completely to flow the MACS GMP Tyto Running Buffer to the negative collection chamber. Hold the plunger down until the buffer level is just above the 3D filter.
13. Unscrew the syringe from the cartridge and unhand the pressure inlet with O-ring.
14. Remove the complete buffer from the input chamber by using the syringe. Reconnect the cap to the input chamber.
15. Remove the buffer from the positive and negative collection chambers by using a capillary pipet tip.
16. Proceed with sample loading (2.4).

2.4 Loading of cells in the MACS GMP Tyto Cartridge

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

▲ 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.

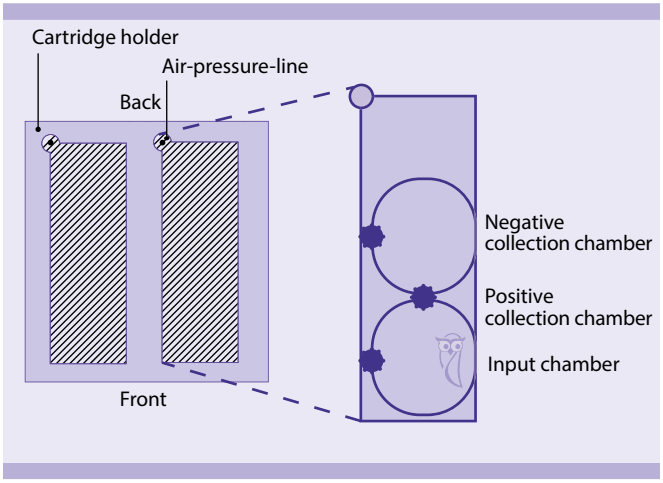
3. Place a 20 µm nylon mesh filter (Pre-Separation Filters (20 µm), # 130-101-812) on top of syringe.
4. Apply cell suspension onto the filter to remove cell clumps (if a 150 ml transfer bag was used, spike the bag with a Luer/Spike Interconnector and remove 10 mL cell suspension using a syringe. If cell suspension was transferred to a 50 mL Falcon, use a serological pipette).
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).

2.5 Loading of cells into the MACS GMP 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. For sort settings, please refer to the MACSQuant Tyto Cell Sorter user manual.
4. After the sort has been completed the MACS GMP Tyto Cartridge can be removed from the instrument and fractions can be retrieved (2.5).

2.6 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.

2.6.1 Positive collection chamber

The sorted cells are contained in the positive collection chamber.

5. (Optional) When volume is <100 µL, add MACS GMP PBS/$MgCl_2$ Buffer or infusion fluid of choice into the positive collection chamber by using a syringe or an extra long pipet tip.
6. Resuspend the sedimented cells by flushing/pipetting up and down.
7. Retrieve the full volume of cell suspension by using the syringe or pipet.
8. Apply cell suspension onto a 20 µm nylon mesh filter.
9. (Optional) For optimal retrieval wash the chamber with appropriate volume of MACS GMP PBS/$MgCl_2$ Buffer or infusion fluid of choice.

2.6.2 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 an extra long pipet tip.

2.7 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.

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

▲ The process requires 2×1 L CliniMACS PBS/EDTA buffer supplemented with HSA to a final concentration of 0.5% (w/v).

1. Weigh an empty Transfer Bag 600 mL 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 of the leukapheresis bag
3. Weigh the bag with the cells and add warm CliniMACS PBS/EDTA Buffer containing 0.5% HSA 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 CliniMACS PBS/EDTA buffer containing 0.5% HSA 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 300xg 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.

Warranty

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