Miltenyi Biotec

Anti-Melanoma MicroBeads

2 ml Anti-Melanoma MicroBeads
For 1×10^9 total cells Order No. 130-090-452

Description of MACS® Anti-Melanoma MicroBeads

MACS colloidal super-paramagnetic MicroBeads conjugated to monoclonal mouse anti-human melanoma-associated chondroitin sulfate proteoglycan (MCSP) antibodies[1-4].
Isotype: mouse IgG2a. Clone: 9.2.27
The product is supplied in a suspension containing 0.1 % gelatine and 0.05 % sodium azide.

Applications

Anti-Melanoma MicroBeads were developed for the enrichment and isolation of melanoma cells. The antigen is a unique glycoprotein-proteoglycan complex consisting of an N-linked glycoprotein of 250 kDa and a proteoglycan component of > 450 kDa which has been found to be expressed on melanoma cells but not on carcinoma cells, fibroblastoid cells and cells of hematopoietic origin.[1-4]

▲ Enrichment of disseminated melanoma cells from peripheral blood,[5] bone marrow, leukapheresis harvest and lymphoid tissue of patients with melanoma for subsequent analysis, e.g. cultivation, RT-PCR etc.

▲ Purification of melanoma cells from single cell suspensions of skin biopsies or primary skin cell cultures.

Principle of MACS Separation

For MACS separation, cells are magnetically labeled with Anti-Melanoma MicroBeads and separated on a column which is placed in the magnetic field of a MACS separator. The magnetically labeled melanoma cells are retained in the column, while the unlabeled cells flow through. After removal of the column from the magnetic field, the magnetically retained melanoma cells can be eluted as the positively selected cell fraction.

How to Use MACS Anti-Melanoma MicroBeads

Anti-Melanoma MicroBeads are suitable for positive selection of melanoma cells from peripheral blood, hematopoietic tissue (single cell suspensions from lymph nodes, bone marrow etc.) and non-hematopoietic tissue (e.g. single cell suspension from skin biopsies). Melanoma cell separation should be performed on MS or LS Columns or by using the autoMACS.

Storage of MACS MicroBeads

Store protected from light at 4°C. Do not freeze.

Strategies for Enrichment and subsequent Detection of Melanoma Cells

Enrichment and Detection

↓

Magnetic separation of melanoma cells by using MS or LS

↓ ↓

Intracellular Staining | **Surface Staining**

↓ ↓

Further enrichment and "Solid Phase Intracellular Staining" on second MS Column | Further enrichment and surface staining on second MS or LS Column

↓ ↓

Anti-Melanoma MicroBeads #130-090-452 and Inside Stain Kit #130-090-477*
*no staining antibody included

Melanoma Cell Enrichment and Detection Kit #130-090-523

Enrichment only

↓

Magnetic separation of melanoma cells by using MS or LS Columns or by using the autoMACS

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(Optional) second magnetic separation by using MS or LS Columns or by using the autoMACS

↓

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Instrument and Reagent Requirement

For magnetic enrichment

- Magnetic cell separator MiniMACS™, MidiMACS™, VarioMACS™ or SuperMACS™ (plus MS or LS Column Adapter)
- MS or LS Columns or autoMACS in combination with autoMACS® columns
- Buffer: phosphate buffered saline pH 7.2, supplemented with 0.5 % bovine serum albumin and 2 mM EDTA (see "Important Notes"). Degas buffer by applying vacuum.
- FcR Blocking Reagent containing human IgG (Order No. 130-059-901)
- Pre-Separation Filters (Order No. 130-041-407) or 30 µm nylon mesh

For the isolation of melanoma cells from lymph nodes

- Scalpels, forceps and Petri dish
- Nylon and steel mesh-screen (mesh size: 30 µm)
- Syringe Plunger
- HEPES buffered cell culture medium (e.g. Iscove's modified Dulbecco medium [IMDM], (e.g. Gibco Life Technologies, Paisley, UK)
- Collagenase (e.g. Collagenase IV, Worthington, Lakewood, NJ, USA)
- DNase (e.g. DNase I, Promega, Madison, Wisconsin, USA)

For the isolation of melanoma cells from bone marrow

- HEPES buffered cell culture medium (e.g. Iscove's modified Dulbecco medium [IMDM], e.g. Gibco Life Technologies, Paisley, UK).
- Heparin (e.g Liquimin from Hoffmann-La Roche AG, Basel, Switzerland)
- DNase (e.g. DNase I, Promega, Madison, Wisconsin, USA)

For Solid Phase Intracellular Staining

- Cytospin centrifuge (e.g. Hettich, Tuttlingen, Germany)
- Silane coated slides, e.g. Histobond® (Marienfeld, Bad Mergentheim, Germany)
- MACS Inside Stain Kit containing Inside Fix and Inside Perm for fixation and permeabilization of the cells (Order No.130-090-477)
- Monoclonal mouse IgG1 anti human melanoma antibody e.g. monoclonal mouse anti human Melan-A, clone: A103, isotype: mouse IgG1 (Dako, Glostrup, Denmark) and/or clone HMB45, isotype: mouse IgG1, (Dako, Glostrup, Denmark)
- Anti mouse IgG1-Alkaline Phosphatase, e.g. goat anti mouse IgG1-Alkaline Phosphatase (Southern Biotechnology Associates, Birmingham, Alabama) or anti mouse IgG1-FITC, e.g. goat anti mouse IgG1-FITC (Southern Biotechnology Associates) and monoclonal mouse anti FITC-Alkaline Phophatase (e.g. Sigma, Deisenhofen, Germany)
- Phosphate Buffered Saline pH 7.2 (PBS) (e.g. Gibco Life Technologies, Paisley, UK)
- SIGMA FASTTM Fast Red TR/Naphtol AS-MX substrate tablets, (Sigma, Deisenhofen, Germany)
- Meyer's hemalum solution (Merck, Darmstadt, Germany)
- Mounting medium, e.g. Faramount Aqueous Mounting Medium (Dako, Hamburg, Germany

Protocols for Cell Preparation

The following protocols describe the preparation of single cell suspensions from peripheral blood, bone marrow and lymphoid tissues and the magnetic enrichment of melanoma cells using MACS technology. For subsequent detection of enriched melanoma cells, use the protocol for: Solid phase Intracellular Staining and the Inside Stain Kit (Order No. 130-090-477; as described in the Appendix) or use the Melanoma Cell Enrichment and Detection Kit (Order No. 130-090-523).

Preparation of Peripheral Blood Cells

- Collect 30-50 ml fresh human blood treated with an anticoagulant, e.g. EDTA, heparin, citrate, ACD-A or citrate phosphate dextrose (CPD).
- Prepare mononuclear cells by Ficoll-Paque™ density gradient centrifugation.
- Resuspend cell pellet in a final volume of 300 µl per 5×10^7 total cells. For less than 5×10^7 total cells, use 300 µl. Proceed to "Magnetic Labeling".

Preparation of Bone Marrow Cells

Collect 2–10 ml bone marrow from the upper iliac crest or sternum using an aspiration needle and place into a 50 ml conical tube containing 2–10 ml HEPES buffered cell culture medium (i.e. IMDM) supplemented with 100 U/ml heparin. The volume of bone marrow aspirate should be about the same as the volume of medium.

- (Optional) Dilute bone marrow cells with one volume of HEPES buffered cell culture medium (e.g. IMDM) containing 100 U DNase I/ml and shake gently at 20–25 °C for 30 minutes.
- Prepare mononuclear cells by Ficoll-Paque™ density gradient centrifugation.
- Resuspend cell pellet in a final volume of 300 µl of buffer per 5×10^7 total cells. For less than 5×10^7 total cells, use 300 µl. Proceed to "Magnetic Labeling".

Preparation of Cells from Lymphoid Tissue

- Directly after surgery place lymph node into a 50 ml conical tube containing HEPES buffered medium (e.g. IMDM).
- Transfer lymph node with medium in a sterile Petri dish.
- Cut lymph node into small pieces using two sterile scalpels. Apply buffer with pieces of lymph node onto sterile steel mesh-screen placed in a fresh Petri dish.
- Carefully push cells through the mesh using the plunger of a sterile syringe using a vertical motion. Do not grate the cells.
- Use sterile technique to transfer the filtered cell suspension into a new 50 ml conical tube.
- Incubate residual pieces of lymph node for 30 min at 37 °C in 5-10 ml of IMDM supplemented with 100 U/ml DNase I and 200 U/ml Collagenase IV.

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- Pass cells through fresh nylon mesh-screen and add these cells to the cell suspension obtained before in the 50 ml conical tube. Discard the rest of the lymph node pieces.
- Fill the tube with IMDM, count the cells, and centrifuge at 300×g.
- Remove supernatant completely and resuspend cell pellet in buffer in a total volume of 300 µl per 5×10^7 total cells. For less cells use 300 µl. Proceed to "Magnetic Labeling".

Protocol for Magnetic Labeling of Melanoma Cells

- Add 100 µl of FcR Blocking Reagent per 5×10^7 **total** cells to block non-specific binding of Anti-Melanoma MicroBeads and mix well. The final volume is 400 µl per 5×10^7 cells.
- Add 100 µl of MACS Anti-Melanoma MicroBeads per 5×10^7 total cells, mix well and incubate for 30 minutes at 6–12 °C. The final labeling volume is 500 µl per 5×10^7 cells.
- Wash cells by adding 10–20× the labeling volume of buffer, centrifuge at 300×g for 10 minutes, remove supernatant and resuspend cell pellet in 500 µl of buffer per 10^8 total cells (for less cells, use 500 µl). Proceed to "Magnetic Separation" or "Magnetic Separation and Solid Phase Intracellular Staining".

Magnetic Separation of Melanoma Cells

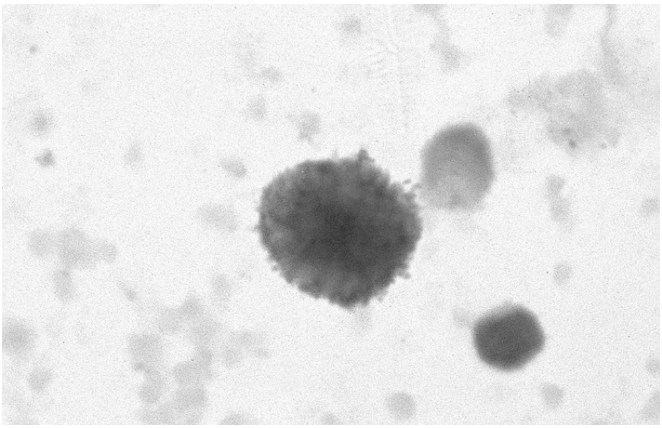
Magnetic separation by using MS or LS Columns

- Choose an MS Column (for up to 2×10^8 total cells and up to 10^7 melanoma cells), or an LS Column (for up to 2×10^9 total cells and up to 10^8 melanoma cells) and place the column in the magnetic field of an appropriate MACS separator (see "Column Data Sheets").
- Prepare column by washing with appropriate amount of degassed buffer (MS: 500 µl, LS: 3 ml; for details, see "Column Data Sheets").
- Pass cells through Pre-Separation Filter (Order No. 130-041-407) or 30 µm nylon mesh to remove any clumps. Wet filters with degassed buffer before use.
- Apply cell suspension in appropriate amount of buffer onto the column (MS: 0.5–1 ml, LS: 1–10 ml). Let the negative cells pass through. Rinse with appropriate amount of buffer (MS: 3×500 µl, LS: 4×3ml).
- Remove column from separator, place column on a suitable collection tube and pipette appropriate amount of buffer (MS: 1 ml; LS: 5 ml) onto the column. Firmly flush out retained cells with pressure using the plunger supplied with the column.
- (Optional) Magnetic separation step can be repeated in order to get higher enrichment rates/purities. Therefore, apply the eluted positive cells to a new prefilled positive selection column (MS: for up to 10^7 melanoma cells; LS: for $>10^7$ melanoma cells), wash as above (MS: 3×500 µl, LS: 4×3ml), and elute retained cells with buffer using the plunger as above (MS: 500 µl; LS: 2.5 ml).

Magnetic separation by using the autoMACS

- Prepare and prime the autoMACS instrument (for up to 4×10^9 total cells and a maximum of 2×10^8 magnetically labeled cells) according to the autoMACS User Manual.
- Pass cells through Pre-Separation Filter (Order No. 130-041-407) or 30 µm nylon mesh to remove clumps.
- Apply magnetically labeled cells to the autoMACS. For higher enrichment rates/purities, choose the separation program POSSELD.

Melanoma cell enriched from blood using the Anti-Melanoma MicroBeads



Melanoma cell from peripheral blood of a patient with malignant melanoma isolated using Anti-Melanoma MicroBeads. Cells were stained according to the "Solid Phase Intracellular Staining" Protocol (see below) using the Inside Stain Kit, an anti-Melan-A antibody, goat anti-mouse IgG1-FITC and Anti-FITC Alkaline Phosphatase.)

Important Notes

▲ Avoid capping of antibodies on the cell surface during staining. Work fast, keep cells cold, use cold solutions only. **Attention:** Working on ice requires increased incubation times for MACS MicroBeads. Incubate in refrigerator at 6°–12°C.

▲ EDTA can be replaced by other supplements such as acid citrate dextrose (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as gelatin, HSA or FCS.

▲ Higher temperatures and longer incubation times for staining and magnetic labeling may lead to unspecific cell labeling.

▲ We strongly recommend using FcR Blocking Reagent to block Fc receptor mediated unspecific labeling.

▲ MACS MicroBeads may bind unspecifically to dead cells. To remove dead cells, we recommend use of MACS Dead Cell Removal Kit (Order No. 130-090-101) or density gradient centrifugation using Ficoll-Paque™.

▲ Large numbers of cells in the starting sample require a larger buffer volume when applying cells onto separation column. Use a maximum cell concentration of 10^8 nucleated cells per 500 µl of buffer.

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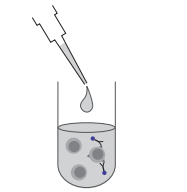
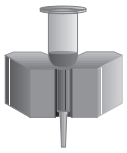
Appendix: Immunocytochemical Detection of magnetically enriched melanoma cells by using the "Inside Stain Kit"

Magnetic enrichment of Melanoma cells can be combined with Solid Phase Intracellular Staining using the Inside Stain Kit. Melanoma cells are magnetically labeled and isolated on a 1st MS Column. The positively selected melanoma cells are eluted, fixed and reapplied onto a 2nd MS Column. While in the column the immobilized melanoma cells are permeabilized and stained for intracellular antigens, e.g. Melan-A. Subsequent analysis can be performed by immunocytochemical detection or flow cytometry after cells are eluted from the column.

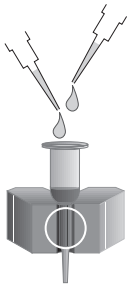


magnetic labeling using Anti-Melanoma MicroBeads

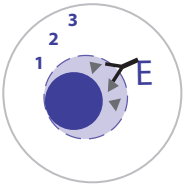


separation on 1st MS Column

elution and fixation with Inside Fix



application to 2nd MS Column and permeabilisation with Inside Perm



Solid Phase Intracellular Column Staining:

1. anti melanoma antibody
2. anti Isotype-FITC
3. anti FITC-alkaline phosphatase

elution, cytocentrifugation and substrate incubation

Magnetic Separation and "Solid Phase Intracellular Staining" of Melanoma Cells

- Choose an MS Column and place the column in the magnetic field of an appropriate MACS Separator (see "Column Data Sheets").
- Prepare MS Column by washing with 500 µl of degassed buffer (for details, see "Column Data Sheets").
- Pass cells through Pre-Separation Filters (Order No. 130-041-407) or 30 µm nylon mesh to remove any clumps. Wet filters with degassed buffer before use.
- Apply cell suspension in 0.5–1 ml of buffer onto the column. Let the non-labeled cells pass through. Wash column with 3×500 µl of buffer.
- Remove the column from separator, place column on a suitable collection tube, pipette 500 µl of degassed buffer onto the column and flush out retained melanoma cells using the plunger supplied with the column.
- Add 500 µl of Inside Fix to the isolated melanoma cells and incubate for 20 minutes at room temperature. The final fixation volume is 1 ml.
- Prepare a new MS Column by washing twice with 500 µl of degassed buffer.
- Apply the fixed positive cells onto the new MS Column, let cell suspension completely enter the column matrix and immediately wash with 500 µl of buffer.
- Permabilize cells by washing the column with 500 µl of Inside Perm.
- Dilute monoclonal anti human melanoma antibody, e.g. anti Melan-A (clone A103, isotype mouse IgG1) and/or clone: HMB45, isotype: mouse IgG1 at appropriate titer (as specified by the manufacturer) in Inside Perm, apply 100 µl of the diluted antibody onto the MACS Column and incubate for 10 minutes at room temperature.
- Rinse column with 2×500 µl of Inside Perm, apply 100 µl of anti mouse IgG1-FITC, e.g. goat anti mouse IgG1-FITC (diluted at appropriate titer in Inside Perm) and incubate for 10 minutes at room temperature.
- Rinse column with 2×500 µl of Inside Perm, apply 100 µl of monoclonal mouse anti FITC-Alkaline Phophatase (diluted 1 to 50 in Inside Perm) and incubate for 10 minutes at room temperature.
- Wash column with 500 µl of Inside Perm and 500 µl of PBS.
- Remove column from separator, place column on a suitable collection tube, pipette 500 µl of PBS onto the column and flush out retained cells using the plunger supplied with the column.

Immunocytochemical Detection of Melanoma Cells

- After magnetic enrichment and "Solid Phase Intracellular Staining" spin cells onto a slide using a cytocentrifuge. Air-dry slide for 2-18 hours at room temperature.
- Using a pen (e.g. DAKO PEN) apply a hydrophobic line around the cell area on slide.
- Wash slide for 2 minutes in PBS in a staining trough.

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- Prepare Fast Red^{TR}/Naphtol AS-MX substrate solution by disolving the TRIS Buffer tablet in 1 ml double destilled water, add substrate tablet and disolve by vigorous shaking.
- Add 50-100 µl of freshly prepared Fast Red TR/Naphtol AS-MX substrate solution to the cell area and incubate for 15 minutes at room temperature.
- Wash slide for 2 minutes in double-distilled water in a staining trough.
- (Optional) Counterstain cells for 1 minute in filtered Meyers hemalum solution (diluted 1 to 2 in 100 mM TRIS-HCl, pH 8.2) in staining trough.
- Wash slide for 2 minutes in double destilled water in a staining trough. Air-dry slide or mount with aquaeous mounting medium.

References

1. Morgan et al. (1981) Hybridoma 1, 27-36.
2. Bumol and Reisfeld (1982) Proc. Natl. Acad. Sci. USA, 79, 1245-1249.
3. Bumol et al. (1984) J. Biol. Chem. 259, 12733-12741.
4. Pluschke et al. (1996) Proc. Natl. Acad. Sci. USA 93, 9710-9715.
5. Benez et al. (1999) J. Clin. Lab. Anal.13, 229-233.

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.

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

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