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1. Description

Components	2 mL CD146 (LSEC) MicroBeads, mouse: MicroBeads conjugated to monoclonal anti-mouse LSEC (clone: ME-9F1; isotype: rat IgG2a) antibody.
Capacity	For 2×10 ⁹ total cells, up to 200 separations.
Product format	CD146 (LSEC) MicroBeads are supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Principle of the MACS® Separation

First, liver sinusoidal endothelial cells (LSECs) are magnetically labeled with CD146 (LSEC) MicroBeads. Then, the cell suspension is loaded onto a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled LSECs are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of LSECs. After removing the column from the magnetic field, the magnetically retained LSECs can be eluted as the positively selected cell fraction.

1.2 Background information

CD146 is a transmembrane glycoprotein and belongs to the IgG superfamily of cell adhesion molecules.¹ CD146 (LSEC) MicroBeads, formerly termed Anti-LSEC MicroBeads, have been developed to isolate mouse liver sinusoidal endothelial cells (LSECs). LSECs are microvascular endothelial cells lining the hepatic sinusoidal wall and are supposed to mainly contribute to the control of immune responses against circulating soluble antigens in the liver. Their strategic positioning favors a tight interaction with lymphocytes migrating through the liver. LSECs possess a high capacity

for antigen uptake and processing but express, in contrast to professional antigen-presenting cells (e.g. dendritic cells), only low levels of costimulatory molecules.²

The CD146 (LSEC) antibody also binds to endothelial cells from a various range of organs, such as skin, liver, kidney, brain, spleen, lymph node, intestine, heart and skeletal muscle as well as on blood vessel structures, such as pulmonary arteries, veins, and the capillary network of the alveolar walls but not on lymphatic endothelium. In contrast to humans that express CD146 also on T cells or follicular dendritic cells, murine CD146 expression was only found on a subset of NK1.1⁺ cells at low levels.³

1.3 Application

- Isolation or depletion of LSECs from mouse liver.
- Isolation of endothelial cells from vascular tissues.

1.4 Reagent and instrument requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as mouse serum, or fetal bovine serum (FBS). Buffers or media containing Ca²⁺ or Mg²⁺ are not recommended for use.

- MACS Columns and MACS Separators: mouse CD146⁺ cells can be enriched by using MS, LS, or XS Columns (positive selection). CD146 (LSEC) MicroBeads can be used for depletion of CD146⁺ cells on LD, CS, or D Columns. Positive selection or depletion can also be performed by using the autoMACS Pro Separator or the autoMACS Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10 ⁷	2×10 ⁸	MiniMACS, OctoMACS, VarioMACS, SuperMACS
LS	10 ⁸	2×10 ⁹	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
XS	10 ⁹	2×10 ¹⁰	SuperMACS
Depletion			
LD	10 ⁸	5×10 ⁸	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
CS	2×10 ⁸		VarioMACS, SuperMACS
D	10 ⁹		SuperMACS
Positive selection or depletion			
autoMACS	2×10 ⁸	4×10 ⁹	autoMACS Pro, autoMACS

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ Separators. For details see the respective MACS Separator data sheet.

- Collagenase D: 2 mg/mL (Collagenase D >0.15 U/mg, e.g. from Roche™ Diagnostics, Germany) in 10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂.
- PBS, pH 7.2.
- RPMI 1640 (# 130-091-440).
- 30% HistoDenz™-PBS solution (e.g. from Sigma-Aldrich®, Germany).
- (Optional) CD146 (LSEC)-FITC (# 130-092-026) or CD146 (LSEC)-Biotin (# 130-092-025).
- (Optional) Anti-Biotin-FITC (# 130-090-857), Anti-Biotin-PE (# 130-090-756), or Anti-Biotin-APC (# 130-090-856) as secondary antibody reagent in combination with CD146 (LSEC)-Biotin (# 130-092-025).
- (Optional) FcR Blocking Reagent, mouse (# 130-092-575) to avoid Fc receptor-mediated antibody labeling.
- (Optional) MACSmix™ tube rotator.
- Cell strainer (100 µm, 70 µm, 40 µm).
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells.
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Sample preparation

1. Wash isolated mouse liver in cold PBS to remove residual blood.
2. Place the liver in a 6 cm petri-dish with sufficient Collagenase D solution to completely cover the bottom of the dish (5 mL/liver).
3. Inject 1 mL Collagenase D solution into different parts of the liver using a 1 mL syringe and a 25G needle, then cut the tissue into small pieces by using sharp scissors.
4. Transfer the entire suspension containing pieces of liver to a 15 mL tube.
5. Incubate the liver suspension in the closed tube for 45 minutes at 37 °C under slow continuous rotation using the MACSmix™ tube rotator, or turn tube every 5 minutes to resuspend settled tissue fragments.
6. Pass the entire material, i.e. remaining fragments and Collagenase D-released cells, gently through a 100 µm, then a 70 µm and a 40 µm cell strainer using a plunger. Collect all cells in a 50 mL tube.
7. Wash cells by filling up the tube with PBS and centrifuge at 300×g for 10 minutes at 4 °C. Repeat this step twice.
8. Resuspend the cell pellet in 2 mL RPMI 1640 and mix with 8 mL of 30% HistoDenz-solution to remove debris and enrich for LSECs.

9. Layer the cell suspension under 2 mL RPMI 1640 in a 15 mL tube and centrifuge at 1500×g for 20 minutes at 4 °C without brake.
10. Harvest the layer of low-density cells at the interface and transfer it to a new tube.
11. Wash cells by filling up with buffer and centrifuge at 300×g for 10 minutes at 4 °C. Repeat this step once.
12. Resuspend cell pellet in 1 mL buffer and proceed to magnetic labeling (2.2).

For using the gentleMACS™ Dissociator see the protocols section at www.miltenyibiotec.com/protocols.

▲ **Dead cells** may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).



2.2 Magnetic labeling

▲ **Work fast, keep cells cold, and use pre-cooled solutions.** This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ **Volumes for magnetic labeling** given below are for up to 10⁷ total cells. When working with fewer than 10⁷ cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10⁷ total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ **For optimal performance** it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ **The recommended incubation temperature** is 2–8 °C. Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 90 µL of buffer per 10⁷ total cells.
4. Add 10 µL of CD146 (LSEC) MicroBeads per 10⁷ total cells.
5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
6. Wash cells by adding 1–2 mL of buffer per 10⁷ cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend up to 10⁸ cells in 500 µL of buffer.
 - ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
 - ▲ **Note:** For depletion with LD Columns, resuspend up to 1.25×10⁸ cells in 500 µL of buffer.
8. Proceed to magnetic separation (2.3).



2.3 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD146⁺ cells. For details see table in section 1.3.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator. For details see the respective MACS Column data sheet.

2. Prepare column by rinsing with the appropriate amount of buffer:

MS: 500 μ L LS: 3 mL

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.

4. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the effluent from step 3.

MS: 3 \times 500 μ L LS: 3 \times 3 mL

▲ **Note:** Perform washing steps by adding buffer aliquots only when the column reservoir is empty.

5. Remove column from the separator and place it on a suitable collection tube.

▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.

6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

MS: 1 mL LS: 5 mL

Magnetic separation with XS Columns

For instructions on the column assembly and the separation refer to the XS Column data sheet.

Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see LD Column data sheet.

2. Prepare column by rinsing with 2 mL of buffer.

3. Apply cell suspension onto the column.

4. Collect unlabeled cells that pass through and wash column with 2 \times 1 mL of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty.

Depletion with CS Columns

1. Assemble CS Column and place it in the magnetic field of a suitable MACS Separator. For details see CS Column data sheet.

2. Prepare column by filling and rinsing with 60 mL of buffer. Attach a 22G flow resistor to the 3-way stopcock of the assembled column. For details see CS Column data sheet.

3. Apply cell suspension onto the column.

4. Collect unlabeled cells that pass through and wash column with 30 mL buffer from the top. Collect total effluent; this is the unlabeled cell fraction.

Depletion with D Columns

For instructions on column assembly and separation refer to the D Column data sheet.

Magnetic separation with the autoMACS[®] Pro Separator or the autoMACS[®] Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS[®] Pro Separator or the autoMACS Separator.

▲ Buffers used for operating the autoMACS Pro Separator or the autoMACS Separator should have a temperature of ≥ 10 °C.

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual.

Magnetic separation with the autoMACS[®] Pro Separator

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.

3. For a standard separation choose one of the following programs:

Positive selection: "Possel"

Collect positive fraction in row C of the tube rack.

Depletion: "DepleteS"

Collect negative fraction in row B of the tube rack.

Magnetic separation with the autoMACS[®] Separator

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1 and pos1.

3. For a standard separation choose one of the following programs:

Positive selection: "Possel"

Collect positive fraction from outlet port pos1.

This is the enriched LSEC fraction.

Depletion: "DepleteS"

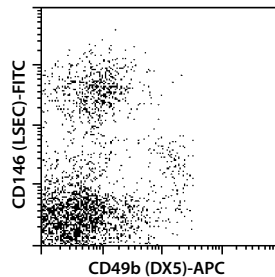
Collect negative fraction from outlet port neg1.

This is the LSEC depleted fraction.

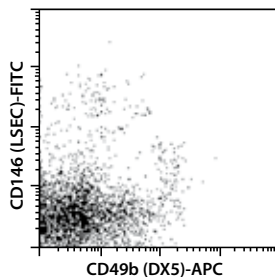
3. Example of a separation using the CD146 (LSEC) MicroBeads

LSECs were isolated from mouse liver cell suspension using CD146 (LSEC) MicroBeads, an MS Column, and a MiniMACS™ Separator. Cells were fluorescently stained with CD146 (LSEC)-FITC (# 130-092-026) and CD49b (DX5)-APC (# 130-091-813) and analyzed by flow cytometry. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

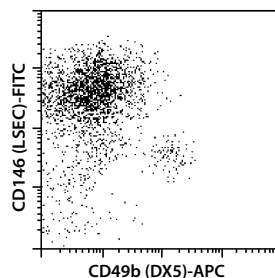
Mouse liver cells before separation



Mouse liver cells depleted of LSECs



Isolated LSECs



4. References

1. Sers, C. *et al.* (1993) Genomic Organization of the Melanoma-Associated Glycoprotein MUC18: Implications for the Evolution of the Immunoglobulin Domains. *Proc. Natl. Acad. Sci. USA* 90: 8514–8518.
2. Diehl, L. *et al.* (2008) Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8⁺ T cell tolerance. *Hepatology* 47: 296–305.
3. Schrage, A. *et al.* (2008) Murine CD146 is widely expressed on endothelial cells and is recognized by the monoclonal antibody ME-9F1. *Histochem. Cell Biol.* 129: 441–451.
4. Hegenbart, S. *et al.* (2006) Efficient isolation of liver sinusoidal endothelial cells (LSECs) by immunomagnetic separation. *MACS&more* 10: 8–10.

All protocols and data sheets are available at www.miltenyibiotec.com.

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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