CD146 MicroBead Kit
human

Order no. 130-093-596

Contents

1. Description

Components	**2 mL CD146 MicroBeads, human:** MicroBeads conjugated to monoclonal anti-human CD146 antibodies (isotype: mouse IgG1). **2 mL FcR Blocking Reagent, human:** Human IgG.
Capacity	For 10^9 total cells, up to 100 separations.
Product format	CD146 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 preparation of the stromal vascular fraction (SVF) from human lipoaspirate

This protocol describes the preparation of CD146$^+$ cells from human thigh or abdomen lipoaspirate obtained from cosmetic surgery. Briefly, the lipoaspirate is first washed thoroughly in phosphate-buffered saline (PBS) before being subjected to enzymatic digestion using collagenase in order to obtain a single-cell suspension. After digestion, the centrifuged cell pellet, termed the stromal vascular fraction (SVF), is resuspended in buffer before serial filtration through 100 µm and then 40 µm nylon filters. The cell suspension is then counted and cells are then ready for separation using the CD146 MicroBead Kit.

1.2 Principle of the MACS® Separation

First, the CD146$^+$ cells are magnetically labeled with CD146 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 CD146$^+$ cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD146$^+$ cells. After removing the column from the magnetic field, the magnetically retained CD146$^+$ cells can be eluted as the positively selected cell fraction.

1.3 Background information

The CD146 MicroBead Kit was developed for the isolation of CD146$^+$ cells. The kit consists of CD146 MicroBeads and FcR Blocking Reagent. The CD146 MicroBeads recognize the human CD146 antigen, also known as MUC18, MCAM, Mel-CAM, and S-Endo-1. CD146 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily and contains five extracellular immunoglobulin (Ig)-like domains.[1,2]

CD146 possesses a limited tissue expression pericytes distribution, including endothelial cells, smooth muscle cells, follicular dendritic cells, melanoma cells, and a sub-population of activated T lymphocytes.[1,3] CD146$^+$ cells isolated from umbilical cord[4], lipoaspirate[5], dental pulp, or endometrial tissue[6] are described to contain cells with clonogenic fibroblastic colony (CFU-F) activity and multilineage differentiation potential, which are termed mesenchymal stromal cells (MSCs).

CD146 functions as a Ca^{2+}-independent cell adhesion molecule which is involved in heterophilic cell-cell interactions[7], and may be involved in the extravasion and/or homing of activated T cells[3]. CD146 expression in melanoma is also directly associated with tumor growth and metastasis.[7,8]

1.4 Applications

- Isolation of CD146$^+$ cells from human tissues.
- Enrichment of CD146$^+$ cells, such as mesenchymal stromal cells (MSCs) from lipoaspirate.
- Isolation of human endothelial cells, such as HUVECs, or pericytes from human endothelial tissue.

1.5 Reagent and instrument requirements

Sample preparation from lipoaspirate

- Sterile phosphate-buffered saline (PBS).
- Collagenase digestion solution (e.g. Collagenase NB 4G Proved Grade, Serva # 17465.02): 0.3 U/mL in sterile PBS (Wünsch units). Resolve enzyme at 37 °C in a water bath.

  ▲ **Note:** For information on the conversion of other catalytic units to Wünsch units, please refer to the appropriate supplement in section General protocols at www.miltenyibiotec.com/protocols.

- Enzyme stop medium: Dulbecco's Modified Eagles Medium (DMEM, # 130-091-437) containing 20% fetal bovine serum (FBS).

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- 500 mL Conical Bottom Centrifuge Tubes with screw caps (e.g. Corning # 431123).
- 1 L storage bottles (e.g. Corning # 430518).
- 50 mL conical tubes (e.g. BD Biosciences # 352070).
- 100 µm cell strainer (e.g. BD Biosciences # 352360).
- 40 µm cell strainer (e.g. BD Biosciences # 352340).
- Orbital shaker with temperature control.
- Water bath, pre-warmed to 37 °C.

MACS® Separation using CD146 MicroBeads

- Erythrocyte lysis buffer (10×): 1.55 M NH_4Cl, 100 mM $KHCO_3$, 1 mM EDTA, pH 7.3. Always dilute freshly before use.
- 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 human serum albumin, human serum, or fetal bovine serum. Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- MACS Columns and MACS Separators: $CD146^+$ cells can be enriched by using LS or XS Columns (positive selection). Manual separation is recommended.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
XS	10^9	2×10^{10}	SuperMACS

▲ **Note:** The capacities of the columns represent guidelines. Depending on the composition of sample the column capacity may be decreased.

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

- Pre-Separation Filters (# 130-041-407) to remove cell clumps.
- (Optional) Fluorochrome-conjugated CD146, CD31, or CD34 antibody for flow cytometric analysis, e.g., CD146-APC (# 130-092-849), CD31-PE (# 130-092-653), CD31-APC (# 130-092-652), or CD34-FITC (# 130-081-001). For more information about other fluorochrome conjugates see www.miltenyibiotec.com.
- (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.

MSC/ADSC cultivation

- NH Expansion Medium (# 130-091-680) for the optimized expansion of selected $CD146^+$ MSCs in culture.
- 75 cm^2 cell culture flasks (e.g. BD Biosciences # 353136) or 25 cm^2 cell culture flasks (e.g. BD Biosciences # 353109).
- (Optional) NH CFU-F Medium (# 130-091-676) for the optimized and reproducible quantification of selected $CD146^+$ MSCs.
- (Optional) NH Differentiation Media, e.g., NH AdipoDiff Medium (# 130-091-677), NH ChondroDiff Medium (# 130-091-679), or NH OsteoDiff Medium (# 130-091-678).

2. Protocol

▲ A special protocol for "Isolation of human umbilical vein endothelial cells (HUVECs) from umbilical cord" is available at www.miltenyibiotec.com/protocols.

2.1 Preparation of the stromal vascular fraction (SVF) from human lipoaspirate

▲ All steps should be performed under sterile working conditions, including the use of sterile reagents and media.

▲ For optimal results, only use aspirate that has been obtained by tumescent liposuction. Other methods, for example ultrasound, can lead to unwanted cell damage.

▲ Lipoaspirate should be stored at room temperature (max. 4 hours) or stored at 2–8 °C (max. 24 hours) before use.[9]

▲ A minimum starting volume of 250 mL of lipoaspirate is required for a sufficient yield of $CD146^+$ cells. Starting with 250 mL of lipoaspirate will result in 1×10^7 to 1×10^8 mononuclear cells in the stromal vascular fraction (SVF).

1. Dilute lipoaspirate sample with an equal volume of PBS and divide evenly between the Conical Bottom Centrifuge Tubes.
2. Centrifuge at 430×g for 10 minutes without brakes. After centrifugation, remove the target cell–containing lipid phase from the top (see figure 1).

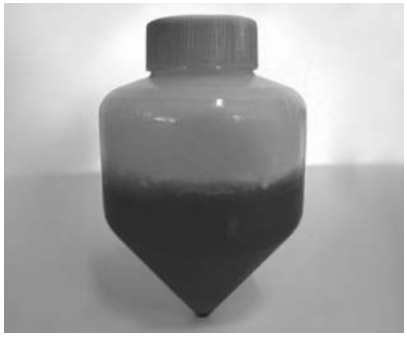

Figure 1: Lipoaspirate preparation after the addition of PBS and centrifugation. Note the upper (yellow) $CD146^+$ cell-containing lipid phase at the top which is to be aspirated and washed a further two times.

3. Apply to a fresh Conical Bottom Centrifuge Tube and dilute with an equal volume of PBS.
4. Repeat steps 2 and 3 twice.
5. Dilute aspirated lipid fraction with an equal volume of the collagenase digestion solution and transfer the mixture to a 1 L storage bottle. Do not transfer more than 500 mL per 1 L bottle.

   ▲ **Note:** Bottles should only be half filled in order to facilitate an optimal mixing during incubation on the orbital shaker.
6. Incubate mixture at 37 °C for 30 minutes on a pre-warmed orbital shaker. A rotation of 250 rpm should be used to ensure thorough mixing and optimal digestion of the cell aggregates.

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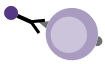
7. After 30 minutes, add an equal volume of the enzyme stop medium to each bottle.
8. Redistribute digested cell preparation into fresh Conical Bottom Centrifuge Tubes and centrifuge at 600×g for 10 minutes. Aspirate and discard the supernatant.
9. Resuspend pellet (the stromal vascular fraction, SVF) in 10 mL of PBS.

   ▲ **Note:** More buffer may be applied if necessary, depending on the size of the pellet.
10. Pass cell suspension through a 100 µm cell strainer and collect the filtrate in 50 mL conical tubes.
11. Centrifuge at 600×g for 10 minutes. Aspirate and discard supernatant and resuspend pellet in 5 mL of PEB buffer.
12. Pass cell suspension through a 40 µm cell strainer and collect filtrate in fresh 50 mL conical tubes.
13. Determine cell number.
14. For isolation according to CD146 expression, proceed to magnetic labeling (2.2).



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 10^7 total cells. When working with fewer than 10^7 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 2×10^7 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 separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column. Wet 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. Transfer 10^7 cells into a 15 mL conical tube.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. (Optional) Lyse red blood cells by adding 1 mL of 1× erythrocyte lysis buffer and incubating cells for 10 minutes at room temperature.
4. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

   ▲ **Note:** Erythrocyte lysis is only necessary when the sample contains significant erythrocyte contamination after preparation of SVF. If very few or no erythrocytes are observed in the sample, proceed directly to step 5 below.
5. Resuspend cell pellet in 60 µL of buffer per 10^7 cells.
6. Add 20 µL of FcR Blocking Reagent per 10^7 total cells.
7. Add 20 µL of CD146 MicroBeads per 10^7 total cells.
8. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
9. (Optional) Add staining antibody, e.g., 10 µL of CD146-APC (# 130-092-849), and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).
10. Wash cells by adding 1 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
11. Resuspend cell pellet in 500 µL of buffer per 10^7 cells.
12. 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 1.5.

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

Magnetic separation with 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 3 mL of buffer.
3. Apply cell suspension onto the column. Always use a MACS Pre-Separation Filter. Collect unlabeled cells that pass through.
4. Wash column with 3×3 mL of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times.

   ▲ **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.
6. Pipette 5 mL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
7. (Optional) To increase the purity of $CD146^+$ cells, the eluted fraction can be enriched over a second LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

   ▲ **Note:** For MSC/ADSC cultivation, it is recommended to flush cells from the column using an appropriate volume of NH Expansion Medium instead of buffer. For further information regarding the cultivation of MSCs or adipose-derived stem cells (ADSCs) see special protocol "Isolation of CD271 ($LNGFR^+$) MSCs/ADSCs from human lipoaspirate" at www.miltenyibiotec.com/protocols.

Magnetic separation with XS Columns

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

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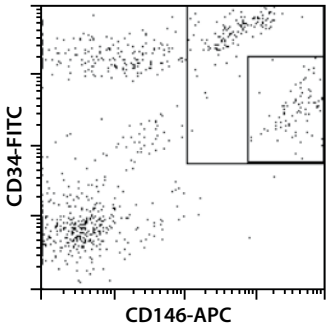
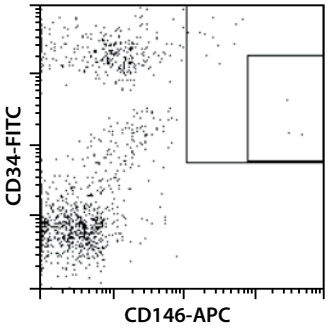
3. Example of a separation using the CD146 MicroBead Kit

CD146$^+$ cells were isolated from lipoaspirate using the CD146 MicroBead Kit, an LS Column, and a MidiMACS™ Separator. Cells are fluorescently stained with CD146-APC (# 130-092-851) and CD34-FITC (# 130-081-001). Cell debris and dead cells are excluded from the analysis based on scatter signals and propidium iodide fluorescence.

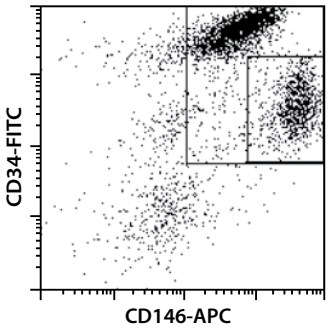
Before separation



CD146$^-$ cell fraction



CD146$^+$ cell fraction



4. References

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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.

Warranty

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