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1. Description

This product is for research use only.

1.1 Background information

The use of lipoaspirate as a source for stem cells with multipotent differentiation potential offers a far less invasive procedure for cell sampling than the aspiration of bone marrow (BM), and numbers of stem cells obtained are reportedly higher in lipoaspirate than in its BM counterpart.¹

Lipoaspirate, an otherwise disposable byproduct of cosmetic surgery, has been shown to contain a putative population of stem cells, termed adipose-derived stem cells (ADSCs), that share many similarities to marrow stromal cells (MSCs) from BM, including multilineage differentiation capacity.² Furthermore, these cells also show high colony-forming unit frequencies¹ as well as an apparent pluripotent ability to differentiate to cells of a neuronal phenotype². Finally, the large quantity in which lipoaspirate can be obtained makes it a very attractive alternative source of MSCs for a broad range of research applications. CD271, also known as LNGFR (low-affinity nerve growth factor receptor), is a well-known marker for the enrichment of nonhematopoietic stem cells from bone marrow aspirates.³⁻⁷ CFU-F activity is contained in the CD271⁺ fraction in bone marrow⁶ and also in lipoaspirate⁸. Furthermore, the phenotype of MSCs in lipoaspirate was described to be positive for CD271, CD105, and CD44, and negative for CD45⁷.

1.2 Principle of the preparation of the stromal vascular fraction (SVF) from human lipoaspirate

This protocol describes the preparation of MSCs 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 expansion medium before serial filtration through 100 µm and then 30 µm nylon filters. The content of mononuclear cells is then counted and cells are then ready for separation using the CD271 MicroBead Kit, human (# 130-099-023).

1.3 Principle of the MACS[®] Separation using CD271 MicroBeads

The CD271⁺ cells are magnetically labeled with CD271 MicroBeads contained within the CD271 MicroBead Kit. Then, the cell suspension is loaded onto a MACS[®] Column placed in the magnetic field of a MACS Separator. The magnetically labeled CD271⁺ cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD271⁺ cells. After removal the column from the magnetic field, the magnetically retained CD271⁺ cells can be eluted as the positively selected cell fraction. To increase the purity, the positively selected cell fraction containing the CD271⁺ cells must be separated over a second column.

1.4 Reagent and instrument requirements

Sample preparation from lipoaspirate

- Sterile phosphate-buffered saline (PBS)
- Collagenase digestion solution (e.g. Collagenase NB 4G Proved Grade, Nordmark Biochemicals #S1746502): 0.3 U/mL in sterile PBS (Wünsch units). Resolve enzyme at 37 °C in a water bath.
 - ▲ **Note:** Please see 5. Appendix for a table detailing the conversion of other catalytic units to Wünsch units.
- Enzyme stop medium: Dulbecco's Modified Eagles Medium (DMEM) containing 20% fetal bovine serum (FBS).
- Expansion medium: StemMACS[™] MSC Expansion Media, human (# 130-091-680), or StemMACS MSC Expansion Media Kit XF, human (# 130-104-182), or MSC-Brew GMP Medium (2000 mL: # 170-076-325 or 500 mL, # 170-076-326)
- 500 mL screw cap conical bottom centrifuge tubes (e.g. Corning[®] # 431123)
- 1 L storage bottles (e.g. Corning # 430518)
- 50 mL conical tubes
- MACS SmartStrainers (100 µm) (# 130-098-463)
- MACS SmartStrainers (30 µm) (# 130-098-458)
- Orbital shaker with temperature control
- Water bath, pre-warmed to 37 °C

MACS[®] Separation using CD271 MicroBeads

- CD271 MicroBead Kit, human (# 130-099-023)
- (Optional) CD271 (LNGFR)-APC

- Red Blood Cell Lysis Solution (10×)(# 130-094-183). 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 Ca²⁺ or Mg²⁺ are not recommended for use.
- MACS Columns and MACS Separators: CD271⁺ 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
LS	10 ⁸	2×10 ⁹	MidiMACS, QuadroMACS, SuperMACS II,
XS	10 ⁹	2×10 ¹⁰	SuperMACS II

▲ **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[™] II Separators. For details refer to the respective MACS Separator data sheet.

- Pre-Separation Filters (30 μm) (# 130-041-407) to remove cell clumps.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells.

MSC/ADSC cultivation

- Expansion medium, e.g., StemMACS[™] MSC Expansion Media, human (# 130-091-680), or StemMACS MSC Expansion Media Kit XF, human (# 130-104-182), or MSC-Brew GMP Medium (2000 mL: # 170-076-325 or 500 mL, # 170-076-326) for the optimized expansion of CD271⁺ MSCs in culture.
- 75 cm² cell culture flasks or 25 cm² cell culture flasks
- (Optional) StemMACS Differentiation Media, e.g. StemMACS AdipoDiff Media, human (# 130-091-677), StemMACS ChondroDiff Media, human (# 130-091-679), or StemMACS OsteoDiff Media, human (# 130-091-678).

2. Protocol

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 use⁹.

▲ A minimum starting volume of 250 mL of lipoaspirate is required for a sufficient yield of MSCs/ADSCs. Starting with 250 mL of lipoaspirate 1×10⁷ to 1×10⁸ mononuclear cells can be expected in the stromal vascular fraction (SVF).

1. Dilute lipoaspirate sample with an equal volume of PBS and divide evenly between the screw cap 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 yellow, MSC-containing lipid phase at the top which is to be aspirated and washed a further two times.

3. Apply to a fresh screw cap 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 a better 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 applied to ensure a thorough mixing and thus digestion of the cell aggregates.
7. After 30 minutes, add an equal volume of the enzyme stop medium to each bottle.
8. Redistribute digested cell preparation into fresh screw cap 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 expansion medium.
 - ▲ **Note:** More medium may be applied if necessary, depending on the size of the pellet.
10. Pass cell suspension through a MACS SmartStrainer (100 μm) 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 expansion medium.
12. Pass cell suspension through a MACS SmartStrainer (30 μm) and collect filtrate in fresh 50 mL conical tubes.
13. Determine cell number.
14. For MSC/ADSC isolation according to CD271 expression, proceed directly to magnetic labeling (2.2). For direct cultivation of MSCs/ADSCs, proceed to MSC/ADSC cultivation (2.4).



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 10^8 total cells, use ten times 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 (30 μm), # 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. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Transfer 10^7 cells into a 15 mL conical tube.
2. Centrifuge cell suspension at 300 \times g for 10 minutes. Aspirate supernatant completely.
3. (Optional) Lyse red blood cells by adding 1 mL of 1 \times Red Blood Cell Lysis Solution and incubating cells for 10 minutes at room temperature.
4. Centrifuge cell suspension at 300 \times g for 10 minutes. Aspirate supernatant completely.
▲ Note: Erythrocyte lysis is only necessary when the sample contains significant erythrocyte contamination after MSC/ADSC isolation. If very few or no erythrocytes are observed in the sample, proceed directly to step 5.
5. Resuspend cell pellet in 60 μL of buffer per 10^7 total cells.
6. Add 20 μL of FcR Blocking Reagent and 20 μL of CD271 MicroBeads per 10^7 total cells.
7. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
8. (Optional) Add staining antibodies, e.g., CD271 (LNGFR)-APC, and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).
9. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300 \times g for 10 minutes. Aspirate supernatant completely.
10. Resuspend up to 10^8 cells in 500 μL of buffer.
▲ Note: For higher cell numbers, scale up buffer volume accordingly.
11. 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 CD271⁺ cells. For details refer to the table in section 1.4.

▲ 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 respective MACS Column data sheet.
2. Prepare column by rinsing with 3 mL of buffer.
3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with 3 \times 3 mL of buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3.
▲ Note: Perform washing steps by adding buffer aliquots as soon as 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 CD271⁺ 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: When cells are to be directly cultivated, it is recommended to flush cells from the column using an appropriate volume of expansion medium instead of buffer.

Magnetic separation with XS Columns

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

2.4 MSC/ADSC cultivation

Cultivation of MSCs/ADSCs directly from stromal vascular fraction (SVF)

1. Adjust the concentration of cells to 1×10^7 mononuclear cells per 15 mL Expansion Medium.
▲ Note: Scale up the volume median accordingly if the lipoaspirate starting material exceeded 250 mL.
2. Apply 15 mL of cell suspension per 75 cm² cell culture flask (or 5 mL of cell suspension per 25 cm² cell culture flask) and cultivate at 37 °C, 5% CO₂, and 95% humidity.
3. Change medium with fresh expansion medium after 24 hours.

Cultivation of CD271⁺ MSCs/ADSCs after MACS[®] Cell Separation

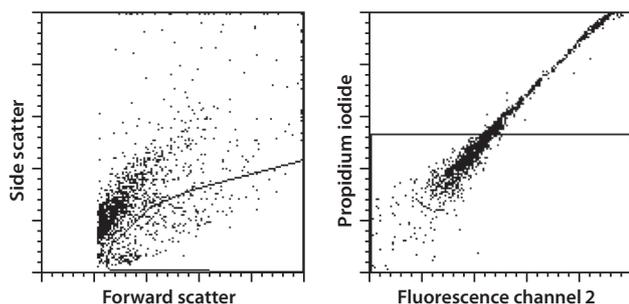
1. After cell separation, resuspend the entire CD271⁺ cell fraction in 5 mL expansion medium.
2. Apply cells to a 25 cm² cell culture flask and cultivate at 37 °C, 5% CO₂, and 95% humidity.
3. Change medium with fresh expansion medium after 24 hours.

3. Example separation of CD271⁺ MSCs/ADSCs using MACS® Technology

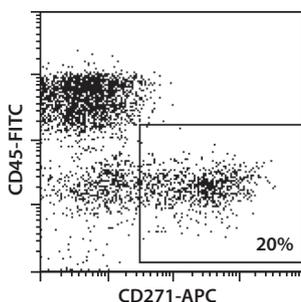
CD271⁺ MSCs/ADSCs were isolated from 2×10⁷ SVF mononuclear cells using the CD271 MicroBead Kit as described above. For fluorescence analysis, cells were also stained with CD45-FITC; CD271⁺ cells can be detected by flow cytometry via the APC moiety of the CD271-APC antibody used for MicroBead labeling.

An MSC/ADSC gating strategy was set using a sample from the unseparated SVF, by where cells with low side scatter properties were gated in R1 and then dead cells excluded after propidium iodide staining in R2 (A). All events in R1 and R2 were then measured in CD271-APC vs. CD45-FITC dot plots for the unseparated (B), CD271-negative (C), and CD271-positive (D) cell fractions. Percentages of CD271⁺CD45^{low} cells (MSCs/ADSCs) were then calculated.

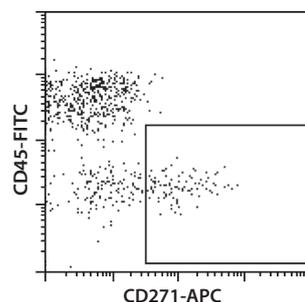
(A) Gating of viable MSCs/ADSCs



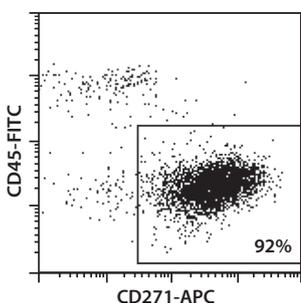
(B) Before separation



(C) CD271-negative cell fraction



(D) CD271-positive cell fraction



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5. Appendix: Conversion of other catalytic units to Wünsch units

The catalytic activity of collagenase can be determined by different methods, including Wünsch units, FALGPA units, and Mandl units. This protocol employs Wünsch units and assistance is provided here for their conversion from FALGPA and Mandl units.

Wünsch units

Collagenase cleaves the substrate PZ-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine to produce a yellow fragment PZ-L-prolyl-L-leucine that can be measured spectrophotometrically.

Unit definition: 1 U catalyzes the hydrolysis of 1 μmol of 4-phenyl azobenzoyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine per minute at 25 °C, pH 7.1

FALGPA units

Collagenase cleaves the synthetic peptide substrate N-(3-[2-furyl]acryloyl)-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA) to yield N-(3-[2-furyl]acryloyl)-L-leucine which can also be measured spectrophotometrically.

Unit definition: 1 U is defined as the hydrolysis of 1 μmol of FALGPA per minute at 25 °C, pH 7.5.

Conversion to Wünsch (U/mg): 1 U/mg Wünsch ≈ 3.9 U/mg FALGPA.

Mandl units (or collagenase degrading units, CDU)

Mandl units are calculated according to the hydrolysis of collagen by collagenase over a 5-hour period and the production of L-leucine equivalent amino acids.

Unit definition: 1 U is defined as the liberation of 1 μmol of L-leucine equivalent amino acid from collagen per 5 hours at 37 °C, pH 7.5.

Conversion to Wünsch (U/mg): 1 U/mg Wünsch ≈ 1000 U/mg Mandl or CDU.

▲ **Note:** Mandl/CDU and Wünsch units cannot be directly correlated due to the basis of Wünsch units mainly on collagenase class II activity and Mandl/CDU on collagenase class I and II collectively.

Source: SERVA Electrophoresis GmbH

Refer to www.miltenyibiotec.com for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.

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