Isolation of CD271 (LNGFR)$^+$ MSCs/ADSCs from human lipoaspirate

Special protocol for cell separation

Contents

1. Description

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 disposible 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 frequencies[1] as well as an apparent pluripotent ability to differentiate to cells of a neuronal phenotype[2]. 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 (LNGFR) 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 marrow[6] and also in lipoaspirate[8]. Furthermore, the phenotype of MSCs in lipoaspirate was described to be positive for CD271, CD105, and CD44, and negative for CD45[7].

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 NH Expansion Medium before serial filtration through 100 µm and then 40 µm nylon filters. The content of mononuclear cells is then counted and cells are then ready for separation using the CD271 MicroBead Kit (APC) (# 130-092-283) or the MSC Research Tool Box – CD271 (APC) (# 130-092-291).

1.3 Principle of the MACS® Separation using CD271 (LNGFR)

CD271$^+$ cells are firstly labeled with the APC-conjugated CD271 (LNGFR) antibody contained within the CD271 MicroBead Kit (APC) or the MSC Research Tool Box – CD271 (APC). Then, the target cells are labeled with Anti-APC MicroBeads. 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 on the column while the unlabeled cells run through. After removal of the column from the magnetic field, the magnetically retained CD271$^+$ cells can be eluted as the positively selected cell fraction.

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, Serva # 17465.02): 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, # 130-091-437) containing 20% fetal bovine serum (FBS).
- NH Expansion Medium (# 130-091-680)*.
- 500 mL Screw Cap Conical Bottom Centrifuge Tubes (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.

* The NH Expansion Medium is supplied in the MSC Research Tool Box - CD271 (APC).

MACS® Separation using CD271 (LNGFR)

- CD271 MicroBead Kit (APC), human (# 130-092-283) or MSC Research Tool Box – CD271 (APC) (# 130-092-291).

  ▲ **Note:** The use of the CD271 MicroBead Kit (PE) and MSC Research Tool Box – CD271 (PE) is not recommended due to MSC/ADSC cellular autofluorescence being detectable in the PE channel in flow cytometry.
- Erythrocyte lysis buffer (10×): 1.55 M NH_4Cl, 100 mM $KHCO_3$, 1 mM EDTA, pH 7.3. Always dilute freshly before use.

SP004201

Miltenyi Biotec GmbH
Friedrich-Ebert-Str. 68
51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0 Fax +49 2204 85197

Miltenyi Biotec Inc.
12740 Earhart Avenue, Auburn CA 95602, USA
Phone 800 FOR MACS, +1 530 888 8871
Fax +1 530 888 8925

- 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 calf serum. Buffers or media containing Ca^{2+} or Mg^{2+} 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
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 VarioMACS™ Separator or SuperMACS™ Separator. For details, see MACS Separator data sheets.

- Pre-Separation Filters (# 130-041-407) to remove cell clumps.
- (Optional) Propidium iodide (PI) or 7-AAD for flow cytometric exclusion of dead cells.

MSC/ADSC cultivation

- NH Expansion Medium (# 130-091-680) for the optimized expansion of $CD271 (LNGFR)^+$ 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 CD271 (LNGFR)-selected 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).

* The NH Expansion Medium is supplied in the MSC Research Tool Box - CD271 (APC).

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 use[9].

▲ A minimum starting volume of 250 mL of lipoaspirate is required for a sufficient yield of MSCs/ADSCs. Starting with 250 mL of lipoaspirate 1×10^7 to 1×10^8 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 NH Expansion Medium.

   ▲ **Note:** More medium 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 NH Expansion Medium.
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 MSC/ADSC isolation according to CD271 (LNGFR) expression, proceed directly to 2.2 Magnetic labeling. For direct cultivation of MSCs/ADSCs, proceed to 2.4 MSC/ADSC cultivation.

SP004201

2.2 Magnetic labeling

▲ The protocol below is applicable for both the CD271 MicroBead Kit (APC) or the MSC Research Tool Box – CD271 (APC).

▲ 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 separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column.

▲ 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 MSC/ADSC isolation. If very few or no erythrocytes are observed in the sample, proceed directly to step 5 below.
5. Resuspend cell pellet in 80 µL of buffer per 10^7 cells.
6. Add 10 µL of FcR Blocking Reagent and 10 µL of CD271-APC 10^7 cells.
5. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
6. Wash cells by adding 1 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in 70 µL of buffer per 10^7 cells.
8. Add 10 µL of FcR Blocking Reagent and 20 µL of Anti-APC MicroBeads per 10^7 cells.
9. Incubate for 15 minutes 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.

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 see table in section 1.4.

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. Always use a MACS Pre-Separation Filter.
4. Collect unlabeled cells that pass through and 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. Only add new buffer 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 $CD271^+$ 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 NH 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 NH 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 cm^2 cell culture flask (or 5 mL of cell suspension per 25 cm^2 cell culture flask) and cultivate at 37 °C, 5% CO_2, and 95% humidity.
3. Change medium with fresh NH Expansion Medium after 24 hours.

   ▲ **Note:** For further protocols describing the cultivation, enumeration, and differentiation of MSCs, please refer to the NH stem cell media data sheet available at www.miltenyibiotec.com.

Cultivation of $CD271^+$ MSCs/ADSCs after MACS® Cell Separation

1. After cell separation, resuspend the entire $CD271^+$ cell fraction in 5 mL NH Expansion Medium.
2. Apply cells to a 25 cm^2 cell culture flask and cultivate at 37 °C, 5% CO_2, and 95% humidity.
3. Change medium with fresh NH Expansion Medium after 24 hours.

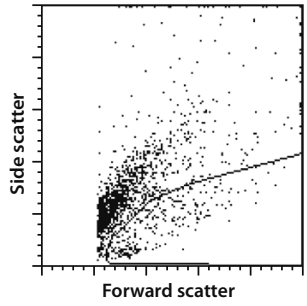
   ▲ **Note:** For further protocols describing the cultivation, enumeration, and differentiation of MSCs, please refer to the NH stem cell media data sheet available at www.miltenyibiotec.com or follow the instructions given in the MSC Research Tool Box - CD271 (APC) data sheet.

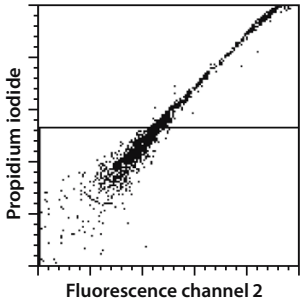
3. Example separation of CD271 (LNGFR)$^+$ MSCs/ ADSCs using MACS® Technology

CD271$^+$ MSCs/ADSCs were isolated from 2×10^7 SVF mononuclear cells using the CD271 MicroBead Kit (APC) 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 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.

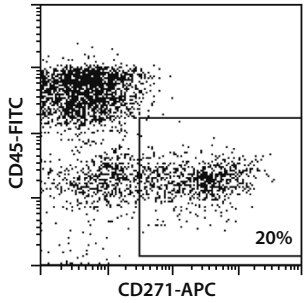
(A) Gating of viable MSCs/ADSCs

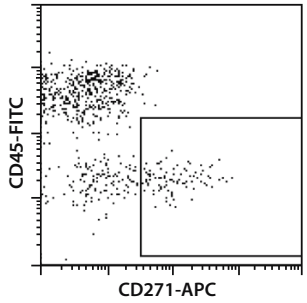




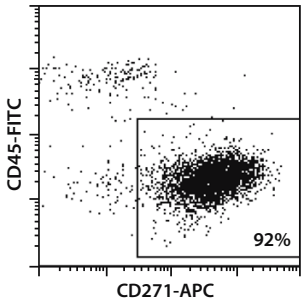
(B) Before separation

(C) CD271-negative cell fraction





(D) CD271-positive cell fraction



4. References

1. Kern, S. *et al.* (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord, or adipose tissue. Stem Cells 24: 1294–1301.
2. Zuk, P. A. *et al.* (2002) Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell 13: 4279–4295.
3. Jones, E. *et al.* (2004) Phenotypical, Morphological and Molecular Analysis of Fresh Human Bone Marrow Mesenchymal/Stromal Stem Sells (MSCs) Enriched by Four Different Methods. 29 ASH Abstract (2337)
4. Jones , E. A. *et al.* (2006) Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow. Cytometry B Clin. Cytom. 70(6): 391–399.
5. Jones, E. A. *et al.* (2007) Purification of proliferative and multipotential marrow stromal cells (MSCs) from bone marrow aspirate by selection for CD271 (LNGFR) expression. MACS&more 11-1: 22–25.
6. Quirici, N. *et al.* (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp. Hematol. 30: 783–791.
7. Meyerrose, T. E. *et al.* (2007) In Vivo Distribution of Human Adipose-Derived Mesenchymal Stem Cells in Novel Xenotransplantation Models. Stem Cells 25: 220–227.
8. Godthardt, K. (2007) Adipose-derived marrow stromal cells from lipoaspirate are CD271-positive. MSC 2007 Adult Mesenchymal Stem Cells in Regenerative Medicine, August 27th - August 29th, 2007 Held at the Cleveland Marriott Downtown at Key Center Poster Nr. 301 Session III: Stem Cells From Adipose Tissue.
9. Matsumoto, D. *et al.* (2007) Influences of preservation at various temperatures on liposuction aspirates. Plast. Reconstr. Surg. 120(6): 1510–1517.

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 azobenzyloxycarbonyl-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

SP004.01

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

The products sold hereunder are warranted only to be free from defects in workmanship and material at the time of delivery to the customer. Miltenyi Biotec GmbH makes no warranty or representation, either expressed or implied, with respect to the fitness of a product for a particular purpose. There are no warranties, expressed or implied, which extend beyond the technical specifications of the products. Miltenyi Biotec GmbH's liability is limited to either replacement of the products or refund of the purchase price. Miltenyi Biotec GmbH is not liable for any property damage, personal injury or economic loss caused by the product.

SP006210