



Special protocol for sample preparation

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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 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.

1.2 Principle of SVF preparation 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 the cell sample taken into culture in NH Expansion Medium. MSCs adhere to plastic surfaces within 24 hours after which the medium should be exchanged with fresh NH Expansion Medium. Optionally, after cell counting, MSCs can be further enriched by magnetic separation using the MSC Research Tool Box – CD271 (LNGFR), CD271 (LNGFR) MicroBead Kits, or CD146 MicroBeads.

1.3 Reagent and instrument requirements

- 1 L 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).

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

Resolve enzyme at 37 °C in a water bath.

▲ **Note:** Please see 4. 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 (Corning # 431123).
- 1 L storage bottles (e.g. Corning # 430518).
- 50 mL conical tubes (e.g. BD Biosciences # 352070).
- 75 cm² cell culture flasks (e.g. BD Biosciences # 353136).
- 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.
- (Optional) MSC Research Tool Box – CD271 (APC), human (# 130-092-291).
- (Optional) CD271 MicroBead Kit (APC), human (# 130-092-283)
- (Optional) CD146 MicroBead Kit, human (# 130-093-596)
- (Optional) CD105 MicroBeads, human (# 130-051-201).
- (Optional) NH differentiation media: NH AdipoDiff Medium (# 130-091-677), NH ChondroDiff Medium (# 130-091-679), and NH OsteoDiff Medium (# 130-091-678).
- (Optional) MACS® Pre-Separation Filter (# 130-041-407).

2. Protocol

▲ 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 using 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 use³.

▲ A minimum starting volume of 250 mL of lipoaspirate is required for a sufficient yield of MSCs. When 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 the lipoaspirate sample with an equal volume of PBS and divide evenly between the Screw Cap Conical Bottom Centrifuge Tubes.
2. Centrifuge at 430 \times g for 10 minutes without brakes. After centrifugation, remove the target cell-containing lipid phase from the top (see figure 1) and apply to a fresh Screw Cap Conical Bottom Centrifuge Tube. Dilute with an equal volume of PBS.





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. Repeat step 2 twice.
4. Dilute the 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.
5. Incubate the 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 preparation.
6. After 30 minutes, add an equal volume of the enzyme stop medium to each bottle.
7. Redistribute the digested cell preparation into fresh Screw Cap Conical Bottom Centrifuge Tubes and centrifuge at 600×g for 10 minutes. Aspirate and discard the supernatant.
8. Resuspend the 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 pellet.
9. Pass the cell suspension through a 100 µm cell strainer, collecting the filtrate in 50 mL conical tubes.
10. Centrifuge at 600×g for 10 minutes. Aspirate and discard the supernatant and resuspend the pellet in 5 mL of NH Expansion Medium.
11. Pass the cell suspension through a 40 µm cell strainer, collecting the filtrate in fresh 50 mL conical tubes, and then count the cells.
12. If MSCs are to be directly isolated using MACS Technology, proceed according to the protocol of the respective product data sheet. For the isolation of MSCs according to the expression of CD271 refer to the special protocol “Isolation of CD271 (LNGFR)⁺ MSCs/ADSCs from human lipoaspirate?”. For the isolation of CD146⁺ cells refer to the CD146 MicroBead Kit data sheet. If MSCs are to be directly cultivated, continue as described in step 13.
 - ▲ **Note:** Apply cells to a MACS Pre-Separation Filter before separation using MACS Technology.
13. Adjust the concentration of mononuclear cells to 1×10⁷ cells per 15 mL of NH Expansion Medium. Apply 1×10⁷ cells per 75 cm² cell culture flask and cultivate at 37 °C, 5% CO₂, 95% humidity. Change the medium with fresh NH Expansion Medium after 24 hours.
 - ▲ **Note:** For further protocols describing the cultivation, enumeration, and differentiation of MSCs, please see the Nonhematopoietic (NH) stem cell media booklet available at www.miltenyibiotec.com.

3. 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. *13*: 4279–4295.
3. Matsumoto, D. *et al.* (2007) Influences of preservation at various temperatures on liposuction aspirates. *Plast. Reconstr. Surg.* 120(6): 1510–1517.

4. 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

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