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## Nonhematopoietic (NH) stem cell media

### for human marrow stromal cells (MSCs)

NH CFU-F Medium	Order no. 130-091-676
NH Expansion Medium	Order no. 130-091-680
NH AdipoDiff Medium	Order no. 130-091-677
NH ChondroDiff Medium	Order no. 130-091-679
NH OsteoDiff Medium	Order no. 130-091-678

For further information refer to our website [www.miltenyibiotec.com](http://www.miltenyibiotec.com)

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### 1. Description

	NH CFU-F Medium <sup>a</sup> 24x5 mL NH Expansion Medium <sup>a,b</sup> 500 mL	NH AdipoDiff Medium <sup>b</sup> for generation of adipocytes 100 mL	NH ChondroDiff Medium <sup>c</sup> for generation of chondrocytes 100 mL	NH OsteoDiff Medium <sup>b</sup> for generation of osteoblasts 100 mL
Product format	DMEM Fetal Bovine Serum L-Glutamine	DMEM Fetal Bovine Serum L-Glutamine supplements		
Storage	-20 °C			
a NH CFU-F Medium and NH Expansion Medium are different formats of the same medium. Therefore, both media can be used for MSC expansion and CFU-F assays.				
b If media are thawed upon arrival, immediately mix them thoroughly (NH Expansion Medium, NH AdipoDiff Medium or NH OsteoDiff Medium), aliquot to the preferred sample volume and store at -20 °C until used.				
c Media is transported frozen. Transfer directly to -20 °C until use.				

The expiration date is indicated on the vial label.

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### 1.1 Background

Bone marrow is a complex cellular arrangement in which hematopoietic stem and precursor cells with hematopoietic differentiation potential exist in close proximity to the heterogeneous group of nonhematopoietic (NH) stem cells with nonhematopoietic differentiation potential.

In early 1970, Friedenstein *et al.* first described the ability of specific cells from bone marrow to slowly proliferate and to be highly adhesive to plastic.<sup>1</sup> These cells were named after their spindle-like morphologic appearance, colony forming unit fibroblasts (CFU-Fs) and showed nonhematopoietic differentiation potential. Starting with unseparated bone marrow cells from iliac crest aspirations of healthy donors, an average of 140 CFU-Fs per 10<sup>7</sup> cells was determined.<sup>2</sup> These cells showed a mesodermal plasticity, i.e. osteogenic<sup>3</sup>, adipogenic and chondrogenic differentiation potential.

The CFU-F progeny was designated in 1997, when proliferated *in vitro*, as marrow stromal fibroblasts (MSFs).<sup>4</sup> However, several additional terms were also used for this heterogeneous cell population, such as mesenchymal stem cells, multipotent stromal cells, mesodermal stem cells or marrow stromal cells (all abbreviated as MSCs), mesenchymal progenitor cells (MPCs), multipotent adult progenitor cells (MAPCs), stromal precursors, bone marrow stromal stem cells (BMSSCs) or bone marrow stromal cells (BMSCs).

This variety arises from the fact that cultures of CFU-Fs are not homogeneous, but consist of a mixture of progenitor cells with differing plasticities. Taking this diversity into account, we decided to use the term **marrow stromal cells (MSCs)** for cells from bone marrow with nonhematopoietic differentiation potential.

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### 1.2 Nonhematopoietic (NH) stem cell media

▲ **MACS® NH Stem Cell Media are for research use only and not for diagnostic or therapeutic use.**

For a better understanding of the processes that mediate the differentiation of MSCs into functional nonhematopoietic cell types and to identify the factors involved, it is essential to establish efficient and reproducible procedures for the isolation and cultivation of target cells. Therefore, it is crucial to use standardized experimental conditions, such as *in vitro* culture systems. For this reason, Miltenyi Biotec offers a range of high-quality media, optimized for human bone marrow cells:

**NH CFU-F Medium** (# 130-091-676): optimized and standardized medium for the quantification of MSCs and quality control of the MSC source and isolation procedure.

**NH Expansion Medium** (# 130-091-680): optimized and standardized medium for the reproducible and reliable expansion of MSCs from human bone marrow.

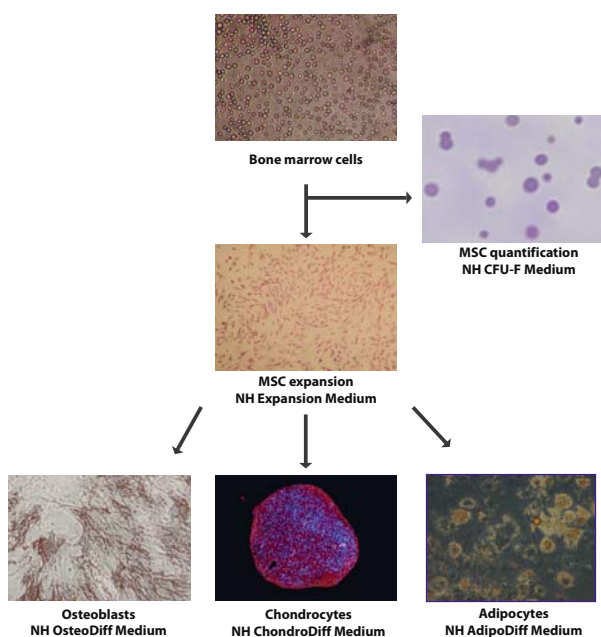
**NH AdipoDiff Medium** (# 130-091-677): optimized differentiation medium for the generation of adipocytes from human MSCs.

**NH ChondroDiff Medium** (# 130-091-679): optimized differentiation medium for the generation of chondrocytes from human MSCs.

**NH OsteoDiff Medium** (# 130-091-678): optimized differentiation medium for the generation of osteoblasts from human MSCs.



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### 1.3 Reagent and instrument requirements

#### General reagents and plastic ware requirements

- Ficoll-Paque™ PLUS
- Phosphate buffered saline (PBS), pH 7.2
- P/E buffer (PBS with 2 mM EDTA), pH 7.4–7.6 (sterile)
- Membrane filter (100 µm) (Becton Dickinson # 352360)
- Sterile 15 mL conical tubes
- Sterile 50 mL conical tubes
- 2 mL reaction tubes
- Tissue culture flasks (T-25, 25 cm<sup>2</sup>) (Becton Dickinson # 353109) or cell culture dishes (ø 60 mm) (Greiner Bio-One # 628160)
- (Optional) MACS® Cell Separation Products (see 7. Related products)
- (Optional) GIBCO® Penicillin-Streptomycin liquid (Invitrogen # 15140-122)
- (Optional) Trypan Blue Stain (Invitrogen # 15250-061), and a Hemocytometer

#### Lab equipment

- Centrifuge
- Laminar Flow Hood (Biohazard Containment Hood)
- CO<sub>2</sub> incubator, 37 °C with 5% CO<sub>2</sub> in air and > 95% humidity
- Microscope
- Water bath (37 °C)

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**Reagents for CFU-F assay procedure**

- NH CFU-F Medium (# 130-091-767) or NH Expansion Medium (# 130-091-680)
- Giemsa staining solution (Giemsa's Azure Eosin Methylene Blue Solution) (Merck # 1.09204)
- Folded filter (Schleicher & Schuell # 10314747)
- Methanol
- Deionized water

**Reagents for MSC expansion procedure**

- NH Expansion Medium (# 130-091-680) or NH CFU-F Medium (# 130-091-676)
- Trypsin-EDTA (0.05%/0.53 mM) (Invitrogen # 25300-054)
- (Optional) Basic Fibroblast Growth Factor (bFGF) (R&D Systems # 234FSE)

**General materials for MSC differentiation and detection**

- Millex®-GV 0.22 µm (Millipore # SLGV033RS)
- Plate shaker
- Oven
- Microtome
- Microscope

**Reagents and materials for adipocyte generation and detection**

- NH AdipoDiff Medium (# 130-091-677)
- Cell culture dishes (35 mm in diameter)
- Oil Red O (Sigma # O9755)
- Isopropanol

**Materials for chondrocyte generation and detection**

- Bio-mold embedding dish (e.g. Medite # 47-2002-00)
- Embedding cassettes (e.g. Medite # 47-1100-00)
- Filter paper for embedding cassettes (e.g. Medite # 46-6200-00)
- HistoBond® slides (Superior # 901236)
- Hydrophobic pen (e.g. Dako # S 2002)
- Coverslips (e.g. Medite # 46-7150-00)
- Fluorescence microscope

**Reagents for chondrocyte generation and detection**

- NH ChondroDiff Medium (# 130-091-679)
- Phosphate buffered saline (PBS)
- Deionized water
- Bovine serum albumin (BSA)
- 37% formalin (e.g. Merck # 1.04003.1000)
- 100% ethanol (e.g. AppliChem # A1613)
- Roti®-Histol (xylol substitute) (Roth # 6640.1)

- Roti®-Plast (paraffin) (Roth # 6642.5)
- Fluoromount-G™ (Southern Biotech # 01001-01)
- Triton® X-100 (e.g. Sigma # 23472-9)
- Normal donkey serum (e.g. Jackson ImmunoResearch # 017-000-121)
- Mouse Anti-Human Aggrecan Antibody (Chemicon # MAB19310)
- Donkey Anti-Mouse IgG (H+L)-Rhodamine (TRITC) (Jackson ImmunoResearch # 715-026-150)
- 4',6-diamidino-2-phenylindole (DAPI) (e.g. Sigma # D-9564)

**Reagents and materials for osteoblast generation and detection**

- NH OsteoDiff Medium (#130-091-678)
- Cell culture dishes (35 mm in diameter)
- SIGMA FAST™ BCIP/NBT Buffered Substrate Tablet (Sigma # B5655)

**2. Preparation of cells****2.1 Preparation of human bone marrow samples**

▲ Use fresh bone marrow only. Avoid freezing and thawing of bone marrow cells and perform all of the following steps under sterile conditions in a laminar flow hood.

**2.1.1 Preparation of human bone marrow mononuclear cells (BM MNCs)**

1. Dilute aspirated human bone marrow at a ratio of 6:1 with sterile PBS containing 2 mM EDTA (P/E buffer), e.g. dilute 30 mL bone marrow with 5 mL P/E buffer to a final volume of 35 mL.
2. Pass cells through a 100 µm membrane filter to remove bone fragments and fat clumps.  
▲ Note: Wet filter with PBS or P/E buffer before use.
3. Carefully layer 35 mL of diluted cell suspension over 15 mL Ficoll-Paque PLUS in a 50 mL conical tube.
4. Centrifuge for 35 minutes at 445×g at room temperature in a swinging bucket rotor (**without brake**).
5. Aspirate the upper layer leaving the mononuclear cell layer undisturbed at the interface.
6. Carefully transfer the bone marrow mononuclear cells (BM MNCs) at the interface to a new 50 mL conical tube.
7. Wash cells by adding up to 40 mL of P/E buffer, mix gently and centrifuge for 10 minutes at 300×g at room temperature. Repeat wash.
8. Aspirate supernatant completely.
9. Resuspend cells in 1–2 mL P/E buffer.
10. Determine cell number and viability using a hemocytometer by Trypan Blue exclusion.

## 2.2 Isolation of human marrow stromal cells (MSCs)

### A. With MACS® MicroBeads

MSCs can be isolated by magnetic cell sorting. The utilization of MSCs separated using MACS® Technology results in a defined cell population as a source for reproducible differentiation experiments. For a list of MACS Products for the enrichment of MSCs, please see 7. Related products. For the MACS Separation procedure, refer to the appropriate data sheet.

### B. With adherence method

As an alternative to the separation of MSCs by MACS Technology, MSCs can be pre-enriched by their ability to adhere strongly to plastic surfaces. The cultivation of human bone marrow samples (see 2.1 Preparation of human bone marrow samples) in NH Expansion Medium will result in a homogeneous layer of MSCs.

## 3. Colony Forming Unit - Fibroblast (CFU-F) assay

The CFU-F assay is a well established method for the quantification of MSCs from a human bone marrow sample. CFU-Fs represent stem and progenitor cells with nonhematopoietic differentiation potential. Therefore, the assay is best suited for quality testing of the MSC source or isolation procedure. CFU-F assays can be performed using the convenient, pre-aliquoted NH CFU-F Medium or NH Expansion Medium.

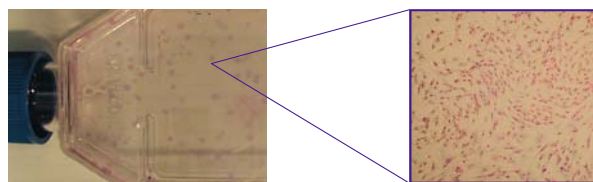


Figure 1: CFU-Fs stained with Giemsa staining solution. A fibroblastoid morphology is typical for all MSCs when cultured at low-density in NH CFU-F Medium, as visualized by light or phase contrast microscopy. MSCs are extremely adherent and will start to adhere within the first minutes of incubation. The majority of MSCs will have attached after a few hours.

## 3.1 Preparing the CFU-F assay

### A. CFU-F assay in dilution series

Bone marrow samples from various donors can differ in numbers and CFU-F proliferation potential. Therefore, it is recommended to use three different cell concentrations for the CFU-F assays. This will ensure that the resulting numbers of fibroblastoid colonies can be scored.

1. Pre-warm NH CFU-F Medium to 37 °C in a water bath or incubator.
  - ▲ **Note:** (Optional) Add 1% Penicillin-Streptomycin to the NH CFU-F Medium to avoid bacterial contamination of the cell culture.
2. Prepare a suspension of human BM MNCs (see 2.1 Preparation of human bone marrow samples) with a concentration of  $2 \times 10^7$  cells/mL in PBS or DMEM.

3. Dilute this BM MNC suspension by pipetting:
  - 50  $\mu$ L cell suspension into a vial with 5 mL NH CFU-F Medium resulting in a final cell concentration of  $2 \times 10^5$  cells/mL NH CFU-F Medium
  - 25  $\mu$ L cell suspension into a vial with 5 mL NH CFU-F Medium resulting in a final cell concentration of  $1 \times 10^5$  cells/mL NH CFU-F Medium
  - 12.5  $\mu$ L cell suspension into a vial with 5 mL NH CFU-F Medium resulting in a final cell concentration of  $0.5 \times 10^5$  cells/mL NH CFU-F Medium
4. Resuspend cells carefully.
5. Transfer the diluted cell samples to 60 mm cell culture dishes (or T-25 tissue culture flasks).
6. Incubate the vessels at 37 °C in an incubator with 5% CO<sub>2</sub> and > 95% humidity for 2 weeks.
  - ▲ **Note:** It is not necessary to change the NH CFU-F Medium during incubation time.

### B. Alternative: CFU-F assay in duplicate

Alternatively, for established bone marrow isolation procedures and predictable donor MSC rates, CFU-F assays can be prepared in duplicate with only one dilution.

1. Pre-warm two vials of NH CFU-F Medium to 37 °C in a water bath or incubator.
  - ▲ **Note:** (Optional) Add 1% Penicillin-Streptomycin to the NH CFU-F Medium to prevent bacterial contamination of the cell culture.

2. Add the pre-defined cell concentration to each of the 5 mL vials with NH CFU-F Medium.
  - ▲ **Note:** The volume of the bone marrow samples added to the NH CFU-F Medium vials should not exceed 0.5 mL.
3. Resuspend cells carefully.
4. Transfer each diluted cell sample to a 60 mm cell culture dish (or T-25 tissue culture flask).
5. Incubate the vessels at 37 °C in an incubator with 5% CO<sub>2</sub> and > 95% humidity for 2 weeks.
  - ▲ **Note:** It is not necessary to change the NH CFU-F Medium during incubation time.

## 3.2 Staining and enumeration of CFU-Fs

1. Remove NH CFU-F Medium from the cell culture vessels.
2. Wash the cell culture vessels with  $2 \times 5$  mL PBS. Afterwards, remove PBS completely.
3. To fix the cells, add 5 mL methanol to the cell culture vessels.
4. Incubate for 5 minutes at room temperature.
5. Decant methanol and air-dry the culture vessels until methanol has evaporated.
6. Dilute Giemsa staining solution 1:20 with deionized water and filter through a folded filter.
7. Add 5 mL Giemsa staining solution and incubate for 5 minutes at room temperature.

8. Remove Giemsa staining solution and wash twice with deionized water.
9. Remove deionized water and air-dry the culture vessels until deionized water has evaporated.
  - ▲ **Note:** Proceed with the scoring procedure within 3 days, since staining will fade.
10. CFU-F colonies are typically between 1–8 mm in diameter and therefore can be scored macroscopically. Count cell clusters with more than 20 cells as a colony.
  - ▲ **Note:** When performing CFU-F assays in dilution series (see 3.1 A. CFU-F assays in dilution series) there should be a linear correlation between the cell numbers plated and the CFU-Fs counted.

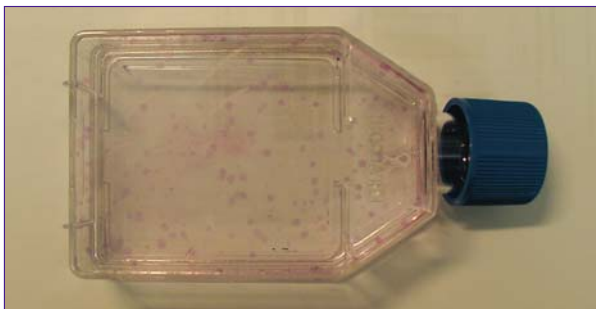


Figure 2: Giemsa staining of human MSCs in a CFU-F assay after two weeks of cultivation. The nucleus and cytoplasm appear blue or pink. For the documentation of the counting process, and to avoid imprecision, it is helpful to mark the counted colonies with a pen.

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#### 4. Expansion of human MSCs

MSCs are present at low frequencies in bone marrow samples. This often necessitates their expansion in order to obtain sufficient numbers for further experiments, such as MSC transplantation studies (animal models), differentiation studies, or gene/protein expression profiling. The NH Expansion Medium is an optimized and standardized medium for the reproducible and reliable expansion of MSCs from human bone marrow.

##### 4.1 Set-up of the expansion procedure

Before using the NH Expansion Medium for the first time, thaw completely, mix thoroughly and pre-aliquot to your preferred sample size. Store aliquots at  $-20^{\circ}\text{C}$  until use.

1. Pre-warm NH Expansion Medium to  $37^{\circ}\text{C}$  in a water bath or incubator.
  - ▲ **Note:** (Optional) Add 1% Penicillin-Streptomycin to the NH Expansion Medium to prevent bacterial contamination of the cell culture.
2. Prepare a suspension of human BM MNCs (see 2.1 Preparation of human bone marrow samples) with a concentration of  $2 \times 10^7$  cells/mL in PBS or NH Expansion Medium.
3. Add 0.5 mL cell suspension with  $2 \times 10^7$  BM MNCs/mL to 5 mL NH Expansion Medium, resulting in a final cell concentration of  $2 \times 10^6$  cells/mL NH Expansion Medium.
  - ▲ **Note:** When working with cells separated by MACS Technology, MSC frequency can be different. Therefore, it might be necessary to adjust the cell concentration.



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- ▲ **Note:** The volume of the bone marrow samples added to the NH Expansion Medium vials should not exceed 0.5 mL.
4. Resuspend cells carefully.
  5. Transfer the diluted cell sample to a T-25 tissue culture flask.
  6. Culture cells at  $37^{\circ}\text{C}$  in an incubator with 5%  $\text{CO}_2$  and  $>95\%$  humidity.
  7. Aspirate the NH Expansion Medium completely after 24 hours of culturing.
  8. Add 5 mL of fresh NH Expansion Medium and continue culturing the MSCs.
  9. Change NH Expansion Medium weekly by removing NH Expansion Medium completely from the T-25 tissue culture flask and adding 5 mL fresh NH Expansion Medium. Continue culturing the cells.
  10. Check your cell culture under a microscope regularly. When MSCs have reached 80% confluency (presumably around day 12), proceed with 4.2 Passaging of MSCs.
    - ▲ **Note:** In some cases, MSCs are distributed unevenly over the growth area of the tissue culture flask. Thus, certain colonies or areas reach 80% confluency earlier than others. A protocol to equally distribute the cells for further cultivation can be found in 6.1 Redistribution of unevenly populated tissue cultures.

##### 4.2 Passaging of human MSCs

1. Pre-warm Trypsin/EDTA (0.05%/0.53 mM), PBS and NH Expansion Medium to  $37^{\circ}\text{C}$  in a water bath or incubator.

2. Remove NH Expansion Medium from the T-25 tissue culture flask.
3. Wash cells with 2 mL PBS to remove residual NH Expansion Medium.
4. Add 1 mL Trypsin/EDTA (0.05%/0.53 mM) to cover cells and incubate at  $37^{\circ}\text{C}$  for 5–10 minutes.
5. Check under a microscope that MSCs are completely dissociated. If not, gently tap flask or increase the incubation time for a few more minutes to facilitate dissociation of the cells.
  - ▲ **Note:** Time of trypsination may vary, but usually cells dissociate within 5 to 15 minutes.
6. Once MSCs are completely detached, add 5 mL NH Expansion Medium or DMEM with 20% FCS, resuspend cells by pipetting and transfer them to a 15 mL conical tube.
7. Wash the T-25 tissue culture flask with an additional 5 mL NH Expansion Medium or DMEM with 20% FCS and collect all cells in the 15 mL conical tube.
8. Centrifuge cells at  $300 \times g$  for 10 minutes at room temperature.
9. Remove supernatant and carefully resuspend cells in 2 mL NH Expansion Medium.
10. Determine cell number and viability using a hemocytometer by Trypan Blue exclusion.
11. Add  $1.5\text{--}2 \times 10^5$  of the harvested cells to 5 mL NH Expansion Medium and transfer the cells to a T-25 tissue culture flask.
  - ▲ **Note:** (Optional) To maintain the proliferation capacity for long term cultivation of MSCs, add bFGF to a final concentration of 0.1  $\mu\text{g}/\text{mL}$  to the NH Expansion Medium, starting with the first passage.

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▲ **Note:** (Optional) Add 1% Penicillin-Streptomycin to the NH Expansion Medium to prevent bacterial contamination of the cell culture.

- Culture cells at 37 °C in an incubator with 5% CO<sub>2</sub> and > 95% humidity.
- Check your cell culture under a microscope regularly. Before MSCs have reached 80% confluency, approx. after 3 to 8 days, repeat the passaging procedure.
- Repeat expansion procedure until a sufficient number of cells is reached.

▲ **Note:** The number of passages that can be achieved while maintaining the full differentiation potential of MSCs is donor dependent.

▲ **Note:** It is possible that MSCs spontaneously differentiate into, for example, osteoblasts. This mainly occurs when MSCs are too dense. If this occurs, split cells before 80% confluency is reached.

▲ **Note:** For freezing and thawing protocols for MSCs, see 6.2 and 6.3, respectively.

## 5. Differentiation

▲ **MACS® NH Stem Cell Media are for research use only and not for diagnostic or therapeutic use.**

Present throughout the whole human life cycle, MSCs are a population of cells with multilineage potential, defined by their ability to differentiate into cells of the osteogenic, chondrogenic, and adipogenic lineages. MSCs have also demonstrated the *in vitro* potential to differentiate into nonhematopoietic cell types, including cells with neuronal, endothelial or myocyte phenotypes.<sup>5</sup>

The remarkable plasticity of MSCs may make these adult stem cells an

invaluable cellular resource for research into cellular therapy of tissue defects and chronic diseases.

The growing interest in the use of MSCs in clinical research necessitates the fundamental understanding of mechanisms and processes underlying the differentiation into specific cell types, as well as to validate the MSC phenotype of cultivated cells. The NH differentiation media can be used to reproducibly differentiate MSCs to adipogenic, chondrogenic, and osteogenic cell types for these research applications.

### 5.1 Harvesting of cultivated cells

- Pre-warm Trypsin/EDTA (0.05%/0.53 mM), PBS and NH Expansion Medium to 37 °C in a water bath or incubator.
- Remove NH Expansion Medium from the T-25 tissue culture flask.
- Wash cells with 2 mL PBS to remove residual NH Expansion Medium.
- Add 1 mL Trypsin/EDTA (0.05%/0.53 mM) to cover cells and incubate at 37 °C for 5-10 minutes.
- Check under a microscope that MSCs are completely dissociated. If not, gently tap flask or increase the incubation time for a few more minutes to facilitate dissociation of the cells.
 

▲ **Note:** Time of trypsination may vary, but usually cells dissociate within 5 to 15 minutes.
- Once MSCs are completely detached, add 5 mL NH Expansion Medium, resuspend cells by pipetting and transfer them to a 15 mL conical tube.

- Wash the T-25 tissue culture flask with an additional 5 mL NH Expansion Medium and collect all cells in the 15 mL conical tube.
- Centrifuge cells at 300×g for 10 minutes at room temperature.
- Remove supernatant and carefully resuspend cells in 0.5 mL NH Expansion Medium.
- Determine cell number and viability using a hemocytometer and Trypan Blue exclusion.
- Proceed directly with the respective differentiation protocol 5.2–5.4 or freeze cells (see 6.2 Freezing of marrow stromal cells (MSCs)).

### 5.2 Differentiation of MSCs into adipocytes and their detection

▲ **MACS® NH Stem Cell Media are for research use only and not for diagnostic or therapeutic use.**

The NH AdipoDiff Medium promotes the differentiation and further maturation of MSCs into adipocytes (fat cells).

Adipocytes play a critical role in the storage of energy as lipids, in the overall regulation of the body's metabolism and as an endocrine organ. Adipocytes have a profound effect on human physiology and have been identified as a risk factor for metabolic diseases such as non-insulin-dependent diabetes, cancer, arteriosclerosis, obesity or cardiovascular diseases (for example hypertension). To understand the development and regulation of adipocytes (adipogenesis) is important for future clinical research into the treatment or prevention of metabolic diseases.

Before using the NH AdipoDiff Medium for the first time, thaw completely, mix thoroughly and pre-aliquot to a sample size of 25 mL for differentiation of MSCs into adipocytes. Store aliquots at –20 °C until use. Throughout the duration of the experiment, store the aliquot at 4 °C.

- Pre-warm NH AdipoDiff Medium to 37 °C in a water bath or incubator.
 

▲ **Note:** To obtain reliable results, the differentiation of MSCs into adipocytes should be performed in duplicate.
- Dilute MSCs to a final concentration of 5×10<sup>4</sup> cells/mL NH AdipoDiff Medium.
 

▲ **Note:** (Optional) Add 1% Penicillin-Streptomycin to the NH AdipoDiff Medium to prevent bacterial contamination of the cell culture.
- Resuspend cells carefully.
- Transfer 1.5 mL of the cell suspension to the 35 mm cell culture dish.
- Culture cells at 37 °C in an incubator with 5% CO<sub>2</sub> and > 95% humidity.
- Change NH AdipoDiff Medium every 3rd day by removing it from the 35 mm cell culture dish completely and adding 1.5 mL fresh prewarmed NH AdipoDiff Medium. Continue culturing the cells.
 

▲ **Note:** Avoid pipetting the medium directly onto the adipocytes and pipette very carefully, since adipocytes are fragile.
- After 2–3 weeks, large vacuoles start to appear. On day 21, proceed with 5.2.1 Detection of adipocytes.
 

▲ **Note:** Terminally differentiated adipocytes do not proliferate, since they save all energy to accumulate lipids.

### 5.2.1 Detection of adipocytes

Adipocytes produce and accumulate neutral lipids. Therefore, adipocytes are rounded and filled with lipid droplets, which might fuse to form vacuoles that can be stained by Oil Red O, a lipophilic red dye.

#### 5.2.1.1 Preparation of the staining reagent

1. Prepare a 0.5% (w/v) Oil Red O stock solution in isopropanol.  
▲ **Note:** Oil Red O will not completely go into solution.
2. Prepare a fresh working solution by diluting 6 mL Oil Red O stock solution with 4 mL deionized water.
3. Filter through a 0.22 µm filter immediately before staining.

#### 5.2.1.2 Staining of adipocytes

1. Aspirate NH AdipoDiff Medium from the 35 mm cell culture dish.
2. Wash cells with 2×2 mL PBS to remove residual NH AdipoDiff Medium.
3. Add 2 mL methanol and incubate for 5 minutes at room temperature.
4. Aspirate methanol completely and wash 35 mm cell culture dish twice with 2 mL deionized H<sub>2</sub>O.
5. Add 2 mL Oil Red O staining reagent to each 35 mm cell culture dish and slowly mix for 20 minutes on a plate shaker at room temperature.

6. Aspirate Oil Red O staining reagent completely and wash 35 mm cell culture dish twice with 2 mL deionized H<sub>2</sub>O.
7. Keep your cells moist. If necessary, add 300 µL deionized H<sub>2</sub>O.
8. Immediately after staining, examine the stained cells under a microscope.

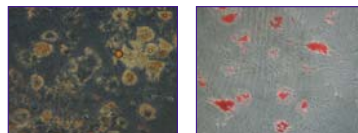


Figure 3: Adipocytes generated from MSCs, after three weeks of cultivation in NH AdipoDiff Medium, stained red for neutral lipids with Oil Red O dye.

### 5.3 Differentiation of MSCs into chondrocytes and their detection

- ▲ **MACS® NH Stem Cell Media are for research use only and not for diagnostic or therapeutic use.**

The NH ChondroDiff Medium promotes the differentiation and further maturation of marrow stromal cells (MSCs) into chondrocytes. Chondrocytes play a vital role in the development and healing of bone as well as other structural tissues in the body and, in conjunction with their complex secreted extracellular matrix (ECM), compose cartilage. A major structural function of cartilage is the mantling of joint surfaces, e.g. the knee, where its unique mechanical load-bearing properties

enables human mobility on a few millimeters of tissue. Due to the small population of cells in functional cartilage, it is difficult to stimulate the tissue to repair itself once damaged. This characteristic has made chondrocytes a focus of tissue engineering research in diseases such as osteochondrodysplasias, non-union fractures, and osteoarthritis. However, the elucidation of the mechanisms of chondrocyte development and regulation (chondrogenesis) remains a crucial focus in such research, and the use of optimized culture media for the efficient and reproducible generation of chondrocytes is requisite.

▲ Before using the NH ChondroDiff Medium for the first time, thaw it completely, mix thoroughly and aliquot in volumes of 20 mL. Store aliquots at -20 °C until use.

▲ Harvest cells as described in 5.1 Harvesting of cultivated cells.

1. Thaw one aliquot (20 mL) of NH ChondroDiff Medium and mix thoroughly.
2. Pre-warm 2.2 mL of the NH ChondroDiff Medium to 37 °C in a waterbath or incubator. Aliquot the remaining medium in 2 mL reaction tubes at 1.1 mL per tube and store at -20 °C.  
▲ **Note:** One aliquot is sufficient for one differentiation experiment. To obtain reliable results, the differentiation of MSCs into chondrocytes should be performed in duplicate.
3. Dilute MSCs to a final concentration of  $2.5 \times 10^5$  cells/mL NH Expansion Medium.
4. Resuspend cells carefully.

5. Transfer 1 mL of the cell suspension to a 15 mL conical tube (polypropylene).  
▲ **Note:** Exclusively use conical tubes composed of polypropylene to prevent cells from attaching to the surface. Do not use polystyrene tubes.
6. Centrifuge for 5 minutes at 150×g at room temperature.
7. Aspirate the NH Expansion Medium completely.
8. Add 1 mL of pre-warmed NH ChondroDiff Medium.
9. Resuspend cells carefully.
10. Repeat step 6. Do not resuspend the cells. Replace cap of tube but do not tighten to permit the circulation of air. Place tubes upright and incubate at 37 °C in an incubator with 5% CO<sub>2</sub> and > 95% humidity.  
▲ **Note:** (Optional) Add 1% Penicillin-Streptomycin to the NH ChondroDiff Medium to prevent bacterial contamination of the cell culture.
11. Change NH ChondroDiff Medium every 3rd day. Carefully remove the medium completely from the pellet and add 1 mL of fresh pre-warmed NH ChondroDiff Medium  
▲ **Note:** Pipetting must be performed very carefully to avoid damaging the nodules. Additionally, ensure that the nodules do not attach to the plastic surface to obtain optimal supply of nutrients for all cells.
13. On day 24, proceed with 3. Detection of chondrocytes.

### 5.3.1 Detection of chondrocytes (in brief)

The differentiation of MSCs into chondrocytes can be characterized by the production of aggrecan. Aggrecan is a large extracellular matrix proteoglycan specifically produced by chondrocytes and is an integral protein in the formation and function of cartilage.<sup>6</sup>

The following protocol for the preparation and staining of chondrocyte nodules for aggrecan is described in brief. For a full protocol, please consult the datasheet entitled "NH ChondroDiff Medium – Differentiation of MSCs into chondrocytes and their detection" which is available from our website [www.miltenyibiotec.com](http://www.miltenyibiotec.com).

#### 5.3.1.1 Preparation of fixation and embedding reagents

1. Dilute formalin with PBS to a final concentration of 3.7% (neutral buffered formalin).
2. Prepare ethanol dilution series (70%, 80% and 90%).
3. Heat Roti-Plast at 60 °C in oven until completely melted.
4. Heat waterbath containing deionized water to 40 °C

#### 5.3.1.2 Preparation of chondrocyte nodules

1. Aspirate all NH ChondroDiff Medium from the micromass culture.
2. Wash cells 1× with PBS.
3. Fix chondrocyte nodules by overnight immersion in neutral buffered formalin. Incubate at room temperature and with agitation.

4. Place nodules in an embedding cassette with filter paper and dehydrate by applying the ethanol in increasing concentrations.
5. Incubate embedding cassette 2× 30 min in Roti-Histol.
6. Incubate embedding cassette 3× 30 min in 58 °C paraffin.
7. Remove chondrocyte nodules from embedding cassette and embed with paraffin in a Bio-mold.
8. Cool overnight at -20 °C.
9. Generate 5 µm thick tissue sections using a microtome and transfer to a 40 °C waterbath.
10. Place the tissue sections on HistoBond slides and incubate at 55 °C for 3 h.
11. Cool to room temperature before proceeding to immunostaining.

#### 5.3.1.3 Preparation of staining reagents

Always work with freshly prepared reagents.

- Washing buffer (1 L): PBS with 1% BSA.
- Blocking buffer (50 mL): Washing buffer with 10% normal donkey serum.
- Permeabilization buffer (10 mL): Blocking buffer with 0.3% Triton X-100.
- Dilute, aliquot and store mouse anti-human aggrecan antibody according to manufacturer's instructions.

- Dilute, aliquot and store rhodamine-conjugated secondary antibody according to manufacturer's instructions.
- Dilute DAPI in distilled water to a final concentration of 5 mg/mL. Store as 200 µL aliquots at -20 °C for no longer than 12 months.

#### 5.3.1.4 Staining of chondrocyte sections

▲ This protocol describes a suggested antibody system for the detection of aggrecan. In principle, aggrecan can be detected with other, user-defined antibody systems, but care must be taken to ensure that the primary antibody is compatible with formalin fixed, paraffin-embedded tissues.

▲ Tissue sections must not be permitted to dry out at any point during the procedure. Should this occur, a reproducible, specific staining may not be obtained.

▲ Reagent volumes can be scaled proportionally to fit the size and number of sections to be stained.

1. Deparaffinize sections using a Roti-Histol and a descending ethanol dilution series.
2. Rinse sections briefly with de-ionized water, then transfer to PBS for 5 min.
3. Incubate sections in permeabilization buffer for 45 min at room temperature.
4. During this time, dilute the primary antibody (mouse anti-human aggrecan) in blocking buffer to a final concentration of 10 µg/mL.

Approx. 150 µL is needed per section.

5. After permeabilization, dab slide dry and encircle section using a hydrophobic pen.
6. Apply primary antibody and incubate overnight at 2–8 °C in a humidified chamber.
  - ▲ Note: To control specific anti-aggrecan reactivity, sections can be incubated in blocking buffer alone without primary antibody.
7. Wash sections 3× 5 min with washing buffer
8. Dilute secondary antibody (donkey anti-mouse IgG-Rhodamine) 1:50 in washing buffer. Approx. 150 µL is needed per section.
9. Apply secondary antibody and incubate in a humidified chamber in the dark for 60 min at room temperature.
10. Dilute DAPI 1:1000 in washing buffer and incubate in a humidified chamber in the dark for 15 min.
11. Wash sections 2× 5 min with washing buffer in the dark.
12. Rinse section 1× with de-ionized water, dab off excess fluid.
13. Apply mounting medium and coverslip. Protect sections from light until analysis by fluorescence microscopy.

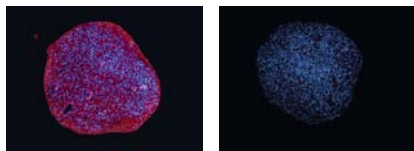


Figure 4: After 24 days of cultivation in NH ChondroDiff Medium, chondrocyte nodules were processed for immunofluorescent detection of aggrecan, a protein marker for differentiated chondrocytes. Left panel: Indirect labeling of aggrecan with a Rhodamine-conjugated antibody (red), nuclei are counterstained by DAPI (blue). Right panel: Aggrecan and DAPI staining of MSCs after cultivation in NH Expansion Medium only (negative control).

#### 5.4 Differentiation of MSCs into osteoblasts and their detection

▲ MACS® NH Stem Cell Media are for research use only and not for diagnostic or therapeutic use.

The NH OsteoDiff Medium promotes the differentiation and further maturation of MSCs into osteoblasts.

Bone is a highly organized structure comprised of calcified connective tissue matrix formed by mature osteoblasts, which develop from the proliferation and differentiation of osteoprogenitor cells. These progenitors are thought to arise from a population of uncommitted marrow stromal cells (MSCs). Counteracting the bone formation, hematopoietically derived osteoclasts resorb the bone matrix. Therefore, most bone diseases result from an imbalance between bone resorption and formation, such

as osteoporosis (local or systemic bone loss), inflammation of bone associated with rheumatoid arthritis or periodontal disease (destruction of tissue anchoring teeth) and developmental disorders such as non-union fractures. Furthermore, there are also genetic disorders of the skeleton, of which osteogenesis imperfecta (brittle bone disease) is an example. To understand the development and regulation of osteoblasts (osteogenesis) in normal and diseased conditions is important for future studies of various bone diseases.

Before using the NH OsteoDiff Medium for the first time, thaw it completely, mix it thoroughly and pre-aliquot the medium to a sample size of 10 mL for differentiation of MSCs into osteoblasts. Store aliquots at  $-20^{\circ}\text{C}$  until use. Throughout the duration of the experiment, store the aliquot at  $4^{\circ}\text{C}$ .

1. Pre-warm NH OsteoDiff Medium to  $37^{\circ}\text{C}$  in a water bath or incubator.
2. Resuspend MSCs to a final concentration of  $3 \times 10^4$  cells/mL in NH OsteoDiff Medium.
  - ▲ Note: (Optional) Add 1% Penicillin-Streptomycin to the NH OsteoDiff Medium to prevent bacterial contamination of the cell culture.
  - ▲ Note: To obtain reliable results, the differentiation of MSCs into osteoblasts should be performed in duplicate.
3. Transfer 1.5 mL of the cell suspension to the 35 mm cell culture dish.
4. Incubate cells at  $37^{\circ}\text{C}$  in an incubator with 5%  $\text{CO}_2$  and  $>95\%$  humidity.

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5. Change NH OsteoDiff Medium every 3rd day by removing NH OsteoDiff Medium completely from the 35 mm cell culture dish. Add 1.5 mL fresh NH OsteoDiff Medium. Continue culturing the cells.
6. On day 10, proceed with 5.4.1 Detection of osteoblasts.

#### 5.4.1 Detection of osteoblasts

Osteoblasts can be identified morphologically by their cuboidal appearance and by their association with newly synthesized bone matrix. Furthermore, committed osteogenic cells are characterized histologically by their expression of high levels of alkaline phosphatase (AP), an enzyme that is involved in the bone matrix mineralization.

#### 5.4.2 Preparation of the staining reagent

Prepare the SIGMA FAST BCIP/NBT substrate as stated in the manufacturer's product information by dissolving 1 tablet SIGMA FAST BCIP/NBT Buffered Substrate Tablet in 10 mL deionized water. Vortex until dissolved.

▲ Note: For best results, solution should be used within one hour of preparation.

#### 5.4.3 Staining of osteoblasts

1. Pre-cool methanol to  $-20^{\circ}\text{C}$ .
2. Aspirate NH OsteoDiff Medium completely from the 35 mm cell culture dish.

3. Wash cells with  $2 \times 2$  mL PBS to remove residual NH OsteoDiff Medium.
4. Add 2 mL pre-cooled methanol and incubate for 5 minutes at  $-20^{\circ}\text{C}$ .
5. Remove methanol completely.
6. Wash cells with 2 mL deionized  $\text{H}_2\text{O}$ .
7. Remove deionized  $\text{H}_2\text{O}$  completely.
8. Add 2 mL SIGMA FAST BCIP/NBT substrate to the 35 mm cell culture dishes and agitate slowly on a plate shaker at room temperature for 10 minutes.
  - ▲ Note: As alkaline phosphatase expressed by osteoblasts processes the substrate, cells stain dark purple.
9. Aspirate the substrate solution.
10. Wash the 35 mm cell culture dishes with 2 mL deionized  $\text{H}_2\text{O}$ .
11. Aspirate the deionized  $\text{H}_2\text{O}$ .
12. Keep your cells moistened. If necessary, add 300  $\mu\text{L}$  deionized  $\text{H}_2\text{O}$ .
13. Immediately after staining, examine the stained cells under a microscope.

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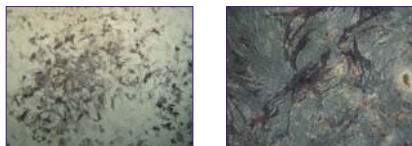


Figure 5: Osteoblasts, generated from MSCs after 10 days of cultivation in NH OsteoDiff Medium, were stained for alkaline phosphatase activity with NBT substrate and appear purple. Increasing spreading of the cells is a sign of the differentiation process. Fully differentiated, the cells show a typical ruptured morphology.

## 6. Appendix

### 6.1 Redistribution of unevenly populated tissue cultures

Cells can be evenly redistributed in culture by trypsinization before further cultivation, as described in steps 1–6 in 4.2 Passaging of human MSCs. However, only 500  $\mu$ L of Trypsin/EDTA (0.05%/0.53 mM) per flask should be used for cell population redistribution.

### 6.2 Freezing of human MSCs

Reagent and cell culture requirements:

- DMSO
- Cryogenic storage vials, e.g. CryoTube™ (1.8 mL) (Nunc # 363401),
- Box, suitable for storage in liquid nitrogen

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- 50 mL conical tubes
- P/E buffer (PBS with 2 mM EDTA), pH 7.2
- Freezing medium: supplement NH Expansion Medium with:
  - 10% FCS
  - 10% DMSO
  - 1% penicillin-streptomycin

1. Pre-cool freezing medium on ice.
2. Perform steps as described in 5.1 Harvesting of cultivated cells.
3. Resuspend cells carefully in freezing medium at a concentration of  $5 \times 10^5$  cells/mL.
4. Immediately aliquot 1.8 mL of cells into appropriate cryogenic storage vials and close the lid tightly.
5. Place the vials in the pre-cooled box and directly store at  $-70$  °C.
6. After 24 hours, transfer the frozen cryogenic storage vials to an ultra-low temperature freezer ( $-150$  °C) or to liquid nitrogen for long-term storage.

### 6.3 Thawing of human MSCs

Perform all following steps under sterile conditions in a laminar flow hood.

1. Fill 50 mL conical tubes with water. Pre-warm to 37 °C.
2. Place frozen vials into 50 mL conical tubes containing water at 37 °C. Do not close the lid of the conical tube.



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3. After thawing, transfer cells to a steril 50 mL conical tube.
4. Wash cells by adding 20 mL P/E buffer, mix gently and centrifuge for 10 minutes at  $300 \times g$  at room temperature.
5. Aspirate supernatant completely.
6. Resuspend cells carefully in the appropriate medium (e.g. NH Expansion Medium or the preferred NH differentiation medium). Information on the adequate cell numbers needed and cultivation procedure is described in chapters 3 to 5.

## 7. Related products

MSC Research Tool Box – CD271 (APC), human	130-092-291
MSC Research Tool Box – CD271 (PE), human	130-092-867
CD271MicroBead Kit (APC), human	130-092-283
CD271MicroBead Kit (PE), human	130-092-819
CD133 MicroBead Kit, human	130-050-801
CD117 MicroBead Kit, human	130-091-332
CD105 MicroBeads, human	130-051-201
Anti-Fibroblast MicroBeads, human	130-050-601
Lineage Cell Depletion Kit, human	130-092-211

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