Miltenyi Biotec

NH ChondroDiff Medium

Differentiation of MSCs into chondrocytes and their detection

Order no. 130-091-679

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1. Reagents and instrument requirements

1.1 Lab equipment for chondrocyte generation and detection

- 15 mL polypropylene conical tube (for micromass culture)
- 2 mL reaction tubes
- Waterbath
- Oven
- Microtome
- Shaker (e.g. MACSMix™ Tube Rotator # 130-090-793)
- 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

1.2 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 # 0100-01)
- 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)

2. Differentiation of mesenchymal stromal cells (MSCs) into chondrocytes

The NH ChondroDiff Medium promotes the differentiation and further maturation of mesenchymal 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 for viable solutions to the regeneration of cartilage. The *in vitro* expansion and differentiation of MSCs to chondrocytes for tissue repair might be an important first step for the future treatment of joint diseases[1], including osteochondrodysplasias, non-union fractures, and osteoarthritis. However, the elucidation of the mechanisms of chondrocyte development and regulation (chondrogenesis) remains a crucial focus in the realization of such therapies, 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. Appendix: 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 2.5×10^5 cells/mL NH Expansion Medium.
4. Resuspend cells carefully.

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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_2 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 section 3. Detection of chondrocytes.

3. Detection of chondrocytes

Differentiated chondrocytes in micromass culture form three-dimensional clusters termed chondrocyte “nodules”. 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[2]. The following protocol describes the preparation and immunostaining of chondrocyte nodules for the detection of aggrecan.
Firstly, nodules are formalin fixed and paraffin-embedded, followed by tissue sectioning, de-paraffination, immunolabeling of aggrecan and then probing with a rhodamine-conjugated secondary antibody. Fluorescent microscopy reveals DAPI stained nuclei in blue as well as aggrecan labeling in bright red. Micromass culture of MSCs using only MSC Expansion Medium can also be performed in parallel, after which aggrecan is not detected (negative control), highlighting the specific induction of MSC differentiaton into chondrocytes by NH ChondroDiff Medium (see figure on page 3).

3.1 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 by diluting ethanol with deionized water to final concentrations of 70%, 80%, and 96% ethanol.
3. Heat Roti-Plast at 58 °C in oven until completely melted.
4. Heat waterbath containing deionized water to 40 °C.

3.2 Preparation of chondrocyte nodules

1. Aspirate all NH ChondroDiff Medium from the micromass culture.
2. Wash chondrocyte nodules with 1 mL PBS.
3. Fix chondrocyte nodules by immersion in neutral buffered formalin for 6 to 12 hours (overnight). Incubate at room temperature and with agitation.

   ▲ **Note:** Nodules should be freely suspended in formalin in order to ensure a uniform fixation.

4. Place nodules in an embedding cassette with filter paper and dehydrate by applying the following ethanol dilution series:
   2×30 min 70% ethanol
   2×30 min 80% ethanol
   2×30 min 96% ethanol
   2×30 min 100% ethanol

   ▲ **Note:** Nodules can be incubated overnight at the second 80% ethanol step, should time limitations inhibit completion of the dilution series.

5. Incubate embedding cassette 2×30 min in Roti-Histol.
6. Incubate embedding cassette 3×30 min in 58 °C Roti-Plast.

   ▲ **Note:** Nodules can be incubated overnight during the second paraffin incubation, should time limitations prevent the completion of all three in one go.

7. Remove chondrocyte nodules from embedding cassette and embed with pre-heated Roti-Plast 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 52 °C for 3 h.
11. Cool to room temperature before proceeding directly to immunostaining or storage at room temperature.

3.3 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 and aliquot mouse anti-human aggrecan antibody according to manufacturer’s instructions and store.
- Dilute and aliquot rhodamine-conjugated secondary antibody according to manufacturer’s instructions and store.
- Dilute DAPI in destilled water to a final concentration of 5 mg/mL. Store as 200 µL aliquots at –20 °C for no longer than 12 months.

3.4 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.

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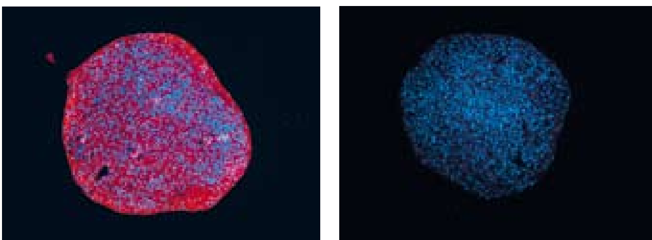
1. Deparaffinize sections using Roti-Histol and a descending ethanol dilution series:
   2×5 min Roti-Histol
   2×5 min 100% ethanol
   2×5 min 96% ethanol
   2×5 min 80% ethanol
   2×5 min 70% ethanol
   2×brief rinse in deionized water
   2×5 min in PBS
2. Incubate sections in permeabilization buffer for 45 min at room temperature.
3. During this time, dilute the primary antibody (mouse anti-human aggrecan) in blocking buffer to a final concentration of 10 µg/mL. Approximately 150 µL is needed per section.
4. After permeabilization, dab slide dry carefully and encircle section using a hydrophobic pen.
5. 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.
6. Wash sections 3×5 min with washing buffer.
7. Dilute secondary antibody (donkey anti-mouse IgG-rhodamine) 1:50 in washing buffer. Approx. 150 µL is needed per section.
8. Apply secondary antibody and incubate in the dark for 60 min at room temperature.
9. Remove antibody by tilting slide and gentle tapping onto a paper towel.
10. Dilute DAPI 1:1000 in washing buffer and apply approximately 150 µL per section. Incubate in the dark for 15 min.
11. Wash sections 2×5 min with washing buffer in the dark.
12. Rinse section 1× with deionized water, dab off excess fluid.
13. Apply mounting medium and coverslip. Protect sections from light until analysis by fluorescence microscopy.

4. Example of stained chondrocyte sections

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).



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5. Appendix: Harvesting of cultivated cells

1. Pre-warm Trypsin/EDTA (0.05%/0.53 mM), PBS and NH Expansion Medium to 37 °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 °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, 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 and collect all cells in the 15 mL conical tube.
8. Centrifuge cells at 300×g for 10 minutes at room temperature.
9. Remove supernatant and carefully resuspend cells in 0.5 mL NH Expansion Medium.
10. Determine cell number and viability using a hemocytometer and Trypan Blue exclusion.

6. References

1. Spagnoli, A. *et al.* (2005) Cartilage disorders: potential therapeutic use of mesenchymal stem cells. Endocr Dev.; 9: 17–30.
2. Kiani, C. *et al.* (2002) Structure and function of aggrecan. Cell Res. 12(1): 19–32.

All protocols and data sheets are available at **www.miltenyibiotec.com**.

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