Product Information

# ChondroMAX Differentiation Medium

**SCM123**

## Introduction

Mesenchymal stem cells (MSCs) are adult stem cells, which have the capacity for multi-lineage differentiation, giving rise to a variety of mesenchymal phenotypes such as osteoblasts (bone), adipocytes (fat), and chondrocytes (cartilage). Articular hyaline cartilage has poor regenerative capacity, and the loss of its function is, in the long term, often painful and debilitating. Therefore, attempts have been made to study chondrogenesis or to replace damaged cartilage tissue with MSC derived stem cells.

ChondroMAX Differentiation Medium is a ready-to-use xeno-free mesenchymal stem cell chondrogenesis differentiation media. ChrondroMAX produces cells with elevated levels of sulfated proteoglycans, analyzed by Alcian-Blue staining, after 3 weeks of MSC pellet differentiation compared to other chrondrogenesis differentiation media. ChondroMAX is an excellent research tool to study chondrocyte differentiation, chondrogenesis and wound healing.

## Safety and Use Statement

This product is for research use only. This product is not approved for human or veterinary use or for use in *in vitro* diagnostics or clinical procedures.

We recommend storing cryopreserved vials in liquid nitrogen vapor phase. Handle cryopreserved vials with caution. Always wear eye protection and gloves when working with cell cultures. Aseptically vent any nitrogen from cryopreserved vials by carefully loosening the vial cap in a biosafety cabinet prior to thawing the vials in a water bath. If vials must be stored in liquid phase, the vials should be transferred to vapor phase storage or −80 °C for up to 24-hours prior to being thawed.

ChondroMAX Differentiation Medium is optimized for the differentiation of human mesenchymal stem cells into chondrocytes.

## Medium Storage

ChondroMAX Differentiation Medium should be stored at −20 °C until ready to use. Once the medium is thawed, it should be stored at 2-8 °C for up to three weeks. Do not use products beyond expiration date. Multiple freeze/thaw cycles are not recommended. Users should take care to protect medium from extended exposure to light.

## Materials Provided

| Description | Volume | Storage | Catalog Number |
|---|---|---|---|
| ChondroMAX Differentiation Medium | 100 mL | Store 20 °C or 2-8 °C once thawed. | SCM123 |

## Basic Aseptic Technique

- The medium and cells should only be used in an aseptic environment, a Class II biological safety cabinet with front access and filtered laminar airflow, or an equivalent device.
- Always wear gloves and eye protection when working with these materials.
- Wipe or spray all bottles and vials with 70% ethanol or isopropanol, especially around the area of the cap, before placing them in the biological safety cabinet. Allow these surfaces to dry completely before opening the bottle or vials.
- Transfer cells or medium with disposable sterile pipettes. Do not mouth pipette! Take up the volume needed into the pipette, being careful not to touch the sterile tip to the rim of the container or any other surface.
- Close the container and open the container into which the transfer is being made, again being careful not to touch any surfaces with the sterile tip. Transfer the material and close the container.
- Wash your hands before and after working with cell cultures.
- Do not block airflow in a laminar flow hood as this may compromise sterility. Ensure that biological safety cabinets are certified routinely and that the HEPA filters are replaced regularly.

## Protocol

### Pre-warming Medium

If using less than 100 mL of medium, we recommend warming only the volume needed in a sterile conical tube. Repeated warming of the entire bottle over extended periods will cause degradation and reduced shelf life of the medium. When warming the entire bottle of medium, we recommend using a water bath sleeve (included with medium) to help protect the medium from contaminants in the 37 °C water bath. Medium will warm to 37 °C in 10 to 30 minutes, depending on the volume. Do not leave medium in water bath for extended periods.

### Thawing and Plating Cryopreserved Cells

Pre-warm fully supplemented ChondroMAX Differentiation Medium, respective to cell type.

1. Remove vial from dewar and check the cap to be sure that the vial is securely sealed.
2. Spray the vial with 70% ethanol or isopropanol and transfer it to a biosafety cabinet. Allow it to dry thoroughly and carefully loosen the cap to vent any liquid nitrogen that may have entered the vial.
3. Recap the vial and hold only the bottom half of the vial in a 37 °C water bath for approximately one minute or until vial is almost completely thawed. A small amount of ice should still be visible.
4. To avoid potential contamination, do not allow the vial cap to make contact with the water. Do not over thaw as this may damage the cells.
5. Dry the vial, spray the exterior of the vial with 70% ethanol or isopropanol and place the vial in a biological safety cabinet and allow it to dry.
6. Carefully remove the cap to avoid contamination or splatter.
7. Gently resuspend the cells in the vial using a 1- or 2-mL sterile pipette.

   **Note:** Do not centrifuge; the cells may be directly plated from the vial.
8. Plate cells into pre-warmed fully supplemented medium (respective to cell type) in the desired culture vessel at a density of 5,000 cells per $cm^2$.

   **Note:** Flasks with vented caps (for example, Corning® flask, canted neck, 150 cm, vented, CLS431465), commonly referred to as filter caps, are strongly recommended.
9. Gently rock the culture vessel from side to side and front to back to evenly distribute cells within the vessel.
10. Place seeded culture vessel in a 37 °C, 5% $CO_2$ incubator. Re-feed the cells after they have attached (approximately 4 to 36 hours after inoculation) to remove cryopreservation reagents.

## Recommended Feeding Guidelines for Undifferentiated Expansion of HMSC

**Guidelines for a T-75 Flask. Adjust volumes according to culture surface area.**

- Every other day, remove medium and feed with 15 mL of fresh supplemented medium.
- Most cultures which are 50% confluent will be ready for passage within 2 to 4 days and should be fed with 15 to 20 mL of supplemented medium.
- Do not use more than 10 mL of medium per 25 $cm^2$ of culture surface to ensure the depth of the medium is at a level where gas diffusion will be sufficient to support the cells' requirements for oxygen.

  **Note:** Gas diffusion gradients through the culture medium to the cells are affected by the depth of the medium. The volumes of medium recommended above result in a range of depths between 2 mm and 5 mm, which is compatible with general recommendations, 30 mL being at the maximum depth allowable (5 mm) for a T-75 flask.

## Passaging Cells

HMSC may be passaged when the culture is 70 to 80% confluent and actively proliferating. We recommend passaging HMSC before reaching confluence since post-confluent cells exhibit morphological changes, slower growth and differentiation.

1. Aspirate the medium from the culture vessel.
2. Rinse the flasks with Phosphate Buffered Saline (PBS) by adding at least 1 mL of PBS per 5 $cm^2$ and gently tilting the flask to cover the surface with PBS.
3. Aspirate the PBS from the culture vessel, repeat the rinse if desired.
4. Add at least 2 mL of 0.05% Trypsin/0.02% EDTA per 25 $cm^2$ to the vessel. Swirl gently to ensure all cells are coated with the Trypsin/EDTA.
5. Observe the cells carefully under the microscope. When the cells round up, they are ready to be released. This normally takes 2 to 3 minutes depending on the confluence of the cells.

   **Note:** Do not over trypsinize as this may damage the cells.
6. Detach the cells by gently striking the culture vessel against your hand several times. Observe the cells under the microscope to be sure they have become detached.
7. Once the cells are fully detached, add Trypsin Neutralizing Solution (TNS) using a volume equal to the amount of Trypsin/EDTA that was originally used. Gently swirl to ensure all the trypsin solution is neutralized.
8. Using aseptic laboratory techniques, pipette the cells into a sterile centrifuge tube. Collect the remaining cells by rinsing the culture vessel with at least 1 mL of PBS per 5 $cm^2$ and pipetting the cells into the sterile centrifuge tube. Check culture vessel under the microscope for cells still attached and repeat steps if necessary to retrieve all the cells from the vessel.

   **Note:** All steps must be completed under aseptic conditions in a biological safety cabinet.
9. Centrifuge the cells at 250 x *g* or 3 to 5 minutes.

   **Note:** For best results, calculate speed for individual centrifuge type. Time may also be centrifuge dependent. Do not over centrifuge cells as this will cause cell damage. After centrifugation, the cells should form a clean loose pellet.

   To calculate RCF ('x *g*'):

   $$RCF = 00001118 \times (rpm)^2 \times r$$

   *r = rotational radius in centimeters*

   *rpm = rotations or revolutions per minute*
10. Aspirate the neutralized trypsin solution from the centrifuge tube and resuspend the cell pellet in pre-warmed culture medium (respective to cell type) by gently pipetting up and down with a 2- or 5-mL pipette.
11. Count cells using a hemacytometer. If expansion of cells is desired, re-plate at 5,000 cells per $cm^2$ (or use a split ratio of 1:6-1:10) in vessel containing the respective pre-warmed culture medium. If differentiation of cells is desired, see Differentiation section (page 3).

## Standard Calculation for Plating of Cells

1. Gently re-suspend the cells evenly in the respective pre-warmed complete ChondroMAX Differentiation Medium.
2. Using a clean hemacytometer and aseptic technique, remove 25 µL of the cell suspension to a separate tube, such as a microcentrifuge tube.
3. Add 75 µL of 0.4% Trypan Blue solution to the cell suspension in the microcentrifuge tube and allow it to sit for up to 1 to 5 minutes.
4. Place 10 µL of the cell suspension into each chamber of the hemacytometer. Count a minimum of 4 quadrants on the hemacytometer Dead and dying cells are permeable to Trypan Blue, viable cells will not be blue.
5. For accurate cell counts, optimal number of cells per quadrant should be 25 to 75 cells. After counting the cells, calculate the average of the number of quadrants counted. Take the cell count average and multiply by the dilution factor and by $10^4$ to get the number of cells per milliliter (mL).
6. Multiply the desired inoculation density (5,000 viable cells per $cm^2$) by the surface area of the well(s) to be inoculated. This will give the total number of cells to inoculate one well.
7. Divide the number of cells needed to inoculate the well(s) by the total number of cells in the cell suspension. This will give the volume of cell suspension with which to inoculate the well(s).
8. Inoculate the cells into the well(s) of the culture vessel(s) prepared with pre-warmed culture medium. Mix gently to evenly distribute the cells and place culture vessel(s) into the incubator at 37 °C, 5% $CO_2$.

### Calculation

**Sample calculation**

Average viable cells per quadrant = 31

31 cells/quadrant x 10,000 quadrants/mL x 4 (dilution factor) = 1,240,000 cells/mL

**Inoculating a T-75 flask at 5,000 cells/$cm^2$**

5,000 cells/$cm^2$ x 75 $cm^2$/well = 375,000 cells/flask

**Calculate volume of cell suspension required to inoculate each flask**

$$\frac{375{,}000 \text{ cells/flask}}{1{,}240{,}000 \text{ cells/mL}} = 0.302 \text{ mL/well}$$

## Alginate Encapsulation and Initiating Chondrogenesis

Chondrogenesis requires a three-dimensional aggregate cell culture. Micromass culture can be used but for the best results, we recommend the use of a scaffolding or matrix, such as alginate, to provide a structure for deposition of proteoglycans.

The following items are recommended to prepare alginate encapsulated cells for chondrogenesis but are not supplied in this kit. All steps must be performed using aseptic technique in a biosafety cabinet.

- Sodium alginate
- Syringe (for example, 3 mL)
- Fine gauge needle (for example, 27-gauge)
- Wide-bore pipette tip
- Vacuum-driven 50 mL filtration system (for example, Steriflip® Vacuum Tube Top Filter)
- 150 mM sodium chloride solution (sterile)
- 100 mM calcium chloride solution (sterile)
- Small sterile magnetic stir bar (for example, 25 mm)
- Sterile 250 mL beaker
- Magnetic stir plate
- Sterile forceps or stir bar extractor

### Preparation of 1.5% (w/v) Alginate Solution in 150 mM Sodium Chloride (NaCl) Solution

1. Add 0.15 g of alginate to 10 mL of 150 mM NaCl solution while stirring rapidly or vortexing to minimize clumping.
2. Agitate the solution on a rocker at room temperature for at least two hours to overnight to completely solubilize the alginate.
3. Filter sterilize (0.22 µm) the solution and store at 4 °C for up to one week.

## Preparation of Alginate Encapsulated Cells (Chondrogenic Microbeads)

We recommend using a minimum of 2.5 x $10^7$ HMSC (approximately 9 to 15 T-75 flasks) to create 1 mL of alginate encapsulated cells (chondrogenic microbeads). The 1.5% (w/v) alginate solution must not be diluted lower than 1.2% (w/v) by the addition of the cells. Therefore, we recommend that the cells are harvested and counted. as described in Standard Calculation for Plating of Cells (page 4).

1. The cell pellet (containing a minimum of 2.5 x $10^7$ cells) is resuspended in 800 µL of the 1.5% (w/v) alginate solution. (The quantities may be adjusted as long as the aforementioned ratios are maintained.)
2. Gently mix the alginate-cell suspension, taking care not to introduce air bubbles into the solution.
3. Transfer 75 mL of sterile 100 mM calcium chloride ($CaCl_2$) solution and a sterile stir bar into a sterile 250 mL beaker. Create a gentle funnel in the $CaCl_2$ solution on a stir plate.
4. Transfer the alginate-cell suspension to a sterile syringe that has a fine gauge (for example, 27 gauge) needle attached.
5. Rapidly dispense the alginate-cell solution (in a single fast stream) into the $CaCl_2$ solution to form the chondrogenic microbeads.
6. Allow the $CaCl_2$ solution containing the newly formed chondrogenic microbeads to stir for an additional 10 minutes to solidify the alginate.
7. Remove the beaker from the stir plate and allow the chondrogenic microbeads to settle.
8. Transfer the chondrogenic microbeads solution into a conical tube and attach to a vacuum-driven 50 mL filter device (for example, Steriflip® Vacuum Tube Top Filter). Immediately break the vacuum as soon as the liquid is removed to prevent damage to the beads.
9. Resuspend chondrogenic microbeads in 2 mL of ChondroMAX Differentiation Medium. Aseptically transfer enough chondrogenic microbeads to cover the bottom surface of a single well of a 48-well plate.

   **Note:** The method described above yields enough chondrogenic microbeads to seed approximately 4 wells of a 48-well plate.
10. After the chondrogenic microbeads settle to the bottom of the well, remove and replace the fluid in each well twice with 0.5 mL ChondroMAX Differentiation Medium to remove residual $CaCl_2$. Incubate the cells in a 37 °C, 5% CO2 incubator. Culturing of the chondrogenic microbeads for chondrogenesis
11. Every 2 to 3 days carefully remove the spent medium from each well, so as to not disturb or aspirate the chondrogenic microbeads. Add 0.5 mL of pre-warmed ChondroMAX Differentiation Medium to each well containing chondrogenic microbeads. Return the plate to a 37 °C, 5% CO2 incubator.
12. After 21 days of differentiation, chondrogenesis is complete and the chondrogenic microbeads can be fixed and stained for proteoglycan deposition or other analysis. We recommend the use of the Alcian Blue Staining Kit (TMS-010-C) to assay for sulfated proteoglycans.

## Differentiation of Chondrocytes from HMSC Using Alginate Encapsulation

### Pre-Differentiation

- Always wash hands before and after working with cell cultures.
- Always wear eye protection and gloves when working with cell cultures.
- When working with cells or medium, always use a certified biological safety cabinet.
- Store cryopreserved cells in liquid nitrogen, vapor phase.
- Handle cryopreserved vials with caution. Aseptically vent any nitrogen from cryopreserved vials in a biosafety cabinet prior to thawing in a water bath.
- Feed cells using pre-warmed culture medium according to feeding guidelines.
- Expand HMSC in the respective ChondroMAX Differentiation Medium as detailed in Passaging Cells (page 3).

### Differentiation

**Day 0**

1. When cells are 70 to 80% confluent and actively proliferating, passage cells using subculture reagents (as detailed in Passaging Cells, page 3).
2. Resuspend pellet of 2.5 x $10^7$ cells in 1.5% alginate solution.
3. Push cell-alginate solution into actively stirring calcium chloride solution to form chondrogenic microbeads.
4. Remove calcium chloride solution and transfer chondrogenic microbeads to 48 well plate.
5. Replace medium on the chondrogenic microbeads twice with 0.5 mL ChondroMAX Differentiation Medium.
6. Incubate multi-well plate(s) at 37 °C, 5% $CO_2$.

**Day 2**

1. Replace medium on the chondrogenic microbeads with 0.5 mL of ChondroMAX Differentiation Medium. Do not aspirate the chondrogenic microbeads from well(s).
2. Incubate multi-well plate(s) at 37 °C, 5% $CO_2$.

**Day 4 or 5 Through Day 21**

1. Replace medium on the chondrogenic microbeads every 2 to 3 days with 0.5 mL of ChondroMAX Differentiation Medium. Do not aspirate the chondrogenic microbeads from the well(s).
2. Incubate multi-well plate(s) at 37 °C, 5% $CO_2$.

**Day 21**

Differentiation is complete.

- Fix the cells then slice and stain for proteoglycan deposition with Alcian Blue Stain.

## Notice

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