



OsteoMAX-XFTM **Differentiation Medium**

Catalog No. SCM121

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

Introduction

Bone undergoes a continual remodeling process that requires the coordinated activity of two cell types. Osteoclasts break down the bone matrix while osteoblasts deposit collagen, calcium, and phosphorous and other minerals to form new bone. The balance between the activity of osteoclasts and osteoblasts determines the mass and density of the bone. Many diseases of bone including osteoporosis, a common age-related phenomenon in post-menopausal women in which the bone mass has been greatly reduced, and osteogenesis imperfecta, also known as brittle-bone disease, are likely caused by the misregulation of osteoblasts and osteoclasts. Understanding the molecular mechanisms that underlie osteogenesis, the process by which new bone is formed is thus of critical importance.

Mesenchymal stem cells (MSC) are defined as a self-renewing population of adherent, multipotent progenitor cells with the capacity to differentiate into several mesenchymal cell lineages. MSC have been isolated from adult bone marrow, adipose tissue, and umbilical cord blood and can be generated in vitro from embryonic and induced pluripotent stem cells. In defined in vitro assays, MSC have been shown to readily differentiate into lineage-specific cells that form bone, cartilage, fat, tendon, and muscle tissues.

EMD Millipore's OsteoMAX-XF™ Differentiation Medium is formulated to readily differentiate human mesenchymal stem cells into the osteogenic lineage as assessed by alkaline phosphatase detection and by alizarin red staining for mineralization. This proprietary formulation was identified using Plasticell's CombiCult™, a patented, bead-based, combinatorial technology specially developed for discovery of novel stem cell differentiation protocols. The unique characteristics of OsteoMAX-XF™ Differentiation Medium include:

- Serum-free (SF), xeno-free (XF), defined medium formulation
- Rapid, robust, and highly efficient mineralization and bone nodule formation in standard tissue culture system by as early as 7 days of differentiation.

Using EMD Millipore's OsteoMAX-XF™ Differentiation Medium, we typically obtain >80% mature osteocytes from human bone marrow derived mesenchymal stem cells. Efficiency of osteogenic differentiation may vary, depending upon the source and quality of the mesenchymal stem cells and if variations to the protocol are introduced.

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Kit Components

1. OsteoMAX-XF™ Basal Medium (Part No. CS210596). One bottle containing 90 mL of basal medium. Store at -20°C.
2. OsteoMAX-XF™ Supplement (10X) (Part No. CS210597). One bottle containing 10 mL of 10X concentrated supplement. Store at -20°C.

Related Products

The following related products are available from EMD Millipore as separate items.

1. Human Mesenchymal Stem Cell Kit (Derived from Bone-Marrow) (Cat. No. SCR108)
2. Cryopreserved Human Mesenchymal Stem Cells (Derived from Bone Marrow) (Cat. No. SCC034)
3. Human Mesenchymal Stem Cells (Derived from MEL-1 Human ESC) (Cat. No. SCC036)
4. Human Mesenchymal Stem Cell Characterization Kit (Cat. No. SCR067)
5. Mesenchymal Stem Cell Adipogenesis Kit (Cat. No. SCR020)
6. Mesenchymal Stem Cell Osteogenesis Kit (Cat. No. SCR028)
7. FibroGRO™ Xeno-Free Human Fibroblast Expansion Medium (Cat. No. SCM037)
8. Human Plasma Fibronectin Purified Protein (Cat. No. FC010)
9. EmbryoMax ES Cell Qualified 0.1% Gelatin Solution, 500 mL (Cat. No. ES-006-B)
10. Alkaline Phosphate Detection Kit (Cat. No. SCR004)
11. Quantitative Alkaline Phosphatase ES Characterization Kit (Cat. No. SCR066)
12. Osteogenesis Quantitation Kit (Cat. No. ECM815)

Materials Required but Not Supplied

1. Human mesenchymal stem cells
2. Human mesenchymal stem cell expansion medium
 - For XF application: use FibroGRO™ Xeno-Free Human Fibroblast Expansion Medium (Cat. No. SCM037)
 - For non-XF application: use Mesenchymal Stem Cell Expansion Medium (Cat. No. SCM015) supplemented with 8 ng/mL basic FGF-2 (Cat. No. GF003).
3. Tissue culture-ware and supplies
4. Extracellular matrix proteins for coating tissue culture-ware:
 - For XF application: use Human Plasma Fibronectin Purified Protein (Cat. No. FC010).

- For non-XF application: use EmbryoMax ES Cell Qualified 0.1% Gelatin Solution, 500 mL (Cat. No. ES-006-B).
5. Phosphate-Buffered Saline (1X PBS) (Cat. No. BSS-1005-B)
 6. Accutase™ Solution (Cat. No. SCR005)
 7. Fixative (e.g. 4% Paraformaldehyde in 1X PBS)
 8. Hemacytometer
 9. Microscope

Storage

OsteoMAX-XF™ Basal Medium: (Part No. CS210596) should be stored at -20°C until ready to use. Use within four months from date of receipt.

OsteoMAX-XF™ Supplement (10X): (Part No. CS210597) should be stored at -20°C until ready to use. Use within four months from date of receipt. The 10X supplement may be aliquoted into 1 mL or other convenient working volumes and stored at -20°C. Avoid further freeze-thaw cycles as activity may be adversely affected.

Preparation of Media

Human Mesenchymal Stem Cell Expansion Medium (not provided in kit):

- For XF applications: human MSC can be expanded in FibroGRO™ Xeno-Free Human Fibroblast Expansion Medium (Cat. No. SCM037).
- For non-XF applications: human MSC can be expanded in serum-containing medium (Cat. No. SCM015) supplemented with 8 ng/mL basic FGF (Cat. No. GF003).

Complete OsteoMAX-XF™ Differentiation Medium:

- Thaw OsteoMAX-XF™ Basal Medium (Part No. CS210596) and OsteoMAX-XF™ Supplement (10X) (Part No. CS210597) at 2-8°C overnight or in a tepid water bath, protected from light..
- Add 10 mL of the OsteoMAX-XF™ Supplement (10X) to 90 mL OsteoMAX-XF™ Basal Medium and mix thoroughly. Penicillin-streptomycin solution may also be added (optional). Filter sterilize the Complete supplemented medium using a 0.22 or 0.45 µm filter (optional).

Alternatively, add 1 volume of 10X Supplement to 9 volumes of Basal Medium, mix thoroughly and filter sterilize using a 0.22 µm Steriflip filter unit (SCGP00525 or SE1M179M6; optional). The Complete Medium may be stored short term at 2 to 8°C in the dark and should be used within 7 days.

Note: This kit contains components that contain human blood, human blood products or other potentially infectious materials. Use universal precautions when handling.

Preparation of Coated Plates

Tissue culture plastic plates should be coated with:

- For XF applications: use 20 µg/mL Human Fibronectin (Cat. No. FC010)
- For non-XF applications: use 0.1% gelatin (Cat. No. ES-006-B)

Human Fibronectin Coating for Xeno-Free Applications:

1. Human Plasma Fibronectin Purified Protein (Cat. No. FC010), 1 mg/mL solution should be diluted to 20 µg/mL in phosphate-buffered saline (1X PBS). For example, add 49 mL 1X PBS to 1 mL of 1 mg/mL solution of Human Fibronectin to make a 20 µg/mL stock solution.
2. Add sufficient 20 µg/mL human fibronectin solution to cover the entire surface of the cultureware plate. Use 10 mL volume for 10-cm plates and T75 flasks and 0.25 mL per well for 48-well plates. Incubate for at least 2 hours at room temperature.
3. Just before use, aspirate the human fibronectin solution from the coated plate or flask. Wash once with 1X PBS buffer.

Gelatin Coating for Non-Xeno Free Applications:

1. Add sufficient 0.1% gelatin solution (Cat. No. ES-006-B) to cover the entire surface of the cultureware plate. Use 10 mL volume for 10-cm plates and T75 flasks and 0.25 mL per well for 48-well plates. Incubate for at least 30 minutes at room temperature.
2. Just before use, aspirate the gelatin solution from the coated plate or flask.

Thawing of Cells

1. Do not thaw the cells until the recommended medium and appropriately coated plasticware are on hand.
2. Remove the vial of Human Mesenchymal Stem Cells (for Bone-Marrow derived, use SCC034; for human ESC-derived, use SCC036) from liquid nitrogen and incubate in a 37°C water bath. Closely monitor until the cells are completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells. **IMPORTANT:** Do not vortex the cells.
3. As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol. Proceed immediately to the next step.
4. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful not to introduce any bubbles during the transfer process.
5. Using a 10 mL pipette, slowly add dropwise 9 mL of Human Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37°C) to the 15 mL conical tube. **IMPORTANT:** Do not add the whole volume of media at once to the cells. This may result in decreased cell viability due to osmotic shock.

- For XF applications: use SCM037
 - For non-XF applications: use SCM015 supplemented with 8 ng/mL basic FGF.
6. Gently mix the cell suspension by slow pipeting up and down twice. Be careful to not introduce any bubbles. **IMPORTANT:** Do not vortex the cells.
 7. Centrifuge the tube at 300 x g for 2-3 minutes to pellet the cells.
 8. Decant as much of the supernatant as possible. Steps 5-8 are necessary to remove residual cryopreservative (DMSO).
 9. Resuspend the cells in a total volume of 10 mL Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37°C).
 - For XF Applications: use SCM037.
 - For non-XF applications: use SCM015 supplemented with 8 ng/mL FGF-2. **Note:** FGF-2 should always be added fresh.
 10. Plate the cell mixture onto an appropriately coated 10-cm tissue culture plate or a T75 tissue culture flask.
 11. Incubate the cells at 37°C in a 5% CO₂ humidified incubator.
 12. The next day, exchange the medium with fresh Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37°C). Exchange with fresh medium every two to 3 days thereafter.
 - For XF Applications: use SCM037.
 - For non-XF applications: use SCM015 supplemented with 8 ng/mL FGF-2. **Note:** FGF-2 should always be added fresh.
 13. When the cells are approximately 80% confluent, they can be dissociated with Accutase (Cat. No. SCR005). Cells may be passaged or alternatively frozen for later use.
 - For XF Applications: use SCM037.
 - For non-XF applications: use SCM015 supplemented with 8 ng/mL FGF-2. **Note:** FGF-2 should always be added fresh.

Subculturing

1. Carefully remove the medium from the 10-cm tissue culture plate or T75 flask containing the confluent layer of Human Mesenchymal Stem Cells.
2. Apply 3-5 mL of Accutase (Cat. No. SCR005) and incubate in a 37°C incubator for 3-5 minutes.

3. Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.
4. Add 5 mL Human Mesenchymal Stem Cell Expansion Medium (prewarmed to 37°C) to the conical tube and resuspend the cells thoroughly. **IMPORTANT:** Do not vortex the cells.
5. Gently rotate the plate to mix the cell suspension. Transfer the dissociated cells to a 15 mL conical tube.
6. Centrifuge the tube at 300 x g for 3-5 minutes to pellet the cells.
7. Discard the supernatant.
8. Apply 2 mL Human Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37°C) to the conical tube and resuspend the cells thoroughly. **IMPORANT:** Do not vortex the cells.
9. Count the number of cells using a hemacytometer.
10. Plate the cells to the desired density into the appropriate flasks, plates or wells in Human Mesenchymal Stem Cell Expansion Medium. Plating ~ 1-2 million cells per 10-cm plate or T75 flask is recommended. Do not exceed a plating ratio of 1:7.

OsteoMAX-XF™ Differentiation (for 48-well tissue culture plates)

1. Prepare coated 48-well tissue culture plates as described in “Preparation of Coated Plates” section on pages 4.
2. Follow steps 1-10 of the protocol outlined in the “Subculturing” section.
3. Plate the cell suspension in Human Mesenchymal Stem Cell Expansion Medium at a density of 20,000 cells per well in the appropriate coated 48-well culture plate with 0.5 mL volume per well.
4. Incubate the cells at 37°C in a 5% CO₂ humidified incubator overnight.

Note: Cells should be attached after overnight incubation.

5. One day after plating, carefully aspirate the medium from each well. **Note:** If serum-containing medium was used (i.e. SCM015), rinse the wells twice with 1X PBS. It is important to wash away any residual serum before initiating the differentiation.
6. Add 0.5 mL Complete OsteoMAX-XF™ Differentiation Medium to each well. This medium change corresponds to differentiation day 1.
7. On day 3, remove 0.25 mL of the medium from each well and replace with 0.5 mL of fresh Complete OsteoMAX-XF™ Differentiation Medium.
8. For all subsequent medium changes, remove 0.5 mL of the medium from each well and replace with 0.5 mL of fresh Complete OsteoMAX-XF™ Differentiation Medium. Medium changes should occur every 3 days for 14-17 days. Depending upon the source of mesenchymal stem

cells (i.e. bone-marrow derived), mineralization may be visible by microscopy as early as 7-10 days after initiation of differentiation.

9. After 14-17 days of differentiation, osteocytes can be fixed and stained for alkaline phosphatase (Cat. No. SCR004) or with Alizarin Red (Cat. No. ECM815) for mineralization.

Results

The following are representative results obtained using OsteoMAX-XF™ Differentiation Medium (SCM121).

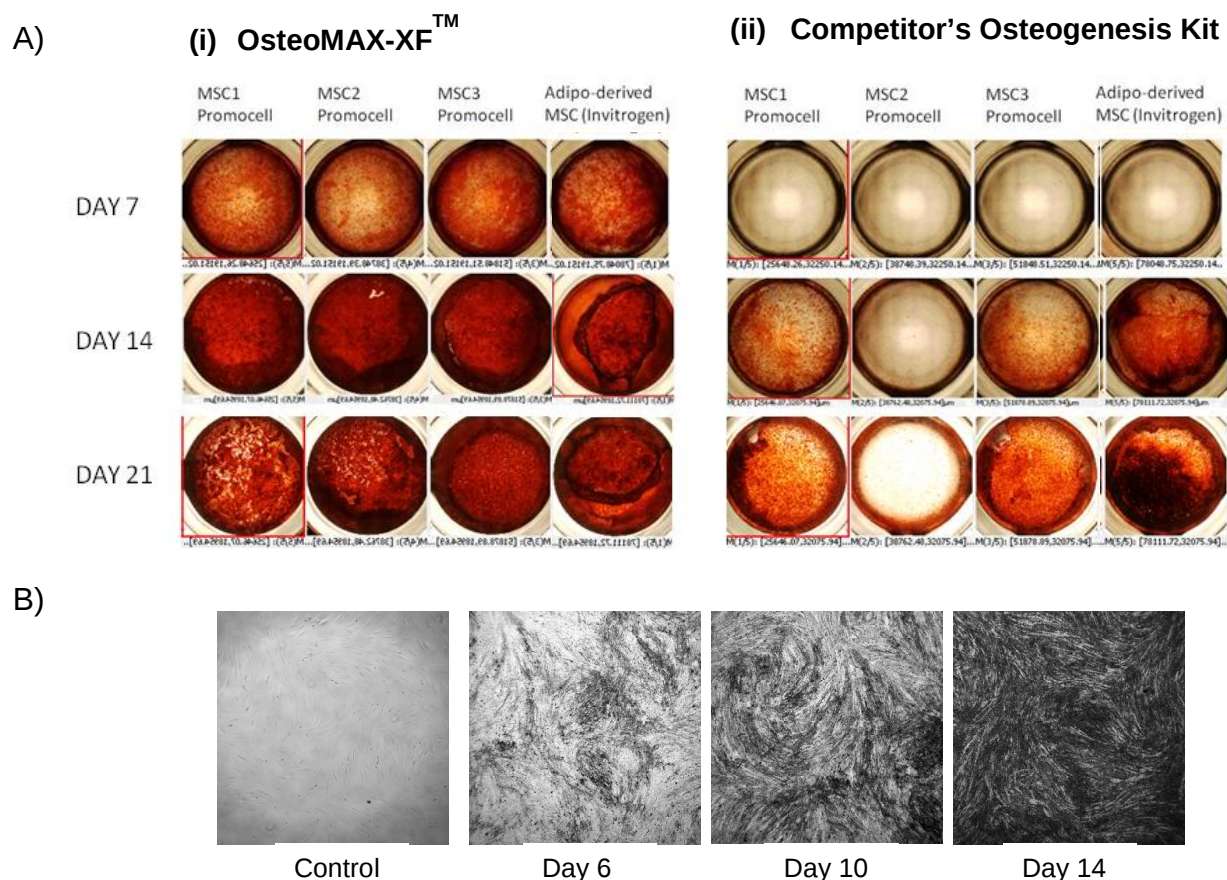


Figure 1. (A) Differentiation of various MSC lines using (i) OsteoMAX-XF™ Differentiation Medium (Cat. No. SCM121); (ii) Competitor's Osteogenesis Differentiation Kit. Differentiation was induced over 21 days in 48-well plate cultures of 4 different human MSC cell lines. Alizarin Red staining of representative wells at day 7, 14 and 21 are shown. (B) Mineralization kinetics of human bone-marrow (BM) derived MSC (Cat. No. SCC034) differentiated in OsteoMAX-XF™.

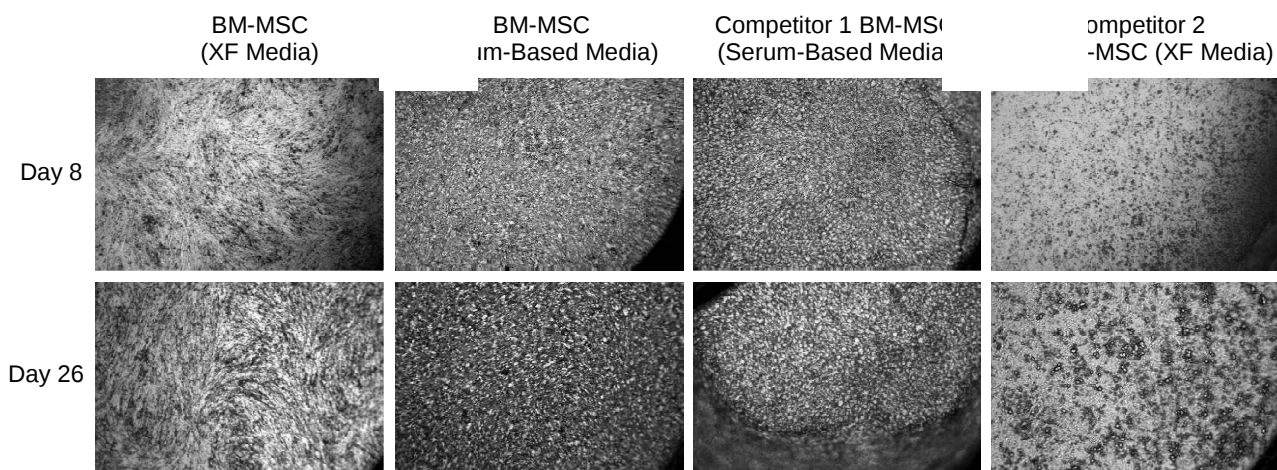


Figure 2. Rapid mineralization of various MSC lines in OsteoMAX-XF™ Differentiation Medium. Cell lines were expanded in serum-based medium (Cat. No. SCM015) or xeno-free medium (Cat. No. SCM037) before being exposed to OsteoMAX-XF™ for 8 and 26 days.

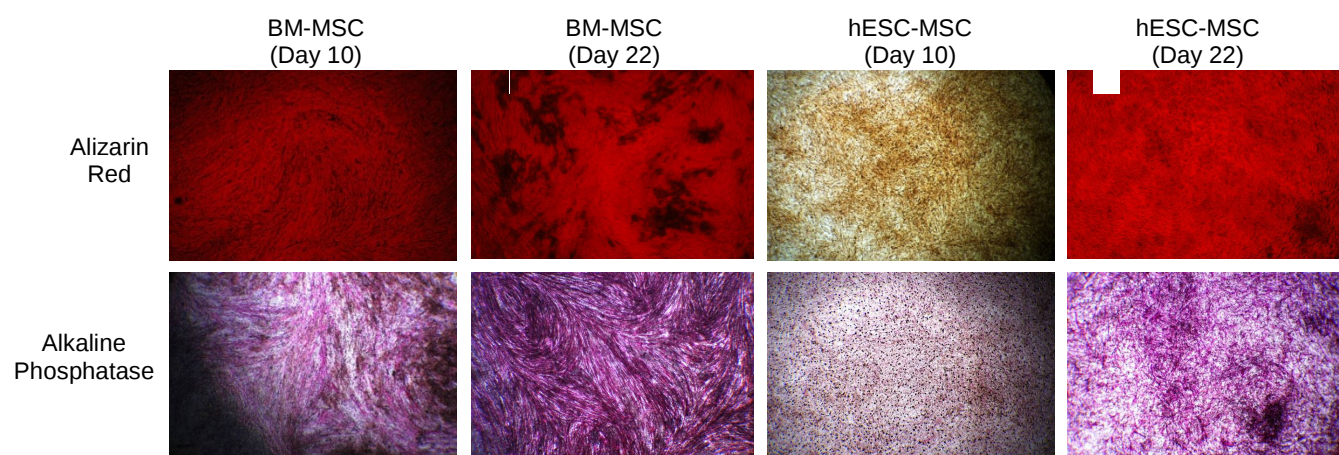


Figure 3. Differentiation kinetics of human BM-derived MSC (Cat. No. SCC034) and human ESC-derived MSC (Cat. No. SCC036) in OsteoMAX-XF™. Human ESC-derived MSC exhibited slower differentiation kinetics as compared to BM-derived MSC. However by day 22-24, maximal differentiation is observed in both cell types.

References

1. Prockop, D. J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**: 71-74.
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3. Alhadlaq, A., and Mao, J. J. (2004) Mesenchymal stem cells: isolation and therapeutics. *Stem Cells and Development* **13**: 436-448.
4. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., and Douglas, R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143-147.

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