User Manual

Mesenchymal Stem Cell Osteogenesis Kit

SCR028

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures. Not for Human or Animal Consumption.

Introduction

Bone undergoes a continual remodeling process that requires the coordinated activity of two types of cells. Osteoclasts break down the bone matrix while osteoblasts deposits collagen, calcium, 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 mis-regulation of osteoblasts and osteoclasts. Understanding the molecular mechanisms that underlie osteogenesis, the process by which new bone is formed is thus of critical importance.

Stem cell technology, particularly embryonic stem cells and/or mesenchymal stem cells, offer attractive sources of osteoblasts for tissue culture studies and for the biochemical dissection of the earliest steps involved in osteoblast cell determination. Mesenchymal stem cells are multipotent progenitor cells that have the capacity to differentiate into several mesenchymal cell lineages, including bone, cartilage, and fat.

The Chemicon® Mesenchymal Stem Cell Osteogenesis Kit contains all the reagents necessary to readily differentiate mesenchymal stem cells to an osteogenic lineage as assessed by Alizarin Red staining. Reagents in the kit include two ECM coating molecules (Collagen Type I and Vitronectin) that have been shown to promote osteogenic differentiation of mesenchymal stem cells[5] along with inducing reagents, dexamethasone, ascorbic acid 2-phosphate and β-glycerophosphate. Also included is Alizarin Red Solution, a staining solution that is used to detect the presence of calcium in bone.

Using the Chemicon® Mesenchymal Stem Cell Osteogenesis Kit, we typically obtain > 50% mature osteocytes from rat bone marrow derived mesenchymal stem cells. Efficiency of osteogenic differentiation may vary, depending upon the quality of the mesenchymal stem cells and if variations to the protocol are introduced.

Materials Provided

- Dexamethasone Solution (Cat. No. 90357): One vial containing 100 µL of 10 mM dexamethasone in ethanol. Store at –20 °C.
- Ascorbic Acid 2-Phosphate Solution (Cat. No. 2004011): One vial containing 500 µL of 100 mM Ascorbic acid 2-phosphate in water. Store at –20 °C.
- Glycerol 2-Phosphate Solution (Cat. No. 2004010): Three vials containing 1 mL of 1 M glycerol 2-phosphate in water. Store at –20 °C.
- Collagen, Type I (Cat. No. 2004013): One vial containing 150 µg Collagen Type I. Store at –20 °C.
- Vitronectin (Cat. No. 2004012): One vial containing 150 µg vitronectin. Store at –20 °C.
- Alizarin Red Solution (Cat. No. 2003999): One bottle containing 50 mL Alizarin Red Solution. Store at 2 to 8 °C.

Materials Required (Not supplied)

- Human or rat mesenchymal stem cell and cell culture reagents
- Mesenchymal Stem Cell Expansion Media (DMEM-low glucose, without glutamine), 10% heat-inactivated fetal bovine serum (Cat. No. ES-009-D), 2 mM L-Glutamine (Cat. No. TMS-002-C) and 1X solution of penicillin and streptomycin (Cat. No. TMS-AB2-C)
- 24–well tissue culture plates
- Phosphate-Buffered Saline (1X PBS) (Cat. No. BSS-1005-B)
- Accutase® (Cat. No. SCR005)
- Fixative (for example, 4% Paraformaldehyde in 1X PBS)
- Hemacytometer
- Microscope

Warnings and Precautions

- Dexamethasone is an irritant and potentially toxic. DMSO is readily absorbed through the skin. Wear a lab coat and gloves when handling these solutions.
- Please refer to the Safety Data Sheet available on the product page at SigmaAldrich.com.

Storage and Stability

- Kit components require two different storage temperatures.
- Dexamethasone Solution, Ascorbic Acid 2-Phosphate Solution, Glycerol 2-Phosphate Solution, Collagen Type I, and Vitronectin should be stored at –20 °C.
- Alizarin Red Solution should be stored at 2 to 8 °C.

Protocol

Preparation

Osteogenesis Induction Media should be made fresh for each use or medium change. Thaw and then heat inactivate Fetal Bovine Serum (Cat. No. ES-009-D) by incubating at 55 °C for 30 minutes.

The recommended amount of medium for a 24-well plate is 1 mL/well.

1. Preparation of Osteogenesis Induction Medium:
   Make up a 1 mM stock solution of dexamethasone by adding 900 µL ethanol to 100 µL of 10 mM dexamethasone solution. Store stock at –20 °C.

Component	Stock Concentration	Amount	Final Concentration
DMEM-low glucose		8.7 mL	~ 87%
Fetal Bovine Serum, heat inactivated (Cat. No. ES-009-D)		1 mL	10%
Dexamethasone Solution	1 mM	1 µL	0.1 µM
Ascorbic Acid 2-Phosphate Solution	0.1 M	20 µL	0.2 mM
Glycerol 2-Phosphate Solution	1 M	100 µL	10 mM
L-Glutamine (Cat. No. TMS-002-C)	100X	100 µL	1X
Penicillin and Streptomycin (Cat. No. TMS-AB2-C)	100X	100 µL	1X

2. Preparation of Mesenchymal Stem Cell Expansion Medium (not supplied in kit):
   Thaw and then heat inactivate Fetal Bovine Serum (Cat. No. ES-009-D) by incubating at 55 °C for 30 minutes. Mix the following sterile ingredients to make 500 mL of medium.

Component	Stock Concentration	Amount	Final Concentration
DMEM-low glucose		440 mL	88%
Fetal Bovine Serum, heat inactivated (Cat. No. ES-009-D)		50 mL	10%
L-Glutamine (Cat. No. TMS-002-C)	100X	5 mL	1X
Penicillin and Streptomycin (Cat. No. TMS-AB2-C)	100X	5 mL	1X

Thawing of Cells

1. Do not thaw the cells until proper media and plasticware are on hand.
2. Remove the vial of mesenchymal stem cells (either rat or human) 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 to not introduce any bubbles during the transfer process.
5. Using a 10 mL pipette, slowly add dropwise 9 mL of Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37 °C) to the 15 mL conical tube.

   Important: Do not add the whole volume of medium at once to the cells. This may result in decreased cell viability due to osmotic shock.
6. Gently mix the cell suspension by slow pipetting 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 3-5 minutes to pellet the cells.
8. Decant as much of the supernatant as possible. Steps 5-8 are necessary to remove the residual cryopreservative (DMSO).
9. Resuspend the cells into a suitable volume of Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37 °C). For a 10 cm tissue culture plate or T75 tissue culture flask, use 10-12 mL volume. For a 6-cm tissue culture plate, use 5 mL volume.

   Important: Do not vortex the Cells.
10. Incubate the cells at 37 °C in a 5% CO_2 humidified incubator.
11. Change to fresh Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37 °C) the next day and every three to four days thereafter.
12. When the cells are 80-90% confluent, they can be dissociated with Accutase® (Cat. No. SCR005) and sub-cultured or alternatively frozen for later use.

Subculturing

1. Culture the cells in a T75 flask in Mesenchymal Stem Cell Expansion Medium until they are 80-90% confluent.
2. Aspirate the media.
3. Wash the flask twice with 5-10 mL of 1X PBS (Cat. No. BSS-1005-B). Aspirate after each wash.
4. Apply 5-7 mL of Accutase® (Cat. No. SCR005) and incubate in a 37 °C incubator for 5-7 minutes.
5. Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.
6. Apply 10 mL of Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37 °C) to the flask.
7. Transfer the dissociated cell suspension into a 15 mL conical tube.
8. Centrifuge the tube at 300 *x g* for 3-5 minutes to pellet the cells.

9. Aspirate and discard the supernatant.
10. Apply 2 mL of Mesenchymal Stem Cell Expansion Medium to the tube and resuspend the cells thoroughly.

    Important: Do not vortex the Cells.
11. Count the number of cells using a hemacytometer.
12. Plate the cells at the desired cell density into appropriate flasks, plates, or wells. Do not exceed a plating ratio of 1:7.

Osteogenesis Differentiation for 24-well tissue culture plates

Studies indicate that coating tissue culture plastic-ware with Vitronectin and Collagen I promote osteogenic differentiation over non-coated plates.[5] Vitronectin and Collagen I are provided in the Kit and we recommend coating tissue culture plastic- or glasswares with these two ECM molecules to aid in the differentiation of mesenchymal stem cells to osteoblasts.

Preparation of Coated 24-well Tissue Culture Plates

1. Dilute Vitronectin and Collagen with 1X PBS to yield final concentrations of 12 µg/mL for each ECM molecule.
2. Add 0.5 mL of the Vitronectin/Collagen mixture to each well of a 24-well plate. Incubate overnight at room temperature.
3. The next day, remove the Vitronectin/Collagen mixture from the wells and rinse the wells once with 1X PBS. Aspirate just before using.

Cell Plating

4. Follow steps 1-11 of the protocol outlined in the Subculturing section.
5. Plate the cell suspension in Mesenchymal Stem Cell Expansion Medium at a density of 60,000 cells per well in the Vitronectin/Collagen coated 24-well culture dish with 1 mL volume per well.
6. Incubate the cells at 37 °C in a 5% CO_2 humidified incubator overnight.

   Note: Cells should be attached and 100% confluent after overnight incubation. If they are not confluent, replace medium every three to four days until the cells are confluent. It is important that the cells be 100% confluent before initiating osteogenesis differentiation.
7. When the cells are 100% confluent, carefully aspirate the medium from each well and add 1 mL Osteogenesis Induction Medium. This medium change corresponds to differentiation day 1.
8. Replace with fresh Osteogenesis Induction Medium every 2-3 days for 14-17 days.
9. After 14-17 days of differentiation, osteocytes can be fixed and stained with Alizarin Red Solution.

Alizarin Red S Staining Protocol

1. Carefully aspirate the medium from each well. Be careful to not aspirate the cells.
2. Fix osteocytes by incubating in iced cold 70% ethanol for 1 hour at room temperature.
3. Carefully aspirate the alcohol and rinse twice (5-10 minutes each) with water.
4. Aspirate the water and add enough Alizarin Red Solution to cover the wells (500 µL to 1 mL per well in a 24-well plate).
5. Incubate at room temperature for 30 minutes.
6. After 30 minutes, remove the Alizarin Red Solution and wash the wells four times with 1 mL water. Aspirate after each wash.
7. Add 1–1.5 mL water to each well to prevent the cells from drying. The plate is now ready for visual inspection and/or image acquisition.

   Note: Osteocytes containing calcium deposits will be stained orange red by the Alizarin Red Solution.

Differentiation Results

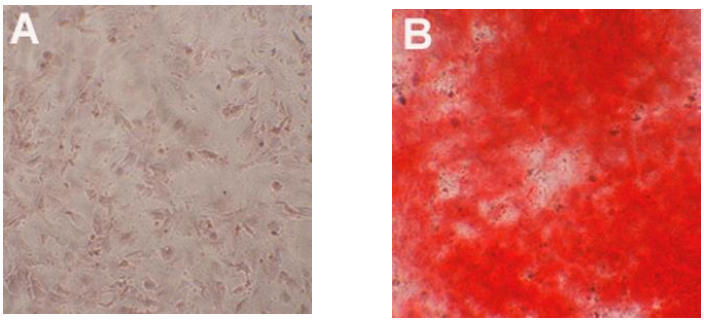

Figure 2. Rat Mesenchymal Stem Cells (Cat. No. SCR026, SCR027) differentiate after 14 days to mature osteocytes. Using the Chemicon® Mesenchymal Stem Cell Osteogenesis Kit (Cat. No. SCR028), rat mesenchymal stem cells readily differentiated to an osteocyte lineage as indicated by Alizarin Red S staining **B.** ARS staining was not observed in control rat skin fibroblasts that were treated in the same manner **A.** Alizarin Red S staining (red) demonstrates mineral deposition throughout the culture.

Related Products

The following stem cell products are available as separate items:

- Rat Mesenchymal Stem Cell Kit: (Cat. No. SCR026)
- Cryopreserved Rat Mesenchymal Stem Cells: (Cat. No. SCR027)
- Mesenchymal Stem Cell Characterization Kit: (Cat. No. SCR018)
- Mesenchymal Stem Cell Adipogenesis Kit: (Cat. No. SCR020)
- Rabbit anti-Human Integrin β1, 100 µL: (Cat. No. AB1952)
- Rabbit anti-Collagen Type I, purified 100 µg: (Cat. No. AB755P)
- Rabbit anti-Rat Fibronectin, purified 100 µg: (Cat. No. AB1954)
- Mouse anti-Human CD54 (ICAM-1), 100 µL: (Cat. No. MAB2130)
- Mouse anti-Rat CD45, purified 0.5 mg: (Cat. No. CBL1502)
- Mouse anti-Human CD14, purified 100 µg: (Cat. No. CBL453)
- Mouse-IgG, purified 10 mg: (Cat. No. PP54)
- Rabbit-IgG, purified 25 mg: (Cat. No. PP64)
- Human Collagen Type I, purified 100 µg: (Cat. No. CC050)
- Human Vitronectin, purified 100 µg: (Cat. No. CC080)

References

1. Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276: 71-74.
2. Pittenger, M. F., and Marshak, D. R. in Stem Cell Biology (Eds Marshak, D. R., Gardner, R. L., & Gottlieb, D.) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001).
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., Douglas, R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147.
5. Salasznyk, R. M., Williams W. A., Boskey, A., Batorsky, A., and Plopper, G. E. (2004). Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells. J. Biomed. Biotechnol. 2004 (1): 24-34.

Notice

We provide information and advice to our customers on application technologies and regulatory matters to the best of our knowledge and ability, but without obligation or liability. Existing laws and regulations are to be observed in all cases by our customers. This also applies in respect to any rights of third parties. Our information and advice do not relieve our customers of their own responsibility for checking the suitability of our products for the envisaged purpose.

The information in this document is subject to change without notice and should not be construed as a commitment by the manufacturing or selling entity, or an affiliate. We assume no responsibility for any errors that may appear in this document.

Technical Assistance

Visit the tech service page at SigmaAldrich.com/techservice.

Terms and Conditions of Sale

Warranty, use restrictions, and other conditions of sale may be found at SigmaAldrich.com/terms.

Contact Information

For the location of the office nearest you, go to SigmaAldrich.com/offices.

Document Template 20769660 Ver 4.0

SCR028 Ver 5.0, Rev 09Apr2024, UD