Mesenchymal Stem Cell Adipogenesis Kit

Cat. No. SCR020

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
Australia +61 3 9839 2000
www.millipore.com

Introduction

Excessive weight gain and obesity pose significant health challenges to many industrialized nations. Many metabolic disorders associated with cardiovascular diseases such as diabetes and atherosclerosis are directly linked to the increased production and size of adipose cells. Understanding the molecular mechanism that underlie adipogenesis, the process by which adipose or fat tissue is formed, is thus of critical importance. Elucidation of the steps involved in adipogenesis, including terminal differentiation, have been facilitated in large part by the development of pre-adipocyte cell lines such as the Swiss 3T3-L1 mouse model (Please refer to CHEMICON®'s Adipogenesis Assay Kit, Catalog No. ECM950). However using these model systems to study some of the earliest events in cellular differentiation are problematic as these cells are already lineage-restricted and pre-determined to become adipocytes.

Stem cell technology, particularly embryonic stem cells and/or mesenchymal stem cells offer attractive alternative sources of adipocytes for tissue culture studies and for the biochemical dissection of the earliest steps involved in adipose 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.

CHEMICON®'s Mesenchymal Stem Cell Adipogenesis Kit contains reagents that readily differentiate mesenchymal stem cells to an adipogenic lineage as assessed with Oil Red O staining of lipid vacuoles in mature adipocytes. These factors include dexamethasone, IBMX, insulin and indomethacin. Along with Oil Red O staining solution, a hematoxylin solution is provided to counterstain the cell nucleus.

Using CHEMICON®'s Mesenchymal Stem Cell Adipogenesis Kit, we typically obtain $\geq$ 30% mature adipocytes from the rat bone marrow derived mesenchymal stem cells. Efficiency of adipogenic differentiation may vary, depending upon the quality of the mesenchymal stem cells and if variations to the protocol are introduced.

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Kit Components

1. Dexamethasone Solution: (Catalog No. 90357) One vial containing 100 µL of 10 mM Dexamethasone in ethanol. Store at –20ºC.
2. IBMX Solution: (Catalog No. 90355) One vial containing 250 µL of 0.5 M 3-isobutyl-1-methylxanthine (IBMX) in DMSO. Store at –20ºC.
3. Insulin Solution: (Catalog No. 90356) Two vials containing 250 µL of 10 mg/mL recombinant human insulin. Store at –20ºC.
4. Indomethacin Solution: (Catalog No. 2003626) Three vials containing 1 mL of 10 mM Indomethacin in methanol. Store at –20ºC.
5. Oil Red O Solution: (Catalog No. 90358) One bottle containing 60 mL 0.36% Oil Red O Solution in 60% isopropanol. Store at room temperature.
6. Hematoxylin Solution: (Catalog No. 2003732) One bottle containing 50 mL Hematoxylin. Store at room temperature.

Materials Not Supplied

1. Human or rat mesenchymal stem cell and cell culture reagents
2. Mesenchymal Stem Cell Expansion Media (DMEM-low glucose, without glutamine, 10% heat-inactivated fetal bovine serum, 2 mM L-Glutamine and 1X solution of penicillin and streptomycin)
3. 24–well tissue culture plates
4. Phosphate-Buffered Saline (1X PBS) (Catalog No. BSS-1005-B)
5. Accutase™ (Catalog No. SCR005)
6. Fixative (e.g. 4% Paraformaldehyde in 1X PBS)
7. Hemacytometer
8. Microscope

Precautions

- Oil Red O stains skin and clothing. IBMX, Dexamethasone and Indomethacin are irritants and potentially toxic. DMSO is readily absorbed through the skin. Wear a lab coat and gloves when handling these solutions.

- Isopropanol is flammable. Keep solutions containing isopropanol (Oil Red O Solution) away from open flames.

Please refer to the Material Safety Data Sheet at www.chemicon.com for further precautions.

Storage

Note: *Kit components require two different storage temperatures.*

Dexamethasone Solution, IBMX Solution, Insulin Solution and Indomethacin Solution should be stored at –20ºC. Oil Red O and Hematoxylin Solutions should be stored at room temperature. Storage of Oil Red O Solution at –20ºC may result in formation of insoluble precipitates and is not recommended. If Oil Red O solution forms a precipitate, remove particulates by passage through a 0.22 or 0.45-micron filter.

Preparation of Reagents

Adipogenesis Induction and Maintenance Media should be made fresh for each use or medium change. Thaw and then heat inactivate Fetal Bovine Serum (Catalog No. ES-009-D) by incubating at 55ºC for 30 minutes.
The recommended amount of medium for a 24-well plate is 0.5 – 1 mL/well. Make 5 mL medium for 5 to 10 wells.

1. Preparation of Adipogenesis Induction Medium:

Mix the following sterile ingredients to make 5 mL of medium. Scale up according to experimental design.

Component	Stock Concentration	Amount	Final Concentration
DMEM-low glucose (Omega, Catalog No. DM21)		4.4 mL	~ 90%
Fetal Bovine Serum, heat inactivated (Catalog No. ES-009-D)		500 µL	10%
Dexamethasone	10 mM	0.5 µL	1 µM
IBMX	0.5 M	5 µL	0.5 mM
Insulin	10 mg/mL	5 µL	10 µg/mL
Indomethacin	10 mM	50 µL	100 µM
Penicillin and Streptomycin (Catalog No. TMS-AB2-C)	100X	50 µL	1X

2. Preparation of Adipogenesis Maintenance Medium:

Mix the following sterile ingredients to make 5 mL of medium. Scale up according to experimental design.

Component	Stock Concentration	Amount	Final Concentration
DMEM-low glucose (Omega Catalog No. DM21)		4.4 mL	~ 90%
Fetal Bovine Serum, heat inactivated (Catalog No. ES-009-D)		500 µL	10%
Insulin	10 mg/mL	5 µL	10 µg/mL
Penicillin and Streptomycin (Catalog No. TMS-AB2-C)	100X	50 µL	1X

3. Preparation of Mesenchymal Stem Cell Expansion Medium (not provided in kit):

Thaw and then heat inactivate Fetal Bovine Serum (Catalog No. ES-009-D) by incubating at 55ºC for 30 minutes.

Mix the following sterile ingredients to make 500 mL of medium.

Component	Stock Concentration	Amount	Final Concentration
DMEM-low glucose (Omega Catalog No. DM21)		440 mL	88%
L-Glutamine (Catalog No. TMS-002-C)	100X	5 mL	1X
Fetal Bovine Serum, heat inactivated (Catalog No. ES-009-D)		50 mL	10%
Penicillin and Streptomycin (Catalog 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 37ºC 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 drop-wise 9 mL of Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37ºC) 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 5-7 are necessary to remove residual cryopreservative (DMSO).
9. Resuspend the cells into a suitable volume of Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37ºC). 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.
10. Incubate the cells at 37ºC in a 5% CO_2 humidified incubator.
11. Change to fresh Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37ºC) the next day and every three to four days thereafter.
12. When the cells are 80-90% confluent, they can be dissociated with Accutase™ (Catalog No. SCR005) and subcultured 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 (Catalog No. BSS-1005-B). Aspirate after each wash.
4. Apply 5-7 mL of Accutase™ (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.
11. Count the number of cells using a hemacytometer.
12. Plate the cells to the desired cell density into appropriate flasks, plates or wells. Do not exceed a plating ratio of 1:7.

Adipogenesis Differentiation

1. Plate the cell suspension in Mesenchymal Stem Cell Expansion Medium at a density of 60,000 cells per well in a 24-well culture dish with 1 mL volume per well.
2. 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 adipocyte differentiation.*

3. When the cells are 100% confluent, carefully aspirate the medium from each well and add 0.5 – 1 mL Adipogenesis Induction Medium. This medium change corresponds to differentiation day 1.

4. Following the differentiation schedule listed below, replace with fresh Adipogenesis Induction or Maintenance Medium (refer to schedule below) every 2-3 days for 21 days. Lipid droplets can be detected by microscopic examination as early as 5 days into the differentiation period.

   Note: *Monolayers of adipogenic cells are fragile and are easily dislodged from their substratum. Disrupted adipogenic cells tend to curl up and form bundles that are difficult to analyze for differentiation. Be extremely gentle and careful with medium changes to avoid disrupting the lipid droplets.*

5. After 21 days of differentiation, adipocytes can be fixed and the lipid droplets stained with Oil Red O Solution.

Differentiation Schedule:

Day	Differentiation Medium
1	Adipogenesis **Induction** Medium
3	Adipogenesis **Induction** Medium
5	Adipogenesis **Induction** Medium
7	Adipogenesis **Maintenance** Medium
9	Adipogenesis **Induction** Medium
11	Adipogenesis **Induction** Medium
13	Adipogenesis **Induction** Medium
15	Adipogenesis **Maintenance** Medium
17	Adipogenesis **Induction** Medium
19	Adipogenesis **Induction** Medium
21	Adipogenesis **Induction** Medium

Oil Red O Staining Protocol:

1. Carefully aspirate the medium from each well. Be careful to not aspirate the cells.
2. Fix adipocytes by incubating in 4% paraformaldehyde for 30-40 minutes at room temperature.
3. Carefully aspirate the fixative and rinse three times (5-10 minutes each) with 1X PBS.
4. Aspirate and rinse twice with water.
5. Aspirate the water and add enough Oil Red O Solution to cover the wells (500 µL to 1 mL per well in a 24 well plate).
6. Incubate at room temperature for 50 minutes.
7. After 50 minutes, remove the Oil Red O Solution and wash the wells three times with 1 mL water.

   Note: *Do not wash with isopropanol as this may remove the Oil Red O stain too rapidly.*

8. Stain cell nuclei with Hematoxylin Solution (0.5 mL volume) for 5 to 15 minutes.

   Note: *Adipocytes containing lipid droplets will be stained red by the Oil Red O solution while the cell nucleus will be stained black/blue from the hematoxylin.*

Differentiation Results:

A) 10x B) 20x C) 40x

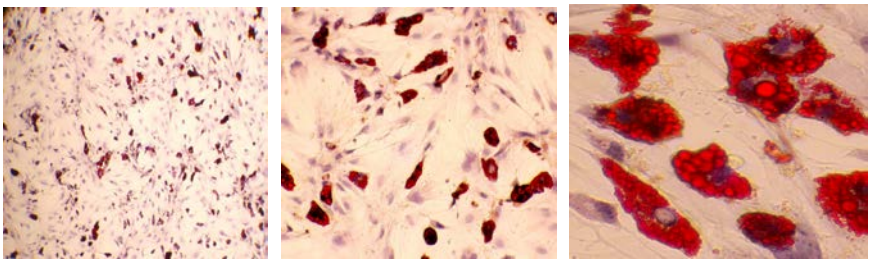

Figure 1. Rat mesenchymal stem cells differentiated after 21 days to mature adipocytes. Lipid vacuoles (red) in the adipocytes were stained with Oil Red O solution. Cell nuclei (purple) were stained with Hematoxylin Solution. **(A)** 10X magnification **(B)** 20X magnification **(C)** 40X magnification

(A) **(B)**

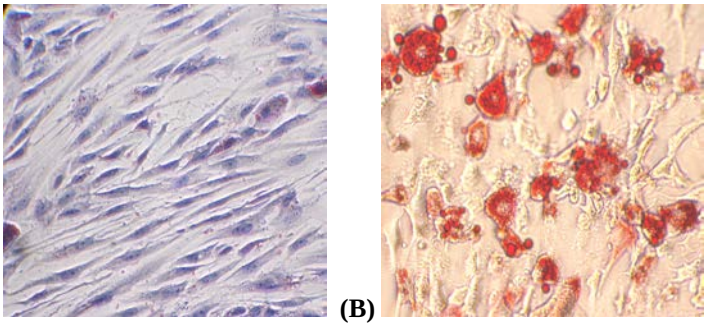

Figure 2. Adipogenic differentiation controls. **(A)** Adult rat skin fibroblasts were differentiated under the same adipogenesis conditions as rat mesenchymal stem cells. Staining with Oil Red O Solution reveals an absence of lipid droplets. **(B)** 3T3-L1 pre-adipocytes differentiated to mature adipocytes. The lipid vacuoles (red) are stained with Oil Red O Solution.

*For colored images, please go to www.chemicon.com

Reference

Pittenger M.F., Mackay A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman M.A., Simonetti D.W., Craig S., Marshak D.R., Multilineage potential of adult human mesenchymal stem cells. (1999) *Science* **284**: 143-7.

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