MILLIPORE

Mouse Embryonic Stem Cell Adipogenesis Kit

10 Reactions

Catalogue No. SCR100

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Adipocyte development has emerged as an attractive area of biomedical/ pharmaceutical research in recent years due to the dramatic increase in obesity and obesity related diseases (type-II diabetes, cardiovascular disease and cancers) worldwide. Current *in vitro* models of adipogenesis rely on freshly isolated adipose tissue, predetermined pre-adipocytes, mesenchymal stem cells or the 3T3-L1 cell line for *in vitro* assays. All of these models have drawbacks that include: timely tissue isolation, the inaccurate use of predetermined cells to study early development and scalability issues.

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of preimplantation embryos and are capable of unlimited, undifferentiated proliferation *in vitro* under appropriate cell culture conditions. These cells have the unique ability to differentiate into cells comprising all three embryonic germ layers (ectoderm, mesoderm and endoderm). Because of their robust growth characteristics and pluripotent nature, embryonic stem cells have emerged as an attractive model to properly study the earliest stages of adipocyte development *in vitro*.

Product Description

Millipore's Mouse Embryonic Stem Cell Adipogenesis Kit (Catalogue No. SCR100) provides a system designed for the differentiation of mouse ES cells into adipocytes. The kit contains all reagents necessary to differentiate mouse ES cells into Oil Red O positive adipocytes *in vitro*. Oil Red O stain, wash and dye extraction solutions are also provided to allow quantification of differentiated adipocytes using common spectrophotometry. The kit provides enough reagents for ten separate differentiation reactions, where each reaction constitutes one well of a 24-well plate. Included in the kit are the following:

(1) One 100 mL bottle of 0.1% Gelatin Solution, a key substrate that provides proper support for cell attachment and spreading.

(2) One 100 mL bottle of Accutase, an enzymatic reagent for the detachment of cells.

(3) Five 100 mL bottles of a specially formulated medium that has been optimized and qualified to support the formation of embryoid bodies.

(4) One vial of Inducer A Solution.

(5) Two 1 mL vials of recombinant insulin and one 200 µL vial of 3,3′,5-Triiodo-L-thyronine (T3), two hormones that aid in the differentiation of mouse ES cells to adipocytes.

(6) One 60 mL bottle of Oil Red O Solution, a positive stain for adipocytes.

(7) One 250 mL bottle of Wash Solution and one 30 mL bottle of Dye Extraction Solution, both used to help quantify Oil Red O positive adipocytes using a spectrophotometer.

Kit Components

Sufficient reagents are provided in the kit for 10 separate differentiation reactions.

1. Embryoid Body (EB) Formation Medium: (Catalogue No. SCM018) Five (5) 100 mL bottles. Store at -20°C.
2. Accutase: (Catalogue No. SCR005) One (1) 100 mL bottle. Store at -20°C.
3. Inducer A Solution: (Catalogue No. CS201483) One 200 µL vial of 500 µM retinoic acid. Store at -20°C.
4. Insulin Solution: (Catalogue No. CS201488) Two (2) 1 mL vials of 85 µM insulin. Store at -20°C.
5. 3,3′,5-Triiodo-L-thyronine (T3) Solution: (Catalogue No. CS201490) One (1) 200 µL vial of 20 µM 3,3′,5-Triiodo-L-thyronine (T3). Store at -20°C.
6. 0.1% Gelatin Solution: (Catalogue No. ES-006-C) One 100 mL bottle of 0.1% gelatin solution. Store at room temperature.
7. Oil Red O Solution: (Catalogue No. 90358) One (1) bottle of 60 mL Oil Red O staining solution. Store at room temperature.
8. Wash Solution: (Catalogue No. 90360) One (1) 250 mL bottle. Store at room temperature.
9. Dye Extraction Solution : (Catalogue No. 90359) One (1) 30 mL bottle. Store at room temperature.

Precautions

- Oil Red O stains skin and clothing.
- Isopropanol is flammable. Keep solutions containing isopropanol (Oil Red O Solution, Wash Solution and Dye Extraction Solution) away from open flames.
- Please refer to the Material Safety Data Sheet at www.millipore.com for further precautions.

Stability and Storage

When stored at the recommended storage conditions (refer to Kit Components), components are stable up to the expiration date listed on the bottle. Do not expose to elevated temperatures. Discard any remaining reagents after the expiration date. ***Note:*** *Kit components requires two different storage temperatures*.

Embryoid Body (EB) Formation Medium: Medium should be stored at -20°C until ready to use. At -20°C the medium is stable for up to six months. Medium is provided in convenient 100 mL bottles. Prior to initial use, thaw frozen media at 2 to 8°C overnight or until it has become completely equilibrated. Upon thawing, medium should be stored at 2 to 8°C and given a 1-month expiration dating.

Accutase: Stable when stored at -20°C. Refer to lot expiration date. Recommended storage upon receipt is -20°C. After thawing, Accutase may be stored for up to 2 months at 2 to 8°C. DO NOT STORE AT ROOM TEMP.

Inducer A Solution: Solution is light sensitive and is readily oxidized upon exposure to air. Inducer A Solution should be stored in working aliquots (5-10 µL) at -20°C for up to 1 year after date of receipt. Prolonged exposure to air and light will result in significant loss of activity. Only thaw aliquots that are required for the experiment. Upon thawing, discard any unused solutions.

Insulin and 3,3′,5-Triiodo-L-thyronine (T3) Solution: Solutions are stable for up to 1 year at -20°C. Avoid repeated freeze/thaw cycles.

0.1% Gelatin Solution: Solution is stable for up to 1 year at room temperature.

Oil Red O Solution, Wash Solution and Dye Extraction Solution: Solutions should be stored at room temperature for up to 1 year. Storage of Oil Red O Solution and Dye Extraction 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.

Materials Required But Not Provided

1. Cryopreserved Mouse Embryonic Stem Cells (Catalogue Nos. SCR011, SCR012, SCC013, CMTI-1, and CMTI-2)
2. EmbryoMax® Complete ES Cell Media w/15% FBS and mLIF; 500 mL (Catalogue No. ES-101-B)
3. ESGRO® mLIF Medium Supplement (Catalogue No. ESG1106, ESG1107)
4. Petri Dishes (BD Catalogue No. 351008)
5. Phosphate-Buffered Saline (1X PBS) (Catalogue No. BSS-1005-B)
6. Hemacytometer
7. Trypan Blue
8. Microscope
9. Spectrophotometer with 520nm or 490nm filter.
10. 24 well tissue culture plate or tissue culture treated plates

Removal of Feeders from Mouse ES Cell Culture

If mouse embryonic stem cells are grown on a mouse embryonic fibroblast feeder (MEF) layer, it is important that the feeder layer is removed before mouse ES cells are differentiated. The following protocol is recommended.

1. Carefully remove the medium used to culture mouse ES cells from the 10-cm tissue culture plate and wash the plate twice with 1X PBS.
2. Apply 5 mL Accutase and incubate in a 37ºC incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of the cells (both ES and MEFs) by gently tapping the side of the plate with the palm of your hand.
4. Apply 5 mL Complete ES Cell Media w/ 15% Serum and mLIF (Catalogue No. ES-101-B) (pre-warmed to 37ºC) to the plate and use this medium to collect the cells onto a 15 mL conical tube.

Note: *For users making their own ES growth medium, the final media composition should be DMEM-High Glucose, 15% FBS, 2 mM Glutamine, 1X Non-Essential Amino Acids, 0.1 mM β-mercaptoethanol, and 1X PSF).*

5. Centrifuge the tube at 300 xg for 2-3 minutes to pellet the cells.
6. Discard the supernatant.
7. Apply 10 mL Complete ES Cell Media w/ 15% Serum and mLIF (pre-warmed to 37ºC) to the conical tube and resuspend the cells thoroughly. **IMPORTANT: Do not vortex.**
8. Transfer the cell suspension to a fresh sterile 10-cm plate that has been pre-coated with 0.1% gelatin solution.
9. Incubate in 37ºC incubator for 30 minutes.
10. After 30 minutes, remove the medium and any non-attached cells and transfer this cell suspension onto another 0.1% gelatin coated 10-cm plate.

    Note: *Mouse embryonic fibroblasts (MEFs) adhere more readily to the tissue-culture plate than undifferentiated mouse ES cells and thus a significant number of MEFs can be removed by this differential adhesion step.*

11. Repeat step 9 and 10 two to three more times.
12. Repeat the whole procedure (steps 1 through 11) for at least three passages to ensure that any residual MEFs are removed from the mouse ES cell culture.

Formation of Embryoid Bodies (2- Stage)

1. Carefully remove the medium used to culture feeder-free mouse ES cells from the 10-cm tissue culture plate and wash the plate twice with 1X PBS.
2. Apply 5 mL Accutase and incubate in a 37ºC incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of the cells (both ES and feeders) by gently tapping the side of the plate with the palm of your hand.
4. Apply 5 mL Embryoid Body Formation medium (pre-warmed to 37ºC) to the plate and use this medium to collect the detached cells and transfer the cell suspension to a 15 mL conical tube.
5. Centrifuge the tube at 300 xg for 2-3 minutes to pellet cells.
6. Discard the supernatant.

7. Apply 2 mL Embryoid Body Formation Medium to the conical tube and resuspend the cells thoroughly. **IMPORTANT: Do not vortex.**
8. Count the number of cells using a hemacytometer.
9. Aliquot 2-3 x 10^6 cells in 10 mL Embryoid Body (EB) Formation Medium and place in a sterile 10-cm bacterial Petri dish or ultra low attachment Petri dish.
10. Incubate the cells in 37ºC, 9-10% CO_2 incubator for two days. After two days, there should be numerous floating embyroid bodies (EBs) formed (refer to Figure 3).

Induction of Adipogenesis with Inducer A Solution (3+ Stage)

1. After a total of 2 days in suspension culture, collect the EBs to a 50 mL conical tube.
2. Wash the 10-cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50 mL conical tube.
3. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube. Set aside.
4. Prepare the Induction Medium by adding 2 µL Inducer A Solution to 10 mL EB Formation Medium. Discard any unused inducer A solution.
5. To the tube containing the EBs, carefully remove and discard the supernatant with a 10 mL or larger pipette.

   Note: *Aspirating the supernatant with a vacuum is* ***not*** *recommended.*
6. Resuspend the EBs in 10 mL Induction Medium (from step 4) and transfer the suspension to a fresh Petri dish.
7. Incubate the dish of cells in 37ºC, 9-10% CO_2 incubator overnight.
8. Repeat steps 2-7 two more times for a total of 3 full days in Induction Medium.

Expansion of Adipocytes from Embryoid Bodies (21+ Stage)

1. From step 8 of section titled "Induction of Adipogenesis with Inducer A Solution," gently swirl the 10-cm Petri dish containing the induced EBs counterclockwise for several circular rotations.

   Note: *Use caution to avoid sloshing the media over the side of the plate as this may increase the risk of cell contamination. The swirling process helps localize all of the EBs to the center of the 10-cm Petri dish.*

2. Immediately place the 10-cm Petri dish under a microscope and count the number of EBs that have localized to the center of the 10-cm Petri dish. This is the total number of EBs in the culture. Typical yields are 50 to 100 EBs per 10-cm Petri dish.

3. Using a 10 mL pipette, collect and transfer the EB suspension to a 50 mL conical tube.

4. Wash the 10-cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50 mL conical tube.

5. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube. Set aside.

6. Prepare Adipocyte Differentiation Medium.

   Note: *Adipocyte Differentiation Medium should be made fresh for each medium change.*

   Mix the following sterile ingredients with 5 mL Embryoid Body Formation Medium to make 5 mL of Adipocyte Differentiation Medium. Scale up or down according to experimental design.

Inducer	Stock Concentration	Final Concentration	Amount of Stock Solution
T3 (Cat. No. CS201490)	20 μM	20 nM	5 μL
Insulin (Cat. No. CS201485)	85 μM	850 nM	50 μL

7. Coat tissue culture treated 24-well plates with 0.1% Gelatin Solution for 30 minutes at room temperature. Remove 0.1% Gelatin Solution before adding cells.

8. Continuing from Step 5, use a 10 mL or larger pipette to remove and discard the supernatant.

9. Resuspend embryoid bodies in prewarmed prepared Adipocyte Differentiation Media (from step 6 above) and transfer to gelatin coated plate (from step 7 above). A seeding density of 10-20 embryoid bodies per well is recommended.

10. Incubate the cells in 37ºC, 9-10% CO_2 incubator for two days.

11. Exchange the medium in each well with 1 mL freshly prepared Adipocyte Differentiation Medium every two days for a total of 21 days. Adipocyte like cells should start to become visible surrounding attached EBs within 14 days of seeding into coated plates.

    Note: *Use extreme care when exchanging the medium as the cells are loosely adherent. Always leave behind a small volume of medium to ensure that the cells do not dry out.*

Staining Protocol (24 well plate format)

1. Carefully remove the medium from each well and wash the wells three times with 1X PBS (5 minutes per wash).

   Note: *Use extreme care when exchanging the medium as the cells are loosely adherent. Always leave behind a small volume of medium to ensure that the cells do not dry out. We do not recommend aspiration with vacuum.*

2. Remove the final 1X PBS wash and add 0.5 mL Oil Red-O Solution to each well of the 24 well plate.

3. Incubate for 15 minutes at room temperature.

4. Remove the staining solution and wash wells 3 times with 1 mL Wash Solution. Cells can be visualized at this point before dye extraction.

5. Add 0.25 mL Dye Extraction Solution to each well.

   Note: *Volume of Dye Extraction Solution may be reduced to achieve a higher absorbance, but care should be taken to ensure that the solution covers the monolayer*

6. Set the plate on an orbital shaker or platform rocker for 15-30 minutes.

7. Transfer extracted dye into a cuvette and read absorbance in a spectrophotometer at 520 nm. Extracted dye can also be transferred to a 96-well plate and quantified in a plate reader. (Although Oil Red O is best quantified at its maximum absorbance of 520 nm, it may be quantified with lower efficiency at 490 nm).

Results

Overview: 26-day protocol for mouse ES cell differentiation to adipocytes

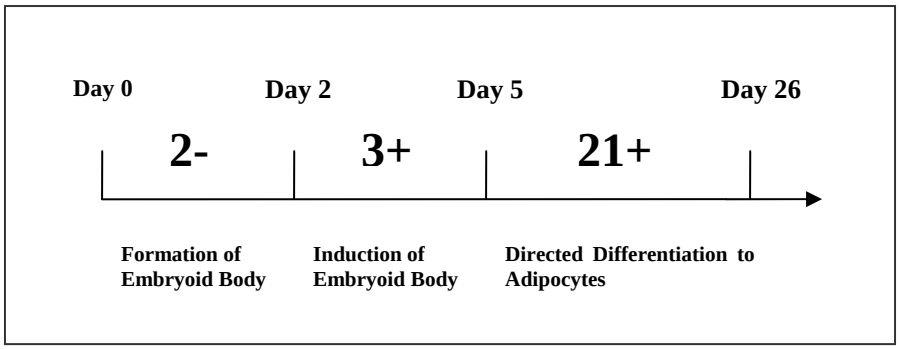

Figure 1. Mouse ES cells are cultured in EB Formation Medium in a non-adhesive 10-cm Petri dish for 2 days in the absence of LIF (**2- Condition**) to help form tight clusters of cells called embryoid bodies (EBs). EB formation is the critical first step in the spontaneous differentiation of ES cells. More directed differentiation of ES cells occurs upon the addition of retinoic acid (Inducer A Solution) to the culture for an additional 3 days (**3+ Condition**). Induced EBs are subsequently transferred to 0.1% gelatin coated wells and cultured in freshly prepared Adipocyte Differentiation Medium for an additional 21 days (**21- Condition**). Adipocytes emanate, migrate and form numerous clusters of lipid containing cells from attached EBs in as little as 14 days after the transfer to coated plates.

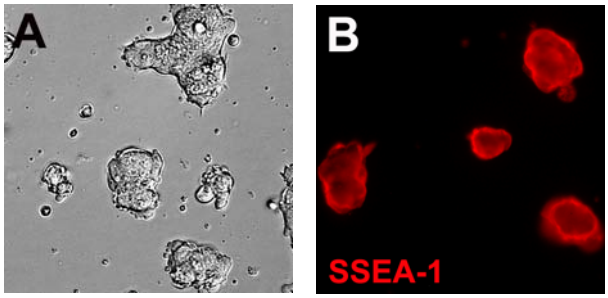

Figure 2. Undifferentiated mouse ES cells (Catalogue No. SCR012) display the characteristic tight round colonies with a high nuclear to cytoplasmic ratio, (**A**, 40X magnification) and stain positively for mouse ES cell marker SSEA-1 (**B,** 40X magnification).

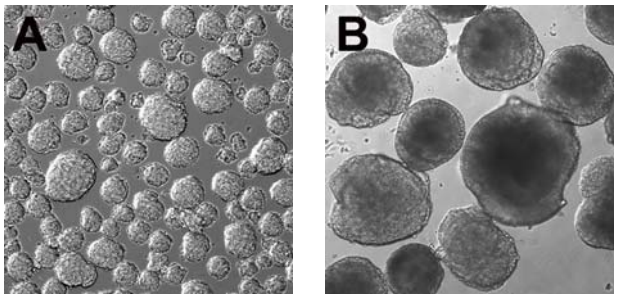

Figure 3. Formation of embryoid bodies (EB) after the culture of dissociated mouse ES cells in EB Formation Medium for 2 days (**A**). Morphology of EBs after treatment with Inducer A Solution for 3 days (**B**). 10X magnification.

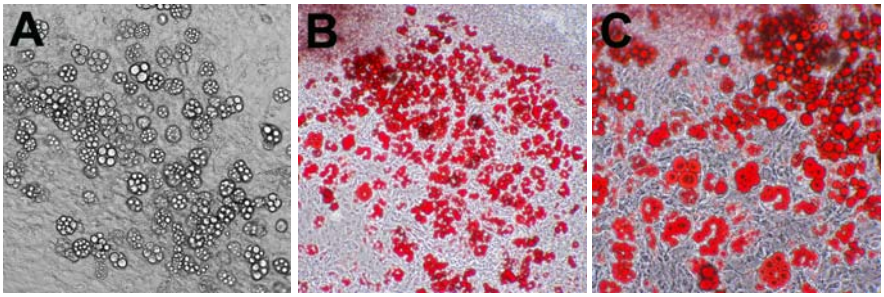

Figure 4. 26-day differentiation of mouse ES cells to Oil Red O positive adipocytes (**A)** bright-field image, 20X magnification. (**B**, **C)** Oil Red O stained images, 10 and 20X magnification, respectively)

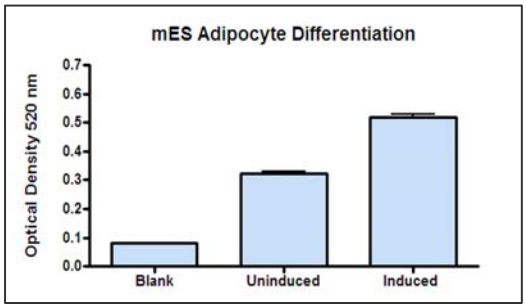

Figure 5. Differentiation of mouse ES cells via T3 and insulin (induced) produced approximately twice the amount of oil red O stained adipocytes as compared to spontaneous differentiation (uninduced). Blank represents the background staining of the plate (no cells included) due to absorbance of plates to the oil red O stain.

*For color images, please go to www.millipore.com.

References

1. C. Dani, A. G. Smith, S. Dessolin, P. Leroy, L. Staccini, P. Villageois, C. Darimont and G. Ailhaud. (1997) Differentiation of embryonic stem cells into adipocytes *in vitro*. *Journal of Cell Science* **110**: 1279-1285.

2. Bost F, Caron L, Marchetti I, Dani C, Le Marchand-Brustel Y, Binétruy B. (2002) Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. *Biochem J.* **361(Pt 3)**: 621-627.

Related Products

The following stem cell products are available from MILLIPORE as separate items:

1. Cryopreserved Mouse Embryonic Stem Cells: (Catalogue Nos. SCR011, SCR012, SCC013, CMTI-1, and CMTI-2)
2. Alkaline Phosphatase Detection Kit: (Catalogue No. SCR004)
3. Quantitative Alkaline Phosphatase ES Cell Characterization Kit: (Catalogue No. SCR066)
4. ES Cell Characterization Kit: (Catalogue No. SCR001)
5. Mouse Embryonic Stem Cell Neurogenesis Kit: (Catalogue No. SCR101)
6. Adipogenesis Assay Kit: (Catalogue No. ECM950)
7. Mesenchymal Adipogenesis Kit: (Catalogue No. SCR020)
8. Adipolysis Assay Kit: (Catalogue No. SCR020)

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