Protocols for Adipogenic Differentiation Assays for Characterization of Adipose Stromal Cells (ASC)

Purpose: To characterize the adipogenic differentiation capabilities of adipose stromal cells.

Overview: Adipogenic Differentiation Assays. As the differentiation capacity of these cells declines with increasing time in culture, differentiation assays of ASC should be determined as soon as possible after recovery. ASC cultures are plated in growth medium on fibronectin-coated multiwell plates and grown to confluence. The growth medium is then replaced with control and differentiation media, and the cultures are further incubated for 14-21 days with regular feedings. After fixation, replicate wells are stained for accumulation of triglyceride by Oil Red O staining (Lillie and Ashburn, 1943). Differentiation may be assessed quantitatively by spectrophotometric analysis to measure lipid accumulation relative to the control samples, or by microscopic examination to determine the percentage of cells that undergo adipogenic differentiation. For microscopic analysis, the cells are counterstained with Hematoxylin to visualize all cells.

***Supplies, Media and Reagents for Adipogenic Differentiation Assays:**

Sterile 0.2 μm pore, 500 ml vacuum filter unit (Corning)
Sterile 0.2 μm pore syringe filters, 13 or 22 mm diameter (Acrodisc)
Sterile 0.2 μm pore nylon syringe filters, 13 mm diameter (Nalgene #195-2520)
Optional: Coverslips, 12 mm or 22 mm *round*, for 24-well or 12-well plates, respectively
Multiwell tissue culture plates, 12-well, (BD Falcon #353043); or 24-well, (BD Falcon #353047); or 48-well, (BD Falcon #353078); or 96-well, (BD Falcon #353072)
Biotin (Sigma #B4639)
Calcium Chloride (ACS reagent grade)
Dexamethasone (Sigma #D4902)
Dulbecco's Modified Eagle's Medium (DMEM), low glucose, with Glutamax™ (Invitrogen #10567-014)
Earle's Balanced Salt Solution (EBSS; Invitrogen #14155-063)
Fibronectin, human plasma, 1 mg/ml (hFN; Chemicon #FC010)
Gelatin (from bovine or porcine skin)
Glycerol (ACS reagent grade)
Hematoxylin Solution (Vector #H3401 or H3404)
Human serum albumin (HSA; Sigma #A1653)
N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES; Sigma #H3375)
3-Isobutyl-1-methylxanthine (IBMX; Sigma #I7018)
Indomethacin (Sigma #I8280)
Insulin, recombinant human (rhIns; Sigma #I2643)
Isopropanol (ACS reagent grade)
Nutrient mixture Ham's F12 (HF12; Invitrogen #11765-062)
Oil Red O (Sigma #O0625)
Paraformaldehyde (Certified or histology grade)
Phenol, water-saturated (ACS or Molecular Biology grade)
Pantothenic acid hemicalcium salt (Sigma #P5155)
Phosphate buffered saline (PBS; from Invitrogen #70011-044, 10X stock)
Transferrin, human (Sigma #T8158)

Triiodothyronine, sodium salt (Sigma #T6397)
Sterile microtubes OR sterile cryovials (Nalgene 2 ml)
Sterile, cell culture quality, water
Whatman Grade 1 filter paper, 15 cm diameter (Whatman #1001-150)

* Suppliers of reagents are listed for the convenience of culture recipients only. Such lists are not intended to be either selective or exhaustive, and the CCR does not recommend specific products or suppliers. It may be necessary to perform dose-response experiments to determine the optimal concentration of human serum albumin from different lots or different suppliers.

Preparation of reagent stocks for differentiation media:

Biotin (FW 244.3): Dissolve 80.62 mg biotin in 100 ml cell culture quality water and filter sterilize to yield 3.3 mM (100X) stock. Dispense 1 ml to 2 ml aliquots in sterile cryovials and store at -20°C or below. Thawed stock can be stored at 4°C for up to 1 week.

Dexamethasone (FW 392.5): Dissolve 0.0196 g (Sigma #D4902) in 10 ml absolute ethanol to yield 5mM stock; store at -80°C. Prepare fresh working stock by diluting ethanol stock 1:500 in DMEM:HF12 (1:1) and filter sterilize to yield 10 µM stock.

3-Isobutyl-1-methylxanthine (IBMX; FW 222.2): Dissolve 100 mg IBMX in 1.8 ml DMSO and sterile filter through 0.2 µm **nylon** filter to make 250 mM (55.55 mg/ml) stock (1000X). Aliquot and store at -20°C or below.

Indomethacin (FW 357.8): Dissolve 0.5367 g indomethacin in 7.5 ml DMSO, and filter through 0.2 µm **nylon** filter to sterilize for 200 mM (1000X) stock. Dispense 1-2 ml aliquots in sterile cryovials, and store in opaque box to protect from light. Store at -70°C or below.

Insulin, recombinant human (rhIns; FW 5807.57): Dissolve 50 mg rhIns in 17.22 ml Earle's Balanced Salt Solution (EBSS) acidified with 0.005 N HCl to yield 500 µM stock, and sterile filter. Dispense 1 ml aliquots in sterile cryovials and store at -70°C or below for up to 2 years. Thawed, working stock can be stored at 4°C for up to 1 month. ‡

Pantothenic acid (FW 238.3 hemicalcium salt): Dissolve 0.405 g pantothenic acid in 100 ml water and filter sterilize to yield 17 mM (1000X) stock. Dispense 2 ml aliquots in sterile cryovials and store at -20°C or below; thawed stock can be kept at 4°C for up to 1 week.

Transferrin, human: Dissolve 100 mg transferrin in 100 ml EBSS to yield 1 mg/ml (100X) stock, and sterile filter. Dispense 1 ml to 2 ml aliquots in sterile cryovials and store at -70°C or below for up to 2 years, and thawed, working stock at 4°C for up to 1 month. ‡

Triiodothyronine, sodium salt (FW 672.96): Dissolve 20.2 mg in a small volume of 0.02 N NaOH, dilute to 150 ml with distilled water and filter sterilize to yield 200 µM stock. Aliquot and store at -20°C or below. Dilute 1:1000 in DMEM:HF12 (1:1) to yield 200 nM (1000X) working stock. Working stock can be stored at 4°C for up to 1 month.

Adipogenic Differentiation Media

Adipogenic Induction Medium (**AIM**) in DMEM: Ham's F12 (1:1) + 1% HSA (25 ml)
33 µM biotin (0.25 ml "100X" 3.3 mM stock)
0.1 µM dexamethasone (0.25 ml of 10 µM stock)†
1 µM Insulin (50 µl "500X" 500 µM stock)‡
200 µM indomethacin (50 µl of 200 mM stock in DMSO)
17 µM pantothenic acid (25 µl "1000X" 17 mM stock)
10 µg/ml transferrin (0.25 ml "100X" 10 mg/ml stock) ‡
0.2 nM triiodothyronine (25 µl "1000X", freshly diluted from 10^6 x 0.2 mM stock)
250 µM isobutyl methylxanthine (IBMX) (25 µl of 250 mM stock)
24.1 ml 1% HSA in DMEM:HF12 (1:1)

Adipogenic Differentiation Medium (**ADM**) in DMEM:HF12 (1:1) + 1% HSA (25 ml)
33 µM biotin (0.25 ml "100X" 3.3 mM stock)
0.1 µM dexamethasone (0.25 ml of 10 µM stock) †
1 µM Insulin (50 µl "1000X" 500 µM stock)‡
200 µM indomethacin (50 µl of 200 mM stock in DMSO)
17 µM pantothenic acid (25 µl "1000X" 17 mM stock)
10 µg/ml transferrin (0.25 ml "100X" 10 mg/ml stock) ‡
0.2 nM triiodothyronine (25 µl "1000X", freshly diluted from 10^6 x 0.2 mM stock)
24.1 ml 1% HSA in DMEM:HF12 (1:1)

†10 µM Dexamethasone must be freshly diluted in DMEM:HF12 from 5 mM stock

‡ Alternatively, substitute 0.25 ml 100X Insulin-Transferrin-Selenium stock (ITS-X; Invitrogen #51500-056 or equivalent) for separate Insulin + Transferrin

Base Medium and Non-Induction (Control) Medium

1% Human Serum Albumin (HSA) in DMEM:HF12 (1:1). Dissolve 5 g HSA in 500 ml DMEM:HF12 (1:1) pre-warmed to 37ºC. Adjust to pH 7.25 if necessary with 0.1 N NaOH and filter sterilize. Store at 4°C for up to six months.

Preparation of 4% Paraformaldehyde/ 1% $CaCl_2$ in PBS with 50 mM HEPES, pH 7.2

Adipose Tissue Fixative (100 ml): This should be prepared in a fume hood.

- Dissolve 4 g of paraformaldehyde in ~60 ml distilled water. This will require warming to about 60ºC (do NOT exceed 70ºC) and stirring, and the addition of 1 or a few drops of 0.1 N NaOH. The solution will clarify upon addition of sufficient NaOH, but there will still be a small amount of particulate material left.
- Remove from heat and let cool.
- Add 10 ml 10X PBS.
- Add 10 ml 0.5M HEPES, pH 7.4
- Add 10 ml 10% $CaCl_2$ (solution will again become cloudy)
- Adjust pH to 7.2
- Bring volume to 100 ml with Distilled water and filter through Whatman filter paper.

Preparation of Oil Red O staining solution:

Oil Red-O Stock Solution

- Oil Red-O 0.30 g (300 mg)
- 2-propanol, 99% 100 ml

Mix well. Transfer to a bottle and cap tightly. Store at room temperature for up to 1 year.

Oil Red-O Working Solution (good for ~3 hrs)

- Oil Red-O, stock solution 24 ml
- Distilled water 16 ml
- Mix well and let stand for 10 minutes. Filter through Whatman paper.

The filtrate can be used for ~3 hours.

Preparation of Glycerol-Gelatin (Optional, for % differentiation only):

Kaiser's Glycerol-Gelatin-1% Phenol Mounting Medium

- Gelatin 10.0 g
- Distilled water 52.5 ml
- Glycerol 62.5 ml
- Phenol 1.25 ml

Measure 52.5 ml distilled water into a 250 ml flask and add a small stir bar. Place on hot plate/ stirrer and heat until steaming. Add the gelatin and continue heating and stirring until dissolved. Turn off the heat and slowly add the glycerol using a 50 ml pipet. Due to the viscosity of glycerol, it will drain slowly from the pipet, and it is necessary to rinse the pipet with the solution to ensure quantitative recovery. Using a glass pipet, add the phenol and continue stirring until dissolved. Store at room temperature in a brown bottle.

Preparation of fibronectin-coated multiwell plates: Adipose Stromal Cells and Mesenchymal Stem Cells are plated in vessels coated with ~1.5 μg/cm^2 human plasma fibronectin (hFN). Fibronectin from Chemicon is a 1 mg/ml solution in screw-cap vials. Each 12-well plate (20 mm wells = 3.14 cm^2) has 37.68 cm^2/plate, and thus requires 56.52 μg fibronectin. Each 24-well plate (16 mm wells = 2.01 cm^2) has 48.24 cm^2/plate, and thus requires 72.36 μg fibronectin. Each 48-well plate (0.75 cm^2 wells) has 36 cm^2/plate and thus requires 54 μg fibronectin. Each 96-well (0.32 cm^2 wells) has 30.72 cm^2/plate and thus requires 46.08 μg fibronectin per plate. One mg hFN will 650-700 cm^2 total area.

- To coat plates, dilute the required amount of 1 mg/ml (1 μg/μl) hFN with PBS or serum-free medium to 4.71 μg/ml for 12-well plates, or to 6.0 μg/ml for 24-well plates. Add 1 ml per well of the diluted solution to each well of 12-well plates, or 0.5 ml per well for 24-well plates.
- Incubate plates in a humidified 5% CO_2-air incubator at 37°C for 30-60 minutes. Alternatively, the plates may be refrigerated immediately upon adding the coating solution, stored for up to one month, and warmed immediately before use.
- Aseptically remove the fibronectin solution with a suctioning pipette and proceed with cell seeding.

Preparation and growth of cells:

Differentiation can be done in slide wells or multiwell plates, depending upon the desired readout method. To quantitate the percentage of cells that undergo adipogenic differentiation, plate the cells in culture slides or 6- or 12-well plates. Inoculate two wells for each cell line: one to serve as control (no induction of differentiation) and the other as experimental (adipogenic induction). To quantitate adipogenic differentiation by spectrophotometric measurement of Oil Red O accumulation, plate the cells in multiwell plates. Inoculate six wells for each cell line: three to serve as control (no induction of differentiation) and the other three as experimental wells (adipogenic induction). Leave 3 wells empty on one plate for use as "blanks" in the spectrophotometric analysis.

1) Inoculate plates at passage of T25 flask at approximately 1 x 10^4 cells/cm^2 for low passage cells, or 2 x 10^4 cells/cm^2 for high passage cells, in 2 ml growth medium per well for 2-well culture slides, 1 ml per well for 12-well plates, 0.5 ml per well for 24-well plates, or 200 µl for 48-well plates. Label with cell line designation(s).
2) Grow cells in complete medium with supplements. Examine cells every day. When cells have reached subconfluency, or are just confluent, remove complete growth medium and replace replicate wells with Adipogenic Induction Medium **(**AIM**)** and with control medium (1% HSA in DMEM: HF12 (1:1)). For subsequent spectrophotometric assay, leave triplicate wells empty for blanks, and triplicate wells of each ASC strain to be tested in non-induction medium (1% HSA in DMEM:HF12 (1:1)).
3) Continue incubation for 3 more days. NOTE: Certain cell strains might continue to grow and become over-dense under these conditions, to the point that the cell monolayer clumps or lifts off. If this happens, it may be necessary to switch the cells to AIM at a lower density.
4) After incubation for three days, the wells with adipogenic induction medium are changed to the Adipogenic Differentiation Medium (ADM).
5) Incubation is continued for two more weeks, carefully changing the medium twice weekly with control medium or ADM.
6) Some cultures may accumulate lipid more slowly than others. If the culture appears to be differentiating, but the putative lipid vesicles are still small after 2 weeks in ADM, the culture may be re-fed with fresh ADM and incubation continued for another 4 days (21 days total in adipogenic conditions).
7) The cells are then washed once with cold D-PBS. If 2-well culture slides are used, aspirate the medium and add 2 ml cold D-PBS per well, or 1 ml per well for 12-well and 24-well plates (use these volumes in all steps below as well).
8) Aspirate the wash and add Formaldehyde/ Calcium solution for a minimum of 10 minutes at room temperature, or a maximum of 24 hours at 4°C. If the Oil Red O staining cannot be done by this time, aspirate the fixative, replace with cold PBS, and store at 4ºC for up to one week until ready to stain.

Quantification Protocols and analysis:

Relative Oil Red O Accumulation by Spectrophotometry:

1) After fixation, gently rinse the wells once with D-PBS. Stain with 1 ml (12-well), 500 µl (24-well), 100 µl (48-well) or 30 µl (96-well) of the Oil Red O working solution for 15 minutes at room temperature.
2) Rinse the wells 3 times with distilled water (1 ml, 1 ml, 300 µl, or 150 µl, respectively).
3) Elute the dye by addition of 1 ml (12-well), 500 µl (24-well), 200 µl (48-well) or 100 µl (96-well) of isopropanol per well. Incubate on a rocker or plate shaker for 15 minutes. For 12-, 24- or 48-well plates, remove 100 µl per well to a clean 96-well plate for reading. If a plate reader with the appropriate filter is available, read the absorbance (O.D.) at 540nm. If a dual-wavelength instrument is available, one can alternatively read the absorbance at 490 nm/570 nm using the wells with no cells as blanks, and the wells in non-induction medium as controls.
4) Calculate the average absorbance of the blank wells, and the control and test wells. The uncorrected absorbance of the control wells should be similar to the blank, and is generally *less than* 2-fold greater than the blank. The absorbance of the control and test wells can be corrected for non-specific absorbance by subtracting the average blank absorbance from the average values of the control and test wells.
5) Cultures showing uncorrected absorbance at 540 nm from the ADM wells ≥2-fold more than that of the control wells are recorded as positive for adipogenic differentiation. Corrected values ≥3-fold more than that of the control wells are recorded as positive for adipogenic differentiation.

Percent Differentiation:

1) Stain the cells with Oil Red O Working Solution for 30 minutes at room temperature.
2) Aspirate the Oil Red O solution and rinse the wells six times (6X) in tap water.
3) Counterstain the cells with hematoxylin for 5 minutes at room temperature.
4) Rinse 5X with tap water and 1X with distilled water. Do NOT wash with alcohol!
5) If desired, it is possible to take photomicrographs of the cells in culture slides or 12-well plates at this time. Image quality is not as good after mounting in glycerol-gelatin.
6) Melt the glycerol-gelatin in a 60°C water bath. Add 2 – 3 drops of glycerol-gelatin per well (for a 12-well plate) and immediately add a 22 mm circular coverslip; do not attempt to coverslip more than 3 wells at a time.
7) Observe with inverted microscope with 10X-40X objectives. The nuclei stained with Hematoxylin appear as bluish discs, while lipid appears as bright red perinuclear vesicles, often as grape-like clusters of small vesicles, but sometimes fewer, larger vesicles are formed. Unilocular (a single, very large lipid vacuole) adipocytes rarely form in attached, *in vitro*-differentiated adipocytes.

8) Observing fields with the 20X objective, count the number of cells in each field with lipid vesicles (red) and the total numbers of nuclei in the same field. Using a dual button counter, count the number of positively staining cells and the total number of cells (nuclei) in each microscopic field. Score a sufficient number of fields to include at least 100 cells. If in the number of positive cells in wells incubated in ADM is either very low (<20%) or very high (>80%), score additional fields to include at least 200 cells.

9) The percentage of cells that underwent adipogenic differentiation = #cells with oil red O ÷ the total number of cells (nuclei) X 100%.

References:

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