

Adipose Stromal Cell (ASC) Culturing Protocol

CAUTION: For laboratory research use only. Not for human use. Not for diagnostic or clinical use given that the safety and efficacy of this product for such uses has not been established. Handle as potentially biohazardous material under at least biosafety level 2 containment. Negative test results for specific pathogens do not imply absence of all potential infectious agents.

***Media and Reagents for Culture:**

L-Ascorbate-2-phosphate (Sigma #A8960)

Bovine serum albumin (BSA; Sigma #A4503)

β -mercaptoethanol solution, 1000X (55 mM, Invitrogen #21985-023)

Dexamethasone (Sigma #D4902)

Dulbecco's Modified Eagle's Medium (DMEM), low glucose, with Glutamax (Invitrogen #10567-014)

Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS; Invitrogen #14190-144)

Recombinant human epidermal growth factor (rhEGF; Peptidech #100-15)

95% (*non*-denatured) ethanol

Fetal bovine serum (FBS; Invitrogen, Sigma, or Hyclone)

Fibronectin, human plasma, 1mg/ml (hFN; Chemicon #FC010)

Ham's Nutrient mixture F12 (HF12), (Invitrogen #11765-062)

Insulin-Transferrin-Selenium solution (Invitrogen ITS-X #51500-056)

Linoleic Acid-BSA (Sigma #L9530)

MCDB 131 (Invitrogen #10372-019) Supplement with L-glutamine (Invitrogen #25030-14).

MCDB 201 (Sigma #M6770)

Recombinant human platelet-derived growth factor-BB (rhPDGF-BB; Peptidech #100-14B)

0.05% trypsin/0.53 mM EDTA (Invitrogen #25300-054)

0.53 mM EDTA in PBS (Invitrogen Versene 1:5000 #15040-066)

Optional: Recombinant human Insulin (Sigma #I2643), and human Transferrin (Sigma #T8158) may be used instead of ITS-X.

***Supplies:**

Sterile 15 ml centrifuge tubes; sterile 125 ml or 250 ml Media bottles

Sterile tips for 20-200 μl micropipettor; sterile 1.5 ml microcentrifuge tubes or screw-cap cryovials

Sterile, tissue culture-quality water

Sterile 0.2 μm pore, 13 mm diameter syringe filters (Acrodisc)

Sterile, disposable syringes (BD)

Sterile 0.22 μm pore, 150 ml filter units (Corning #431153)

Sterile 0.22 μm pore, 500 ml filter units (Corning #431097)

Vent-cap T25 or T75 tissue culture flasks (Corning)

* Specific catalog numbers and 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 bovine serum albumin from different lots or different suppliers.

Preparation of reagent stocks for 0.5% FBS ASC Medium:

L-Ascorbate-2-phosphate: To make 10 mM stock, dissolve 0.384 g L-Ascorbate-2-phosphate in 150 ml DMEM (LG): MCDB 201 (2:1) and filter sterilize. Store at 4°C for up to three months.

2% BSA stock: Dissolve 3.0 g bovine serum albumin in 150 ml DMEM: MCDB 201: MCDB 131 (60:20:20). The albumin is added with constant stirring to medium pre-warmed to 37°C. Adjust to pH 7.25 if necessary with 0.1 N NaOH and filter sterilize. Store at 4°C for up to six months.

0.25% BSA in 10 mM acetic acid in PBS (For EGF & PDGF stocks): Add 18.75 ml 2% BSA stock to 128.25 ml D-PBS without Ca²⁺, Mg²⁺ plus 3 ml sterile 0.5 M acetic acid. Store at 4°C for up to one year.

Dexamethasone: Dissolve 0.0196 g dexamethasone in 10 ml 95% (*non*-denatured) ethanol to yield 5 mM 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. Store working stock at 4°C for up to one week.

Epidermal Growth Factor: Dissolve 100 µg recombinant human EGF in 10 ml 0.25% BSA + 10 mM acetic acid in D-PBS and filter sterilize using 13 mm diameter filter; aliquot and store at -20°C to -80°C. Thawed stock is good at 4°C for up to one month.

MCDB 201: Prepare powder according to the package insert directions. Use only tissue culture quality water and adjust to pH 7.25 with 0.1 N NaOH or HCl as necessary before filter sterilization.

Platelet-Derived Growth Factor-BB: Dissolve 10 µg recombinant human PDGF-BB in 1 ml 0.25% BSA in D-PBS + 10 mM acetic acid (or 100 µg in 10 ml) and sterile filter (13 mm diameter filter). Aliquot and store at -20°C to -80°C.

<u>0.5% FBS ASC Medium</u>	<u>per 250 ml</u>
0.5% FBS	1.25 ml
1X ITS-X	2.5 ml 100X stock
0.2% Bovine serum albumin (BSA)	25 ml 2% stock
1X Linoleic Acid-BSA	2.5 ml 100X stock
100 µM L-Ascorbate-2-phosphate	2.5 ml 10 mM stock
100 µM β-mercaptoethanol	0.45 ml 55 mM stock
10 ng/ ml rhEGF	0.25 ml 10 µg/ml stock
5 ng/ml rhPDGF-BB	125 µl 10 µg/ml stock
1 nM dexamethasone	25 µl 10 µM stock
<u>in DMEM: MCDB 201: MCDB 131 (60:20:20):</u>	<u>215.4 ml</u>
DMEM (low glucose)	129.2 ml
MCDB 201	43.1 ml
MCDB 131	43.1 ml

Preparation of culture substrate: ASC are plated in vessels coated with $\sim 1.5 \mu\text{g}/\text{cm}^2$ human plasma fibronectin (hFN). Wipe the cap of the Fibronectin vial with 70% ethanol before opening. One mg hFN will coat 650 - 700 cm^2 total area. To coat 75 cm^2 flasks, dilute 1 mg hFN with PBS or serum-free medium to 44.4 ml (22.5 $\mu\text{g}/\text{ml}$) and add 5 ml per flask. To coat 25 cm^2 flasks, dilute 1 mg hFN to 80 ml (12.5 $\mu\text{g}/\text{ml}$) and add 3 ml per flask.

- 1) Add indicated volumes to flasks required.
- 2) Incubate flasks in a horizontal position in a humidified 5% CO_2 -air incubator at 37°C for 30-60 minutes for immediate use *or* store flasks at 4°C overnight (cold flasks can be kept for up to 2 months).
- 3) Allow flasks to come to room temperature.
- 4) Aseptically remove the coating solution with a suctioning pipet immediately before use.

Sub-culturing procedure: ASC should be sub-cultured prior to, or immediately upon, reaching confluence. (Volumes indicated are for use with T25 flasks; double volumes for T75 flasks)

- 1) Warm growth medium in a water bath to 37°C. Warm only as much medium as will be required for immediate use; do NOT warm the whole bottle.
- 2) Aspirate medium and rinse culture with 3 ml D-PBS.
- 3) Aspirate PBS and rinse cell monolayer with 3 ml room-temperature 0.53 mM EDTA solution.
- 4) Aspirate 0.53 mM EDTA solution and add 1 ml trypsin/EDTA warmed to 37°C.
- 5) Incubate flasks at room temperature, inspecting cells under the microscope periodically and gently rocking the flasks to redistribute the trypsin. ASC usually detach within 2-3 minutes. If the cells are not rounded up and coming off after 5 minutes, the flasks may be placed in the 37°C incubator for 2-minute intervals. Once the cells are rounded, gently rap the flasks to dislodge the cells.
- 6) Add 5 ml of growth medium to stop trypsin action and pipette gently to obtain a single cell suspension.
- 7) Transfer cell suspension to a 15 ml centrifuge tube, and remove a 0.5 ml aliquot to count using an automated cell counter *or* a hemacytometer using trypan blue dye exclusion viability stain.
- 8) Centrifuge cell suspension at 100-150 x g for 10 minutes.
- 9) Aspirate medium and resuspend pellet in growth medium, pre-warmed in water bath at 37°C. Aspirate fibronectin solution from new flasks *immediately before use*. Inoculate flasks at 2×10^3 to 2×10^4 cells/ cm^2 . The total plating volume in each flask should be 5-6 ml. If T75 flasks are used for culture expansion, medium volume at plating should initially be 10-12 ml, increasing to 20 ml as cell density increases.
- 10) Gently move the flasks back and forth to ensure an even dispersion of cells. Incubate flasks at 37°C in a horizontal position in a humidified 5% CO_2 -air incubator.
- 11) Change growth medium twice weekly while culture is sparse; change medium three times a week as density increases.

Notes: These cells will grow in 10% FBS in DMEM, but growth in this medium will adversely affect subsequent multilineage differentiation. Cultures should be sub-cultured before confluency is attained (usually 5-7 days). Cultures plated at the higher density of 2×10^4 cells/ cm^2 will typically need to be split or harvested within 3-5 days. If a culture is held at confluence, subsequent growth and differentiation will be adversely affected. **Differentiation capacity declines with increasing passage.**

References:

Moscatello, D.K. Cell culture media, kits, and methods of use. U.S. Patent Application 11542863, filed 10/04/2006; U.S. Provisional Patent Application No. 60/723,804, filed 10/06/2005.

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