PROCEDURE

EasySep™

NEGATIVE SELECTION

V1.1.0

STEMCELL TECHNOLOGIES

ISOLATION OF MESENCHYMAL PROGENITORS FROM MOUSE COMPACT BONE

(FOR CATALOG #19771)

THE FOLLOWING IS THE RECOMMENDED PROCEDURE TO ISOLATE MOUSE MESENCHYMAL PROGENITOR CELLS FROM COMPACT BONE PRIOR TO EASYSEP™ ENRICHMENT.

1. Clean a mortar and pestle with 70% isopropanol. Remove the isopropanol from the mortar and pestle, and allow to air dry in a sterile biohazard safety cabinet for 30 minutes. Rinse mortar and pestle with sterile Phosphate Buffered Saline (PBS) just prior to use.
2. Wipe scissors and forceps with an alcohol wipe and air dry. Ensure they are completely dry as residual alcohol may reduce marrow cell viability.
3. After sacrificing the mouse, wet the pelt thoroughly with 70% isopropanol, then clip and peel it back to expose the hind limbs. Using sterile sharp scissors, make a small incision parallel and as close to the spine as possible. Hold the foot and pull the leg towards the head to remove the entire intact leg (Iliac Crest, femur and tibia) from the animal. Repeat for remaining leg. Using a scalpel, cut through the tibia just above the ankle to remove the foot. Position the leg such that the Iliac crest is facing up, and holding the femur near the knee, scrape the scalpel down the femur towards the Iliac crest to dislocate the hip joint. The femoral head should be visible once it has dislocated from the hip. Cut remaining muscle to remove Iliac crest. Cut the knee joint in the center and remove ligaments and excess tissue.
4. Using a scalpel, scrape bones thoroughly to remove muscle, and cut to remove epiphyses. Ensure that the bones are cleaned thoroughly with no remaining muscle tissue attached.
5. Place clean bones in the mortar containing 10 mL PBS with 2% FBS (Catalog #07905) and 1 mM EDTA. The solution of PBS with 2% FBS and 1 mM EDTA is now referred to as 'Buffer'.
6. Crush bones gently with pestle, using only enough force to crack open the bones. Agitate gently to free bone marrow (BM) from bone fragments and pipette Buffer off. Buffer containing BM can be filtered through a 70 µm cell strainer (Falcon, Catalog #352350) and used for other applications (for example, culture expansion or to perform the CFU-F assay).
7. Add 10 mL fresh Buffer and repeat agitation and removal of BM. Repeat wash step an additional 4 times (for a total of 6 washes) or until the majority of the BM has been removed (bone fragments turn white in color).

   A loss of cell viability and excess debris will be generated when bones are harshly ground. It is important to only use gentle pressure to crack open the bones.
8. Transfer the bone fragments to a 100 mm dish. Add 2 mL of 0.25% Collagenase Type I in PBS containing 20% FBS (Catalog #07902). Ensure bones are completely covered in solution. Let sit for 3 - 5 minutes.

   This step softens the bone, allowing it to be chopped more easily.
9. Using a scalpel, chop the remaining bone fragments into fine pieces (1 – 2 mm fragments).

   Proper bone fragmentation is required to release sufficient amounts of cells for cell separation.
10. Transfer the bone fragments and collagenase solution to a 50 mL polypropylene tube and add further 0.25% Collagenase Type I (Catalog #07902) to a final volume of 2 mL per mouse used, or a minimum of 10 mL.
11. Seal lid with Parafilm® and place tube in a shaking 37°C waterbath at maximum speed for 45 minutes. If using a bacterial culture shaker, set speed to ~200 rpm.
12. After 45 minutes, remove the tube from the shaker and add Buffer (refer to Step 6) to a final volume of 30 mL. Collect supernatant and filter through a 70 µm cell strainer (Falcon, Catalog #352350). Wash bone fragments by mixing with an additional 10 mL of Buffer and allowing fragments to settle for 3 - 4 minutes. Filter the wash through the 70 µm strainer, combining with the previously collected cells (for a final volume of 40 mL).
13. Centrifuge at 300 x *g* for 10 minutes at room temperature (15 - 25°C) with the brake on. Remove the supernatant and resuspend the cell pellet in ~200 - 500 µL of medium (note that small particles and debris may be visible in the cell suspension):

    a. For cell culture, resuspend cells in Complete MesenCult™ Medium (Mouse) (Catalog #05511).

    b. For cell separation experiments, resuspend cells in the recommended medium described in the Mouse Mesenchymal Progenitor Enrichment Kit for Compact Bone Product Information Sheet (Catalog #29122, provided).

    Transfer the cells into a smaller tube (e.g. Falcon 5 mL polystyrene round-bottom tubes (BD Biosciences, Catalog #352058).
14. Place cells on ice until ready for use.
15. Remove a small aliquot of cells and dilute 1/20 to 1/100 in 3% Acetic Acid with Methylene Blue (Catalog #07060). Count nucleated cells using a hemacytometer.
16. Expected cell recovery: 1.5 - 3.5 x 10^6 cells per mouse (2 femurs and 2 tibias). If the cell yield is >5 x 10^6 cells/mouse, this is an indication that the marrow was not depleted sufficiently.

CFU-F assay and culture expansion protocols are described on the reverse side.

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CFU-F ASSAY FOR COMPACT BONE CELLS

1. Mouse CFU-F assays initiated from compact bone cells should be initiated at 3 different cell concentrations, to ensure that there are sufficient colony numbers to accurately assess the progenitor cell content of the mouse bone cells.

   a. For cells from compact bone prepared according to the protocol described on the reverse side and grown in a hypoxic environment (5% O_2/ 10% CO_2 /85% N_2 in a Hypoxia Chamber, Catalog #27310):
   - In 2 mL Complete MesenCult™ Medium (Mouse) (Catalog #05511) plate 1,000, 5,000 and 10,000 cells/cm^2 in duplicate in a 6-well plate (e.g. plate 10,000, 50,000 and 100,000 cells/well).

   b. For cells from compact bone prepared according to the protocol described on the reverse side and grown in a normal environment (20% O_2 /5% CO_2):
   - In 2 mL Complete MesenCult™ Medium (Mouse) (Catalog #05511) plate 10,000, 20,000, and 40,000 cells/cm^2 in duplicate in a 6-well plate (e.g. plate 100,000, 200,000 and 400,000 cells/well).

   c. For enriched compact bone cells (e.g. enriched for mesenchymal progenitor cells using the EasySep™ Mesenchymal Progenitor Enrichment Kit Catalog #19771) grown in a hypoxic environment (5% O_2/ 10% CO_2 /85% N_2 in a Hypoxia Chamber, Catalog #27310):
   - In 2 mL Complete MesenCult™ Medium (Mouse) (Catalog #05511) plate 50 - 250 cells/cm^2 in duplicate in a 6-well plate (e.g. plate 500 - 2,500 cells/well).

   c. For enriched compact bone cells (e.g. enriched for mesenchymal progenitor cells using the EasySep™ Mesenchymal Progenitor Enrichment Kit Catalog #19771) grown in a normal environment (20% O_2 /5% CO_2):
   - In 2 mL Complete MesenCult™ Medium (Mouse) (Catalog #05511) plate 200 - 1000 cells/cm^2 in duplicate in a 6-well plate (e.g. plate 2,000 – 10,000 cells/well).

2. Culture cells for 10 - 13 days. No medium change is required.

   Cells from compact bone grown in the CFU-F assay occasionally undergo spontaneous differentiation to form adipocytic or chondrogenic cells. For additional information, please contact STEMCELL Technologies Technical Support (techsupport@stemcell.com).

EXPANSION PROTOCOL FOR CELLS ISOLATED FROM COMPACT BONE

1. Harvest and process mouse compact bone cells as described in the protocol on the reverse side.

2. For cells grown in a hypoxic environment (5%O_2/ 10% CO_2/85% N_2 in a Hypoxia Chamber, Catalog #27310):
   - In 2 mL Complete MesenCult™ Medium (Mouse) (Catalog #05511) plate 2 - 5 x 10^5 cells in 1 well of a 6-well plate, or
   - In 10 mL Complete MesenCult™ Medium (Mouse) plate 6 x 10^5 - 1.2 x 10^6 cells in a T-25 cm^2 tissue culture flask.

   For growth at normal oxygen levels, more cells must be plated. We recommend starting with 5 - 10X more cells, and optimizing for your own experimental conditions.

3. For enriched compact bone cells (e.g. enriched for mesenchymal progenitors using the EasySep™ Mouse Mesenchymal Progenitor Enrichment Kit, Catalog #19771) grown in a hypoxic environment (5% O_2/10% CO_2 /85% N_2 in a Hypoxia Chamber, Catalog #27310):
   - In 2 mL Complete MesenCult™ Medium (Mouse) (Catalog #05511) plate 1 - 5 x 10^4 cells in 1 well of a 6-well plate, or
   - In 10 mL Complete MesenCult™ Medium (Mouse) plate 4 x 10^4 - 1.2 x 10^5 cells in a T-25 cm^2 tissue culture flask.

   For growth at normal oxygen levels, more cells must be plated. We recommend starting with 5 - 10X more cells, and optimizing for your own experimental conditions.

4. Grow cells at 37°C in desired atmosphere for 8 - 14 days until an adherent cell layer has formed. After 8 days, if the color of the medium has become orange, a half-medium change can be performed.

   For growth at normal oxygen levels, grow cells for 10 - 21 days.

5. Observe mesenchymal cells under the microscope after 7 days to determine confluency. Once the cells have reached 80% confluency, they are ready to be passaged. 80% confluency may be reached in 7 - 16 days.

6. Passage according to the protocol provided in the MesenCult™ Technical Manual (Mouse; Catalog #28374).

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