Preparation of Mouse Bone Marrow Stromal Cells

A single-step stem cell purification method using adhesion to cell culture plastic was employed as described in the Reference.

Briefly, neonatal and adult male mouse bone marrow stromal cells were collected from bilateral femurs and tibias after sacrifice by removing the epiphyses and flushing the shaft with complete media (Iscove's Modified Dulbecco's Medium (GIBCO Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (GIBCO Invitrogen, Carlsbad, CA)) using a syringe with a 26G needle.

Cells were disaggregated by vigorous pipetting several times, and were passed through a 30-Km nylon mesh to remove remaining clumps of tissue.

Cells were washed by adding complete media, centrifuging for 5 min at 300 rpm @ 24°C, and removing supernatant.

The cell pellet was then resuspended and cultured in 75 cm2 culture flasks with complete media at 37°C in 5%CO2 in air.

BMSCs preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded.

Fresh complete media was added and replaced every three or four days thereafter.

When the cultures reached 90% of confluence, the BMSC culture was passaged; cells were recovered by the addition of a solution 0.25% trypsin-EDTA (GIBCO Invitrogen, Carlsbad, CA) and replated in culture flasks.

Cells were utilized for experimentation between passages 3-9.

Reference:

Peister A, Mellad JA, Larson BL, et al. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood 2004;103:1662-1668.

Isolation of Bone Marrow Cells

Note: Animals should be sacrificed using procedures approved by your institution.

1. Wet the pelt thoroughly with 70% isopropyl alcohol, then clip and peel back to expose hind limbs. Using sterile sharp scissors (to avoid splitting of the bone), cut the knee joint in the center and remove ligaments and excess tissue.
2. Remove the femur and tibia by severing them from the animal at the hip and ankle respectively.
3. Trim the ends of the long bones to expose the interior of the marrow shaft.
4. Collect the marrow cells in 1 to 2 mL of Iscove's MDM containing 2% fetal bovine serum (refer to Catalog #07700). Flush the marrow from the femoral shaft using a 21g needle attached to a 3 mL syringe. A smaller needle (22g or 23g) may be required to flush marrow from the tibia. Use the same medium to flush bones from one to three animals.
5. Make a single cell suspension by gently aspirating several times using the same needle and syringe.
6. Keep the cells on ice. For most applications, it is not necessary to ficoll or wash the cells.
7. Remove small aliquot of cells and dilute 1/50 to 1/100 in 3% acetic acid (refer to Catalog #07060). Count nucleated cells using a hemocytometer.

   Expect cell recovery: 1 - 2 x 10^7 per femur
   6 x 10^6 per tibia
   3 - 5 x 10^7 per mouse (2 femurs and 2 tibias)

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Procedure for Murine Colony Forming Unit Fibroblast (CFU-F) Assay

Media and Supplies

1. MesenCult™ Basal Media (refer to Catalog #05501).
2. Mesenchymal stem cell stimulatory supplements (refer to Catalog #05502).
3. Thaw mesenchymal stem cell stimulatory supplements and add contents to MesenCult™ Basal Media (complete media).
4. 6-well **tissue culture treated** plates

Cells

1. Dilute murine bone marrow cells to 1.0 x 10^7 cells/mL
2. For duplicate cultures add 2.5 x 10^5 cells/mL (5 x 10^5 cells/well), add 100 µL of 1.0 x 10^7 cells/mL to 3.9 mL complete media.
3. Vortex the tube and then plate 2 mL in each of 2 wells.
4. Ensure the cells are plated in **tissue culture treated** dishes.
5. For duplicate cultures at 5.0 x 10^5 cells/mL (1.0 x 10^6 cells/well), add 200 µL of 1.0 x 10^7 cells to 3.8 mL complete media.
6. Vortex the tube and then plate 2 mL in each of 2 wells.
7. Ensure the cells are plated in **tissue culture treated** dishes.
8. It is recommended to perform assays at 2 different cell concentrations as described above.
9. Dilute murine fetal liver cells to 4 x 10^6 cells/mL.
10. For duplicate cultures at 1.0 x 10^5 cells/mL (2.0 x 10^5 cells/well) add 100 µL of 4 x 10^6 cells/mL to 3.9 mL of complete media.
11. Vortex the tube and then plate 2 mL into each of 2 wells.
12. Ensure the cells are plated in **tissue culture treated** dishes.

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Staining and Enumeration of Murine CFU-F

At day 14, remove media from 6 well plate by decanting into waste containing bleach. Wash each well with PBS 2 times to ensure that residual FBS is removed. Allow to air dry for 5 minutes.

Add enough methanol to cover each well and incubate at room temperature for 5 minutes. Decant methanol and allow to air dry for 5 minutes.

Add enough Giemsa stain to completely cover the well and incubate at room temperature for 5 minutes.

Remove Giemsa stain. Wash with low stream tap water to remove non-bound stain. Allow to air dry and count colonies microscopically. (Examples of CFU-F provided at the back of the procedure of manual.)

Expected CFU-F Counts

Murine Bone Marrow: 20 ± 10 (5×10^5 cells/well)
(mean ± SD, n = 12)

Fetal Liver: 20 (2×10^5 cells/well)
(Preliminary experiments confirmed that complete media supports the proliferation of CFU-F from murine fetal liver).

6-well tissue culture treated dishes: Falcon Product Number B-D 353502
Corning Product Number 3506

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Helpful Hints

- Thawing of mesenchymal stem cell stimulatory supplements should preferably be performed overnight under refrigeration. If this is not possible, thawing of supplements in a 37°C water bath is permissible. (Note: do not thaw supplement in a 56°C water bath)
- Once prepared, the complete media is stable at 4°C for 1 month. If the volume of the complete media exceeds your monthly requirements, it is possible to aliquot the supplements and store these at -20°C. Therefore, smaller volumes of complete media can be prepared ensuring the supplements represent one-fifth of the total volume (i.e. 10 mL of supplements to 40 mL of basal medium).
- Cell counts should be performed in 3% acetic acid to obtain an accurate white cell count.
- Dilution and suspension of cells into complete media is recommended prior to plating in 6-well **tissue culture treated** dishes to ensure equal distribution of colonies on well surface.
- **Tissue culture treated** dishes must be used to support the proliferation of CFU-F.

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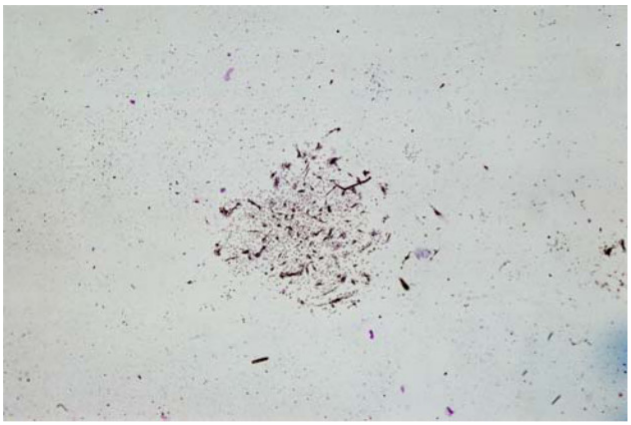
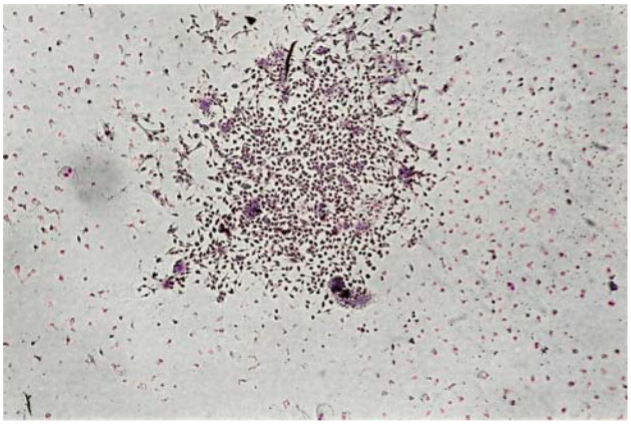
Examples of CFU-F Cultured in MesenCult™ Basal Medium for Murine Mesenchymal Cells (Catalog #05501) containing Mesenchymal Stem Cell Stimulatory Supplements (Catalog #05502)

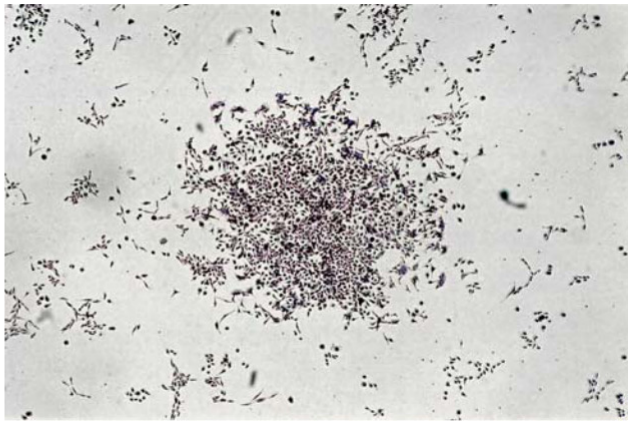


CFU-F (50X)

CFU-F (50X)



CFU-F (125X)



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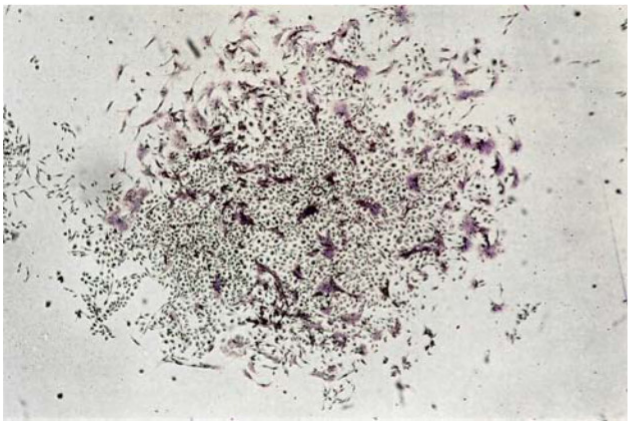
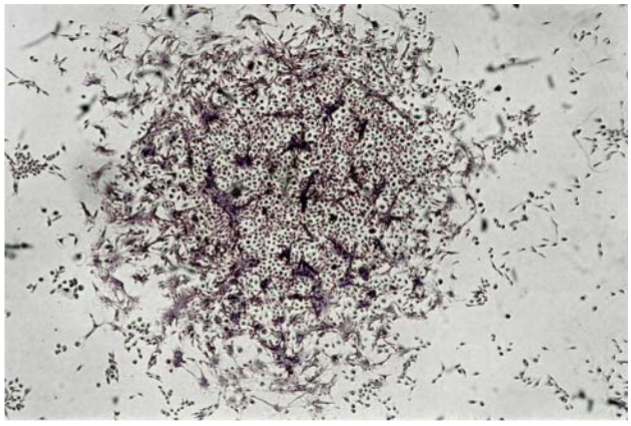


CFU-F (125X)



CFU-F (125X)