Bone marrow stromal cell culture

Reagents:

PBS:	**PBS (Ca, Mg free) +2% P/S**
Washing media:	**DMEM + glu +P/S + 2-mercaptoethanol (2-ME) (Gibco BRL cat#21985-023 0.5ml→500ml)**
Culture Media:	**MEM □-media (Gibco Cat# 12571-063) + L-gln + 20% FBS+P/S + 2-mercaptoethanol (2-ME) (Gibco BRL cat#21985-023 0.5ml→500ml)**

Procedure:

A. Preparation of bone bone marrow cells:

1. **Euthanize mice, remove the legs of mice, and then put them in a 50ml conical tube in procedure room. Keep the tube on ice when take the legs to tissue culture room.**
2. **Aseptically remove femurs/tibia in hood.**
3. **Cut off ends of each bone to expose marrow. Flush out marrow with PBS into a clean petri dish with syringe.**
4. **Filter marrow soup through a cell strainer (Falcon, Cat# 352350) into a 50ml conical tube, and then put the tube on ice before counting and plating.**

For CFU-F formation assay: Low density 1 or 2 million cells per flask (25cm^2)

For bone marrow cell growth: Low density 10-15-20 million cells /100mm dish
Plate 5ml of cell suspension into each 100mm dish

B. Preparation of feeder cells:

1. **Sacrificed 4-6 week old Hartley male guinea pig(s) (in building 14), remove the legs, and then put them in a 50ml conical tube in procedure room. Keep the tube on ice when take the legs to tissue culture room.**

2. **Aseptically remove femurs/tibia in hood.**

3. **Cut off ends of each bone to expose marrow. Flush out marrow with PBS into a clean petri dish with syringe.**

4. **Filter marrow soup through a cell strainer (Falcon, Cat# 352350) into a 50mlconical tube, and then put the tube on ice before counting and plating.**

5. **Resuspend pellet in culture media, □-irradiate (Caesium-137) for 7.5 min. with 6000 cGy by a Gemmacell irradiatioor. Count the cells.**

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7. **Aseptically remove femurs/tibia in hood.**

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9. **Filter marrow soup through a cell strainer (Falcon, Cat# 352350) into a 50mlconical tube, and then put the tube on ice before counting and plating.**

10. **Resuspend pellet in culture media, -irradiate (Caesium-137) for 7.5 min. with 6000 cGy by a Gemmacell irradiatioor.**

11. **Count the cells.**

CFU-F formation assay:

Reagents:
Culture Media: MEM □-media (Gibco cat# 12571-063) + L-gln + 20% FBS+P/S + 2-mercaptoethanol (2-ME) (Gibco BRL cat#21985-023 0.5ml→500ml)
PBS: w/o Ca, Mg
Saturated methyl violet (sigma): Dissolve methyl violet into water and filter through Whatman paper filter.

1. **Add 5 ml of mouse bone marrow stromal cells in each 3-5 of 25cm^2 tissue culture flasks; incubate for 3 hours to allow adherent cells to attach.**
2. **Wash the cells twice with 5 ml PBS DMEM medium to remove non-adherent cells.**
3. **Add 5 ml of feeder cells (10 million each flask) with or without TGF-□.**
4. **Incubate for until colonies are formed (12-14 days).**
5. **Wash the cells twice with 5 ml PBS w/o Ca and Mg++.**
6. **Fix the cells for 20min.with 3 ml of absolute methanol.**
7. **Stain the cells with alkaline phosphatase (sigma) for 20 min.**
8. **Wash with distilled water until water is clear.**
9. **Count the number of colonies.**
10. **Stain the cells with saturated methyl violet (sigma) for 20 min.**
11. **Wash with distilled water until water is clear.**
12. **Count the number of colonies.**

Passing stromal cells

Reagents:
Media: MEM-□ w/ 20%FBS, P/S L-glu + Dex (10^{-8})
Collagenase: 250u/ml in PBS w/o Ca++ and Mg++ + TLC

1. **Aspirate media.**
2. **Wash the cells with 5 ml PBS w/o Ca++ and Mg++ with EDTA.**
3. **Add 5ml of collagenase, incubate for about 20-25 minutes, and then transfer media to a 50ml conical tube with 10ml media.**
4. **Spin suspension for 5mins. at 1000 rpm, 4^0C.**
5. **Resuspend pellet in media, count**

1. **Avg count per sample Add 4ml cell solution to 60mm dishes for pass the cells.**
2. **Add 2ml cell solution to each well of a 12-well plate for bone formation assay**
3. **Add 2ml cell solution to each well of a 6-well plate for western assay**
4. **Add 300ul to each well of 8-well chamber slice for apoptosis assay.**
5. **Add 300ul to each well of 8-well chamber slice for proliferation (BrdU) assay.**
6. **Add 200ul to each well of 96-well plate for Ca^{2++} deposition assay.**
7. **Culture at 37^0C 5% CO_2.**

Proliferation assay (MTT)
Bone marrow Stromal cells
Media: MEM-□ w/ 20%FBS, P/S L-glu + Dex (10^{-8})
MTT kit: Boehringer Mannheim cat# 1465 007

1. **Aspirate media.**
2. **Wash the cells with 5 ml PBS w/o Ca++ and Mg++ with EDTA.**
3. **Add 5ml of collagenase, incubate for about 20-25 minutes, and then transfer media to a 50ml conical tube with 10ml media.**
4. **Spin suspension for 5mins. at 1000 rpm, 4^0C.**
5. **Resuspend pellet in media, count cells**
6. **Add 100ul cell solution to wells of 96-well plate (Labeled 2000cells/well).**
7. **Add 50ul media 50ul cell solution to wells that labeled 1000 cells/well).**
8. **Culture for 1, 2, 3, 4, 5, 6, 7 days**
9. **Add 10ul MTT labeling solution into each well, incubate for 4 hours, and add 100ul solubilization solution and incubate for overnight**
10. **Measure OD at 560nm.**