Mouse Embryonic Stem Cell Culturing Protocols

General Guidelines for Handling Mouse ES cells

• mES cells are cryopreserved in plastic cryovials and shipped on dry ice. If storing the mES cells before thawing, store in liquid nitrogen vapor. Storage directly in liquid nitrogen may result in cracking of the o-rings.

• It is highly recommended that a small number of vials are cryopreserved as a master stock before beginning any experimentation

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Table 1. Media and Reagents

MEF medium	**mES growth medium** (Make medium in small batches to avoid glutamine breakdown)	**mES cryopreservation medium**
DMEM 10% FBS mM Sodium Pyruvate 100 µM Non-essential amino acids 2mM L-glutamine	High glucose DMEM 15% Fetal Calf (Bovine) Serum Glutamax 1mM Sodium Pyruvate 100 µM Non-essential amino acids 0.1 mM 2-mercaptoethanol Stock 1.0 µg/ml Puromycin 0.2 µg/ml Doxycycline 10^3 units/ml LIF	High glucose DMEM 30% FCS (heat activated) 10% DMSO

Plating Mouse Embryonic Fibroblasts (MEFs)

DR4 irradiated MEFs may be obtained from a number of qualified vendors. Optimal MEF density per well of a 6-well plate should be determined by individual labs each time a new lot of MEFs is obtained. When determining MEF density, thaw MEFs and plate at varying densities between 6 x to 5 x 10^5 cells per well. Passage mES cells onto MEFs and maintain for 2 days. Assess the ability of MEFs to maintain mES cells in the undifferentiated state using morphology, growth rate and alkaline phosphatase expression.

1. Coat wells with 0.1% gelatin and incubate for a minimum of 20 minutes at 37°C in an incubator.
2. Thaw one vial of DR4 MEFs by swirling in 37°C water bath and mix in 10 ml of warm MEF media.
3. Centrifuge at 1100 rpm for 2 minutes.
4. Aspirate medium and resuspend pellet in 10 ml of MEF medium.
5. Remove gelatin-coated plate from incubator, aspirate gelatin and replace with 2 ml/well of MEF media.

6. Mix cell suspension and count viable cells by Trypan blue dye exclusion.
7. Using viable cell number, calculate and aliquot appropriate number of cells to yield predetermined optimal cell density into each well of a 6-well plate (be sure to mix cell suspension several times during plating to avoid settling of cells).
8. Place in incubator and shake plate back/forth and left/right.
   NOTE: Failure to gently shake plate back and forth may result in uneven seeding.
9. Incubate overnight or up to 5 days before using as feeder layer for mES cells.

Thawing murine ES cells

For optimal thawing result of murine ES cells, MEFs should be plated on gelatin-coated plates at least 1 day prior to use to allow adherence and flattening prior to mES cell plating. Seed mES cells up to 5 days after plating MEFs.

1. Remove ES cells from liquid nitrogen/dry ice and thaw quickly in 37°C water bath.
2. Transfer cell suspension to sterile 15 ml tube containing 10 ml warm growth medium and mix cells.
3. Centrifuge conical tube containing cells at 1100 rpm for 2 minutes at room temperature.
4. Aspirate freeze medium and resuspend cells into 2 ml of warm ES growth medium.
5. Plate cells in 1- 2 wells of a 6-well plate (1.0×10^6 to 1.8×10^6 cells per well) containing MEF feeder cells.
6. Maintain cells by daily medium exchange.
7. Passage cells at 75%-90% confluence (Image 1).

Passaging of murine ES cells

Passage cells every 2-3 days depending upon the growth rate of cells. The optimal condition is to maintain cells at approximately 80% confluency on day 2 or 3 (Image 1). To avoid spontaneous differentiation, do not allow cells to become confluent (Image 2). Split ratios range from 1:4 to 1:10. Optimal cell number for seeding in a 6 well plate is between 1.0×10^6 and 1.8×10^6 cells per well. (approximately 1.0×10^5 cells/cm^2).

1. Remove spent medium from culture and rinse with PBS.
2. Add 1 ml/well accutase and place in incubator for 5 minutes or until cells begin to dissociate from plate.
3. Triturate accutase solution 2-3 times to dissociate cells from plate.
4. Transfer the detached cell aggregates to a 15 ml conical tube containing 5 ml mES growth medium.
5. Rinse each well with an additional 1 ml of growth medium to collect any remaining aggregates. Add the rinse to conical tube containing cells.
6. Take small aliquot of cells for cell count.

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7. Centrifuge conical tube containing cells at 1100 rpm for 2 minutes at room temperature.

> **NOTE**: When using accutase, cells can be plated directly into culture vessel without centrifuging to pellet cells as long as accutase is inactivated by the addition of the growth medium at a 3:1 ratio (medium to accutase).

8. Remove the supernatant from conical tubes and resuspend cells in appropriate volume of growth medium such that there is an appropriate cell density for cell culture vessel **(**1.0 X 10^6 to 1.8 x 10^6 cells per well of a 6-well plate (approximately 1.0 x 10^5 cells/cm^2).

9. Seed cells onto prepared MEF-containing plates. Rock plates gently back and forth, sideways and diagonally to achieve uniform cell distribution.

> **NOTE:** Failure to gently shake plate back and forth may result in uneven seeding.

Cyropreservation of murine ES cells

1. Remove spent medium from culture and rinse with PBS.
2. Add 1 ml/well accutase and place in incubator for 5 minutes or until cells begin to dissociate from plate.
3. Triturate accutase solution 2-3 times to dissociate cells from plate.
4. Transfer the detached cell aggregates to a 15 ml conical tube containing 5 ml mES growth medium.
5. Rinse each well with an additional 1 ml of growth medium to collect any remaining aggregates. Add the rinse to conical tube containing cells.
6. Centrifuge conical tube containing cells at 1100 rpm for 2 minutes at room temperature.
7. Remove supernatant and resuspend cells in appropriate volume of pre-cooled freeze medium (1 ml per well of a 6-well plate).
8. Transfer 1 ml of cell suspension to cryovials on ice.
9. Place cryovials in isopropanol freezing container and store at -80°C overnight.
10. Transfer vials to liquid nitrogen vapor for permanent storage.

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Table 2. Troubleshooting Tips

Problem	Observation of problem	Possible Causes and solutions
Spontaneous differentiation within culture (Image 2)	Differentiation can look different depending on culture and cause of differentiation: 1. flattened cells that have dark and spiky boundaries 2. colonies appear as individual cells instead of one large syncial mass 3. flattened colonies	1. Low feeder layer quality- this can be prevented by testing optimal density for feeder layers prior to using in an experiment 2. Inappropriate feeder layer density- when testing feeder layers for trophic support of ES cells, test at varying densities to determine appropriate density per lot of feeder layers 3. Low LIF concentration 4. Over confluence (allowing cultures to exceed 90% confluence by day 3
Dying or differentiating cells	Large colonies with necrotic centers Cells will appear healthy and then appear to round up and lift off plate	Cells have not been passaged for 4 or more days • Passage cells and plate at higher density to ensure 70-90% confluence by day 3
Non-uniform distribution of colonies with culture vessel	Areas within culture vessel that are highly confluent mixed with areas that do not contain colonies	Cells were not distributed evenly throughout culture vessel or culture vessel was disturbed following plating • Rock plates following plating- usually rocking plates back and forth and then side to side produces a fairly uniform distribution
Cells are growing but do not appear to be doubling as expected	Cells are not reaching 70-90% confluence by day 3	Plating at lower than optimal cell density Suboptimal culturing conditions; check the following reagents 1. FCS lot 2. Glutamine 3. MEF Feeders

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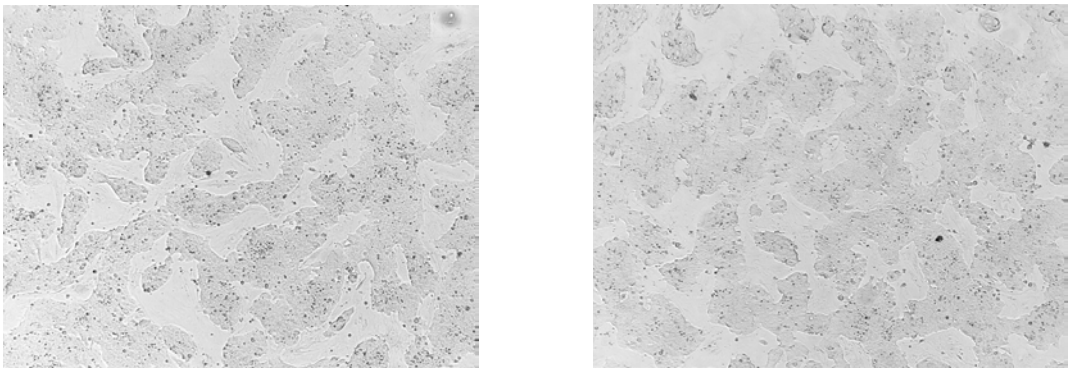

Image 1: mES cultures at 60 to 80% confluence. Note round morphology with distinct borders.

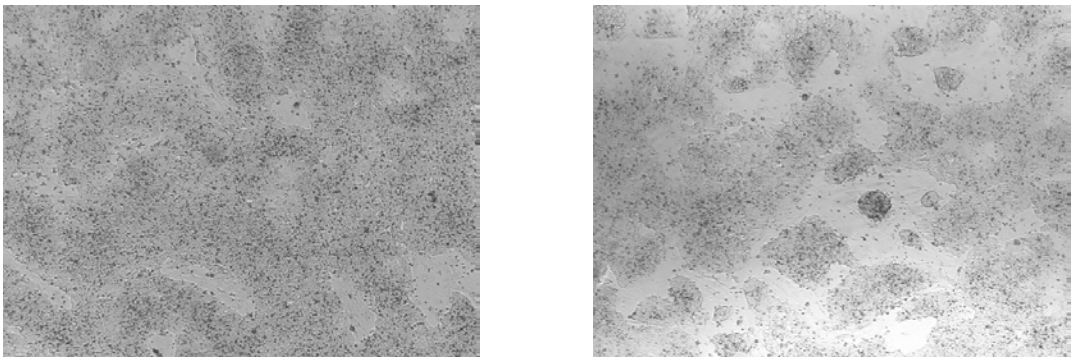

Image 2: Overly confluent mES cultures with areas of spontaneous differentiation.