

Maintenance of mouse Embryonic Stem cells in culture

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Embryonic Stem (ES) cells are grown on a feeder layer of mitotically inactivated Mouse Embryonic Fibroblast (MEF) cells in order to keep them pluripotent. You need to use gelatin-coated tissue culture dishes in order to facilitate and increase ES cell adhesion.

Gelatin coating procedure

To prepare gelatin-coated tissue culture dishes, distribute a thin layer of 0.1% gelatin solution to cover the bottom of the plate and incubate 1 hr at room temperature. Then, remove the gelatin solution and keep the plate at room temperature for at least 1 hr before use them. Several gelatin-coated plates can be prepared and stored at room temperature for several months.

Maintenance of MEF cells

MEF cells are maintained in complete MEF medium (high glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine). Usually, when thawing MEF cells, the content of a cryo vial is transferred to one 100-mm tissue culture dish (you don't need to use gelatin-coated tissue culture dishes to culture MEF cells). At confluence, subculture MEF cells 1:4 (or 1:5) using the following procedure:

1. Remove MEF medium and rinse twice with 4-5mL of 1X PBS.
2. Add 2 mL of Trypsin solution (0.05%) to each 100-mm tissue culture dish and then incubate in the humidified incubator at 37°C for 2-3 min.
3. While incubating the cells, fill 4 tissue culture dishes with MEF medium. Add 8 mL of MEF medium for each 100-mm dish.
4. Remove the plate from the incubator and swirl to dislodge the cells from the bottom of the plate.
5. Add 6 mL of MEF media to floating cells and gently pipet up and down several times to obtain a single-cell suspension.

6. Plate 2 mL of cell suspension into each of the four 100-mm dishes. Gently agitate the plates back-and-forth and side-to-side to distribute the cells. Incubate in the humidified incubator at 37°C.

Track the passage number of MEF cells. MEFs can be sub-cultured up to 1:5 once a week and maintained in culture up to the 25th passage before new batches/vials of cells are thawed.

Mitomycin C treatment of MEF to prepare feeder layers for ES cells

At confluence, MEF cells are treated with Mitomycin C to halt the division of the cells when they are still able to condition the medium. Prepare plates of Mitomycin C-treated MEF cells once a week, to provide a fresh substrate for undifferentiated ES cells. Use the following procedure:

1. To one 100 mm-dish of confluent MEF cells: remove medium, add 6 mL of fresh MEF medium containing 50 µl of Mytomycin C solution (1mg/mL).
2. Incubate at 37°C for at least 3 h.
3. Alternatively: remove medium and add 10 mL of fresh MEF medium containing 10 µl of Mytomycin C solution and incubate overnight.

For subculture:

1. Remove Mytomycin C-containing medium and rinse with 5 mL of 1X PBS. Repeat this step 2 times.
2. Add 2 mL of 0.05% trypsin solution and return the plate to the incubator for 2-3 min.
3. Remove the plate from the incubator and swirl to dislodge the cells from the bottom of the plate. Add 8 mL of MEF medium and collect all the cells.
4. Prepare 5 gelatin-coated 100 mm-plates; add 8 mL of MEF medium to each plate. Dispense 2 mL of cell suspension to each plate. Gently move the plates back-and-forth and side-to-side to distribute the cells. Incubate at 37°C. Now the cells are ready to be used as feeder layers. Mitomycin C-treated MEF cells can be stored at 37°C in a humidified incubator up to 1 week.

Maintenance of ES cells

It is important do not keep ES cells in culture for long periods in order to maintain the pluripotency. Extensive culturing can result in inconsistent or uncontrolled differentiation. To sub-culture ES cells 1:5 in 60-mm dishes:

1. Remove 1 plate of sub-clonfluent ES cells from the incubator and wash the cells twice with 2 mL of 1X PBS.

2. Add 1 mL of 0.05% trypsin solution.
3. Return the plate to the incubator for 1-2 min.
4. While incubating, remove five 60-mm tissue culture dishes of Mitomycin C-treated MEFs from the incubator. Aspirate MEF medium and add 4 mL of ES medium (high glucose DMEM medium supplemented with 15% FBS, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin/streptomycin and 10^3 U/mL LIF) to each plate.
5. Remove the plate containing ES cells from the incubator and swirl to dislodge the cells from the bottom of the plate.
6. Add 1 mL of ES medium to the plate and pipet up and down with a 1-mL pipetman in order to obtain a single-cell suspension.
7. Distribute 0.4 mL of the ES cell suspension to each of the 5 dishes. Right after plating ES cells, gently move the plate back-and-forth and side-to-side and incubate at 37°C. The ES media must be changed every day and ES cells sub-cultured every 2-3 d. Track passage number of ES cells.

Freezing ES cells

1. Grow cells to the exponential phase in a 60-mm dish.
2. Wash twice with 2-3 mL of 1X PBS.
3. Add 1 mL of 0.05% Trypsine solution and incubate 1-2 min at 37 °C.
4. Remove the plate from the incubator and swirl to dislodge the cells from the bottom of the plate.
5. Add 1 mL of complete ES growth medium, harvest cells and collect in a 15 mL Corning tube.
6. Pellet by centrifugation (800g, 4-5 min) and gently resuspend in an appropriate amount of freezing medium (10% DMSO-90% FBS). Typically, we use 0.5 mL/60-mm dish.
7. Dispense into 0.5 mL aliquots using freezing vials.
8. Store the vials at -80°C for 24 h.
9. Transfer the tubes to liquid nitrogen for long-term storage.