

# ES Cell Protocols

## Thawing:

1. Quickly transfer the vial of cells from the liquid nitrogen freezer/or dry ice to a 37°C water bath. Thaw the vial in the 37°C water bath (about 2 minutes).
2. After the liquid is completely thawed, centrifuge the cryovial at 1000 rpm for 5 minutes.
3. When you remove the vial from the centrifuge you will see a small pellet of cells at the bottom of the vial. Sterilize the outside of the vial with ethanol prior to opening. Pipette off / aspirate the supernatant carefully without disturbing the pellet.
4. Add 1 mL of M15 medium to the vial and break up the pellet by gently pipetting up and down with a 1000µL micropipettor + tip until the cells are evenly in suspension.
5. Transfer the entire volume of cells to a 6cm feeder plate, fed 1 hour in advance with 4 mL M15.
6. Gently swirl the plate along the X and Y axes to evenly distribute the cells.
7. Incubate at 37°C, 5%CO<sub>2</sub>, 95% RH

On average, cultures take 2-3 days after thawing to reach confluency sufficient for passage to a 10cm plate.

## Transfer from 6cm to 10 cm feeder plate:

1. Pre-feed the 10cm feeder plate AND the ES cells on the 6cm plate 2-3 hour in advance.
2. To trypsinize the ES cells, wash the plate with 5mL PBS--aspirate off the PBS, then add 0.7 mL trypsin solution and distribute evenly across the surface. Incubate at 37°C for 7 minutes.
3. After 7 minutes, add 4 mL M15 medium, and triturate the cells using a transfer pipette (40x). The goal is to produce a suspension of cells with NO clumps. Please keep in mind that C57Bl/6 cells are tightly adherent and require vigorous trituration and perhaps even increased incubation in trypsin (up to 20 min) to break up clumps. This improves the distribution of the cells on the plate and is critical for preventing differentiation.
4. Transfer the ¼ to ½ the volume of cells to the pre-fed 10cm plate, and swirl along the X and Y axes to evenly distribute the cells.

## **Freezing ES Cell Cultures:**

1. Pre-feed the 10cm plate with M15 2-3 hours prior to starting the freezing process.
2. Wash the ESC culture plate with 10 mL PBS (2x) —aspirate off the PBS.
3. Add 1 mL trypsin solution, distribute across the surface of the cells, and incubate for 7 minutes at 37°C.
4. Add 5 mL M15 to the plate, and triturate the cells (40x) with a transfer pipette.
5. Transfer the cell suspension to a 15 mL conical centrifuge tube, centrifuge at 1000 rpm for 5 minutes.
6. Pipette off the supernatant without disturbing the cell pellet.
7. Using a transfer pipette, add 1.5 mL M15 and break up the pellet so the cells are evenly in suspension.
8. Using a transfer pipette, SLOWLY add 1 mL 2X FM (Freezing Medium) 0.25 mL, 0.25 mL and 0.5 mL, mixing between additions.
9. Transfer 0.65 mL to each of 3 cryovials (internal threads).
10. Freeze at  $-86^{\circ}\text{C}$  in a close fitting Styrofoam container, to approximate  $-1^{\circ}\text{C}$  per minute cooling rate.
11. After 24 hours, transfer the vials to  $\text{LN}_2$  liquid immersion storage.

## Reagents and Media Preparation

The ES cells must be grown in the presence of **LIF** (Leukemia Inhibitory Factor), either added to the culture medium or produced by feeder cells engineered to secrete LIF.

**FBS:** fetal bovine serum qualified for maintenance of ES cell lines and feeders, evaluated for performance in the following assays: plating efficiency, cytotoxicity screen, growth promotion, morphology and differentiation.

**DMEM:** Dulbecco's modified Eagle's medium (liquid) containing 4500 mg/L D-glucose, plus sodium bicarbonate but NO sodium pyruvate. Qualified for use with ES cell lines (see FBS above).

**GPS:** 100X Amino acid/antibiotics solution  
200mM L-glutamine  
5,000 U/mL penicillin G, sodium salt  
5,000 µg/mL streptomycin sulfate

**PBS,** Phosphate buffered saline, without calcium and magnesium.

**Trypsin:** 0.25% trypsin and 0.04% EDTA - 2Na in PBS.

**β-ME:** 100X =  $1 \times 10^{-2}$  M β-mercaptoethanol in PBS

### M15 Medium

500 mL

500 mL DMEM  
92 mL FBS  
6.1 mL GPS  
6.1 mL 100x β-ME

1000 mL

1000 mL DMEM  
184 mL FBS  
12.2 mL GPS  
12.2 mL 100x β-ME

### 2X FM Freezing Medium

Combine in this order:

20 mL DMEM  
20 mL FBS  
10 mL DMSO

Filter sterilize, store at 4°C. Use within 1 week.