



Growing feeder-independent embryonic stem cells§

We use feeder-independent ES cell lines derived from the 129/Ola strain of mice (Nichols et al., Development 110, p.1341, 1990). These cells are easy to maintain and significantly reduce the amount of tissue culture required. Parental cell lines (CGR8 and E14Tg2A) were established from delayed blastocysts on gelatinized tissue culture dishes in ES cell medium containing leukocyte inhibitory factor (LIF) (Nichols et al., 1990). Sublines were isolated by plating cells at a single-cell density, picking and expanding single colonies, and testing several clones for germline competence. The majority of BayGenomics cell lines are derived from the E14Tg2A.4 subclone.

Tissue culture reagents

1. Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (Gibco-BRL).
2. 2 mercaptoethanol stock solution: Add 70 μ l of beta-mercaptoethanol (Sigma) to 20 ml of distilled, deionized water (Gibco-BRL). Filter sterilize and store at 4°C for up to 2 weeks.
3. ES cell medium: 1 \times GMEM medium (Sigma, G5154) supplemented with 2 mM glutamine (Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 1 \times nonessential amino acids, 10% (v/v) fetal bovine serum (characterized, Hyclone), a 1:1000 dilution of beta-mercaptoethanol stock solution, and 500-1000 units per ml of leukocyte inhibitory factor (Chemicon catalog # ESG1107).
4. Freezing medium: Add DMSO (Dimethyl Sulphoxide, tissue-culture grade, Sigma) to ES cell medium to a final concentration of 10% (v/v). Filter sterilize. Make fresh before use.
5. Trypsin solution: Add 100 mg of EDTA tetrasodium salt (Sigma) to 500 ml of PBS. Filter sterilize and add 10 ml (for 1 \times) or 20 ml (for 2 \times) of 2.5% trypsin solution (Gibco-BRL) and 5 ml of chicken serum (Gibco-BRL). Store in 20 ml aliquots at -20°C. (Note: 2.5% trypsin solution should be aliquoted and stored at -20°C to avoid multiple freeze-thawing cycles.)
6. 0.1% gelatin: Add 25 ml of 2% bovine gelatin solution (Sigma) to 500 ml of PBS. Store at 4°C.
7. Geneticin (Gibco-BRL): Dissolve powder in PBS to make a 125 mg/ml stock solution (active concentration). Filter sterilize and store at -20°C. Add 0.56 ml to each bottle (560 ml) of ES cell medium. (Note: The concentration of Geneticin

should be titrated to determine the minimum concentration that will kill nontransfected ES cells in 5 days.)

Thawing ES cells

ES cells are frozen in medium containing 10% DMSO. Since DMSO can induce the differentiation of ES cells, we advise thawing the cells late in the day and changing the medium the following morning to minimize the effects of residual DMSO.

1. Coat a 25 cm² tissue culture flask with 0.1% gelatin and aspirate off immediately before use.
2. Thaw ES cells (approximately 5×10⁶ cells, equivalent to one confluent 6-well or 1/2 of a confluent 25 cm² flask) in a 37°C water bath and dilute into 10 ml of prewarmed ES cell medium.
3. Pellet the cells by spinning for 3 minutes at 1200 rpm in a bench-top clinical centrifuge.
4. Aspirate off medium and gently resuspend cells in 10 ml of prewarmed medium.
5. Transfer cell suspension to a 25 cm² flask and grow at 37°C in a humidified 6% CO₂ incubator.
6. Change medium the following day to remove dead cells and residual DMSO.

Passage and expansion of ES cell cultures

ES cells are routinely passaged every 2 days, and the medium is changed on alternate days. Thus, ES cells require daily attention. In our experience, feeder-independent ES cells grow rapidly and quickly acidify the medium, turning it yellow. Allowing the cells to acidify the medium (by not changing the media every day or by passaging the cells at too low a dilution) will cause the cells to undergo crisis, triggering excess differentiation and cell death, after which their totipotency cannot be guaranteed. Plating cells at too low a density, insufficient dispersion of cells during passage, or uneven plating can cause similar problems, as the cells will form large clumps before reaching confluence and the cells within these clumps will differentiate or die. Germline transmission is a significantly reduced in cells that have been mistreated, even when they appear healthy at the time of injection.

1. For a confluent 25 cm² flask of cells aspirate medium off and wash with 5-10 ml of prewarmed PBS, pipetting it away from the cells. Rock flask gently and aspirate medium. Repeat.
2. Cover cells with 1 ml of 1× trypsin solution and return to 37°C incubator for 1-2 minutes or until cells are uniformly dispersed into small clumps.
3. Add 9 ml of medium to inactivate the trypsin.

4. Count cells and add 1×10^6 cells (usually 1/10 of a 25 cm² flask) to a freshly gelatinized flask.
5. To expand ES cells for electroporation (requiring a total of 1×10^8 cells), seed 3×10^6 cells (1/3 to 1/4 of a confluent 25 cm² flask) into a gelatinized 75 cm² flask and add 30 ml of medium. Add 20 ml of medium on the following day. Once the cells reach confluence, trypsinize the contents of the 75 cm² flask and add 5×10^6 cells (1/5 of a confluent 75 cm² flask) to each of three 175 cm² gelatinized flasks containing 50 ml of medium. Add an additional 30 ml of medium the following day.

Freezing ES cells

1. Trypsinize a confluent 25 cm² flask of cells (approximately 1×10^7 cells) as described above.
2. Collect trypsinized cells in 9 ml of medium and pellet for 3 minutes at 1200 rpm.
3. Aspirate off medium and resuspend cell pellet in 1 ml of freshly prepared freezing medium. Aliquot 0.5 ml of cells into two cryotubes.
4. Freeze the vials at -80°C overnight and transfer to liquid nitrogen for long-term storage.

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