

Supplementary Methods

Preparation of human ES medium

For 100ml hES medium (Amit et al., 2000)

1. Add 10ml PBS (w/o Ca⁺⁺, Mg⁺⁺) to 0.146g L-glutamine in a 15ml tube.
2. Add 7_1 of β -mercaptoethanol to the L-glutamine/PBS mix well.
3. Into a 225ml 0.2 micron cellulose acetate filtering unit add:
 - 80ml knockout DMEM
 - 20ml Knockout SR.
 - 1ml L-Glutamine/ β -mercaptoethanol solution
 - 1ml 100X non-essential amino acid solution
 - 200_1 of 2_g/ml bFGF stock.
 - Antibiotics (optional, e.g. Gentamicin)
4. Filter.
5. Store at 4°C and use within two weeks.

Final Concentration	Stock Concentration	Cat #
80% Knockout DMEM		Gibco 10829-018
20% GIBCO knockout SR		Gibco 10828-028
1% Non-essential amino acid solution	100X MEM non-essential amino acid solution	Gibco 11140-035
1mM L-glutamine	0.146g in 10ml PBS	Gibco 21051-016
0.1mM β -mercaptoethanol	14.3M β -mercaptoethanol	Sigma M-7154
4ng/ml human bFGF	2_g/ml in PBS (w/o Ca ⁺ , Mg ⁺⁺) with 0.1% BSA	Gibco 13256-029

Solutions and Notes:

- Human bFGF: 10_g in 5ml of 0.1% BSA in PBS (w/o Ca⁺, Mg⁺⁺). Aliquot and store at -20°C

Passage of hES cells

1. Aspirate medium from flask.
2. Add 2ml of Collagenase per T25 and incubate at 37°C in CO₂ for 8-10min. – until the edges of the hES colonies start to curl.
3. Gentle scrape with glass beads (or, if using plates, the tip of a glass Pasteur pipette rounded off with a flame).
4. Add a 5ml of hES medium and gently titurate. Transfer to a 10ml centrifuge tube.
5. Spin at 50 xg for 3min at 4°C.
6. Whilst the cells are spinning, remove the medium from the fresh flasks of MEF feeders and wash once with PBS.
7. Aspirate supernatant, leaving hES cell pellet.
8. Remove PBS from MEFs.
9. Gently flick tube to disperse hES pellet.
10. Gently re-suspend hES cell pellet in an appropriate volume of hES medium (e.g. 4ml for a 1:4 split into 4 x T25s (at 1ml per T25)) and distribute between flasks of feeders. Add 4ml hES medium per T25.
11. Carefully place in a 5% CO₂ incubator at 37°C, maintaining an even distribution of cells across the flask.

Notes:

- Human ES cells require feeding every day with fresh hES medium.
- **Collagenase IV solution** is made by adding 1mg/ml collagenase type IV (Gibco cat no17104-019) in DMEM/F12 and sterilised with a 0.2 micron cellulose acetate filter. Store at 4°C. Use with a 2 weeks.
- **MEFs:** Strain MF1 12.5 dpc embryos. Mitomycin-c inactivated and seeded at 6×10^3 per cm² of pre-gelatinised tissue culture plastic.
- **Feeder-Free conditions:** HES lines were grown on Matrigel (Becton Dickinson, Bedford, MA, USA) instead of layers of MEFs in non-conditioned hES medium.

Freezing Human ES cells.

1. Aspirate medium from flask
 2. Add 2ml of Collagenase per T25 and incubate at 37°C in 5% CO₂ for 8-10min. – until the edges of the hES colonies start to curl.
 3. Gentle scrape with glass beads (or, if using plates, the tip of a glass Pasteur pipette rounded off with a flame)
 4. Add a 5ml of hES medium and very gently titurate – larger clumps survive the freezing process better than smaller clumps. Transfer to a 15ml centrifuge tube.
 5. Spin down at 50 xg for 3 minutes.
 6. Aspirate medium and very gently flick tube to disperse pellet. Wash the pellet by re-suspending pellet in 5ml hES medium.
 7. Spin down at 50 xg for 3 minutes.
 8. Carefully re-suspend the pellet in 600_1 ice-cold Freezing Medium and transfer 200_1 per cryovial. We typically freeze one T25 into 3 cryovials. Keep cryovials on ice and when ready place in a freezing container¹ and transfer to a -70°C freezer.
 9. The next day transfer cryovials from -70°C to liquid nitrogen for long-term storage.
- **Freezing Medium:** 90% fetal calf serum:10% DMSO
 - ¹e.g. Nalgene Cryo 1°C Freezing Container, cat no 5100-0001