

Standard Operating Procedure (SOP) for human ES/iPS cell culture on mouse feeders

Reagents

- Irradiated CF1 MEFs: GlobalStem cat# 6001G
- DMSO: Sigma cat# D2650-100ML
- Defined FBS: Hyclone cat# SH30070.01
- FBS: Invitrogen cat# 16000-044
- 0.1% geletin: Millipore cat# ES-006-B
- 1x PBS: Invitrogen cat# 14190-136
- bFGF: Invitrogen cat# PHG0023
- ROCK inhibitor Y27632: Calbiochem cat# 688000
- 7.5% BSA solution: Invitrogen cat# 15260-037
- DMEM: Invitrogen cat# 11965-118
- Pen/strep: Invitrogen cat# 15140-155
- L-glutamine: Invitrogen cat# 25030-156
- Non essential amino acid (MEM-NEAA): Invitrogen cat# 11140-050
- DMEM/F12: Invitrogen cat# 11330
- Knockout Serum Replacement (KOSR): Invitrogen cat#10828
- Beta-Mercaptoethanol (BME): Sigma cat# M7522 -100ml
- Collagenase IV: Invitrogen cat# 17104-019

Media

MEF feeder media (500 mls):

- DMEM (450 ml)
- 10% FBS (50 ml)
- Pen/strep (5ml)
- L-glutamine (5ml)
- MEM-NEAA (5 ml)

Filter sterilize

hES cell media (500 mls):

- DMEM/F12 (400 ml)
- KOSR (100 ml)
- Pen/strep (5 ml)
- L-glutamine (5 ml)
- MEM-NEAA (5 ml)
- BME (3.5 ul)
- bFGF (10 ng/ml final concentration)

Filter sterilize

Reagent setup

Gelatin-coated culture dishes Add 0.1% gelatin to cover the dish and incubate at room temperature for 20 minutes. Aspirate excess liquid and wash with 1xPBS. Use right away.

bFGF Prepare 0.1% BSA in PBS by diluting from the 7.5% solution and sterile filter with a 0.2 μm low protein binding syringe filter. Reconstitute bFGF to 20ug/ml and store at -80°C in aliquots. Stable up to 6 months.

2x ES/iPS cell-freezing medium 20% DMSO, 80% defined FBS. Filter sterilize.

Collagenase IV Resuspend powder to 1mg/ml in DMEM/F12. Filter sterilize with a 0.2 μm low protein binding syringe filter.

Plating MEF feeders

ES/iPS cells are co-cultured with MEF feeders on gelatin-coated tissue culture dishes. We typically plate 1×10^6 feeders on 10-cm tissue culture dishes or 1.6×10^5 cells per well for 6-well plates and culture overnight before seeding ES/iPS cells. The feeders should be used within 2 days.

Thawing ES/iPS cells

Each vial of ES/iPS cells should be thawed in 1 well of a 6-well plate. These cells should be kept in as large of clumps as possible to increase survival efficiency so one must minimize the amount of pipetting when thawing these vials.

1. Set up 2x 15 ml cell culture tubes. In tube 2, add 10 ml of pre-warmed hES media including 10 μM of ROCK inhibitor Y27632 (**ROCK inhibitor increases the initial survival of cells after thawing and should be included in the thawing media and the culture media before the first media change.**)
2. Partially thaw the frozen vial of ES/iPS cells in 37°C water bath, until there is a small piece of ice remaining. Spray the vial with 70% ethanol to sterilize.
3. Taking 1 ml of media from tube 2, slowly add the pre-warmed media drop wise to the vial and transfer the liquid content with cells into tube 1. Add the remaining media from tube 2 to tube 1 slowly while moving tube 1 back and forth to mix the

cells.

4. Spin tube 1 at 200g for 4 min.
5. Meanwhile, wash with 1x PBS one well of a 6-well plate that was plated with feeders and add 2 ml hES media.
6. Aspirate media from tube 1 and gently resuspend the pellet with 1 ml of hES media, trying to avoid disrupting the chunks of cells, and transfer to one well of a 6-well plate.
7. Change the medium after 36 to 48 hours. **Omit ROCK inhibitor starting at this point.**
8. Feed cells daily with 2 ml media. Colonies should emerge anywhere from 5 to 10 days.

Passaging ES/iPS Cells

Newly thawed ES/iPS cells should be split manually for the first 1 or 2 passages until cells are growing well. Then enzymatic passaging can be used as described below. For manual passaging protocols and additional protocols, please visit the WiCell website:

http://www.wicell.org/index.php?option=com_content&task=section&id=23&Itemid=273

Optional: ROCK inhibitor can be added to the media when cells are passaged to improve seeding efficiency, but should be omitted after the first media change.

1. Mark differentiated colonies with pen on the bottom of the plate before splitting.
2. Wash cells with either warm hES medium or 1x PBS.
3. Add 1 ml of Collagenase IV per well of a 6-well plate and incubate at 37°C for 5-10 minutes (expect to see visible curling or thickening of colonies around the edges)
4. Aspirate off the enzyme and remove the marked differentiated colonies via slow-vacuum aspiration or pipet scraping. Make sure cells do not dry out if using vacuum method.
5. Gently wash the cells on the plate with warm 1x PBS or hES medium and aspirate off the wash.
6. Add 1 ml of fresh hES medium to the cells and scrape the entire well to lift the colonies using a cell lifter (i.e. Corning #3008).

7. Add the cell suspension to a 15-ml cell culture tube.
8. Wash the well with 2 ml of hES medium and add the wash to the same tube.
9. Triturate the cell suspension to get medium-small fragments (~50-200 cells per fragment). Avoid over-triturating since that will lead to cell death, especially when colonies are broken down to single cell suspensions.
10. Plate 1 ml of cells into 1 well of a 6-well plate of MEFs that was pre-washed with 1x PBS and containing 1 ml of hES media to give a splitting ratio of 1:3.
11. We recommend splitting 1:3 if the cells are close to being confluent; but depending on cell growth rate, splitting ratios between 1:3 and 1:10 should be maintained.
12. Refresh with hES media everyday until cells are ready to be split again (usually between 5-7 days).

Freezing ES/iPS Cells

ES/iPS cells should be frozen in as large clumps as possible to increase survival efficiency. When resuspending cells, all attempts should be made not to break up cell colonies too much.

1. When cells are confluent, perform steps 1-4 above.
2. Add 1ml of hES media to each well of a 6-well plate and scrape the entire well to lift the colonies using a cell lifter (i.e. Corning #3008).
3. Collect cell suspensions from all wells to a 15-ml cell culture tube and spin at 200g for 4 min.
4. Aspirate off the media and add 0.5 ml of fresh media per well of cells collected and resuspend the cells 2-3 times without breaking up the cell clumps too much.
5. Slowly add an equal volume of 2x freezing media to cells drop wise while gently shaking the tube to mix.
6. Resuspend the cells 1-2 times more to mix and aliquot 1 ml per one cryotube.
7. Place cryotubes in cell freezing container at -80°C overnight and transfer to liquid nitrogen the next day for long-term storage.