Madeline A. Lancaster

Culturing feeder-dependent human iPS/ES cells

Modified from Harvard Stem cell protocols and WiCell protocols

**Materials**

* DMEM/F12: Invitrogen cat#11330-032
* KOSR: Invitrogen cat# 10828-028
* GlutaMAX: Invitrogen cat#35050-038
* MEM-NEAA: MEM-Non-essential amino acids: Sigma cat#M7145
* 2-Mercaptoethanol
* bFGF: Peprotech cat# 100-18B
	+ 10ug/ml solution made by reconstituting 50ug in 5ml PBS+0.1%BSA
* DMEM: Invitrogen cat#11965-092
* ES-quality FBS: Tested batch from stem cell core
* 0.05% Trypsin-EDTA
* Mitomycin C: Sigma cat# M0503
	+ 50mg powder of which 2mg is Mitomycin C
* DMSO
* Collagenase IV: Invitrogen cat#17104-019
* Dispase: Invitrogen cat#17105-041
* Rock inhibitor Y27632: VWR cat# 688001-500
* Gelatin: Sigma cat#G1890-100G
* Sterile D-PBS w/Ca and Mg
* Sterile D-PBS w/o Ca and Mg
* Mr. Frosty
* Cell lifters: Corning cat#3008

**Media**

hES media (500 ml) – Store at 4C up to 2 weeks

* 400 ml DMEM/F12
* 100 ml KOSR
* 15 ml ES-quality FBS
* 5 ml GlutaMAX
* 5 ml MEM-NEAA
* 3.5 ul 2-Mercaptoethanol
* Filtered using 0.22 um filter
* 20 ng/ml bFGF (Add just before use)

MEF Media (500 ml) – Store at 4C up to 2 weeks

* 450 ml DMEM
* 50 ml FBS (ES-quality just before using for stem cells)
* 5 ml GlutaMAX
* Filtered using 0.22 um filter

Gelatin coating solution (500 ml) – Store at 4C up to 1 year

* 0.5 g Gelatin
* Sterile water for tissue culture
* Filtered using 0.22 um filter

Mitomycin C 200X stock solution (10 ml) – Store at 4C up to 1 week

* 2 mg Mitomycin C (50 mg powder mixture with NaCl)
* 10 ml DMEM
* Filtered using 0.22 um filter
* Throw out if precipitate forms

Collagenase IV solution (50 ml) – Aliquot and store at 4C for 2 weeks or -20C up to 6 months

* 50 mg Collagenase IV
* 50 ml DMEM/F-12
* Filtered using 0.22 um filter

Dispase solution (40 ml) – Aliquot and store at -20C up to 6 months

* 20 mg Dispase
* 40 ml DMEM/F-12
* Filtered using 0.22 um filter

MEF Freezing Media (10 ml) – Store at -20C up to 6 months

* 5 ml DMEM
* 4 ml ES-quality FBS
* Filtered using 0.22 um filter
* 1 ml DMSO

hES media containing Rock inhibitor (13 ml) – Make fresh

* 13 ml hES media
* 26 ul of 5 mM Rock inhibitor Y-27632

2X iPS Freezing Media (10 ml) – Store at -20C up to 6 months

* 8 ml ES-quality FBS
* Filtered using 0.22 um filter
* 2 ml DMSO

2X ES Freezing media (10ml) – Store at -20C up to 6 months

* 6 ml ES-quality FBS
* 2 ml hES media
* Filtered using 0.22 um filter
* 2 ml DMSO

Procedures

**Gelatin coating plates**

1. Add 2 ml Gelatin coating solution to each well of a 6-well plate to be used for MEF plating.
2. Place in incubator for 30 min to 1 hour.
3. Remove Gelatin and immediately add media to avoid drying the well.

**Preparing MEF feeders**

1. Culture MEFs in MEF media
2. Split MEFs by first washing with D-PBS w/o Ca and Mg
3. Add 2 ml 0.05% Trypsin-EDTA to each 10 cm flask
4. Incubate at 37C ~5 min.
5. Add 8 ml MEF media and triturate to remove cells
6. Take 5 ul to count cell number
7. Spin at 270xg for 5 min
8. Resuspend in 10 ml media
9. Plate ~170,000 MEFs per well of 6-well plate
10. When cells have attached (following ~4 hrs) treat in MEF media containing mitomycin C solution diluted 1:200 for 2 hr in the incubator.
11. Aspirate mitomycin C solution and wash feeders at least 4 times thoroughly with warm D-PBS w/Ca and Mg.
12. Wait one day before plating hES or hiPS cells to verify cell cycle arrest of feeder cells. MEFs can also be frozen at this point.

**Freezing MEF feeders**

1. Follow protocol for preparing MEF feeders steps 1 – 6
2. Resuspend cell pellet in appropriate volume of MEF feeder freezing media
	1. For example, for 1.5x10^6 cells, resuspend in 4.5 ml freezing media
3. Aliquot 500 ul per cryovial (1.7x10^5 cells) to be thawed in 1 well of a 6-well plate, or 1 ml per cryovial to be thawed in 2 wells of a 6-well plate.
4. Place in Mr. Frosty in -80C
5. Move to liquid N2 after 24-48 hrs
6. Freezing can also be performed 1 day following growth inactivation

**Thawing MEF feeders**

1. Thaw vial at 37C until a sliver of ice remains.
2. Transfer to 15ml conical containing 10ml MEF media
3. Spin at 270xg for 5 min.
4. Resuspend pellet in 2 ml MEF media for each well of 6-well plate, and transfer all 2 ml to one Gelatin coated well.
5. If mitotically inactivated, plate stem cells on top the following day (not before 4 hours after plating feeders), and ideally use within 2 days.

**Thawing human iPS/ES cells**

1. Wash one well of 6-well plate containing MEF feeder cells with PBS w/Ca and Mg.
2. Add 2 ml hES media containing Rock inhibitor to the well
3. Set up 2x15 ml conical tubes:
	1. Tube 1 with 1 ml hES media containing Rock inhibitor
	2. Tube 2 with 9 ml hES media containing Rock inhibitor
4. Partially thaw hES or hiPS cells until sliver of ice remains
5. Transfer 1ml from tube 2 dropwise onto the cells then transfer that 1 ml to Tube 1
6. Repeat with 1 ml at a time until the total has been transferred to Tube 1
7. Spin at 1000rpm (200xg) for 2 min
8. Add 1 ml hES media containing Rock inhibitor to pellet and carefully resuspend by pipetting slowly 1-2 times. Do not break up colonies in the process.
9. Transfer the 1 ml to the well containing 2 ml media
10. Change 2ml of the media after 36 to 48 hours. Change to media still containing Rock inhibitor
11. Then change media daily without Rock inhibitor

**Routine maintenance and feeding of hiPS/ES cells**

1. Feed cells daily by replacing media with 2 ml fresh hES media.
2. When colonies begin to get larger, feed by replacing media with 2 ml of media in the morning, and adding 1 additional ml of fresh hES media in the evening.
3. Routine tests for mycoplasma should be performed using a combination of PCR and MycoAlert assays. This should be performed at least every month.

**Routine passaging of hiPS/ES cells**

1. Once colonies are ~80% confluent or the colony sizes have reached maximal size, it’s time to split (usually every 5-7 days)
2. Before splitting, remove differentiated cells by first marking on a tissue culture microscope, then using vacuum aspiration or pipet scraping
3. Wash cells with warm D-PBS w/o Ca and Mg
4. Add 1 ml Collagenase IV solution and incubate at 37C for 5-10 min
5. When edges of colonies begin to detach, remove collagenase solution, wash with D-PBS w/o Ca and Mg, and add 1 ml hES media
6. Using a cell lifter, scrape the well and pipet into a 15 ml conical tube.
7. Wash the well with an additional 1 ml hES media and pipet into tube.
8. Spin at 1000 rpm (200xg) for 2 min
9. During spin, wash a well of a 6-well plate containing MEF feeder cells in D-PBS w/Ca and Mg. Add 1 ml hES media to well.
10. Resuspend hiPSC pellet in 1 ml hES media per new well of 6-well plate and triturate gently with P1000 tip to get medium-small fragments.
11. Transfer the 1 ml hES/hiPS cells to well containing feeders and 1 ml media.
12. Typically, splitting 1:3 is adequate but can range from 1:2 to 1:6.

**Freezing hiPS/ES cells**

1. Prepare cells as described in steps 1-8 of routine passaging hiPS cells.
2. Resuspend the pellet in 250ul hES media for every vial you intend to freeze (should freeze one 80% confluent well of 6-well plate per vial).
3. Add 250ul 2X hES or hiPS freezing media and quickly transfer to vial without disturbing colonies too much since they should be as large as possible to increase survival.
4. Transfer to Mr. Frosty and place in -80 C within 3 minutes.
5. 24-48 hours later, transfer to liquid N2.

**Manual passaging hES/hiPS**

1. Prepare sterile microscope enclosure by moving tissue culture microscope to laminar flow hood and exposing to UV for 20-30 min.
2. Under the microscope, use a sterile syringe needle to gently cut around the edges of each colony and cut colonies that are very large into smaller pieces.
3. Don’t let the plate remain outside the incubator for more than 5-10 min at a time, and periodically re-warm the plate by placing back in the incubator for 5-10 min.
4. Once all colonies have been cut, wash the well with warm D-PBS w/Ca and Mg, then add 1 ml fresh hES media.
5. Using a P20 pipet tip, gently scrape hES/hiPS colonies inside the outlines to dislodge them into the media.
6. Transfer this 1 ml media containing scraped colonies to a fresh well of a 6-well plate containing feeders wash with D-PBS w/Ca and Mg.
7. Wash the old well with another 1 ml hES media and transfer to new well.

**Removing hES/hiPS cells from MEF feeders using dispase**

1. Remove media from cells and add 1 ml Collagenase solution. Incubate at 37C for 10 min.
2. When colonies are nearly peeled off dish (can take up 30 min) add 1 ml dispase solution without removing collagenase solution.
3. Incubate for another 5-10 min at 37C until colonies have completely peeled off well.
4. Spray well using P1000 tip to remove colonies (careful not to disrupt colonies) and transfer the collagenase/dispase solution containing hES/hiPS colonies to a 15 ml conical tube and allow to settle for 3-5 min.
5. Remove supernatant with a pipet (NOT by vacuum aspiration).
6. Wash colonies with 0.5 ml fresh hES media and again allow to settle and remove supernatant by pipetting.
7. Reconstitute pellet in 2 ml hES media per well to be plated, triturate to medium sized colonies and transfer to new well of 6-well plate containing MEF feeder cells that have been washed with D-PBS w/Ca and Mg.
8. Do not perform dispase treatment immediately before freezing since this will lower survival.