ES Cell Culture

ES cells are grown on gelatinized plates on feeder layers.

Prepare Gelatinized Plates

1. Add enough 0.1% gelatin to dishes/wells to completely cover bottom
2. Let sit under hood at room temperature for at least 2 hrs.
3. Remove gelatin solution.
4. Use immediately or let further dry under hood O.N.
5. Store plates at room temperature, wrapped in plastic bags, labeled clearly as "gelatinized", for several months.

3.5cm-dish:	~ 1 ml
6cm-dish:	~ 2 ml
10cm-dish:	~ 4 ml
96-well plate:	~ 50 µl/well [knock at side of plate to insure liquid is distributed over bottom surface]
24-well plate:	~ 0.5 ml/well

Prepare Feeder Plates

ES cells are grown on feeder layers of mouse embryonic fibroblasts (MEFs), mitotically inactivated by either irradiation or by Mitomycin C treatment (cells can be purchased as mitotically arrested cells).
Thaw appropriate number of vials of mitotically arrested MEFs at 37°C for 1 - 2 minutes (until just a small pellet of ice remains), transfer to appropriate amount of MEF medium (ES medium if used for parallel plating), and plate on gelatinized dishes.

If vial of irradiated MEFs contains 2 x 10^6 cells, use as follows:

	6cm-dish	10cm-dish	96-well plate	24-well plate	3.5cm-dish
1 vial	20 ml MEF	20 ml MEF	20 ml MEF	20 ml MEF	20 ml
	4 ml/dish = 5 dishes	10 ml/dish = 2 dishes	100 µl/well = 2 plates	1 ml/well = <1 plates	2 ml/dish = 10 dishes

Prepare feeder plates 1 or several days before seeding ES cells.

If parallel plating (seeding ES cells and feeders cells at the same time), resuspend all cells in ES medium.

Culturing ES cells

1. Seed ES cells on feeder layers (ES cells are either thawed or passaged from previous plate)
2. Change medium every day!!!
3. If medium is very yellow when passing, change medium 2 - 3 hrs before trypsinization (to bring pH to neutral)
4. For passing, trypsinize, spin to pellet (1,200 rpm [50], 7 min.), count, and transfer appropriate number of cells to new feeder plate
5. cells are passed every 2 (best) - 4 days

For a 6cm-dish:	expect 1-4 x 10^6 cells (80% confluency)
For a 6cm-dish, plate:	2 x 10^5 for harvesting 2 days after plating 1 x 10^5 cells for harvesting 3 days after plating 0.5 x 10^5 cells for harvesting 4 days after plating

Trypsinize

1. Aspirate medium
2. Wash with room temperature PBS
3. Add Trypsin-EDTA
4. Put dish in 37°C incubator for 7 minutes (if cells don't visibly detach when dish is tilted and tapped gently, extend incubation for another 2 minutes)
5. Add culture medium to dish (same volume as Trypsin-EDTA), pipette up and down a few times to break up clumps, and transfer to 15ml tube. Completely re-suspend cells by pipetting up and down 5-10 times – do not introduce bubbles (i.e., as little as possible).
6. Spin to pellet. Aspirate SUP with Pasteur pipette and vacuum.
7. Resuspend pellet in 0.2 ml medium. Pipette up/down 40-50 times (using blue tip).
8. Count and passage or freeze or electroporate

Volumes for trypsinization:	1.5 mL for 6cm-dish 2.5 mL for 10cm-dish 5 mL for 15cm-dish 0.5 mL for 24-well-plate (individual wells) 0.05 mL for 96-well-plate (individual wells)
Volumes for re-suspension:	add 2.5 mL for 6cm-dish to total 4 ml (1.5 + 2.5) add 5 ml for 10 cm dish for total 7.5 ml
Dilution for counting:	1:10 (5 ul + 45 ul); then 1:5 (5 ul + 20 ul)

Freeze/Thaw

***Freezing**:*

1. Spin cells down to pellet (1,200 rpm [50], 7 minutes)
2. Re-suspend in culture medium at 5 x 10^5/0.25 mL
3. While continuously swirling cells, add 2 x freezing medium dropwise to equal volume of cell suspension
4. Immediately aliquot into freezing vials, 0.5 mL/vial
5. Place vials between two 15 ml tube-styrofoam-holders
6. Put into -80°C O.N.
7. Store in liquid nitrogen the next day

***Thawing**:*

1. Rapidly thaw the vial of ES Cells in a 37ºC water bath. (Place from liquid nitrogen/dry ice/ or -80°C directly into 37°C water bath for 1 - 3 minutes. Watch vials; take out as soon as only small amount of ice left – longer times at 37°C kill cells). Thoroughly decontaminate the exterior of the vial with a 70% ethanol solution.

2. Using a sterile pipette, transfer the cells to a clean 15 ml tube containing 4 ml of ES Cell Medium. Wash vial with medium from the tube. Gently pipette to make a uniform cell suspension.

3. Aspirate the Feeder Medium from 60 mm feeder plate prepared the day before. Dispense 4 ml of the ES cell suspension per feeder plate. Gently swirl the plates in bi-directional (i.e., along the *x* and *y* axes) figure eight patterns to evenly distribute the cells across the dish surface. Incubate the plates at 37ºC.

4. No sooner than 24 hours after plating the ES cells aspirate the old medium from the plates and replenish with 4 ml of ES Cell Medium per plate. This will rid the plates of floating dead cells and any residual dimethyl sulfoxide (DMSO) from the Freezing Medium. Return the plates to the incubator.

5. Expand ES cells to numbers required.

Media for ES Cell Culture

Growth Medium for Routine ES Cell Culture

	stock	final			
KO-DMEM	1x		122.25	406.6	500
FCS		15%	22.5	75	90
Glutamax	200 mM	2 mM	1.5	5	6
Pen-Strep	10,000U or µg/ml	50U or µg/ml	0.75	2.5	3
NEAA	100x	1x	1.5	5	6
Nucleosides	100x	1x	1.5	5	6
LIF/ESGRO	10^7 U/ml	10^3 U/ml	0.015	0.050	0.060
β-Mercaptoethanol	55mM	0.1mM	0.270	0.9	1.08
			150	500	600

Growth Medium for MEF Cell Culture & Plating

	stock	final			
DMEM	1x		122.25	406.6	500
FCS		10%	15	50	60
Glutamax	200 mM	2 mM	1.5	5	6
Pen-Strep	10,000U or µg/ml	50U or µg/ml	0.75	2.5	3
NEAA	10mM	0.1mM	1.5	5	6
Sodium Pyruvate					
			150	500	600

Media Stocks

KNOCKOUT DMEM Gibco 10829
1 x formulation DMEM, optimized for ES cells
Contains glucose + sodium pyruvate; needs all other ingredients

DMEM Gibco 11960
1 x formulation high glucose DMEM
Contains glucose; needs all other ingredients

PBS

Without calcium chloride, without magnesium chloride (Gibco # 14190-029)

Trypsin-EDTA

1X (0.25% Trypsin, 1 mM EDTA), Gibco #25200-056

keep at 4°C for no longer than 1 week; aliquot and keep frozen

Gelatin

0.1% gelatin in PBS (Sigma #G1890); autoclave and store at 4°C or room temperature

Freezing Solution

2 x Freezing Solution:
40% ES medium + 40% FCS + 20% DMSO; mix, filter sterilize (0.2 um), keep at 4°C for no longer than 2 weeks

DMSO
Sigma, cat # D2650