MMRRC Culturing Protocol for JM8 and JM8.F6 ES Cell Clones

Revised on 2/19/09

Cell Line Information

JM8 (derived from C57BL/6N mice) and the sub-line JM8.F6 are considered to be feeder-dependent.

*Feeder cells: Mouse Embryonic Fibroblasts (MEF)

Reagents and Supplies

Item	Vendor	Catalog Number
Knockout DMEM™, high glucose	Gibco	10829-018
GlutaMax™-I, 100X	Gibco	35050-061
D-MEM, high glucose	Gibco	11971-025
Hepes-Buffered D-MEM	Gibco	12430-054
D-glucose	Sigma	G7528
EDTA	Sigma	E6511
NE Amino Acids	Gibco	11140-050
Trypsin EDTA, 2.5%	Gibco	15090-046
Chicken serum	Gibco	16110-082
Trypsin EDTA, 0.05%	Gibco	25300-054
LIF (ESGRO)	Gibco	13275-029
FBS (ES cell tested)	Hyclone	SH300 70.03
PBS (1X without Ca or Mg)	Gibco	14190-144
DMSO, 100 ml	Sigma	D2650
Mitocycin C 10x2mg	Sigma	M0503
2(β)-Mercaptoethanol	Sigma	M-7522
Gelatin, 2%	Sigma	G1393

MEF Feeder Medium (500 ml)

Reagent	Stock Conc	Final Conc	Total
DMEM™		1x	435ml
FBS	100%	10%	50ml
GlutaMax™-I	200mM	2mM	5ml
Sodium Pyruvate	100mM	1mM	5ml
Pen/Strep	10,000U/ml	100U/ml	50µl
1000x βME	5.5×10^{-6} M	1µM	0.5ml

Sterile filter through 0.2µM filter unit

1000x 2(β)-Mercaptoethanol

- To 10 ml PBS add 70µl 2-Mercaptoethanol
- Store at 4°C and make fresh every 2 weeks

JM8 and JM8.F6 ES Cell Medium (500 ml)

Reagent	Stock Conc	Final Conc	Total
KO DMEM™		1x	414.5ml
FBS	100%	15%	75ml
GlutaMax™-I	200mM	2mM	5ml
NE Amino Acids	100mM	1mM	5ml
LIF	10^7 U/ml	1000U/ml	50µl
1000x βME	5.5×10^{-6} M	1µM	0.5ml

Sterile filter through 0.2µM filter unit

0.1% Trypsin

- To 475ml PBS add 0.1g EDTA and 0.5g D-glucose
- Add 5ml Chicken Serum
- Add 20ml 2.5% Trypsin
- Filter sterilize (0.22 µm)
- Aliquot 20ml into centrifuge tubes
- Store at -20°C

0.1% Gelatin

- Add 25 ml of 2% solution to 475ml of PBS
- Filter sterilize (0.22 µm)
- Store at 4°C

MEF Inactivation Media (200 ml)

Reagent	Stock Conc	Final Conc	Total
MEF Feeder medium	1x	1x	200ml
Mitomycin C	200mg	10ug/ml	2mg

Sterile filter through 0.2µM filter
May be stored at -20^{o}C for up to 6 months

Microinjection Medium (500 ml)

Reagent	Stock Conc	Final Conc	Total
Hepes-buffered D-MEM	1x	1x	475ml
FBS	100%	5%	25ml

Sterile filter through 0.2µM filter, and make 4 ml aliquots
May be stored at -20 to -80^{o}C for up to 1 year

2X Freezing Medium

20% DMSO
20% FBS
60% ES cell medium (see above recipe)

Thawing, Expanding and Treating Active MEF Cells

1. Thaw 1 vial of MEF cells (approximately 1.5-2 x 10^6 cells/vial in 0.5ml) in a 37°C water bath and dilute into 4.5 ml of pre-warmed MEF feeder medium.
2. Pellet the cells by spinning for 4 minutes at 1000 rpm in a bench-top clinical centrifuge.
3. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed MEF feeder medium.
4. Transfer cell suspension to a 6 cm gelatinized dish, and grow at 37°C in a humidified 5% CO_2 incubator (should be confluent within 2-3 days).
5. When confluent, aspirate medium off and wash with 5 ml of pre-warmed PBS, pipeting it away from the cells. Rock dish gently and aspirate medium. Repeat.
6. Cover cells with 1 ml of 0.05% trypsin solution and incubate at 37°C for 4 minutes or until cells are uniformly dispersed into small clumps.
7. Add 5 ml of MEF Feeder medium to inactivate the trypsin, and pipette vigorously to make single cell suspension.
8. Spin for 4 minutes at 1000 rpm.
9. Aspirate off medium and gently resuspend cells in 20 ml of pre-warmed MEF Feeder medium.
10. Split the cell suspension onto two gelatinized 10 cm tissue culture dishes, and grow at 37°C in a humidified 5% CO_2 incubator (should be confluent within 2-3 days).
11. To mitotically inactivate, replace medium with 10 ml Inactivation medium, and incubate in a 37°C humidified 5% CO_2 incubator for 2.5 hours. Aspirate Inactivation medium, and rinse three times with pre-warmed PBS; aspirating completely between rinses. These dishes are now ready to use.

12. If you wish to freeze the cells for later usage, trypsinize and pellet the cells as before, but with 1.5 ml of 0.05% trypsin solution, and inactivate the trypsin with 5 ml medium.
13. For each 10 cm dish, count cells, and resuspend in an equal volume of MEF Feeder medium and 2X Freezing medium; to a density of 1.5-2 x 10^6 cells/0.5 ml. Decant 0.5 ml aliquots into labeled cryovials.
14. Immediately place cryovials in a Styrofoam container or temperature controlled freezing vessel.
15. Freeze vials in a -80^{o}C freezer. After 24 hours, transfer cryovials to liquid or vapor-phase nitrogen for longer term storage.

Preparing Mitotically Inactive MEF Feeder Cells

1. One day before thawing ES cells, prepare one gelatinized 6-w and one 6 cm dish with treated feeders. (Coat dish with 0.1% gelatin and aspirate off before adding feeder cells.)
2. Thaw 1 vial of inactive feeder cells (approx. 1.5-2 x 10^6 cells) in a 37°C water bath and dilute into 10 ml of pre-warmed feeder cell medium.
3. Pellet the cells by spinning for 4 minutes at 1000 rpm.
4. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed feeder cell medium.
5. Transfer cell suspension to gelatinized dish, and grow at 37°C in a humidified 5% CO_2 incubator. (See Plating Density Guide below for suggested densities.)
6. The feeders are ready for use after 6-12 hours, or may be maintained in the incubator for a maximum of 8-10 days.

MEF Treated Feeder Cells – Plating Density Guide

Plate/Dish	Feeder Cell Density
96 well	1.5 x 10^4
24 well	1 x 10^5
3.5 cm dish	8 x 10^5
6 cm dish	1.5-2 x 10^6
100 mm dish	3.5-4 x 10^6

Thawing JM8, JM8.F6 ES cell Clones

1. Thaw 1 vial of ES cells (approximately 3 x 10^6 cells/vial) in a 37°C water bath and dilute (drop wise) into 4.5 ml of pre-warmed JM8 ES cell medium in an universal tube.
2. Pellet the cells by spinning for 5 minutes at 1000 rpm.
3. Aspirate supernatant, determine well size to use based on pellet size.Thaw onto:
   6-well: medium sized pellet, about 2 mm in size
   6cm dish: large pellet, about 2.5 to 3 mm in size
4. Aspirate off medium and gently resuspend cells in 3 ml for thawing onto 6-w plate (or 5 ml for thawing onto 6 cm plate) of pre-warmed JM8 ES cell medium.
5. Aspirate the old medium from your 6-w or 6 cm mitotically inactive MEF Feeder dish.
6. Transfer the ES cell suspension to the feeder dish, and grow in a 37°C humidified 5% CO_2 incubator.
7. Change medium the following day to remove dead cells and residual DMSO.
8. Change medium daily until 80% confluent; should take 2-3 days.
9. When confluent, feed cells 3 to 4 fours before splitting. The dish may be split in two; half for microinjection and half to expand for freezing.

Expansion of JM8, JM8.F6 ES Cell Clones for Microinjection and Future Use

1. 1 day prior, prepare one 10 cm MEF Treated Feeder dishes (using the plating density guide below.)
2. The next day, aspirate off the old medium prior to plating ES cells.
3. On the day, wash the confluent 6 cm ES cell dish once with 5 ml PBS.
4. Cover the cells with 1 ml of 0.1% trypsin with chicken serum and incubate at 37°C for 15 minutes or until cells are uniformly dispersed into small clumps.
5. Add 5 ml of ES cell medium; to inactivate the trypsin, and pipette vigorously to make single cell suspension (we recommend 10-15 times).
6. Split the cell suspension in half, placing 2.5 ml each into 15 ml centrifuge tubes (labeled ‘Expansion’ and ‘Microinjection’).
7. Spin both tubes for 4 minutes at 1000 rpm.
8. For the ‘Expansion’ cells; aspirate off the supernatant and resuspend the pellet in 10 ml JM8 ES cell medium. Transfer the cell suspension onto the 10 cm MEF Treated Feeder dish prepared the day before. Grow in a 37°C humidified 5% CO2 incubator. Change medium daily until 80% confluent (should take 2-3 days).
9. For the ‘Microinjection’ cells; aspirate off the supernatant and resuspend the pellet in 150-400 µl microinjection medium. Immediately place the cell suspension on ice, and microinject within 1-2.5 hours.

Freezing JM8, JM8.F6 ES Cell Clones

1. Wash the confluent 10 cm ES cell dish once with 10 ml PBS each.

2. Cover the cells with 1.5 ml of 0.1% trypsin with chicken serum and incubate at 37°C for 15 minutes or until cells are uniformly dispersed into small clumps.
3. Add 5 ml JM8 ES cell medium to inactivate the trypsin, and pipette vigorously to make single cell suspension (we recommend 10-15 times).
4. Spin for 4 minutes at 1000 rpm.
5. Aspirate supernatant and resuspend the pellet in JM8 ES cell medium, and add equal volume of 2X Freezing medium (we would recommend 8-10 vials containing 0.5 ml aliquots; per 10 cm dish). Decant into labeled cryovials.
6. Immediately place cryovials in a Styrofoam container or temperature controlled freezing vessel.
7. Freeze vials in a -80^{o}C freezer. After 24 hours, transfer cryovials to liquid or vapor-phase nitrogen for longer term storage.