Culturing JM8 and JM8.F6 ES Cell Clones

Revised July 2014

Cell Line Information

JM8 and **JM8.F6** sub-lines are derived from C57BL/6N mice and are considered to be **feeder dependent**. Typically, Murine Embryonic Fibroblasts (MEFs) are used as feeder layers but other options include the SNL cell line, DR4 embryonic fibroblasts or others. MEFs may be purchased or derived in house as needed from mid-gestation pregnant mice.

This protocol is based on Sanger procedures as adapted by the Mouse Biology Program (Pettitt, S.J., et al, Agouti C57BL/6N embryonic stem cells for mouse genetic resources. Nature Methods 6, 493-495, 2009.)

For procedures using 2i reagents, protocol is adapted from Gertsenstein, M., et al, Efficient Generation of Germ Line Transmitting Chimeras from C57BL/6N ES Cells by Aggregation with Outbred Host Embryos. PloS ONE 5, e11260, 2010.

Reagents and Supplies

Item	Vendor	Catalog Number
Knockout DMEM™, high glucose	Gibco	10829-018
L-Glutamine (200 mM, 100X)*	Gibco	35030-081
NE Amino Acids	Gibco	11140-050
LIF**	Millipore	ESGRO (ESG 1107)
FBS (ES cell tested)***	Gibco	10437-028
2(β)-Mercaptoethanol	Sigma	M3148
Penicillin/Streptomycin****	Gibco	15140-122
DMSO	Sigma	D2650
Trypsin EDTA, 2.5%	Gibco	15090-046
Trypsin EDTA, 0.05%	Gibco	25300-054
Chicken serum	Gibco	16110-082
EDTA	Sigma	E6511
D-glucose	Sigma	G7528
Hepes-Buffered D-MEM	Gibco	12430-054
PBS (1X without Ca or Mg)	Gibco	14190-144
Gelatin, 2%	Sigma	G1393
Sodium Pyruvate	Gibco	11360-070
Mitomycin C	Sigma	M4287
2i reagent: MEK inhibitor PD0325901	StemGent	04-0006
2i reagent: GSK inhibitor CHIR99021	StemGent	04-0004

*****Glutamax, Gibco 35050-061, may also be used.

******An alternate supplier of LIF is GlobalStem (GSR-7001) 100 ug/vial.

***Other suppliers of FBS may be used, e.g. Hyclone but serum should always be pre-tested to be ES Cell qualified prior to use.

****It's generally preferable not to include antibiotics when culturing cells but we routinely include Pen/Strep because of our high volume and multiple sources of ES cells.

1000x 2(β)-Mercaptoethanol (14.3 M, Sigma M3148)**

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

JM8 and JM8.F6 ES Cell Medium (500 ml) Sterile filter through 0.2µm filter unit

Reagent	Stock Conc	Final Conc	Quantity
KO DMEM™		1x	409.5 ml
FBS	100%	15%	75 ml
Glutamine	200 mM	2 mM	5 ml
NE Amino Acids	100 mM	1 mM	5 ml
LIF*	10^7 U/ml	1000 U/ml	50 ul
2(β)-ME**	1000X	0.1 mM	0.5 ml
Pen/Strep	10,000 U-ug/ml	100 U-ug/ml	5 ml

*For GlobalStem LIF, the 100 ug vial is reconstituted in 1 ml, used at 25 ul/500 ml media for a final concentration of 1000 units/ml

**2-B-Mercaptoethanol is also available from Invitrogen, 21985-023 and may be used directly, 1 ml/500 ml media.

KO DMEM + KOSR + 2i (from TCP Formulation) (500 ml)

Reagent and Ordering Information	Stock Conc	Final Conc	Quantity
KO DMEM (Invitrogen 10829-018)			400 ml
KO Serum Replacement (Invitrogen 10828-028)		15%	75 ml
Glutamax (Invitrogen 35050-061)	200 mM	4 mM	10 ml
NE Amino Acids	100 mM	1 mM	5 ml
Sodium Pyruvate	100 mM	1 mM	5 ml
2(β)-ME	1000X	0.1 mM	0.5 ml
Pen/Strep	10,000 U-ug/ml	100 U-ug/ml	5 ml
LIF (Millipore ESG 1107)	10^7 U/ml	200 U/ml	10 ul
Insulin (Sigma I0516)	10 mg/ml	5 ug/ml	250 ul
2i reagent: MEK inhibitor PD0325901*****	5 mM	1 uM	100 ul
2i reagent: GSK3 inhibitor CHIR99021******	3 mM	3 uM	500 ul

*****To prepare StemGent MEK inhibitor, resuspend 2 mg vial in 830 ul of DMSO
******To prepare StemGent GSK inhibitor, resuspend 2 mg vial in 1.4 ml of DMSO

Chicken Serum Trypsin (0.1% Trypsin)

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

Note: We recommend the use of chicken serum trypsin which tends to be gentler on these cells but standard 0.25% trypsin-EDTA (Gibco 15050-06) may also be used.

0.1% Gelatin (500 ml)

- Add 25 ml of 2% solution to 475 ml of PBS
- Filter sterilize (0.22 µm)
- Store at 4°C
- To prepare culture dish, add 0.1% gelatin to cover, remove after minimum of ~10 minutes

Alternatively, 0.1% Gelatin, ready to use, may be purchased from Millipore (ES-066-B.)

MEF Feeder Medium (500 ml) Sterile filter through 0.2 um filter unit

Reagent	Stock Conc	Final Conc	Quantity
DMEM*		1x	435 ml
FBS	100%	10%	50 ml
Glutamine	200 mM	2 mM	5 ml
Pen/Strep	10,000 U-ug/ml	100 U-ug/ml	5 ml
Sodium Pyruvate	100 mM	1 mM	5 ml

*****For feeder media, KO DMEM may be used, or alternatively, standard high glucose DMEM, Gibco 11971-025 may be used.

MEF Inactivation Media (200 ml)

To 200 ml of MEF Feeder Media, add Mitomycin C to a final concentration of 10 ug/ml.) Sterile filter through 0.2 um filter. May be stored at -20 °C for up to 6 months.

Note: Feeder cells can also be inactivated using **gamma irradiation** with 6,000 rads to achieve mitotic arrest. However, this would require a radiation source which may not be available.

Microinjection Medium (500 ml)

- Add 25 ml of FBS to 475 ml of HEPES-buffered D-MEM
- Filter sterilize (0.22 um) and make 4 ml aliquots. May be stored at -20 to -80^{o}C for up to 1 year

2X Freezing Medium

- 60% JM8 ES Cell Medium (see above recipe)
- 20% FBS
- 20% DMSO

Note: Add FBS to Media before addition of DMSO. Filter sterilize unless sterile reagents are used. May be used for ~ a week when stored at 4° C but freshly made is preferable.

IMPORTANT SAFETY NOTE: When you remove the vial from a nitrogen tank, please loosen the lid immediately to release the pressure and thereby reduce the chance of an explosion. We recommend that you thaw tubes rapidly in a 37° water bath, submerging just the bottom half of the vial. Gently swirl until just a small ice crystal is left.

Manufacturer's Alert: **To prevent cryogenic vials from exploding** please review the following. Always use full face shields, heavy safety gloves and laboratory protective apparel when removing vials from cryogenic storage. Manufacturer recommends you review appropriate procedures outlined in the Nalge Nunc International Cryopreservation Manual located at in the FAQ. *We store vials in vapor phase nitrogen*, and recommend that you place the vials from the dry ice into your -80 freezer if you will be thawing within the next 3 weeks. If you will not be thawing within 3 weeks we recommend long term storage in a nitrogen vapor storage tank. If you choose to store in liquid nitrogen, **please use caution.**

Thawing, Expanding and Treating Active MEF Cells

1. Rapidly thaw 1 vial of MEF cells (approximately 1-2 x 10^{6} cells/vial) in a 37° C water bath and dilute into ~5 ml of pre-warmed MEF feeder medium. Pellet the cells by centrifuging at 1000 rpm for 5 minutes. Aspirate off medium and resuspend cells in 4-5 ml of pre-warmed MEF feeder medium.
2. Transfer the cell suspension to a gelatinized 6 cm dish (or we prefer to use 1 well of a gelatinized 6 well dish) and grow at 37° C in a humidified 5% CO2 incubator (should be confluent within 2-3 days.)
3. To pass the cells when confluent, aspirate off the media and carefully wash with 5 ml of pre-warmed PBS. Cover cells with 1-2 ml of 0.05% trypsin and incubate at 37° C for ~4 minutes or until cells are uniformly dispersed into small clumps. Add 5 ml of MEF Feeder Medium to inactivate the trypsin and pipette ~7-10 times to make a single cell suspension.
4. Centrifuge as above, aspirate off the medium and gently resuspend cells in 20 ml of pre-warmed MEF Feeder Medium. Split the cell suspension onto two gelatinized 10 cm tissue culture dishes and grow at 37° C (should be confluent within 2-3 days.)
5. **To mitotically inactivate**, replace medium with 10 ml Inactivation Medium (contains mitomycin C) and incubate for ~2.5 hours. Aspirate media and rinse carefully three times with pre-warmed PBS; aspirating completely between rinse. The cells are now ready to use but can also be trypsinized and frozen for later use. Large batches of feeders may be treated and frozen.

6. To freeze cells for later use, trypsinize and inactivate the trypsin, resuspend the cells in MEF Feeder Medium and add an equal volume of 2X Freezing medium, to a final density of 1 – 2 x 10^6 cells/1.0 ml. Pipette 0.5 - 1.0 ml aliquots into labeled cryovials (we recommend Nunc 377267.)
7. Immediately place cryovials in a Styrofoam container (tape closed) or other temperature controlled freezing container and place in a -80° C freezer. After 24 hours, transfer cryovials to liquid or vapor-phase nitrogen for long term storage.

Preparing Mitotically Inactive MEF Feeder Cells for Use

1. One day before thawing ES cells, prepare gelatinized dishes for use with treated feeders. Coat dish with 0.1% gelatin for ~30 minutes at room temperature in the hood and aspirate off before adding feeder cells.
2. Thaw 1 vial of inactivated feeder cells (~1-2 x 10^6 cells) in a 37° C water bath and dilute into 10 ml of pre-warmed Feeder Cell Medium. Pellet the cells by spinning for 4 minutes at 1000 rpm. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed Feeder Cell Medium.
3. Transfer cell suspension to gelatinized dish and grow at 37° C in a humidified 5% CO2 incubator. (See Plating Density Guide below for suggested approximate densities.)
4. The feeders are ready for use after ~2-4 hours or may be maintained in the incubator for approximately 8-10 days.

MEF Treated Feeders – Plating Density Guide

Plate/Dish	Feeder Cell Density
96 well	1.0 x 10^4
24 well	1.0 x 10^5
35 mm dish	8.0 x 10^5
6 cm	1.5 x 10^6
10 cm dish	2.0 x 10^6

Thawing JM8 and JM8.F6 ES Cell Clones

1. Thaw one vial of ES cells (~1-3 x 10^6 cells/vial) rapidly in a 37° C water bath and dilute drop wise into 5 ml of pre-warmed JM8 ES Cell Medium. Centrifuge 4 minutes at 1000 rpm. Aspirate the supernatant and gently resuspend cells in ~3 ml of ES Cell Medium for transfer into one well of 6 well plate. Adjust volume for other sized dishes. Aspirate the feeder medium from the dish of inactivated feeders and transfer the ES cell suspension to the feeder dish. Grow in a 37° C humidified 5% C02 incubator.
2. Change medium the following day to remove dead cells and residual DMSO.
   Note: Cells may also be cultured by gently adding the thawed cells directly to the ES Cell Medium in the feeder dish without centrifuging, followed by incubation and changing of the medium when the cells have attached or following overnight incubation.

   (Ultra-low passages may be supplied in micro-vials which contain ~5 x 10^5 cells/vial. These smaller vials should be thawed as described above and added to 0.5 ml of media in one well

of 48 well dish of feeders. Pass when ready to a 24 well and then to a 6 well following the procedures outlined below.)

3. Change medium daily or as needed until ~80% confluent (approximately 1-2 x 10^7 cells); should take 2-3 days but some clones may be slower to become confluent.
4. When confluent, feed cells 3-4 hours before splitting. The well may be split in two, half for microinjection and half for expansion or freezing.
5. If the 2i reagents are added to the media, we've found it preferable to pass the cells with media without 2i and resume using media plus 2i for daily feeding.

NOTE: We are currently expanding cells for blast injection or aggregation in the above KO-DMEM + KOSR + 2i medium. Cells are passed 2-3 times as necessary up to 24 hours before injection at which point media (either KOSR or JM8 ES Cell Media) without the 2i reagents is used until the cells are injected. Accutase (Millipore SF006 or Invitrogen StemPro A1110501) may be used instead of trypsin when KOSR is used since there is no FBS in the media to inactivate the trypsin; Accutase does not require inactivation and may be spun out or diluted out.

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

1. One day prior, prepare 10 cm feeder dish. The next day, aspirate off the old medium prior to plating the ES cells. On the day, wash the confluent ES cell well or dish once with 3 ml PBS.
2. Cover the cells with 0.5 ml of 0.1% trypsin with chicken serum and incubate at 37° C for ~10-15 minutes or until cells are uniformly dispersed into small clumps.
3. Add 3 ml of ES cell medium to inactivate the trypsin and pipette gently to make a single cell suspension (~10-15 times.)
4. For '**Expansion**' half the cell suspension may be added to ~8 ml of pre-warmed media in the 10 cm feeder dish for a final volume of ~10 ml/dish. Grow in a 37° C humidified 5% CO_2 incubator. Change medium daily. Cells should be passed or frozen when 75 - 85% confluent. Alternatively, KO-DMEM + KOSR + 2i medium may be used for expansion or feeding of the cells, **particularly if any differentiation is seen**.
5. For the '**Microinjection**' cells, add 5 ml of media to the remaining trypsinized cells and centrifuge in a 15 ml tube for 4 minutes at 1000 rpm. Aspirate off the supernatant and re-suspend the pellet in ~200 ul of Microinjection Medium. Place the vial containing the cells on ice and microinject within 1-2.5 hours.

Freezing JM8 and JM8.F6 ES Cell Clones

1. Wash the confluent 10 cm ES cell dish once with 10 ml PBS.
2. Cover the cells with 1.5-2.0 ml of 0.1% trypsin with chicken serum and incubate at 37°C for 10-15 minutes or until cells are uniformly dispersed into small clumps. Add 10 ml of ES Cell Medium to inactivate the trypsin and pipette gently to make single cell suspension (10-15 times.)
3. Centrifuge for 4 minutes at 1000 rpm.
4. Aspirate supernatant and re-suspend the pellet in ES cell medium. Add equal volume of 2X Freezing Medium (we would recommend 6-8 vials containing 0.5 ml aliquots from a 10 cm dish,
3-4 vials from a 6 well or 6 cm dish.) So for 8 vials, re-suspend pellet in 2 ml of media and add

2 ml of 2X Freezing Media for a total of 4 ml or 8 samples of 0.5 ml each. Dispense into labeled cryo vials (we use Nunc cryotubes, 377267.)

5. Immediately place cryo vials in a Styrofoam container or temperature controlled freezing container.
6. Freeze vials in a -80° C freezer. After 24 hours, transfer cryo vials to liquid or vapor phase nitrogen for longer term storage.

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