Culturing Protocol for JM8.N4 ES Cell Clones

Revised July 2014

Cell Line Information

The **JM8.N4** subline is derived from the JM8 parental line and are considered to be feeder independent. These cells are derived from C57BL/6N mice. We are currently using feeder free conditions for growth of these cells but if you prefer to use feeders please see Protocol for JM8.F6 for details.

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	M-7522
PBS (1X without Ca or Mg)	Gibco	14190-144
Penicillin/Streptomycin***	Gibco	15140-122
Trypsin EDTA, 2.5%	Gibco	15090-046
Chicken serum	Gibco	16110-082
EDTA	Sigma	E6511
Hepes-Buffered D-MEM	Gibco	12430-054
D-glucose	Sigma	G7528
DMSO, 100 ml	Sigma	D2650
Gelatin, 2%	Sigma	G1393
MEK inhibitor PD0325901 (2i reagent)	StemGent also Cedarlane	04-0006 Axon 1408
GSK3 inhibitor CHIR99021 (2i reagent)	StemGent also Cedarlane	04-0004 Axon 1386

*****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

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

JM8.N4 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

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) (500 ml)

- 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.)

Microinjection Medium (500 ml)

- Add 25 ml of FBS to 475 ml of HEPES-buffered D-MEM
- Filter sterilize (0.22 µm) and make 4 ml aliquots. Store at -20 to -80^{o}C up to 1 yr

2X Freezing Medium

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

Note: Add FBS to Media before addition of DMSO

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 JM8.N4 ES cell Clones

1. Rapidly thaw 1 vial of ES cells (approximately 2 x 10^6 cells/vial) in a 37°C water bath and dilute (drop wise) into 3 ml of pre-warmed Medium. 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. Pass when ready to a 24 well and then to a 6 well following the procedures outlined below.
2. Transfer the ES cell suspension to gelatinized 6 cm dish (or we prefer to use 1 well

of gelatinized 6 well dish) and grow in a 37°C humidified 5% CO_2 incubator.

3. Change medium the following day to remove dead cells and residual DMSO. Alternatively, thawed cells may be centrifuged after adding contents of vial to warm media followed by resuspending the cells in media and plating as above.
4. Change medium daily until 80% confluent (approximately 1.5-2 x 10^7 cells); should take 2-3 days but some clones may be slower to become confluent.
5. When ~80% confluent, the well or dish may be split in two; half for microinjection and half for expansion or freezing.

6. If the 2i reagents are added to the media, we've found it preferable to pass the cells in 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.N4 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.N4 ES Cell Clones for Microinjection and Future Use

1. 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 ~7 minutes or until cells are uniformly dispersed into small clumps.
3. Add 3 ml of JM8 medium to inactivate the trypsin and pipette gently to make a single cell suspension (~7-10 times).
4. For '**Expansion**' half the cell suspension may be added to ~8 ml of pre-warmed media in a 10 cm gelatinized dish for a final volume of ~10 ml/dish. Grow in a 37°C humidified 5% CO_2 incubator. Change medium daily until 80% confluent. Alternatively, KO-DMEM + KOSR + 2i medium may be used for expansion or feeding of the cells, **particularly if any differentiation is seen**.
   Note: The JM8 lines are very sensitive to over-confluence. Cells should be passed or frozen between 75 and 85% confluence.
5. For the '**Microinjection**' cells, add 5 ml of media to the remaining trypsinized cells and centrifuge in a 15 ml tube for 5 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.N4 ES Cell Clones

1. Wash the confluent 10 cm JM8.N4 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 6-7 minutes or until cells are uniformly dispersed into small clumps.
3. Add 10 ml JM8.N4 ES cell medium to inactivate the trypsin and pipette gently to make single cell suspension (~7-10 times).
4. Centrifuge for 5 minutes at 1000 rpm.
5. 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). 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. Decant into labeled cryo vials (we use Nunc cryotubes, 377267.)

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

We use Nunc Cryo Tube vials, for which the manufacturer recommends that for storage in LIQUID N_2 you place them in Nunc Cryoflex Tubing (Catalogue # 343958). Please note that we have had reports of vials exploding if stored in liquid, therefore if you choose not to use Cryoflex tubing you may want to do the following to try and avoid injury: When you remove the vial from liquid nitrogen, unscrew the top immediately to release the pressure and thereby *reduce the chance of an explosion*. And again, always use proper personal protective equipment, such as full face shields, when handling items stored in liquid nitrogen.