

Culturing Protocol for JM8.N4 ES Cell Clones

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Cell Line Information

The **JM8.N4** subline is derived from the JM8 parental line and is considered feeder independent. We are currently using feeder free conditions for growth of these cells but if you prefer to use feeders please see Protocols for JM8 and 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.)

Reagents and Supplies

Note: Vendors listed are our current suppliers but other ES cell qualified sources may be used as well.

<u>Item</u>	<u>Vendor</u>	<u>Catalog Number</u>
Knockout DMEM™, high glucose	Gibco	10829-018
L-Glutamine (200mM, 100X)	Gibco	25030-081
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	Sigma	D2650
Gelatin, 2%	Sigma	G1393

*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 70µl of the 14M Mercaptoethanol.
Store at 4°C, and make fresh every 2 weeks.

JM8.N4 ES Cell Medium (500ml) (sterile filter through 0.2 um filter unit)

<u>Reagent</u>	<u>Stock</u>	<u>Final</u>	<u>Quantity</u>
KO DMEM	1x		409.5 ml
FBS	100%	15%	75 ml
Glutamine	200 mM, 100X	2 mM	5 ml
NE Amino Acids	100 mM	1 mM	5 ml
LIF*	10 ⁷ U/ml	1000 U/ml	50 ul
2-BME (1000X)	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

Chicken Serum Trypsin (0.1% trypsin)

- To 475 ml PBS add 0.1g EDTA and 0.5g D-glucose.
- Add 5 ml Chicken Serum.
- Add 20 ml 2.5% Trypsin.
- Filter sterilize (0.22 um 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 um).
- 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, Catalog No. ES-066-B.

Microinjection Medium (500ml)

- Add 25 ml of FBS to 475 ml of HEPES-buffered D-MEM.
- Filter sterilize (0.22 μ m) and make 4 ml aliquots.
- May be stored at -20 to -80° C for up to a year.

2X Freezing Medium

60% JM8.N4 DMEM (see above recipe)
20% FBS
20% DMSO

Thawing JM8.N4 ES cell Clones

1. Thaw 1 vial of ES cells (1-2 x 10⁶ cells/vial) in a 37°C water bath and dilute gently into 10 ml of pre-warmed JM8.N4 ES cell medium.
2. Pellet the cells by spinning for 4 minutes at 1000 rpm.
3. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed JM8.N4 ES cell medium.
4. Transfer the ES cell suspension to gelatinized 6 cm dish (or 1 well of gelatinized 6 well dish) and grow in a 37°C humidified 5% CO₂ incubator.
5. Change medium the following day to remove dead cells and residual DMSO.
Note: Cells may also be thawed by gently adding the thawed cells directly to the medium in the dish without centrifuging, followed by incubation and changing the medium when cells have attached. For the micro-vials which contain about 200 μ l each, contents may be added to one well of 48 well plate.
6. Change medium daily or as needed until ~80% confluent (approx. 1.0-2 x 10⁷ cells from 6 well dish); most clones will take 2-3 days to become ~80% confluent but some may take 5-7 days.
7. When confluent, the dish or well may be split 1:2 to provide half for microinjection and half for further expansion and freezing.

Expansion of JM8.N4 ES Cell Clones for Microinjection and Future Use

1. Wash the confluent 6 well ES cell dish once with ~5 ml PBS.
2. Cover the cells with 1 ml of 0.1% trypsin with chicken serum and incubate at 37°C for 12-15 minutes or until cells are uniformly dispersed into small clumps.
3. Add 5 ml of JM8.N4 ES cell medium to inactivate the trypsin and pipette vigorously to make single cell suspension (we recommend 10-15 times).
4. Split the cell suspension in half, placing 2.5 ml each into 15 ml centrifuge tubes (labeled 'Expansion' and 'Microinjection').
5. Spin both tubes for 4 minutes at 1000 rpm.
6. For the 'Expansion' cells; aspirate off the supernatant and resuspend the pellet in 10 ml JM8. N4 ES cell medium. Transfer the cell suspension onto a 10 cm gelatinized dish. Grow in a 37°C humidified 5% CO₂ incubator. Change medium daily or as needed until 80% confluent (should take 2-3 days for most clones).

7. For the 'Microinjection' cells; aspirate off the supernatant and resuspend the pellet in 150 ul of microinjection medium. Immediately place the cell suspension 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 12-15 minutes or until cells are uniformly dispersed into small clumps.
3. Add 5 ml JM8.N4 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.N4 ES cell medium and add equal volume of 2X Freezing Medium (we recommend 8-10 vials; $1.5 - 2 \times 10^6$ /vial, per 10 cm dish). Dispense into labeled cryovials.
6. Immediately place cryovials in a Styrofoam container or temperature controlled freezing vessel.
7. Freeze vials in Styrofoam container in a -80°C freezer. After 24 hours, cryovials may be transferred to liquid or vapor-phase nitrogen for longer term storage.