MMRRC Culturing Protocol for JM8A (Agouti) ES Cell Clones

Revised April 2012

Cell Line Information

JM8A sub-lines (JM8A3, JM8A3.N1, JM8A1.N3) are derived from the JM8 parental line and are considered feeder independent. These cells are derived from C57BL/6N mice and the Agouti allele has been modified to correct the black mutation. Mice derived from these cells will have Agouti coat color and are heterozygous for the Agouti allele (A/a). We recommend using feeder free conditions for growth of these cells; however, if you prefer to use feeders, please see the MMRRC Protocol for Culturing 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

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

* 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 2-Mercaptoethanol
- Store at 4°C, and make fresh every 2 weeks

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JM8A ES Cell Medium (500 ml) Sterile filter through 0.2µm filter unit.

Reagent	Stock Conc	Final Conc	Volumn
KO DMEM™		1x	414.5ml
FBS	100%	15%	75ml
Glutamine	200mM	2mM	5ml
NE Amino Acids	100mM	1mM	5ml
LIF*	10^7U/ml	0.1mM	50µl
2(β)-ME	1000X	1µM	0.5ml
Pen/Strep	10,000 U-µg/ml	100 U-µg/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 475ml PBS add 0.1g EDTA and 0.5g D-glucose
- Add 5ml Chicken Serum
- Add 20ml 2.5% Trypsin
- Filter sterilize (0.2 µm filter unit)
- Aliquot 20ml 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 475ml of PBS
- Filter sterilize (0.2 µm)
- Store at 4°C
- To prepare culture dish, add 0.1% gelatin to cover, remove after ~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 (0.2 um) and make 4 ml aliquots. May be stored at -20 to -80^{o}C for up to 1 year.

2X Freezing Medium

- 60% JM8A ES Cell Medium (see above recipe)
- 20% FBS
- 20% DMSO

Note: Add FBS to Media before addition of DMSO

Thawing JM8A 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 JM8A ES Cell 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.
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.

Expansion of JM8A 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 JM8A ES cell 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. Note: The Agouti lines are very sensitive to over-confluency and cells should be passed or frozen when reach about 75 - 85% confluency.
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.

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Freezing JM8A ES Cell Clones

1. Wash the confluent 10 cm JM8A 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 JM8A 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 JM8A 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 vessel.
7. Freeze vials in a -80ºC freezer. After minimum of 24 hours, transfer cryo vials to liquid or vapor phase nitrogen for longer term storage.