

Culturing Protocol for JM8.A (Agouti) ES Cell Clones

Revised on 10/27/09

Cell Line Information

JM8.A sub-lines are derived from the JM8 parental line and is 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).

Reagents and Supplies

Item Vendor Catalog Number

Knockout DMEM™, high glucose Gibco 10829-018

GlutaMax™-I, 100X Gibco 35050-061

NE Amino Acids Gibco 11140-050

LIF (ESGRO) Gibco 13275-029

FBS (ES cell tested) Hyclone SH300 70.03

2(β)-Mercaptoethanol Sigma M-7522

PBS (1X without Ca or Mg) Gibco 14190-144

Trypsin EDTA, 2.5% Gibco 15090-046

Chicken serum Gibco 16110-082

EDTA Sigma 02650

Hepes-Buffered D-MEM Gibco 12430-054

D-glucose Sigma G7528

DMSO, 100 ml Sigma D2650

Gelatin, 2% Sigma G1393

1000x 2(β)-Mercaptoethano l

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

JM8.A ES Cell Medium (500 ml) (sterile filter through 0.2μM filter unit)

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 10⁷ U/ml 1000U/ml 50ul

1000x βME 5.5 x 10⁻⁶ M 1μM 0.5ml

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 μ m).
- Store at 4°C.
- To prepare culture dish, add 0.1% gelatin to cover, remove after ~10 minutes.

Microinjection Medium (500 ml)

(Sterile filter through 0.2 μ M filter, and make 4 ml aliquots. May be stored at -20 to -80°C for up to 1 year).

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

2X Freezing Medium

20% DMSO

20% FBS

60% JM8.A Knockout Media (see above recipe)

*Note: Add FBS to Media before addition of DMSO

Thawing JM8.A ES cell Clones

1. Thaw 1 vial of ES cells (approximately 3×10^6 cells/vial) in a 37°C water bath and dilute (drop wise) into 10 ml of pre-warmed JM8.A ES cell medium.
2. Pellet the cells by spinning for 5 minutes at 1000 rpm.
3. Aspirate off medium and gently re-suspend cells in 5 ml of pre-warmed JM8.A 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.
6. Change medium daily until 80% confluent (approx. $1.5-2 \times 10^7$ cells); should take 2-3 days.
7. When confluent, the dish or well may be split in two; half for microinjection and half to expand for freezing.

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

- 1.** Wash the confluent 6 cm 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 6-7 minutes or until cells are uniformly dispersed into small clumps.
- 3.** Add 5 ml of JM8.A ES cell medium; to inactivate the trypsin, and pipette gently to make single cell suspension (we recommend 7-10 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 5 minutes at 1000 rpm.
- 6.** For the 'Expansion' cells; aspirate off the supernatant and re-suspend the pellet in 5 ml JM8.A 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 until 80% confluent (should take 2-3 days). *Special Note: The Agouti lines are very sensitive to over-confluence. Cells should be passed between 75 and 85% confluence.
- 7.** For the 'Microinjection' cells; aspirate off the supernatant and re-suspend the pellet in 150-400 µl microinjection medium. Immediately place the cell suspension on ice, and microinject within 1-2.5 hours.

Freezing JM8.A ES Cell Clones

- 1.** Wash the confluent 10 cm JM8.A 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.A ES cell medium to inactivate the trypsin, and pipette gently to make single cell suspension (we recommend 7-10 times).
- 4.** Spin for 5 minutes at 1000 rpm.
- 5.** Aspirate supernatant and re-suspend the pellet in JM8.A ES cell medium, and add equal volume of 2X Freezing medium (we would recommend 8 vials containing 0.5 ml aliquots; per 10 cm dish). Decant into labeled cryo vials.
- 6.** Immediately place cryo vials in a Styrofoam container or temperature controlled freezing vessel.
- 7.** Freeze vials in a -80°C freezer. After 24 hours, transfer cryo vials to liquid or vapor phase nitrogen for longer term storage.