

Electroporation of JM8.F6 cells and selection of targeted clones

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UC Davis

Cell Line Information

JM8.F6 is a subline of JM8 which is derived from C57BL/6N mice. This cell line is feeder dependent.

JM8.F6 (p12) has 3×10^6 cell per vial.

Reagents and Supplies

<u>Item</u>	<u>Vendor</u>	<u>Catalog Number</u>
Knockout DMEM™, high glucose	Gibco	10829-018
Penicillin/Streptomycin, 100 u/ml	Gibco	15140-122
GlutaMax™-I, 100X	Gibco	35050-061
Sodium Pyruvate	Gibco	11360-070
NE Amino Acids	Gibco	11140-050
Trypsin EDTA (1X, 0.25%) (supplement the 0.25% with 1% Chick Serum)	Gibco	25300-054 (0.05%) 25200-072 (0.25%)
LIF (ESGRO)	Gibco	13275-029
Knockout Serum Replacement™	Gibco	10828-028
PBS (1X without Ca or Mg)	Gibco	14190-144
DMSO, 100 ml (2X Freezing medium: FBS with 20% DMSO – make fresh as required)	Sigma	D2650
<u>Mitocycin C 10x2mg</u> (10µg/ml Inactivation media: add 2mg of Mitomycin C to 200 ml MEF Feeder medium -may be stored at 20°C for up to 6 months)	Sigma	M0503
<u>2(β)-Mercaptoethanol</u> (1000x working soln: add 70µl 2- Mercaptoethanol to 9.93ml PBS. Store at 4°C, and make fresh every 2 weeks)	Sigma	M-7522
<u>Gelatin, 2%</u> (0.1% working soln: add 25 ml of 2% solution to 475ml of PBS. Store at 4°C)	Sigma	G1393

MEF Feeder Medium (sterile filter through 0.2µM filter unit)

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final conc.</u>	<u>Total: 500ml</u>
DMEM™		1x	435ml
FBS	100%	10%	50ml
GlutaMax™-I	200mM	2mM	5ml
Sodium Pyruvate	100mM	1mM	5ml
Pen/Strep	10,000U/ml	100U/ml	5ml

JM8 ES Cell Medium (sterile filter through 0.2µM filter unit)

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final conc.</u>	<u>Total: 500ml</u>
KO DMEM™		1x	414.5ml
K/O SR™	100%	15%	75ml
GlutaMax™-I	200mM	2mM	5ml
NE Amino Acids	100mM	1mM	5ml
LIF	10 ⁷ U/ml	1000U/ml	50ul
1000x βME	5.5 x 10 ⁻⁶ M	1µM	0.5ml

MEF Inactivation Medium (sterile filter through 0.2µM filter unit)

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final Conc.</u>	<u>Volume</u>
MEF Feeder medium	1x	1x	200ml
Mitomycin C	2mg powder	10ug/ml	2mg
Total Volume			200 ml

2X Freezing Medium**20% DMSO****80% FBS**

Thawing JM8.F6 ES Cells

- 1.** One day before thawing, prepare one 6-w dish with inactivated MEF cells.
- 2.** Thaw 1 vial of ES cells (approximately 3×10^6 cells/vial) in a 37°C water bath and dilute (drop wise) into 9.5 ml of pre-warmed JM8 ES cell medium.
- 3.** Pellet the cells by spinning for 5 minutes at 1000 rpm.
- 4.** Aspirate off medium and gently resuspend cells in 3 ml of pre-warmed JM8 ES cell medium.
- 5.** Aspirate the old medium from your 6-w inactivated MEF feeder dish.
- 6.** Transfer the ES cell suspension to the feeder dish, and grow in a 37°C humidified 5% CO₂ incubator.
- 7.** Change medium the following day to remove dead cells and residual DMSO.
- 8.** Change medium daily until 80% confluent; should take 1-2 days.
- 9.** When confluent, passage cells with 1:6 ratio.

Expansion of JM8.F6 ES Cell Clones

- 1.** 1 day prior, prepare one 10 cm dish with inactivated MEF feeder cells.
- 2.** The next day, aspirate off the old medium prior to plating ES cells.
- 3.** On the day, wash the confluent 6-w ES cell dish once with 3 ml PBS.
- 4.** Cover the cells with 1 ml of 0.25% trypsin solution and incubate at 37°C for 10 minutes or until cells are uniformly dispersed into small clumps.
- 5.** Add 3 ml of JM8 ES cell medium; to inactivate the trypsin, and pipette vigorously to make single cell suspension.
- 6.** Spin for 5 minutes at 1000 rpm.
- 7.** Aspirate off the supernatant and resuspend the pellet in 10 ml JM8 ES cell medium. Transfer the cell suspension onto the 10 cm MEF Treated Feeder dish prepared the day before. Grow in a 37°C humidified 5% CO₂ incubator. Change medium daily until 80% confluent (should take 2-3 days).

Electroporation and selection of targeted clones

- 1) Expand JM8 cells (p13) to 4 x 150 cm flasks:
 - p14: 6-well to 25cm² flask
 - p15: 25 cm² flask to 2 x 75 cm² flask
 - p16: 75 cm² flask to 3 x 150 cm² flask
- 2) Change medium 2-4 hours prior to electroporation
- 3) Wash cells 2 x PBS (pre-warmed)
- 4) Trypsinize cells for 7.5 min (5 ml/150cm² flask)
- 5) Add medium (15 ml/150cm² flask), disperse cells and transfer to centrifuge tube
- 6) Spin cells (1300 rpm, 3 min)
- 7) Combine and resuspend cells in 20 ml PBS (room temp)
- 8) Count aliquot of cells in hemocytometer
- 9) Spin cells (1300 rpm, 3 min)
- 10) Resuspend cells in PBS at a density of 0.5-1 x 10⁷ cells/70 microliters
- 11) Add cells to 50 microliters of linear DNA in PBS (1-2 micrograms)
- 12) Electroporate in BTX 96-well electroporation apparatus (25 cell cuvette/ 0.2 cm gap):
 - 700 V, 25 microF, 400 Ohm, time constant (RC) = 100-150 microsec
- 13) Plate cells onto a 10 cm dish containing 10 ml media (no G418)
- 14) Change media containing 100 micrograms (active)/ml Geneticin (G418) on day 1, 2, 3, 4, 6 and 8 days post-electroporation
- 15) Pick colonies on day 9-10 into 30 microliters trypsin in U-bottom 96-well plate
- 16) Incubate at 37 C for 15 min
- 17) Add 170 microliters media containing G418, disperse cells and transfer to 96-well tissue culture plate
- 18) Change media (+G418) every 2 days until most of the wells reach confluence

Freezing cells on 96-w plates for genotyping and expansion

1. When 80% of the wells are approaching 80 % confluency, they are ready to pass to genotyping and master plates.
2. For each genotyping plate, gelatinize (no feeder cells!) a 96 well flat bottom plate. Aspirate gelatin and add 180 ul selection medium to each well.
3. Prepare one round bottom 96 well plate per plate of cells with 40 ul 2x freezing medium (master plate).
4. Wash the 96-well plates 2x with PBS and trypsinize with 30 ul trypsin for 5 minutes at 37°C. Add 30 ul ES cell medium (or selection medium) and break up the colonies by pipetting up and down
5. Add 40 ul of the cell suspension to the master plate and 20 ul to the genotyping plate. Pipette up and down to mix for each addition, but mix the master last so you don't get DMSO in the duplicate plate. Do not create bubbles in the Master plate.
6. Put the duplicate in the incubator.
7. Wrap the master plate in paraffin and place in a Styrofoam box. Put the Styrofoam box in -80°C freezer for storage. Can be stored at -80°C for 3-4 months.
8. Allow the cells on the gelatin-coated plate (duplicates) to grow until they turn the media yellow every day (4 to 5 days).
9. Rinse the wells twice with PBS and wrap with paraffin. Freeze in the -20°C freezer for DNA extraction.