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Culturing Bruce4 ES Cell Clones

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Cell Line Information

Bruce4 ES cells are feeder-dependent.

Parental ES cells: Bruce4, isolated from C57BL/6 mouse strain
Injection of cells into C57BL/6 blastocysts will produce agouti chimeras. These cells are mycoplasma free.

Feeder cells: MEF feeder cells express very small amounts of Leukemia Inhibitory Factor (LIF) and neomycin phosphotransferase (*Neo*). These cells are is mycoplasma free.

Reagents and Supplies

Item	Vendor	Catalog Number
DMEM, high glucose, with glutamine	Gibco (Invitrogen)	11965-092
Penicillin/Streptomycin (10,000 U/ml Penicillin and 10,000 µg/ml Streptomycin)	Gibco (Invitrogen)	15140-122
L-Glutamine	Gibco (Invitrogen)	25030-081
Sodium Pyruvate	Gibco (Invitrogen)	11360-070
NE Amino Acids	Gibco (Invitrogen)	11140-050
Trypsin (2.5%)	Gibco (Invitrogen)	15090-046
Chicken Serum	Gibco (Invitrogen)	16110-082
EDTA	Sigma	E6511
D-glucose	Sigma	G7528
LIF (ESGRO)	Millipore	ESG1107
Fetal Bovine Serum Other suppliers may be used, but the serum should be evaluated on ES cells before use.	Hyclone or Invitrogen	Hyclone SH30070.03E Invitrogen 10437028
PBS (1X without Ca or Mg)	Gibco (Invitrogen)	14190-144
DMSO (2X Freezing medium: 20% FBS + 20% DMSO + 60% ES Media – make fresh)	Sigma	D2650
Mitocycin C (2mg) (10µg/ml Inactivation media: add 2mg of Mitomycin C to 200 ml MEF Feeder medium -may be stored at -20^{o}C for up to 6 months)	Sigma	M0503
2(β)-Mercaptoethanol (1000x working soln: add 70µl 2-Mercaptoethanol to 9.93ml PBS. Store at 4°C, and make fresh every 2 weeks)	Sigma	M-3148
Gelatin, 2% (0.1% working soln: add 25 ml of 2% solution to 475ml of PBS. Store at 4°C)	Sigma	G1393
Trypsin (0.05% + EDTA)	Gibco (Invitrogen)	25300-054
Trypsin (0.25% + EDTA)	Gibco (Invitrogen)	25200-056

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

Reagent	Stock Conc.	Final Conc.	Volume
DMEM	1x	1x	435ml
FBS	100%	10%	50ml
L-Glutamine	200 mM	2 mM	5 ml
Sodium Pyruvate	100 mM	1 mM	5 ml
Pen/Strep	10,000 U/ml & 10,000 μg/ml	100 U & 100 μg/ml	5 ml
Total Volume			**500 ml**

Bruce4 Cell Medium (sterile filter through 0.2μM filter unit)

Reagent	Stock Conc.	Final Conc.	Volume
DMEM	1x	1x	435 ml
FBS	100%	15%	75 ml
L-Glutamine	200 mM	2 mM	5 ml
Sodium Pyruvate	100 mM	1 mM	5 ml
NE Amino Acids	100 mM	1 mM	5 ml
Pen/Strep	10,000 U/ml & 10,000 μg/ml	100 U & 100 μg/ml	5 ml
LIF	10^7 U/ml	1000 U/ml	50 ul
1000x βME	5.5×10^{-6} M	100 μM	0.5 ml
Total Volume			**500 ml**

0.1% Trypsin with Chicken Serum

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, aliquot and store at -20°C.

We find that chicken serum trypsin is more gentle on the cells and may be left on the cells longer. Alternatively, standard 0.25% trypsin EDTA may be used.

Inactivation of MEF

This is an example of inactivation of feeder cells using mitomycin C; alternatively, cells can be inactivated by gamma irradiation if a source of radiation is available. We are currently using the irradiation method here at UC Davis.

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

Reagent	Stock Conc.	Final Conc.	Volume
MEF Feeder medium	1x	1x	200 ml
Mitomycin C	2 mg powder	10 μg/ml	2 mg
Total Volume			**200 ml**

Preparing MEF Feeder Cells

Thawing, Expanding and Treating Active MEF Cells

1. Thaw 1 vial of MEF cells (typically 1.5-2 x 10^6 cells/vial) in a 37°C water bath and dilute into 10 ml of pre-warmed MEF feeder medium. See chart at end of protocol for plating density guide.

2. Pellet the cells by spinning for 4 minutes at 1000 rpm in a bench-top clinical centrifuge (or cells may be plated directly and media changed as soon as cells are attached.)

3. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed MEF feeder medium.

4. Transfer cell suspension to 6 cm gelatinized dish, and grow at 37°C in a humidified 5% CO_2 incubator (should be confluent within 2-3 days).

5. When confluent, aspirate medium off and wash with 5 ml of pre-warmed PBS, pipetting it away from the cells. Rock dish gently and aspirate medium.

6. Cover cells with 1 ml of 0.05% trypsin solution and incubate at 37°C for 4 minutes or until cells are uniformly dispersed into small clumps.

7. Add ~5 ml of MEF Feeder medium to inactivate the trypsin, and pipette vigorously to make single cell suspension (we recommend 15 times).

8. Spin for 4 minutes at 1000 rpm.

9. Aspirate off medium and gently resuspend cells in 20 ml of pre-warmed MEF Feeder medium.

10. Split the cell suspension onto two gelatinized 10 cm tissue culture dishes, and grow at 37°C in a humidified 5% CO_2 incubator (should be confluent within 2-3 days).

11. To mitotically inactivate, replace medium with 10 ml Inactivation medium (2 mg of Mitomycin C to 200 ml MEF feeder medium), and incubate in a 37°C humidified 5% CO_2 incubator for 2.5 hours. Aspirate Inactivation medium, and rinse three times with pre-warmed PBS; aspirating completely between rinses. These dishes are now ready to use.

12. If you wish to freeze the cells for later usage, trypsinize and pellet the cells as before, but with 1.5 ml of 0.05% trypsin solution, and inactivate the trypsin with ~5 ml medium.

13. For each 10 cm dish, count cells, and resuspend in an equal volume of MEF Feeder medium and 2X Freezing medium; to a density of 1.5-2 x 10^6 cells/0.5 ml. Decant 0.5 ml aliquots into labeled cryovials.

14. Immediately place cryovials in a Styrofoam container or temperature controlled freezing vessel.

15. Freeze vials in a -80ºC freezer. After 24 hours, transfer cryovials to liquid or vapor-phase nitrogen for longer term storage.

Plating Mitotically Inactive MEF Feeder Cells

1. Coat a 6 cm tissue culture dish with 0.1% gelatin and aspirate off immediately before use.

2. Thaw 1 vial of mitotically inactive MEF Feeder cells (approx. 1.5-2 x 10^6 cells) in a 37°C water bath and dilute into 10 ml of pre-warmed MEF feeder cell medium.

3. Pellet the cells by spinning for 4 minutes at 1000 rpm.

4. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed MEF Feeder cell medium.

5. Transfer cell suspension to the 6 cm gelatinized dish, and grow at 37°C in a humidified 5% CO_2 incubator.

6. The feeders are ready for use after 6-12 hours, or may be maintained in the incubator for a maximum of 8-10 days.

Thawing Bruce4 ES Cell Clones

1. Thaw 1 vial of ES cells (approximately 2 to 5 x 10^6 cells/vial) in a 37°C water bath and dilute (drop wise) with 10 ml of pre-warmed Bruce4 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 Bruce4 cell medium.

4. Aspirate the old medium from your 6 cm mitotically inactive MEF Feeder dish.

5. Transfer the ES cell suspension to the feeder dish, and grow in a 37°C humidified 5% CO_2 incubator (10% CO_2 incubator may be used for Bruce4-derived cell lines).

6. Change medium the following day to remove dead cells and residual DMSO.

7. Change medium daily until 80% confluent (approx. 1.5-2 x 10^7 cells); should take 2-3 days.

8. When confluent, the 6 cm dish may be split in two, half for microinjection and half to expand for freezing.

Expansion of Bruce4 ES Cell Clones for Microinjection and Future Use

1. 1 day prior, prepare one 10 cm MEF Treated Feeder dish, using the plating density guide below.

2. The next day, aspirate off the old medium prior to plating ES cells.

3. On the day, wash the confluent 6 cm ES cell dish once with 5 ml PBS.

4. Cover the cells with 1 ml of 0.25% trypsin or Chicken serum trypsin solution and incubate at 37°C for 4-5 minutes or until cells are uniformly dispersed into small clumps.

5. Add ~5 ml of Bruce4 cell medium; to inactivate the trypsin, and pipette vigorously to make single cell suspension (we recommend 10-15 times).

6. Split the cell suspension in half, placing 2.5 ml each into 15 ml centrifuge tubes (labeled 'Expansion' and 'Microinjection').

7. Spin both tubes for 4 minutes at 1000 rpm.

8. For the 'Expansion' cells; aspirate off the supernatant and resuspend the pellet in 10 ml Bruce4 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% CO_2 incubator (10% CO_2 incubator may be used for Bruce4-derived cell lines). Change medium daily until 80% confluent (should take 2-3 days).

9. For the 'Microinjection' cells; aspirate off the supernatant and resuspend the pellet in 150-400 µl microinjection medium (Hepes Buffered DMEM with 5% FBS; filtered through 0.2 µM filter unit, can be aliquoted and stored at -20°C for up to 12 months). Immediately place the cell suspension on ice, and microinject within 1-2.5 hours.

Freezing Expanded Bruce4 ES Cell Clones

1. Wash the confluent 10 cm ES cell dish once with 10 ml PBS each.

2. Cover the cells with 1.5 ml of 0.25% trypsin or Chicken serum trypsin solutions and incubate at 37°C for 4-5 minutes or until cells are uniformly dispersed into small clumps.

3. Add ~5 ml Bruce4 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 an equal volume of Bruce4 cell medium and 2X Freezing medium (we would recommend 8-10 vials containing 0.5 -1.0 ml aliquots; per 10 cm dish). Decant into labeled Nunc cryovials.

6. Immediately place cryovials in a Styrofoam container (taped closed) or temperature controlled freezing vessel.

7. Freeze vials in a -80ºC freezer. After 24 hours, transfer cryovials to liquid or vapor-phase nitrogen for longer term storage.

MEF Treated Feeder Cells – Plating Density Guide

Plate/Dish	Feeder Cell Density
96 well	1.5×10^4
24 well	1×10^5
3.5 cm dish	8×10^5
6 cm dish	$1.5\text{-}2 \times 10^6$
100 mm dish	$3.5\text{-}4 \times 10^6$