Mouse Embryo Fibroblast (MEF) Feeder Cell Preparation

The production of mouse mutants using homologous recombination and blastocyst-mediated transgenesis requires the maintenance of mouse embryonic stem (ES) cells in an undifferentiated state. Many investigators rely on feeder layers to prevent ES cell differentiation; feeder layers prepared from mitotically inactivated primary mouse embryo fibroblasts (MEFs) are used most commonly. This unit describes a simple method to isolate and store MEFs (Basic Protocol 1 and Support Protocol) and two common techniques for mitotic inactivation: γ-irradiation (Basic Protocol 2) and mitomycin C treatment (Alternate Protocol).

NOTE: All cell culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

ISOLATION OF PRIMARY MOUSE EMBRYO FIBROBLASTS

BASIC PROTOCOL 1

MEFs are isolated from 12.5 to 13.5 postcoitum (p.c.) mouse embryos. The embryos are dissociated and then trypsinized to produce single-cell suspensions. After expansion, aliquots can be frozen and stored in liquid nitrogen indefinitely. Alternatively, MEFs suitable for ES cell culture may be obtained from commercial sources (see *http://www.biosupplynet.com* for a current list of suppliers). Commercial MEFs may be useful to researchers new to ES cell culture or to those who will grow ES cells on a limited scale.

Materials

Mouse embryos, 12.5 to 13.5 days postcoitum (Hogan et al., 1994)
DPBS (see recipe), sterile
Trypsin/EDTA solution (see recipe)
MEF medium (see recipe) with penicillin/streptomycin

Laminar flow hood
Inverted microscope
100-mm tissue culture dish
Dissecting forceps and fine scissors, sterilized by autoclaving or ethanol flaming
10-ml syringe and 16-G needle
100-mm tissue culture plates or 75-cm^2 flasks

Additional reagents and equipment for passaging and freezing MEFs (see Support Protocol)

1. Dissect mouse embryos (12.5 to 13.5 days postcoitum) into 10 to 20 ml sterile DPBS in a 100-mm tissue culture dish. Process embryos from one mouse together. Remove embryonic internal organs from the abdominal cavity using dissecting forceps. Transfer the carcass to a clean dish with fresh DPBS.

   Organ removal can be done crudely. Initial dissection can be performed at the bench. Subsequent procedures should be performed in a laminar flow hood.

   Any mouse strain can be used as an embryo source. However, outbred mice or F1 hybrids will usually produce more embryos per mating than inbred mice. If possible, use mice maintained in a viral-antibody-free (VAF) facility to reduce the chance of contamination. If the feeder cells will be used during the selection of antibiotic-resistant ES cells, use embryos from a transgenic mouse expressing the appropriate selectable marker. Appropriate transgenic mice may be obtained from the ES cell community or from standard mouse vendors such as The Jackson Laboratory and Taconic.

Contributed by David A. Conner
Current Protocols in Molecular Biology (2000) 23.2.1-23.2.7

2. Rinse embryos again in 10 to 20 ml DPBS. Transfer the embryos to a clean 100-mm tissue culture dish containing 3 to 5 ml trypsin/EDTA solution.
3. Dissociate embryos by aspirating into a 10-ml syringe through a 16-G needle and expelling the contents. Repeat two to four times.

   Too many repetitions will reduce the cell yield. The embryos should be dissociated to the extent that they can be easily aspirated into a 5- or 10-ml pipette.
4. Add trypsin/EDTA solution to 10 ml. Mix contents by trituration, return to dish, and incubate 5 to 10 min in a 37°C incubator.
5. Mix again by trituration and incubate for an additional 5 to 10 min at 37°C.
6. Transfer contents to a 50-ml conical tube and add an equal volume of MEF medium with penicillin/streptomycin. Let stand 3 to 5 min at room temperature to allow large tissue pieces to settle to the bottom.
7. Remove solution, avoiding large tissue pieces, and place in a fresh 50-ml tube. Centrifuge 5 min at 1000 × *g*, room temperature.
8. Remove supernatant and resuspend pellet in 10 to 50 ml fresh MEF medium with penicillin/streptomycin. Plate cells on 100-mm tissue culture plates or 75-cm^2 flasks, using approximately one embryo per plate or flask. Add medium to a final volume of 10 to 15 ml/plate.
9. Grow cells until confluent (2 to 5 days). Monitor cell density using an inverted microscope. Change medium after the first day and every other day thereafter.
10. Passage cells by trypsinizing (see Support Protocol, steps 1 to 4), resuspending the cell pellet in 10 to 50 ml MEF medium with penicillin/streptomycin, and plating at a dilution of 1:5 to 1:10. Add medium to a final volume of 10 to 15 ml per 75-cm^2 flask or 10-mm plate.

    Using this dilution, fibroblasts from each original embryo are now plated on five to ten 75-cm^2 flasks or 100-mm plates.
11. Grow again until confluent (3 to 5 days) and freeze (see Support Protocol, steps 1 to 7) at ~5×10^6 cells/ml.

    A representative vial should be thawed to check for viability. It is also a good habit to check each new batch for mycoplasma contamination using a commercial service or a PCR-based screening kit (e.g., Pan Vera, Stratagene; also see Coté, 2000)). In addition, MEFs made from previously untested transgenic mice should be grown in the appropriate antibiotic to ensure that they are resistant to the concentration of antibiotic used for selection.

BASIC PROTOCOL 2

MITOTIC INACTIVATION OF MEFS WITH γ-IRRADIATION

MEFs must be inactivated prior to use as a feeder layer for mouse ES cells. Mitotic inactivation prevents the dilution of ES cell lines with dividing fibroblasts. MEFs can be inactivated using γ-irradiation, as described here, or mitomycin C treatment (see Alternate Protocol). This procedure is faster and less labor intensive, but requires a convenient radiation source. Both methods produce feeder layers suitable for the maintenance of undifferentiated ES cells.

Materials

Frozen MEF culture (see Basic Protocol 1)
MEF medium (see recipe) without penicillin/streptomycin
Ca^{2+}- and Mg^{2+}-free HBSS (see recipe)

100-mm tissue culture plates or 75-cm^2 flasks
150-cm^2 tissue culture flasks
100-mm Petri dishes
γ-Radiation source

Additional reagents and equipment for passaging, freezing, and thawing MEFs (see Support Protocol)

1. Thaw a vial of frozen MEFs as described below (see Support Protocol, steps 8 to 10) and plate in a 75-cm^2 tissue culture flask or 100-mm plate. Grow until confluent (3 to 5 days). Change medium after the first day and every other day thereafter.
2. Passage cells by trypsinizing (see Support Protocol, steps 1 to 4), resuspending the cell pellet in 10 to 50 ml MEF medium without penicillin/streptomycin, and plating at a 1:10 dilution. Add medium to a final volume of 10 to 15 ml per 75-cm^2 flask or 100-mm plate.
3. Grow until confluent (3 to 5 days), and passage at a 1:5 to 1:10 dilution, using twenty-five to fifty 150-cm^2 flasks.

   Primary fibroblasts undergo a limited and variable number of cell divisions. Further passaging may be possible, but the rate of cell division slows quickly. Thawing a fresh vial is usually the fastest way to generate more feeders.
4. Remove medium from confluent flasks, rinse with 15 ml Ca^{2+}- and Mg^{2+}-free HBSS, and trypsinize again (see Support Protocol, steps 1 to 4).

   MEFs from ten to fifteen 150-cm^2 flasks can be processed together.
5. Resuspend pellet in 10 ml MEF medium without penicillin/streptomycin and transfer suspension in a 100-mm Petri dish.

   Use a constant volume of medium regardless of the number of cells to ensure consistent results. Use a Petri dish rather than a cell culture plate to prevent adherence of the MEFs to the surface during irradiation.
6. Expose cells to 4000 rads from a γ-radiation source.
7. Dilute suspension to 50 ml with MEF medium without penicillin/streptomycin. Count the number of cells and freeze as described below (see Support Protocol, steps 4 to 7).

MITOTIC INACTIVATION OF MEFS WITH MITOMYCIN C

ALTERNATE PROTOCOL

MEFs may be inactivated by mitomycin C treatment if a γ-radiation source is not available. Although this method is more time and labor intensive, the inactivated feeders are equally suitable for ES cell culture.

Additional Materials *(also see Basic Protocol 2)*

1 mg/ml mitomycin C (Sigma) stock solution, filter sterilized (store at 4°C protected from light)

1. Expand a vial of MEFs as described above (see Basic Protocol 2, steps 1 to 3).
2. Add 1 mg/ml mitomycin C stock solution to the medium to a final concentration of 10 µg/ml. Return plates to the incubator for 2 to 3 hr.
3. Rinse plates twice with 10 to 15 ml Ca^{2+}- and Mg^{2+}-free HBSS.
4. Trypsinize as if passaging (see Support Protocol, steps 1 to 4).

5. Add an equal volume of MEF medium without penicillin/streptomycin.
6. Count and freeze cells (see Support Protocol, steps 4 to 7).

SUPPORT PROTOCOL

FREEZING AND THAWING MEFS

Freezing MEFs, particularly inactivated fibroblasts, is a great convenience. Large stocks of active or inactivated fibroblasts can be prepared at any time, obviating the need to coordinate MEF preparations with ES cell manipulations. Cells are frozen slowly in medium containing 10% (v/v) dimethyl sulfoxide and thawed rapidly.

Materials

Plates containing MEFs (see Basic Protocols 1 and 2; see Alternate Protocol)
Ca^{2+}- and Mg^{2+}-free HBSS (see recipe)
Trypsin/EDTA solution (see recipe)
Freezing medium (see recipe)
MEF medium (see recipe) with or without penicillin/streptomycin
Cryovials

Additional reagents and equipment for counting cells with a hemacytometer (*APPENDIX 3F*)

Freeze cells

1. Remove MEF medium and rinse plates with 10 to 15 ml Ca^{2+}- and Mg^{2+}-free HBSS.

   Washing the plates removes residual serum that will inhibit trypsin.

2. Remove HBSS and add trypsin/EDTA solution to cover the surface of the cells (e.g., 3 to 5 ml in a 75-cm^2 flask). Incubate 3 to 5 min at 37°C and tap the flask to release the cells.

   When trypsinization is complete, tapping and rocking the plate should release the cells. Loose sheets of cells are visible to the naked eye. Incubate at 37°C for an additional 1 to 2 min if the cells do not slough off.

3. When the cell layer has loosened, add an equal volume of MEF medium and mix by trituration to produce a single-cell suspension.

   MEF medium should contain penicillin/streptomycin when preparing initial MEFs (i.e., Basic Protocol 1, step 10), but not for other protocols. Addition of medium containing serum will inhibit further trypsinization.

4. Count cells using a hemacytometer (*APPENDIX 3F*) and then pellet by centrifuging 3 to 5 min at $1000 \times g$, room temperature.
5. Resuspend pellet at 3×10^6 cells/ml in freezing medium and mix by trituration.
6. Dispense into cryovials in 1-ml aliquots, place cryovials in an insulated container at −80°C, and leave overnight.

   Under these conditions, the cells will freeze slowly enough to maintain viability.

7. Transfer cryovials to liquid nitrogen.

   Cells should not be quick-frozen in liquid nitrogen. Vials can be stored at −80°C for several months and in liquid nitrogen for years.

Thaw cells

8. Thaw cells rapidly by placing vials in a 37°C water bath.

9. Add cells to a tube with 10 ml MEF medium without penicillin/streptomycin. To remove DMSO, pellet by centrifuging 3 to 5 min at 1000 × *g*, room temperature.

10. Resuspend in a volume appropriate for the surface area of the plate (e.g., 10 to 15 ml for a 75-cm^2 flask or 100-mm plate).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

NOTE: Prepare all cell culture solutions from tissue culture–grade reagents. Use tissue culture–grade water (high resistance and endotoxin free). Sterilize all final solutions by filtration or prepare from sterile stocks. Use disposable sterile plasticware to prevent microbial and detergent contamination.

Dulbecco's phosphate-buffered saline (DPBS)

0.1 g/liter anhydrous $CaCl_2$
0.1 g/liter $MgCl_2 \cdot 6H_2O$
0.2 g/liter KCl
0.2 g/liter KH_2PO_4
8.0 g/liter NaCl
2.16 g/liter $Na_2HPO_4 \cdot 7H_2O$
Adjust pH, if necessary, to 7.0 to 7.2 with 1 N HCl or 1 N NaOH
Sterilize by filtration or by autoclaving
Store at 4°C (stable indefinitely)

Freezing medium

Dulbecco's modified Eagle medium (DMEM) with high glucose (4500 mg/liter), L-glutamine, sodium pyruvate, and pyridoxine hydrochloride or pyridoxal hydrochloride (e.g., Life Technologies, JRH Biosciences, or Sigma) containing:
20 mM HEPES, pH 7.3
20% (v/v) FBS, heat inactivated for 30 min at 56°C
10% (v/v) dimethyl sulfoxide (DMSO)
Store up to 1 year at −20°C

Freezing and thawing should be avoided. Commercial freezing media based on 10% DMSO can be used. Consult UNIT 23.3 regarding the selection of FBS.

Hank's balanced salt solution (HBSS), Ca^{2+} and Mg^{2+} free

0.4 g/liter KCl
0.06 g/liter KH_2PO_4
8.0 g/liter NaCl
0.35 g/liter $NaHCO_3$
0.048 g/liter Na_2HPO_4
1.g/liter D-glucose
0.01 g/liter phenol red
Adjust pH, if necessary, to 7.0 to 7.4 with 1 N HCl or 1 N NaOH
Store up to 6 months at 4°C

Mouse embryo fibroblast (MEF) medium

Dulbecco's modified Eagle medium (DMEM) with high glucose (4500 mg/liter), L-glutamine, sodium pyruvate, and pyridoxine hydrochloride or pyridoxal hydrochloride (e.g., Life Technologies, JRH Biosciences, or Sigma) containing:
10% (v/v) FBS, heat inactivated for 30 min at 56°C
1× MEM nonessential amino acids (from 100× stock; Life Technologies)
2 mM L-glutamine (from 100× stock; Life Technologies)
0.1 mM 2-mercaptoethanol
20 mM HEPES, pH 7.3
1× penicillin/streptomycin when indicated (from 100× stock; Life Technologies)
Store up to 2 weeks at 4°C

Consult UNIT 23.3 regarding the selection of FBS. Penicillin/streptomycin should be included during the initial isolation of MEFs. It is not needed after Basic Protocol 1.

Trypsin/EDTA solution

Ca^{2+}- and Mg^{2+}-free HBSS (see recipe) containing:
2.5 g/liter porcine trypsin (0.25%)
0.38 g/liter EDTA·$4H_2O$
Store aliquots up to 1 year at −20°C

Aliquots can be thawed and stored at 4°C for up to 1 week. Repeated freezing and thawing should be avoided.

COMMENTARY

Background Information

Historically, ES cells have been cultured under a variety of conditions to prevent differentiation (Wurst and Joyner, 1993; *UNIT 23.3*). The most common method utilizes feeder layers prepared from mouse embryo fibroblasts or from SIM mouse embryo fibroblasts resistant to thioguanine and oubain (STO cells; Hogan et al., 1994; Martin and Evans, 1975). Whereas MEFs are primary cultures with a limited mitotic potential, STO fibroblasts will divide indefinitely. STO cells resistant to G418 are readily available from the ES cell community. STO cells can be grown and inactivated using the protocols for MEFs. For more details see Robertson (1987), Wurst and Joyner (1993), or Hogan et al. (1994).

The advantage of MEFs is that they provide a more consistent source of feeder cells. Early passage cells with reproducible characteristics must be used because they rapidly lose their ability to divide. Long-term propagation of STO cells can lead to changes that result in characteristics that are less favorable for ES cell growth. Because of these potential problems, researchers who are new to ES cell culture and who wish to generate ES cell clones capable of germ-line transmission will probably have more success with primary MEFs.

Critical Parameters and Troubleshooting

The major problems encountered during the isolation and inactivation of MEFs are typically a result of poor aseptic technique, low cell yields during isolation, or inefficient mitotic inactivation. Special attention should be paid to the use of aseptic technique. After the initial primary cultures have been frozen, antibiotics should not be used; antibiotics can mask bad aseptic technique. Each new batch of MEFs should be checked for mycoplasma after growth without antibiotics (Coté, 2000). Inadvertent contamination will spread to ES cells and reduce their germ-line potential. Contaminated batches should be disposed of; it is best not to try to treat them. Contamination should be a rare occurrence.

Aseptic technique is also important in the preparation of media and solutions. All solutions should be prepared using tissue culture–grade reagents and water (high resistance and endotoxin free). Although they are more expensive, solutions that have been screened for toxicity and mycoplasma are available from many companies. Laboratories with little cell culture experience will probably have the greatest success with commercial, prescreened solutions.

Occasionally low cell yields are observed after the initial embryo dissociation. Plates from the initial dissociation should reach con-

fluence in 2 to 5 days. If this does not occur, it is likely due to low trypsin activity or cell lysis during dissociation with the needle and syringe. Some cells may be recovered by passaging without dilution or by concentrating the cells from several plates onto a single plate. Under these circumstances, the cells may still be used as long as they resume dividing and reach confluence. During future preparations, fresh trypsin solutions should be used and the number of times each embryo is passed through the needle should be reduced.

Occasionally evidence of cell division is observed in cultures of mitotically inactivated MEFs. When preparing inactivated feeders for the first time, check to make sure that the treatment was effective. This can be accomplished by plating out an aliquot of the inactivated feeders at a density of ~6×10^4 cells/cm^2. The cells are then cultured for 10 to 14 days, with periodic changes of medium, and cultures are assessed for increases in cell density and foci of mitotic activity. Foci can be observed by eye as opaque splotches on the plate, but should be confirmed with a microscope. No growth should be seen on a 100-mm plate; however, feeders may still be used if there are only a few foci. If there is a dramatic change in cell density or there are many colonies of dividing cells, the preparation should be discarded and the procedure repeated with newly expanded cells. If mitomycin C was used, a fresh solution should be prepared. If γ-irradiation was used, the radiation source should be properly calibrated. Distance and shielding can affect the dose dramatically.

In addition to these concerns, it is important to remember that MEFs are primary cells with limited mitotic potential. Expanding the cells more than suggested may work, but the rate of growth will decrease. Plates can still become confluent because the cell size will increase; however, the number of cells per plate will decrease and the time to reach confluence will increase. Feeder layers made under these conditions may not be ideal for ES cell culture.

Anticipated Results

Expect 10 to 30 vials of frozen cells from each embryo. Each vial can be expanded to produce 50 to 100 vials containing 1 ml of mitotically inactive feeders. Each vial should be sufficient for one targeting experiment.

Time Considerations

Processing a single litter of embryos (six to ten) should take 1 to 2 hr. After plating the dissociated embryos, cultures should reach confluence within 2 to 5 days. Cells can be frozen at this point, but the total yield will be dramatically reduced. After passaging, the cells should be ready to freeze in 3 to 5 days. Thawed vials should reach confluence in 3 to 5 days. After each passage the cells should take 3 to 5 days to reach confluence. The cells can be inactivated at any stage, but the yield will be lower with less expansion.

Literature Cited

Coté, R. 2000. Assessing and controlling microbial contamination in cell cultures. *In* Current Protocols in Cell Biology (J.S. Bonifacino, M. Dasso, J.B. Harford, J. Lippincott-Schwartz, and K.M. Yamada, eds.) pp. 1.5.1-1.5.18. John Wiley & Sons, New York.

Hogan, B., Beddington, R., Constantini, F., and Lacy, E. 1994. Manipulating the Mouse Embryo: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Martin, G.R. and Evans, M.J. 1975. Differentiation of clonal lines of teratocarcinoma cells: Formation of embryoid bodies in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 72:1441-1445.

Robertson, E.J. 1987. Embryo-derived stem cell lines. *In* Teratocarcinomas and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.) pp. 71-112. IRL Press, Oxford.

Wurst, W. and Joyner, A.L. 1993. Production of targeted embryonic stem cell clones. *In* Gene Targeting: A Practical Approach (A.L. Joyner, ed.) pp. 33-61. IRL Press, Oxford.

Key References

Hogan et al., 1994. See above.

Provides additional or alternative protocols and defines the context for use of MEFs during gene targeting in ES cells.

Internet Resources

www.biosupplynet.com

Search this Web site for "embryonic stem cell reagents" to obtain a current list of suppliers that provide medium and MEFs suitable for ES cell culture.

Contributed by David A. Conner
Harvard Medical School
Boston, Massachusetts