



Standard Operating Procedure

Standard Operating Procedure

Protocol:	ESC02	Version:	1.0
Protocol Section:	Embryonic Stem Cells	Effective Date:	1-Jan-2008
Protocol Title:	Preparation and inactivation of primary mouse embryonic fibroblasts as feeders for ES cell culture	Date Reviewed:	15-Dec-2007
		Date Revised:	13-Jun-2008

This standard operating procedure (SOP) was adapted from the protocols developed by the ES Cell Core Facility at the Samuel Lunenfeld Research Institute (SLRI), Mount Sinai Hospital, Toronto, Canada. This SOP describes the protocol for used to make primary mouse embryonic fibroblasts (MEFs) used as feeders for embryonic stem (mES) cell culture at the CMMR. The CMMR obtains its MEFs from the ES Cell Core Facility at SLRI.

To maintain pluripotency, mES cells are cultured on feeder cells; either MEFs, STO fibroblast cell line; or for feeder-free mES clones on gelatinized plates in the presence of leukaemia inhibitory factor (LIF). Growth media and culture conditions should be used as suggested for each ES cell line. Mitotically inactivated MEFs are used as feeders only for a long-term culture of R1 ES cells [1], typically before and after cryopreservation. Otherwise, culture of R1 ES cells for electroporation and during the selection is done on gelatinized plates. MEF cells can be made from any strain of mice including transgenic mice that express bacterial neomycin or hygromycin genes depending on the choice of selectable markers used for altering the ES cell genome. Neo^R and Hygro^R (JR2356, JR2354) as well as the DR-4 strain (Jackson Laboratories #003208) bearing neo, puro, hyg resistance genes and a deletion of the Hprt gene are available from Jackson Laboratories. Detailed protocols for preparation of MEF stocks and mitomycin C treated feeders for ES cell culture are presented in many publications (e.g. [2]). Our brief protocols are given below. MEF are also commercially available (e.g. Specialty Media #PMEF).

Related Protocols:

Protocol ESC01 describes the culture and cryopreservation of mES cells. Protocol ESC03 describes the pathogen testing protocol used at the CMMR.

Materials:

15.5-17.5 dpc pregnant mice
 Sterile dissection instruments
 Autoclaved 3-5 mm diameter glass beads
 Autoclaved 1-2 inch stir bar
 50-ml sterile conical tubes [BD Falcon 352070, or equivalent]
 10-cm & 15-cm tissue culture dishes [BD Falcon 353003 & 353025, or equivalent]
 DMEM [Invitrogen 11960-044 (liquid), 12100 (powder)] with 10% FBS [ES cell qualified]
 1X PBS, Ca²⁺, Mg²⁺ free [Invitrogen 14190-144]
 10 mg/ml DNaseI [Sigma D4527]
 0.05% Trypsin/0.53 mM EDTA [Invitrogen 25300]

Trypan blue [Flow labs # 16-910-49]

Freezing medium [80% ES-DMEM (see ESC01), 10% FBS, 10% DMSO] prepared fresh and kept on ice

Mitomycin C [Sigma M0503]

Sterile disposable pipettes and pipet-aid

Cryovials [Wheaton 98574500, Naglene 5000-0012, or equivalent]

Controlled rate freezer [Bel-Air F18844-0000, or equivalent]

Protocol:

Section 1. Preparation & Freezing of Primary MEFs

- 1.1. Aseptically dissect fetuses from 1 or 2 mice in 10-cm Petri dish containing 1X PBS.
- 1.2. Transfer dissected embryos into a new dish with 1X PBS; remove heads and all internal organs.
- 1.3. Transfer embryos to a 50-ml tube. Remove as much blood as possible by washing carcasses at least twice with 50 ml of 1X PBS.
- 1.4. Mince carcasses into small pieces with sterile scissors in minimal volume of 1X PBS in a 50-ml tube with the top cut off or in a petri dish.
- 1.5. Add 10 ml of Trypsin/EDTA to minced tissue. Transfer suspension into a fresh 50-ml tube.
- 1.6. Add approximately 5 ml of glass beads and place a stir bar inside the tube. If solution becomes viscous, add 100 μ l of 10 mg/ml DNase per 10 ml of suspension.
- 1.7. Incubate at 37°C for 30 minutes with stirring.
- 1.8. Add another 10 ml of Trypsin/EDTA. Incubate at 37°C for another 30 minutes with stirring.
- 1.9. Repeat step 1.8 one more time (final volume ~30 ml).
- 1.10. Allow any remaining pieces of tissue to settle by letting tube rest a few minutes without stirring. Transfer equal volumes (~15 ml/tube) of cell suspension into two 50-ml tubes each containing 3 ml of FBS.
- 1.11. Wash the original tube twice with DMEM+10% FBS and add to the tubes with cell suspension, leaving behind any pieces of tissue.
- 1.12. Collect cells by centrifugation at 270 xg for 5 minutes.
- 1.13. Resuspend the pellet in DMEM + 10% FBS.
- 1.14. Count viable nucleated cells by trypan blue exclusion. Normal yield is approximately 5×10^7 – 10^8 cells from 10 fetuses.
- 1.15. Plate 5×10^6 nucleated cells (approximate yield from 1.25 embryos) per 15-cm dish. Incubate at 37°C/5% CO₂.
- 1.16. Change the medium the next day.
- 1.17. When confluent (in 2-3 days) remove media from cells. Wash cells with ~20 ml room temperature 1X PBS. Aspirate PBS.
- 1.18. Add 10-ml Trypsin/EDTA and incubate at 37°C/5% CO₂ for 5 to 10 minutes, or until cells lift off plate. If cells lift off as a single sheet, then pipette up and down to make a single cell suspension. Add 20-30 ml DMEM+10% FBS.
- 1.19. Transfer cell suspension to a 50-ml tube. Collect cells by centrifugation at 270 xg for 5 minutes. Aspirate supernatant.
- 1.20. Resuspend cells in ~30 ml DMEM+10% FBS. Transfer equal volumes of cell suspension into each of six (6) 15-cm plates. Top up media to 25 ml and disperse cells evenly across plates. Incubate at 37°C/5% CO₂.
- 1.21. When these plates reach confluency, cells from each plate can be cryopreserved for later use. Trypsinize cells as in steps 1.17 to 1.19.
- 1.22. Resuspend cell pellet in 1-ml freezing media/15-cm plate of feeders. Transfer cells from one 15-cm plate (~1-ml) to one cryovial on ice.

- 1.23. Transfer cryovials into a controlled-rate freezer and freeze to -86°C . Once frozen, transfer cells to the vapour phase of liquid nitrogen for long-term storage.
- 1.24. Prepared MEF stocks should be tested for mouse pathogens (eg. RADIL IMPACT I). Reserve a small amount of MEFs and passage and expand as appropriate for pathogen testing.

Section 2. Preparing MEFs for Mitomycin C treatment

- 2.1. Prepare a 50-ml tube by adding ~20 ml of DMEM+10% FBS.
- 2.2. Thaw a vial frozen primary MEFs (as prepared in Section 1) in a 37°C water bath or heat block until only a few ice crystals remain.
- 2.3. Aseptically transfer cell suspension into the tube prepared in step 2.1.
- 2.4. Collect cells by centrifugation at 270 xg for 5 minutes. Aspirate supernatant.
- 2.5. Resuspend pellet in ~30 ml DMEM+10% FBS
- 2.6. Seed cells onto five or six 15-cm plates. Top up media to 25 ml per plate. Incubate at $37^{\circ}\text{C}/5\% \text{CO}_2$.
- 2.7. When cells are confluent (in about three days), they can either be
 - a. split one more time before being treated with Mitomycin C; or
 - b. treated with Mitomycin C and used directly as feeders for ES cell culture; or
 - c. treated with mitomycin C, frozen in cryovials and used as feeders later (alternative cost-effective way for laboratories with small volume needs).

Section 3. Mitomycin C treatment

- 3.1. Aspirate media from a confluent plate of MEFs.
- 3.2. Add 10 ml DMEM+10% FBS and 100 μl of 1 mg/ml Mitomycin C stock solution. Incubate for 2 hours at $37^{\circ}\text{C}/5\% \text{CO}_2$.
- 3.3. Aspirate Mitomycin C media. Rinse plate twice with 25 ml room temperature 1XPBS.
- 3.4. Add 10-ml Trypsin/EDTA and incubate at $37^{\circ}\text{C}/5\% \text{CO}_2$ for 5 to 10 minutes, or until cells lift off plate. If cells lift off as a single sheet, then pipette up and down to make a single cell suspension. Add 20-30 ml DMEM+10% FBS.
- 3.5. Transfer cell suspension to a 50-ml tube. Collect cells by centrifugation at 270 xg for 5 minutes. Aspirate supernatant.
- 3.6. Either freeze cells from one 15-cm plate into a single 1-ml cryovial (steps 1.22-1.23); or resuspend cells in DMEM+10% FBS at 2×10^5 cells/ml and seed onto tissue culture plates for immediate use.

One confluent 15-cm Mitomycin C-treated MEF plate can generate approximately the following number of feeder plates for ES cell growth:

- 5 x 10-cm plate (10 ml each)
 - 12 x 6-cm plates (5 ml each)
 - 25 x 35-mm plates (2 ml each)
 - 25 x 4-well plates or 4-5 x 24-well plates (0.5 ml/well)
 - 6 x 96-well plates (0.2 ml/well)
- 3.7. Incubate at $37^{\circ}\text{C}/5\% \text{CO}_2$.
Feeders are preferably incubated overnight, or for at least a few hours, before plating ES cells. The medium is changed to ES-DMEM prior to use for ES cells. Mitomycin C treated feeders can be used within 7-10 days (medium is changed every 3-4 days).

Additional Notes:

More information about ES cell resources available from the CMMR can be found at <http://www.cmmr.ca>.

References:

1. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C., (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A, **90**, 8424-8.
2. Joyner, A., (ed.) (1999) Gene Targeting: A Practical Approach. 2nd ed , Oxford University Press: New York.