



Standard Operating Procedure

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Protocol:	ESC01	Version:	1.0
Protocol Section:	Embryonic Stem Cells	Effective Date:	1-Jan-2008
Protocol Title:	Culture and cryopreservation of mouse embryonic stem cells	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, Mount Sinai Hospital, Toronto, Canada and by Tammy Reid at the Centre for Modeling Human Disease (CMHD) Gene Trap Core led by Dr. William L. Stanford, University of Toronto, Canada. This SOP describes the protocol for culture and freezing of mouse embryonic stem (mES) cells.

Culture conditions are critical for maintaining pluripotent ES cells. The reagents used in this protocol should be matched as close as practicably possible. In particular, fetal bovine serum (FBS) lots can show great variation with respect to their ability to maintain pluripotency in ES cells. FBS lots used at the CMMR are pre-screened for their ability to enable the generation of mice by tetraploid aggregation, that is completely ES-cell derived embryos, before use in production.

Related Protocols:

Protocol ESC02 describes the production of MEF feeders. Protocol ESC03 describes the pathogen testing protocol used at the CMMR.

Materials:

6-cm & 10-cm tissue culture dishes [BD Falcon 353002 & 353003, or equivalent]
 15-ml sterile conical tubes [BD Falcon 352096, or equivalent]
 Sterile disposable pipettes and pipet-aid
 1-ml cryovials [Wheaton 98574500, or equivalent]
 Controlled rate freezer [Bel-Air F18844-0000, or equivalent]

Reagents:

Embryonic Stem Cell Medium (ES-DMEM)

Use a 500ml bottle of Dulbecco's modified Eagle's medium (DMEM) with high glucose [Invitrogen/Gibco #11960-044(liquid), #12100 (powder), #10829-018 (Knockout DMEM)].

**media should be made fresh every 2-3wks and stored at 4°C.*

To prepare medium, add:

- 6ml GlutaMAX - stock 200mM, final concentration 2mM [Invitrogen #35050]
- 6ml 2-mercaptoethanol – stock 10mM, final 0.1mM [Sigma #M7522]
Note: 70ul of 2-ME (14.3M) is diluted in 100ml of PBS or water to make 100X stock (10mM).
- 6ml Sodium Pyruvate – stock 100mM, final 1mM [Invitrogen #11360]
Note: Knockout DMEM already contains sodium pyruvate
- 6ml Non-Essential Amino Acids – stock 10mM, final 1mM [Invitrogen #11140]

- 3ml Penicillin/Streptomycin – stock 10,000U, final 50U/ml [Invitrogen #15140]
- 50ul LIF – stock 1000U/ml [Chemicon #ESG1107]
- 95ml FBS – final 15%

Note: FBS should be used within 4 months of thawing otherwise prepare aliquots and re-freeze.

Use FBS that is ES cell qualified. Samples of lots should be ordered and tested for plating efficiency to indentify the batch of serum that gives optimal growth of ES cells. Some facilities also test serum in the generation of chimeric mice and/or tetraploid complementation assays. Suppliers include: Hyclone (hyclone.com), Wisent Inc. (wisent.ca), Gemini Bio-Products (gembio.com), Invitrogen (invitrogen.com), Sigma (sigma-aldrich.com), Specialty Media (specialtymedia.com) and Chemicon (chemicon.com).

- Selection antibiotic – vector-dependent

Note: When passaging a Genetrap line that has a selection gene (G418, puromycin, etc.), add selection antibiotic to the medium. (G418 = 165ug/ml final, puromycin = 2ug/ml final for CMHD clones). The CMHD genetrap vectors require G418 added to the medium; except for those clones having the pGTlox series of vectors. The G418 (Geneticin) can be ordered from Invitrogen #10131-035 (use 2mls per 500ml bottle of medium).

Freezing Medium

Medium should be made fresh and should always be kept cold.

- 50% ES-DMEM
- 40% FBS
- 10% DMSO [Sigma #D265]

Trypsin

0.25% Trypsin/EDTA [Invitrogen #25200-0720] **OR**

0.05% Trypsin/EDTA [Invitrogen #25300-054]

1X PBS, pH 7.0

Ca²⁺, Mg²⁺ free [Invitrogen #14190-144]

Gelatin

Add 0.1% w/v gelatin [Sigma #G2500] to water or 1X PBS. Autoclave to dissolve gelatin and store at 4°C.

Mouse Embryonic Fibroblasts (MEFs/Feeders)

To maintain pluripotency, R1, G4 or C2 mES cells must be maintained on a lawn of mitotically-arrested primary mouse embryonic fibroblasts (MEFs). Thaw mES cells onto a plate coated with inactivated MEFs. For details about preparation of mitotically inactivated MEFs, consult CMMR protocol ESC02 and references 1 & 2 below. MEFs are plated >24 prior to addition of ES cells and allowed to adhere to the tissue culture plate. MEFs are grown in feeder medium and this must be removed and replaced with ES-DMEM before ES cells are added to the plate.

Protocol:

Section 1. Thawing Cells

- 1.1. Prepare a 6-cm feeder dish by removing feeder medium and adding 1-2 ml of ES-DMEM.
- 1.2. Prepare a 15-ml conical centrifuge tube by adding 5 ml of ES-DMEM. Allow to warm to room temperature.
- 1.3. In a 37°C waterbath, thaw the vial of ES cells until there are only small pieces of ice visible in the tube.
- 1.4. Transfer the vial contents to the prepared 15-ml conical centrifuge tube containing 5 ml ES-DMEM. Mix well by pipeting.
- 1.5. Collect cells by centrifugation at 1000 rpm (~300 xg) for 5 minutes.

- 1.6. Remove supernatant by aspiration.
- 1.7. Resuspend cells in 3-4 ml ES-DMEM medium by gentle pipetting.
- 1.8. Transfer cells to feeder dish prepared in step 1 and resuspend well to evenly disperse ES cells.
- 1.9. Incubate at 37°C/5% CO₂.

Section 2. Feeding Cells

Cells should be fed with 5 ml of ES-DMEM for 6-cm dishes and 10 ml for 10-cm dishes.

- 2.1. Remove medium from ES cell plate by aspiration.
- 2.2. Rinse plate with room temperature 1X PBS.
- 2.3. Remove PBS by aspiration.
- 2.4. Add fresh ES-DMEM to the plate.
- 2.5. Incubate at 37°C/5% CO₂.

Section 3. Passaging Cells

Cells should be passaged when a dish reaches 80% confluency, which usually occurs every 2-3 days.

- 3.1. Remove medium by aspiration.
- 3.2. Rinse plate with room temperature 1XPBS.
- 3.3. Remove PBS by aspiration.
- 3.4. Add trypsin (1 ml for 6-cm dish, 2 ml for 10-cm dish) to dish.
- 3.5. Incubate dish at 37°C/5% CO₂ for 5 minutes.
- 3.6. Add 5 volumes of ES-DMEM to the trypsinized plate.
- 3.7. Resuspend cells by pipetting.
- 3.8. Dispense approximately 1/5 of volume into a new MEF-containing dish and resuspend by pipetting.
- 3.9. Top up ES-DMEM, if necessary, to completely and evenly cover surface of dish.
- 3.10. Incubate at 37°C/5% CO₂.

Section 4. Cryopreserving Cells

- 4.1. Remove medium by aspiration.
- 4.2. Rinse plate with room temperature 1X PBS.
- 4.3. Remove PBS by aspiration.
- 4.4. Add trypsin (1 ml for 6-cm dish, 2 ml for 10-cm dish) to dish.
- 4.5. Incubate dish at 37°C/5% CO₂ for 5 minutes.
- 4.6. Add 5 volumes of ES-DMEM to the trypsinized plate.
- 4.7. Resuspend cells by pipetting.
- 4.8. Transfer cell suspension to 15-ml conical centrifuge tube.
- 4.9. Centrifuge cells at 1000rpm (~300 xg) for 5 minutes.
- 4.10. Aspirate supernatant.
- 4.11. Resuspend cell pellet in freezing media (1 ml for every vial you are freezing – typically 5 vials from a 10-cm plate).
- 4.12. Transfer 1 ml into a cryovial and place on ice immediately. Freeze cells in a controlled rate freezer to -86°C. For long term storage, transfer vial to liquid nitrogen after 24 to 48 hours at -86°C.

Additional Notes:

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

References:

1. Maise, M.P., W. Auerbach and Joyner, A.L. (1999) Production of targeted embryonic stem cell clones. In *Gene Targeting: A Practical Approach*, (ed. A.L. Joyner, Second Edition) Oxford University Press Inc, New York, pp. 101-132.
2. *Manipulating the Mouse Embryo: A Laboratory Manual*. 3rd Ed. (2003) by Nagy, A., Gertsenstein, M., Vintersten, K., Behringer, R. Cold Spring Harbor Laboratory Press. New York.