Mouse ES Cell Culture and Manipulation Protocols

Protocols courtesy of :
Ute Hocheschwender, MD
Director, Neurogenetics Transgenic Program
Duke University, Chapel Hill, NC

Editing and additional protocols:
Cindy McKinney, Ph.D.

This collects all the protocols and additional/supplemental mES protocols in one document.

METHODS FOR mES CELL CULTURE

General Comments: Mouse embryonic stem (mES) cells are "picky" ---they like to grow in small colonies (cells are mostly nucleus with very little cytoplasm) that appear as round to ovoid clusters with dark centers and very defined margins on top of embryonic fibroblast feeder cells. The feeder cells supply undetermined nutrients that help the cells maintain the pluripotency needed for targeted genetic manipulations. Cultures in dishes/flasks **must be refed every day** and **split every 1-2 days** or they will begin to differentiate. Plates should be **no more** than 80% confluent---mES colonies distributed evenly over the surface area of the plate and NO colonies growing into each other. Note if you observe cells growing out of the margins of an ES cell colony they have begun to differentiate.

References:

1 .**Gene Targeting : A Practical Approach**, edited by Alexandra L. Joyner Oxford University Press, 2000 (ISBN 0-19-963792 –X papberback)
2: **Manipulating the Mouse Embryo: A Lab Manual** (A.Nagy, M.Gerttsenstein, K.Vintersten, R. Behringer) Cold Spring Harbor Press, 2003 (ISBN 0-87969-574-9 cloth).
3. International Transgenic Society website---many methods available for review if a member. More up o date for newer techniques and sources of reagents. Also a list serve for questions to active members. Can be found on LinkedIn.

***Procedure: Preparation of Gelatin covered plates**

Use Porcine Gelatin Type A (Sigma #G1890)

1) make 0.5% stock in distilled DI water. (2.5 g gelatin into 500 ml DI water)
2) Autoclave for 30 minutes ---cool to room temperature
3) Store at room temperature

Working Dilution:

Make 0.1% stock:
100 ml of 0.5% stock into 400 ml of DPBS = 0.1% Gelatin for plates
(DPBS Gibco #14190-029 w/o calcium or magnesium)

Coat plates with gelatin **prior** to setting mouse embryonic fibroblasts (MEFs) or mES cells directly on to gelatin. Make sure surface area of plate is covered by solution. After covering plates they can set in hood 15 minutes or O/N in incubator.

Plate Size	Amount of 0.1% Gelatin
6 cm	5 ml
10 cm	10 ml
T-25 flask	10-12 ml
T-75 flask	15-20 ml
96 well plate	50 μL each well
24 well plate	0.5 ml each well

*** Procedures for E14TG2a ES cells- Feeder Independent**

This line of mES cells has been culture adapted to grow without MEF feeders but the procedures are as described below. Care and caution must be taken (since they do not grow on feeders) change the medium ***everyday*** even if the ES cells are not turning the medium yellow. It will be much easier for these cells to begin to differentiation if the culture conditions and manipulations are not rigorous.

Procedure: Preparation of Mouse Embryonic Feeder (MEF) Plates

MEFs for feeder cells are available from commercial vendors (Applied Stem Cell – www.appliedstemcell.com Neo^R irradiated MEFs (AS1116) Neo^R non-irradiated either already irradiated or treated with mitomycin C and ready to use OR can be made from E14 embryos. There are also commercial suppliers for non-irradiated MEFs (Applied Stem Cell- Neo^R non-irradiated AS1102s). Purchase of non-irradiated MEFs (usually at P3) requires expansion to collect as many feeders as possible and then irradiated to compete preparation for mES cell growth.

1) Plating irradiated MEFs

Thaw appropriate number of vials of MEFs to cover the number of plates needed for mES culture.

To Thaw: pull vials from LN2 on dry ice and then quickly place 1-2 vials at a time into 37 C water bath with just the frozen cells and medium in the 37 C water. HAZARD- LN in vials can expand and the vial explode so should wear eye protection when doing this procedure. Thaw until the cells are in liquid with just a few ice crystals. This step(s) should be done as quickly as the DMSO in the thawed medium begins to damage cells immediately.

Pull all vials of thawed MEFs into one tube and gently spin at 1200 rpm for 5-10 minutes. Remove DMSO containing freezing medium and resuspend in feeder medium.

Plate appropriate number of MEF /size of plate needed on *gelatinized plates.*
Check cell concentration/vial usually 2x10^6 irradiated cells/vial

	6 cm dish	10 cm dish	96 well	24 well	T-25 flask	T-75 flask
1 vial MEFs	20 ml MEF 4 ml/dish makes 5 dishes	20 ml MEF 10 ml/dish makes 2 dishes	20 ml MEF 100 μL/well makes 2 plates	20 ml MEF 1 ml/ well makes < 1 plate	20 ml MEF 8 ml/flask makes 2 T-25s	20 ml MEF 20 /ml flask makes 1 T-75

Prepare feeders in feeder medium at *least one* day in advance of thawing or splitting mES cells.

Plates can be kept for approx. 2 weeks in incubator and still used.

NOTE: When planning has not been good---one can thaw MEFs and mES resuspend in mES medium and plate together but *this is not recommended.*

Recipe for MEF/Feeder Growth Medium (500 ml)—filter sterilize (Note 1):

Reagent	Source /Catalog #	Amount
DMEM	Invitrogen	500 ml
10% **HI** FBS (Note-2)	Invitrogen	60 ml
Na Pyruvate 100X	Invitrogen	6 ml
Pen/Strep 100X	Invitrogen	6 ml
NEAA	Invitrogen	6ml
L-Glutamine 100 X (Note-3)	Invitrogen	6 ml

Note 1: Use 500 ml 0.2 μm low protein binding filter flask to sterilize medium once all components have been added. Test an aliquot in incubator to ensure that medium is sterile.

Note 2: Any lot of FBS that has been tested for good cell growth works here. It should be heat – inactivated (**HI**)—thaw serum in refrigerator, warm to 37 C and then place in 56 C water bath for 30 minutes. Remove from 56 C and cool to room temperature. Use as needed. Or, freeze 88 ml aliquots if the HI serum won't be used very frequently.

Note 3: L-glutamine in solution has a 2 week shelf life. Consequently, it should be thawed and used to make medium and then frozen in 6.5 ml aliquots at -20 C to preserve its activity. Each time medium is made thaw the appropriate number of aliquots. Alternately, sue GlutaMax which has longer shelf life.

Procedure: Preparation of primary mouse embryonic fibroblasts (MEF)

These are the non-irradiated MEFs stock vials. (Alternatively purchase these MEFs from commercial vendor and proceed to expansion for irradiation.)

1. Set timed pregnancies for female mice with Neomycin resistant genotype.

2. Euthanize pregnant female(s) at E13.5-14.5 day of gestation/pregnancy. Dip carcass in ethanol and harvest the uterine tract containing embryos and place in sterile PBS.

3. Transfer embryos to TC hood and rinse in PBS again. Dissect out each embryo from extra embryonic membranes.

4. Place embryo in 15 cm dish with PBS and remove head with scalpel. Remove internal organs with dissection. Repeat for each embryo placing dissected one in PBS.

5. When dissections are complete, mince each embryo as finely as possible with scalpels. This can be done as a pool. The tissue will stick together but that is acceptable.

6. Rinse blood away from minced tissue and place in STERILE spinner flask with 0.25% Trysin EDTA approximately one ml/embryo and add enough trypsin-EDTA to bring up solution to 2 ml/embryo,

7. Place embryos on warm plate (37 C) with spinning for 30 minutes (alternatively a 50 ml tube in 37 C water bath). Pipette up and down frequently ---every 5-10 minutes to help bring the suspension into single cells.

8. Add equal volume of MEF medium (serum halts trypsin-EDTA action) mix and let particulates settle to bottom of flask.

9. Pull off supernatant (without particulates) adjust volume to number of original embryos (e.g., 10 embryos collected = 10 ml final volume)

10. Plate onto gelatinized T-75 flasks---1 ml embryo suspension (i.e., 1 embryo /flask) + 20 ml MEF medium place in incubator and allow to grow to extreme confluency.

11. Harvest fibroblasts in flasks and freezer cells ---1 flask/vial = 1 embryo/vial. Resuspend in freezing medium (MEF+10% DMSO) Cells should not sit in freezing medium longer than 10 minutes. Freeze slowly in -70 and then store in LN2

***Procedure: Expansion of MEF stocks for Irradiated Feeder cells**

These IR-MEFs are the feeder cells that are plated on gelatinized plates for mES cell growth.

The MEFs produced from the embryos in the previous procedure are expanded for irradiation to produce non-dividing fibroblasts that act as feeder cells for the MEFs.

1. Thaw one vial of non-irradiated MEF stock and plate in one T-150 flask (50 ml MEF medium) or two T-75 flasks (25 ml MEF medium)

2. Grow each flask to extreme confluency and then trypsinize and split each flask 1:5.

3. Repeat step 2 for each flask splitting 1:5 each time until you have MEFs growing to confluency in 30 flasks.

4. Trypsinize cells each of the 30 flasks and pool into appropriate number of 50 ml conical tubes. Spin cells out of trypsin containing medium (1200 rpm x 10 minutes) Resuspend cells in MEF medium—pull into one 50 ml conical.

5. Make appointment with Dr. Bob Paulson's Lab (schedule ahead) and transport for irradiation. Irradiate each tube with a dose of 3000 rads.

6. Return to tissue culture hood. Count cells. Spin IR-MEFs down. Resuspend in MEF freezing medium (MEF medium +10% DMSO). Cells should not sit in DMSOP medium more than 10 minutes/ Slow freeze in -70 and then transfer to LN2.

7. Freeze at 2 x 106 cells/vial. Label vials with IR-MEF and date.

***Procedure: Growth of mES cells**

These cells are used for gene targeting or other experiments that require undifferentiated embryonic cells. These cells are preferably grown on gelatin treated MEF plates but can be grown(carefully) on gelatin coated plates directly.

Recipe for mES growth medium (see note 1):

Reagent	Source/ Catalog#	Amount
DMEM	Invitrogen	470 ml
15% **HI** KO FBS (see #2)	Invitrogen	87 ml
NEAA 100x	Invitrogen	6 ml
Pen/Strep 100 X	Invitrogen	6 ml
β ME STOCK (see #3)	Sigma	6 ml
mLIF	Millipore/ESGRO 10^5 U	58 µL
L-glutamine (see #4)	Invitrogen	6 ml

Note 1: Use 500 ml 0.2 µm low protein binding filter flask to sterilize medium once all components have been added. Test an aliquot in incubator to ensure that medium is sterile.

Note 2: FBS must be ES cell tested for good growth. It should be heat –inactivated (**HI**)—thaw serum in refrigerator, warm to 37 C and then place in 56 C water bath for 30 minutes. Remove from 56 C and cool to room temperature. Use as needed.

Note 3: 2-mercaptoethanol should be tested for endotoxin and tested on hybridomas for cell toxicity (Sigma 63689 or M3148). To prepare β ME Stock: add 70 µL β ME to 100 ml DPBS and filter sterilize. Invitrogen sells an already diluted stock used in the E14 ES cell line medium.

Note 4: L-glutamine in solution has a 2 week shelf life. Consequently, it should be thawed and used to make medium and then frozen in 6.5 ml aliquots at -20 C to preserve its activity. Each time medium is made thaw the appropriate number of aliquots. Alternately, sue GlutaMax which has longer shelf life.

***Thawing mES cell Vial:**

1. Pre-warm to 37 C any solutions/medium needed for daily procedures.
2. MEF plates needed should be fed with 10-12 ml mES medium approx. 4 h before use or the night before.

2a: If not growing mES cells on MEF plates; prepare gelatin coated plates and then add 12 ml mES medium before plating cells.

3. Pull mES cell vial from LN2 freezer onto dry ice---hold on dry ice until moment ready to quick thaw in 37 C. (Note: LN2 trapped in vials may explode wear protective eye shield. Vial cap can be carefully loosened to relieve pressure but then recap before thawing.)
4. Quick thaw cells in vial in 37 C water bath ---DMSO in freezing medium will damage cells so need to do these steps quickly:
    a. swish vial in 37 C water bath until barely thawed
    b. spray vial with 70% ETOH , move to TC hood
    c. remove cap and slowly (drop by drop) add 1 ml fresh mES cell medium to vial. Pipette gently up and down. Dilutes DMSO.
    d. Move the 2 ml in vial to sterile 15 ml conical---slowly add 2 ml mES medium. Cap and rotate to equilibrate medium.
    e. Add 6 ml mES medium to conical—cap and rotate to mix cells and medium again.
    f. Centrifuge cells gently at 1200 rpm x 10 minutes.
5. Carefully and gently resuspend cell pellet in 1 ml mES medium. Gently pipette cells up and down against side of tube until a homogenous suspension is reached
6. Plate the mES cell suspension onto 10 cm feeder plate(s) into mES medium. Swirl plates to distribute the cell suspension evenly.
   Note: can also plate on to gelatinized plates without feeders.
7. Incubate overnight at 37 C, 5% CO_2. Check growth and stage of confluency the next morning. Change medium **every day** for optimal growth.

Note: If medium is yellow or tinged yellow-change medium immediately to bring pH back to neutral. Also, change medium 2-3 h before trypsinization.

8. mES cells should be split at least 1:5 (one plate into 5 plates) when they reach 70-80% confluency (plate coverage) as the colonies begin to touch and merge the chances for differentiation increase dramatically.

   Note: for a 6 cm plate expect 1-4 x10^6 cells (@80% confluency)
   As a guide--Seed (in 6 cm plates):
   For harvest 2 days after plating, seed 2 x 10^5 cells/plate
   For harvesting 3 days after plating, seed 1 x 10^5 cells/plate
   For harvesting 4 days after plating, seed 0.5 x 10^5 cells/plate

***Procedure: Trypsinizing Cells from plates/Splitting ES cells**

1. Feed plates to be harvested 2-6 h before trypsin treatment with mES complete medium
   Note: It's easy to schedule refeed in AM and split plates/flasks in PM

2. Wash plates with room temperature DPBS

3. Add enough 0.25% trypsin- 1 mM EDTA solution (Gibco/Invitrogen #25200-056) to barely cover plate-swirl. (Note: Keep at 4 C. Some labs recommend using w/in 1 week, or aliquoting and freezing at -20 C until needed)

4. Place dish in 37 C incubator until cells begin to visibly detach from plate expect 2-5 minutes for this to happen

5. Add same amount of culture medium to plate with trypsin (serum in medium swamps action of trypsin on cells)---pipette up and down into plate to break up clumps of cells. Then transfer medium and cells to 15 ml conical tube.

6. Add culture medium to 10 ml volume and completely re-suspend cells by Pipetting up and down against side of tube ---do not induce bubble formation.

7. Centrifuge gently to pellet ES cells (1200 rpm x 10 minutes)

8. Remove supernatant with pipette attached to vacuum trap.

9. Resuspend cell pellet in 1-5 ml medium depending on number of plates pooled.
10. Prepare appropriate cell dilution (1:10 or 1:100) in trypan blue and count viable cells (dead cells take up trypan blue).
11. Count on hemocytometer

12. Passage or freeze or electroporate

Hemocytometer cell count:
Count all the cells in 5 of the large squares (4 corners of the hemocytometer area and center square that has multiple smaller lines) ---if a cell touches a line- count those that touch the top and left side of the smaller squares.

Cell count calculation:
cells/ml= total cells from 5 counted squares/5 x dilution factor x 10^4

Example: 5 plates were pooled and cells pelleted and resuspended in 5 ml of medium. A 1:10 dilution of the cells was made (0.8 ml of DPBS+ 0.1 ml of trypan blue and 0.1 ml of homogenous cell ssuspension). Cells loaded under cover glass of hemocytometer. The cell counts/large square were: 26, 32, 19, 29, 35.

Mean cell count (26+32+19+29+35)=141/5= 28.2 x 10 (dilution) x 10^4= 28.2 x 10^5
Or 2.82 x 10^6 cells/ml x 5 ml of cell suspension=1.41 x 10^7 cells total in the 5 ml suspension.

***Procedure: Freezing mES cells**

1. Refeed cells 2-6 h before tryspsinization

2. Collect cells into pools from designated number of plates.
   Note: Nunc-star footed tubes /6 cm plate and 4 nunc tubes/10 cm plate.
   Or, count and aliquot cells @ 5 x 10^5 cells /ml/nunc tube

3. Gently pellet cells (1200 rpm x 10 minutes)

From here on steps must be done quickly:

4. Resuspend in 1X freezing medium (1x culture medium +10% DMSO). Use 1 ml per vial.

5. Move to isopropanol freezer cans. Place in -80 C overnight.

6. Move to LN2 freezer next day.

7. Vials should be labeled with the cell line, the passage # and the date.

Alternate: use 2x freezing medium (Recipe: DMEM Knockout + 40 % serum + 20% DMSO)—dilute cells in pool with ES cell medium with equal volume 2x freezing medium (DMSO now becomes10%) and aliquot to Nunc tubes. Nunc tubes should be star footed (so they are stable in blue holders) and internal threads.

Note: DMSO is from Sigma D2650 (endotoxin tested and tested on hybridomas for cell growth).

***Procedure: Transfection of ES cells with Targeting Construct DNA**

Note: See Procedure for preparing DNA for electroporation before starting this procedure.

1. Approximately 48 h before electroporation, prepare mES on feeders so that they will be in exponential growth phase (70% confluent---all colonies small and not growing into other colonies) at harvest for electroporation.
2. Prepare 10 cm feeder plates 72-48 h before electroporation to have ready for plating targeted mES after electroporation. (**Note:** make sure the MEFs are

drug resistance to your selection of choice: neomycin (G418), hygromycin or puromycin, etc).

3. Day of electroporation:
    a) 2 h before mES cell collection---refeed all plates that will be used to collect cells
    b) 2 h before electroporation, reefed all feeder plates with mES cell medium
    c) Prepare a 50 ml conical with 8 ml of mES culure medium, cap lightly and hold in 37 C incubator.
4. Minimum of 2 h after refeeding mES plates, trypsinize and collect mES cells as previously described. Collect mES cells into 50 ml conical tube, vigorously triturate the cells up and down against side of tube (avoiding air bubbles) to achieve homogenous single cell suspension.
5. Pellet cells by centrifugation (1200 rpm x 10 minutes)
6. Re-suspend pellet in 12 ml 1X DPBS. Pool in 15 ml concial tube. Pipette up and down to obtain homogenous single cell suspension.
7. Pellet cells again (1200 rpm x 10 minutes).
8. Siphon off DPBS (careful the cell pellet will be slippery in PBS).
9. Resuspend the cell pellet in 2 ml of Electroporation Buffer—prepare a dilution of cells from this pool for cell count.
10. Adjust volume of cells in Electroporation Buffer to obtain a cell concentration of 20 x 10^6 cells/0.8 ml (volume of 0.4 cm gap electroporation cuvette) and add 0.8 ml to three electroporation cuvettes.
11. Add 25 to 30 μg DNA/electroporation cuvette (Note: DNA for electroporation should be prepared as described---precipitated, washed in 70% ETOH, dried at room temperature and re-suspended in "clean" water or electroporation buffer at 1 μg targeting DNA /μL . Mix DNA into cell suspension thoroughly. [Alternatively, transfer appropriate [DNA] to cell suspension and then transfer 0.8 ml to electroporation cuvette.
12. Set BioRad GenePulser II to program 2. 800 volts, 0.2 msec with 4 mm gap no resistance. Electroporate each cuvette.
13. Leave cuvettes at room temperature for 20 minutes.
14. Carefully and gently transfer the electroporated mES cells into the warmed 50 ml conical, rinse each cuvette with 0.8 ml medium. Gently pipette to obtain homogenous suspension.
15. Remove MEF plates from incubator, to each of the feeder plates add 2 ml of electroproated mES cell suspension from the collection tube. Swirl plates in x and y axis to uniformly distribute cell across the dish.
16. Incubate dishes for 36 hours then apply selection medium. Control plate is mES cells at same concentration plated on feeders. When this plate has no colonies (7-12 days) then pick mES colonies from selection plates. [Alternatively: use the cells left in electroporation buffer ; add 4 ml to MEF plates

    A: Control A=G418 + FIAU (Gancyclovir) selection+ counter selection
    B: Control B= G418 selection only

After 7-12 days of selection there should be 2-10 times more colonies on G418 plates compared to doubly selected plates.]

***Procedure: Picking mES Clones to 96 well plates (after DNA targeting)**

Potentially targeted ES cell colonies are ready to "pick" when the control cells in selection are dead (ie, no visible colonies on the plates-only MEFs). This is about 7-12 days following electroporation and plating.

1. Prepare gelatin-coated **flat bottom** 96 well plates about 4-5 days before selection ends. Seed with MEFs.
2. medium-return plates to incubator. (Multichannel aspirator can sue same tips for all plate columns.)
3. Prepare **round bottom** 96 well plates with 30 μL 0.25% trypsin-EDTA in each well.
4. Need a TC scope to see colonies to pick on plates---this can be done on bench top but most people like to move microscope under hood.
5. Remove one electroporated plate with colonies from incubator; aspirate off the mES medium and replace with 10 ml DPBS.
6. Using a sterile 20 μL pipette/colony. Set pipette to 10 μL, under microscope visualize the ES colony to be picked-dark center clean margins , circle it with the pipette tip "cutting" he MEF layer then aspirate into pipette tip and transfer to round bottom trypsin plate----one colony/well. You will see the colony move into the tip. Aspirate colony up and down in trypsin to begin making a single cell suspension.
7. Continue to pick colonies (one colony/well) –align pipette tip box (96) to plate to keep track of your location.
8. Fill 96 well trypsin plate with colonies (approximately 5 minutes) ---then place plate in incubator for 5-10 minutes. Add 70 μL ES cell medium to wells with multi-channel pipette, vigorously triturate up and down. Then move aspirates to the MEF flat bottom plate. Remove PBS from 10 cm selection plates, replace with medium and return to incubator ---can check for colonies next day.
9. Once all the disaggregated picked colonies have been transferred , place plate in incubator for 48 h undisturbed. Disguard the trypsin plate.
10. Continue until all the colonies that need to be picked are done---making new 96 well trypsin plates as needed. Pick 4 x 96 plates.
11. After 48 h, gently aspirate medium from picked colony plates, reefed with 150 μL ES medium. Cells should be cultured until 90% of colonies are turning the medium pink to yellow. Estimate 3-5days for color change. But will depend on size of colonies picked.
12. Repeat is more colonies are needed from selection plates returned to incubator.

***Procedure: Plate Expansion for master plate and duplicates for DNA (PCR/mini-Southern)**

This procedure produces three "master" plates for:

a) 96 well plates of clones grown on MEFs for later use to expand positive clones for microinjection.
b) Two 96 well plates with mES cells from picked clones grown on gelatin for DNA isolation and detection of positive clones by PCR or mini-Southern.

Note: need to keep orientation of all plates the same---usually the A-H rows on left side facing TC hood. Allows identification of all clones by row and column. For example: plate 2, row H , column 10 is clone 2H10.

Note: *Always change tips between rows to avoid cross contamination of picked clones.*

1. Aspirate the medium from 96 well plate using 8 row aspirator with sterile tips for each row/column of eight wells. Add 150 μL D-PBS using a multichannel pipettor and one set of tips that do not touch the bottom of the plates. Aspirate the D-PBS from wells.
2. Add 25 μL trypsin+EDTA each well---if you add drop at top of well and and then tap all down to bottom ---you can use one set of tips.
3. Incubate plates with trypsin at 37 C for 10 minutes.
4. Prepare 2 plates that have been gelantinized by adding 150 μL KO medium to each well of 96 well flat bottom plate(s).
5. After 10 minutes remove mES cell plate from incubator, add 25 μL KO medium to each well—break up cell clumps by pipetting up and down 40X---change tips between rows.
6. Once all the mES cells clumps have been broken up in the master plate, add 50 μL of 2X freezing medium to each well pipetting up and down (clean tips each row!) to equally distribute the medium to 1X. Once the 20 μL has been removed this is the **master** plate(s) to recover positive clones from.
7. Remove 20 μL from this master plate and add to first set of rows of duplicate gelatinized plates made in step #4 above. Pipette up and down to distribute cells and then transfer 20 μL to second geltantized plate.

NOTE: MAKE SURE THE *ORIENTATION* OF ALL THE PLATES IS THE SAME OR YOU WILL LOSE THE IDENTITY OF THE CLONES (A1-8 FOR EXAMPLE) SHOULD BE THE SAME ROW ON PLATES 1-3.

8. Incubate the gelatinized plates will duplicate cells for 4-6 days at 37 C.
9. Change medium after 48 h—aspirate carefully and refeed with 150 μL of KO medium. And feed again as the medium turns yellow.
10. Seal the master plate(s) Zip lock bag or seal a meal bag and place in Styrofoam box with fitted lid in -70 C freezer. (Ideally the temperature

should drop 1C/minute O/N. Next day remove from box and leave at -70 C until correctly targeted clones have been identified.

11. When medium on duplicate plates turns yellow in most wells daily. Aspirate medium , wash with D-PBS , seal plate with tape of parafilm tightly. Place at -70 C until ready to isolate DNA. This procedure "cracks" the cells and makes it easier to get DNA.

Note: Alternative is to transfer one gelatinized plate to 24 well plates and grow to confluency. Then wash and freeze plate for DNA preparation. With the advent of screening by PCR this amount of DNA is not usually needed but is an option.

***Procedure: DNA Preparation from Duplicate DNA plates**

The 96 well gelatinized ES cell plate from above---only one of duplicates made is used to prepare DNA for PCR and identification of clones that were correctly homologously recombined/targeted.

Recipe: Lysis Buffer for DNA isolation

Final Concentration from stocks:

10 mM Tris pH 7.5
10 mM EDTA pH 8.0
10 mM NaCl
0.5% Sarcosyl (DO NOT USE SDS!)

Prepare 500 ml and store sterile at RT. When ready to use add Proteinase K to final concentration 1 mg/ml of lysis buffer.

1. Remove plate from -70 thaw for 5 minutes and then add 50 μL of Lysis +Proteinase K to each well of 96 plate and resuspend cells in buffer by pipetting up and down several times to mix lysed cells with digestion buffer.
2. Incubate overnight at 60 C in a humidified chamber. (Example plastic food container with wet towel on bottom.)
3. Next day spin down plates in at low speed to capture condensation on lids. In Microtitier carrier –1200 rpm for 1-2 minutes.
4. Add 100 μL of -20 C absolute ETOH to each well. Hold on table at RT for at least 30 minutes or until you can see DNA under low power microscope.
5. The DNA will adhere to polystyrene plate so look fro "spider web" structures on perimeter of each well.
6. Gently invert plate over sink (do not shake) and then blot onto paper towels to absorb ETOH. DNA will remain adhered to plate is you do this gently.
7. Wash DNA with 100 μL 70% ETOH. Repeat the blotting procedure – moving to new stacks of paper towels until no liquid remains. Wash in 70% ETOH a total of 3x.
8. Let samples dry with lids off for0 .45-1 h at RT. (IT IS ESSENTIAL THAT DNA IS DRY AND *FREE OF ETOH* OR IT WILL *NOT* CUT!)

Proceeding to PCR:

1. Add 100 μL sterile ddH_2O/well incubate the plates over night in humidified chamber (see 2 above) at 37 C.
2. The next day DNA is ready for PCR---use 1 μL prepared DNA /reaction

May need to adjust DNA but 1 μL is a good place to start.
Run on agarose gel identify putative positive clones.

Proceeding to Southerns:

1. After step 8, add 100 μL sterile dd H_2O using multichannel pipettor—make sure you don't touch bottom of wells with tips. Incubate overnight in humidified container with plate inside (or sealed plastic bag with wet towels inside).
2. Next day add 10 μL 10X digestion buffer for the enzyme being used to cut DNA/well and 5 μL of restriction enzyme. Use multichannel pipettor and thoroughly mix reagents in wells. Use new sterile tips for each column of eight wells. Incubate overnight at 37C in humidified container or bag.
3. Next day run 40 μL of digest/agarose gel well.
4. Blot gel as with your standard procedure.
5. Identify putative positive clones

***Procedure: Expansion of Targeted Clone for Further Use**

Once putative positive clones (successfully targeted following electroporation and selection) are identified by PCR or Southern, these clones must be pulled from its master 96 well plate and expanded for further use (microinjection etc). Each clone is considered an independently derived cell line so you need a naming procedure. One usually adapted is the abbreviation of the targeted gene and the location of the clone on the plate. For example rd8 is a gene name and the colne was on plate 1, row C and column 6….clone name is then "rd81C6". Also since each clone is independently derived they may exhibit different growth characteristics, consider this when deciding on strategy for expansion and preservation. Finally, it is important to avoid cross contamination of clones with each other and especially with non-targeted clones. Non-targeted clones are usually wildtype and will wreak havoc if mixed with a targeted clone---they confound genotyping and may even over grow the targeted clone cuing you to lose the clone you want.

1. Day before clone expansion from master 96 well plate—prepare 24 well plates –one well/targeted clone to be recovered—with IR feeders.
2. About 2 h before you want to thaw the 96 well master plates, reefed feeder 24 well plates with mES cell medium—2 ml medium/well.
3. Have master 24 well sheet diagram to identify which clones are placed in which wells—retain identity from master plate because that is how they are identified on PCR or Southern data.
4. Remove 96 well mater plates from -70 C freezer, place in 37 C incubator until all wells are thawed---no more than 10 minutes because DMSO at 37 C will harm cells causing viability issues.
5. Using sterile pipette tips and working quickly pull medium for 96 well plate (pipette gently up and down to capture clone ES cells) on putative positive clones and transfer to appropriately identified well on a 24 well plate with pre-equilibrated medium--one clone/well.
   Note: For maximum recovery of ES cells remember that cells will accumulate at margins of well when they were prepared for freezing so gently pipette freezing medium from well and then wash each selected well with an additional 100 μL of medium to achieve maximum recovery of cells.
6. Incubate the 24 well plates containing the putative targeted ES cell clones at least 48 h before changing medium. Carefully aspirate off 1.5 ml ---do not touch cells in well; refeed with 1.5 ml ES medium.
7. As wells reach 75-90% confluency , split between and 24 well feeder (freezing) plate and 24 well gelatin (DNA) plate. REMEMBER ORIENTATION AND CLONE IDENTIFCATION SCHEME.

Freezing Plate:

1. Remove medium from confluent wells and wash with 1.5 ml of DPBS.
2. Add 150 μL Trypsin+EDTA and incubate at 37 C for 5 minutes.
3. Add 350 μL ES cell medium and pipette un and down approx. 40 times to disperse nests of ES cells.
4. Add 500 μL 2X freezing medium, dropwise mixing continuously.
5. Aliquot 500 μL of cells to each of 2 cryo tubes (LABELLED!) –freeze at 1 C/Minutes drop in Styrofoam or freezer container.

DNA Plate:

1. Incubate and change medium daily until wells are confluent.
2. Remove medium –avoiding cross contamination between wells.
3. Wash each well to be harvested with 1.5 ml DPBS.
4. Add 150 μL 0.25% trypin+EDTA to each well; incubate at 37 C for 5 minutes
5. Add 350 μL ES cell medium; pipette un and down approx. 40 X to disperse ES cell nests; transfer all volume to 1.5 ml Eppendorf tube.
6. Centrifuge 8K x 5 minutes to pellet cells.
7. Remove all supernatant with pipette tips.

8. Freeze pellets at -80 overnight before processing for DNA. **Note:** if procedures will require a lot of DNA these cells can be transferred to 6 well plates and other procedures adjusted as necessary.

DNA Preparation from Eppendorf Tube:

1. Thaw eppendrof tubes; resuspend cell pellet in 50 μL $T_{10}E_1$ by pipetting.
2. Add 400 μL lysis buffer + 1 mg/ml proteinase K
3. Close tubes tightly; rotate overnight at 55 C (or invert tubes every 1 h)
4. Add 900 μL ETOH:NaCl (2X starting volume) (Stock: 450 μL 5M NaCl into 15 ml of 100% ETOH)—invert tubes DNA will fall out.
5. Pick DNA with yellow pipette tip into eppendorf with 1 ml 80% ETOH—let sit for 20 minutes—gently invert tubes a few times to "wash".
6. Pick DNA into dry eppendorf---if DNA is wet, spin at 10K x 30 seconds and pick off ETOH with pipette tip. (Any remaining ETOH inhibits digestion and other steps downstream).
7. Let DNA dry at RT with tops open—1 h.
8. When pellets are dry add 50 μL $T_{10}E_1$-flick tube to dislodge pellet.
9. Incubate overnight at 37 C.
10. Next day gently pipette up and down to make sure DNA is dissolved.
11. Use DNA as necessary to further confirm correct gene targeting.

***Procedure: Transfection of ES cell with Targeting DNA Construct**

This needs careful planning since the DNA must be cut and prepped the day before electroporation and then ES cells need to be growing exponentially the day of electroporation. (**Ref:** Tompers and Labosky, Electroporation of Murine ES cells, Stem Cells 22: 243-249, 2004. Good pictures and description of one procedure for success.)

1. Approximately 48 hours before electroporation split enough mES cells (need 20 x 10^6 ES cells /electroporation) onto feeder plate to do 2 electroporation/DNA construct.
2. 24 h before electroporation prepare 4 feeder 10 cm plates/electroporation x # electroporations planned. These feeder cells should be resistant to the selection agent (G418, hygromycin FIAU etc).
3. On the day of electroporation, refeed the ES cell plates with new ES cell medium; refeed prepared feeder plates with ES cell medium.
4. Also prepare 8 ml KO medium in 50 ml conicals ---place in incubator with cap loosen for gas exchange.
5. At least 2 h after fresh medium was placed on ES cell plates; rinse ES plates in DPBS and harvest plates by trypsin+EDTA.
6. Resuspend trypin cells in ES medium and move to 15 ml conical tube(s). Vigorously triturate the cells to disperse the ES cell nests

and make a homogeneous single cell suspension. Avoid making bubbles in suspension.

7. Centrifuge cells into pellet 1000 rpm x 7 minutes.
8. Resuspend cells in DPBS and triturate vigorously into single cells suspend again.
9. Centrifuge cells again
10. Carefully remove all supernatant from cells without dislodging cell pellet(it's slippery after PBS wash). Resuspend cells in 400 μL electroporation buffer (BioRad Cat # 165-2677)s. Resuspend the cells with a blue tip—40X.
11. Count small aliquot of cell suspension with hemocytometer and determine the total number of cells by using the total cell volume.
12. Adjust volume of cell suspension with electroporation buffer to obtain a cell density of 20 $x10^6$ cell/0.8 ml. Cells are held at rppm temperature for following manipulations.
13. Add 25 μg targeting DNA (suggest a range of cuvettes containing 25, 35 and 40 μg DNA/cuvette) in electroporation buffer for each 20 $x10^6$ cell. **Note:** DNA is phenol/chloroform treated, precipitated, washed in 70% ETOH, dried at room temperature , and resusended at 1 μg/μL gently and thoroughly.
14. Transfer 0.8 ml of cells +DNA to 4 mm gap electroporation cuvette.
15. In BioRad Gene Pulser II with capacitance extender (program 2), place cuvette in apparatus and electroporate at 800 volts, 0.2 msec, 4mm gap—no resistance.
    Note: Other labs electroporate at different settings for BioRad apparatus: 230 V, 500 μF Time constant between 5.6-8.0 msec.
16. Slowly, transfer the cells from cuvette with sterile glass pipette to 50 ml conical from incubator. Rinse cuvette with 1 ml KO medium and transfer back to conical tube. Gently, pipette to make a uniform suspension (there will be white "crud" on top of cuvette from process don't worry about it and transfer along with other material—in this step one expects that 50% of the cells will die and lyse).
17. Remove 4 feeder plates from incubator add two ml of collected ES +DNA suspension to each feeder plate---gently swirl in x and y directions to evenly distribute cells---want well spaced ES cell nest when they start to grow.
18. Incubate plates for 24-36 h before switching to selection medium. (If using a Cre construct no selection is necessary) Approximately 66% of cells will die---but those ES cells that contain the selection cassette will begin to grow nests and when big enough they can be picked to 96 well master plates to establish independent lines . They will then need to be checked by PCR or Southern for correct targeting at both the 3' and 5' end of the anticipated homologous recombination.

19. Drug selection can begin 24-36 hours after electroporation. First two day, use 200 µg/ml of G418 (0.4 ml of 50 mg/ml active form G418 in 100 ml ES medium Sigma G8168), and then 150 µg/ml G418 (0.3 ml G418 per 100 ml medium) and 10 mM Gancyclovir (10 µl to 100 ml of ES cell medium). For negative selection if targeted contains the HSV-Tk cassette.
20. Colonies may be picked as early as 8 days, are best around 10-12 days, but may be recovered up to 18-21 days after the electroporation.

***Procedure: Preparation of DNA for Electroporation/Gene Targeting**

A large scale Qiagen preparation of DNA is recommended.

1. The DNA to be used for electroporation should be *high-quality* and *clean*. Use linearized DNA (targeting vectors), so do a large-scale, overnight digest on your DNA. Vector sequences can be retained in targeting construct since they will be lost when homologous recombination occurs—for targeting DNA open at 5' end of construct. Otherwise vector sequences may cause issues and should be removed by digesting at 5' and 3' end to remove DNA insert of interest. Targeting construct should contain positive selection cassette.

2. Extract the large-scale digest once with an equal volume of phenol/chloroform and once with an equal volume of chloroform. Precipitate the DNA with ethanol, dry down (residual EtOH will cause problems downsteeam), and resuspend in sterile TE or water. It's handy if the DNA is at 1 µg/µl and proceed. This procedure and prep should be done the day before electroporation so the DNA ends are not degraded.

***Alternate Procedure: DNA Preparation for ES Cell Electroportion**

(Ref: Transgenic Core, Toronto Centre for Phenogenomics 25 Orde Street, Toronto, Ontario)

Expect to prepare 100-150 ug of linearized vector at a concentration of 1.0 µg/ul. This is necessary for several rounds of electroporations. Prepare at least 6 eppendorf tubes with 20 µg of digested vector each. *The purity of DNA is critical for the successful electroporation.*

1. Purify vector DNA (e.g. CsCl gradient centrifugation or anion-exchange resins such as Qiagen #12143 or 12362; or Invitrogen # K2100-04).

2. Digest plasmid DNA with an appropriate enzyme(s).

3. Run a sample of cut DNA on mini-gel to check for complete linearization.

4. Last step purification:

   Option 1:

   (a) Extract DNA with phenol : chloroform : isoamylalcohol (25:24:1) (e.g. Invitrogen #15593-031) OR with an equal volume of 1:1 phenol : chloroform, then equal volume of chloroform

   (b) Precipitate with two volumes of absolute ethanol on ice, spin down, can be stored at -20 C from this point

Option 2:

   (a) Precipitate the DNA with NaAc/EtOH [1/10 vol. of NaAc 3M pH 5.2 and 2 vol. of 100% EtOH]

   (b) Wash two-three times in 0.5-1 ml of 70% ethanol. Drain off as much 70% ethanol as possible and allow the remainder to evaporate in a sterile laminar flow hood, leaving the lid of the tube open for 30-60 min (optional)

   (c) Re-suspend in ultrapure sterile water, take an aliquot for measurements and freeze the rest at – 20C.

(d) Quantify OD260nm:280nm. If possible, use NanoDrop to quantify and assess the purity of the final prep. Please, refer to the NanoDrop Nucleic Acid Purity ratios.

(e) Prepare aliquots as described above and bring them to TCP on dry ice.

APPENDIX:

Addition information or alternate sources for embryonic stem cell culture/reagents.

***MEF Tissue culture media:**

DMEM (Gibco 11995-065)
1:100 Gentamicin (Gibco 15710-064)
1:100 Glutamine (Gibco 25030-149)
1:100 MEM non-essential amino acids (Gibco 11140-050)
1:1000 2-mercaptoethanol (Gibco 21985-023)
10% fetal calf serum (Gibco growth screened but need not be KO FBS)

Filter sterilize/ Use for 2 weeks or less, fresh is best.

***Protocol for Mitotic Inactivation of MEFs by Mitimycin C.**
Adapted from Wesselschmidt, R. L. Primogenix, Inc.

For research use only

Background: Mouse embryonic fibroblasts (MEFs) have been used as feeder cell layers for the culture of embryonic stem cells (ESCs) since the first mouse ESCs were derived in 1981. MEFs continue to be the most commonly used feeder cell type for the culture and maintenance of mouse and human derived ESC lines. MEFs provide a complex, but unknown mixture of nutrients and substrata for the long term growth and proliferation of undifferentiated pluripotent ESCs.

Required Materials

1. Flask of MEFs

2. MEF medium

3. MEF inactivation medium (mitomycin C added) to MEF medium

4. General cell culture supplies ***Media Preparation*** Table 1: MEF Mediu

General Considerations

5. MEFs require careful culture and maintenance. Keeping MEFs in a healthy proliferating state and producing the entire matrix and growth factor support for ESCs, is an important goal. Since MEFs are primary cells, they have a limited lifespan in culture (1-5 passages). If they begin to elongate and doubling time increases significantly, they are beyond their useful state. They need to be carefully monitored to avoid over growing the culture, which results in early senescence. The following criteria are recommended when sourcing MEFs. They should be:

Isolated from 12.5- 13.5 day mouse embryos

Mycoplasma free

Mouse Antibody Production (MAP) tested

6. MEFs must be healthy and actively dividing prior to inactivation and their subsequent use as a feeder layer for ESCs. After the MEFs have been inactivated, replace the MEF media every other day. Inactivated MEFs may be cryopreserved for future use (see SC Protocol Sheet 00004). If inactivated MEFs are fed every other day, they make good feeders for mouse ESCs for 5-7 days. For human ESC culture, it is best to inactivate MEFs the day before passaging the hESCs.

7. Do not inactivate MEFs until they are needed. It is not possible to expand mitotically inactivated MEFs.

8. Exposure to Mitomycin C (Fisher) at 10 μg/mL for 2-3 hours is sufficient to mitotically inactivate MEFs. Inactivated MEFs are counted and plated on tissue culture vessels to give the desired MEF concentration for the culture of the ESC line.

9. Mitomycin C inhibits DNA synthesis and nuclear division. It is toxic and possibly carcinogenic. Therefore handle with caution by covering bare skin and wearing protective gloves and safety glasses. Work in an area with good ventilation. Read MSDS from provider before handling and for proper disposal methods.

Inactivation Protocol

1. Beginning with a confluent layer of MEFs, remove the growth medium from the flask and replace it with 10 mL/75 cm2 of recently thawed or freshly prepared inactivation medium (Table 2) to cover the monolayer. Place the flask in incubator for approximately 2-3 hours.

2. Remove inactivation medium from the flask. Properly dispose of the inactivation medium.

3. Wash the monolayer of cells at least three times with a minimum of 10 mL/75 cm2 of HyClone ES Qualified DPBS.

4. Add Thermo Scientific HyClone Trypsin (SH30236.01) or HyQTase (SV30030.01) to cover cells (1-5 mLs). If using Trypsin, incubate until the cells detach from the plate (3-5 minutes). If using HyQTase, use at room temperature until the cells detach from the plate (3-5 minutes).

5. Add equal amounts of MEF medium (equal to amounts of Trypsin or HyQTase used) and break up cell aggregation by pipetting up and down.

6. Count cells.

7. Plate 4.0 to 6.0 x 104 cells/cm2. For example plate 1 x 106 to 1.5 x 106 cells per T-25 flask.

8. Incubate overnight and use as feeder layers the next day and up to day six. Change medium every other day.

***2X Freezing Medium:**

40% ES medium +40% FBS +20% DMSO (TC certified) : mix filter sterilize (0.2 μm) keep at 4 C no longer than 2 weeks.

***Additional Sources of mES cells besides MMRC.**

Penn State University Transgenic Facility (lines J1 p15, BK4 p6, AB2.2 p3 (Allan Bradley from commercial source), HM-1 (p16 soucre Dr. Sarah Bronson Hershey), RW4 p5, R1 p3 (A. Nagy, Toronto) GS-1 (commercial). Tom Salada showed germline transmission without genetargetting for HM-1, RW4, R1, and J1 with and without gene targeting manipulation.

ATCC (wwwatcc.org/vivocyte): sources for several known mES cell lines with information about genetic background (e.g. which strain of 129 they were derived from and the newer C56Bl/6 mES cells lines which are favored now for gene targeting since you can use the albino C57Bl/6 line and keep the genetic background stable.)

***Alkaline Phosphatase Assay (for undifferentiated mES cells)**

Stemgent AP staining kit: 00-0009 (www.stemgent.com)

***Karyotyping (WiCell Research Institute; www.wicell.org/cytogenetics)**

current contact is : Seth Taapken, Cytogenetics Lab Manager, 608-316-4145