PVR, Jan07

Culture of ES cells with feeders

For routine culture, cells should be passaged twice a week (ideally on a Monday - Friday rota), splitting them between 1:6 and 1:12, depending on conditions. Medium change should take place every other day as long as culture is thin, later on change medium daily. They should not be plated too thin as this encourages excessive differentiation. Do not allow cultures to become over-confluent, for the same reason. Any drastic change in cell doubling times and /or cell morphology is indicative of unwanted damage to the cells, eg. chromosomal aberrations. Typical doubling time is about 22-24 hrs.

In order to retain their excellent germline transmission frequency, it is essential to keep passage number of HM-1 cells as low as possible. Good germline transmission rates boil down to 1) tested sera, media, plasticware etc., 2) limiting passage number, 3) experimental skills.

We use cell culture plastic from Falcon (Becton Dickinson), cell culture grade water and reagents and test all batches of sera for germline transmission.

If you plan an electroporation experiment, thaw 1 aliquot of cells on a single 25 cm^2 dish(day 1). This becomes confluent 2-3 days later. Trypsinize and plate on a single 75 cm^2 dish(if necessary, 2 flasks are possible). These should be confluent 3-4 days later (day 6-8). Then trypsinize and use cells for experiment. Don’t use spare cells for another electroporation experiment as their passage number has increased.

Thawing and plating cells

Gelatinize dish with 3-5 ml of gelatin per 6 cm and 7-10 ml per 10 cm dish (for any other vessel, make sure that surface is completely covered with gelatin). Leave at RT for at least 10 min. Aspirate off before plating cells. Seed feeders on gelatin-coated dishes 1-2 h before plating ES cells. Co-plating with ES cells is ok also. A density of 50.000 cells /cm^2 is required. Feeder layers last about one week after treatment.

After each round of trypsinization, fresh feeder cells must be added.

Prepare centrifuge tube with 10 ml of pre-warmed medium. Thaw cells quickly in 37° C bath and transfer into pre-warmed medium. Spin for 3 min at 1.200 rpm. Remove medium, replace with 5 ml of fresh medium containing Pen/Strep, if necessary, and replate all cells on 6 cm dish. Next day, replace with fresh medium without antibiotics. Routine culture without antibiotics is strongly recommended.

To subculture, aspirate the medium and wash cells with 1 ml ES cell-Trypsin (pre-warmed) per 6 cm dish (2 ml per 10 cm). Remove and replace with fresh trypsin and leave in 37°C incubator until cells are dislodged by gentle agitation. It is a good sign if this happens after about 2 minutes but may take up to 5 minutes. Don't over-trypsinize as this can damage the cells. Add 4 ml of medium per 1 ml of trypsin and pipette up and down against dish wall about 10 - 20 x (you may vary the distance between pipet tip and dish wall between 0.5 and 2.5 cm). It is not essential to obtain a single cell suspension for routine culture but large cell clumps must be avoided as those will lead to differentiation. Check suspension under microscope before transferring cells into a centrifuge tube. There should be a uniform size distribution of cells/cell aggregates.
In the meantime, gelatinize new dish (es) and plate feeders.
Spin for 2 min at 1.200 rpm (RT), aspirate off spnt, resuspend cell pellet gently in a few ml of medium. Replate at desired density and add 6-8 ml of medium to small and 25-30 ml for a large dish. A 6 cm dish yields about 7-10 million and a 10 cm dish about 25-30 million cells.

Electroporation of ES cells (first step gene targeting)

The conditions recommended will work with a Biorad gene pulser and cuvettes of 0.4 cm electrode gap.
Setting 800 V, 3 microfarads (standard). They will work with any ES line and are relatively insensitive against changes in cell number and amount of DNA. They do not yield the highest possible number of transformed cells but are gentle to cells.
Other settings with higher efficiencies are 250 V, 500 microfarads (optional). Under these conditions, use 25 µg of DNA and 10 mio. cells per electroporation. Changes in DNA amounts and cell number alter efficiency and cell viability.
Optimal results are currently achieved if the DNA is purified with *Nucleobond* or *Qiagen* columns. EndoFree purified DNA is not necessary but may improve transfection efficiency.
For a standard targeting experiment, trypsinize about 20-30 million cells. Determine cell number in a counting chamber. Spin as usual and resuspend cells in 10 ml of fresh medium. Dissolve cells by gentle shaking and spin as before. This step gets rid of feeders. Resuspend ES cells in 0.8 ml of 1x HBS buffer. Add cells to 200 µg linearized DNA in 100 µl **sterile** TE in Eppendorf tube (Phenol/Chloroform-extracted, Ethanol-precipitated, remove 70 % Ethanol in hood!) and mix by pipetting up and down using a 1 ml cell culture pipette. Transfer into electroporation cuvette. Apply one pulse (standard or optional!) and stand for 10 min. at RT.

Add cells to appropriate amount of medium in a 500 ml bottle, depending on the number of dishes to be used altogether.

Selection plates no dot contain feeder cells! Plate about 1 - 2 million cells per 10 cm dish in 10 ml of medium. Start positive, eg. HAT selection 12-16 h after electroporation (G 418 is 350 µg/ml; hygromycin is 150 µg/ml; puromycin is 1 µg/ml; if you use these selective agents, resistant STO feeders must be prepared!). Change medium. After 3-5 days, change medium again. Colonies will appear after 8-10 days. Don't allow colonies to become too big!

Prepare 24 well plates with feeder cells.

Isolate colonies as described in *ES cell colony isolation protocol*, use 1/2 for PCR and leave the remaining cells in 24 well plate with appr. 1 ml of medium. After a few days, multiple colonies should have appeared. Aspirate off medium, wash with 0.2 ml of trypsin, aspirate off again. Add 2-3 drops of trypsin, incubate for 3-5 min at 37°C. Add 0.5 ml of medium and break up cell clumps using a blue-tip Gilson pipet. Transfer cells to a 6 well plate and add 5 ml of medium. Grow to near-confluency. Trypsinize and plate onto two 25 cm^2 flasks adding 2/3 on one and 1/3 onto the other flask. When confluent, use 2/3 dishfor freezing cells (2-3 vials) and the other one for preparation of genomic DNA.

Maintain selection until targeted ES cells are expanded and characterized by Southern blotting. To remove cells from HAT selection, replace HAT with HT for at least 2 days before growing cells in normal medium. Cells will grow slightly faster in the absence of selection.

For HSV TK negative selection, the final conc. of ganciclovir is 2 µM; for HPRT negative selection, the final conc. of 6-thioguanine is 5 µg/ml.

For correctly targeted ES cells, thaw an aliquot on 25 cm^2 flask, grow until confluency and passage on 1 75 cm^2 flask. Prepare 6-8 freezing aliquots for blastocyst injections.

Second step- gene targeting (double replacement using HPRT minigenes)

Remember the fluctuation test (Luria and Delbrück, 1943) and keep passage number as low as possible in order to minimize the accumulation of 6-TG-sensitive cells before electroporation.

Transfer cells in normal medium as described. Electroporate about 20 million cells as before and plate onto six 75 cm^2 flasks. Apply 6-TG selection at day 6. Most likely, it will be necessary to split cells before. In this case, split in such a way that you end up with 6 near-confluent flasks at day 6. Then, trypsinize cells. From each flask, plate 6 x 1-1.5 million cells

onto 10 cm dishes in medium containing 6-TG. Change medium after 2 and 4 days, respectively. Colonies will appear around day 10 (there will only be 1-5 colonies per dish).

Further handling of resistant colonies

Passage colonies when they are about 2-3 mm in size, using yellow Gilson tips and pipet adjusted at 150 µl. Transfer colony to Gelatin-coated 24 well plate and pipet up and down a few times, removing a cell aliquot for PCR. When cells are becoming confluent, trypsinize and transfer to a 6-well plate. From there, passage onto two 25 cm^2 flasks as described before. Remember to keep passage number as low as possible and be sure to trypsinize cells well. Avoid prolonged growth of clumpy cell aggregates (See protocol below for PCR genotyping).

Freezing ES cells

Freeze cells from near-confluent flasks. One aliquot should contain 2-4 mill. cells (ie. 2-3 per small and 5-8 from a large flask. It is beneficial to freeze cells as concentrated as possible, eg. in 0.5-1 ml per vial.
Add 250 ml isopropanol in Nalgene freezing container.
Trypsinize cells as usual. Resuspend them gently in x ml of normal medium. Add x ml of 2 x freezing medium (dropwise). Mix gently and transfer instantly to Nalgene freezing container and place into -80° C ON (or at least 4 hrs). Thereafter, transfer cells to liquid nitrogen container.

Preparation of HM-1 cells for blastocyst injections

A subconfluent 25 cm^2 flask of cells is sufficient. Ideally, thaw cells 4 days before injection. About 24 h before microinjection, they should be near-confluent. Trypsinize and replate 70-100 % of cells.
Change medium early in the morning of the injection. Start trypsinization only when everything at the microinjection facility is ready to go. Keep in mind that cells become fragile after 1.5-2 hrs. If microinjection takes a long time, it is better to trypsinize a second flask of cells 1.5-2 hrs after the first one.

- Remove the medium and wash the cells1x with 1ml of ES cell trypsin.

- Add 1ml of trypsin and incubate for 1-3 minutes at 37ºC (Control under the microscope for cell rounding and detachment on slight agitation)
- Stop the trypsin reaction by adding an equal amount of trypsin inhibitor and 4 ml of serum free medium **or** 5ml of serum containing ES cell culture media.
- Carefully resuspend the cells by pipetting them gently up and down three to five times in order to produce a single-cell suspension.
- If cells are cultured on feeders, in order to remove the feeders, place the suspension in a tissue culture dish and incubate the cells for 10 min in a 37°C incubator (alternative 20 min at RT). Most of the feeders will loosely attach to the plastic surface, whereas the ES cells will be in suspension. Move the dish carefully, not to disturb these layers! Carefully tilt the dish and collect the media containing the ES cells, leaving the feeders attached to the dish. Transfer the ES cells to a 15-ml Falcon Tube.
- Alternative to remove the MEFs: After ES cells have been resuspended in ES cell media after trypsinization, transfer the cell suspension (usually 4-6 ml) into a 15-ml Falcon tube. Let the MEFs settle down for 20 min in an upright position in the tube at RT. Collect max. 50% of the media volume containing the ES cells and transfer to a new tube.
- Centrifuge the ES cell suspension at 1000 rpm for 5 min and remove the supernatant.
- Resuspend the cell pellet in 500 ul ice-cold M2 media (**Sigma M7167**) and add one droplet of 1 µl ultrapure DNase I (20-40 u/µl). Store the cell suspension on ice. Cells should be used within 3-4 hours.

HM-1 culture medium

Glasgow Modification of Eagles Medium (BHK-21 medium)
w/o Tryptose Phosphate Broth
with L-Glutamine
Gibco BRL 041-01710
Sodium Pyruvate 100 mM Gibco BRL
Non-Essential Amino Acids 100 x Gibco BRL
10 % Serum: 5 % Fetal Calf Serum (at present from Sigma, Australian / New Zealand origin)
5 % Newborn Calf Serum (at present from Sigma, Australian / New Zealand origin)

Note that all batches of sera should be tested for ES cell viability and germline competence in comparison to existing good batches.

Preparation of complete HM-1 culture medium

Always prepare appropriate amount of medium (i.e. sufficient for up to 1 week)!

Add the following to a 500 ml bottle of Glasgows Medium:
5.6 ml Sodium Pyruvate
5.6 ml Non-Essential Amino Acids
5.6 ml 200 mM Glutamine (= 100 x conc.)
28 ml Fetal Calf Serum
28 ml Newborn Calf Serum
910 µl Monothioglycerol
570 µl LIF (this amount will vary from batch to batch) if using self-prepared LIF. For commercially available LIF, follow manufacturer's recommendations. The 2002 stock is diluted 1:1.000.
Avoid unnecessary warm-up cycles in waterbath!!!
Store complete medium at 4° C and use for up no more than 1 week.

Preparation of Monothioglycerol

Add 1 ml of Mtg (Sigma M6145, cell culture grade) to 99 ml of sterile water (stock is 9.24 M) and freeze in small aliquots at –20°C. This gives a conc. of 92.4 mM. The final concentration in the medium is 0.15 mM.

Alternative to Mtg: Preparation of beta-Mercaptoethanol

Add 0.2 ml of beta-MeOH to 28.2 ml of water and filter through 0.1 µm pore filter. Store at -20 °C. Avoid more than 20 freezing/thawing cycles. The final concentration in the medium is 10^{-4} M.

10 x Hepes-Buffered-Saline (HBS) for electroporation

16 g NaCl

0.74 g KCl

0.252 g Na_2HPO_4

2 g D-Glucose (Dextrose)

10 g HEPES

Dissolve all in 180 ml cell culture grade water, adjust pH to 7.2, bring up to 200 ml and filter through 0.1 µm pore filter. Store at -20° C. To prepare 1 x HBS, dilute with sterile water which brings pH to 7.05.

Preparation of Gelatine solution

Use Sigma G2500 swine skin Type I gelatin. Prepare a 1 % sol in cell culture grade water. Autoclave. Leave overnight and autoclave again the following day. Store at 4° C. Warm up before preparing 0.1 % working dilution.

Preparation of Trypsin

For 500 ml of Trypsin Solution:

400 ml cell culture grade sterile PBS

100 ml sterile EDTA (1.85 g/l)

5 ml Trypsin (Gibco BRL 25090)

5 ml Chicken Serum (Gibco BRL 16110)

Mix and store as 100 ml aliquots at -20° C. Once frozen, keep aliquots at 4° C.

Preparation of ES cell freezing medium

Prepare fresh

1 x freezing medium contains

complete ES medium plus 10 % serum (20 % final conc.) and 10 % DMSO. ***To freeze ES cells, prepare 2 x freezing medium*** as follows:

<u>20 ml</u>

10 ml Complete ES medium

4 ml DMSO (ultrapure)

6 ml FCS

HAT and HT media are bought as 50 x stocks from Sigma but used as if they were 100 x concentrated.

To prepare 100 x HAT yourself, mix

272.2 mg Hypoxanthine
48.4 mg Thymidine
4 ml of 1 mM Aminopterin

Dissolve Hypoxanthine in 2 ml 1 N NaOH. Add 8 ml cell culture grade water and mix well. Add another 10 ml of water and mix until most of the Hypoxanthine is dissolved. Add 1 ml of 1 N NaOH and mix until Hypoxanthin is dissolved. Add 75 ml of water, then add Thymidine, followed by a further 100 ml of water. Then add Aminopterin and mix again (Aminopterin is a poison, watch material safety sheet).
Filter through 0.1 µm filter and store as 20 ml aliquots at -20° C.

To prepare 1 mM Aminopterin, weigh out 100 mg and dissolve in 45 ml of cell culture grade water. Add about 1 ml 1 N NaOH to dissolve Aminopterin. Sterilize as above and store at -20° C.

Additional compounds

200 x Pen/Strep is bought from Gibco BRL and stored as 1 ml aliquots at -20° C.

G 418 is bought from Gibco BRL or any other manufacturer. It is prepared at 40 mg/ml in PBS and filter sterilized. Store at -20° C. Final concentration is 350- 400 µg/ml.

Tylosin is used against mycoplasms as recommended by supplier.

Ganciclovir (M_r 255.23) is prepared as a 1000 x stock (ie. 2 mM). Dissolve 5.1 mg in PBS and filter sterilize. Store in 5 ml aliquots at - 20° C.

6-Thioguanine (6-TG) is prepared as a 1000 x stock (ie. 2 mM). Dissolve 50 mg, dissolve in small volume of 1 N NaOH. Add water up to 25 ml, dissolve completely and filter sterilize. Store as 5 ml aliquots at - 20° C.

LIF is prepared according to the protocol below

TMM, 5-02,rev.

Making and testing LIF

Making:

For a large batch of LIF, use 15 flasks (75 ml) of COS 7 cells grown to confluency. This makes 500 ml of LIF. It requires about 1.200 µg of pC10 DNA (no need for linearization; prepare by NucleoBond, Qiagen, or any other high purity column purification method). Grow COS 7 cells in ES cell medium without LIF/ mercapto-ethanol.

1. Trypsinize flasks in batches of 3. No need to count cells. Pool the cells from 3 flasks, spin down (3 min 1.000 rpm) and resuspend in 1.6 ml of HBS buffer.

2. Electroporate 0.8 ml of cells with ~120 µg of pC10 DNA; 200 V, 500 µFd.

3. Leave cells for 10 min., then transfer each 0.8 ml of electroporated cells into a separate 50 ml blue cap tube with 10 ml of medium and plate into a 140 mm dish. Add a further 20 ml of medium without antibiotics to each dish. This yields 10 dishes in total.

4. The next day, the dishes should be nearly confluent. Aspirate medium and add 25 ml of fresh medium. Leave 3 days.

5. Collect the medium and add another 25 ml. Leave for further 2 days and collect again.

6. As a precautionary measure, collect the medium into 3 separate bottles. Know which bottle is being used to collect medium from a particular dish. After collection is completed, and if there is no evidence of any culture contamination, pool the medium and filter. Use pre-filter, 0.45 µm and 0.1 µm filters. Pipette 5 ml aliquots into tubes and store at -20°C. Set up a sterility test on the first and last aliquots.
   In our experience, a preparation can be used for up to 5 years without a drop in quality.

Testing:

1. Trypsinize a healthy culture of HM-1 cells in the morning. Plate 1 000 cells/well into a gelatinized 24-well microtitre plate. Also, plate 250 cells/well into a separate plate.

2. After ~4-5 hrs, aspirate medium and replace with 1 ml/well of complete medium containing the new batch of LIF at dilutions of 1.000-, 2.000-, 5.000- and 10.000-fold. Also, set up a row of 4 wells with no LIF, and with the current batch of LIF at the appropriate dilution.

3. Fix and stain after 7 days. Compare plating efficiency, colony size and, most importantly, level of differentiation in the dilutions of the new batch of LIF against the standard, which should show only a low level (no more than 5-10 %) of differentiation.

10 x Hepes-Buffered-Saline (HBS) for electroporation

16	g NaCl
0.74	g KCl
0.252	g Na_2HPO_4 dihydrate
2	g D-Glucose (Dextrose)
10	g HEPES

Dissolve all in 180 ml cell culture grade water, adjust pH to 7.2, bring up to 200 ml and filter through 0.1 µm pore filter. Store at -20° C. To prepare 1 x HBS, dilute with sterile water which brings pH to 7.05.

TMM, 8-02

ES cell colony isolation protocol for PCR based genotyping

Coat 24 well plate with gelatin and remove by aspiration after 10-20 min.

Pick colonies along with approximately 150 µl of medium using a P 200 gilson pipette.

Transfer colony to microtiter plate, disperse by pipetting up and down 3-4 x.

Leave half of cells in well and transfer other half into an Eppendorf tube. Add 1 ml of selective medium and grow cells until 80 % confluency.

Spin down cells 2 min full speed. Aspirate off supernatant.

Add 50 µl of lysis buffer (check below for the lysis buffer used with Taq polymerase from Invitrogen). Choose the buffer corresponding to Taq polymerase you are going to use. it must contain appr. 0.5 % of N-P 40 or Triton X-100 for cell lysis) including 200 µg/ml proteinase K. Vortex briefly and incubate 1 h at 65 °C, preferably at slow motion (rot. wheel).

Heat 10 min at 95 °C. Spin briefly and use 2- 5 µl of cell lysate for 25 µl PCR reaction. **Carry out PCR immediately after having the extract ready**!!!
If PCR can't be carried out instantly freeze extracts ON at -80°C. Prolonged storage can decrease efficiency of detection down to nothing.

Lysis buffer (for 10ml): 10x PCR buffer – 1 ml
100% Triton-X (0.1% end concn) – 10µl
50mM MgCl2 (1.5mM end concn) – 300µl
Sterile water – 8590µl
Add 100 µl proteinase K fresh

Karyotyping of ES cells

Before using a clone for a second electroporation or for blastocyst injections, the number of chromosomes should be determined by karyotype analysis. A high degree of euploidy (greater than 70 %) is necessary but does not guarantee germline competence. Therefore, it is preferable to characterize a sufficient number of euploid clones to increase the probability of germline transmission. Starting with confluent 25-cm^2 flasks, the cells are arrested in metaphase by incubation them for 1 h at 37°C, 5% CO_2 with 1 ml fresh medium containing 10 µl Colcemid (10 µg/ml); Roche – 10295892001 ; toxic-handle and dispose according to safety guidelines). Remove medium and wash twice with PBS. Add 1 ml of trypsin solution and incubate for 4 min at 37°C, stop trypsinization by adding 5 ml medium, spin down cells,

remove medium, and add 1 ml of 0.56% KCl dropwise to the cell pellet. Resuspend cells by flicking, then add another milliliter of 0.56% KCl and mix by flicking. Incubate at room temperature for 8-10 min. Spin down gently (700 rpm, 5 min) and remove supernatant. Add dropwise 2-3 ml of chilled fixation solution (methanol/acetic acid 3:1, freshly prepared) onto the pellet, resuspend by flicking, incubate 5 min at room temperature, spin down gently for 5 min at 700 rpm, and remove supernatant. Repeat fixation procedure once or twice. Finally resuspend pellet in 0.5-1 ml fixation solution, drop the solution onto clean slides from a distance of 15-30 cm, and air-dry slides. Check under the microscope if the broken nuclei are sufficiently separated from each other to ensure that every chromosome can be assigned to its metaphase spread. Incubate slides for 1 min in Giemsa staining solution (Giemsa stain, modified, 0.4%, Sigma). Wash twice with distilled water, air-dry, and mount the slides. Count the number of chromosomes in metaphase spreads of the nuclei. Since direct counting under the microscope is tedious and error prone, it is recommended that pictures be taken on which the counted chromosomes can be marked.

PV, 3-04

Preparing and culturing mouse embryo fibroblasts (MEF)

Preparation

Materials

1. Mouse embryos (13.5 – 16.5 days p.c) dissected in a 10-cm sterile glass or plastic Petri dish
2. Glasgow Modification of Eagles Medium (BHK-21 medium)w/o Tryptose Phosphate Broth
3. 15 and 50 ml Falcon tubes
4. 0.05% trypsin / 0.02% EDTA in phosphate buffered saline
5. 10% New born calf serum (NCS)
6. Rotating wheel maintained at 37°C
7. Sterile razor blade
8. 10 cm sterile plastic tissue culture dishes containing Glasgow Modification of Eagles Medium with 10% serum
9. Sterile forceps and scissors for dissection

Procedure (The following procedure is to isolate MEF's from 10 embryos. Calculate accordingly)

1. Dissect mouse 13.5-16.5 days p.c. Collect the uterine horns in a Petri dish and transfer them into a 10-cm sterile Petri dish containing Glasgow Modification of Eagles Medium (without serum) under the hood. Wash once and transfer to another dish containing Glasgow Modification of Eagles Medium (without serum). Dissect the uterine horns and collect the embryos in a fresh dish containing medium without serum.
2. Remove the limbs and scoop out the internal organs. Leave the lower head on, but remove the brain and upper part of the head containing the brain.
3. Place the carcasses into a 50 ml Falcon tube containing sterile DMEM medium. Rinse three times with sterile medium.
4. Place the embryos in a sterile Petri dish and mince the embryos with a sterile razor blade until the are the consistency of a sludge.
5. Place the minced embryos into a 15 ml Falcon tube containing approximately 10ml of trypsin/EDTA in PBS.
6. Rotate the tube end over end at 37°C for 10 minutes. Remove 5 ml aliquot from the incubated tube and place it in a 50 ml sterile Falcon tube containing 5 ml of medium with 10% NCS.
7. Add another 5 ml of trypsin / EDTA in PBS to the first tube (step 5) and continue to incubate it end over end for an additional 10 minutes. Remove another 5 ml aliquot and add it to the 50 ml Falcon (step 6) and add 5 ml of medium containing serum.
8. Repeat step 7 for approximately five incubations. By this time, only soluble cartilage etc will be left in the 15ml tube.
9. Centrifuge contents of the 50ml tube and resuspend in approximately 100 ml of medium with serum and 0.1% Tylosin.
10. Seed cells into ten 10-cm dishes.
11. The next day, change medium and allow cells to grow until the dishes are confluent. Many cell types will be seen in additional to fibroblasts. These will not survive the subculture. Split the cells 1:10 and allow them to grow to confluency continuing to maintain the cultures in the presence of Tylosin. When confluent, freeze each dish in one cryo vial.
12. Thaw one vial of frozen cells in one 10 cm dish. And prepare them for inactivation either by γ-irradiation or mitomycin C treatment.

Inactivation and culture

Feeder cells should be grown in the same medium as ES cells, except for LIF and monothioglycerol. For serum-free ES cell culture, grow feeders in serum-containing medium. After trypsinization, wash once in serum-free medium and use them.

There are two ways to inactivate feeder cells, mitomycin C-treatment and γ-irradiation.

Mitomycin C-treatment:

1. Add 10 μg/ml mitomycin C to a confluent culture of feeder cells and place in incubator for 2-3 h.
2. Wash the dishes 5 x with PBS and collect cells by trypsinization (1-2 min.). Spin cells 3 min at 1.000 rpm (room temp.), discard spnt., resuspend 1 x in medium and respin.
3. Resuspend cells in ES cell medium and count them. Adjust at appropriate density, eg. 10^6/ml and use them. Mitomycin C-treated feeder cells can be frozen, following the same regimen as described for ES cell freezing.

γ-Irradiation:

1. Grow cells to near confluency. Trypsinize cells from 10-15 75 cm^2 flasks, spin down 3 min at 1.000 rpm. Discard supernatant. Resuspend cells in 10-12 ml of culture medium in a 50 ml plastic tube.
2. Irradiate cell suspension at 15 gray for 7-8 min.
3. Replate irradiated cells on same number (as before) of gelatinised flasks O/N. Trypsinize and freeze in aliquots or use in experiment. Don’t refreeze unused feeders.

For use in ES cell growth, plate feeders on gelatin-coated dishes 1-2 h before plating ES cells. Alternatively, seed them together with ES cells.

A density of 50.000 cells /cm^2 is required. Feeder layers last about one week after treatment.