ES cell culture protocols (2005 update)

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Introduction

The culture conditions and protocols described below were established and are currently used in SLRI for R1 ES cells (1) as well as G4 F1-hybrid ES cells. To maintain pluripotency, ES cells are cultured on feeder cells: mitotically inactivated primary mouse embryonic fibroblasts (MEF) and in the presence of leukaemia inhibitory factor (LIF). R1 ES cells can be grown on gelatinized plates in the presence of LIF during selection and replication of clones in 96well plates. G4 ES cells should be grown on MEF at all times. Both ES cell lines have been expanded and frozen in liquid nitrogen. Vials from the same pool are thawed every other week for experimental use in the Core facility.

MEFs can be made from any strain of mice including transgenic mice that express bacterial neomycin or hygromycin resistance genes depending on the choice of selectable markers used for altering the ES cell genome. Neo R and Hygro R mice are available from Jackson Laboratories (JR2354, JR2356). We currently use MEFs prepared from TgN (DR4)1 Jae (#003208 http://jaxmice.jax.org/), a strain that is resistant to G418, 6-thioguanine, puromycin and hygromycin. The protocols for preparation of MEF stocks and mitomycin C treatment can be found elsewhere (2, 3). MEFs are also commercially available from Specialty Media (#PMEF) and ATCC Stem Cell Center http://stemcells.atcc.org/ (#SCRC-1045).

ES cells grow rapidly and should be kept at optimal densities to prevent differentiation. The cells within ES cell colonies adhere tightly to one another, making it difficult to see individual cells. A tight border should surround the colony. ES colonies could be of various shapes, such as oblong or circular. The cells should be fed daily, and subcultured every other day. It is important to trypsinize the cells well to ensure a single cell suspension. The passage ratio should be kept between 1:4 and 1:8 depending on growth speed, so
that the cultures become sub-confluent every other day (intermediate size colonies close but not in direct contact with each other). It is very important not to let the ES cells overgrow as cells in the middle of very large colonies tend to differentiate. After electroporation and selection, stable undifferentiated clones are carefully picked for subsequent expansion and DNA analysis.

**Equipment**

- Tissue culture facility, preferably used only for ES cells, including a laminar flow cabinet, humidified incubator (37\(^0\) C, 5% CO\(_2\)), inverted phase-contrast microscope with 4x, 10x, 20-25x objectives for routine observations, stereo microscope with transmitted light base for picking colonies, table-top centrifuge, -70\(^0\) C freezer, liquid nitrogen tank. Microscope equipped with fluorescence, appropriate filters and camera (e.g. Leica MZFLIII or universal fluorescence light source from BLS Ltd, Hungary, [www.bls-ltd.com](http://www.bls-ltd.com), e-mail: bls@euroweb.hu) is necessary if fluorescent proteins are to be used as reporters.
- Electroporation apparatus with capacitance extender (e.g. Bio-Rad Gene Pulser®)
- Sterile disposable tissue culture grade plasticware (100, 60, 35 mm dishes, 6-well, 24-well, 4-well, flat and V-bottom 96-well plates, centrifuge tubes, cryovials).
- Sterile disposable reagent reservoirs for multichannel pipettor (e.g. Costar #4870). Various size sterile disposable plastic or reusable detergent-free glass pipettes.

*Note: users of SLRI ES cell core facility, bring the following:*

- 0.4 cm electrode gap electroporation cuvettes (e.g. Bio-Rad, # 165-2088).
- Multichannel pipettor with volume adjustable up to 200 \(\mu\)l, sterile pipette tips.
- Multichannel aspirator system (optional) (e.g. Inotech Biosystems Vacuset®).
- Isopropanol freezing container (optional), styrofoam box with lid, styrofoam boxes for cryovials.

All reagents and other consumables are provided by the ES cell core facility. It is of paramount importance that no reagents, cell cultures, or not properly sterilized equipment are ever brought in to the ES cell culture facility!

**ES cell culture medium and other reagents**

It is recommended to use only tissue culture grade or, preferably, ES cell qualified reagents (e.g. Invitrogen/Gibco, Specialty Media) whenever available. The water quality is a critical factor for optimal culture. It should be obtained from a regularly maintained Milli-Q (Millipore) filtration system preferably pre-treated by deionization. Large quantities of media and solutions can be prepared from powder and filter-sterilized as it is currently done at SLRI.

The quality of FBS is one of the most important factors for successful ES cell culture. ES cell qualified pre-tested FBS is available from some suppliers (e.g. Invitrogen/Gibco, Specialty Media). We test FBS before ordering large quantities. Samples of various lots are obtained from different suppliers such as HyClone [www.HyClone.com](http://www.HyClone.com), Wisent Inc. [www.wisent.ca](http://www.wisent.ca), Gemini Bio-Products [www.gembio.com](http://www.gembio.com), Invitrogen Life Technologies [www.invitrogen.com](http://www.invitrogen.com), Sigma [www.sigma-aldrich.com](http://www.sigma-aldrich.com), Specialty Media [www.specialtymedia.com](http://www.specialtymedia.com) and tested for plating efficiency and toxicity as described in (3,4) to identify a batch of serum that gives optimal growth of ES cells. Optimally the serum should also be tested for germline transmission.
ES cell culture medium (ES-DMEM)

Dulbecco's modified Eagle's medium (DMEM) (high glucose, 4500 mg/liter): e.g. Invitrogen Life Technologies [www.invitrogen.com](http://www.invitrogen.com) GIBCO™ Cell Culture #12100-061 (powder) – currently used at SLRI or #11960-044 (liquid), 10829-018 (KnockOut-DMEM) or Specialty Media: [www.specialtymedia.com](http://www.specialtymedia.com) (SLM-220B). When preparing DMEM from the powder either glass containers with absolutely no trace of detergents or preferably disposable tissue culture ware and freshly drawn Milli-Q-purified (Millipore) water are used. For ES cell culture, DMEM is often buffered with 2.2 g/liter of sodium bicarbonate, not 3.7 g/liter as per the manufacturer. This will allow proper pH equilibration in a 5% CO2 incubator. The osmolality of the completed medium (without serum) should be tested for each batch and should be approximately 290-300 mmol/kg. Prior to use, DMEM should be supplemented with the components listed below and stored in the dark at 4º C. The complete media should not be stored for longer than 4 weeks and supplemented with additional 2 mM L-Glutamine from 100X stock and LIF after 2 weeks. Adding L-Glutamine is not necessary if GlutaMAX™ (L-glutamine in a stabilized form of the dipeptide) is used.

To a 500 ml bottle of high glucose DMEM prepared by SLRI Media Prep add the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cat. #</th>
<th>Stock</th>
<th>Storage</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine OR</td>
<td>Invitrogen 25030</td>
<td>200mM</td>
<td>-20 C</td>
<td>6 ml</td>
<td>2mM</td>
</tr>
<tr>
<td>GlutaMAX™</td>
<td>Invitrogen 35050</td>
<td>200mM</td>
<td>-20 C</td>
<td>6 ml</td>
<td>2mM</td>
</tr>
<tr>
<td>2-mercaptoethanol (BME)</td>
<td>Sigma M7522</td>
<td>10 mM</td>
<td>-20 C</td>
<td>6 ml</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

*Note: 70 µl of BME (14.3 M) is diluted in 100 ml of PBS or water to make 100X stock*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cat. #</th>
<th>Stock</th>
<th>Storage</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM Non-essential</td>
<td>Invitrogen 11140</td>
<td>10mM</td>
<td>+4 C</td>
<td>6 ml</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Amino-acids (NEAA)</td>
<td>Invitrogen 11360</td>
<td>100mM</td>
<td>+4 C</td>
<td>6 ml</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

*Note: KnockOut-DMEM already contains Sodium Pyruvate*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cat. #</th>
<th>Stock</th>
<th>Storage</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pyruvate</td>
<td>Invitrogen 15140</td>
<td>10,000</td>
<td>-20 C</td>
<td>3 ml</td>
<td>50 U (µg)/ml each</td>
</tr>
</tbody>
</table>

*Note: DMEM from SLRI Media Prep already contains Pen-Strep*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cat. #</th>
<th>Stock</th>
<th>Storage</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIF</td>
<td>Chemicon ESG1107</td>
<td>-20 C</td>
<td>1000 U/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: Aliquots of LIF in SLRI ES cell facility are made for 600 ml of complete medium.*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cat. #</th>
<th>Stock</th>
<th>Storage</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS</td>
<td>ES cell qualified</td>
<td>-20/+4 C</td>
<td>90 ml</td>
<td>15%</td>
<td></td>
</tr>
</tbody>
</table>

*Note: It is best to use FBS within 4 months after thawing. If you are not planning to use the whole bottle during that time, prepare aliquots and re-freeze it.*
Other solutions

Supplied by the ES Cell Core facility:

**PBS Ca\(^{2+}\) Mg\(^{2+}\) free** (SLRI Media Prep or Invitrogen #21600-044 (powder) or #14190-144 (liquid))

**Trypsin**: Originally 0.1% with EDTA from Difco DF0153-61-1 (new cat # 215320); Alternatively 0.25% Trypsin, 1 mM EDTA (Invitrogen # 25200-072) or 0.05% Trypsin, 0.53 mM EDTA (Invitrogen #25300-054)

**Selection reagents**:

Suggested concentrations for selection reagents are below. The exact working concentration must be determined by performing killing curve experiments. We currently use ~160 µg/ml G418 for R1 and G4 ES cells (2 ml of 50 mg/ml Geneticin \(\text{\textregistered}\) per 600 ml bottle of complete ES-DMEM).

- Geneticin \(\text{\textregistered}\) (G418) (Gibco # 10131 (liquid), # 11811 (powder); Sigma G9516) 150-200 µg/ml
- Ganciclovir (Sigma G2566) 2 µM
- FIAU (Moravek Biochemical MC251) 0.2 µM

*Provided by the user:*

- Puromycin (Sigma # P8833) 1-1.5 µg/ml
- Hygromycin B (Calbiochem) 100-150 µg/ml

**Gelatin** (Sigma G2500): 0.1% solution in water, autoclaved and stored at 4°C or Specialty Media #ES-006B.

**Passage of ES cells**

It is important to carefully follow the ES cells culture protocol to maintain the pluripotency of ES cells and their ability to contribute to the germline. Typically, ES cells are kept at a relatively high density and should be passaged when they reach a sub-confluent state of 70-80% i.e. tightly packed intermediate size colonies close to but not touching each other. Media should be changed every day. For regular maintenance, ES cells should never be seeded too sparsely (when 4-5 days is required to reach subconfluency), nor should they grow past 90% confluence before passage. Both conditions induce cell differentiation. Usually, ES cells are split at 1:4 to 1:8 ratio depending on their growth rate, ideally every other day (about 1 x 10\(^6\) cells are seeded onto 60 mm plate, 2 x 10\(^6\) cells onto 100 mm plate). Cells should be trypsinized to a single cell suspension at every passage as large clumps differentiate. The passage number of ES cells must be kept as low as possible with a stock of frozen vials kept in liquid nitrogen.

- Prepare the necessary number of gelatinized plates by coating them with 0.1% gelatin: rinse the plate with 0.1% gelatin solution covering the whole surface (~5ml per 100mm plate), leave for a few minutes,
Aspirate and allow to dry for a few minutes), add ES-DMEM, and place in the incubator. Alternatively, replace the medium on the appropriate number of prepared feeder plates to ES-DMEM.

- Aspirate the growth medium, rinse twice with PBS, add trypsin (1.5-2 ml per 100 mm, 1-1.5 ml per 60 mm, 0.5 ml per 35 mm dish, 0.25-0.3 ml per well for 4- or 24-well plates); place the plate in the incubator for 5 minutes.
- Check that the trypsin treatment has reached the desired level by swirling the plate to detach clumps from the bottom of the plate. Add an equal volume of ES-DMEM to neutralize the trypsin, pipette up and down several times; transfer the suspension into a 12ml tube. Pellet the cells by low-speed centrifugation (~1000 rpm) for 5 minutes at room temperature.
- Aspirate the supernatant, add 1 drop of ES-DMEM to the pellet; flick the tube to gently resuspend the cells before adding ES-DMEM (optional). Add 5-7 ml of ES-DMEM to the tube, pipette gently to mix well, and split the contents into the new plates containing a sufficient volume of medium (e.g. 5 ml per 60 mm, 10 ml per 100 mm plate).
- Change the medium next day and split every other day.

*Freezing and thawing of ES cells in cryovials*

**ES cell freezing medium**

Freezing medium is prepared fresh immediately prior to use and kept on ice. We commonly use ~20% FBS and 10% DMSO as a final concentration. It is possible to increase the concentration of FBS in a freezing medium up to 40% for better recovery of small amount of cells in 96-well plates (see below). ES cells are usually frozen at ~5 x 10^6 cell/ml of 1X freezing media (approximately 5-6 vials from sub-confluent 100mm dish, 2-3 vials from 60 mm dish, 1-2 vials from 35 mm dish). A vial frozen in such a way can be thawed onto a 60 mm plate. If a smaller amount of cells is frozen in a vial, smaller surface area should be used for thawing. Freezing and thawing are usually counted as one passage.

| 2X: 60% ES-DMEM, 20% FBS, 20% DMSO |
| 1X: 80% ES-DMEM, 10% FBS, 10% DMSO |

**Cryovials** (e.g. Nalge 5000-0012)

**DMSO** (Sigma # D5879, D4540)

**Freezing**

- Change growth medium 2-3 hours before freezing the cells (optional but highly recommended).
- Freshly prepare 2X or 1X freezing medium, keep on ice.
- Harvest the cells in a 12 ml tube containing ES-DMEM as described. Pellet the cells at 1000 rpm for 5 min at room temperature.
- Remove the supernatant, re-suspend cells gently in half of the final volume required using ES-DMEM, gradually add an equal volume of cold 2X freezing medium while shaking the tube, mix by pipetting up and down several times. Alternatively, gently re-suspend the pellet in cold 1X freezing media.
- Quickly aliquot 1 ml of the cell suspension in freezing medium into labeled cryovials and put them on ice. Place the vials in a styrofoam box that will allow them to cool down gradually. Alternatively, isopropanol containers (e.g. Nalge #5100-0001) can be used for the same purpose.
• Immediately place the container in a –70°C freezer for 1-2 days, then transfer cryovials into a liquid nitrogen tank for long term storage.

**Thawing**

• Remove the vial from liquid nitrogen (LN2) and place it on dry ice. Thaw the vial by quickly warming it in the hand or in the incubator.

**Caution:** Handle the vials with care because LN2 may be trapped inside and cause an explosion. It helps to hold the vial in the vapour phase to allow LN2 to escape

• When the ice crystals have almost disappeared, aseptically transfer the cell suspension into a 12 ml tube using a pipette filled with 4 ml of ES-DMEM to slowly dilute the DMSO.
• Pellet the cells at ~1000 rpm for 5 minutes and aspirate the supernatant.
• Re-suspend the pellet in fresh ES-DMEM, plate on a dish with feeders, gently swirl the plate bi-directionally to evenly distribute the cells, and place in the incubator.
• Remove floating dead cells next day and change the media (if there are a lot of floating cells, rinse the cells with PBS before replacing the medium). If the correct procedure was used, cells should be ready for passage in 2 days.

**Introduction of DNA into ES cells by electroporation**

ES cells are routinely passaged one or two days prior to electroporation. Usually, one 100 mm plate at approximately 70% confluency (15-20 x 10^6 cells/ml), will provide enough cells for 1 or 2 electroporations. We regularly electroporate 20-40 µg of DNA into cells at the density of ~7-10 x 10^6 cells/ml.

Vector DNA is purified by CsCl gradient centrifugation or anion-exchange resins (e.g. Qiagen #12143 ;Omega #D6905-02 [www.omegabioktek.com]; Canadian distributors www.ubi.ca); Invitrogen #K2100-04) and linearized by restriction enzyme digestion. DNA is extracted with phenol : chloroform : isoamyl alcohol (25:24:1) (e.g. Invitrogen #15593-031), ethanol precipitated, washed twice in 0.5-1 ml of 70% ethanol and re-suspended in sterile PBS or ultrapure water at a concentration of 1 µg/µl. For transient expression of Cre recombinase, circular plasmid is used.

• Change medium on ES cells at least 2 hours prior to electroporation.
• Switch on the electroporation apparatus and set up conditions in advance. We routinely use one pulse of 250 V at 500 µF for the BioRad GenePulser for R1 and G4 ES cells. It is also possible to use two pulses of 220 V for G4 ES cells.
• Prepare the appropriate number of gelatinized or feeder-coated plates. The number of plates will depend on the cells’ survival, the specific vector used, selection approach and desired density of colonies. We routinely plate cells from one cuvette onto 1.5-2 x 100 mm plates. For transient expression of Cre recombinase, cells are plated very sparsely (~1000 cells per 100 mm dish).
• Harvest cells by trypsinization as described above. It is critical to get a single cell suspension. Pool the cells from all dishes into one tube.
• Re-suspend the pellet in a minimal volume of ice-cold PBS (~ 1ml per 100 mm plate). Determine the cell density with a haemocytometer and dilute with a volume of PBS or electroporation buffer (Specialty Media #ES-003-D), that will give the required density of a cell suspension (electroporation buffer gives better recovery of electroporated ES cells than PBS). Keep the suspension on ice.
• Gently mix 0.8 ml of the ES cell suspension and 20-40 µg of DNA in a pre-cooled cuvette. Electroporate the cells. Transfer electroporated cells from the cuvette into a tube with the appropriate volume of ES-DMEM. Cells from several cuvettes electroporated with the same construct can be pooled into one tube and gently mixed by pipetting into a uniform suspension. Transfer the cell suspension onto prepared plates, and swirl bi-directionally to evenly distribute the cells across the surface. Incubate overnight.

• Change the medium next day. Start drug selection 24-48 hours after electroporation, depending on the survival rate (colony size). Prepare only the amount of selection medium that will be used within 2 weeks. For short-term selection (e.g. puromycin), do not store selection drug containing media at all. Change the selection media every day for the first few days, then every other day. Continue the selection until colonies become apparent and ready to pick, i.e. visible to the naked eye.

Growing drug-resistant ES cell clones

Picking ES cell clones into 96-well plates

Well-separated colonies of similar size, with a defined perimeter, a compact centre with undistinguishable individual cells should be picked. Large colonies with flat borders and those with large, distinguishable cells are differentiating and should be avoided if possible. Colonies can either be picked with the naked eye, or by using a stereomicroscope with transmitting light placed in the hood. Colonies can also be circled on the bottom of the plate with a marker for easier visualization. R1 ES cell colonies can be picked into gelatinized or MEF covered 96-well plates, while G4 ES cells should be picked only on MEF containing plates. Clones growing in 96-well plates right after picking are considered passage 1. Make sure to keep track of passage number when replicating 96-well plates.

Note: If G4 ES cells are used, make sure to order MEF suspension in time and have 96-well plates with MEF ready for picking.

• Using the multichannel pipette, prepare the appropriate number of gelatinized or feeder flat bottom 96-well plates. Aliquot 30-50 µl of trypsin into V-bottom 96-well plates. It is also possible to aliquot 20 µl of PBS into the 96-well plate and add trypsin only after all colonies have been picked to avoid the long exposure of cells to trypsin.

• Aspirate the growth media from the plate with ES colonies, rinse twice with PBS, and add 6-8 ml of PBS to completely cover the dish.

• Using drawn-out Pasteur pipettes or Gilson P20 or P200 set at 15 µl, carefully dislodge the colony from the dish and pull it into the pipette tip with as little volume of PBS as possible (usually 3-5 µl).

• Transfer each individual colony into one of the wells of a V-bottom 96-well plate containing trypsin.

• Using a new tip for each colony, proceed with the rest. This process should not take longer than 30-60 minutes. 48 or 96 colonies should be picked at a time, depending on the picking speed.

• Place the 96-well plate in a 37°C incubator for 5 minutes.

• Working row by row with a multichannel pipetet, add 50-70 µl of selective ES-DMEM to each well with trypsin (to 100 µl/well). Gently pipette up and down several times to disaggregate the cells, check under the microscope if cells are in single-cell suspension. Transfer the suspension to the equivalent row of gelatinized or feeder plates. Alternatively, add ES-DMEM to all wells to neutralize trypsin first, and then proceed with pipetting and transfer.

• Using the multichannel pipette, wash each well of V-bottom plate with another 100 µl of medium and add to the equivalent row in the flat bottom 96-well gelatinized plate (total volume of 200 µl/well). Place the plate into the 37°C incubator.

• Change the media daily until the cells are ready for the passage (70-80% confluent).
Passage of ES cell clones in 96-well plates

Optimally, three or four days after picking colonies into the 96 well plates, the cells should reach the density required for passage. Often cells in different wells do not grow at a synchronous rate (especially on gelatin). Some wells might be subconfluent but rather have one or two large colonies. Such wells can be trypsinized individually to allow more even growth after plating back the single-cell suspension in the same original well. The time for the passage should be chosen when the majority of the wells have reached 70-80% confluency. 96-well plates are split into two or three replica plates, which can then be passaged again 2-3 days later. Replica plates are used for creating frozen stocks, preparation of DNA for screening, X-gal staining, differentiation assays etc. It is generally recommended to keep one or two (optional) replica plates as a frozen stock at minimum passage number for future recovery and at least two confluent replica plates for DNA preparation. See the scheme for two suggested options in the Appendix.

- Prepare the required number of flat 96-well dishes (plate MEF suspension or gelatinize). Add 150 µl of ES-DMEM per well and place in a 37°C incubator.
- Aspirate the medium from the plate to be split, wash twice with 200 µl of PBS per well by using the multichannel pipettor.
- Add 30-50 µl of trypsin per well. Incubate at 37°C for 5 min. The cells should detach with gentle tapping on the plate.
- Add 50 µl of medium per well into each of the wells to stop trypsinization. Pipette up and down at least 5 times to mix well. Working row by row, transfer the cell suspension into 2 or 3 new plates containing ES-DMEM, it is also possible to add the medium to the suspension left in the original plate. Place in the 37°C incubator. Change the media next day.

Freezing and thawing of ES cell clones in 96-well plates

Freezing

- Freshly prepare 2X freezing medium and keep it on ice.
- Trypsinize the cells of an 80% confluent 96-well plate as described. Stop the trypsin reaction by adding ES-DMEM. The volume of the cell suspension is now 100 µl per well.
- Working quickly on ice, aliquot 100 µl of 2X freezing media into each well. Pipette the cells up and down several times to get a homogeneous suspension. Alternatively, transfer the cell suspension into the new V-bottom 96-well plate, containing 2X freezing medium kept on ice.
- Add 50µl of cold sterile mineral oil (e.g. Sigma M8410, or Specialty Media #ES-005-C) on top of each well (optional).
- Wrap the plates in parafilm. Place in a pre-cooled styrofoam box, and store in a −70°C freezer, preferably not longer than two months. It is also possible to use blue pads or foil to wrap the plates instead of Styrofoam, either method that allows the cells to cool down gradually would work.

The first frozen plates are considered master plates. The replica plates are used for characterization of the clones. After identification of positive clones, the frozen stock is thawed and expanded for further analysis.

Thawing

- Prepare the necessary number of 4- or 24-well feeder plates (depending on number of potentially positive clones), containing ES-DMEM.
• Remove the plate containing identified clones from the freezer. Unwrap the plate and warm quickly (place in the incubator).
• When the ice crystals have almost disappeared, wipe the outside of the plate with 70% ethanol.
• Add 100 µl of warm ES-DMEM to the wells under the oil and transfer the content of the wells into wells of newly prepared plates.
• Rinse the original wells of the 96-well plate with more ES-DMEM and transfer to the same wells. Change media after overnight culture and thereafter daily.
• Passage the cells when they become 70-80 % confluent to a larger plate (e.g. 35 mm dish). If cells do not reach confluency in a few days but form only a few colonies in a well, they can be trypsinized, broken into smaller cell clumps and plated back into the original well (same surface).
• When there are enough cells recovered after thawing (e.g. 35 mm dish) it is advisable to expand them in selection medium to make sure they are drug-resistant and not derived from a colony mixed with wild type cells.
• Passage cells on a larger surface (60 or 100 mm plate) in selection medium and freeze them in vials (3-5) as described (keep passage number to a minimum and record it).

DNA isolation from ES cell clones in 96-well plates

This procedure was established by Ramirez-Solis et al. (6) and allows cell lysis, DNA precipitation and the restriction digestion in the original 96-well plate where ES cells have been grown. Usually, two replica plates are used for DNA isolation. One is processed for Southern blot analysis, leaving the second plate as a back up. The cells should be lysed and genomic DNA isolated from them when the majority of clones are confluent. The orange colour of the media within 24 hours after its change indicates the correct high density of cells. Not all enzymes cut well with this DNA preparation. It is highly recommended to test the enzyme of choice before starting experiments.

Lysis buffer: 10 mM Tris-HCl, pH 7.5; 10 mM EDTA; 10 mM NaCl; 0.5% sarcosyl; 1 mg/ml Proteinase K added directly before use.

NaCl/Ethanol: 150 µl of 5 M NaCl per 10 ml of cold 100% ethanol (prepared fresh).

Restriction digestion mix (per well): 1X appropriate restriction buffer, 1mM spermidine, 100 µg/ml BSA, 50-100 µg/ml RNase, 10-20 units of enzyme. Use 35-40 µl per sample.

• Aspirate media from each well, wash twice with PBS.
• Add 50 µl of lysis buffer to each well.
• Incubate the plates overnight at 55°C in a humid atmosphere (wrap the plates sealed with the parafilm with wet paper towels and place in a plastic container or sealed plastic bag).
• The next day, carefully add 100 µl of cold NaCl/ethanol mixture to each well.
• Leave the plate undisturbed at room temperature for 30-60+ minutes or until the precipitated DNA attached to the dish is visible against a dark background (“spider-web” appearance on the bottom of the well).
• Gently invert the plate on a paper towel to drain the liquid.
• Rinse three times with 150-200 µl of 70% ethanol per well, inverting the plate each time. After this step, DNA can be stored in 70% ethanol at –20°C.
• Invert the plate after the final wash and allow to air dry for 10-15 minutes (it is important for all ethanol to dry out).
Add 30-40 µl of restriction digest mix per well, mix well, seal the plate and incubate overnight at the appropriate temperature in a humid atmosphere.

Proceed with Southern blot analysis.

**DNA isolation from ES cells in 24-well plates**

After identification of potentially positive clones, cells from the 96-well master plates are thawed, expanded and frozen in cryovials. During this process, new DNA samples are prepared to confirm the genotype of ES cell clones before introducing them into mice.

**Lysis buffer:** 100 mM Tris-HCl (pH 8.5); 5 mM EDTA; 0.2 % SDS; 200 mM NaCl; 100 µg/ml Proteinase K added directly before use

It is possible to add lysis buffer directly to the cells in the plate as described below or to the Eppendorf tube with cell suspension harvested by trypsinization.

- Aspirate the medium from each well containing confluent ES cells, rinse the cells once with PBS
- Add 500 µl of lysis buffer per well and incubate overnight at 55°C in humid conditions
- Shake the plate on a shaker for 15 minutes, add 500 µl of isopropanol per well and shake until DNA precipitate becomes visible (15-30 minutes).
- Lift DNA from the solution by using a disposable tip and transfer it to an eppendorf tube. Centrifuge briefly.
- Wash the DNA pellet two times with 70% Ethanol and dry for 10 minutes at room temperature.
- Resuspend the DNA pellet in 50-100 µl TE buffer, and incubate for 3-4 hours at 55°C to dissolve. Store at 4°C
- Use 15 µl of DNA for restriction enzyme digestion.

**Preparation of ES cells for aggregation**

It is important to maintain optimal ES cell culture conditions all the time, but particularly for ES cell clones to be used for generation of chimeric animals.

1. Three or four days prior to the aggregation experiment, thaw a vial of ES cells on MEF.
2. Change the medium the next day.
3. One or two days prior to aggregation, passage subconfluent ES cells on gelatin-coated plates as described below. 24 hours growth is enough for most clones; 48 hours may be necessary for slower growing clones. Sparser than usual passage (e.g.1:10-1:50) one or two days before aggregation produces small colonies of 8-15 cells required for aggregations.

- Remove the medium and rinse the cells with PBS. Add trypsin and incubate for 5 minutes at 37°C. Resuspend the cells by gentle pipetting after adding ES cell medium. Ensure to achieve a single cell suspension. Spin down the cell suspension (~1000 rpm, 5 minutes), remove the supernatant. Resuspend the pellet in ES cell medium. Leave the tube undisturbed for 10 minutes to allow for the majority of ES cells and feeders to settle. Alternatively, place the cell suspension back onto the original plate and place in the incubator for 20 minutes to allow the MEF to reattach.
- Seed the ES cells from the top portion of the cell suspension into a few gelatinized plates, using different dilutions. For example, re-suspend cells from subconfluent 60 mm dish in 5 ml, using a 1ml pipette seed
0.1 ml, 0.3 ml and 0.6 ml of suspension from top portion on 3 x 60 mm plates. Check the cell density under a microscope and adjust if necessary. Seed the rest of cells on one or more plates, they will serve as a back up and may be re-frozen if necessary.

4. On the day of the experiment, after preparation of the embryos small colonies of 8-15 cells are lifted by gentle trypsinization immediately before the aggregation experiment.

- Remove the medium, and rinse the cells with PBS, then with trypsin (optional but helps to loosen up cells and minimize the amount of trypsin in the next step).
- Add a minimal amount of trypsin to just cover the cells (e.g. 0.5 ml per 60 mm plate), place in the incubator for 1-2 minutes or leave at room temperature. Watch under the microscope, gently swirl the plate to detach the colonies and tap at the microscope stage until all of them are lifted. Do not over-trypsinize, as cells will become sticky and hard to manipulate.
- Add ES cell medium. Do not pipette. However, if a lot of clumps are much larger than 8-15 cells, a very gentle pipetting can be used. Loosely connected clumps of ES cells are now ready for aggregation during the next two hours. Keep the plate at room temperature, as they will start attaching to the plate if placed in the incubator.

If ES cells were grown on MEF up until aggregation experiment very short trypsinization at room temperature should be used to lift the ES cell colonies leaving the majority of feeders behind. Transfer floating ES cell clumps into a new dish with medium, gently pipette if necessary to reach the clumps of the right size.

References

Appendix: Scheme of replicating 96-well plates

Option 1

1. Electroporation
2. Pick colonies into 96-well plates
3. Grow and freeze for storage (I)
4. Passage 1:2
5. Grow for DNA Screen
6. Grow and freeze for storage (II)
7. Thaw positive clones
8. Option 2
9. Electroporation
10. Selection
11. Pick colonies into 96-well plates
12. Grow to passage
13. Passage 1:2
14. Grow for DNA Screen
15. Passage 1:2
16. Grow and freeze for storage (II)