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Rapid genetic modification of mouse embryonic stem cells by inducible cassette exchange recombination

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Summary

Embryonic stem cell (ESC) differentiation is a useful tool by which to develop large quantities of cells *in vitro* representing early stages of embryonic development. A conditional gene expression system allows interrogation of factors at specific time points in the differentiation of ES cells to defined cell types. We have developed a method for rapidly generating conditional inducible murine ES cells by targeting genes into an Inducible Cassette Exchange (ICE) locus. The ICE locus encodes a doxycycline-inducible floxed Cre, which replaces itself with an incoming floxed gene of interest. The derivative cell lines, selected in G418, thus bear doxycycline-inducible transgenes. We provide detailed methods for performing ICE recombination and generating derivative doxycycline-inducible cell lines.

Keywords

ESC; Cre; Recombination; ESC targeting; inducible system

1.Introduction

To identify factors involved in Embryonic Stem (ES) Cell differentiation, an inducible gain of function or loss of function approach is extremely useful. This allows for up- or down-regulation of genes of interest in temporal windows during *in vitro* differentiation in order to probe gene activity during embryonic development (1), or to generate progenitor cells *in vitro* for therapeutic purposes (2, 3).

We have developed a murine embryonic stem cell line that can be targeted with a specific incoming plasmid vector to generate a derivative inducible gene expressing ES cell line (4). We have targeted an ICE locus into the DNA 5' of the HPRT gene on the X chromosome in A17 mES cells (5), a derivative of the male cell line, E14Tg2a (6) in which rtTA has been inserted into the *Rosa26* locus. Downstream of the floxed *cre* is a G418 resistance gene lacking a start codon and promoter, referred to as Δneo , (7). The region upstream of the HPRT gene is a site at which transgenes express reliably (8-11), presumably because HPRT is a housekeeping gene, active in all cell types and therefore embedded in constitutively open chromatin. At this site, cre-mediated recombination is exceptionally high, producing recombinants after nucleofection at a rate of > 1/1,000 in our hands (4). This is about 3 orders of magnitude higher than conventional homologous recombination. Recombinants can be selected in G418 because after recombination, the Δneo gene is repaired.

2. Materials

2.1 mESC growth and maintenance

mESC medium: Knockout™ DMEM, Optimized for ES cells (Gibco C# 10829018), supplemented with 15% Fetal Bovine Serum (FBS) qualified for ES cells, 1X GlutaMax (Gibco C# 35050-079), 1X Penicillin/Streptomycin, 1X MEM Non-Essential Amino Acid, 100 µM β-Mercaptoethanol, and 500 to 1000 U/ml Leukaemia inhibitory factor (ESGRO® Lif, Chemicon).

10% FBS DMEM: high glucose DMEM supplemented with 10% FBS, 1X GlutaMax (Gibco C# 35050-079) and 1X Penicillin/Streptomycin.

2X Freezing Medium: 60% FBS and 20% DMSO in the appropriate cell culture medium.

Additional supplies for tissue culture:

- A2lox.Cre mES cells
- Primary Mouse Embryonic Fibroblasts (MEFs), irradiated
- PBS without Ca⁺⁺ and Mg⁺⁺ (sterile);
- Trypsin-EDTA 0.25% (sterile);
- Tissue culture treated plates, 12-well and 6-well;
- Tissue culture treated flasks, T25 and T75;
- 60 mm tissue culture treated petri dishes;
- 0.1% gelatin (solubilized in water, sterilized by autoclaving);
- Serological pipettes (sterile);
- 15 ml conical tubes (sterile);
- Tissue Culture Incubator (5% CO₂, ambient O₂, 37° C)
- 1.5 ml Cryotubes

2.2 Recombination of the p2Lox plasmid in mESC

- Primary MEFs, Neo Resistant (Millipore C# PMEF-N)
- Electroporator
- Cuvettes
- G418 (Gibco)
- Evos microscope.
- Doxycycline (Sigma) 1 µg/mL (solubilized in H₂O, sterile filtered, and stored in dark at -20° C or 4° C)
- Amaxa nucleofactor shuttle for 96 well cuvettes.
- Amaxa Mouse ES Cell 96-well Nucleofection Kit

2.3 Analysis of Recombined Cell Lines

- Loxin-f primer sequence: 5' – ATA CTT TCT CGG CAG GAG CA – 3'
- Loxin-r primer sequence: 5' – CTA GAT CTC GAA GGA TCT GGA G – 3'

- TRE forward primer sequence: 5' – ACC TCC ATA GAA GAC ACC G – 3'
- EB Differentiation Medium: Iscove's Modified Dulbecco's Medium with GlutaMAX (Gibco C# 31980-030), supplemented with 15% FBS, 0.5X GlutaMAX, 1X Penicillin/Streptomycin, 450 μ M monothioglycerol, 200 μ g/ml bovine Holo-Transferrin (Sigma C# T1283), and 50 μ g/ml Ascorbic acid .

3. Methods

3.1 Preparation of p2lox targeting plasmid

To integrate the gene of interest into A2lox.Cre cells, the gene needs to be subcloned into the p2Lox plasmid (Fig. 1). Once the gene is properly inserted, the plasmid should be purified to a fairly high level for optimal recombination efficiency. Particularly for plasmids containing large DNA inserts, we recommend the Qiagen QIAfilter Plasmid Midi Kit (C# 12243). For targeting DNA to the A2lox.Cre cells with electroporation, 20 μ g of each plasmid is recommended, in a final volume not exceeding 25 μ L; for targeting via nucleofection, 4 μ g plasmid in a volume not exceeding 2 μ L is recommended (see Note 1).

3.2 A2Lox.Cre cell culture and maintenance

A2lox.Cre cells are grown on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs), which provide support and secrete cytokines that prevent differentiation. As MEFs are irradiated to prevent growth, they must be replaced with fresh cells at every passage. The A2Lox.Cre cells grow as colonies sitting on the feeder layer. When mES colonies increase in size, they begin to differentiate; therefore, to maintain mES cells in an undifferentiated state, cells must be maintained as small colonies. This is accomplished by trypsinizing the cells every two days, fully dissociating each colony into individual cells that will form new colonies. It is also important to replace the medium with fresh mESC medium each day, including at least one hour before harvesting the cells for a passage.

All cell culture procedures must be performed in a laminar flow hood under sterile conditions. Centrifugation steps are done at 800 RCF for 4 minutes.

3.2.1: Preparation of feeder cells—Prepare a MEF-coated tissue culture flask the day before, or morning of, thawing or passaging A2Lox.Cre cells. MEFs will attach to the plate after approximately 1 hour, but they will not acquire the stretched fibroblast shape for several hours. Before plating the ES cells, make sure that the MEFs are fully confluent, covering the entire surface of the plate.

1. Coat the bottom of a T25 flask with 5 ml of 0.1% gelatin and incubate for 20' in the cell incubator at 37°C/5% CO₂ to allow collagen I in the gelatin solution to coat the plate. Aspirate excess gelatin with a sterile glass pipet.
2. Transfer 10 ml of 10% FBS DMEM into a 15 ml centrifuge tube. Thaw a tube of irradiated MEFs (approximately 6-8 *10⁵ cells) at 37°C, quickly transfer the contents of the vial into the 15 ml conical tube, and centrifuge.
3. With a sterile glass pipet, aspirate the liquid from the cell pellet; then re-suspend the cells in 5 ml fresh 10% FBS DMEM. Gently mix the cells in the liquid to ensure a homogeneous suspension, and transfer the suspension to the gelatin-coated T25 flask.

¹Amaza suggests adding DNA in a volume no more than 1/10th the final volume. We routinely add up to 10 μ l of DNA to the 20 μ l of nucleofection mix, and achieve successful recombination; however, we have not optimized this protocol and we do not know how this may affect efficiency. We suggest following the manufacturer's protocol if possible.

4. Incubate the cells in the cell culture incubator at 37°C/5% CO₂ until they acquire the fibroblastic morphology and are fully confluent.

3.2.2: Growing A2Lox.Cre cells

1. Day 0: Thaw and pellet cells as described in section 3.2.1, with the following modifications: Re-suspend A2Lox.Cre cells in mESC medium. With a glass pipette, aspirate the culture medium from the MEF pre-coated T-25 flask and gently replace with the ES cell suspension. Place the cells in the incubator overnight.
2. Day 1: Observe the cells under the microscope: small clumps of cells growing as colonies should be present on the top of the layer of MEFs (Fig. 2B). Replace the medium with 5 ml of fresh mESC medium. Prepare a T75 flask for the next day by coating with gelatin and plating fresh MEFs as described in section 3.2.1, correcting for the larger area of a T-75 flask.
3. Day 2: Colonies should have more than doubled in size and be ready to be separated. To passage the cells, aspirate the medium and wash with 5 ml of PBS. Remove the PBS, replace with 0.5 ml of Trypsin-EDTA, and incubate at 37° C for 3 to 4 minutes. After incubation, inspect the cells under a microscope to ensure that the MEFs have lifted off the plate and that ES colonies have detached. It will be apparent that the ES cells have not yet separated, but each single cell is now visible in the colony. Add 5 ml of 10% DMEM to quench the trypsin, and dissociate the cells by pipetting the liquid up and down against the bottom of the flask, avoiding frothing, but making sure that the tip of the pipette is in full contact with the bottom of the flask (Fig. 2D). Usually pipetting up and down 8 to 10 times is sufficient to release individual cells from the colonies. When first using this technique, it is recommended to check under the microscope after this step to be sure that all cells have been released. Dissociation of colonies into single cells is critical to maintain an undifferentiated culture. Transfer the cell suspension to a 15 ml conical tube, centrifuge, aspirate the medium and re-suspend the cells in 3.5 ml of mESC medium. Remove medium from the MEF-coated T75, and replace with 1.5 ml of the A2Lox.Cre cell suspension together with an additional 11.5 ml of fresh ES medium. See Note 2 for more information on dilutions.
4. The remaining cells can be frozen to replace your stock. Add an equal volume of ice cold 2X Freezing medium to the remaining mES cell suspension. Distribute in 1 ml aliquots into 2 ml cryovials and transfer vials to -80° C. It is recommended that cells are transferred to liquid Nitrogen after 24 hours.

3.2.3: Preparation of selection plate with neo-resistant feeder cells—

Recombination will be selected on G418, therefore neomycin-resistant MEFs are necessary for the next step. We recommend Neo Resistant EmbryoMax® Primary MEFs (Millipore). One vial of EmbryoMax® MEFs contains 5-6*10⁶ cells, which we have found sufficient to fully coat eight 60-mm tissue culture dishes. Cells should be plated one day before harvesting A2Lox.Cre cells for recombination. One 60 mm dish will be needed for each p2lox plasmid that will be introduced, as well as an additional dish to serve as a selection

²The procedure for passaging A2Lox.Cre cells describes a standard dilution of 1:6 every two days, which we have usually found sufficient to generate the next confluent plate in two days. The appropriate dilution of the cells depends on the confluency of the initial plate, so this may require higher (1 to 8 or 1 to 10) or lower dilutions. Usually 2-3*10⁵ cells plated in a fresh T25 flask will yield the optimal concentration of 2-3*10⁶ cells after two days growth. When mES cells become too confluent, the colonies may merge with each other.

control. If there are fewer than eight conditions in total, excess cells may be plated out into a T-flask(s) and then frozen down for future use the following day.

1. Pre-coat dishes or flasks with 0.1% gelatin, as described in section 3.1, then add 3 ml 10% FBS DMEM to each dish (4 ml for a T25 flask, 10 ml for a T75 flask).
2. Thaw Neo Resistant MEFs as described in section 3.2.1, and re-suspend the cell pellet in 8 ml 10% FBS DMEM. Add 1 ml of MEF suspension to each 60 mm dish or T25 flask, or 3 ml MEF suspension to a T75 flask. Incubate the cells in the cell culture incubator at 37°C/5% CO₂.
3. After 1 day of culture, aspirate the medium from the 60 mm dishes, and replace with 5 ml of mESC medium.
4. At this point, excess Neo Resistant MEFs may be harvested and frozen down. Wash cells in a T25 flask with 5 ml PBS (10 ml for a T75 flask) and incubate with 0.5 ml (2.0 ml) 0.25% trypsin for 5 minutes at 37° C. Add 4.5 ml (8 ml) 10% FBS DMEM, transfer cell suspension to 15 ml flasks, and centrifuge. Re-suspend the cell pellet in 0.5 ml (1.5 ml) of 10% FBS DMEM, add an equal volume of 2X Freezing medium, and freeze in 1 ml aliquots as described in section 3.2.2(4). To use these cells for a later experiment, thaw cells and plate each vial into one 60 mm tissue culture dish.

3.3 ICE recombination

When starting from a frozen stock of mES cells, we recommend expanding the cells as described in section 3.2 for at least one passage before preparing cells for targeting. Cells may be transfected with the p2lox-GFP plasmid by either electroporation (3) or nucleofection using the Amaxa Nucleofector Shuttle (4). Nucleofection is the recommended procedure when generating multiple inducible mES cell lines. It is also recommended in cases where the p2lox plasmid contains a large insert, or where you want to minimize the number of starting cells or amount of DNA.

1. The day before harvesting A2Lox.Cre cells for recombination, induce Cre expression by adding 0.5 µg/ml of doxycycline when the mESC medium is changed. Plate Neo Resistant MEFs on 60mm tissue culture dishes (section 3.2.3).
2. The following day, replace the medium with fresh mESC medium containing 0.5 µg/ml of doxycycline at least one hour before harvesting cells.
3. On the plate of neo-resistant MEFs, aspirate the medium and replace with mESC medium *without doxycycline*.
4. Recombination by Electroporation: A2Lox.Cre cells are harvested as described in section 3.2.3(3) with the following modification: After dissociating individual cells, count the cells with a hemocytometer, and separate into aliquots of 2×10^6 cells per condition. After centrifuging the cells, re-suspend each pellet in 800 µL room-temperature PBS and transfer the cells to a sterile electroporation cuvette at room temperature. Add 20 µg of p2lox plasmid for each condition, or an equal volume of PBS for the no-DNA control. Perform the electroporation at the following condition: 0.25 Kw; 500 µF. We usually obtain a time constant between 6 and 7.
5. (alt) Recombination using the Amaxa Nucleofector Shuttle. A2lox.Cre cells are harvested as described in section 3.2.3(3) with the following modifications: For the nucleofection procedure, it is necessary to remove MEFs from the ES culture prior to counting cells. This step improves the efficiency of nucleofection by ensuring that the p2Lox plasmid is not titrated out due to introduction of DNA into the MEFs, which are much larger than ES cells. Before harvesting cells, pre-treat a T25

flask with 0.1% gelatin. After ES cells are trypsinized, dissociated, and centrifuged, re-suspend the cell pellet in 5 ml of mESC medium, transfer the cell suspension to the pre-coated T25 flask, and store in the tissue culture incubator for 30 minutes. During this time the MEFs will attach to the plate; however, ES cells, which attach more slowly, will remain in suspension. After 30 minutes, collect the ES cells by gently washing the medium over the bottom of the flask. Transfer the MEF-depleted ES cell suspension to a 15 ml conical tube. Count the cells with a hemocytometer and separate out enough cells to have 2.5×10^5 cells per condition. Prepare the nucleofection reagent by mixing 17 μ l mix with 3 μ l supplement for each condition. Spin down the cells, aspirate all liquid, and resuspend the pellet in the nucleofection reagent. Pipette 20 μ l of nucleofection solution containing cells into 96 well cuvettes, one well per condition. Add 4 μ g of p2lox plasmid (do not exceed 30 μ l in total volume), or an equal volume of TE for the no-DNA control, to the cells, mix gently, and incubate for 5 minutes at room temperature. Nucleofect, using the waveform program 96-CG-104.

6. After the samples have been electroporated or nucleofected, use a P200 pipette to transfer the cells dropwise onto the medium covering one plate of Neo-resistant MEFs. Swirl gently to mix, and transfer the plate to 37° C/5% CO₂.

3.4 G418 Selection

After recombination, continue to change the medium covering mES cells every day. Two days after electroporation or nucleofection (day 4), begin selecting for recombined cells by adding G418 to the mESC medium at a concentration of 300 μ g/ml. After 5 to 7 days of selection, all mES colonies in the no-DNA control condition should be dead or clearly dying. Once this has occurred, colonies with positive recombination events can be picked for expansion when they have reached the size shown in Figure 3A. Choosing appropriately sized colonies is important at this stage: if colonies are too large they will have already begun differentiation; if too small, they will have a limited replating efficiency. In addition, karyotypically abnormal subclones that may arise in the stock will tend to grow faster and thus give larger clones. It is therefore recommended to avoid the largest clones and pick from the clones of middle size. Colonies will be visible by the naked eye if the dish is held up to a light source and examined from underneath. Circle visible colonies with a marker on the bottom of the plate. Once all colonies are circled, inspect them under the microscope and choose 6 to 8 colonies per line for further expansion, identifying them by marking the bottom of the plate. Ideal colonies will be moderately sized, with a nice oval shape and clearly defined, non-differentiated edges. At this point, selection can be withdrawn: replace the medium with fresh mESC medium that does not contain G418 at least 1 hour prior to picking colonies.

3.5 Picking and expanding recombined colonies

Single colonies will be picked and dissociated into individual cells, and plated on 12-well plates. They will be expanded as separate clones onto 6-well plates, then T25 flasks. During expansion, cells are maintained and passaged as described in section 3.2, adjusting volumes and MEF cell numbers to reflect the reduced surface area.

1. Prepare 12-well plates for the newly picked clones on the day before or morning of picking colonies. Each colony will be placed in one well of a 12-well plate. Change the medium covering the MEFs to mESC medium (without G418) just prior to harvesting colonies.

2. Transfer a microscope into the laminar flow hood, so that cells may be picked under sterile conditions. We recommend the use of a portable microscope with a flat screen display, such as the AMG Evos microscope.
3. Prepare 1.7 ml microcentrifuge tubes (one for each colony to be picked) by adding 25 μ l of 0.25% trypsin-EDTA.
4. Pick clones: as trypsinization is time-sensitive, perform the entire procedure for each 60 mm dish before moving onto the next dish. Wash A2Lox.Cre cells with PBS and aspirate the dish. Using a P200 pipette, place a drop of 0.25% trypsin-EDTA on top of each colony selected for expansion (visualized by the circle marked underneath). Incubate at room temperature while observing the cells under the microscope. After 3 to 4 minutes, you should see the MEFS start to detach. Although the colonies have not detached, at this point the trypsin should be diluted and plate prepared for picking. Slowly add 5 ml of PBS to the edge of the plate, letting the PBS gently cover the bottom of the dish. This step must be performed gently so as not to displace or disrupt the colonies. Do not add medium at this stage; completely quenching the trypsin will prevent the dissociation of colonies into individual ES cells. Purge air from a P20 pipette and place the tip near but not touching the marked colony, then pull the colony into the pipette tip. Multiple attempts may be required before the colony comes loose. If the colony is too tightly attached to be pulled into the pipette tip, it is possible to fill the tip with PBS and gently pipette this towards the colony, loosening it enough to then pull the colony away from the plate. However, this must be done very carefully, or the colony will become fully detached and can be blown away. Once a colony is in the pipette tip, transfer the contents of the tip into a microcentrifuge tube containing 25 μ l of 0.25% trypsin-EDTA, and proceed to pick the other marked colonies on that plate.
5. Use a P200 pipette to dissociate the cells by pipetting up and down several times against the bottom of the tube. Add all the contents from each tube into the medium covering MEFs on one well of a 12-well plate. Each colony is a single clone and must be maintained separately; this is a good time to give each clone a discrete name. After colonies have been picked, dissociated, and plated on 12-well plates, place cells in the 37°C/5% CO₂ incubator.
6. The following day, change medium covering the cells to fresh mESC medium, and plate fresh MEFs on gelatin-coated 6-well plates for the next expansion step. Two days after picking colonies, the plate should be confluent with ES colonies (if not, see Note 3). Trypsinize and dissociate the mES cells as described in section 3.2.2. After centrifugation, re-suspend cells in 1 ml mESC medium and freeze half of the cell suspension as one vial of P0 cells. Add 3 ml mESC medium to the other half of the cell suspension, and use this to replace the medium covering MEFs on one well of the prepared 6-well plate. From a confluent well of a 6 well plate, freeze half in 2 or 3 vials of P1 cells, and expand the remaining cells into a T25. A confluent T25 flask can be frozen into 6 to 8 vials.

³If the ES cells are present but not confluent two days after picking colonies, harvest the cells as described above, with the following modification: after centrifugation, re-suspend the cell pellet in 2 ml mESC medium and replate all of the cell suspension on one well of a 12-well plate that has been pre-treated with 0.1% gelatin. It is not necessary to plate fresh MEFs as this is not a dilution. This additional procedure will allow for the formation of new colonies from each dissociated cell, and should lead to a fully confluent well after another two days. At this time, the well can be harvested, a vial of P1 cells frozen, and the remainder expanded on a 6-well plate as described.

3.6 Genotyping the clones

This procedure can be performed any time during the expansion of clones, although we recommended waiting until the cells have been expanded at least to a 6-well plate, in order to minimize the ratio between MEFs and ES cells. When performing a passage, transfer a small number of cells from each clone (around 1/20th) to one well of a 12-well plate. As the cells do not need to be maintained as pluripotent, they can be plated directly onto a gelatin-coated plate in 10% FBS DMEM. After one day of growth, aspirate the medium, add DNA extraction buffer to solubilize the cells and proceed to DNA purification. There are two methods to identify correct integration:

1. Generic: If correctly integrated, the Loxin primers described in section 2.3 will produce a band at 430 bp.
2. Gene-specific: Alternatively, generate an insert-specific PCR using a forward primer in the distal portion of the TRE promoter (TRE-f primer, described in section 2.3), and a reverse primer based on sequence near the 5' part of the inserted gene. This method is recommended when generating many cell lines, as it can distinguish between cell lines as well as showing correct integration in the HPRT locus.

3.7 Induction of the gene of interest during EBs differentiation

This procedure requires the differentiation of ES cells into Embryoid Bodies (EBs) via the Hanging Drops procedure.

1. Harvest the mES cells as described in section 3.2.2(3) and remove MEFs from the mES culture as described in section 3.3(4). After counting mES cells, re-suspend them to a final concentration of 1×10^4 cells/ml in EB Differentiation Medium.
2. Place the cells into a 50 ml reservoir vessel and use an 8-channel pipette to place 10 μ l drops of the cell suspension on the bottom of a 150 mm bacterial Petri dish. It is important not to use a tissue culture plate for this procedure, as drops form best on hydrophilic plastic. Each drop of suspension will contain an average of 100 cells, which will aggregate to form the EBs. Continue to place rows of drops on the plate, alternating the pipette tips to place each drop in between two previous drops (Fig. 4). The drops should be placed as close to each other as is possible; a larger volume within the dish will help reduce evaporation rate of the drops. Continue until the plate is full; each full plate should contain approximately 3.5 ml of suspended cells.
3. After each plate is full, replace the cover and flip the plate upside down so that the drops are suspended. Incubate the hanging drops for two days in a cell culture incubator at 37°C/5% CO₂.
4. After two days of differentiation, harvest the EBs from the hanging drops by washing the drops off the bottom of each dish with 10 ml PBS. Two plates can be sequentially washed with the same PBS. Transfer the EBs into a 15 or 50 ml centrifuge tube and let the EBs settle to the bottom of the tube (approximately 5 minutes). Carefully aspirate the medium away from the EBs, and re-suspend in fresh EB differentiation media, using 10 ml of EB differentiation medium for every two 150 mm dishes. Plate these into 100 mm bacterial Petri dishes (again, not tissue culture dishes) and incubate for 1 day at 37°C/5% CO₂. Add 10 μ g of Doxycycline to one of the plates to induce the EBs. Twenty-four hours after inducing, the EBs will be ready to harvest for protein and RNA extraction.

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Figure 1. ICE Recombination

The ICE locus is 5' of the *HPRT* gene on the X chromosome. At this locus, a doxycycline-inducible promoter (TRE) drives expression of a *Cre* transgene, which is flanked by heterologous loxP sites. When cells are grown with doxycycline, Cre is expressed. After a pulse of doxycycline, cells are transfected with a p2Lox plasmid derivative, shown above, which carries the incoming transgene and polyA sequence, as well as a PGK promoter and ATG with heterologous loxP sequences arranged such that after recombination the incoming gene is placed downstream of the TRE and the PGK-Promoter and ATG elements are introduced upstream of a deleted *neo* gene (lacking a promoter and ATG). Derivative cells are thus rendered G418-resistant and dox-inducible for the transgene of interest.

Figure 2. MEF and ES colony morphology

A. Appropriate confluency of MEFs prior to plating ES cells

B. ES cells the day after thawing.

C. ES cells appropriate for passaging (generally day 2, although after the initial thaw they may require an additional day to recover to this density).

D. ES cell colonies being disrupted. Note that the pipette is pressed flat against the floor of the T25 flask. Liquid is being expelled equally in all directions. This should be done in a controlled manner, avoiding froth.

Figure 3. Picking colonies

A. Morphology of a colony appropriate for picking.

B. Set-up of a flat panel portable microscope in a tissue culture hood.

Figure 4. Generating hanging drop cultures
Drops are arrayed in parallel lines, offset to maximize density.