

LePage DF, Conlon RA. (2006) Animal models for disease: knockout, knock-in, and conditional mutant mice. *Methods Mol Med.* 129:41-67.

Animal Models For Disease--Knockout, Knockin And Conditional Mutant Mice

David F. LePage and Ronald A. Conlon

Abstract

Diseases with a genetic basis can be modeled with knockout, knockin and conditional mutant gene targeted mice. In the following, we provide detailed protocols for gene targeting. Gene targeting of embryonic stem cells can be accomplished by laboratories equipped for tissue culture. Alternatively, many gene targeting services divide the work of targeting with a customer lab. In this collaborative situation, knowledge of the entire process helps ensure a successful outcome. The construction of chimeras for germ line transmission is not described here since this procedure is beyond the means of most laboratories, typically is provided by transgenic core facilities, and is best learned through hands-on demonstration.

Key Words: gene targeting, homologous recombination, ES cells, knockout, knockin, conditional mutant, animal model of disease, genetically engineered mice.

1. Introduction

The generation of mutant mice by gene targeting takes advantage of the remarkable ability of embryonic stem (ES) cell lines (*1, 2*) to participate in the formation of germ cells of mice when the cells are put back into an early embryo (*3*). Cell lines which have undergone gene targeting are enriched by the incorporation of selectable markers into the targeting vector (*4*). Out of this enriched set of cell lines, the desired homologous recombination event is identified by molecular analysis of genomic DNA. Targeted ES cell lines which have a normal number of chromosomes are identified and selected to make chimeras.

Targeting can be used to generate knockouts, knockins or conditional alleles. Knockouts, knockins and conditional alleles are generated in a similar fashion using the protocols given here. Knockouts are used to define the overall requirement for a gene and to model loss of function mutations. Knockins introduce heterologous coding sequences such as reporters or DNA recombinases, or to incorporate changes in DNA sequence to "humanize" a mouse gene or to generate point mutations. Conditional mutations are used to define the organ, tissue or cellular autonomy of mutant effects, to circumvent embryonic lethality, or to model somatic mutations.

The two major hurdles for the generation of gene targeted mice are obtaining homologous recombination and germ line transmission. Although it has been almost twenty years since the first gene targeted mice were constructed (4, 5), not all parameters which affect the frequency of homologous recombination and germ line transmission are known. Nonetheless, we give criteria for the parameters which are known, provide advice on how to ensure success, and provide recovery strategies and trouble shooting guidance.

The protocols assume a working knowledge of basic tissue culture technique (6).

1.1 Targeting Vector Design

1. General Principles

Our purpose is to present the most common and widely applicable vector designs (Figure 1). There are a large number of variations possible in the design of targeting vectors and the uses they are put to (7, 8). The following concentrates on the most common applications.

For all targeting vectors, the following considerations apply: 1) the vector needs to be linearized outside of the arms of homology, so provision must be made for a unique recognition sequence for a restriction enzyme at an appropriate place; 2) a strategy to detect gene targeting by Southern blot analysis of genomic DNA must be developed; 3) the greater the amount of sequence match, the more likely the targeting is to succeed.

2. Targeting Vectors for Null Alleles

We recommend that a targeting vector for construction of a null allele be constructed with genomic DNA from the 129 strain of mice, with at least 7 kb of total homology split in two arms which are positioned to delete early or critical coding exons of the gene (Figure 1). Two selectable markers are incorporated into the vector (**4**). The neomycin resistance gene (neo) should be flanked by loxP sites, and located between the two arms. The herpes simplex virus (HSV) thymidine kinase (tk) gene, should be placed outside one of the arms of the vector. The neomycin resistance gene is removed in ES cells through the activity of Cre recombinase acting on the loxP sites after gene targeting.

3. Targeting Vectors for Knockin Alleles

To create a knockin allele (Figure 1), a sequence change or inserted coding sequence is incorporated into one of the arms of the targeting vector (**9**). The goal is to minimize other disruption of gene function. The neomycin resistance gene is flanked by loxP sites so that it can be excised by expression of Cre recombinase and is typically inserted into an intron. In designing knockins, particularly for those generating small or subtle changes, provision needs to be made to detect if the knockin change itself has been integrated as was intended--this is necessary because a homologous recombination exchange could occur internal to the intended change and not incorporate the altered sequence. The neo marker is removed in ES cells before constructing chimeras.

4. Targeting Vectors for Conditional Alleles

A conditional allele has wild type function but can be mutated to a null allele in cells in which Cre recombinase is expressed (**10**). To maintain wild type function, no part of the gene is deleted, and loxP sites are inserted into introns away from sequences which might function in splicing and transcription (Figure 1).

Targeting vectors for conditional alleles should be constructed as for null alleles, except that the neomycin resistance gene with its flanking loxP sites is inserted into an intron, and a loxP is inserted into different intron, such that deletion of sequences between the most distal loxP sites will remove an essential coding exon.

The neomycin resistance gene is removed with Cre recombinase in ES cells, and alleles with the two loxP sites flanking the essential exon are identified. The conditional allele is introduced into mice through chimeras, and the gene is mutated to a null allele through the action of Cre recombinase on the two remaining loxP sites, deleting the sequences between them.

1.2 Construction Of Vectors

1. Isogenic DNA

The frequency of homologous recombination depends on the degree of sequence match and the length of the matching sequences (*11, 12*). Greater than 7 kilobases of DNA of perfect sequence match usually is needed to obtain homologous recombination at practical frequencies. The relationship between sequence divergence and targeting rates has not been examined systematically, but about 0.5% sequence divergence was shown to result in a 20-fold decrease in targeting frequency for constructs targeting the Rb locus (*11*). In a targeting vector, the exact sequence match typically is interrupted by a gap and/or an insertion with each of the two parts of the match approximately halved. The apparent paradox that the sequence match with the target gene must be near exact whereas the gap or insertion can be as large as 10 kb probably arises because there are two independent homologous recombination events which occur in gene targeting, one for each arm.

The genomic sequences of different inbred strains of mice diverge enough that DNA from the same strain as the ES cells is needed to construct the targeting vector. Most ES cells are made from the 129 strain of mice, thus targeting vectors are made from 129 genomic DNA. There are several different substrains of the 129 strain, but differences between them are unlikely to be great enough to affect gene

targeting. Genomic libraries of 129 mice are available in lambda phage vectors from commercial vendors (Stratagene 946313), and in bacterial artificial chromosome (BAC) vectors from the BACPAC Resources Center <<http://bacpac.chori.org>>.

Alternatively, long range PCR can be used to isolate construct arms from ES cell genomic DNA. DNA recovered by PCR should be sequenced to ensure that PCR did not introduce unintended mutations into the targeting vector, particularly for knockin and conditional gene targeting.

2. Selectable Markers

Heterologous genes are incorporated into the targeting vector for enrichment of targeting events. Between the arms of the targeting vector a gene consisting of a promoter, coding sequence for a drug resistance protein and a polyadenylation signal is incorporated. The neomycin resistance coding sequence is typically used, as this confers resistance to the neomycin analogue G418. Outside of one arm, a gene to express Herpes Simplex Virus thymidine kinase (tk) is placed. In a successful homologous recombination, the tk gene is not integrated into the genome and is lost. Cells in which tk has integrated into genomic DNA can be killed by selection with the drug FIAU. In most transfections, the majority of cells incorporating the targeting vector do not do so by homologous recombination, so most cells incorporate the tk selectable marker, and the FIAU selection kills these cells. Alternative drug selection genes are available as well (**8**).

The orientation of the selectable marker gene transcription units relative to each other and the targeted gene do not appear to be important.

3. Avoiding Unintended Consequences

It is important to anticipate the consequences of gene targeting to ensure that the targeted gene lacks all function as intended. Examine the splicing patterns which might be expected to occur in the targeted allele, looking for in-frame splicing which could result in aberrant protein products with altered

activity. Avoid designs which might give rise to proteins with dominant negative or other gain of function activities.

The neomycin resistance coding sequence and its PGK1 promoter can have unintended consequences on the targeted gene and on adjacent genes if they are left in the gene (*13-16*). The neomycin resistance gene contains cryptic splice acceptors and donors which can be utilized by transcripts from targeted gene. In addition, the function of genes adjacent to the targeted gene can be altered by the neomycin gene inserted at the targeted locus. In one case this interference has been shown to be due to transcription from the PGK1 promoter and aberrant splicing of neo sequences into the adjacent gene, but other mechanisms are possible, including interference through enhancer competition. If the targeting vector is designed with recognition sequences for a site-specific DNA recombinase flanking the neo gene as describe above, it can be removed after gene targeting by expression of the DNA recombinase. The Cre site-specific DNA recombinase recognizes a 34 base pair sequences, the loxP site. Sequences between a pair of matched sites in direct repeat orientation are deleted, leaving a single loxP sequence.

4. Planning For Detection Of Gene Targeting

A strategy for identifying the targeted locus by Southern blot analysis must be developed in parallel with vector design (Figure 1). Two DNA probes from the gene on each side outside the targeting vector must be able to detect a change in fragment size.

Avoid the use of restriction enzymes which have recognition sequences which have 5'CG3' dinucleotides because this sequence is most often methylated in ES cell genomic DNA and will be resistant to cutting. For example, the recognition sequence for Xho I is 5'CTCGAG3' and thus Xho I should be avoided; Hind III recognizes the sequence 5'AAGCTT3' and is suitable. Before transfecting ES cells with the targeting vector, test the probes for Southern blot analysis on a genomic digest of ES cell DNA to verify that they work well.

It is important to verify the homologous recombination event from both sides with probes external to the targeting vector since recombination can occur on one side only (*17, 18*). In practice, the initial identification of homologous recombination events is done with one external probe, then the candidate targeted cell lines are thawed, larger amounts of genomic DNA are prepared, and targeting is verified by extensive genomic Southern blot analysis using multiple probes and digests.

5. Vector Linearization

Targeting vectors are linearized before transfection, so provision needs to be made for a unique recognition sequence to linearize the vector. Ideally, this site is placed such that when the vector is cut, the vector has at one end one arm of homology, and at the other end the vector backbone is external to the tk gene.

6. Assembling The Targeting Vector

We do not provide a protocol describing construction of the targeting vector, but the technology involved is that common to most molecular biology subcloning. Alternatively, companies which provide targeting vector construction services include inGenious, genOway and Genomatix. Plasmids carrying the neo selectable marker flanked by loxP sites, pflox (*19*), and the tk selectable marker, pPNT (*20*), can be obtained from the labs they originated in.

2. Materials

The key material for successful gene targeting is an early passage, germ line-competent ES cell line. Because germ line-competency can be lost during culture, and because the only way to assay germ line-competency is by the construction of chimeras, it is strongly recommended that ES cells be obtained from a lab which is active in gene targeting. Most ES cell lines are from the 129 inbred strain of mice, which historically was most amenable to the establishment of lines (*21*), and which may be more stable

in culture (22). There are a number of different substrains of 129, designated as "129" followed additional letters. A number of cell lines have been established more recently which derive from the embryos of a cross between 129 and a second strain of inbred mice (23-25). These F1 hybrid cell lines are generally more robust in their growth and contribution to chimeras and may come to be widely used in the future.

Our experience with a number of different ES cell lines for about two decades lead us to recommend the R1 ES cell line, a 129 ES cell line established by the Nagy lab (26). This line is robust and has maintained germ line competency in a large number of different labs, and has adapted to growth without feeder cells multiple times. The methods we describe have been optimized for the growth of R1 cells in the absence of feeder cells.

On obtaining an ES cell line, a stock of frozen vials of early passage cells should be prepared to provide cells for future targeting projects.

General

A lab equipped for tissue culture: a laminar air flow hood, 5% carbon dioxide humidified 37°C incubator, inverted microscope, tabletop clinical centrifuge, water bath, -20°C and -80°C freezers, 4°C refrigerator, a liquid nitrogen freezer which can accept freezer boxes and a source of high quality water suitable for tissue culture.

2.1 Thawing ES Cells

1. 10 mM 2-mercaptoethanol. Add 70 µl of 2-mercaptoethanol (14.3 M 2-mercaptoethanol Sigma-Aldrich M7522) to 100 ml of high quality water and filter sterilize through a 0.2 µm filter. Make 10 ml aliquots and store at 4°C.
2. Fetal Bovine Serum (FBS). Serum of different lots is tested by comparison against serum from a lot known to be satisfactory. ES cells are grown at normal (20%) and high (30%) serum

concentrations, and the lot of serum with the highest plating efficiency and growth at 30% FBS is selected. Serum is kept frozen at -80°C for long term storage, and heat-inactivated as needed. To heat-inactivate, thaw a 500 ml bottle of serum overnight at 4°C in a refrigerator. Put the bottle into a 37°C water bath such that the water comes to the level of the serum inside the bottle. Allow to thaw until only a large ice cube remains (approximately 1 hour). Heat-inactivate the serum in a water bath set at 56°C for 30 minutes, mixing occasionally. At the end of heat-inactivation, mix and aliquot the serum into 40 ml aliquots and freeze at -20°C. Do not refreeze. Some white particulate matter floating in the serum after heat-inactivation is normal.

3. ES Cell Medium: To make 100 ml of ES cell medium, combine in the order listed
 - 80 ml Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen 12440-046)
 - 20 ml heat-inactivated Fetal Bovine Serum
 - 1 ml 10 mM 2-mercaptoethanol
 - 1 ml 10 mM MEM Non-Essential Amino Acids Solution (Invitrogen 11140-050)
 - 1 ml 50 U/ml Penicillin 50 µg/ml Streptomycin (Invitrogen 15070-063)
 - 10 µl of 10⁷ U/ml leukemia inhibitory factor (LIF, Chemicon ESG1107) or recombinant protein purified from E. coli as described (27).

Store protected from light at 4°C for less than a week. Before use, warm in the tissue culture hood at room temperature for 10-20 minutes protected from light.

4. Gelatinized 60 mm tissue culture plate (Falcon 1007). Briefly cover dish with 0.1% gelatin solution (Sigma G9382, autoclaved in water) and then aspirate off. Allow to air-dry in the tissue culture hood for at least a half hour. Use within 24 hours.
5. If feeder cells are required to thaw a cell line obtained from another lab, it may be preferable to purchase them from (Chemicon/Specialty Media PMEF-N) rather than to prepare them yourself (28).

2.2 Passaging ES Cells

1. ES Cell Medium (2.1.3)
2. Gelatinized tissue culture plates (2.1.4)
3. PBS: Dulbecco's Phosphate-Buffered Saline, calcium and magnesium-free, made from powder (Invitrogen 21600-010) in high quality water, stored at 4°C, and brought to room temperature before use.
4. Trypsin: 0.25% trypsin 0.02% EDTA in Hanks' Balanced Salt Solution (JRH Biosciences 59428), stored frozen at -20°C, used freshly thawed at room temperature and not refrozen.

2.3 Freezing ES Cells From 35 mm Plates Or Larger

1. ES Cell Medium (2.1.3)
2. PBS (2.2.3)
3. Trypsin (2.2.4)
4. Cryoprotective Medium (CPM): Iscove's Modified Dulbecco's Medium (Invitrogen 12440-046) containing 10% Fetal Bovine Serum and 10% DMSO (Sigma-Aldrich D2650). Add the serum first, and mix, then add the DMSO and filter sterilize through a 0.2 µm filter. Aliquots can be stored frozen at -20°C, protected from light. Before use, thaw and bring to 4°C.
5. Cryovials: 1.8 ml screw cap round bottom tubes for liquid nitrogen storage (Nunc 363401).
6. Nalgene slow freeze container (Nalgene 5100-0001) filled with isopropanol. Replace the isopropanol after five uses.

2.4 Gene Targeting

1. ES Cell Medium (2.1.3)
2. A device suitable for electroporation of eukaryotic tissue culture cells--the models sold by Bio-Rad are widely used in gene targeting labs. The current model is the Gene Pulser Xcell electroporation device, and requires the CE (capacitance extender) module for electroporation of ES cells.
3. G418: 50 mg/ml Geneticin (G418) solution (Invitrogen 10131-019) frozen at -20°C in 1 ml aliquots. Because activity varies with lot and ES vary in their sensitivity, batches are purchased in bulk from the same lot number and the activity of the lot is determined empirically. Mock electroporated (electroporation without DNA) cells are plated and subjected to G418 selection at different concentrations for seven to ten days. The minimum concentration which yields 100% killing in seven to ten days is chosen. For R1 ES cells grown in IMDM without feeders, a final concentration of 100 µg/ml has proven the optimal concentration for most lots of G418.
4. FIAU: 1-2'-deoxy-2'-fluoro-β-D-arabinofuranosyl-5-iodouracil (Moravek Biochemicals M-251). To make a 100 mM stock, add 388 mg of FIAU to 9 ml of PBS and add NaOH until the FIAU has dissolved. Make total volume to 10 ml. Dilute in PBS to give a 200 µM (1000X) stock. Aliquot and store at -20°C. The working concentration in ES cell medium is 0.2 µM FIAU.
5. 500 µg purified, linearized targeting vector: purify the targeting vector using a maxi-prep protocol, for example a Qiagen kit protocol. Cut the vector to completion with the appropriate restriction enzyme, purify by phenol/chloroform and chloroform extraction and ethanol precipitation. Dissolve the purified, linearized plasmid in low EDTA TE (10 mM Tris pH 8.5, 0.1 mM EDTA) at a concentration of 2 µg/µl.
6. 0.1% (w/v) erythrosin B (Sigma-Aldrich E-7379) in PBS.
7. Hemacytometer/counting chamber (Fisher 02-671-10).
8. Gelatinized 100 mm tissue culture plates (Falcon 3003).

9. Methylene blue/basic fuchsin: 0.33% (w/v) methylene blue (Fisher M291), 0.11% (w/v) basic fuchsin (Fisher F98) in methanol.

2.5 Picking Colonies Into 96 Well Plates

1. ES cell medium (2.1.3)
2. G418 (2.4.3)
3. Gelatinized 96 well flat bottom tissue culture plates (Falcon 3072).

2.6 Tryplating 96 Well Plates

1. ES cell medium (2.1.3)
2. G418 (2.4.3)
3. PBS (2.2.3)
4. Trypsin (2.2.4)
5. 8 well multichannel pipettor, adjustable, 10 μ l maximum volume
6. 8 well multichannel pipettor, adjustable, 50 μ l maximum volume
7. 8 well multichannel pipettor, adjustable, 300 μ l maximum volume
8. 8 well multichannel aspirator (Inotech IV-596)

2.7 Splitting ES Cells For DNA And Freezing In 96 Well Plates

1. U bottom polypropylene 96 well plates (ABgene AB-0796) and sealing mat lids (ABgene AB-0674).

2. 2X freezing medium: Iscove's Modified Dulbecco's Medium containing 20% fetal bovine serum and 20% DMSO (Sigma-Aldrich D2650)—made fresh and placed on ice.
3. Mineral oil, mouse embryo tested (Sigma-Aldrich M8410).

2.8 Preparing Genomic DNA From ES Cells Grown In 96 Well Plates

1. 96 well plate lysis buffer: 10 mM Tris-HCL pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% Sarkosyl, 1 mg/ml proteinase K
2. Plastic wrap
3. Humidified container: a plastic container large enough to hold the 96 well plates with wet paper towels on the bottom
4. 55°C incubator
5. ethanol + salt made fresh: combine 10 ml of 95% ethanol and 0.15 ml of 5 M NaCl stock, put on ice
6. Paper towels
7. 70% EtOH
8. Low EDTA TE: 10 mM Tris pH 8.4, 0.1 mM EDTA

2.9 Digestion of Genomic DNA In 96 Well Plates For Southern Blot Analysis

1. Restriction enzyme cocktail:

(genomic DNA in low TE in well	20 μ l)
10X restriction enzyme buffer	4 μ l
Water	to bring total volume to 40 μ l

100 mg/ml BSA	0.4 μ l
50 mM Spermidine	0.8 μ l
10 mg/ml DNase-free RNase A	0.2 μ l
Restriction enzyme	20-40 Units

2. Humidified container.
3. 37°C oven.

2.10 Recovering Candidate Targeted Clones And Verifying Homologous Recombination

1. 37°C water bath
2. Gelatinized 24 well plates (Falcon 3047).

2.11 Extracting Genomic DNA From ES Cells In Quantity

1. Large Scale Lysis Buffer: 200 mM NaCl, 100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS,
100 μ g/ml proteinase K
2. Nutator (Becton Dickinson 421105)
3. Orbital shaker

2.12 Digestion Of High Molecular Weight DNA

1. Restriction Enzyme Cocktail
(Genomic DNA in low EDTA TE 10 μ l)

10X Restriction Enzyme	20 μ l
Buffer	
Water	to bring volume to 200 μ l total
10 mg/ml BSA	2.0 μ l
50 mM Spermidine	4 μ l
High Concentration Restriction Enzyme	20 to 100 Units

2. 5 M NaCl
3. Microcentrifuge
4. 70% and 95% EtOH.

2.13 Counting ES Cell Chromosomes

1. ES cell medium (2.1.3)
2. Colcemid: 3.125 μ l of 10 μ g/ml colcemid (Invitrogen 15212-012) per ml of medium
3. 37°C water bath
4. 0.075 M KCl warmed to 37°C.
5. Methanol/Acetic Acid Fix: 2 ml glacial acetic acid and 5 ml methanol, prepared fresh.
6. Microscope slides: plain, pre-cleaned slides (Fisher 12-549) labeled with etching tool
7. Coplin jar (Fisher 08-813)
8. Giemsa: 2.5 ml of Giemsa solution (Invitrogen 10092-013) in 47.5 ml of Gurr's pH 6.8 buffer (Invitrogen 10582-013)
9. Glass coverslips
10. Permount® mounting medium (Fisher SP15-100)
11. Compound microscope with 40X and 100X objectives

2.14 Transient Transfection Of ES Cells With Cre Recombinase To Excise The Neo Cassette

1. Supercoiled pOG231 plasmid (**29**) for the expression of Cre Recombinase, 25 µg per cell line.
2. ES cell medium (2.1.3)
3. Gelatinized 100 mm plates.

3. Methods

ES cells lose their ability to colonize the germ line with increased time in culture. Because the only reliable means of testing germ line competency is by constructing and breeding chimeras, and because gene targeted cell lines represent a large investment of time and money, great care must be taken in the culture of the ES cells to avoid subjecting the cells to less than optimal culture conditions. Avoid overgrowth by passaging ES cells before they become confluent, change media frequently, and keep the total passage number as low as possible.

ES cells grow as a colony of cells tightly apposed to each other through extensive cell-cell contacts. When ES cells differentiate, they attach and spread on the surface of the tissue culture plate. Differentiated cells have lost the stem cell character, cannot be propagated, and will not colonize the germ line of chimeric mice.

3.1. Thawing ES Cells

1. Loosen the cap of the vial and quickly warm in a 37°C water bath (*see Note 1*). Before all the ice has disappeared, transfer the cells to a tube containing 8 ml of ES cell media and gently invert the tube a few times to mix.
2. Recover the cells by centrifugation at 300 g for 5 minutes.

3. Promptly resuspend the cells in 3.5 ml of ES cell medium and plate on a 60 mm gelatinized tissue culture plate.

3.2 Passaging ES cells

1. Replace the medium daily, and passage the cells every second day. ES cells are split typically into 6 equivalent plates (*see Table 1* for plate sizes, medium and trypsin volumes). Aspirate the medium from the plate.
2. Add an equal or greater volume of PBS to the plate.
3. Aspirate the PBS from the plate.
4. Add trypsin and place the plates in the incubator for 5 minutes.
5. Using a plugged Pasteur pipet with a bulb, vigorously pipette each plate in order to break the colonies up into single cells. With a circular stream, pipet all around the dish, avoiding bubbles as much as possible. The goal is an even suspension of single cells. Examine the plates on the microscope to see that the colonies are well broken up and mostly single cells (*see Note 2*).
6. Transfer the suspension of cells to a tube with an equal volume of medium. Draw the suspension up and down in the pipet three times to mix.
7. Centrifuge at 300 g for 5 minutes.
8. Promptly aspirate the medium and thoroughly disperse the cells in the appropriate amount of ES cell medium.
9. Gently add the cells to gelatinized plates, splitting the original plate onto 6 equivalent plates (*see Note 3*).

Table 1: Culture Plate Size, Area And Volumes

<u>Plate Size</u>	<u>Surface Area (cm²)</u>	<u>Medium Volume (ml)</u>	<u>Trypsin Volume (ml)</u>
96 well	0.2/well	0.2 - 0.3/well	
24 well	2/well	0.5/well	
35 mm	9.5	1.5 - 2.0	0.7 - 1
60 mm	21	3.5	1.5 - 2
100 mm	56	10	3 - 4

3.3 Freezing ES Cells From 35 mm Plates or Larger

1. Four hours before freezing, replace the medium on the cells with fresh medium and place them back in the incubator (*see Note 4*).
2. Bring a sufficient volume of cryoprotective medium (CPM) and the slow cool freezing container to 0-4°C on ice (*see Note 5*).
3. Trypsinize the cells exactly as for passaging cells in the previous protocol (3.2), and add the cells in trypsin solution to a tube with an equal volume of ES cell culture medium.
4. Pellet the cells by centrifugation at 300 g for 5 minutes.
5. Promptly remove the supernatant and resuspend the cells in ice cold CPM, using 1.5 ml CPM for each 35 mm confluent plate equivalent. Add the cells to labeled vials on ice.
6. When all vials are ready to freeze, place them in the prechilled Nalgene slow cool freezing container.
7. Promptly place the slow cool freezing container in a -70°C freezer, and leave in the freezer for at least 24 hours.
8. Transfer the vials to liquid nitrogen storage.

3.4 Gene Targeting

1. Thaw a vial of early pass ES cells onto a 60 mm plate as described in the preceding protocol (3.1, *see Note 6*).
2. On the second day, replace the medium.
3. On the third day, passage the plate onto two 100 mm plates (a 1 to 6 split) as described above (3.2).
4. On the fourth day, replace the medium.
5. On the fifth day, passage the cells (3.2), splitting them 1 to 2.
6. Early on the sixth day, replace the medium.
7. Four hours later, remove the medium, wash the cells with PBS, and trypsinize each plate with 3 to 4 ml of fresh trypsin as described above to obtain a single cell suspension, mix with an equal volume of medium. Recover the cells by centrifugation at 300 g for 5 minutes, and promptly and gently resuspend the cells in 1 ml of cold PBS for each plate of cells. Pool the cells, storing on ice.
8. Remove 0.1 ml of cells and add to 0.9 ml of 0.1% erythrosin B in PBS. Mix, and apply a small volume to the assembled hemacytometer and count the number of cells. Adjust the concentration of the pooled cells to 7×10^6 cells/ml by adding cold PBS.
9. There should be enough cells for 6 to 12 electroporation cuvettes containing 0.8 ml of cell suspension each. Place the cuvettes on ice, and add 0.8 ml of cells and 40 μ g of linearized vector to each.
10. Electroporate at 240 V, 500 μ F, and place the cuvettes on ice for 20 minutes.
11. Using a plugged Pasteur pipet, gently resuspend the cells of each cuvette and pool with 1.5 ml of prewarmed medium per cuvette. Wash each cuvette with 1 ml of medium and add to the pool, and mix.

12. Mark a single 100 mm gelatinized plate to monitor transfection efficiency, then add 0.3 ml of the cell suspension to 9.7 ml of medium in the dish: this plate will be used for monitoring transfection efficiency as the 1/10th quantitation plate. For the remainder of the plates, add 3 ml of cells to 7 ml medium in each 100 mm plate.
13. Approximately 24 hours after the cells were plated out, start the drug selection by replacing the medium with medium with G418 and FIAU together, or G418 alone for the plate used to monitor transfection efficiency.
14. Feed the cells daily with media supplemented with the appropriate fresh drugs. Most colonies will grow, die, and a small number of colonies will survive. For a brief period of time it may appear that nothing has survived under double selection, but after 7 to 10 days of selection colonies should be clearly visible.
15. After 7 to 10 days of selection, when colonies are visible to the naked eye as specks on the plate and debris has been cleared from the plates, feed the cells every other day with medium containing only G418 (no FIAU).
16. Pick cells (see the next protocol, 3.5) at 10 to 14 days, depending on the rate of growth. When the plate for monitoring transfection efficiency has grown clear colonies, remove the medium from the plate, wash with PBS, fix/stain with methylene blue/basic fuchsin for 5 minutes, wash with water and air dry (*see Note 7*).

3.5 Picking Colonies Into 96 Well Plates

1. For each 100 mm plate, prepare a 96 well gelatinized plate with 180 μ l/well of medium with G418. Warm the 96 well plates for 10 to 15 minutes in the incubator (*see Note 8*).
2. Remove one 100 mm plate and one 96 well plate from the incubator. Using an inverted or stereo microscope, pick each colony into one well of the 96 well plate, and mark the well with an air

bubble. To pick a colony, use a small volume (20 or 10 μ l maximum volume) pipettor with a yellow tip set at 10 μ l and dislodge and pick up an intact colony. Place the pipet tip in the corner of the well of the 96 well plate, and draw the colony up and down in the pipet tip to break the colony into 3 to 5 pieces.

3. Use a fresh tip for the each colony. Repeat for 10 to 20 colonies from the same plate, then place the paired 100 mm dish and 96 well plate into the incubator and pick another plate for 10 to 20 colonies.
4. Place the picked cell lines in the incubator, and discard any 100 mm plates which have been completely picked.
5. Leave the plates undisturbed in the incubator for about 48 hours.

3.6 Tryplating 96 Well Plates

1. Aspirate the medium from a 96 well plate using a multichannel aspirator (usually the clumps of cells will collect on one side of all the wells, and the aspirator tips can be inserted on the opposite side, and there will be no need to change tips between columns) (*see Note 9*).
2. Add 180 μ l of PBS to each well with a multichannel pipet, and aspirate.
3. Add 50 μ l of fresh trypsin to each well, and place the plate in the incubator for 5 minutes.
4. Stop the trypsin digestion by adding 180 μ l of medium to each well. The chunks of colonies should be floating.
5. With a multichannel pipet set at 150 μ l, disperse the cells to a single cell suspension by vigorously drawing the cells of a column up and down five or six times. Change the tips for each column.
6. Examine the plate carefully to ensure that a single cell suspension has been achieved. If cells are not completely dispersed, continue to triturate the cells until dispersed. If a single cell suspension

cannot be obtained by trituration, let the cells attach and repeat the tryplating procedure the following day. Place the plates in the incubator.

7. Early the next day, replace the medium on the cells. Grow the cells for 3 to 5 days with daily changes of medium containing G418 until the cells are confluent.

3.7 Splitting ES Cells For DNA And Freezing In 96 Well Plates

1. Replace the medium on the cells with fresh medium without G418 two to four hours before trypsinizing the cells (*see Note 10*). Prepare the plates as described in the next item while the cells are feeding.
2. Prepare two gelatinized 96 well plates, and one freezing plate for each of the original plates. In the gelatinized plates, place 100 μ l of medium without G418 in each well and place these plates in the incubator. To each well of the U-bottom polypropylene freezing plates, add 30 μ l of 2X freezing medium. Overlay each well with 100 μ l of mineral oil. Put the labeled mat cap on loosely and place the plate on ice protected from light.
3. Remove the fed cells from the incubator and aspirate the medium from the wells.
4. Wash each well of the plate once with 100 μ l of PBS.
5. Add 25 μ l of fresh trypsin to each well. Place the plates in the incubator for 5-10 min. Gently agitate the plates. The colonies should have detached from the plate.
6. Apply 25 μ l of medium per well to stop the trypsinization. From this point on, work with two plates at a time.
7. Put the appropriate pair of labeled freezing plates on a shallow tray with flat compacted ice. Set the multichannel pipettor to 30 μ l and vigorously break up a column of colonies. Transfer 30 μ l to the appropriate column of the freezing plate, stabbing under the oil and mixing gently by drawing up and down 3 times. Repeat using fresh tips for each column. Fully place the matt cap

on the freezing plate and return it to ice. Process the second plate. When both freezing plates are finished, transfer both sealed freezing plates to a styrofoam box and place the box inside the -80°C freezer. Place the original 96 well plates (which have 20 µl/well remaining) back in the incubator.

8. Repeat the processing steps until all the plates are in the -80°C freezer.
9. Now return to the original plates and add 80 µl of medium to each well. Return them to the incubator temporarily.
10. Remove an original plate and two gelatinized 96 well plates to split it into (Do not attempt to do more than one original plate at a time).
11. Gently mix the original plate and transfer 50 µl of suspension to each replicate, mixing gently each time. The replicates should now have all the cells. Return the replicates to the incubator. Discard the original. Repeat for each original plate. The replicate plates for DNA can usually be ignored the next day, fed the second day and fed every day thereafter (without drug selection).
12. After the freezing plates have been in the -80°C freezer for 2 days, they can be transferred to liquid nitrogen. Wrap each plate in aluminum foil (so that the lid will not float off in the liquid nitrogen) and place on dry ice for transport to the liquid nitrogen freezer. Two capped plates can fit into a large freezer box.

3.8 Preparing Genomic DNA From ES Cells Grown In 96 Well Plates

1. Grow the cells to very high density, changing medium (without G418) daily. When the cells are at sufficiently high density, the media will have turned yellow daily for four to five consecutive days (*see Note 11*).
2. Aspirate the medium from each well, and wash each well with 180 µl PBS.
3. Aspirate the PBS from each well, and wash again with 180 µl PBS per well.

4. Add 50 μ l of 96 well lysis buffer to each well, and cover the plate with plastic wrap followed by the lid. Stack plates and wrap the stack with plastic wrap. Place the stack in a humidified container in a oven at 55°C overnight.
5. Add 100 μ l of ethanol + salt to each well **without mixing**. Put the lid back on.
6. Leave the plates undisturbed at room temperature for 30-60 minutes, preferably on a dark background to visualize the DNA. The DNA should become visible as a white filamentous network or a white sheet.
7. Drain the plates by gently inverting the plates onto a stack of paper towels.
8. Wash gently three times with 150 μ l of 70% EtOH per well, inverting gently each time onto fresh paper towels.
9. After the last wash, make certain that all the ethanol has drained, then air dry the plates tilted upside down for 20 minutes at room temperature. Do not over-dry the plates as this makes the DNA hard to dissolve.
10. Add 20 μ l of low EDTA TE to each well and cover the plate with plastic wrap. Put the lid back on and stack the dishes, then wrap the stack in plastic wrap. Store overnight at 4°C. The DNA is stable for several months at 4°C.

3.9 Digestion of Genomic DNA In 96 Well Plates For Southern Blot Analysis

1. Add 20 μ l of the digestion cocktail to each well. Stir the column of wells with the tips of the multichannel pipettor, and slowly and gently draw the mixture up and down once or twice. Take note of the degree of viscosity of the DNA, because the viscosity will be lost when the DNA is cut, so it can be a helpful sign that digestion has occurred (*see Note 12*).
2. Seal each plate with plastic wrap and put the lids on. Stack several plates and wrap the stack with plastic wrap. Place in a humidified container and place in a 37°C oven overnight.

3. The next day, check the state of digestion of a few wells by drawing them up in a yellow pipet tip and seeing that they drip down, and that the viscosity is lost. If they seem too viscous add more enzyme up to but not exceeding a volume of 4 μ l of enzyme per well when all enzyme additions are considered. Alternatively, run a few microliters from a row on a mini-gel to see if the DNA forms the continuous smear indicative of a complete digest (*see Note 13*).

3.10 Recovering Candidate Targeted Clones And Verifying Homologous Recombination

1. Set up a 37°C water bath for thawing 96 well plates by stacking empty yellow tip racks such that there are only a few millimeters of warm water above the rack (you don't want the water to be so high that it seeps into the lid--practice with an old plate). Prepare sufficient gelatinized 24 well plates to accept all the candidate lines and a few extra lines as negative controls. Add 0.5 ml of medium (without G418) to each well of the 24 well plate (*see Note 14*).
2. Work with one plate at a time. Put the plate on the rack in the 37°C water bath. To keep it in close contact with the water put a weight on it. It will take 8 to 10 minutes for the plate to thaw.
3. Once you can see that the majority of clones have thawed (the wells will be translucent) remove the dish from the water bath. Place the plate on top of 70% EtOH-soaked tissues and wipe dry all around the top and edges.
4. Clearly mark all your positive clones. Also mark one or two non-targeted lines to serve as negative controls.
5. Using a 200 μ l pipettor set at 105 μ l, stab under the oil and gently stir to mix. Draw up the entire volume of medium plus a little oil. Place the cells in a well of the 24 well. Draw medium up and down in the tip to disperse cells evenly and remove 105 μ l of the 24-well and put it back under the oil of the well of the 96 well plate the cells came from. Draw up repeatedly to mix and get most of the cells. Return this to the 24-well. Repeat with each cell line you wish to recover.

6. Culture at 37°C.
7. Replace the medium on the cells daily. The growth of the individual cell lines will vary. Those lines which are growing well can be passaged to a 35 mm dish (a 1 to 5 split). Those lines which are growing more slowly can be passaged to one or two wells of a 24 well plate. Passage all lines by the fourth day.
8. After each line is established in a 35 mm plate, the next split is into two 60 mm plates and two 35 mm plates. The 60 mm plates will be used to freeze the line into four vials (protocol 2.3), one 35 mm plate will be used to prepare DNA (protocol 3.11), and one 35 mm plate will be used to obtain a chromosome count (protocol 2.13, pausing at step 7 until targeting has been confirmed).

3.11 Extracting Genomic DNA From ES Cells In Quantity

1. Grow the cells until they are confluent (*see Note 16*).
2. Aspirate medium and wash twice with PBS.
3. Add Large Scale Lysis Buffer using the volumes listed in **Table 2**.
4. Wrap the dishes in plastic wrap and enclose inside a humidified container. Incubate overnight at 55°C.
5. For 24 well plates, the precipitation can be done in the dish. For larger plates, transfer the lysate to either a 15 ml (35 or 60 mm) or a 50 ml (100 mm) conical capped plastic tube, using a cut blue tip or large bore pipet.
6. Add an equal volume of isopropanol to the dish or tube without making an effort to mix.
7. Cover the 24 well plates with plastic wrap and put lids on. Tape the dish to an orbital shaker, and start the shaker at low speed and gradually turning up until going the fastest possible without spilling. The tubes are mixed on a rocking or rotating mixer like a nutator.

8. Mix for at least 20 min at room temperature. A large white "jellyfish" of precipitated DNA will begin to appear at the interface of the alcohol with the lysate. The longer you rotate the tighter and easier to manage the "jellyfish" will become.
9. Using a pasteur pipet, carefully remove the fluid leaving the DNA precipitate behind. Add an equal volume of 70% EtOH and rotate for another 20 minutes at room temperature.
10. Lift the precipitate out with a clean pipet tip. Put the DNA into a tube with the appropriate amount of low EDTA TE (*see Table 2*). Sometimes the precipitate sticks to the tip. Carefully scrape the DNA off on the side of tube.
11. Incubate the tubes at 50 to 60°C for 30 minutes with lids off to evaporate trace ethanol.
12. Place the tubes in a refrigerator at 4°C overnight or longer before use. The DNA can be stored for months at 4°C.

Table 2: Volumes for DNA Digestion by Plate Size

<u>Dish Size</u>	<u>Lysis Buffer Volume (ml)</u>	<u>Low EDTA TE Volume (ml)</u>
One well of a 24 well plate	0.5	0.05
35 mm	2.0	0.2
60 mm	4.0	0.4
100 mm	10.0	1.0

3.12 Digestion Of High Molecular Weight DNA

1. Assemble a 200 µl digestion reaction for each sample and incubate overnight at the temperature appropriate for the restriction enzyme.

2. Check to see if the DNA is still viscous by drawing it up with a pipettor and dripping it back down into the tube drop-wise. If the DNA is still viscous, add more enzyme, keeping the total enzyme added to less than 10% of the total volume and continue incubating. Once you are satisfied that the DNA is cut, proceed to the next step.
3. To each reaction add 8.3 μ l of 5 M NaCl and mix.
4. Add 530 μ l 95% EtOH and precipitate 20 minutes at -20°C .
5. Spin down 10 minutes at top speed in a microcentrifuge and remove the supernatant.
6. Wash the pellet with 530 μ l of 70% EtOH and spin down 5 minutes.
7. Remove supernatant.
8. Dry the pellet by placing the open tube in a dry bath at 37°C for 5 min.
9. Dissolve the DNA pellet in 36 μ l of TE, and add 4 μ l of 10X gel loading dye.
10. Load the entire reaction in a well of a 0.7%/0.5X TBE gel and perform Southern blot analysis.

3.13 Counting ES Cell Chromosomes

1. Culture cells for 24 hours after they have been passaged onto a 35 mm gelatinized plate.
2. Culture for four hours in medium containing colcemid (*see Note 17*).
3. Remove the medium from the plate and transfer it to a 15 ml conical capped tube. Wash the plate with 2 ml PBS, and add the wash to the tube. Trypsinize the plate with 1.5 ml trypsin, and add the cell suspension to the tube and mix.
4. Pellet the cells by centrifugation at 300 g for 5 minutes. Aspirate the supernatant and flick the tube to disperse the cells in the residual supernatant.
5. Add 37°C 0.075 M KCl drop by drop, flicking the tube to mix the cells with each drop for the first 10-15 drops. Bring the total volume up to 7 ml with 0.075 M KCl, invert several times, and incubate in the 37°C water bath for 10 minutes.

6. Centrifuge at 300 g for 5 minutes.
7. Aspirate the supernatant and flick the tube to loosen the pellet. In the next step, be careful to disperse the cells thoroughly in the fix. Add fresh methanol/acetic acid fix drop by drop, flicking the pellet with every drop for the first 10 to 15 drops, bringing the total volume to 7 ml. Cap and incubate at 4°C overnight. The fixed cells can be stored at 4°C indefinitely.
8. Centrifuge at 300 g for 5 minutes and aspirate the old fix. Resuspend the cells in 0.5 to 0.75 ml of fresh methanol/acetic acid fix.
9. Dip labeled glass slides in water, leaving a puddle of water on the slide. Gently draw the cell suspension up in a pasteur pipet. Each tube will have enough cells to make 4 to 5 slides.
10. From a height of about 1 foot above the slide, drop 3 drops of cell suspension per slide.
11. Leave the slides to air dry.
12. Stain the slides for 10 minutes with 50 ml of Giemsa in a Coplin jar.
13. Rinse with water 3-4 times until the excess stain is removed.
14. Thoroughly air dry the slides.
15. Mount glass coverslips with Permount.
16. After the Permount has dried, count the chromosomes in the metaphase spreads of widely separated fields. Scan slides with a 40X objective and count the chromosomes with a 100X objective. Five metaphase spreads on each of 4 different slides gives a good sample. A cell line with a majority of metaphases with 40 chromosomes is a good candidate for germ line transmission (*see Note 18*).

3.14 Transient Transfection Of ES Cells With Cre Recombinase To Excise The Neo Cassette

1. Thaw (protocol 3.1) and grow (protocol 3.2) a targeted line which has been thoroughly characterized by Southern blot analysis and has been shown to have 40 chromosomes (protocol 3.13) (*see Note 19*).
2. Electroporate one cuvette of cells (protocol 3.4, steps 1-10) with 25 μ g supercoiled pOG231 Cre expression plasmid, resuspending the cells in 10 ml of ES cell medium (*see Note 20*).
3. From the 10 ml of electroporated cells, plate out 0.1 ml, 0.250 ml and 0.5 ml into 10 ml of medium each on gelatinized 100 mm plates.
4. Allow the cells to grow in ES cell medium, changing the medium every second day, for 10 to 14 days in the tissue culture incubator.
5. Select a plate with well separated colonies, and pick (protocol 3.5) about 96 colonies into a 96 well plate.
6. Tryplate (protocol 3.6) and propagate the lines in the 96 well plate and split (protocol 3.7) to freeze and prepare DNA.
7. Prepare DNA from the 96 well plates (protocol 3.8), analyze by Southern blot or PCR, and retrieve the desired cell lines from the frozen plate (protocol 3.10) (*see Notes 21 and 22*).

4. Notes

1. Frozen cells need to be thawed rapidly and removed quickly from the freezing medium. Thawed cells should be returned to the culture conditions they experienced before freezing, so if a frozen cell line is obtained from a commercial source or another lab, use the culture conditions (media, supplements and feeder cells) the cells are accustomed to before attempting to passage them into new conditions, for example, growth without feeder cells.

2. ES cells grow as clonal colonies. In passaging ES cells it is important to disperse the colonies down to single cells so that growth of the culture is even and rapid, and so that differentiation is prevented.
3. For routine splitting into 60mm or 100mm dishes, add a bolus of concentrated cells to one side of a dish that is covered with medium. Resuspend the cells in a convenient volume of medium--say 0.5 or 1.0 ml for each plate. Since the cells are coming out of a stressful spin, be gentle. Mix cells by running them along the side of the tube. When thoroughly resuspended, apply the cells to one side of the receiving dish and gently rock the plate back and forth so that a wave front goes from side to side. When first starting out, do only one plate at a time and keep the dish down on the table--eventually you can do three or four plates by rocking them gently in the air. Do not swirl the dishes as this concentrates the cells in the middle. 35 mm dishes (and smaller) are too small to rock back and forth--gently resuspend cells with their full volume of medium and apply to the dish. Incubate plates overnight and evaluate your technique the next morning. If there are large numbers of clumps, or cells are not evenly distributed, then repeat the passaging onto fresh plates. ES cells have a plating efficiency of only about 20%, therefore expect a lot of floating debris the day after plating.
4. ES cells are slowly frozen in a cryoprotective medium (CPM) in cryovials and stored indefinitely in liquid nitrogen, or stored for less than 6 months at -70°C . Each vial contains the equivalent of one confluent 35 mm dish (or 2 vials per 60 mm dish and 6 vials per 100 mm dish).
5. A cold Styrofoam box with a lid can be used in place of the Nalgene slow cool freezing container.
6. A total of about 400 μg of linearized targeting vector is electroporated into 6×10^7 ES cells (about 5 dense 100 mm plates of ES cells) in several electroporations. A day after plating the cells, drug selection is started. Several hundred colonies should survive the double selection.

7. The expected outcome is that each plate under double selection will have about 40 colonies, and the 1/10th quantitation plate under G418 selection alone will have about 50 colonies, representing a 12.5-fold enrichment ($50 \times 10 / 40 = 12.5$). A low transfection efficiency indicated by a substantially lower number of colonies on the 1/10th quantitation plate indicate that the neo gene is either defective, or the context of the targeting arms do not allow for efficient expression of the neo gene. A low transfection efficiency may need to be corrected by construction of a new targeting vector. Enrichments which are too high (2-fold) or too low (100-fold) can indicate that something is awry with the selection. Although extremes in the enrichment ratio can still result in gene targeting, most often a fresh round of electroporations will result in a more typical enrichment ratio and bona fide gene targeting events. Targeting frequencies in the enriched cells surviving double selection typically ranges from 1/10 to 1/100. Therefore to ensure that several targeted cell lines are recovered, several hundred colonies are picked, cell lines are grown and screened for gene targeting. If thorough analysis of several hundred cell lines fails to identify targeted cell lines, and the transfection and enrichment are close to the expected values, consider building a new targeting vector with a greater length of sequence match or with neo in a different position. The targeting frequency of a targeting vector is reproducible. Low frequencies may be due to a failure for homologous recombination to occur, or poor expression of neo from the targeted locus.
8. Colonies which survived the double selection are propagated as individual cell lines. Several hundred lines of cells should be analyzed to ensure that several gene targeted cell lines can be identified. Each colony is used to establish a cell line propagated in a well of a 96 well plate. It takes about an hour for an experienced individual to pick a full 96 well plate.
9. The key to propagating cells in 96 well plates is ensuring comparable growth rates in all wells by dispersing colonies to individual cells by the technique of tryplating about 48 hours after picking

colonies. Tryplating disperses the roughly broken colonies from picking into a single cell suspension. The cells are allowed to attach to the same plate.

10. The cell lines in 96 well plates are split three ways into one freezing plate and two DNA plates. Analysis of the DNA is used to retrieve targeted cell lines from the frozen copy, so be sure to clearly mark the plates so that the correct cell lines can be retrieved. G418 selection is removed just before splitting.
11. In order to obtain enough DNA from each cell line, and because the cells in these replicates won't be used to make chimeras, the DNA replicate plates are grown to very high density. If the plates and their lids are not all clearly marked in order that sib cell lines in the freezer can be easily identified, do so now. The DNA preparation protocol involves lysis, digestion, DNA precipitation and washing in situ in the plate the cells grew in.
12. The genomic DNA of the ES cell lines is digested in the wells of the plate. Each plate should have enough DNA for two Southern blots. Typically, only one external probe is used to identify candidate cell lines from this round of Southern blot analysis. The strategy is to narrow down the cell lines to a smaller number which can be thoroughly analyzed and to freeze the lines in a format where there is near certainty that they can be reestablished after thawing. We do not provide a detailed protocol for performing Southern blots, as there are many good descriptions to be found elsewhere, and there are no exceptional requirements for the analysis of ES cell genomic DNA other than that a large number of samples (several hundred) will be analyzed. Thus it is useful to have large gel boxes which can accommodate multiple combs with many wells, for example 12 x 8 inch gels with three combs with 36 well combs (thus an entire plate can be loaded onto a gel with 4 x 8 columns = 32 samples with room for markers on each third of the gel).

13. Once the Southern blot analysis is complete, and the films have been exposed long enough to identify all possible candidate targeted clones, thaw and recover the candidate cell lines from the frozen 96 well plates as soon as possible.
14. The cells are grown without selection from the recovery from freezing in the 96 well plate to freezing again in vials. The cells grow faster in the absence of selection and this improves the chances of recovering all lines. Cells are also grown without selection after thawing from the vials up to injection into blastocysts, since there are data which indicate that growth in G418 is detrimental to the chimeras. However, long term growth without selection can lead to loss of the targeted allele in some or all of the cells of a line. If cells are propagated in culture beyond the number of passes indicated here, it would be best to grow them under G418 selection at least for one passage to kill any cells which have lost the targeted allele, or which survived the original selection.
15. The yield of DNA prepared by this protocol is about 0.5 $\mu\text{g}/\mu\text{l}$. For analysis by PCR, make a 1:5 dilution with water and store frozen at -20°C . Use 1 μl of the diluted DNA for each PCR reaction. For analysis by Southern blot, cut 10 μl of undiluted DNA in a 200 μl reaction as described in Protocol 3.12.
16. Since the cells on these DNA plates will not be propagated further, it is OK to let them grow to a density beyond which would be desirable for cells which would be used to make chimeras. Thus, if multiple cell lines are progressing at different rates, the fast growers can be allowed to grow very dense until the slow growers are at sufficient density such that all dishes can be processed at one time.
17. Cells are grown in the presence of colcemid to arrest them at metaphase. The colcemid treatment causes cells to detach from the plate, so the medium from the cells is collected from the plates after the colcemid treatment, in addition to the cells released by trypsin treatment.

18. A normal number (40) of chromosomes is not a guarantee that a cell line is euploid, but identifying cell lines with a normal number of chromosomes should greatly enrich for euploid cell lines. Two or three targeted cell lines with 40 chromosomes should be used to generate chimeras, in order that the probability of germ line transmission is high.
19. For the construction of conditional mutants and knockins, the neo cassette can be removed in ES cells by transiently transfecting a targeted line which has a normal number of chromosomes with an expression plasmid for Cre recombinase. Although the neo cassette could be removed in mice by crossing to mice transgenic for Cre recombinase expressed in the germ line or early embryo, there are advantages to removing the neo cassette early. There is no need to maintain an additional line of mice, and because the neo cassette has cryptic splice acceptors and donors, the possibility that the targeted allele has an unintended gain-of-function activity which might prevent colonization of the embryo or germ line is avoided.
20. ES cells are transiently transfected by electroporation with supercoiled expression plasmid for Cre recombinase. Because transient transfection and recombination driven by Cre are efficient, no selection is necessary to find the desired recombinant. In those instances where there are three loxP sites at the targeted locus, recombinants between each pair of loxP sites (in addition to nonrecombinants) are recovered.
21. The typical result is that 30 to 50% of the cell lines have undergone a Cre-mediated recombination. In the case of conditional alleles, amongst those lines which have undergone recombination, some proportion will have undergone recombination only between the two loxP sites flanking neo, leaving loxP sites flanking the sequence to be conditionally deleted in mice.
22. Chimeras made by injection of ES cells into host embryos are mosaics of the heterozygous mutant cells, and the host embryo cells. The contribution of the ES cells to the chimera is reflected by the coat color of the chimera in most cases, with the ES cells contributing brown (agouti) hairs, and the host black (recessive nonagouti, the mutated version of agouti). Most ES

cells used for targeting are male, and the male ES cells will co-opt most female host embryos into participating in male development through the nonautonomous action of the hormone testosterone. Male chimeras of the brown/black coat color combination are mated to black mice like C57BL6. Both brown and black offspring are possible, and the brown offspring indicate that a sperm derived from an ES cell fertilized the C57BL6 egg (agouti over recessive nonagouti, so a brown mouse). Because sperm are haploid and the ES cells are heterozygous, a brown pup could be either heterozygous mutant or wild type for the targeted gene. Thus, the brown offspring need to be analyzed for their genotype at the targeted locus, preferably by one of the Southern blot assays developed by targeting. Once a few heterozygous founder mice are obtained, a PCR assay for the allele can be developed with confidence. The best PCR assays identify a unique DNA junction specific to the mutant. Heterozygous mutant offspring of the chimeras can be bred to each other to obtain homozygotes for analysis. Quite often, the heterozygous mutants are bred for nine or more generations to C57BL6 mice to place them fully on the standard inbred genetic background to minimize the effect of segregation of other gene variants.

References

1. Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* **78**, 7634-8.
2. Evans, M. J., and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-6.
3. Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255-6.
4. Thomas, K. R., and Capecchi, M. R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503-12.

5. Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S., and Smithies, O. (1987) Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330**, 576-8.
6. Davis, J. (Ed.) (2002) *Basic cell culture: a practical approach*. Oxford University Press, Oxford University Press, Oxford.
7. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulating the mouse embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
8. Hasty, P., Abuin, A., and Bradley, A. (2000) Gene targeting, principles, and practice in mammalian cells, in *Gene Targeting. A Practical Approach*. (Joyner, A. L., Ed.), Vol. 212, Oxford University Press, Oxford, pp. 1-35.
9. Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B., and Joyner, A. L. (1995) Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. *Science* **269**, 679-82.
10. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103-6.
11. te Riele, H., Maandag, E. R., and Berns, A. (1992) Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc Natl Acad Sci U S A* **89**, 5128-32.
12. Hasty, P., Rivera-Perez, J., and Bradley, A. (1991) The length of homology required for gene targeting in embryonic stem cells. *Mol Cell Biol* **11**, 5586-91.
13. Meyers, E. N., Lewandoski, M., and Martin, G. R. (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat Genet* **18**, 136-41.
14. Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A. K., Purity, M., and Rossant, J. (1998) Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr Biol* **8**, 661-4.

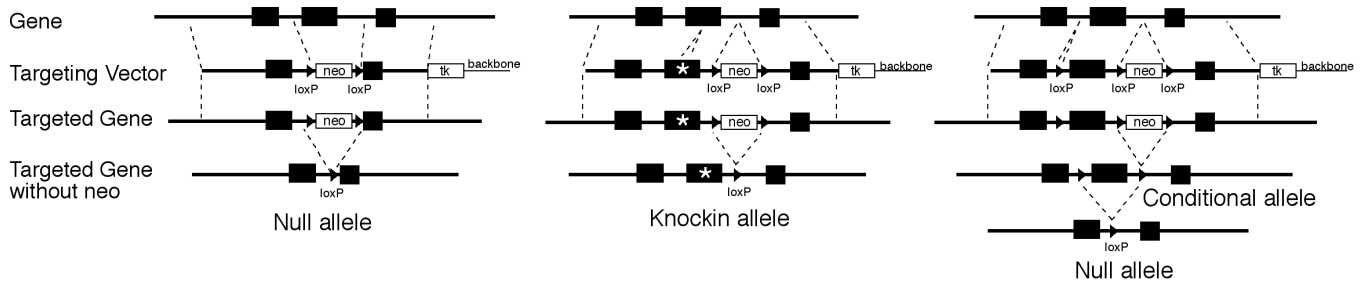
15. Olson, E. N., Arnold, H. H., Rigby, P. W., and Wold, B. J. (1996) Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* **85**, 1-4.
16. Ren, S. Y., Angrand, P. O., and Rijli, F. M. (2002) Targeted insertion results in a rhombomere 2-specific Hoxa2 knockdown and ectopic activation of Hoxa1 expression. *Dev Dyn* **225**, 305-15.
17. Hasty, P., Rivera-Perez, J., Chang, C., and Bradley, A. (1991) Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. *Mol Cell Biol* **11**, 4509-17.
18. Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L., and Rossant, J. (1992) A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. *Genes Dev* **6**, 691-704.
19. Chui, D., Oh-Eda, M., Liao, Y. F., Panneerselvam, K., Lal, A., Marek, K. W., Freeze, H. H., Moremen, K. W., Fukuda, M. N., and Marth, J. D. (1997) Alpha-mannosidase-II deficiency results in dyserythropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. *Cell* **90**, 157-67.
20. Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991) Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* **65**, 1153-63.
21. Robertson, E. J. (1987) in *Teratocarcinomas and embryonic stem cells: a practical approach* (Robertson, E. J., Ed.), Vol. 212, Oxford University Press, Oxford, pp. 71-112.
22. Auerbach, W., Dunmore, J. H., Fairchild-Huntress, V., Fang, Q., Auerbach, A. B., Huszar, D., and Joyner, A. L. (2000) Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. *Biotechniques* **29**, 1024-8, 30, 32.
23. You, Y., Bergsram, R., Klemm, M., Nelson, H., Jaenisch, R., and Schimenti, J. (1998) Utility of C57BL/6J x 129/SvJae embryonic stem cells for generating chromosomal deletions: tolerance to gamma radiation and microsatellite polymorphism. *Mamm Genome* **9**, 232-4.

24. Thomas, J. W., LaMantia, C., and Magnuson, T. (1998) X-ray-induced mutations in mouse embryonic stem cells. *Proc Natl Acad Sci U S A* **95**, 1114-9.
25. Brook, F. A., Evans, E. P., Lord, C. J., Lyons, P. A., Rainbow, D. B., Howlett, S. K., Wicker, L. S., Todd, J. A., and Gardner, R. L. (2003) The derivation of highly germline-competent embryonic stem cells containing NOD-derived genome. *Diabetes* **52**, 205-8.
26. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci U S A* **90**, 8424-8.
27. Mereau, A., Grey, L., Piquet-Pellorce, C., and Heath, J. K. (1993) Characterization of a binding protein for leukemia inhibitory factor localized in extracellular matrix. *J Cell Biol* **122**, 713-9.
28. Matisse, M. P., Auerbach, W., and Joyner, A. L. (2000) Production of targeted embryonic stem cell clones, in *Gene Targeting. A Practical Approach*. (Joyner, A. L., Ed.), Vol. 212, Oxford University Press, Oxford, pp. 101-32.
29. O'Gorman, S., Dagenais, N. A., Qian, M., and Marchuk, Y. (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc Natl Acad Sci U S A* **94**, 14602-7.

Figure Legend

Figure 1 Vector design and detection of targeting. A) Vector designs for gene targeted null, knockin and conditional alleles are shown (left to right). The wild type allele with three exons is shown at top, the targeting vector on the next line, the correctly targeted allele on the next line and the targeted allele after excision of the neo cassette by transfection of Cre recombinase in ES cells. B) Strategy for detection of correctly targeted alleles. DNA probes for Southern blot analysis are selected which are in the gene to be targeted, but external to the targeting vector (5' and 3' probes). After targeting, random insertions of the vector which survive the selection do not show alteration of the endogenous gene (lanes 1 and 3) but correctly targeted lines show a wild type allele and the predicted fragment for a targeted allele. Excision of neo is also verified by observing the predicted changes in fragment size. (neo and tk indicate the minigenes consisting of promoters, coding sequences and polyadenylation signals for the neomycin resistance and HSV thymidine kinase genes respectively.)

A



B

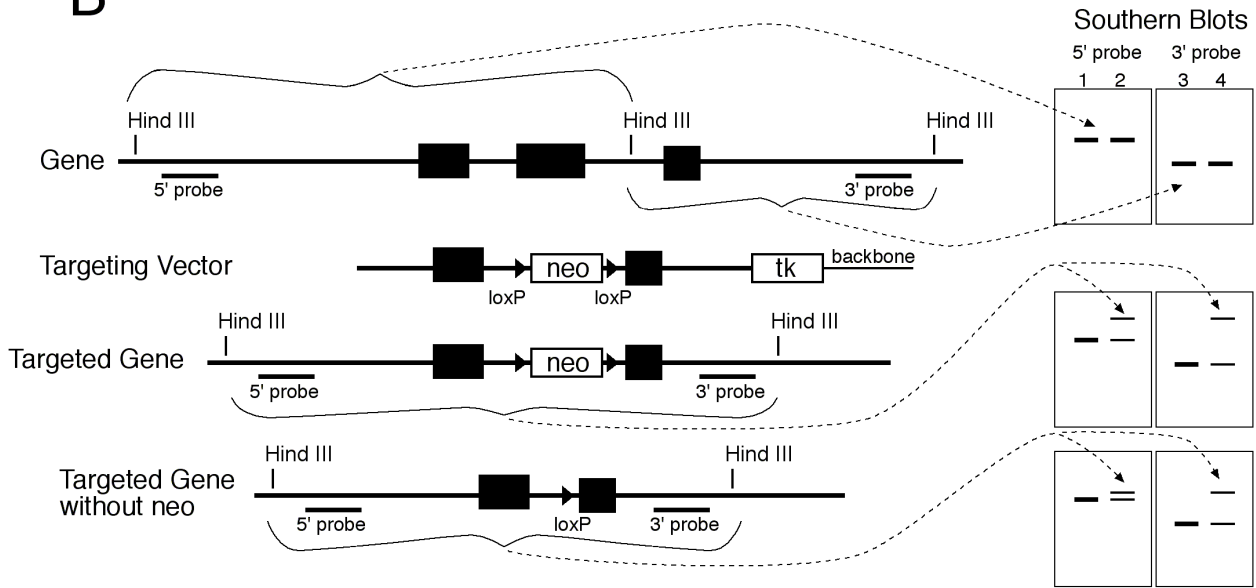


Figure 1