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Cre Recombinase Mediated Alterations of the Mouse Genome Using Embryonic Stem Cells

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1. Introduction

The introduction and establishment of transgenic, and in particular embryonic stem (ES) cell-based gene “knockout” technologies have made the mouse a key player in studying embryonic development and disease (1,2). In recent years, methods for the production of more complex genomic alterations have become increasingly widespread, hinting at an ability to manipulate and study a mammalian genome to an extent never previously thought possible. Such methodologies often partner homologous recombination-mediated gene targeting or random integration with site-specific recombination events.

This chapter is concerned with the utilization of the bacteriophage P1 derived site-specific recombinase protein Cre (3–5), and its employment as a means to catalyze modifications in homologously recombined and randomly integrated target sites within the mouse genome.

Cre is a 38-kDa protein that recombines DNA between two loxP target sites. loxP sequences are 34 basepairs (bp) long comprising two 13-bp inverted repeats flanking an asymmetric 8 bp core sequence. The recombination between two loxP sites with same orientation on the same DNA leaves two products each containing a single loxP site (6) (Fig. 1). This type of site-specific recombination, of which there are several other well-characterized systems in addition to the Cre/loxP, generates precise rearrangements of DNA but dispenses with the requirement for extensive homology between DNA partaking in the recombination. Recombination occurs through the recognition of the target sites by the recombinase, which then catalyzes strand exchange between them by precise breakage and rejoining events that are restricted to an internal region of identical sequence contained within the specific sites (6).

In addition to the Cre/loxP system, another one of the many recombinase that does not require *cis*-elements, but utilizes short recognition sites for recombination is the yeast FLP/FRT system. This system has also been widely used and applied to genome alterations (7–10), though to date the Cre protein has been shown to be more amenable to use in mammalian cells, and is therefore currently favored by most laboratories for use in ES cells and transgenic mice (11). As a consequence, we will solely refer to the Cre/loxP system in the strategies we present, though it should be noted that if the FLP/FRT were to be as efficient as the Cre/loxP system it could be substituted in all methodologies. Additionally, it might also be anticipated that future experiments may require a multistep, site-specific recombination strategy, thereby requiring the use of two separate recombination systems.

This chapter will illustrate and provide the methodologies for some of the applications of such a site-specific recombination system to experiments aimed at analyzing mouse embryonic development and/or disease conditions, from single-gene alterations, lineage-restricted and/or conditional gene ablations or ectopic expression through to chromosome engineering, and finally the use of such a system for lineage analysis.

1.1. Combining Homologous and Site-Specific Recombination

Homologous recombination in ES cells allows the precise disruption (knockout) of a target gene. Many new approaches require a defined alteration of a gene or the genome. By combining the homologous and site-specific recombinations, we are now in the position of creating most desired alterations in the mouse genome (12,13). In the following section, we will briefly introduce some of the most important current applications. The list will not be complete, since novel applications for the use of this system are continuously being reported.

1.1.1. Eliminating Any Regional Effect of a Knockout: Removal of a Selectable Marker—To identify targeted events, an introduction of a positive selectable marker, usually neomycin, into the targeted locus is required. Recently, there has been an increasing concern regarding the repressor effect of the selectable marker cassette on the genes in the vicinity of its insertion. Therefore, removal of the marker from all targeted genes is advisable. This can most easily be performed by flanking (floxing [flanking with *loxP*]) the selectable marker cassette by *loxP* sites, which on introduction of the Cre recombinase will result in the removal of neomycin.

1.1.2. Introducing Subtle Changes in a Gene of Interest—Cre/*loxP* type approaches can also be used to introduce subtle changes into any gene, including point mutations (13a) or small deletions into genes of interest, particularly if domain deletions (14) or domain swaps within the protein coding regions of a particular gene are desired. Such an approach is illustrated in Fig. 2.

1.1.3. Introducing Specific Chromosomal Changes—Over the past few years, strategies have been developed for chromosome engineering in ES cells. Such approaches have been used to design novel chromosomal variants, or to mimic altered chromosomes associated with human disease or metastasis (15). Such approaches rely on the sequential targeting of two *loxP* sites, either in *trans* (i.e., to different chromosomes) or in *cis* (some distance apart on the same chromosome), followed by the transient expression of the Cre protein in order to mediate the site-specific recombination event between the *loxP* sites, leading to the formation of the new chromosomal variant (15,16) (Fig. 3). In this case, usually three ES cell electroporations and resulting screening for the alteration are required: the two end points are targeted separately, and are followed by the introduction of the recombinase, which will mediate the recombination event between them.

This type of strategy can be used to create almost all cataloged forms of chromosomal aberration. Additionally, application of this technology could allow the creation of multiple large-scale chromosomal alterations, for example, a set of nested hemizygous deletions (also known as deficiencies) covering an entire chromosome. These could then be used to reveal novel tumor suppressor genes or functional haploinsufficiencies mapping within the deleted DNA. If a panel of deficiencies is available, screens for interesting phenotypes can be carried out either in culture, or in mice, the latter being particularly amenable to ES cell ↔ tetraploid embryo aggregation (17,18) as a means of creating completely ES cell-derived embryos, therefore bypassing the germline for accessing embryonic phenotypes (19).

Since a recombination event between two *loxP* sites some distance apart or positioned on different chromosomes, is relatively rare, presumably because of physical constraints, a reflection of chromosome architecture and decreased proximity, such strategies are designed incorporating a binary positive selection system that is only activated after a successful recombination recreates the cassette. Thus, the desired recombination event will reconstruct the selectable genetic marker, from two silent portions placed adjacent to each of the *loxP* sites. The most commonly used selection systems include the reconstitution of a human

hypoxanthine phosphoribosyltransferase (HPRT) minigene (16–20), or the juxtaposition of a strong promoter upstream of a selectable marker.

1.1.4. Creating Lineage and/or Inducible Gene Alteration

1.1.4.1. Lineage-Specific Gene Knockouts: The combination of a lineage-restricted promoter and the Cre/loxP system can be used to create a modified locus that is restricted to a certain spatiotemporal domain within the mouse. This has recently been demonstrated using a keratin 5 promoter-driven Cre to ablate the X-linked pig-a gene in skin (21) and α Camkinase II promoter-driven Cre to ablate the NMDAR1 gene in a subset of postnatal cells of the CNS (22). Combinations of conditional and inducible Cre/loxP gene targeting regimes can be utilized for a more sophisticated assessment of gene function in the developing embryo and adult animal.

1.1.4.2. Lineage-Specific Gene Repair: This approach allows one to study specific cellular phenotypes over restricted time-points or spatial locations during development or adult life. Here, the targeted allele should contain the original gene structure designed to be silent or compromised (13a) owing to the interruption by the loxP-flanked “stop” sequence (resulting in no or compromised gene expression). The Cre recombinase will be expressed from a transgene in a restricted set of cells, thereby resulting in the excision of the loxP-flanked region in these cells (resulting in normal gene expression). Consequently, the original gene structure will be restored solely in cells expressing the Cre transgene, but the remaining population will still be deficient.

If the reparable allele phenotype is characterized, and found to be embryonically lethal, then the primary responsible lineage/organ can be identified, and a proper Cre transgenic line made (or selected from the existing lines), which expresses the recombinase only in the primarily affected lineage. When this lineage-specific Cre-expressing line is crossed over the homozygous mutant genotype, the Cre recombinase repairs the mutant allele in the primary lineage, rescuing the primary deficiency, therefore allowing for the manifestation of secondary defects. This approach is expected to be less sensitive to a possible mosaic action of the Cre recombinase, since in many cases, a mosaic repair is sufficient for complete rescue. On the other hand, in almost all cases, high-fidelity lineage-specific deletion is necessary for lineage-specific knockout.

1.1.4.3. Inducible Gene Knockout: Conditional lineage-restricted ablations can be obtained through the incorporation of an inducible system into a transgenic regime. Here the Cre protein can be induced where and when appropriate. This can either be achieved by placing the Cre gene under the control of an inducible promoter (either ubiquitous or lineage-specific) or to construct the Cre cassette as an inducible fusion protein.

Several approaches have been utilized for inducible gene expression in both experimental animals and in culture. Initially, inducible systems involved the use of heat shock, isopropylthio- β -D-galactoside (IPTG), and heavy metals as inducing agents (23,24), but owing to their lack of specificity and toxic side effects, these systems are primarily restricted to use in prokaryotes, yeast, and *Drosophila*. Unfortunately, at present there is no totally satisfactory inducible system available for use in transgenic mice, though recently several laboratories have reported the successful use of drug- and hormone-inducible systems in mammalian cell culture (25,26). A common aspect of these various approaches is that the majority comprise binary systems involving the use of chimeric transcription factors that can reversibly bind target gene sequences in response to the administered drug or hormone. Modifications of the bacterial tetracycline system (27), the *Drosophila* ecdysone receptor system (26), and molecular

dimerizer systems based on FK506 or its analog rapamycin (28) have been shown to work in cells in culture and are presently being developed for use in transgenic mice.

1.1.4.4. Requirement for Lineage-Specific or Inducible Cre Transgenic Lines: A Cre Transgenic Mouse Database: All of the above technologies rely on the availability of properly working lineage-specific or inducible Cre transgenic lines. To this end, we are coordinating the assimilation of information concerning the available and planned Cre transgenic lines, and have compiled them into a continually updated database, which can be accessed through the World Wide Web at www.mshri.on.ca/develop/nagy/nagy.htm.

1.2. Future Directions

1.2.1. Fate Mapping—A great deal of interest has centered around the fate of individual cells within the developing embryo (29,30). In lower organisms, following the fate and genesis of cells is less complex than in a mammal such as the mouse embryo, where marking a single cell by injection or transplantation and following its descendants during the course of development is technically demanding, requiring expensive equipment and expertise.

ES cell-mediated transgenic technologies utilizing the Cre/loxP system may be able to facilitate fate mapping the embryo greatly. Here a lineage-restricted promoter is used to drive the Cre recombinase. A second transgene containing a reporter gene flanked by the loxP sites is also required. The second transgene should contain a “stop” sequence between the ubiquitous promoter and the marker gene to keep the gene silent. Double transgenic animals will neither express Cre nor the marker gene until the specific developmental stage permitting Cre expression and subsequent recombination, resulting in marker gene activation. This will result in activation of the recombinase, resulting in the excision of the “stop” sequence, and expression of the marker in all the progenitors cells, regardless of the later expression status of the Cre-driving promoter. If conditions are optimized, then the Cre can be used for noncomplete excision and, thus, result in excision in a very limited number of cells or even in single cell. Another possible improvement would be the utilization of an inducible Cre recombinase, allowing the precise regulation of excision frequency and timing, thereby making it relatively straightforward to follow the fate of individual cells and their descendants based on the expression pattern of the marker.

1.2.2. Gene Trap—It is feasible that gene insertion strategies utilizing loxP sites may gain popularity in certain gene-trap experiments, the goal of such experiments being to identify novel ubiquitous and/or lineage-restricted promoter/enhancer elements or genes.

Using a vector carrying a splice acceptor sequence placed upstream of a reporter gene (such as *lacZ* or GFP [green fluorescent protein]), different types of regulatory or gene sequences can be trapped (31–33). ES cell-chimeric embryos are stained for the histochemical marker to reveal expression domains of the trapped elements (31–33). On the basis of the expression pattern information gained on the trap cell lines, a subset is chosen for further study. If a specially designed trapping vector is used, such as that illustrated in Fig. 4, the trapped locus can be retargeted via loxP sites, and different transgenes can be knocked-in leading to their spatiotemporal expression being governed by the trapped element. Later, the expression can also be abolished by introducing the Cre recombinase into the system.

2. Materials

For all procedures, solutions should be made to the standard required for molecular biology using molecular biology-grade and/or “tissue-culture-tested” reagents. All solutions should be made using sterile double-distilled or MilliQ water, and where appropriate, autoclaved or filter-sterilized.

2.1. ES Cell-Culture Media and Solutions

1. DMEM+: For all procedures, we use Dulbecco's Modified Eagle's Medium (DMEM —Flow Labs [Finland], powder, cat. no. 430–1600) supplemented with the following:
 - a. 0.1 mM nonessential amino acids (100X stock, Gibco, Grand Island, NY, cat. no. 320–1140AG; *see Note 1*).
 - b. 1 mM sodium pyruvate (100X stock, Gibco cat. no. 320–1360).
 - c. 100 mM β -mercaptoethanol (100X stock stored as aliquots at -20°C , Sigma, St. Louis, MO, cat. no. 600564AG).
 - d. 2 mM L-glutamine (100X stock, stored as aliquots at -20°C , Gibco cat. no. 320–5030AG).
 - e. 15% Fetal calf serum (FCS) (*see Note 2*).
 - f. Penicillin and streptomycin (final concentration 50 $\mu\text{g/mL}$ each, Gibco cat. no. 600–564AG).
 - g. Leukemia inhibitory factor (different sources, for example, Gibco) 1000 U/mL. The supplemented DMEM used for propagation of ES cells is referred to as DMEM+ (*see Notes 1–3*).
2. 0.1% Gelatin: 1 g (w/v) gelatin is 0.1% gelatin (Sigma or BDH) made up in 1 L water, autoclaved and stored at 4°C (*see Note 4*).
3. 2X ES cell-freezing medium: 2X ES cell-freezing medium should be made up fresh each time it is to be used, and should comprise freshly prepared 60% DMEM+, 20% FCS, and 20% DMSO (Sigma, cat. no. D-5879).
4. Phosphate-buffered saline (PBS): For all tissue-culture work, we use PBS without calcium and magnesium. This is made from 10 g NaCl, 0.25 g KCl, 1.5 g Na_2HPO_4 , 0.25 g KH_2PO_4 , pH 7.2. The solution is autoclaved and stored at 4°C .
5. Trypsin (0.1%): Dissolve 0.5 g trypsin powder (Gibco, cat. no. 0153-61-1) in 500 mL saline/EDTA solution. Adjust the pH to 7.6, sterilize through a 0.22- μm filter, and store at -20°C . This constitutes a 5% stock, which needs to be diluted to 0.1% on defrosting.
6. Saline/EDTA solution: 1 L of saline/EDTA solution comprises 0.2 g EDTA, 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4 , 0.01 g phenol red, 0.2 g glucose, pH 7.2. The solution should be sterilized through a 0.22- μm filter, and stored at room temperature.
7. Tissue-culture-treated plasticware: We routinely use NUNC, Corning, and Falcon plasticware.
8. Humidified incubator: This is maintained at 37°C and 5% CO_2 .
9. Electroporation apparatus: Use apparatus, such as a Bio-Rad GenePulser, and appropriate cuvettes (Bio-Rad, Hercules, CA, cat. no. 165–2090).
10. Selection reagents: There are drugs, such as G418 (Gibco, cat. no. 11811-031), gancyclovir (Syntex, cat. no. 00865516), puromycin (Sigma, cat. no. P8833), and 6-thioguanine (Sigma, cat. no. A4660) or HAT (Gibco, cat. no. 31062-037) for HPRT-negative or positive selection, and mitomycin C (Sigma, cat. no. M-0563).
11. Cre recombinase-expression vectors: Cre recombinase-expressing vectors are described in detail in a number of publications (34–36).

3. Methods

3.1. Preparation of Tissue Culture Plates

Tissue-culture plates require special treatment prior to the plating of ES cells. This can either be by coating them with gelatin or mitotically inactivated fibroblast cells.

3.1.1. Gelatinized Plates—In our experience, R1 ES cells (17) can be propagated on both types of plates without losing their totipotency or their ability to contribute to the germline (*see Note 7*).

1. To prepare gelatinized plates rinse the surface of tissue-culture dishes with a 0.1% gelatin solution (approx 100 μ L/well of 96-well plates, or 3 mL for 6-cm plates, 5 mL for 10-cm plates).
2. Aspirate off the excess gelatin, and then allow the surface of the plates to dry a little (2–4 min).
3. Add fresh medium to the plates, and place them into the incubator until required.

3.1.2. Plates with Feeder Cells—Primary mouse embryonic fibroblast (EMFI) cells (37) or the STO fibroblast cell line (38) is the most commonly used feeder layers. Details for the preparation of a stock of EMFI or STO cells are described elsewhere (39). A brief protocol for the preparation of EMFI feeder layers will be given here.

1. Quickly defrost a vial of EMFI or STO cells, and then transfer the cell suspension to a sterile 15-mL tube containing 10 mL prewarmed feeder cell media (DMEM supplemented with 10% FCS). Then spin at 1000 *g* for 5 min, at room temperature.
2. Aspirate the supernatant, and then gently resuspend the cell pellet in 10 mL media.
3. Plate the cell suspension onto five 15-cm plates each containing 25 mL media, and place in an incubator.
4. When the cells form a confluent monolayer (usually takes 3 d) they are ready to be treated with mitomycin C.
5. Briefly, medium is aspirated from the confluent plates and replaced with 10 mL media containing 100 μ L of 1 mg/mL mitomycin C, and then placed in an incubator for 2–2.5 h.
6. The medium is aspirated, and the plates washed twice each with 10 mL PBS, followed by the addition of 10 mL of trypsin/EDTA/dish.
7. Plates are placed in an incubator until the cells begin to detach.
8. Ten milliliters of media are added to each plate, and the cell suspension broken up by gentle pipeting.
9. The cell density is determined (hemocytometer) and adjusted to 2×10^5 cells/mL. Then the cells are plated directly onto dishes suitable for ES cell culture. We routinely plate approx 1×10^6 R1 cells per 10-cm dish (*see Notes 5–7*).

3.2. Passaging ES Cells

Optimally ES cells should be fed every day and split every second day (by which time they should be 70–80% confluent). It is important not to let them overgrow, since this may induce them to differentiate.

1. If the cells are split into gelatinized plate, the plates should be prepared (as detailed previously) before starting the trypsinization.
2. Trypsinize the cells by first aspirating the medium off the dishes and then rinsing twice with PBS.
3. Aspirate off any remaining PBS, and add trypsin (0.1%) to the cells. For 10-cm plates, we use 2.5 mL trypsin. This volume should be scaled according to the size of plate used.
4. Place the dish containing the cells in trypsin in a 37°C incubator for 3–6 min.
5. Check under an inverted microscope to see if the cells have detached. When they have, add 5 mL medium to the dish.
6. Resuspend the cells and transfer them to a 12-mL tube.
7. Spin down at 1000 *g* for 5 min at room temperature.
8. Aspirate the medium, and then add 1 drop of PBS to the pellet.
9. Flick the tube hard in order to resuspend the cells.
10. Add 5 mL of medium to the tube, pipet again to mix, and split the contents at 1:5 or 1:7 ratio in new plates containing sufficient volume of medium.

3.3. Electroporation of ES Cells

Cells are routinely passaged two days prior to electroporating. Cells are ready for electroporating when their density is optimal. Usually one 10-cm plate at approx 80% confluency will provide enough cells for 1–2 electroporations. Our standard electroporation protocol is given below.

1. Gelatinize 10-cm plates, and then add 10 mL medium to each.
2. Place them in a 37°C incubator until they are required.
3. Switch on the electroporation apparatus.
4. Harvest the cells by trypsinization.
5. Resuspend the cell pellet in ice-cold PBS (1 mL for each 10-cm plate).
6. Determine the cell density (hemocytometer), and dilute with PBS to the required density for electroporation. We regularly electroporate at a relatively high cell density: 7×10^6 cells/mL (this number varies between different labs).
7. For each electroporation, mix together 20–40 µg vector DNA (for an approx 10-kb vector; *see* **Notes 8 and 9**) and 0.8 mL of the ES cell suspension in an electroporation cuvet (Bio-Rad, cat. no. 165–2088) (*see* **Note 8**).
8. Set up the electroporation conditions prior to placing the cuvet into the electroporation chamber. We routinely use 250 V, 500 µF for the Bio-Rad GenePulser (*see* **Note 9**).
9. Zap the cuvet, then place it on ice for 20 min to 1 h.
10. Transfer the cells from the cuvet into the prewarmed medium containing dishes. (The contents of one cuvette are routinely seeded into two 10-cm dishes).
11. Change medium daily.
12. If drug selection is required, start this on the second day after electroporation (*see* **Note 10**).

13. Continue the selection until colonies become apparent, and grow to a size that is amenable to picking (usually takes 7–10 d) (*see Note 10*).

3.4. Picking Colonies After Selection

This can either be done with the naked eye or by placing a dissecting microscope (such as a Lietz M3B) into the laminar flow hood.

1. Colonies are ready for picking if they are large and well separated (usually 7–9 d after electroporation). For picking start by gently washing the plate twice with PBS.
2. After the second rinsing, leave a little of the PBS behind in the dish (about 1 mL) in order to keep the surface of the plate wet, therefore preventing the colonies from drying out.
3. Choose a colony to pick. The colony is optimal if it is neither too small nor too big, and contains nondifferentiated ES cells with characteristic morphology. Fill a P200 pipeter with approx 20 μ L PBS, and gently pour this over the colony thereby rinsing it. Retain about 5–10 μ mL of the PBS in the pipet, and with this, try to “suck up” the colony from the bottom of the plate. Hold the pipet perpendicular (if picking with the naked eye) or at 45° (if picking under the dissecting microscope) to the surface of the plate, since this facilitates the lifting of the colony from the plastic.
4. Using the P200, transfer each individual colony into separate wells of a 96-well plate containing 50 μ L trypsin in each well. In doing so, pipet the colony up and down several times in order to dissociate the cells. Be careful to avoid creating too many air bubbles.
5. When all the colonies from a plate have been picked and transferred to the trypsin, the 96-well plate is placed in a 37°C incubator for 5–10 min.
6. During this time a new gelatinized 96-well flat-bottom plate with medium (200 μ L/well, containing the selection agent) is prepared.
7. Working row by row with a multichannel pipeter the cell-trypsin solution is transferred to the gelatinized plate.
8. Pipet thoroughly, without creating too many air bubbles, so as to promote the formation of a single cell suspension.
9. Return the plate to the 37°C incubator.
10. Change the media daily until the cells are ready to passage (80% confluency).
11. When passaging the cells, split them into two or three new plates. These can each be used for the preparation of DNA for genotype screening and for creating frozen stocks, which can then be used for thawing the required clones.

3.5. Passaging Cells in 96-Well Plates

Optimally 3 or 4 d after picking colonies into the 96-well plates, the cells are at a density required for splitting. Since cells in different wells generally exhibit different growth potential, they will not grow at a synchronous rate. Therefore the optimal time for splitting the whole plate needs to be determined. It is best to choose a time when the majority of the cells have reached 80–90% confluency. Another more laborious alternative is to passage the clones at different stages (pooling them into groups depending on their growth rate) and replating them into different 96-well plates.

1. To passage cells in 96-well plates, first prepare several gelatinized 96-well plates.

2. Add 200 μ L medium/well, and place plate in a 37°C incubator.
3. Aspirate the medium from the plate to be split, and then wash with PBS by multipipeting 200 μ L of PBS into each well followed by aspiration.
4. Remove all traces of PBS, and then add 50 μ L trypsin per well.
5. Incubate at 37°C for 5–10 min. The cells should detached with gentle tapping on the plate.
6. Multipipet 50 μ L medium/well into each of the wells. Pipet up and down about five times so as to resuspend completely (*see Note 11*). Then split them (working row by row) into two or three newly gelatinized plates.
7. Return these plates to the 37°C incubator (*see Notes 12 and 13*).

3.6. ES Cell Freezing

3.6.1. In Cryovials—The general protocol for freezing cells grown in a standard 10-cm dish at 70% confluency is given below (*see Notes 14 and 15*):

1. Change media 2–3 h before freezing the cells.
2. Freshly prepare 2X freezing media.
3. Harvest the cells in a 15-mL tube containing DMEM+ after trypsinization (*see Note 15*).
4. Spin down at 1000 *g* for 5 min at room temperature.
5. Remove the supernatant, and then add one or two drops of DMEM+ to the tube. Shake gently, but thoroughly to disperse the cells.
6. Add an additional DMEM+ medium to a total volume of 1.5 mL, and disperse the cells carefully so that they comprise a single-cell suspension.
7. Add an equal volume (1.5 mL) of 2X freezing medium, and mix by pipeting several times.
8. Quickly aliquot the cell suspension into three vials, and immediately place them in a styrofoam box (this will allow them to cool down gradually). Alternatively, special boxes dedicated to this task can be purchased from a number of manufacturers (for example, Stratagene) (*see Note 16*).
9. Place the box in a –70°C freezer for 1–2 d, and then transfer the individual cryovials into a liquid nitrogen container for long-term storage (*see Note 16*).

3.6.2. In 96-Well Plates

1. Working one row at a time using a multichannel pipetter, change the medium 2–3 h prior to freezing.
2. Freshly prepare 2X cell-freezing media.
3. Aspirate the medium from each well, and wash the cells with PBS (approx 200 μ L).
4. Add 50 μ L trypsin to each well, and then place plate in an incubator for 5–10 min.
5. Working on ice, preferably in a wide, flat container, aliquot 50 μ L of DMEM+ into each well. Pipet the cells several times in order to get them into a homogenous suspension.
6. Then add 100 μ L 2X cell freezing media to the wells, and again pipet to mix.

7. Finally add 80–100 μL sterile mineral oil (Sigma, cat. no. M-8410) to cover the cell/freezing medium mixture.
8. Wrap the plates in parafilm, place in a styrofoam box, and store in a -70°C freezer until such time as the desired clones have been identified and need to be recovered (*see Note 17*).

3.7. Thawing ES Cells

1. Prepare the appropriate-sized feeder layer containing plates for the cells that are to be recovered (24-well plates for 96-well frozen plates or 6 cm plates for cryovials containing approx $5\text{--}10 \times 10^6$ cells).
2. Add medium to the plates, and prewarm them in the incubator at 37°C .
3. The following steps vary with the type of tissue-culture plates used (*see Note 18*).

3.7.1. From 96-Well Plates

1. Remove the sample from the freezer, and as it begins to melt, carefully aspirate the overlying oil from the cell/medium mixture.
2. Using a multichannel pipetter and working one row at a time, quickly, but gently multipipet the cells twice in order to resuspend them thoroughly.
3. Change the pipet to a P200 (set to 200 μL), and then quickly transfer the well contents one at a time to the individual wells of a 24-well plate. Pipet quickly to resuspend the cells in the 96-well plate. Then transfer. Once the sample is transferred to the 24-well plate, pipet again to resuspend the cells and distribute them evenly in the prewarmed fresh media.
4. Aliquot another 200 μL into the now empty well of the 96-well plate to rinse it, and remove any remaining cells.
5. Transfer this additional 200 μL to the equivalent well of the 24-well plate.
6. Repeat the above for each required well of the 96-well plate.
7. Place the 24-well plate containing the newly transferred cells in a humidified CO_2 incubator at 37°C .
8. Change the media after 8 h or the following morning (if defrosting is carried out late in the evening), and then daily until the cells are ready to passage.

3.7.2. From Cryovials

1. Defrost the cells quickly, then transfer the cell suspension to a sterile 15-mL tube containing prewarmed media (approx 10 mL), and then spin at 1000 g for 5 min at room temperature.
2. Aspirate the supernatant, and then add 1 drop of PBS.
3. Resuspend the cells by either flicking the bottom of the tube or by gently pipeting up and down.
4. Add few milliliters of media to the tube, and again gently pipet up and down to dissociate the cells.
5. Plate the cell suspension onto a 6-cm plate, and place in an incubator.
6. Change the media after 8 h or the following morning (if defrosting is carried out late in the evening), and then daily until passaging is required.

7. When the cells reach approx 70% confluence (usually taking 2 d), they are ready to passage.

3.8. Removal of loxP-Flanked Short Genomic Segment in ES Cells by Transient Expression of Cre Recombinase

1. Electroporate the correctly targeted ES cell line with 50 µg/mL of circular plasmid containing the Cre recombinase gene driven by an ES cell transcriptionally active promoter, for example, the pBS185 plasmid, which contains Cre under the control of the hCMV promoter (35).
2. After electroporation, plate the cells very sparsely (approx 1000 cells/10-cm dish) onto gelatinized 10 cm plates each containing 10 mL of DMEM+.
3. Change media daily until the colonies have attained a size that is ready to pick (usually takes 7 d).
4. Pick colonies into 96-well plates, and expand into two or more plates. One plate is frozen, and the others are used for PCR or Southern screening to detect the required recombination event. A subset of the colonies will be mosaic for the Cre-mediated excision, therefore requiring further subcloning for the derivation of pure lines (*see Notes 19 and 20*).

3.9. Deletion of loxP-Flanked Large Genomic Segment or Selection for Site-Specific Chromosomal Alteration in ES Cells by Transient Expression of Cre Recombinase

This is carried out in much the same manner as in **Subheading 3.8.**, except that cells are usually subjected to selection, since a bipartite cassette that will be reconstituted after the desired recombination is routinely used (for example, HAT for the reconstitution of the HPRT minigene). Routine selection is applied 2 d after the electroporation. As a consequence, the cells need to be plated at regular density (one cuvet into one or two 10-cm plates) after the electroporation.

3.10. Introducing ES Cells into Mice Preparation of Cells for Aggregation with and Injection into Embryos

When genetically altered ES cell lines are identified, thaw the 96-well plates, or cells from vials, onto feeders, and grow them up. Avoid taking them through too many passages, since with increasing passage number, the possibility of cells losing their germline-transmitting ability may increase. Our standard protocol for preparing cells for aggregation is given below. Preparation of cells for blastocyst injection is performed in a similar manner, except that for this procedure, the cells need to be seeded at the usual density on d 3, trypsinized slightly longer, spun down and washed in PBS to obtain single-cell suspension on d 5 (*below*).

Day 1: Thaw cells 4 d prior to aggregation on a feeder cell layer containing plate.

Day 2: Change the medium.

Day 3: Split cells onto gelatinized plates, but instead of the usual 1:5 ratio, pass them 1:50 or even more dilute (*see Notes 21 and 22*).

Day 4: Change the medium.

Day 5: Trypsinize the cells briefly, until the colonies lift up as a loosely connected clump of cells. Stop trypsin by adding DMEM+ to the plate. Select clumps directly from the plate for aggregation (*see Note 23*).

Protocols and descriptions detailing the introduction of cells into mice by aggregation of ES cells with preimplantation embryos are provided elsewhere (40). Our own lab protocols can

be obtained through the World Wide Web at
<http://www.mshri.on.ca/develop/nagy/nagy.htm>.

3.11. Removal of loxP-Flanked Short Genomic Segment In Situ by Crossing Germline-Transmitter Chimeras with a Stable Cre Transgenic Line

This approach requires the availability of a Cre-expressing transgenic line. In our laboratory, we have established a Cre recombinase-expressing transgenic mouse line (tgCre-1) by pronuclear injection of the hCMV Cre gene. Briefly, the insert purified from pBS 185 plasmid (34) was directly injected at a concentration of 5 ng/mL into the pronucleus of zygotes fertilized by germline-transmitting males. This line is now routinely used to remove the loxP-flanked genomic piece efficiently from a targeted locus, by crossing this line with the targeted mouse (13a). This and other lines with similar properties (41) can be found in the Cre transgenic database mentioned previously (**Subheading 1.1.4.4.**).

1. Males homozygous or heterozygous for the loxP-flanked DNA sequence are crossed with Cre transgenic females.
2. Offspring are screened for the required recombination event by PCR (from ear punch-derived sample DNA) or Southern analysis (from tail biopsy sample-derived DNA).

3.12. Removal of loxP-Flanked Genomic Segment In Situ by Transiently Expressing the Cre Recombinase in F1 Preimplantation Stage

An alternative method for excising loxP-flanked DNA is to express the Cre recombinase in an early embryo transiently (42).

1. Briefly, germline transmission of the primary loxP-flanked DNA sequence is achieved.
2. Then a Cre-expressing plasmid is injected in circular form into the pronucleus of zygotes produced by a cross between males carrying the loxP-flanked sequence and wild-type females.
3. Injected embryos are transferred into pseudopregnant recipients.
4. Offspring are screened for the required excision event.

4. Notes

4.1. Cell-Culture Media and Solutions

1. Because glutamine and LIF are unstable, DMEM+ that is kept at 4°C for a period exceeding 2 wk needs to be supplemented with a new aliquot of 100X glutamine stock and LIF.
2. The quality of the FCS is critical to the propagation and maintenance of ES cells. We recommend that several batches be tested for plating efficiency and toxicity from different suppliers for their ability to support growth of pluripotent ES cells, and that then a bulk order of the best (to last approx 1 yr) be purchased, and the bottles stored at -20°C for up to 2 yr.
3. LIF helps maintain ES cells in an undifferentiated state especially when they are growing on gelatinized plates in the absence of feeder cell layers. However, it should be noted that too high a concentration of LIF can be deleterious for the cells. We suggest using twice the lowest concentration in which the cells stay undifferentiated. For our cell line, R1 it is 1000 U/mL.

4.2. Preparation of Tissue-Culture Plates

1. Gelatinized Plates: Using R1 ES cells, it is possible to carry out all ES cell manipulations on gelatinized plates from the initial passaging through to the introduction into mice. After replating cells from feeder containing plates onto gelatin plates, their characteristic morphology may be seen to change. This is usually only transient, and in time, they will revert to their usual appearance.
2. After mitomycin C treatment, the feeders are plated onto dishes for ES cell culture, the cells usually take an overnight incubation to attach they are then ready for use.
3. The medium should be changed from feeder cell medium (DMEM+ 10% FCS) to ES cell medium (DMEM+) before the addition of the ES cells.
4. An alternative to mitomycin C treatment is to treat the cells with 6000–10,000 rad of γ -irradiation.

4.3. Electroporation of ES Cells

1. The targeting vector needs to be linearized for electroporation. The DNA is prepared by standard “maxiprep” procedures, for example, cesium chloride gradient centrifugation, or popular kits (such as Qiaex, Promega Wizard, Geneclean). Ethanol-precipitate the digested DNA before the electroporation, and dissolve the pellet in sterile TE. The concentration of the DNA should be around 1 $\mu\text{g}/\mu\text{L}$.
2. For the transient Cre electroporation, plasmid DNA can be used straight after “maxiprep” purification. In this case, the electroporation procedure is carried out according to the standard protocol provided. The Cre expressing vector can be coelectroporated along with a second selectable marker containing vector, or can be introduced alone (35). If a selection-based coelectroporating strategy for identifying cells taking up the Cre plasmid is used, then the cells should be plated at normal density (one cuvet into one or two 10-cm plates). Transient expression of the Cre recombinase in ES cell culture results in almost 100% excision between loxP sites placed a few kilobases apart (14).
3. When selecting for loss or gain of HPRT function, the cells should be maintained in the positive or negative selection prior to the selection switch that will assay the altered HPRT activity. The transiently expressed Cre-mediated excision is often mosaic. Therefore, a PCR screen should be carefully designed to detect such situations. Additionally, Southern blot analyses should be performed as a final check on candidate clones identified by PCR.

4.4. Passaging ES Cells

1. The cell pellet should be carefully resuspended. If this is not carried out, the ES cells will grow as large clumps, containing necrotic centers and differentiated cells at the periphery.
2. ES cells are growing optimally if they are ready for passing on each alternate day, and if their morphology does not change during this time. The typical ES cell morphology is when single cells are not visible, and they grow as characteristically shaped small clumps.
3. The number of cells to be plated at each passage and length of growth between two passages is critical. Cells should not be left to overgrow, and care should be taken not to split cells too diluted or too dense.

4.5. ES Cell Freezing

1. We usually freeze ES cells in cryovials at a density of $5\text{--}10 \times 10^6$ cells/mL of freezing medium. A single 10-cm dish usually gives approx three cryovials each containing 1 mL of cells.
2. To freeze cells from different-sized dishes, proportionally altered volumes of cell medium are used (for example, a 6-cm dish requires 1 mL, and a 3.5-cm dish requires 0.5 mL).
3. It is important to note that the cells should be frozen down gradually (in a styrofoam box or isopropanol container), and not to be kept at -70°C for too long a period of time. Cryovials should be transferred to liquid Nitrogen for long-term storage.
4. Unfortunately, 96-well plates can only be kept at -70°C . We do not recommend keeping the plates for more than 2 mo at this temperature. Therefore, all screening for the required alleles needs to be performed within this time period.

4.6. Thawing ES Cells

1. It is important to thaw cells as rapidly as possible in order to avoid long crystal formation as the frozen vial passing through critical temperatures. Immediately after removal from frozen storage, the cell-containing vials (cryovials or 96-well plates) are placed at 37°C in a water bath until they are almost completely defrosted (1–3 min).

4.7. Removal of loxP-Flanked Short Genomic Segment in ES Cells by Transient Expression of Cre Recombinase

1. No selectable marker is needed for this Cre-mediated excision, since 2–5% of cells are picking up the Cre expressing DNA. Practically in almost all these cells excision occurs between loxP sites placed a few kilobases apart. As a consequence, transient expression of the Cre recombinase in ES cells results in a 1 in 30 average frequency of excision.
2. If the region of DNA to be deleted contains a drug selection marker, then the newly acquired sensitivity to the marker can be tested when replica 96-well plates containing cells are available. Here, however, one should be aware of the frequent mosaic type of excision. Therefore this step does not replace the PCR or Southern blot screening.

4.8. Introducing ES Cells into Mice

1. In general cells should be replated on the appropriate size plate (96- or 24-well plate when passaging from 96-well plate, 6-cm dish when passaging from a cryovial), and grown up so that there are enough for freezing into cryovials and introducing into mice.
2. A separate plate should be prepared for aggregation as was described in the protocol. The reason for highly diluted single-cell plating is to produce clumps of 10–25 loosely connected cells prior to aggregation. Then the required-size colonies are predominantly found on the plate. Care should also be taken in order not to disaggregate the cell clumps (by pipeting the cells too vigorously or overtrypsinization).
3. For blastocyst injection purposes, cells do not need to be maintained as clumps. Therefore they should be completely disaggregated.

4.9. Removal of loxP-Flanked Short Genomic Segment In Situ by Crossing Germline-Transmitter Chimeras with a Stable Cre Transgenic Line

In our experience, not all offspring that inherit the Cre transgene will undergo excision as shown in Fig. 4, indicating that although the efficacy of such a transgene is very high (complete excision in 90% of animals), it is not active in all embryos that inherit it.

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Fig. 1.
The Cre recombinase has a 34-bp recognition site comprising two inverted repeats and a core sequence (A). It can catalyze a site-specific recombination event leading to the deletion of the intervening DNA (B).

Fig. 2.

Simple gene alterations—introducing a subtle change into a gene of interest. In this example, a point mutation, small deletion, or domain swap (*) is introduced into an exon of a gene using a replacement type vector, containing a floxed neomycin resistance cassette. Transient expression of Cre results in removal of the region between the loxP sites, and as a result the neo selection cassette is removed, thereby rendering the genomic structure identical to wt with the exception of the introduced change and the single loxP site remaining in the first intron.

Fig. 3. Chromosomal modifications. Targeting of the two loxP sites some distance apart, either in *cis* (A) or in *trans*, in the same (B), or different chromosomes (C) can give rise to various chromosomal modifications.

Fig. 4.

Trapping an endogenous locus with the option of introducing a new gene. Here two electroporation steps are required. The first is used to identify a locus of interest, and the second is used to introduce the new gene into that locus. Two alternative alleles are created on the second electroporation, one doubly drug-resistant, the other singly. Thus, the drug selection will determine which clones can grow. P, promoter driving gene expression in ES cells; SA, splice acceptor; β geo, β gal-neo gene fusion; puro, puromycin resistance cassettes; IRES, internal ribosome entry sites.