Generation and Validation of Tissue-Specific Knockout Strains for Toxicology Research

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Tissue-specific knockout mice are widely used throughout scientific research. A principle method for generating tissue-specific knockout mice is the Cre-*loxP* system. Here, we give a detailed description of the steps required to generate and validate tissue-specific knockout mice using the Cre-*loxP* system. The first protocol describes how to use gene targeting in mouse embryonic stem cells to generate mice with conditional alleles. Subsequent protocols describe how to recover Cre transgenic mice from cryopreserved sperm using in vitro fertilization and present a breeding strategy for obtaining tissue-specific knockouts. Finally, methods are provided for validating the knockout mice using PCR of genomic DNA, reverse-transcription PCR and quantitative reverse-transcription PCR of mRNA, and immunoblot analysis of proteins.

Keywords: Cre-*loxP* • knockout mice • tissue-specific

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INTRODUCTION

The use of knockout mice to study gene function is a staple in toxicology research. Conditional knockout strains, in which gene function is altered in a cell- or tissue-specific manner, are particularly powerful (Taylor et al., 2019). The Cre-*loxP* system is widely used for generating conditional knockouts (Papaioannou & Behringer, 2005). In this system, Cre recombinase, derived from bacteriophage P1, catalyzes recombination between two 34-bp *loxP* recognition sequences (Klos, 2004; Papaioannou & Behringer, 2005). To make mice with a conditional allele, *loxP* sites oriented in the same direction are introduced into the mouse genome to flank a critical exon or exons of a particular gene (these genes are called "floxed"). The expression of Cre recombinase in a specific cell type results in deletion of the sequence between the two *loxP* sites. A schematic of the Cre-*loxP* system is shown in Figure 1.

Conditional knockout mice are an ideal model to study organ-specific gene function and human disease. For example, to understand how loss of the manganese efflux transporter SLC30A10 induces neurological disease, we used the Cre-*loxP* system to generate

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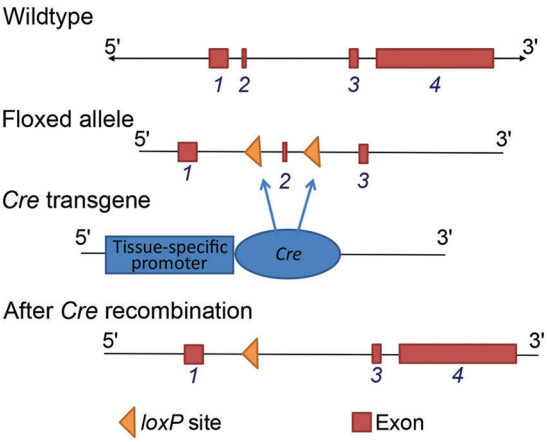

Figure 1 Overview of Cre-*loxP* system. Inserted *loxP* sites flank the target region (here, exon 2 of the targeted gene). When Cre is expressed under the control of a specific promoter, it binds to the *loxP* sites and Cre recombination occurs, knocking out the targeted region.

full-body and tissue-specific *Slc30a10* knockout mice. Full-body *Slc30a10* knockout mice exhibited extremely elevated manganese in the brain, blood, and liver (Hutchens et al., 2017; Liu et al., 2017; Taylor et al., 2019). These knockouts were also hypothyroid, highlighting a previously unappreciated relationship between manganese toxicity and thyroid function (Hutchens et al., 2017; Liu et al., 2017; Taylor et al., 2019). Interestingly, brain-specific *Slc30a10* knockouts had no change in brain manganese levels under basal conditions, whereas liver- and digestive-system-specific knockouts showed moderate and extreme elevations in brain manganese, respectively (Taylor et al., 2019). Comparing the phenotypes of full-body and tissue-specific *Slc30a10* knockout mice resulted in the unexpected discovery that brain manganese homeostasis is regulated by manganese efflux in the digestive system (Taylor et al., 2019). This application of the Cre-*loxP* system gave critical insight into the mechanisms of manganese homeostasis and manganese-induced neurological disease and underlines the power of using tissue-specific knockouts in research.

Here, we provide a detailed protocol for generating and validating tissue-specific knockout mice using the Cre-*loxP* system. Basic Protocol 1 describes the generation of mice containing *loxP* sites that flank a specific gene. Basic Protocol 2 describes how to recover mice expressing the Cre transgene. Basic Protocol 3 describes a breeding strategy for obtaining tissue-specific knockouts. Finally, Basic Protocols 4-6 detail four methods for validating the knockouts generated: (1) PCR to confirm the deletion in genomic DNA, (2) RT-PCR and (3) qRT-PCR to confirm loss of the expected transcript, and (4) immunoblot analysis to confirm loss of the target protein.

STRATEGIC PLANNING

Primer Design for Genotyping Mice

Primer set 1: Detection of 5′ loxP site. The forward primer should begin just before the start of the 5′ *loxP* site and the reverse primer should begin just after the 5′ *loxP* site. Because each *loxP* site is 34 bp, the total PCR product when *loxP* sites are present should be longer than the wildtype allele, which has no *loxP* sites (Klos, 2004). The PCR product from heterozygous mice will show two bands, whereas the product from homozygous mice will show one longer band. Figure 2 provides a schematic for placement of primer sets 1 and 2.

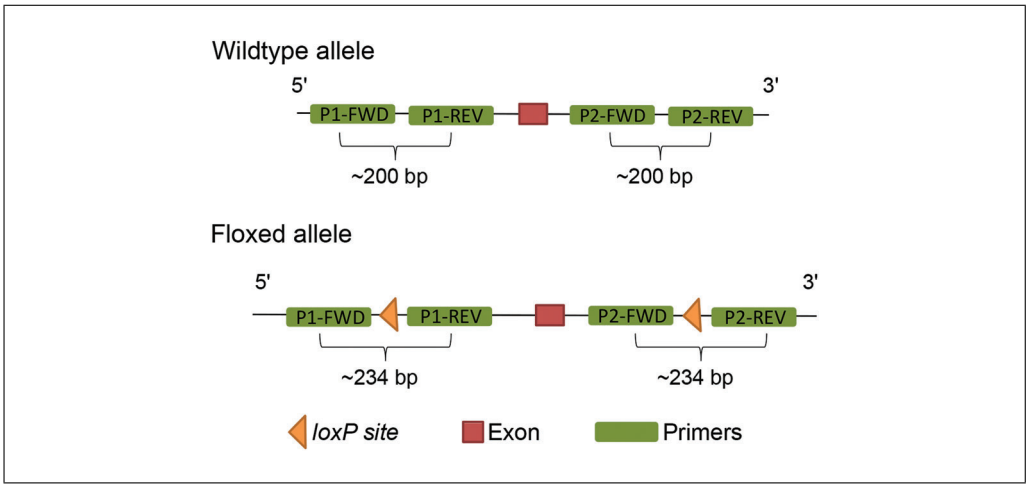

Figure 2 Primer placement for genotyping. The forward and reverse primers for primer set 1 (P1-FWD and P1-REV) should be placed around the 5′ *loxP* site. The forward and reverse primers for primer set 2 (P2-FWD and P2-REV) should be placed around the 3′ *loxP* site. Since each *loxP* site is 34 bp, the resulting PCR product from a floxed allele should be 34 bp longer than the PCR product from a wildtype allele.

Primer set 2: Detection of 3′ loxP site. The forward primer should begin just before the start of the 3′ *loxP* site and the reverse primer should begin just after the 3′ *loxP* site. The total PCR product when *loxP* sites are present should be longer than the wildtype allele, which has no *loxP* sites. The PCR product from heterozygous mice will show two bands, whereas the product from homozygous mice will show one longer band.

Primer set 3: Detection of Cre. Forward and reverse primers can be designed to probe any part of the Cre transgene. This primer set will produce a single band when Cre expression is present and no band if there is no Cre.

Primer Design for PCR Using Genomic DNA

Validation using genomic DNA requires assessing the presence of the knockout allele and the presence of Cre. Thus, two separate primer sets are needed: one to detect if recombination occurred, indicating knockout of the targeted gene, and the other to detect if Cre is expressed. In each set, the forward primer consists of the exact base-pair sequence at the beginning of the desired sequence region and the reverse primer consists of the reverse complement of the base-pair sequence at the end of the desired region. Primers should be ~15-20 bp in length and have unique sequences. Below are recommendations for designing primer sets needed to validate knockouts using genomic DNA. Once the primer sequences are determined, primers can be ordered through Sigma-Aldrich or other online services.

Primer Set 1: Detection of recombination. The forward primer should end at least 50-100 bases before the start of the 5′ *loxP* site. The reverse primer should begin 50-100 bases after the 3′ *loxP* site. With recombination, the 3′ *loxP* site and sequence between the *loxP* sites are removed. Figure 3 shows primer placements with and without recombination. The primers should be designed such that with recombination, the PCR product is ~250-500 bps, but without recombination the product size is greater than 1 kb (which is too large for the PCR conditions set). Thus, with this primer set, a PCR product from a knockout allele will produce a single band, while PCR product from a wildtype allele will produce no band.

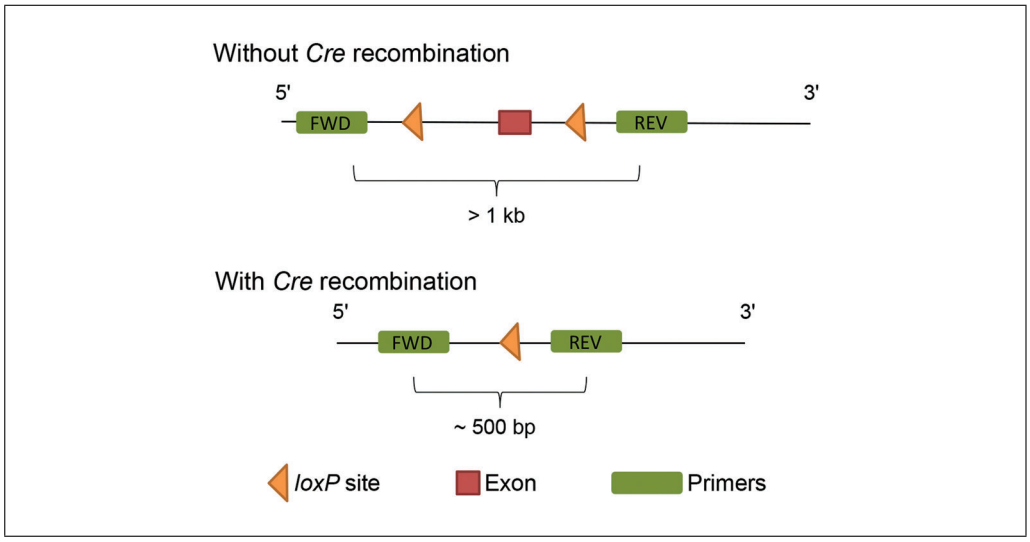

Figure 3 Primer placement for detecting the recombination in genomic DNA. The forward primer should be placed just before the 5′ *loxP* site, and the reverse primer should be placed just after the 3′ *loxP* site. Without recombination, the PCR product size is too large for the PCR conditions set. However, with recombination, the region between the *loxP* sites is excised, making the PCR product short enough for amplification using the PCR conditions described.

Primer Set 2: Detection of Cre. Forward and reverse primers can be designed to probe any part of the Cre transgene. This primer set will produce a single band when Cre expression is present and no band if there is no Cre.

Primer Design for RT-PCR and qRT-PCR

Validation using RT-PCR or qRT-PCR requires detecting the presence of the targeted gene and a control gene. Only two primer sets are required: one for the target gene and one for the control gene. Each primer should be ~15-20 bp and the resulting PCR product for both reactions should be ~500 bp or less. Once the primer sequences are determined, primers can be ordered online.

Primer Set 1: Detection of the target gene. The forward and reverse primers should begin just before and after, respectively, a sequence within the target gene. The PCR product should be present for control tissue and absent for knockout tissue.

Primer Set 2: Detection of the control gene. The forward and reverse primers should begin just before and after, respectively, a sequence within the control gene. The PCR product should be present for all tissue.

BASIC PROTOCOL 1 GENERATING MICE WITH CONDITIONAL ALLELES BY GENE TARGETING IN MOUSE EMBRYONIC STEM CELLS

Mice with a conditional allele, in which a critical exon(s) is flanked by *loxP* sites oriented in the same direction (floxed), can be made by gene targeting in mouse embryonic stem (ES) cells or by CRISPR-Cas9 genome editing. CRISPR-Cas9 can be faster and less labor intensive, but there are a number of limitations, including varying efficiencies and unintended mutations (Kosicki, Tomberg, & Bradley, 2018; Lanza et al., 2018). A discussion of the CRISPR-Cas9 system is provided in Background Information. Here, we present gene targeting in ES cells. This approach involves four main steps: (1) electroporation of a gene-targeting vector into ES cells, (2) selection and picking of ES cell clones, (3) identification of correctly targeted clones by Southern analysis, and (4) expansion of correctly targeted clones for microinjection. In more detail, ES cells are first expanded on a layer of SNL76/7 feeder cells, electroporated with the targeting vector,

and selected with G418 sulfate. The resulting colonies are picked, disaggregated with trypsin, and cultured using 96-well feeder plates. The 96-well plates are then split to make one plate for isolation of DNA to be used for screening and another plate that is frozen for later clone retrieval.

General considerations and strategies for designing gene-targeting vectors to make mice with conditional alleles are reviewed in Papaioannou & Behringer (2005). Targeting vectors can be designed online and purchased commercially (VectorBuilder, Cyagen). It is vital to design a Southern screening strategy to detect homologous recombination and identify single-copy probes that lie outside of the homology arms of the targeting vector before electroporating the vector into cells. The design of the screening strategy should be coincident with the design of the targeting vector. Restriction enzyme sites at the locus and DNA fragments that may be suitable to use as single-copy probes for Southern analysis can be identified using the UCSC Genome Browser. Although Southern analysis is generally more informative for identifying clones that are correctly targeted, an Alternate Protocol for identifying targeted clones by long-range PCR is also provided. The cell culture protocols described here can also be used to culture EUCOMM and KOMP C57BL/6N ES cell clones that have conditional alleles. EUCOMM and KOMP ES cell clones for a specific gene can be searched for at the International Mouse Strain Resource (IMSR). The IMSR links provide allele details, quality control information, and ordering information for the clones selected.

Materials

Feeder cells: SNL 76/7 STO cells (ATCC, SCRC-1049)
STO medium (see recipe)
0.1% (w/v) gelatin (Millipore, ES-006-B)
Mitomycin C (Sigma, M0503)
Dulbecco's phosphate-buffered saline (DPBS) without calcium, magnesium (Hyclone, SH30028.02)
0.25% (w/v) trypsin (Gibco, 25200-056)
ES cells: V6.5 129/B6 hybrid mouse ES cells (Novus Biologicals, NBP1-41162)
1 mg/ml linearized targeting vector
ES cell medium (see recipe)
G418 sulfate (Geneticin, Gibco 11811-023)
2× freezing medium (see recipe)
Lysis buffer (see recipe)
NaCl/ethanol mix: 150 μl of 5 M NaCl in 10 ml 100% ethanol
70% (v/v) ethanol
Restriction enzymes (*Eco*RI, *Eco*RV, *Bam*HI, *Bgl*II, *Pst*I, *Asp*718, and/or *Sst*I) with appropriate buffers
Agarose gel
0.4 N NaOH
0.25 M HCl
20× SSC
5′ and 3′ single-copy hybridization probes
Megaprime DNA labeling system (GE Healthcare, RPN1604)
^{32}P-dCTP (NEN, BLU013H)
G50 Sephadex syringe column
Scintillation counter
Hybridization buffer (see recipe)
10% (w/v) SDS
ES cell injection medium (see recipe)

15- and 50-ml centrifuge tubes
Clinical centrifuge
Tissue culture dishes: 6-cm (Falcon, 353004), 10-cm (Corning, 25020), 4-well (Nunc, 179820), 12-well (Falcon, 353043)
Hemocytometer
Gene Pulser Xcell electroporation system (Bio-Rad) with capacitance extender and Gene Pulser cuvettes (0.4-cm electrode, Bio-Rad, 165-2088)
Stereomicroscope
20-200 µl, 8-channel pipettor (VWR, 89079-948)
8-channel aspirator (Corning, 4931)
96-well flat-bottom plates (Falcon, 353072)
Parafilm
Plastic food storage bags
Styrofoam box for freezing 96-well plates of cells
Tupperware-type container
42°, 60°, and 65°C shaking water baths
Owl Model A2 large gel system (Thermo Fisher Scientific)
20 × 24 cm UVT gel tray (Thermo Fisher Scientific, A2-UVT)
24-well 1.5-mm comb (Thermo Fisher Scientific, A2-24D)
Large glass baking dishes
Large sponges
Blotting paper (grade 703, VWR, 28298-020)
Amersham Hybond-XL blotting membrane (GE Healthcare, RPN203S)
10-ml glass pipette
Glass plate (*optional*)
Hybridization bags (VWR, 95059-394)
Tabletop Impulse Sealer, 12 inch (Uline H-190)
95°C heat block or thermocycler
Plastic wrap
X-ray film cassette with intensifying screen
Carestream Biomax XAR film (Sigma, F5513)
2-ml cryogenic vials (Corning, 430488)
Cell freezing container (Bel-Art, F18844-000)

Additional reagents and equipment for injecting blastocysts and transplanting into female mice

Electroporate ES cells

To maintain their germline potential, mouse ES cells should be grown at high density on mitotically inactivated SNL76/7 feeder cells. The feeder cells provide leukemia inhibitory factor (LIF) and other unknown factors that keep the cells in an undifferentiated state. ES cells need to be passaged ~1:6 every second or third day to prevent them from overgrowing and differentiating. During the trypsinization step, cell clumps must be disrupted by repeated pipetting to ensure a single-cell suspension, as cell clumps will differentiate. Cells should be fed every morning, and the medium should not be allowed to become yellow. The passage numbers of ES and SNL76/7 cells should be recorded. SNL76/7 cells should not be used after passage 20. V6.5 (C57BL/6 × 129/Sv) ES cells are very robust, and the stocks that we use for electroporation are passage 26. If 129 strain ES cells are used instead of the hybrid V6.5 line, it is recommended to start with cells that have passage numbers in the low to mid teens. It is also imperative to plan for the quantity of feeder plates needed for the experiment so there are enough SNL76/7 cells on hand to make the feeder plates. A confluent 10-cm plate contains ~7–8 × 10^6 cells. Feeder plates should be prepared at least 1 day before use. They are good for

~2 weeks, but should be inspected before use to make sure the feeder layer is intact. Fresh 1× glutamine should be added to the medium after 2 weeks.

Prepare feeder cell plates

1. Remove a 1-ml vial of SNL76/7 cells (5×10^6 cells/ml, passage number <16) from liquid nitrogen storage, loosen the cap, and thaw in a 37°C water bath for ~5 min.

   CAUTION: *Cryovial caps should be loosened immediately after removal from liquid nitrogen to allow any liquid nitrogen that might have gotten into the vial to evaporate. Failure to do so may result in the tube exploding. A lab coat, cryo gloves, safety glasses, and a face shield should be worn when removing clones from liquid nitrogen storage.*

2. Transfer thawed cells to a 15-ml tube containing 5 ml STO medium to dilute the cryoprotectant medium. Spin in a clinical centrifuge for 10 min at 400 × *g* and remove medium.

3. Resuspend cell pellet in 5 ml STO medium, transfer to a 10-cm gelatin-coated plate containing 7 ml STO medium, and place in a 37°C, 5% CO_2 incubator. When cells become confluent (2-3 days), passage cells 1:5 in five 10-cm gelatin-coated plates.

   Plates are gelatin-coated by pipetting enough 0.1% gelatin solution to cover the plate. The gelatin is removed after 10-15 min. Gelatin-coated plates are used the same day that they are prepared.

4. Make a 0.5 mg/ml mitomycin stock by adding 4 ml DPBS to a bottle containing 2 mg mitomycin. Add mitomycin to the required amount of STO medium to a final concentration of 10 µg/ml.

   The mitomycin stock can be stored at 4°C for 2 weeks in its original dark vial.

   CAUTION: *Mitomycin is a suspected carcinogen, and should be handled carefully. Stocks and working solutions should be disposed as chemical waste as directed by the institution's chemical safety department.*

5. When SNL76/7 plates are confluent, remove medium, replace with 6 ml STO medium containing mitomycin, and return plates to the incubator for 2 hr.

6. Aspirate off the mitomycin-containing medium (collect it as chemical waste) and wash plates two times with DPBS.

7. Add 1 ml of 0.25% trypsin to each plate and incubate at 37°C for 5-10 min.

8. Add 5-10 ml STO medium (no mitomycin) to each plate to inactivate the trypsin. Disaggregate cells by pipetting up and down approximately ten times.

9. Pool cells from the five plates between two 50-ml tubes and centrifuge 10 min at 400 × *g*.

10. Remove medium and wash cell pellets twice with fresh STO medium to remove all traces of mitomycin.

11. Resuspend cells in 10 ml STO medium per tube and count a 10-µl aliquot of cells using a hemocytometer. Adjust concentration to 3.5×10^5 cells/ml.

12. Prepare one 6-cm gelatin-coated feeder plate (4 ml cells/plate) and eight 10-cm gelatin-coated feeder plates (12 ml cells/plate). Incubate feeder plates overnight to allow mitomycin-treated cells to attach and recover.

    For this example, a total of 35×10^6 mitomycin-treated cells are required. A confluent 10-cm SNL76/7 plate contains 7–8 × 10^6 cells, so approximately five SNL76/7 plates (step 3) are required to make the necessary feeder plates.

Electroporate ES cells

13. Thaw and spin down a vial of V6.5 ES cells as described for SNL76/7 cells (steps 1-2). Resuspend cells in 4 ml ES cell medium and plate on a 6-cm feeder plate. Feed cells the following morning.

14. When cells are ~70% confluent (2-3 days), passage ES cells 1:2 to a 10-cm feeder plate. Feed cells the following morning.

15. In the afternoon (~4 hr after feeding), trypsinize ES cells for 10 min and gently disaggregate the cells to make a single-cell suspension.

16. Spin down cells and resuspend in 10 ml DPBS. Count a 10-µl aliquot using a hemocytometer and adjust concentration to 11×10^6 cell/ml in DPBS.

17. Place 0.9 ml cells in a Gene Pulser cuvette and add 25 µl linearized targeting vector (1 mg/ml). Electroporate cells at 230 V and 500 µF and let recover on ice for 5 min.

18. Replace medium on six 10-cm feeder plates with ES cell medium and then aliquot the electroporated cells between the six feeder plates. Place in incubator for 24 hr.

Select and pick ES cell clones

19. After 24 hr, start selection by feeding cells daily with selection medium for 4-5 days, then feeding every day or every other day, depending on the number of clones on the plate. For V6.5 ES cells, use ES cell medium containing 200 µg/ml G418.

    Cells should be maintained on G418 selection medium until clones are picked. Clones can be picked 8-10 days after electroporation.

    For other ES cell lines, various concentrations of G418 should be tested to determine the optimal concentration for selection.

20. Prepare a 96-well feeder plate as described above (see steps 1-12) using 150 µl of 3.5×10^5 mitomycin-treated cells per well.

    Feeder cells should be prepared so that the 96-well plate is seeded at least one day before clones are to be picked.

21. When ready to pick clones, rinse plates twice with DPBS and cover with 10 ml DPBS.

    The steps for picking clones (steps 21-25) are illustrated in Figure 4.

22. Using a stereomicroscope at low power, identify clones with a rounded morphology. Pick colonies using a pipetter set to 2 µl and place in individual wells of a 96-well plate containing 50 µl/well of 0.25% trypsin.

    Avoid clones with a flat phenotype. The rounded morphology should be visible by eye.

    This procedure can be done outside of the cell culture hood, but in a quiet area of the lab to prevent contamination.

23. Once a full 96-well plate has been picked (~30 min), place the plate of clones in the incubator for 10 min. Pick two to three plates of clones.

24. Use a multichannel pipettor to add 50 µl ES cell medium to each well and break up clones by pipetting up and down ~20 times.

25. Transfer cells from each well to the 96-well feeder plate. Feed cells daily for 3-4 days until the wells become almost confluent.

26. Aspirate off medium and wash cells twice with DPBS.

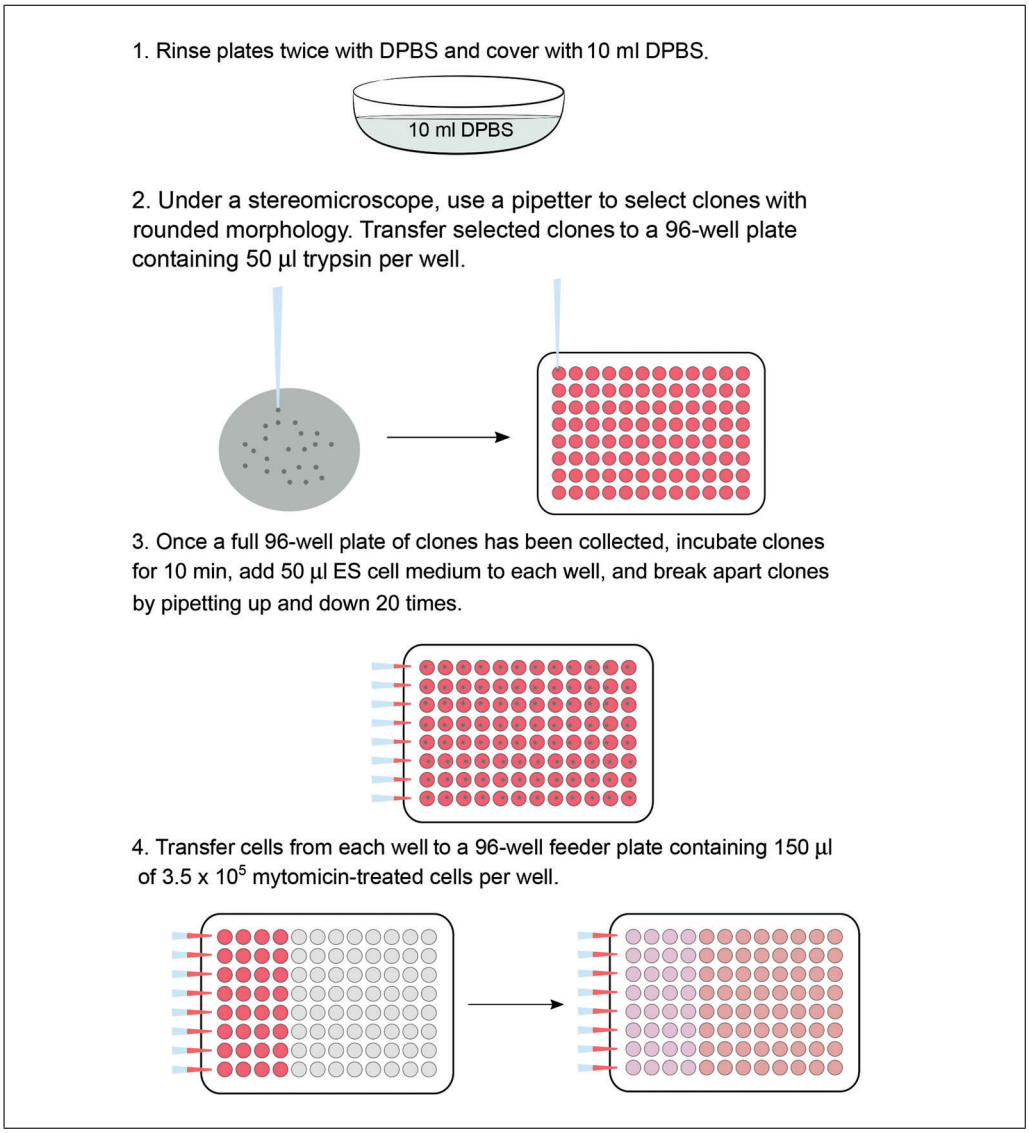

Figure 4 Steps in picking ES cell clones.

27. Add 50 µl of 0.25% trypsin/well and incubate for 10 min at 37°C. Add 50 µl ES cell medium and disaggregate the cells.

28. Transfer 50 µl cells from each well to a gelatin-coated 96-well plate and culture for 4-5 days.

    This plate will be used for DNA isolation.

29. To the remaining 50 µl cells, add 50 µl of 2× freezing medium and mix several times. Wrap the plate in Parafilm and place in a food storage bag. Put plates in a small Styrofoam box and place in a −80°C freezer.

Isolate DNA and perform Southern analysis

DNA from the ES cell clones is isolated by a simple extraction technique and digested with a restriction enzyme directly in the 96-well plate (Ramirez-Solis, Davis, & Bradley, 1993). *Eco*RI, *Eco*RV, *Bam*HI, *Bgl*II, *Pst*I, *Asp*718, and *Sst*I give complete digestions using this isolation procedure. Following restriction digestion, the DNA is run on a gel containing four combs of 24 wells each, so that one 96-well plate equals one gel. The DNA is then transferred to a nylon membrane by Southern blotting and hybridized with radioactively labeled 5′ and 3′ single-copy probes that lie outside the homology arms of

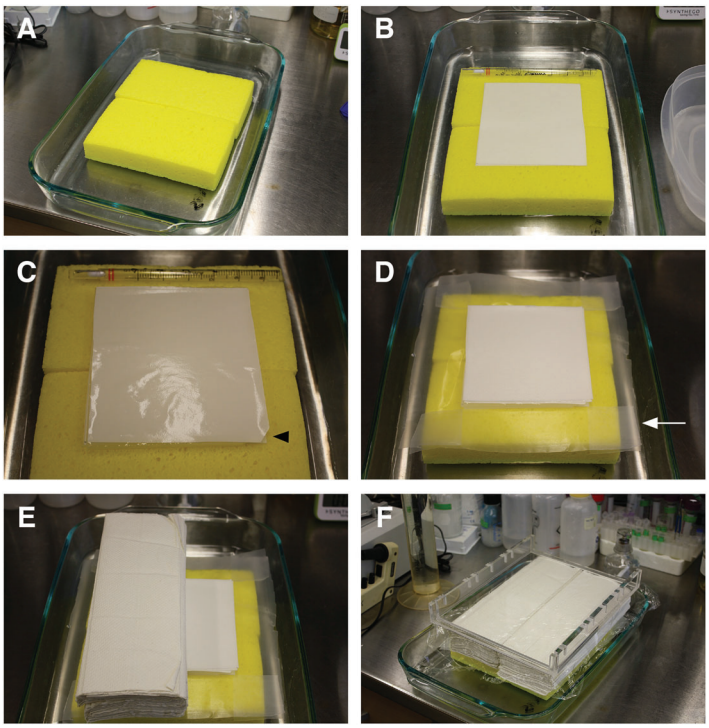

Figure 5 Assembly of Southern blot stack for screening ES cell clones. Refer to Basic Protocol 1, steps 35-45.

the vector. Blots are then washed and exposed to X-ray film to detect clones that have undergone targeting.

Isolate DNA

30. Wash 96-well plates (step 28) twice with 100 μl/well DPBS and then add 50 μl/well freshly prepared lysis buffer. Seal plates with Parafilm and place in a Tupperware-type container containing a wet paper towel to provide humidity. Place container in a 60°C water bath overnight.

31. Precipitate ES cell DNA by adding 100 μl NaCl/ethanol mix to each well and letting stand for 30 min.

    The precipitated DNA will form a white meshwork in the well.

32. Slowly pour off ethanol, keeping the DNA in the well. Gently rinse plates five to six times with 70% ethanol and then air-dry.

33. Digest DNA by adding 30 μl restriction enzyme mixture containing 30 U of enzyme. Seal plates with Parafilm and incubate overnight in a sealed humidified container.

34. Run DNA samples on an appropriate percentage agarose gel containing 24 wells across and four rows of wells (20 × 24 cm gel tray). Run gel for several hours at 60-70 V until the bromophenol blue of the loading dye migrates to just before the next set of wells. Photograph the gel.

Transfer DNA to membrane

35. Place three large sponges in a large glass baking dish (Fig. 5A).

36. Cut four pieces of blotting paper the size of the gel.

37. Cut one piece of the Hybond-XL blotting membrane the size of the gel and pre-wet the membrane by placing it in a tray of distilled water.

38. Flood the sponges in the baking dish with 1 L of 0.4 N NaOH.

    The 0.4 N NaOH is used as transfer solution because it denatures DNA in the gel, allowing it to bind to the Hybond-XL membrane.

39. Place two sheets of blotting paper on top of the sponges (Fig. 5B).

40. Flood with more 0.4 N NaOH and smooth out any bubbles in the blotting paper by rolling a 10-ml glass pipette across the surface.

41. In another glass baking dish, nick the DNA in the gel by incubating the gel in 0.25 M HCl (~500 ml) until the bromophenol blue dye band starts to turn yellow (typically ~7 min, depend on gel thickness).

    The nicking step aids in the transfer of high-molecular-weight DNA out of the agarose gel.

42. Carefully pour off the HCl solution and rinse the gel several times with tap water. Place the gel on the blotting paper-covered sponges.

43. Flood the gel with more 0.4 N NaOH and place the blotting membrane over the gel (Fig. 5C). Cut the bottom right corner of the gel and membrane for orientation purposes (arrowhead in Fig. 5C).

44. Flood again with 0.4 N NaOH and place the remaining two blotting papers on top of the membrane (Fig. 5D). Place two strips of Parafilm around the sides, top, and bottom of the papers (arrow in Fig. 5D) to prevent short-circuiting of the transfer solution between the sponges and the paper towels.

45. Add a 4-in. stack of paper towels on top of the blotting paper (Fig. 5E). Compress the paper towels lightly by adding a glass plate or the acrylic gel tray (Fig. 5F) and let transfer proceed for 4 hr.

    For illustrative purposes, the stack of towels is shown to one side. In practice, the paper towels should fully cover the blotting paper.

    Do not use heavy items on top of the paper towels, as this may adversely affect transfer.

46. Disassemble the transfer setup, rinse the Hybond-XL membrane twice with 2× SSC in a baking dish or tray, and allow membrane to air-dry.

    The bromophenol dye band transferred to the blotting membrane will turn blue again when the membrane is neutralized.

47. For easy handling during hybridization and washing, label the membrane with a pencil and cut into four strips representing each well tier, and then place in a hybridization bag until use.

Perform hybridization

48. Label 100-200 ng of 5′ and 3′ single-copy hybridization probes using the MegaPrime labeling kit and ^{32}P-dCTP (NEN BLU013H).

49. Remove unincorporated ^{32}P-dCTP by spinning the reaction through a G50 Sephadex syringe column and then count 1 μl in a scintillation counter.

    Probes should have a specific activity of $0.5-1 \times 10^9$ cpm.

50. Pre-hybridize membrane strips in 50 ml hybridization buffer in a heat-sealed hybridization bag for 1 hr at 42°C in a shaking water bath.

51. Pour out hybridization buffer and add 20 ml fresh hybridization buffer.

52. Denature probe by heating to 95°C in a thermocycler for 5 min, then place on ice.

53. Add probe to hybridization buffer in the bag. Smooth out any air bubbles by carefully rolling a glass pipette across the bag, then seal the bag. Place in a shaking water bath at 42°C and hybridize for 24 hr.

    The hybridization bag can be weighted down using a glass plate or small weights placed on the edges of the bag.

Wash blots

54. Pour off radioactive hybridization solution and dispose as directed by your institution's safety department.

55. Transfer blots to a Tupperware-type container with 500 ml of 2× SSC/0.1% SDS and rinse once.

56. Transfer blots to a second container of the same solution and wash for 30 min at 65°C with gentle shaking.

    Dispose of the radioactive hybridization bag and your gloves as radioactive solid waste for disposal by your safety department. Monitor all work areas and your person with a GM counter for potential contamination with radioactivity.

57. Wash blots twice in 0.1× SSC/0.1% SDS for 30 min each time at 65°C.

58. Rinse blots with 0.1× SSC and air-dry until just damp.

59. Wrap blot strips neatly in plastic wrap and orient according to their position on the original gel. Place in an X-ray film cassette with an intensifying screen and expose Biomax XAR X-ray film for at least 24 hr.

Expand clones and prepare cells for microinjection

Targeted clones are thawed and gradually expanded on consecutively larger feeder plates. Clones are fed daily with ES cell medium and passaged when ~70% confluent. Cells from a 6-cm plate are frozen in cryovials and stored in liquid nitrogen before microinjection. For V6.5 ES cell clones, three to six targeted clones are expanded and at least three vials of cells are frozen per clone. To begin, three targeted clones are injected per project.

60. Prepare one or two 4-well feeder plates, one 12-well feeder plate, and two 6-cm feeder plates as described above (see steps 1-12). Use 0.5 ml of mitomycin-treated cells per well for the 4-well plate(s), 2 ml per well for the 12-well plate, and 4 ml for the 6-cm dishes.

    Feeder cells should be prepared so that plates are seeded the day before use.

61. Remove a 96-well plate containing targeted clones from −80°C and place in the CO_2 incubator for 15-20 min or until clones are thawed.

62. Transfer cell contents (~100 μl) from the appropriate wells to the 4-well feeder plate(s) containing 1 ml ES cell medium per well.

63. Feed cells the next morning to remove DMSO and oil.

64. Feed cells daily and passage the entire well to a 12-well feeder plate, usually in 3-4 days before cells become confluent. When cells are 70% confluent, passage 1:2 to two 6-cm feeder dishes.

    Do not let cells overgrow and do not passage them too sparsely.

65. Trypsinize cells from the 6-cm plates and count cells with a hemocytometer. Adjust cell concentration to 10^7 cells/ml and add an equal volume of 2× freezing medium.

66. Prepare at least three 2-ml cryogenic vials of cells per clone, place in a cell freezing container, and store at −80°C for 24 hr. Transfer to liquid nitrogen for long-term storage.

Perform microinjection

67. Remove a vial of cells from liquid nitrogen and thaw to a 6-cm feeder plate. Feed cells daily and passage 2-3 days later to a new 6-cm feeder plate so that the dish will be ~60% to 70% confluent after 2 days. Feed cells 4 hr before injection.

68. Trypsinize cells for 7-10 min and then disaggregate them into a single-cell suspension.

    If a second injection will be needed, passage the cells at 2 days ~1:6 to a new 6-cm feeder plate. Take the remaining cells, place them in a 15-ml tube, and centrifuge for 5-10 min at 400 × g. Gently resuspend the cell pellet in 2-3 ml ES cell injection medium and place the tube on ice for injection.

69. Place ~200-300 μl cells into an injection chamber containing ES cell injection medium. For V6.5 cell clones, inject ~30 C57BL/6N blastocysts with 10-12 ES cells each.

    For 129 cell clones, inject ~30 C57BL/6N blastocysts with 12-15 ES cells each. For KOMP and EUCOMM C57BL/6N ES cell clones, inject 40-50 blastocysts with 12-15 ES cells each. Use C57BL/6 albino strain blastocysts when injecting C57BL/6N KOMP and EUCOMM clones.

70. Transfer six to seven injected blastocysts to the oviduct or uterine horn on each side of each recipient mouse.

    Potential chimeric mice are born 17-19 days later.

71. Pick three to four high percentage chimeric male mice based on coat color for each clone and breed them with C57BL/6N females to test for germline transmission.

    For V6.5 or 129 ES cell clones, if germline transmission has occurred, one or more pups will have an agouti coat. Genotype the agouti pups to identify mice that are heterozygous for the mutation. Chimeric males generated using EUCOMM or KOMP C57BL/6 JM8A3 cells, in which the agouti mutation has been corrected, can also be crossed with C57BL/6N female mice to test for germline transmission by observing the coat color. For C57BL/6N JM8 clones, chimeras can be mated with C57BL/6 albino strain mice to identify germline transmitters. As all commercial C57BL/6 albino strains are J substrain and the EUCOMM and KOMP ES cells are N substrain, breed the identified germline transmitters with C57BL/6N females and genotype all the pups to identify heterozygous pups.

SCREENING ES CELL CLONES BY LONG-RANGE PCR

ALTERNATE PROTOCOL

As an alternative to Southern analysis, ES cell clones can be screened by long-range PCR to detect clones that have undergone homologous recombination. DNA for PCR is prepared using the same protocol as for Southern analysis. Clones with a targeted allele are identified using a primer to a unique cassette sequence in the targeting vector, such as *neo*, and a second primer located outside the region of homology arm of the targeting vector. Sequence information for designing PCR strategies and primers can be obtained using the UCSC or Ensembl genome browsers.

This protocol is based on the LongAmp polymerase from New England Biolabs. If a different polymerase is used, the manufacturer's recommendations should be followed.

Additional Materials *(also see Basic Protocol 1)*

LongAmp *Taq* 2× Master Mix (New England Biolabs, M0287S)

1. Resuspend DNA in the 96-well plate (see Basic Protocol 1, step 32) with 50-100 µl DNase/RNase-free water or TE buffer per well.
2. Prepare the LongAmp PCR cocktail as follows:

   5 µl LongAmp *Taq* 2× Master Mix
   0.5 µl 10 µM cassette primer
   0.5 µl 10 µM genomic primer
   3 µl water

   Make enough master mix for the number of clones to be screened plus 10%.
3. Add 1 µl ES cell DNA to 9 µl PCR cocktail for each 5′ and 3′ PCR reaction.
4. Run PCR using the following program for amplifying DNA bands up to 4 kb:

   95°C for 2 min
   35 cycles of:
      95°C for 30 s
      60°C for 15 s
      65°C for 3 min
   5 min extension at 65°C
5. Run 5 µl PCR product using the same gel set up as for Southern analysis (4 × 24-well combs, see Basic Protocol 1, step 34).

BASIC PROTOCOL 2

RECOVERY OF CRE TRANSGENIC LINES BY IN VITRO FERTILIZATION USING CRYOPRESERVED SPERM

The second critical step in generating a tissue-specific knockout mouse using the Cre-*loxP* system is obtaining the desired Cre-expressing mouse. Tissue specificity is established by the promoter controlling the expression of Cre recombinase. For example, the Sox2-Cre strain can be used to generate global knockouts, while the Nestin- and Albumin-Cre strains can be used to generate brain- and liver-specific knockouts, respectively (Hutchens et al., 2017; Liu et al., 2017; Taylor et al., 2019). In the Nestin-Cre strain, Cre expression is under the control of the Nestin promoter, which is expressed in neuronal and glial precursors (Tronche et al., 1999). Conversely, in the Albumin-Cre strain, Cre activity is under the control of the Albumin promoter, which is expressed in hepatocytes (Postic et al., 1998). Another important factor in choosing a Cre strain is the developmental day on which Cre expression begins. For example, in the Nestin-Cre strain, recombinase activity is observed by embryonic day 11 (Tronche et al., 1999). Thus, beginning embryonic day 11, cells derived from neural cells expressing Cre will have the gene deleted. There are also tamoxifen-inducible Cre strains, in which Cre activity is induced following exposure to tamoxifen (El Marjou et al., 2004; Feil et al., 1996). This provides temporal control over full-body or organ-specific Cre recombination. A final factor to consider when selecting a Cre strain is any phenotype associated with that strain that may be related to the transgene integration site (Goodwin et al., 2019). It is essential to be aware of how strain-specific phenotypes may influence experimental results.

Many Cre strains are readily available through mouse repositories. These repositories often list original papers describing the strain, the expression pattern of the recombination, any strain-associated phenotypes, and recommendations for control animals. If the live animal is available, the desired Cre strain can be ordered and bred. If the live animal is not available, the transgenic line must be recovered (detailed below).

Sperm cryopreservation has become the standard method to archive transgenic mouse lines. Straws or vials containing aliquots of cryopreserved sperm can be shipped from repositories or core facilities to university core labs and the animals recovered by *in vitro* fertilization (IVF). This protocol is based on the IVF protocol developed at the Center for Animal Resources and Development (CARD) at Kumamoto University (Takeo & Nakagata, 2011). A kit based on the CARD IVF protocol is also available from Cosmo Bio Co., Ltd. It is critical to adhere to the given times when performing the IVF steps. Additionally, the quality of the paraffin oil is vital for successful IVF. The oil should be tested before use in IVF by culturing one-cell embryos overnight under the oil to determine the percentage of embryos that develop to the two-cell stage. If the oil is good, nearly all embryos will advance to the two-cell stage.

Materials

Pregnant mare serum gonadotrophin (PMSG, ProSpec, HOR-272)
Human chorionic gonadotrophin (HCG, Sigma, 1063)
Water, embryo tested (Sigma, W1503)
Sterile saline
Female mice, ~8-12 weeks of age
HTF medium, high calcium with BSA (Cytospring, mH0114)
Paraffin oil (Nacalai USA, 2613785)
CARD FERTIUP PM MEDIUM set (Cosmo Bio Co., Ltd. KYD-006-EX)

1.5-ml microtubes (Sarstedt, 72.690)
Syringes with 27-G needles
35-cm plastic dishes
Humidified incubator
50-ml tube
Dissecting tools
200-µl pipette tips for handling embryos

1. Prepare PMSG and HCG aliquots for superovulation by reconstituting with water to a concentration of 1000 IU/ml. Aliquot 50 µl hormone per tube and store at −80°C for up to 1 year. Immediately before superovulation, thaw an aliquot and add 950 µl sterile 0.9% saline to make a 50 IU/ml injection stock.
2. Superovulate female mice by injecting 100 µl (5 IU) PMSG at 6:00 pm on Day –3 and 100 µl (5 IU) HCG at 6 pm on Day –1. Perform injections intraperitoneally using a 27-G needle.

   For each experiment, use six female mice approximately 8-12 weeks of age.
3. On Day –1, prepare a dish with four 150-µl drops of HTF medium covered with paraffin oil and place in a CO_2 incubator. Also, place a 50-ml tube of water in a beaker in the incubator.
4. At 7:30 am on Day 0, prepare a plate with a 90-µl drop of FERTIUP, cover with paraffin oil, and place in the incubator. Make a second dish with a 90-µl drop of CARD medium, cover with paraffin oil, and place in the incubator.

   Follow the kit instructions for preparing the individual FERTIUP and CARD reagents.
5. At 8:00 am, euthanize a female mouse by cervical dislocation. Remove the oviducts and place into the paraffin oil alongside the CARD medium. Use forceps to tear the oviducts and release the oocyte-containing cumulus masses into the oil. Drag the cumulus masses into the CARD medium drop.

   Figure 6 provides an image of this process.

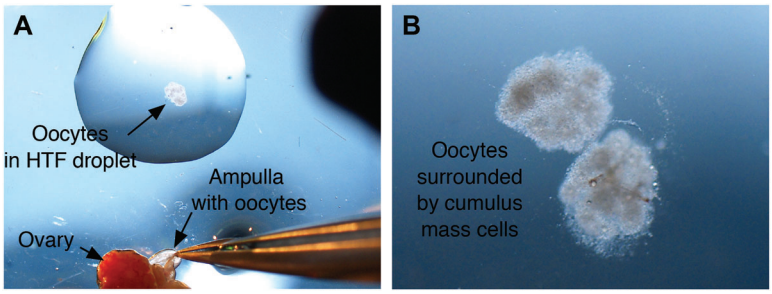

Figure 6 Isolation of mouse oocytes for IVF. (**A**) The ovary with the attached oviduct is shown towards the bottom of the image. The oocytes are removed from the ampulla of the oviduct by tearing the oviduct wall with forceps. The released oocytes surrounded by cumulus mass cells are then dragged into the HTF drop. (**B**) Higher magnification image of isolated oocytes surrounded by cumulus mass cells in the HTF droplet.

It is crucial to work with one mouse at a time and to work as quickly as possible. The entire process for one mouse should take less than a minute.

6. Repeat oviduct collection steps with the remaining mice and then return the dish with oocytes to the incubator.
7. At 8:30 am, remove the straw containing the sperm sample from liquid nitrogen. Immerse the straw in the 50-ml tube of prewarmed water in the incubator and leave undisturbed for 10 min.
8. Remove the straw and dry the outside with a paper towel. Cut the ends of the straw and carefully expel only the sperm aliquot (~10 µl) into the 90-µl drop of FERTIUP. Incubate for 30 min at 37°C in the CO_2 incubator.
9. At 9:00 am, remove 10 µl of thawed sperm from the FERTIUP medium using a 200-µl pipette tip cut at an angle to give a larger bore. Remove the sperm from the edge of the drop, to collect the more highly motile sperm. Add the sperm to the oocytes in CARD medium and incubate for 3 hr in the CO_2 incubator.
10. Wash oocytes through three of the drops of HTF medium. Move oocytes to the fourth drop and culture overnight.
11. Count the number of two-cell embryos and transfer them to the oviducts of pseudo-pregnant recipient mice.

The protocol described above is for sperm cryopreserved using the CARD method, which is widely used by mouse repositories and university transgenic core labs because of its simplicity and efficiency. For working with sperm prepared by older methods, use a slight modification of the protocol described by CARD (Nakagata et al., 2014). Transfer the thawed sperm to a 1.5-ml microtube using a wide-bore pipette tip and centrifuge the sample at 300 rpm for 5 min. Remove the supernatant and resuspend the sperm sample to ~100 µl using FERTIUP medium. Place the sperm solution in a 35-mm dish and top with oil that has been pre-equilibrated in the CO_2 incubator for at least 12 hr. Incubate the sperm suspension for 30 min in the CO_2 incubator and add it to the oocytes as described step 9 above.

BASIC PROTOCOL 3

GENERATION AND GENOTYPING OF TISSUE-SPECIFIC KNOCKOUT MICE

Once both the floxed mouse and Cre-expressing mouse are obtained, two rounds of breeding must occur in order to produce knockout mice. Generating a tissue-specific

knockout mouse requires accurate genotyping of animals maintained for experimental crosses.

Materials

Homozygous floxed mouse (*loxP* sites flanking target region)
Cre strain
Ear punching tool for mice

1. Breed the Cre strain with a homozygous floxed mouse. Genotype progeny.

   This breeding pair will produce animals that are heterozygous for the loxP sites, with or without Cre. This first breeding pair will not produce knockout mice.

2. Breed a homozygous floxed mouse with a mouse heterozygous for the *loxP* sites and expressing Cre.

   This breeding pair will produce mice that are heterozygous or homozygous for the loxP sites, with or without Cre. The mice homozygous for the loxP sites and expressing Cre are the knockout mice.

3. Genotype progeny.

   The protocol for genotyping is very similar to the protocol used for validating a knockout using genomic DNA. See Basic Protocol 4 for DNA extraction, PCR, and gel electrophoresis.

   The key differences are the tissue used for DNA extraction and the primers. Tissue collected from ear punches (or tail snips) should be used for DNA extraction. Only 50 μl of DNA extraction reagent and DNA stabilization buffer are needed, as ear punches are much smaller than tissue samples. Because ear punches or tail snips may not be the target tissue for Cre recombination, there is no need to conduct PCR for the knockout allele, as it should not be present in non-target tissue. Instead, PCR is used to detect the loxP sites and Cre. Considerations for primer design are detailed in Strategic Planning.

IDENTIFICATION OF EXON DELETION IN TARGET TISSUE

BASIC PROTOCOL 4

When the Cre-*loxP* system is used to generate tissue-specific knockout mice, Cre expression should only be found in the targeted tissue(s). For example, if Nestin-Cre is used to generate a pan-neuronal/glial knockout, the Cre should be expressed in neurons and specified glial cells (oligodendrocytes and astrocytes), but not in other tissue, such as liver. Additionally, the recombined allele should only be expressed in target tissue of mice homozygous for the floxed allele and expressing Cre. These limitations on where the Cre should be expressed and active allow for the use of genomic DNA from target and off-target tissues to validate a successful and specific knockout of the desired gene.

Broadly, the procedure for validating a knockout using genomic DNA involves designing appropriate primers (see Strategic Planning), extracting DNA from necessary tissue, conducting PCR using designed primers, and electrophoresing the PCR products on an agarose gel. The following protocol was used to validate knockout of the manganese efflux transporter *Slc30a10* in specific tissue in mice (Taylor et al., 2019).

Tissue collection for this protocol should be done with RNase- and DNase-free materials. Once the tissue is extracted, it may be placed in a 1.5-ml microcentrifuge tube. If tissue will not be used immediately, it can be flash frozen in liquid nitrogen and stored at −80°C until ready for use. The protocol for DNA extraction and PCR are based on information provided by Quantabio. Please see reagent/kit manuals for instructions if not using the same reagents as those listed below.

Materials

DNase/RNase removal solution
70% (v/v) ethanol in DNase/RNase-free ultrapure water
Fresh or fresh-frozen tissue: target and off-target tissue from both a knockout and control mouse
DNA extraction reagent (Quantabio, 84158)
DNA stabilization buffer (Quantabio, 84159)
AccuStart II 2× PCR SuperMix (Quantabio, 95136)
DNase/RNase-free ultrapure water
100 µM primers (see Strategic Planning)
Agarose
1× TAE buffer (see recipe)
5 mg/ml ethidium bromide
DNA ladder of appropriate size (Thermo Fisher Scientific, SM0244 for 100 bp, SM0314 for 1 kb)

Delicate task wipes
1.5-ml microcentrifuge tubes
95°C heat block
0.2-ml PCR tubes
Thermocycler
Agarose gel casting kit (Wide Mini Handcasting Kit, Bio-Rad)
Submerged horizontal electrophoresis cell (Wide Mini-Sub Cell GT Cell, Bio-Rad)
Power supply (PowerPac Universal Power Supply, Bio-Rad)
Gel Imaging System (GE Amersham Imager 600QC)

NOTE: Before beginning, gloves and the working surface should be cleaned with DNase/RNase removal solution, wiped with a delicate task wipe, and wiped down again with 70% ethanol in ultrapure water.

Extract DNA

1. Add 100 µl DNA extraction reagent to ~50 mg of each tissue sample in separate 1.5-ml microcentrifuge tubes. Ensure each sample is clearly labeled.
2. Centrifuge at 13,800 × *g* for 30 s to ensure the tissue is submerged in the extraction reagent.
3. Place samples in a 95°C heat block for 30 min.
4. Centrifuge again at 13,800 × *g* for 30 s.
5. Add 100 µl DNA stabilization buffer to each tissue sample and place on ice.

   Samples can be stored at −20°C for several months until use.

Perform PCR

6. Thaw PCR SuperMix and primers on ice.
7. Make a PCR master mix for each primer set. Combine the following per reaction:

   10 µl 2× PCR SuperMix
   10 µl RNase/DNase-free ultrapure water
   0.2 µl 100 µM forward primer
   0.2 µl 100 µM reverse primer

   Make enough master mix to complete all necessary reactions and still have some remaining. Vortex to mix and keep on ice.

8. Aliquot 20 μl of each master mix to one empty 0.2-ml PCR tube for each sample. Keep on ice.

   For four tissue samples (target and off-target from knockout and control mice) and three primer sets (and thus three master mixes), there should be twelve total tubes.

9. Add 2 μl of each DNA sample to the appropriate tubes. Pipette to mix and then centrifuge briefly.

10. Run PCR using the appropriate conditions. For PCR products of ~250-500 bp:

    94°C for 3 min
    30 cycles of:
        94°C for 30 s
        60°C for 30 s
        72°C for 1 min
    72°C for 5 min
    4°C for ∞

    PCR conditions are based on primers and the length of the PCR product. The annealing temperature should be ~5°C less than the lowest T_m of the primers. For AccuStart II PCR SuperMix, 1 min per kb PCR product is recommended for the extension time.

Perform electrophoresis

11. When PCR has ~30 min remaining, make the agarose gel. Weigh 2 g agarose into a microwaveable flask, add 100 ml of 1× TEA, and microwave as follows:

    Microwave for ~45 s, then swirl to mix
    Microwave for ~15 s, then swirl to mix
    Microwave for ~30 s, then swirl to mix

    When the agarose is fully dissolved, the solution will be clear. If it is not, microwave an additional 20 s and repeat as necessary until the agarose is fully dissolved.

    The percent of agarose should be based on the size of the PCR product. This 2% solution is recommended for PCR products of 100-2000 bp.

    CAUTION: *As the solution heats, it will boil. Do not let the mixture boil over.*

12. Allow agarose mixture to cool until it can be touched by hand. While it is cooling, assemble the agarose gel casting kit. Be sure to place the combs in the casting tray.

    Comb size is based on the total number of samples to be run. Ensure there are enough wells for all samples and the DNA ladder(s). Two sets of combs can be used in the same gel.

13. Add 10-15 μl of 5 mg/ml ethidium bromide to the agarose mixture (final ~0.5-0.75 μg/ml) and swirl to mix.

14. Pour the agarose mixture into the gel cast. Use a pipette tip to remove/move any bubbles. Allow at least 20 min for the gel to fully harden.

    There should be no bubbles near the wells or near where sample will run.

15. Remove the gel casting tray with the gel and combs from the casting gate, and place in the horizontal electrophoresis cell.

    In the electrophoresis cell, black is negatively charged and red is positively charged. DNA is negatively charged, so the wells need to be placed closer to the negatively charged part of the electrophoresis cell (black). This allows the DNA to flow towards the positive (red).

16. Fill electrophoresis cell with 1× TAE and carefully remove the combs from the gel.

17. Add ~5 µl DNA ladder to one well for each set of wells. Add ~10 µl of each sample to a well.

18. Place the top on the electrophoresis cell, connect the power supply, and run gel at a constant 100 V until the yellow front is ~3/4 of the way down the gel.

    If there are two sets of the wells in one gel, run the gel until the yellow front from the top row of wells is ~3/4 of the way to the bottom wells.

19. Transfer the gel tray with gel (or the gel alone) to the gel imager and image the gel using the UV light setting. Save image.

20. Dispose of the gel in a waste container designated for items containing ethidium bromide.

BASIC PROTOCOL 5

VALIDATION OF TRANSCRIPT REMOVAL USING RT-PCR AND qRT-PCR

In tissue-specific knockouts generated using the Cre-*loxP* system, Cre expression should be specific to the target tissue and Cre activity should be specific to the floxed allele, so that only the target tissue lacks expression of the floxed allele. The ideal method to validate a tissue-specific knockout is to perform immunoblotting (see Basic Protocol 6) to ensure that the targeted protein is depleted only in organs that express Cre. However, if antibodies are not available to detect the target protein, RT-PCR and qRT-PCR may be used to assess whether the knockout was both successful and specific.

RT-PCR involves extracting RNA from a tissue sample and reverse transcribing that RNA into cDNA. That cDNA product is amplified using PCR, and the PCR product is separated and visualized using gel electrophoresis as described in Basic Protocol 4. If a knockout is both successful and specific, the targeted gene should be absent in the target tissue but present in all other tissue. Additionally, expression of a ubiquitously expressed control gene should be analyzed to compare expression levels. The following protocol was used to assess expression of SLC30A10 and the mouse housekeeping gene 18S in tissue-specific *Slc30a10* knockout mice (Taylor et al., 2019).

Tissue collection should be completed using RNase- and DNase-free materials. Once extracted, tissue should be placed in 1.5-ml microcentrifuge tubes and immediately flash frozen in liquid nitrogen in order to preserve the quality of RNA. Tissue can then be processed for RNA extraction (detailed below) or stored at −80°C until use. This protocol is based on the PureLink RNA Mini Kit and the cDNA synthesis reagents from Thermo Fisher Scientific. Please see kit instruction manuals if not using the same materials described here.

Additional Materials *(also see Basic Protocol 4)*

PureLink RNA Mini Kit (Thermo Fisher Scientific, 12183025)
GeneAmp 10× PCR Buffer II and 25 mM $MgCl_2$ (Thermo Fisher Scientific, 1710071)
10 mM dNTP Mix (Thermo Fisher Scientific, 2079395)
50 µM random hexamers (Thermo Fisher Scientific, N8080127)
RNase inhibitor (Thermo Fisher Scientific, 2032166)
Multiscribe Reverse Transcriptase (Thermo Fisher Scientific, 00751040)
10 and 100 µM primers (see Strategic Planning)
SYBER Green (Bio-Rad, 172-5120)

Pellet pestles
3-ml sterile syringes (two per sample)
18- and 25-G needles (one of each size per sample)
Spectrophotometer capable of providing $A_{260/280}$ and $A_{260/230}$ ratios

96-well PCR plate and seal
Centrifuge for 96-well PCR plates
Real-time PCR machine

NOTE: Before beginning, gloves, the working surface, and all pellet pestles should be cleaned with DNase/RNase removal solution, wiped with a delicate task wipe, and wiped down again with 70% ethanol in ultrapure water. Pellet pestles should be covered with a delicate task wipe until use.

Isolate RNA

1. Isolate RNA using the PureLink RNA Mini Kit according to manufacturer's instructions. Use a separate pellet pestle and syringe for each tissue sample.
2. Determine RNA quality using a spectrophotometer. Ideal values are $A_{260/280} \geq 1.8$ and $A_{260/230} \geq 1.5$.

   RNA of lesser quality can be used if a control gene is used in addition to the target gene. RT-PCR and qRT-PCR results from the control gene can be used to determine if a particular sample is too poor to use.

   RNA can be stored up to 1 year at −80°C before use.

Synthesize cDNA

3. Thaw 10× PCR Buffer, $MgCl_2$, dNTPs, and random hexamers on ice.

   Keep RNase inhibitor and reverse transcriptase at −20°C until immediately before use.

4. Dilute RNA samples to 20 ng/µl. Test the quality and record the diluted concentration for each sample.

   For more accurate comparisons, the concentrations for all samples being run together should be within 5 ng/µl of each other.

5. Prepare cDNA master mix by combining the following per reaction:

   4.5 µl 10× PCR buffer
   6.5 µl 25 mM $MgCl_2$
   1.25 µl 10 mM dNTPs
   1.25 µl 50 µM random hexamers
   7.35 µl RNase/DNase-free ultrapure water
   0.50 µl RNase inhibitor
   0.65 µl reverse transcriptase

   Make enough master mix to complete all necessary reactions and still have some remaining. Pipette to mix (do not vortex) and keep on ice.

   RNase inhibitor and reverse transcriptase should be added last and should not be removed from the freezer until immediately before adding to the master mix.

6. Aliquot 20 µl master mix to one labeled 0.2-ml PCR tube per sample.
7. Add 5 µl of the respective diluted RNA sample to each tube. Centrifuge gently for ~1 s to ensure all liquid is at the bottom of the tubes.
8. Run cDNA synthesis PCR program:

   25°C for 10 min
   48°C for 30 min
   95°C for 5 min
   4°C for ∞

This program is based on the cDNA kit used in this protocol. If using different reagents, consult the instruction manual(s).

cDNA can be stored up to 1 year at −20°C before use.

Perform RT-PCR and gel electrophoresis

9. Perform RT-PCR and agarose gel electrophoresis as described (see Basic Protocol 4, steps 6-20) using the appropriate primers (see Strategic Planning for design of primers).

Perform qRT-PCR

10. Thaw SYBR Green and primers on ice.

11. Prepare qRT-PCR master mix for each primer set by combining the following per reaction:

    10 µl SYBR Green
    8.2 µl RNase/DNase-free ultrapure water
    0.40 µl 10 µM forward primer
    0.40 µl 10 µM reverse primer

    Make enough master mix to complete all necessary reactions and still have some remaining. Vortex to mix and keep on ice.

12. Aliquot 19 µl of each master mix into triplicate wells of a 96-well plate. Check each well visually to ensure equal volume across all wells.

    To improve the reliability of results, reactions should be done in triplicate. Thus, there should be three wells of each master mix per sample. If there are two primer sets (target gene and control gene), there should be six wells per sample.

13. Add 1 µl cDNA per well and pipette to mix.

14. Place seal over plate and ensure that the seal adheres fully by gently going over each well with a delicate task wipe.

15. Centrifuge at 770 × *g* for 5 min at 4°C.

16. Place plate in real-time PCR machine and run qRT-PCR program:

    95°C for 5 min
    40 cycles of 95°C for 15 s and 60°C for 30 s
    Default melt curve (4°C for ∞)

    This program is based on the reagents, primers, and machine used in this protocol. For other reagents or equipment, see the instruction manual(s).

17. Use the C_T values obtained to calculate relative expression using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001).

BASIC PROTOCOL 6

VALIDIATION OF TISSUE-SPECIFIC KNOCKOUT USING IMMUNOBLOTTING

In addition to validating tissue-specific knockouts at the genomic and transcript levels, it is also beneficial to validate knockouts at the protein level. If an antibody for the targeted protein is available, protein levels in animal tissue can be assessed using immunoblotting. If a knockout was successful, there should be decreased protein levels in the target tissue of knockouts compared to controls, and comparable levels of protein in non-target tissue of knockouts and controls.

Immunoblotting involves isolating protein from a tissue samples, measuring the protein concentration, separating proteins using gel electrophoresis, transferring proteins to a nitrocellulose membrane, and probing a specific protein using antibodies. The following protocol is a general protocol for immunoblotting developed in the Mukhopadhyay Lab.

Tissue collection should be performed using RNase- and DNase-free materials. Once extracted, tissue should be placed in a preweighed 1.5-ml microcentrifuge tube and the wet weight of the tissue should be recorded. Tissue can be processed immediately for protein isolation (detailed below) or flash-frozen in liquid nitrogen and stored at −80°C.

Materials

10× lysis buffer (see recipe)
PBS (see recipe)
Protease inhibitor mini tablet (Thermo Fisher Scientific, A32953)
Fresh or fresh-frozen tissue
Protein assay dye reagent concentrate (Bio-Rad, 5000006)
2 mg/ml protein standard
Deionized water
30% degassed acrylamide/bis (Bio-Rad, 1610158)
1.5 M Tris·Cl, pH 8.8
0.5 M Tris·Cl, pH 6.8
10% (w/v) sodium dodecyl sulfate (SDS)
10% (w/v) ammonium persulfate (APS)
Tetramethylethylenediamine (TEMED)
70% (v/v) ethanol
2× sample buffer (see recipe)
SDS-PAGE buffer (see recipe)
Protein ladder
Transfer buffer (see recipe)
Ponceau S stain (see recipe)
1× TBST (see recipe)
Non-fat dry milk (Bio-Rad, 1706404)
Primary antibody against target protein
Secondary antibody
Detection Reagents 1 and 2 for Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, 32106)

Sonicator
1.5-ml microcentrifuge tubes
Spectrophotometer
Gel-loading pipette tips
Mini-PROTEAN Tetra Cell (Bio-Rad, 1658003) with 1.0-mm comb
Power supply (e.g., PowerPac Universal Power Supply, Bio-Rad)
Small spatula
∼9 × 9–in. (or larger) glass dish
Nitrocellulose membrane (GE, 10600033)
Filter paper (Sigma, Z270857)
Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, 1703930)
Small roller
Large ice bucket (able to fit electrophoresis transfer cell)
Square plastic Petri dish (Electron Microscopy Sciences, 70691)
Table rocker
Gel imaging system (GE Amersham Imager 600QC)

Isolate protein

1. Prepare 10 ml of 1× lysis buffer by diluting 10× stock in PBS and place on ice. Add one protease inhibitor mini tablet and dissolve.
2. Place tube with tissue sample on ice and add 100 µl of 1× lysis buffer for every 1 mg tissue.

   If the wet weight of the tissue is more than 10 mg, transfer the tissue to a larger tube before adding lysis buffer. Alternatively, cut and reweigh the tissue before adding lysis buffer.

3. Homogenize sample by sonicating at 40% output twice for 1 min with a 1 min interval between. Keep sample on ice to prevent it from warming.
4. Centrifuge at 9600 × *g* rpm for 10 min at 4°C.
5. Transfer supernatant to a new 1.5-ml tube (or larger) on ice.

Measure protein concentration (Bradford assay)

6. Dilute protein assay dye reagent concentrate to 1× and vortex to mix.
7. Label five 1.5-ml microcentrifuge tubes as 0, 1, 2, 4, and 8 µl. Label additional 1.5-ml tubes for tissue samples.
8. Add 1 ml of 1× protein assay dye to each tube.
9. Add 0, 1, 2, 4, and 8 µl of 2 mg/ml protein standard to the five labeled tubes. Add 1 µl tissue sample supernatant to the appropriate tubes. Vortex all to mix.

   When using a 2 mg/ml protein standard, these volumes provide 0 to 16 µg protein. Alter volumes as necessary to ensure sufficient protein if a different standard concentration is used.

10. Transfer mixtures to individual cuvettes and measure absorbance at 595 nm using the "0 µl" tube to blank the instrument. Record values.
11. Calculate the protein concentration of each tissue sample:

    a. Calculate a linear equation ($y = mx + b$) using the values from the protein standard, where x is µg protein and y is absorbance.
    b. Ensure the calculated slope is between 0.96 and 1. This is an indicator of accuracy and whether results from the Bradford assay can be trusted.
    c. For each tissue sample, use the respective absorbance value as y and solve for x (protein concentration in µg/µl).

Cast SDS-polyacrylamide gel

12. Assemble the gel casting apparatus using a gel cast for 1.0-mm well size.

    If needed, ensure the apparatus is properly sealed by filling the gel cast with water and waiting ~15 min. If no water leaks out, pour out the water and use a delicate task wipe to absorb any remaining water before proceeding. If water has leaked out, reassemble the apparatus and test with water again.

13. Prepare the following gel solutions:

    For 10% resolving gel:

    2.05 ml deionized water
    1.65 ml degassed acrylamide/bis
    1.25 ml 1.5 M Tris·Cl, pH 8.8
    50 µl 10% SDS

For 5% stacking gel:

2.85 ml deionized water
850 µl degassed acrylamide/bis
1.25 ml 0.5 M Tris·Cl, pH 6.8
50 µl 10% SDS

The gel percentage (percent of acrylamide) required depends on the sizes of the proteins being separated. Alter the amount of acrylamide and deionized water if a different gel percentage is required.

14. Add 50 µl of 10% APS and 5 µl TEMED to the 10% resolving gel mixture and pipette to mix.

15. Using a 1000-µl pipetter and tip, slowly add the 10% resolving gel mixture to the gel cast. Fill up to ~1 inch from the top, so there is ample room for the stacking gel and wells. Be careful not to introduce any bubbles into the gel. Keep any remaining gel mixture.

16. Add 70% ethanol to the gel cast to help level the resolving gel. Wait ~20 min for the resolving gel to harden.

    Use any remaining gel liquid to check if the resolving gel has solidified.

17. Pour out the 70% ethanol.

18. Add 50 µl of 10% APS and 5 µl TEMED to the 5% stacking gel mixture and pipette to mix.

19. Using a 1000-µl pipetter and tip, slowly add the 5% stacking gel mixture to the gel cast, filling to the top of the gel cast. Be careful not to introduce any bubbles into the gel. Keep the remaining gel mixture.

20. Add a 1.0-mm well comb and wait ~20 min for the stacking gel to harden.

    Use any remaining gel liquid to check if the resolving gel has solidified.

Load and run gel

21. While waiting for the gel to harden, prepare samples by mixing 10 µl of 2× sample buffer with 10 µl protein sample and boiling in a boiling water bath for ~10 min.

22. Once the gel has hardened, remove the gel cast plates from the gel casting stand and frames. Do not remove casting plates from the gel.

23. Secure the casting plates (with gel) in the electrode assembly. If only one gel is being used, place the buffer dam on the other side of the electrode assembly. Place in the electrophoresis cell tank. Be sure to align black to black and red to red.

    To ensure that the casting plates and electrode assembly are properly sealed, fill the space between the casting plates (or casting plate and buffer dam) with SDS-PAGE buffer (see recipe) and allow to sit for a few minutes. If buffer leaks into the tank, the assembly is not well sealed. Pour out remaining buffer, unclip the casting plate(s), and reassemble. Repeat test until a proper seal is achieved.

24. Fill the tank to the max line with SDS-PAGE buffer. Carefully remove well comb from the gel.

25. Load ~5 µl protein ladder into one well using a gel-loading pipette tip. Then load the necessary volume of boiled samples (~30-50 µg protein) into the desired wells.

26. Place the top on the electrophoresis cell tank, aligning black to black and red to red. Insert electrodes into the power supply and run gel at ~160 V for 60 min, or until the dye front reaches the bottom of the gel.

    Do not let the dye front run off of the gel.

Transfer proteins

27. Disassemble the SDS-PAGE electrode assembly and carefully remove the short plate from the gel.

    Use a small spatula to gently lift the top of the short plate. This will lightly loosen the short plate from the gel and make it easier to remove.

28. Cut off the stacking gel and place the separating gel (with spacer plate) into a 9 × 9–in. glass dish containing ~500 ml transfer buffer. Remove the gel from the spacer plate and float it in the transfer buffer.

    Use the same spatula to gently lift the top of the gel from the spacer plate, and then gently remove the spacer plate. Be careful not to rip the gel.

29. Soak two foam pads and two pieces of filter paper (cut to the size of the color-coded cassette) in transfer buffer.

30. Place the color-coded cassette from the Mini Trans-Blot transfer cell into the glass dish with the gel, with the clear side against the bottom of the dish. Be careful to avoid the gel so it does not get damaged.

31. Place one pre-soaked foam pad on top of the clear side of the cassette, followed by one piece of pre-soaked filter paper.

32. Soak the nitrocellulose membrane (cut to the size of the color-coded cassette) in transfer buffer and place on top of the filter paper. Pour transfer buffer over the membrane to remove any bubbles.

33. Gently place the gel on top of the nitrocellulose membrane.

34. Place the second pre-soaked filter paper on top of the gel, followed by the second pre-soaked foam pad. Use a small roller to roll over the foam pad once from left to right to remove any bubbles.

35. Place the black side of the cassette down and clamp the transfer apparatus using the white clip.

36. Place the transfer apparatus into the electrode module with the black sides facing each other. Place the electrode module into the electrophoresis cell tank and then place a frozen ice pack into the electrophoresis cell tank.

37. Fill tank with transfer buffer and place the entire apparatus into a large ice bucket.

38. Place the top on the electrophoresis cell bucket, aligning black to black and red to red, and run transfer at 100 V for 60 min.

Perform immunoblotting

39. Disassemble transfer apparatus and place the nitrocellulose membrane face-up in a square plastic Petri dish.

40. Pour Ponceau S over the membrane and stain for ~2 min on a rocker.

41. Pour out Ponceau S and wash membrane with water until protein bands are visible to ensure successful protein transfer. Wash any remaining Ponceau S with 1× TBST.

42. Prepare 100 ml each of 1% and 5% (w/v) solutions of non-fat dry milk in 1× TBST.

43. Pour out remaining 1× TBST and cover membrane with 5% dry milk solution. Leave at room temperature on a rocker for 60 min to block nonspecific binding.
44. Approximately 5 min before the end of blocking, prepare a 1:1000 dilution of primary antibody in 10 ml of 1% dry milk solution (alter the dilution ratio if needed). Store remaining 1% dry milk solution at 4°C.
45. Pour out 5% dry milk solution and cover membrane in diluted primary antibody. Incubate overnight at 4°C on a rocker.
46. Pour out antibody solution and wash membrane in 1× TBST for 60 min on a rocker at room temperature, changing to new 1× TBST about every 12 min (five times).
47. Prepare a 1:3000 dilution of secondary antibody in 10 ml of 1% dry milk solution (alter dilution ratio if necessary).
48. Pour out 1× TBST and cover membrane in diluted secondary antibody. Incubate for 40 min at room temperature on a rocker.
49. Pour out antibody solution and wash membrane in 1× TBST for 60 min on a rocker at room temperature, changing to new 1× TBST about every 12 min (five times).
50. Pour out 1× TBST and cover membrane in ∼4 ml of a 1:1 solution of Detection Reagents 1 and 2.
51. Image on the gel imaging system using the chemiluminescense setting.

REAGENTS AND SOLUTIONS

ES cell injection medium

88 ml knockout DMEM (Thermo Fisher Scientific, 10829018)
10 ml fetal bovine serum (FBS, defined, Hyclone, SH30070.03)
2 ml HEPES
Store up to 1 week at 4°C

ES cell medium

500 ml knockout DMEM (Thermo Fisher Scientific, 10829018)
91.5 ml FBS (defined, Hyclone, SH30070.03)
6.1 ml 100× L-glutamine (Gibco, 25030-081)
6.1 ml 100× Pen-Strep (Gibco, 15140-122)
6.1 ml 10 mM 2-mercaptoethanol
Store up to 1-2 months at 4°C
Every two weeks, add fresh L-glutamine at 1×

The 10 mM (100×) stock of 2-mercaptoethanol (Sigma, M3148) should be filter sterilized and then stored up to 1 year at 4°C.

Freezing medium, 2×

12 ml knockout DMEM (Thermo Fisher Scientific, 10829018)
4 ml FBS (defined, Hyclone, SH30070.03)
4 ml dimethyl sulfoxide (Sigma, D2650)
Make fresh

Gel buffer, 5×

151 g Tris Base
720 g glycine
Bring to 10 L with Milli-Q or deionized water
Store up to 6 months at room temperature

Hybridization buffer

25 ml 20× SSC
10 ml 10% SDS
2 ml 1 M $NaPO_4$, pH 7.0
2 ml 5 mg/ml herring sperm DNA
500 mg dry milk
50 ml formamide
11 ml sterile water
Make fresh

Lysis buffer for immunoblotting, 10×

20 ml 1 M Tris, pH 7.4
10 ml Triton X-100
10 ml 10% (w/v) SDS
Bring to 100 ml with 1× PBS (see recipe)
Store up to 1 year at 4°C

Lysis buffer for Southern analysis

For each plate:
0.05 ml 1 M Tris, pH 7.5
0.01 ml 5 M NaCl
0.1 ml 0.5 M EDTA
0.25 ml 10% Sarkosyl
0.5 ml 10 mg/ml proteinase K (Roche, 03115879001)
4.09 ml sterile water
Make fresh

Phosphate-buffered saline (PBS), 10× and 1×

For 10× stock:
80 g NaCl
2 g KCl
9.16 g Na_2HPO_4
2 g K_2HPO_4
Bring to 1 L with Milli-Q water
For 1× PBS: Dilute with Milli-Q water and adjust pH to 7.3
Store 10× and 1× PBS up to 6 months at room temperature

Ponceau S stain

1 g Ponceau S
50 ml acetic acid
Bring to 1 L with Milli-Q water
Store up to 1 year at 4°C

Sample buffer, 2×

500 µl 6× sample buffer (see recipe)
1 ml 8 M urea
100 µl 0.25 M dithiothreitol (DTT)
75 µl 2-mercaptoethanol (Sigma, M3148)
Store up to 1 week at room temperature or long-term at −20°C

Sample buffer, 6×

25 ml glycerol
5 g SDS

7.8 ml 1 M Tris, pH 6.8
25 mg bromophenol blue
Store up to 1 month at room temperature

SDS-PAGE buffer

200 ml 5× gel buffer (see recipe)
10 ml 10% (w/v) SDS
Bring to 1 L with Milli-Q water
Store up to 6 months at room temperature

STO medium

500 ml knockout DMEM (Thermo Fisher Scientific, 10829018)
38.5 ml FBS (defined, Hyclone, SH30070.03)
5.5 ml 100× L-glutamine (Gibco, 25030-081)
5.5 ml 100× Pen-Strep (Gibco, 15140-122)
Store up to 1-2 months at 4°C
Every two weeks, add fresh L-glutamine at 1×

Transfer buffer

200 ml 5× gel buffer (see recipe)
200 ml methanol
Bring to 1 L with Milli-Q or deionized water
Store up to 6 months at room temperature

Tris-acetate/EDTA (TAE) buffer, 1×

For 50× stock:
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA, pH 8.0
Bring to 1 L with Milli-Q water
For 1× TAE buffer: Dilute with Milli-Q water
Store 50× and 1× buffer up to 6 months at room temperature

Tris-buffered saline with Tween 20 (TBST), 1×

8.7 g NaCl
10 ml 1 M Tris, pH 7.4
0.5 ml Tween 20
Bring to 1 L with Milli-Q water
Store up to 6 months at room temperature

COMMENTARY

Background Information

Developed in the 1980s and 1990s, the Cre-*loxP* system has become indispensable for the generation of tissue-specific knockout mice. The Cre enzyme and *loxP* sites do not occur naturally in mammalian genomes, and were initially derived from bacteriophage (Klos, 2004; Sauer, 1998). In the late 1980s, Dr. Brian Sauer first introduced the Cre-*loxP* system to yeast, demonstrating site-specific recombination in a eukaryotic cell (Sauer, 1987). He and Dr. Nancy Henderson then introduced the Cre-*loxP* system to a mouse cell line, demonstrating functional site-specific recombination in mammalian cells (Sauer & Henderson, 1988). These studies provided the initial evidence that the bacteria-derived Cre-*loxP* system could be used to target specific genes in a variety of cell types in vitro, providing a basis for testing its function in whole organisms.

One of the first tissue-specific knockout mice was generated in the early 1990s by Dr. Jamey Marth. Dr. Marth showed that the Cre enzyme could be inserted in the mouse genome under the control of a tissue-specific promoter, the thymus promoter *lck*, and that *loxP* sites

could be integrated to flank a specific gene, β-galactosidase. The transgenic mice expressing both Cre and the floxed gene exhibited loss of β-galactosidase in thymus cells (Orban, Chui, & Marth, 1992). The successful generation of a tissue-specific knockout mouse using the Cre-*loxP* system provides a novel and revolutionary method for modeling human disease and studying the tissue-specific functions of proteins.

In addition to gene targeting in mouse ES cells as in the Cre-*loxP* system, it is also possible to generate mice with conditional alleles using CRISPR-Cas9 (Yang et al., 2013). Two benefits of using CRISPR-Cas9 are that (1) it is less labor intensive to make potential founder mice compared to gene targeting, where extensive ES cell culture and screening work is required at the start, and (2) possible founder animals can be screened as early as four to six weeks after microinjection. However, CRISPR-Cas9 does have limitations. The efficiency of obtaining founder mice with two independent *loxP* sites integrated correctly on the same chromosome can vary (our unpublished data and personal communications from other transgenic core labs). Low efficiencies are likely due in part to nonhomologous end joining (NHEJ) repair being more favored over homology-directed repair following double-strand DNA cleavage by Cas9 (Singh, Schimenti, & Bolcun-Filas, 2015). A second difficulty with the CRISPR approach is that, once potential founders are born, detailed PCR and sequence analysis is required along with time-consuming breeding steps. Founder animals are usually mosaic and can transmit multiple alleles (Yen et al., 2014). Thus, founder animals need to be extensively characterized by PCR and sequence analysis to identify animals that contain the correct floxed allele and no other alterations (Kosicki et al., 2018). Lastly, there is also the possibility of off-target mutations in CRISPR mice, although they appear to be rare (Iyer et al., 2015). Overall, the CRISPR-Cas9 system provides an alternative method for creating conditional alleles, but efficiencies can vary and extensive validation is required to ensure only the intended alterations have occurred.

Critical Parameters

In order to validate tissue-specific knockouts using RT-PCR or qRT-PCR, preserving the quality of RNA is vital. RNA is easily and quickly degraded, and poor quality or quantity of RNA decreases the reliability of RT-PCR and qRT-PCR. All working surfaces and materials must be cleaned with a DNase/RNase removal solution. Isolated RNA must also be kept on ice while in use and at −80°C for long-term storage to slow down degradation. If results from RT-PCR or qRT-PCR are inconsistent, the RNA concentration and quality should be measured again to ensure that excessive degradation has not occurred.

For both PCR and RT-PCR, if the band(s) for the PCR product are not readily detectable, troubleshoot by altering the PCR conditions. If optimizing the PCR conditions does not work, re-check the PCR primers and design new primers if needed.

When conducting qRT-PCR, accurate pipetting is necessary to ensure reliable results. Because only 1 μl of cDNA is used for each reaction, it is critical that each reaction has exactly 20 μl of solution (1 μl cDNA and 19 μl master mix). If qRT-PCR results are variable but the RNA quality is not a concern, assess the accuracy and precision of pipetting.

For immunoblotting, it is important to remember that the SDS-polyacrylamide gel is delicate. Precautions must be taken to ensure the gel does not rip during the procedure. Submerging the gel in liquid during removal and transfer help with this.

Statistical Analyses

Statistical analysis is needed to validate results obtained by qRT-PCR. Depending on the total number of groups being analyzed, varying statistical tests may be appropriate. For this protocol, relative expression is compared from only two groups (control and knockout animals), and thus a *t*-test is appropriate.

Understanding Results

For gene targeting, the number of targeted clones obtained will vary depending on the gene and the targeting vector, but efficiencies of 5%-20% are typical. The percentage of clones that contribute to the germline in chimeric mice can also vary, but our experience is that more than half of hybrid or 129 ES clones go germline and ∼40% of C57BL/6N KOMP/EUCOMM clones go germline. Given this frequency, it is recommended to purchase at least three KOMP or EUCOMM clones for your gene of interest if they are available.

The efficiency of the IVF will depend on the quality of the cryopreserved sperm. Asking the providing repository for information on the fertilization rate of the sperm can help you plan for how many mice to use for oocyte recovery. For C57BL/6 mice, we estimate to recover ∼20 oocytes per superovulated mouse. For sperm with a reported fertilization rate of

50%, we would plan to superovulate six mice to get about 120 oocytes. At this fertilization rate, there should be ~60 two-cell embryos the day after IVF. The embryos are then transferred to three or four 0.5-day pseudopregnant female mice. About 30 mice should be recovered from the transfers.

The results obtained for validating tissue-specific knockouts rely on the presence or absence of PCR products or a protein bands. Results should indicate that mice designated as knockouts (Basic Protocol 3) exhibit loss of the target gene only in the tissue associated with the promoter controlling Cre expression (Basic Protocols 4-6). An example of results obtained from Basic Protocols 4-5 can be found in Taylor et al., 2019.

Time Considerations

It takes ~9 to 12 months to generate a mouse with a floxed allele by gene targeting in ES cells if everything goes as planned. The IVF procedure takes 1 week to complete, and 19 days are needed for pups to be born after embryo transfer. Recovered Cre transgenic lines are ready for crossing to floxed allele mice in 8-10 weeks. Once that litter is born and is ready for breeding (~3 months), heterozygous mice are then crossed with floxed mice to produce the knockout strain (~3 weeks).

Validation of the generated knockouts takes considerably less time. Validation using genomic DNA takes 4 hr. RNA extraction requires 1 hr and cDNA synthesis requires 2.5 hr. RT-PCR and qRT-PCR can then be completed in 2 hr and 3 hr, respectively. Immunoblot analysis takes 6 hr.

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Internet Resources

www.en.vectorbuilder.com
VectorBuilder (Cyagen).

http://genome.ucsc.edu/
University of Santa Cruz (UCSC) genome web browser.

www.ensembl.org
Ensembl genome browser.

www.findmice.org
International Mouse Strain Resource (IMSR).

https://www.jax.org/mouse-search
The Jackson Laboratory mouse search.

https://www.mmrrc.org/
Mutant Mouse Resource and Research Centers.