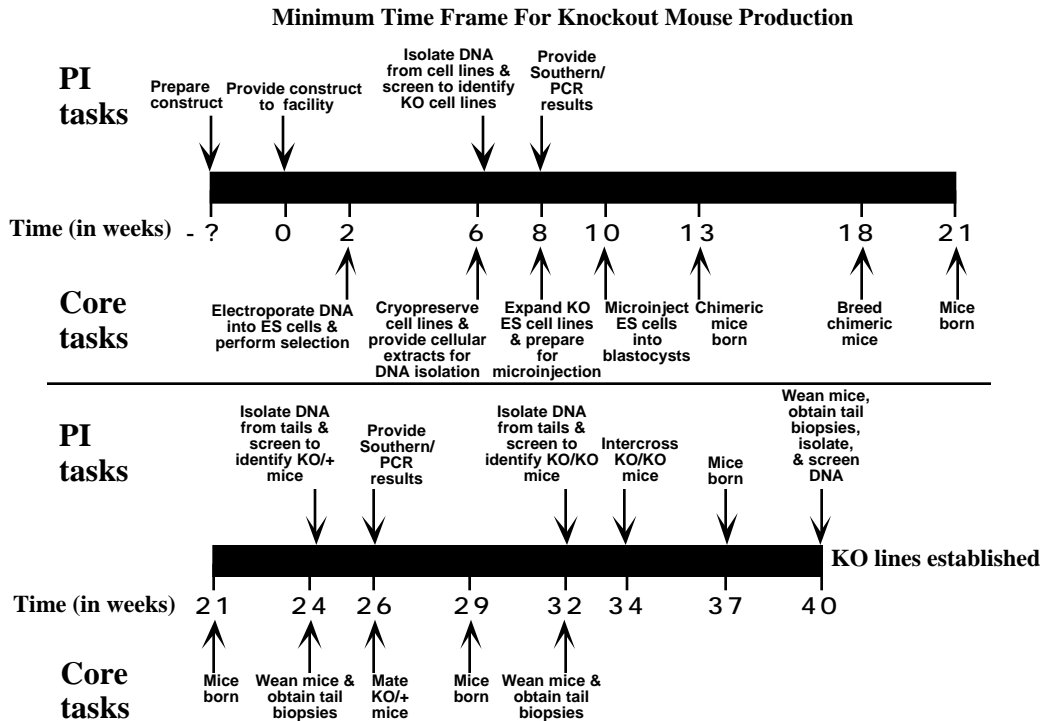


Knockout Mouse Production

1. Time course. This is a time course for generating knockout mice with a genetic mutation introduced by homologous recombination (gene replacement, gene targeting, induced mutation, knockout, etc.). The investigator must isolate and characterize genomic clones from a mouse 129/SV genomic library, and then design and make the appropriate construct that contains selectable markers. The Core will electroporate the construct into embryonic stem (ES) cells and isolate ES cell lines that have integrated the construct by homologous recombination. We will then microinject knockout ES cell lines into mouse blastocysts to generate chimeric mice. We will breed chimeras to obtain offspring that are heterozygous for the knockout allele. Finally, we will breed the heterozygous offspring to obtain mice that are homozygous for the knockout allele. The minimum time frame for the generation of knockout mouse lines is shown below. Contact Maki Wakamiya (Ph. 409-772-2811, Email: mawakami@utmb.edu) with any questions.



2. Experimental design. Decide what you want to accomplish: 1) Gene inactivation by replacement of essential coding sequences with a neomycin (Neo) cassette, 2) Introduction of a point mutation or other type of alteration in the gene, 3) Introduction of loxP sites so that the gene can be selectively inactivated in Cre recombinase expressing cells (i.e. a tissue specific knockout), or 4) A knock-in with a homologous gene. Once these decisions have been made, a detailed map of the genetic locus is needed.

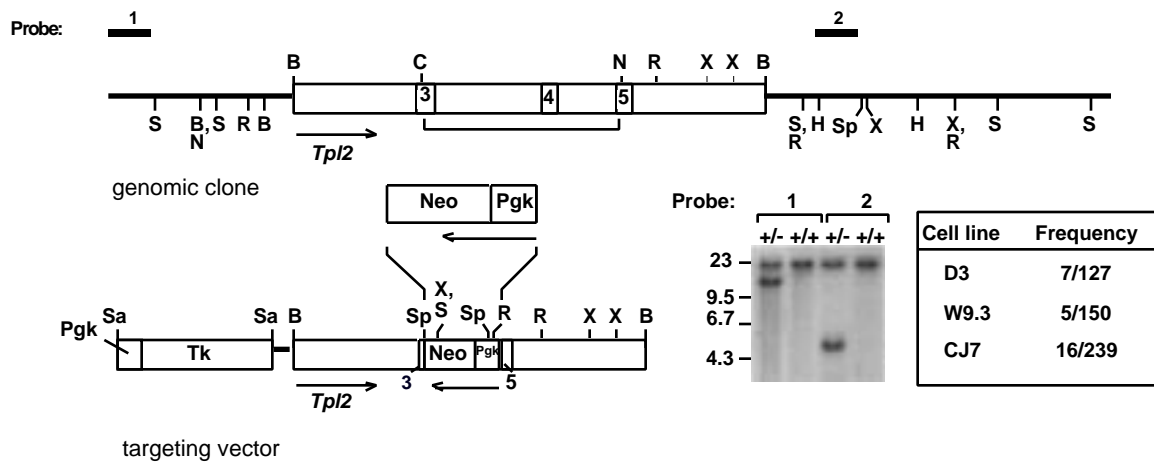
3. Cloning and mapping the gene. Screen a 129/Sv mouse library and obtain genomic clones of your gene. Once you have isolated genomic clones, develop a detailed

restriction map and the intron/exon structure of the gene. Determine which regions of the gene contain important domains that are essential for its function. Sequencing of the exons and intron/exon boundaries may be valuable for determining where important domains are located. You will use this information for planning a gene targeting strategy and for designing a construct. The best strategy for obtaining a null mutation is to delete important regions of the gene. **Devising a strategy for screening for the homologous recombination event is essential prior to beginning the experiment.** Screening can be conducted by either Southern blot analyses or by PCR. The ideal probe for Southern blot analyses is one that maps outside of the targeting vector and is able to distinguish between homologous versus nonhomologous recombination.

4. Making the targeting vector. The most common method of selection for homologous recombination is positive-negative selection (i.e. positive selection for Neo and negative selection for thymidine kinase). The targeting construct contains the Neo and thymidine kinase (TK) selectable markers and 6-10 kb of genomic DNA from the targeted gene (to ensure an adequate targeting efficiency). The goal is to replace the normal gene on the chromosome with the targeting construct by homologous recombination. In experiments designed to cause a loss of function mutation, this is referred to as a gene knockout.

If the investigator does not wish to make the targeting vector, the Recombinant DNA Laboratory (directed by Dr. Tom Wood, Ext. 70387) can perform this service. **We have the PGK-neo and PGK-TK plasmids. Since we have established conditions for selection, please use our selectable markers when making your targeting construct, if possible.** Restriction maps for these plasmids are available upon request.

An example of a knockout vector with selectable markers is shown below. For this knockout construct, a 3-kb deletion was made between exon 3 and exon 5, and replaced with the PGK-Neo selectable marker. PGK-TK was inserted upstream of exon 3. The probes used for screening by Southern blot analyses (Note that these probes were not part of the targeting vector), the Southern blot hybridization, and the targeting efficiency for this vector are illustrated.



5. Isolating knockout ES cell lines. The Core will introduce the targeting vector into ES cells by electroporation and will pick a few hundred clones that survive selection. Investigators may wish to perform this step, but should be aware that ES cells must be cultured under conditions that maintain their pluripotent capacity and inhibit their differentiation; this procedure requires impeccable tissue culture technique and specialized reagents. We will cryopreserve cell lines that survive selection and provide investigators with cellular extracts from these lines for DNA isolation for analyses by Southern blots or PCR.

6. Identifying targeted clones. The investigator will analyze DNAs from the cell lines by Southern blot hybridization or PCR to identify cell lines that have undergone homologous recombination with the targeting vector (see protocols for “DNA from ES cells” and “Southern blots” on our web page). The frequency of homologous recombination following electroporation of the targeting vector into ES cells and subsequent selection can range from 1:10 to 1:500 (or higher).

7. Chimera production. After knockout ES cell lines are identified, the Core will thaw, expand, and microinject the ES cell lines into C57BL/6 mouse blastocysts. We will then transfer the blastocysts into foster mothers. The mice born from these procedures are referred to as chimeras, as they are composed of cells derived from the C57BL/6 embryo and the 129/SV ES cells. C57BL/6 mice have a black coat color, and 129/SV mice have an agouti coat color. We can estimate the percentage of cells derived from 129/SV ES cells by examining the coat color of the chimeric mice.

We will perform ES cell injections in one-week blocks. Generation of a sufficient number of chimeric mice will require at least 2-3 weeks of injection. We will microinject multiple ES cell lines to maximize the chances of generating excellent chimeric mice. Due to the intrinsic variability in ES cell clones, the Core cannot guarantee that any given clone will produce germline chimeras.

8. Germline transmission and analysis. We will select the best chimeric mice and mate these mice to C57BL/6 mice in order to transmit the knockout allele through the mouse germline. We will provide the investigator tail biopsies from the offspring. The investigator will then extract DNAs from the tail biopsies and analyze these DNAs by either Southern blot analyses or PCR to identify mice that are heterozygous for the knockout allele. We will then intercross the heterozygous knockout mice to generate homozygous knockout mice. When the offspring of the intercross are 20 days of age, we will transfer care and maintenance of these mice to the investigator. At the time of weaning, my technicians will conduct an orientation to the investigator's staff (if needed) about how to collect tail biopsies, and how to maintain knockout lines. If the investigator wishes, the facility can maintain mouse stocks for \$25.00/hr¹. This service will be available as long as the facility is not overwhelmed with maintaining too many knockout stocks. In this case, priority will be given to the newest knockout stocks and to collaborative projects.

For recommendations how to maintain lines, see “Maintenance of transgenic/knockout stocks” in the protocols section of our web page.

9. Analyzing the phenotype of the knockout mice. The final stage in the process is to determine whether homozygous knockout mice have any detectable phenotype. One possible result is that deletion of the gene results in embryonic lethality. It is also possible that deletion of the gene results in neonatal lethality. Knockout mice for some genes may have an easily detectable phenotype, but for other genes may not show any phenotype.

Commonly Asked Questions

What services are offered by the Transgenic Core?

- (1) Microinjection of DNA into the pronucleus of fertilized eggs to generate transgenic mice.
- (2) Isolation of ES cell lines that have targeted mutations (knockout cell lines) by electroporation of DNA into ES cells.
- (3) Microinjection of ES cells into mouse blastocysts to generate knockout mice.
- (4) Rederivation of specific pathogen free mice from pathogen infected mice.
- (5) Cryopreservation of mouse embryos and mouse sperm for investigators who wish to stop breeding a line or to preserve a mouse line from accidental loss.
- (6) Regeneration of a mouse line from cryopreserved embryos or sperm.
- (7) Special services: Isolation of DNAs from tail biopsies of founders, screening for transgenic founders by Southern hybridization, maintenance of transgenic lines generated by the facility, and ovary transfers on mouse lines that do not breed well naturally to maintain such lines.

What is a knockout mouse?

A knockout mouse is derived from ES cells that have integrated a knockout construct by homologous recombination. This construct is cloned in the lab using recombinant DNA technology. The targeting construct precisely replaces a segment of genomic DNA (hence the name "gene targeting") in the ES cell. The knockout ES cells are then microinjected into normal mouse blastocysts where they mix with cells from the normal embryo to form a chimeric mouse (that contains cells derived from both the host embryo and knockout ES cells). Up to 100% of the resulting mouse chimera can be formed from cells derived from knockout ES cells. The chimeric mice are bred to produce mice that are heterozygous for the knockout gene. The knockout allele can now be transmitted as a Mendelian trait.

What is involved making knockout mice?

The investigator obtains and maps a 129/Sv genomic clone of the gene of interest. The investigator then designs and makes the targeting vector and purifies it for electroporation into ES cells. The Core will then electroporate the DNA into ES cells and perform cell selection of potential cell lines that may have undergone homologous recombination. The

investigator will be provided cellular extracts from several hundred ES cell lines, isolate DNAs from these cell lines, and perform Southern blot/PCR to identify cell lines that have undergone homologous recombination. The Core will then expand these cell lines, microinject the ES cell lines into blastocysts, and then transfer the embryos into foster mothers. We will breed the chimeric animals, and provide tail biopsies of the offspring for DNA isolation by the investigator. We will mate heterozygous knockout mice to each other. When the offspring of this intercross are 3 weeks of age, we will transfer the maintenance of the knockout mouse lines to the investigator.

How much does it cost to generate knockout mice?

(1) Identification of knockout ES cell lines: \$5400/electroporation¹.

(2) Microinjection of ES cells into mouse blastocysts: \$4200/week of injection + \$850 set up for the purchase of C57BL/6 male mice¹.

The cost of knockout mouse generation can vary from construct-to-construct and will depend upon: (1) whether more than one electroporation is required to generate the knockout embryonic stem cell lines and (2) the number of weeks of microinjection of the embryonic stem cell lines requested by the investigator. The investigator pays for the per diem mouse cage charges, starting with the day that microinjected blastocysts are transferred into foster mothers. This cost is currently \$0.69/cage/day, and is billed to the investigator directly by ARC.

Why is it important to use 129/SV genomic DNA for constructing the targeting vector?

Most ES cell lines were derived from 129/SV mice. The frequency of homologous recombination is significantly higher when the DNA used to make the targeting vector matches the strain of the ES cells (because there should be 100% homology at the DNA sequence level).

What can I do to maximize a successful gene-targeting outcome?

The frequency of homologous recombination following electroporation of the targeting vector into ES cells and subsequent selection can range from 1:10 to 1:500 (or higher). It is not entirely clear why there is such dramatic variability. Steps that the investigator can take to maximize targeting efficiency are to: (1) include 6-10 kb of genomic DNA in the targeting vector, (2) use genomic DNA isolated from a 129/SV genomic library, (3) provide highly purified DNA to the Core for electroporation. Because of the intrinsic variability in ES cell clones, we can not guarantee that the resulting mice will transmit your targeted gene through the germline. Contact Jeffrey Ceci for more information on gene targeting projects.

What paperwork must be completed?

The UTMB Animal Care and Use Committee (IACUC) and Biological Safety Committee (use of recombinant DNA) must approve your project. The paperwork for IACUC approval can be obtained from the IACUC web site (see "Links to other sites" on our web page), and the paperwork for Biological Safety Committee approval can be obtained by calling Biological and Chemical Safety (Ext. 21781). **We have a sample IACUC**

protocol that is available upon request. In addition, you must also fill out a request for knockout mouse production. We begin projects in the order that we receive this paperwork, along with the construct. If multiple constructs are received from the same investigator, it may be necessary to alternate projects between different investigators.

What additional steps are required for a tissue specific knockout (conditional knockout)?

Your targeting constructs must contain site-specific recombination sites (LoxP or Flp recognition sequences) flanking the part of the gene that you would like to delete. You must decide how you would like to express the recombinase such that your gene is deleted in the desired tissue(s) at the appropriate time during development. Possible methods include (1) a transgene expressing the recombinase (Cre or Flp) linked to the appropriate promoter, (2) a transgene expressing the recombinase controlled by an inducible system (i.e. tetracycline, RU-486, or ecdysone regulated gene expression systems), or an adenovirus vector expressing the recombinase introduced into the appropriate tissue(s).

Is consultation available?

We provide advice on all aspects of the development of knockout mouse models from experimental design to mouse breeding. Knockout projects often require a significant amount of interaction with the Director and should be conducted as collaborations. Please call Maki Wakamiya to schedule an appointment (Ph. 409-772-2811).

¹Fees are subject to change without notice (You will be informed at the time of the service request if fees have changed).