Gene Targeting and Transgenic Resource
Roswell Park Cancer Institute
Targeted Mutagenesis Service Request Form

Please fill out as completely as possible. Provide as many of the details in section II that apply to your construct. Return to Aimee Stablewski (aimee.stablewski @roswellpark.org or fax 845-5908; tel 845-5843).

I. Investigator Name: _____

Institution: ☐ Roswell Park ☐ SUNYAB

II. Gene targeting construct to be use:

1. Construct name: _____
2. Gene: _____
3. Is this locus X-linked? ☐ No ☐ Yes ☐ Don't know
4. Source of DNA for the targeting vector (*Include strain of mouse and species if non-mouse DNA is present):* _____
5. Description of the mutation: _____

   _____
6. Length of 5' and 3' homologous arms: 5" _____ 3"_____
7. Selectable markers present: _____
8. Other features (*Promoter trap, poly A trap, GFP fusion, LoxP sites etc)*:_____

   _____
9. Vector type: ☐ Replacement ☐ Insertion
10. Cloning vector:_____
11. Insert size: _____
12. Restriction enzymes used to linearize the vector (*For replacement type vectors (the most commonly used ones), the site chosen is outside the region of homology. It is perfectly fine if plasmid sequences remain attached to one or both homologous arms, in fact it is best if plasmid sequences remain attached to any external negatively-selected markers)*:

    _____
13. List other vector features that are relevant to the targeted mutation _____

    _____
14. Please attach a detailed map of the clone.

III. Success Issues:

1. Has a construct like this been previously used, anywhere, to make targeted mutants?
   ☐ Yes ☐ No

2. Do you have any reason to anticipate viability problems with chimeras?
☐ Yes ☐ No

3. Do you have any reason to anticipate viability problems with homozygous mutants?
☐ Yes ☐ No

IV. Animal Issues:

1. What protocol number will the mice be under when they are transferred to you? _____
Approval date _____

2. Do you have approved space to house these animals from Lab Animal Resources? _____

V. Detection of Targeted Mutants

1. How will gene targeting be confirmed in mutant ES cells?
List restriction enzymes and the probes to be used and indicate on your map where the sites and probes are located.
Southern analysis with external probes is strongly preferred using enzymes that give mutant bands smaller than the wild-type band. An internal probe is also needed to verify single copy integration of the vector (typically one that hybridizes to the selectable marker will suffice). Ideally, probes at the 5' and 3' ends will be used to confirm proper recombination at each end, but a single external probe will suffice as long as there are digests, that in combination with the internal and external probes, test for correct targeting at both the 3' and 5' ends.
External probes must be tested to ensure they are single copy before making your vector.
Fill in as much detail as is known.

Location relative to vector	DNA Fragment To use as probe	Probe Size	Enzyme(s) used in Southerns	WT band	Mutant band	Has this probe been tested?
5' end						
3' end						
internal						

VI. Getting us going on your electroporation

1. Use your preferred method for preparing DNA *but be sure to do a CsCl gradient*.
2. Linearize approximately 60 µgm of plasmid.
3. Phenol chloroform extract the DNA, ethanol precipitate it and perform an ethanol wash.
4. Fill the tube containing DNA after the ethanol wash to the top with fresh 70% ethanol and bring it to us for electroporation into ES cells.

PERFORMING HOMOLOGOUS RECOMBINATION IN EMBRYONIC STEM CELLS
Vector Design Considerations for Making Null Mutations

The most common mutation people want to make is a simple “knockout” that ablates the function of a specific gene. More commonly though, people want to make “knock-ins” in which the endogenous coding or regulatory sequence is replaced by another, or “conditional mutants” in which the mutation is tissue- or timing-specific. This summary of gene targeting is not meant to be exhaustive, but highlights the general considerations investigators need to be aware of before designing a vector for gene targeting. Before starting, please discuss your project with RPCI Gene Targeting and Transgenic Resource personnel. We may have some thoughts that are of use to you.

The first step is to isolate a genomic mouse clone containing your locus of interest. ES cells used by the RPCI Gene Targeting and Transgenic Resource are J1 and derived from mouse strain 129 Sv ^{Jae} and the genomic clone used to build the vector must come from a 129 strain as well for efficient targeting. The DNA Microarray Resource at RPCI has BAC libraries from that strain. The next step is to do some restriction mapping of the locus and identification of exonic sequences for targeting. During this process, candidate fragments for use in Southern analysis will be identified.

The most commonly used vector design for generating null mutants is a replacement vector consisting of a 5' homologous sequence from the gene of interest, a positively selectable marker such as the *Neo* gene conferring G418 resistance and a 3' homologous sequence from the gene of interest. A counter-selectable marker such as *HSV-TK* is often placed on the distal end of one homologous arm of the vector. The *Neo* marker insertion usually replaces essential exonic sequences internal to the arms of homology from the gene of interest. The combination of the deletion and *Neo* insertion usually creates a null mutation. The length of the 5' and 3' homologous sequences can range from 500 nt to several kb. There is no strict correlation between frequency of homologous recombination and the length of homology. Often one homologous sequence is longer than the other, and *TK* is placed distal to the short arm. The vector is linearized prior to electroporation such that the plasmid sequences are adjacent to the *TK* marker. Core vectors requiring only insertion of 5’ and 3’ homologous arms can be provided for your use in building your vector.

Vector design must take into account the method for screening ES cells for homologous recombinants. This is done by Southern analysis which requires the availability of probes outside the regions of homology and restriction site polymorphisms between the mutant and wild-type alleles. Ideally, the mutant allele will produce a band that is smaller than the wild-type allele. This minimizes confusing false positives caused by incomplete restriction digestion. The most reliable methods to screen for homologous recombinants include use of probes 5' and 3' of the targeting vector as well as a *Neo* probe, and multiple restriction sites. Before building a vector, make sure that external probes for screening are first tested to ensure they do not hybridize with repeated sequences.