# Beginners Guide to Gene Targeting
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- Step 8: Test your Southern strategy with mouse genomic DNA to get a wild type band
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## Step 1: Gather the latest information about your gene & protein from the literature

- What is the size of the protein in kD?
- How many amino acids?
- Where are the functional domains, binding domains, localization sequences?
- Do you have full-length cDNA or EST sequence?
- Is there any intron/exon information from mouse or human?
- Are there any enhancers located in introns?
- Are any products made with splice variants from the gene?

A great place to start gathering data is the CMC Knowledgebase: http://data.cellmigration.org/cmckb/

Sometimes, this information is available for the human gene, but not for the mouse. Therefore, make sure you check publications for both species to gather the most information possible. You can search publications using PubMed at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed

Do not rely completely on public websites for all your information because these can be out of date. Publications are your safest bet. It is possible that nothing is known about enhancers or splice variants for your gene, but if this info is available, it will help with your design.

If you have limited experience with Molecular Biology techniques (restriction cloning, PCR, etc), there are good animations explaining these at the Dolan DNA Learning Center website (http://www.dnalc.org/ddnalc/resources/animations.html)

## Step 2: Get exon/intron sequence information about your gene

You can usually find your gene of interest in a genomic database.
PLEASE NOTE:  The mouse sequence in these databases is from a C57BL/6J mouse because this is the mouse strain whose genome was sequenced.  Traditionally, mouse targeting has been done in the 129Sv background, which can be quite different at some loci. Gene targeting is now performed regularly in both

strain backgrounds.  If you are not using the MIT Transgenic Facility (its services are only available to the MIT/CMC community), you should make sure that you have access to a B6 ES cell line and that your facility has proven success with B6/white chimeras going germline before you dedicate yourself to using B6 DNA for your targeting construct. B6 ES cells can have a lower success rate for generating mice, but they do result in a mouse that has the C57B6 background which can be very important to some projects.  Please make sure you use DNA from only one strain or the other for your construct and then use ES cells of the same background for your targeting.  More information about this is included in Step 4.

Some information you should be sure to collect at this step are:
1   How many exons in your gene?
2   How many Kb does your gene encompass?
3   What is your gene location? (for example, GIT2 is located at 113842523  - 113888603 bp (113.8 Mb) on chromosome 5 according to Ensembl and EntrezGene)
4   Is there an Ensembl Gene ID? (for example, ENSMUSG00000041890)

- Ensembl http://www.ensembl.org/Mus_musculus/
- EntrezGene  http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
- Mouse Genome Informatics http://www.informatics.jax.org/
- NCBI Mouse Genome Resource http://www.ncbi.nlm.nih.gov/genome/guide/mouse/
- UCSC Genome Browser http://genome.ucsc.edu/cgi-bin/hgGateway

Often, databases cross-reference one another, and if one database gives you an Ensembl #, click on that. Ensembl has easy-to-use exon information which is critical for the design of your construct. Also, these web sites usually have some information about structural/functional domains, so make sure this information is added to the data you collected in Step 1.  The information listed on these web sites can be minimal and outdated, so do not rely on them for collection of data in Step 1.

Make sure this information is complete, as compared to the info in Step 1.  Sometimes the databases do not include all the exons for a gene!!!

## Step 3: Develop a general strategy – which exons to remove

If you are collaborating with the Cell Migration Consortium for your mouse, this is a good place to first contact Aurora (aaburds@mit.edu) if you have not done so already. Please note that the MIT Transgenic Facility is not able to offer electroporation/targeting services to investigators outside of the MIT/CMC community.
If you are unsure whether to make a tradiotinal or a conditional knockout, you can learn about your options on the Cell Migration Transgenic and Knockout Mouse Approaches page:
http://www.cellmigration.org/resource/komouse/komouse_approaches.shtml

Generally, try to remove one or more early exon(s) so that the remaining exons are out of frame and produce many stop codons.

Keep in mind the information from Step 1
- If there is an important functional domain coded for in Exon1, you should seriously consider removing it.
- If there is alternate splicing, you'll want to remove exons upstream/downstream of the splicing to ensure that you eliminate all gene products.

• If there is an enhancer in an intron, avoid placing your selection cassette there.

## Step 4: Order/Acquire the DNA

Get plasmid with LoxP, Frt, and selection cassettes from Aurora.  This plasmid is called pPGKNeo.F2L2.DTA from the laboratory of Philippe Soriano.  Please acknowledge this kind gift in any publications for the mouse.

> PGK is a strong promoter
> Neo confers resistance to Geneticin (G418) for positive selection
> F2 stands for the two Frt sites in the plasmid
> L2 stands for the two LoxP sites in the plasmid
> DTA is the diphtheria toxin, for negative selection

You can get the DNA for your locus by PCR from mouse cells or by finding a BAC with your locus (see below for  a lot of BAC info).  Remember to use ONLY DNA from a pure 129Sv background or from a pure C57B6 background!  Many mouse cell lines are of a mixed or unknown background and should not be used as a source of DNA for your construct.  Primary cells/tissue is the best source of DNA because cell lines have accumulated mutations.

If you will PCR your gene from genomic DNA, do it now, getting larges pieces (5-10kB) of your gene into a stable plasmid for further sequencing and use.  To PCR from genomic DNA, Platinum Pfx DNA Polymerase from Invitrogen works well (cat# 11708-013).  This is a high-fidelity polymerase that efficiently amplifies pieces that are up to 12kb long. This polymerase also comes with a PCRx Enhancer Solution for problematic or G/C rich templates, which has proven necessary for many researchers.  I also recommend Invitrogen's Elongase Amplification System (cat#10481-018) or Clontech's Advantage-GC Genomic PCR Kit (cat# 639118). For PCR from genomic DNA, it is better to design a primer that is 23-25bp long, with an annealing temperature around 65 rather than a shorter one that has an annealing temperature of 55.

Genomic mouse DNA is often obtained as a circular BAC (Bacterial Artificial Chromosome).  This is like a huge plasmid that holds 100-300kb of genomic DNA in each BAC.  Because each BAC is so large, it cannot be cloned using standard bacteria.  Generally, BACs are shipped and maintained in DH10B electrocompetent cells called GeneHogs (also known as HS996), (Invitrogen Catalog# C8080-10).

Thanks to the sequencing of the C57BL/6J mouse genome, you can now use BLAST and the Web to find and purchase BAC clones (on a BL/6 background) that contain your gene of interest.  Please make sure you use only 129Sv or only BL/6 DNA for your constructs, and then you must target matching ES cells.

Mouse Genome Resource:  http://www.ncbi.nlm.nih.gov/genome/guide/mouse/
> Then use Clone Finder or BLAST
TIGR BAC finder http://www.tigr.org/tdb/bac_ends/mouse/bac_end_intro.html

The RPCI-23 BAC library was assembled from a female 129S6/SvEvTac (Taconic) mouse.
The RPCI-23 BAC library was assembled from a female C57BL/6J (Jackson Labs) mouse.
The RPCI-24 BAC library was assembled from a male C57BL/6J (Jackson Labs) mouse.

The RPCI (Roswell Park Cancer Institute) BAC (Bacterial Artificial Chromosome) Libraries are assembled from DNA in one or more tissues of a mouse.  This genomic DNA was isolated and partially digested with a combination of EcoRI and EcoRI Methylase. Size selected DNA was cloned into the pBACe3.6 vector

between the EcoRI sites. The ligation products were transformed in DH10B elecrocompetent cells. Average insert size is 155 Kb, with a maximum of 300 Kb. The library has been arrayed into 384-well microtiter dishes and also gridded onto 22x22cm nylon high density filters (Amersham Hybond N+) for screening by probe hybridization. Each hybridization membrane represents over 18,000 distinct mouse BAC clones, stamped in duplicate. The RPCI-22 library has 10.9X genomic redundancy.

If the web cannot help you find a BAC with your gene, you will have to screen through one of the RPCI BAC Libraries to find the BACs that contain your gene of interest. Screening involves looking for hybridization of a radioactive DNA probe (generally 200-500 bp of an exon that will be included in your targeting construct) to the BACs. Since each library conatains upwards of 10,000 BACs, this is no small task.

The library can be purchased from CHORI or from Invitrogen. The CHORI site (http://bacpac.chori.org/mouse22.htm) has a lot of great information, and is now priced the same as Invitrogen. You can also buy half the library at half the cost, hoping to get a few good BACs from that. Invitrogen BAC Mouse CTT (9 Membranes), catalog # RPCI22.M, $2400 You can also pay CHORI to screen their library for the BAC(s) that you want. It costs about $1000 and takes 4-6 weeks, based on talking to people who have done it.

For comments and hints on screening the BAC filters:
http://www.biocompare.com/prorev.asp?profrevid=142
CHORI also has protocols for using their filters and BACs: http://bacpac.chori.org/protocols.htm

Once you have found BAC clones that probably contain your gene of interest, you can order the individual clones from:

CHORI          http://bacpac.chori.org/ordering_information.htm
or from Invitrogen      http://clones.invitrogen.com/bacpacsearch.php

Once you receive the BACs, confirm that they have your gene by PCRing small pieces of your exons from the BACs. Introns can be somewhat variable from mouse strain to mouse strain and may contain random DNA, so PCR of the exons is more specific for your gene.

Mouse genomic DNA can also be obtained in the form of:
        YACs (Yeast Artificial Chromosomes)
        Lambda phage

You must inform the ES cell facility whether you used 129 DNA or BL/6 DNA for your construct. ES cells from a 129 or BL/6 embryo will be used, respectively, for different constructs to achieve maximal homologous recombination. There is also one line of ES cells called v6.5 that is 50% 129 and 50% B6, which can be used to target with constructs from either background. I have successfully used a B6 construct to target 129 ES cells, so it is not impossible, just more difficult.
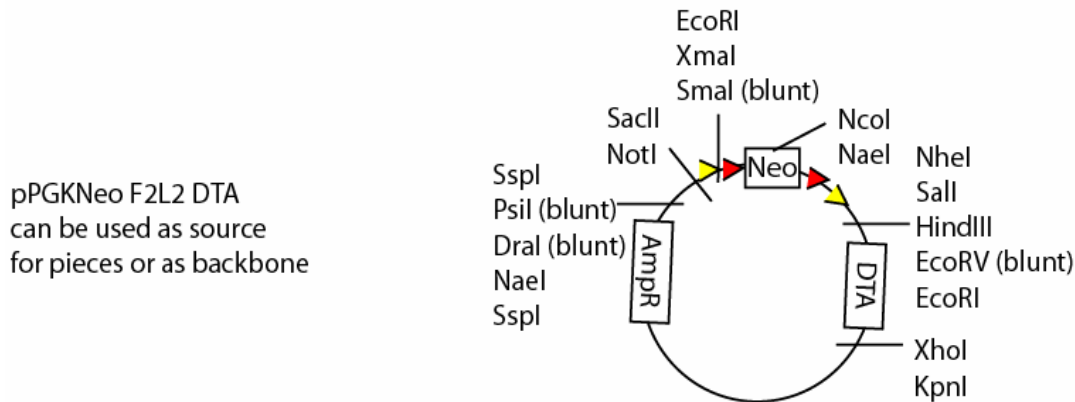
## Step 5: Design the construct
Note -Do not build your construct until you have completed steps 6, 7 and 8!
If you are using DNA from a BL/6 source, use the databases to place about 20kb of the genomic sequence surrounding the genes to be deleted (10kb on either side of where you plan on placing LoxP sites) into a program to find the restriction endonuclease sites in the area.
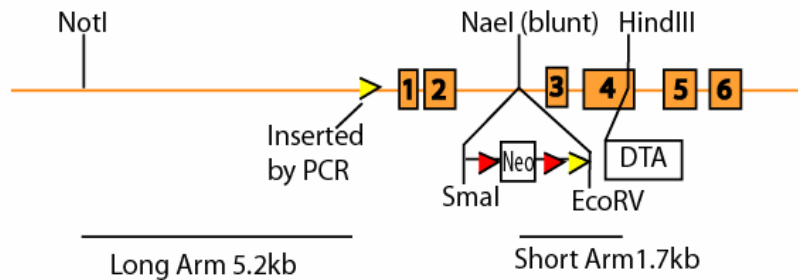
If you are using 129 DNA, you will need to sequence the regions of your gene that will be included in the construct. Generally, there is little variation between 129 and BL/6 in the exons, so you can design PCR oligos to anneal in the exons of your gene. Sequencing will allow you to get the restriction site information for your construct.

Use sequence information and restriction site information to select 5' and 3' arms of homology and location you will place the Neo selection cassette. Here are some examples to help you with arm selection and the location of inserted elements
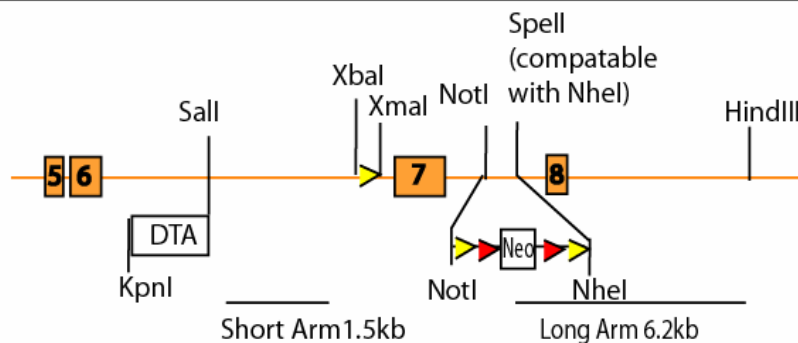
## Some Examples of Vector Assembly Plans



pPGKNeo F2L2 DTA
can be used as source
for pieces or as backbone

**Example 1**
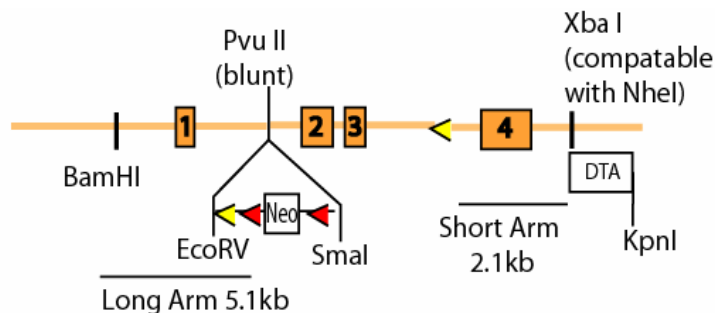-To Remove
Exon 1
(using pF2L2
as backbone)

**Example 2**
- To Remove
a late Exon
(using other
plamid as backbone)

Neo  Neomycin Resistance Gene for Positive Selection   ▷ LoxP Site
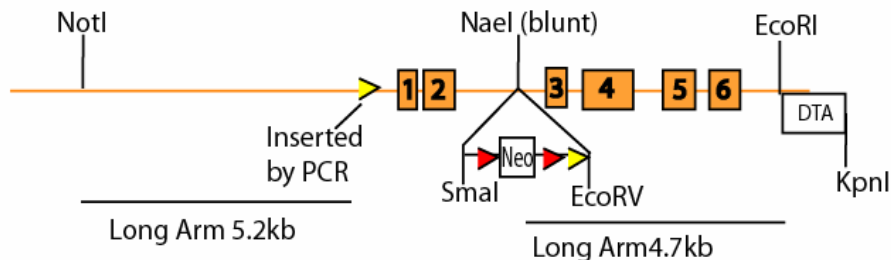DTA  Diptheria Toxin Gene for Negative Selection   ▶ Frt Site

**Example 3**
- To Remove Early Exons, but not Exon 1 (using other plamid as backbone)

Pvu II (blunt)

Xba I (compatable with NheI)

BamHI

1 | 2 | 3 | 4

DTA

EcoRV | Smal

Short Arm 2.1kb

KpnI

Long Arm 5.1kb

Neo

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**Example 4**
- using two Long Arms for increased rate of homologous recombination

NotI

Nael (blunt)

EcoRI

1 | 2 | 3 | 4 | 5 | 6

DTA

Inserted by PCR

Smal | EcoRV

KpnI

Long Arm 5.2kb

Long Arm 4.7kb

Neo

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One homology arm should be at least 4kb while the other can be a small as 1.2-2.5kb.  This short arm enables a PCR-screening strategy.  While PCR screening is much quicker than Southerns, using a shorter arm will reduce the frequency of homologous recombination.  If you do not mind doing many Southerns, or if your locus is known to be difficult to target, you may wish to have both arms of length that is a least 4kb.  If you use one small arm, place the PGK DTA adjacent to the small arm.  Having negative selection next to the shorter arm is very important and reduces the number of clones you will have to screen by about 3 fold.  To improve the odds of successful recombination even more, you can also place a PGK DTA adjacent to the long arm.  This double-end selection seems to end up with a total 4-fold improvement as compared to no DTA at all.

It is not a good idea to place the NEO piece upstream of Exon1 (in the promoter) as this will probably interfere with expression of the gene.

Develop a step-by-step cloning strategy for building your construct.  Many people find that BACs contain so much DNA that a normal digestion to get a 2-5kb piece will give so many pieces that you cannot isolate the piece you want.  The easiest way to get around this is to PCR up the piece, placing the primers 50-200bp outside of the desired restriction sites, isolating the PCR product, then restriction digestion to use the fragment for cloning.  To PCR from genomic DNA, Platinum Pfx DNA Polymerase from Invitrogen works well (cat# 11708-013).  This is a high-fidelity polymerase that efficiently amplifies pieces that are up to 12kb long. This polymerase also comes with a PCRx Enhancer Solution for problematic or G/C rich templates, which has proven necessary for many researchers.  I also recommend Invitrogen's Elongase Amplification System (cat#10481-018).  Some labs have been very successful with Clontech's Advantage-GC Genomic PCR Kit (cat# 639118) though this is more expensive than the others. For PCR from genomic DNA, it is better to design a primer that is 23-25bp long, with an annealing temperature around 65 rather than a shorter one that has an annealing temperature of 55.

If you PCR across any exons, you must sequence them to ensure that you have not introduced any mutations.

You can use any standard cloning plasmid to be the backbone of your construct.  You just want to select one that has the best restriction sites for your cloning.  Some examples:  pUC19, pBS, pBR322 etc.

Some people are lucky and they can use the pPGKNeo.F2L2.DTA as their backbone. However, most cloning strategies require using several donor plasmids along the way to assemble the final targeting construct. It is not unusual for the cloning strategy to consist of 6-12 different steps, many of which can be done in parallel, for building the construct.

For ES cell targeting, you will need to linearize your final construct. Therefore, make sure you have at least one enzyme that will cut efficiently in the backbone (but not in the inserted mouse genomic, LoxP, Frt, Neo or DTA sequences).
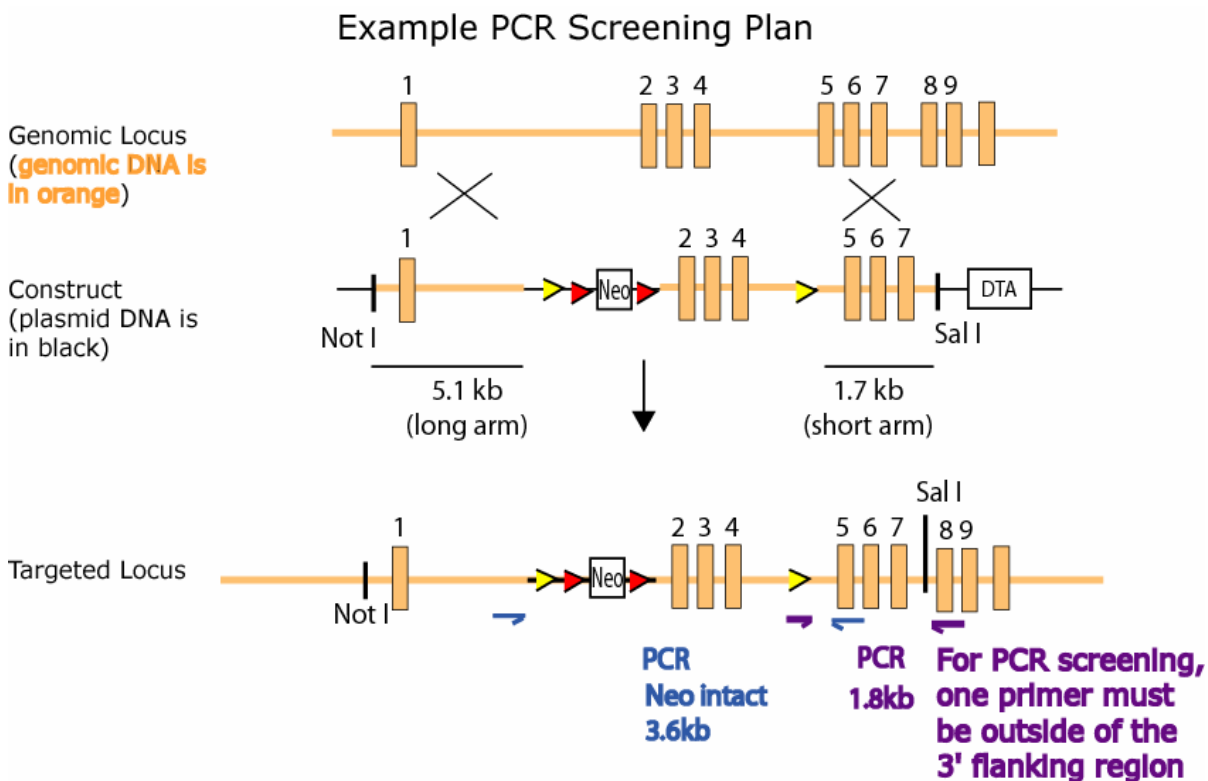
If you insert the Neo Cassette with blunt cuts (SmaI & EcoRV) you must perform restriction digests to verify orientation and to verify that only one Neo has been ligated into that location. Orientation of the LoxP sites is extremely important. The lone LoxP site (not with the Neo) must be in the same configuration as the LoxP with the Neo for your desired exons to be removed.
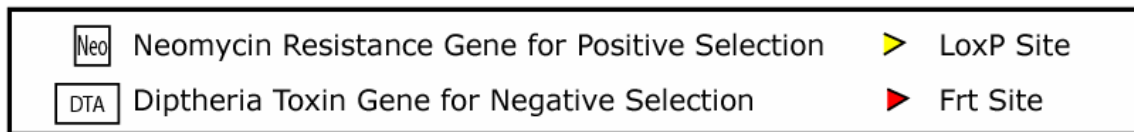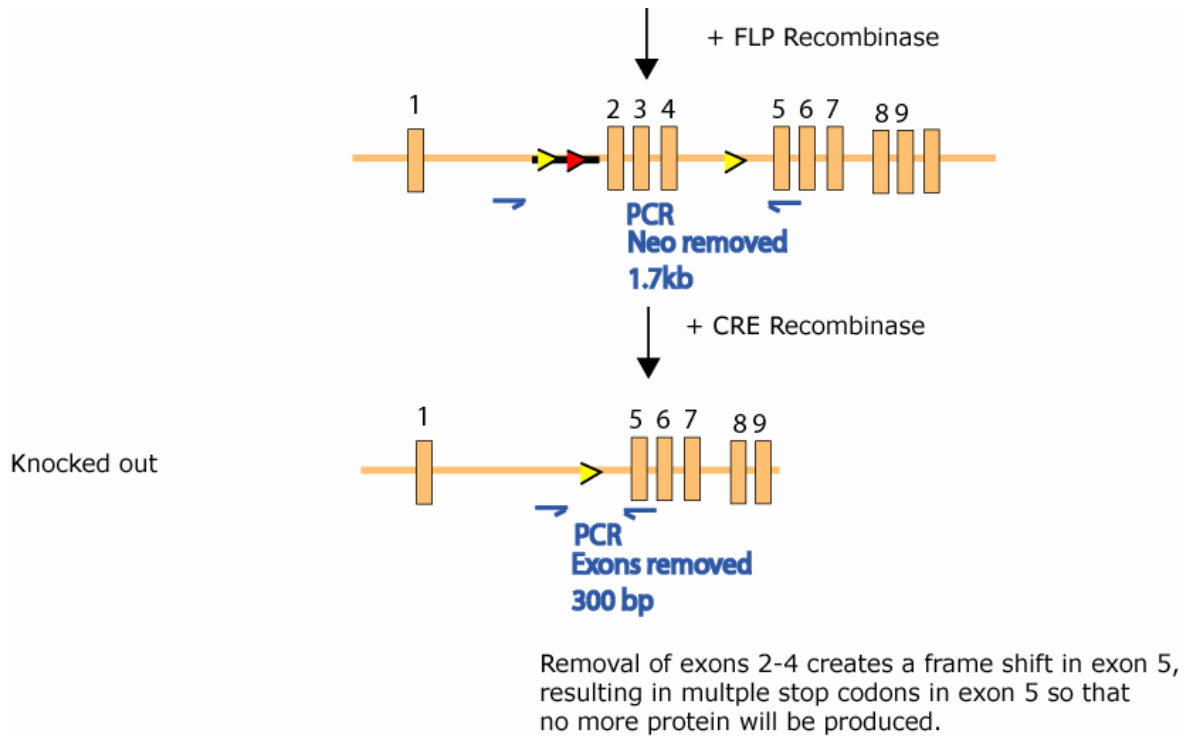
Here is the LoxP orientation (non-palindromic sequence is underlined) as it is drawn on the map for pPGK Neo.F2L2.DTA :

5' ATAACTTCGTATAATGTATGCTATACGAAGTTAT 3' is drawn ">"
5' ATAACTTCGTATAGCATACATTATACGAAGTTAT 3' is drawn "<"

## Step 6: Develop the PCR screening strategy for the small arm and recombinations



Example PCR Screening Plan

Removal of exons 2-4 creates a frame shift in exon 5, resulting in multple stop codons in exon 5 so that no more protein will be produced.

| Neo | Neomycin Resistance Gene for Positive Selection | > | LoxP Site |
| DTA | Diptheria Toxin Gene for Negative Selection | ▶ | Frt Site |

One primer for the PCR should anneal to a region of the gene that is NOT included in your construct, and the other should anneal to the Neo or to the lone LoxP site (if it is the inserted piece nearest your short arm). If you are annealing to Neo, you have many options for good oligos. However, designing oligos at the LoxP site is more difficult. The LoxP (and Frt) sites are palindromes with a non-palindromic spacer in the middle, so your primer should span the connection between the LoxP site and your genomic DNA. If you must go significantly into the palindromic region, try to extend your primer in to the non-palindromic spacer.
For example, with genomic DNA in green and LoxP in yellow (with the non-palindromic region underlined), if your construct has this sequence,

5'CGAAGGTTCTCGCACTAAAAGTGGAATAACTTCGTATAATGTATGCTATACGAAGTTATAGCTCTAAGGTACAGA
TCCAATGTTTAAGTGTGCCAGCCATCTGTTGTTTGCC3'

Then design a primer to overlap the junction: 5' CGCACTAAAAGTGGAATAACTTCG 3'

This oligo has 24 bp, and an estimated annealing temp of $68^o$. The estimated annealing temp based on $4^o$ for each G or C and $2^o$ for each A or T

To check your primers for self-annealing or hairpin structures, run them through the OligoTech analysis software program from OligosEtc: http://www.oligosetc.com/analysis.php#TECHANALYSIS

Running the above oligo on OligoTech says that it will create hairpins below $34^o$ and homodimers below $3.8^o$. This is acceptable because a PCR reaction would never get as cold as 34, but other oligos may work better. In general, oligos that do not form structures at room temperature are the most desirable.

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Let's try:

5' GGGTGGAATAACTTCGTATAATGTATGC 3'

Oligo tech finds no stable hairpins or homodimers arising from this oligo.
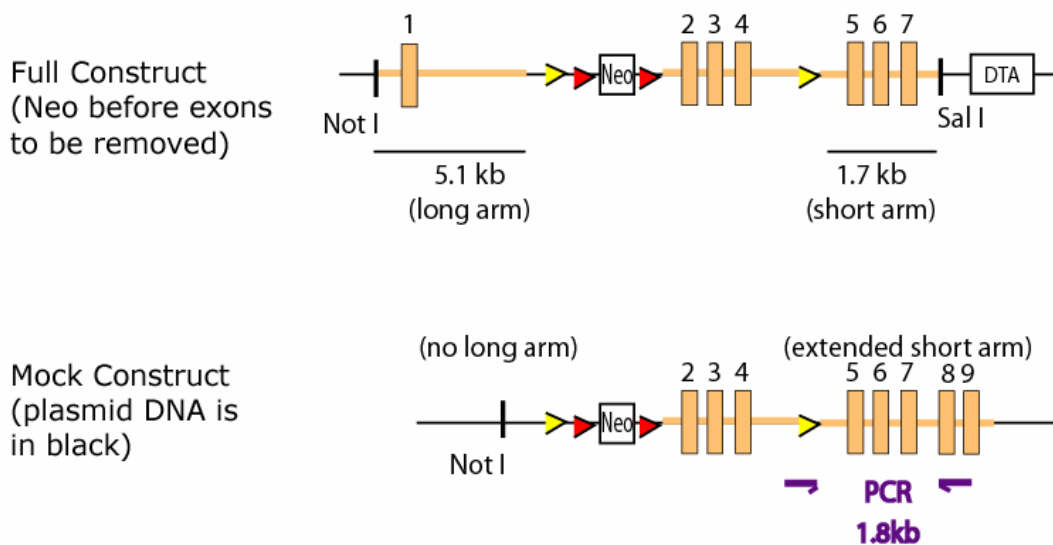
Similarly, this oligo would also work well:
5' GAAGTTATAGCTCTAAGGTACAG 3'

Also design primers that will allow you to test these oligos using either wild type DNA or your construct as the template. This will enable you to get a robust PCR for your screen.
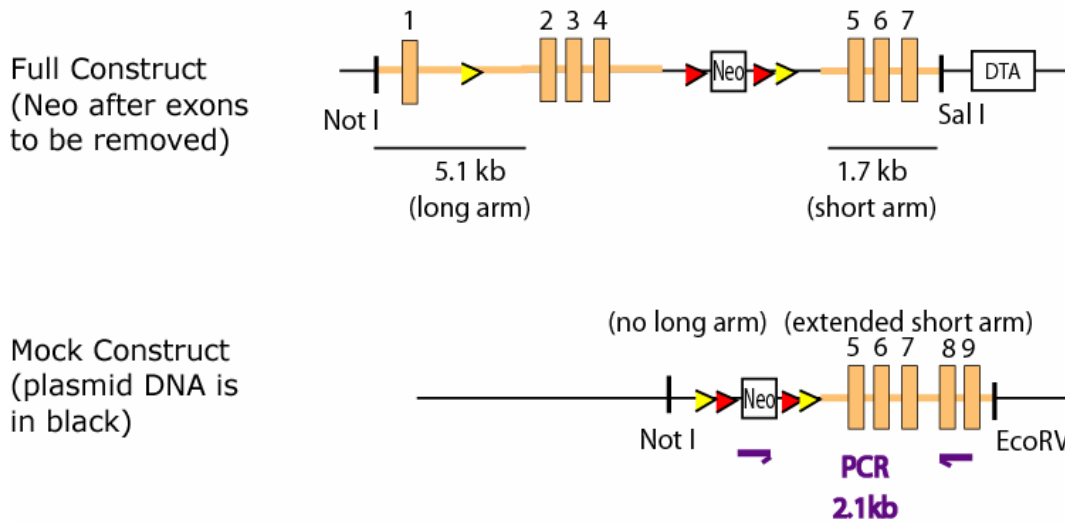
While you are designing oligos, you should also design primers that will allow you to confirm the removal of the Neo by recombination of Frt site and a PCR that confirms removal of your target exon(s) after recombination of the LoxP sites.

Test all PCR reactions to optimize amplification conditions. This is most easily done after you have completed your construct in step 9. Testing of the short-arm PCR is best to optimize with a "mock" construct that includes the Neo/FRT/LOX as need and also an extended arm

## Using a Mock construct to optimize PCR screening of the short arm



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## Step 7:  Design a Southern strategy for the long arm(s)

Select restriction enzymes for your Southern that might cut appropriately in your target gene, using a probe that hybridizes outside of the sequence used for your long arm.  To test for recombination using a Southern, you want to get a significant change in the size of the band of DNA resulting from a restriction enzyme digest.  This can be an increase in size or a decrease in size.  Generally, to clearly resolve the two bands on the gel, you want at least a 20% difference in the size of the two bands.

A good schematic representation of good Southern Screening can be found at the website of the Transgenic Mouse Core Facility at the University of Virginia:
http://www.healthsystem.virginia.edu/internet/transgenic-mouse/kodesignTMCF.cfm
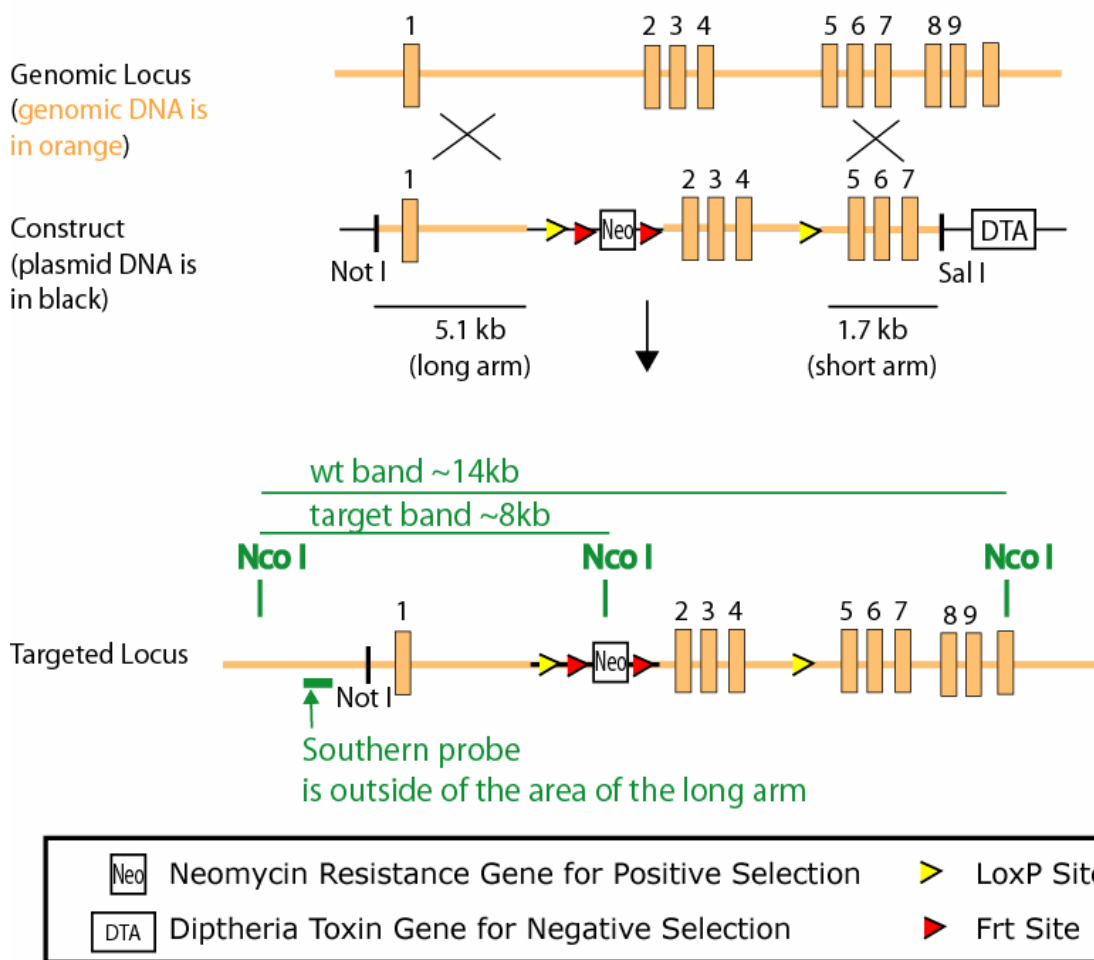
The largest band I have seen used successfully is a 30kb band, which became a 16kb band with correct targeting. the smallest I have seen is 3kb (from a 7kb wild type band).  Remember, the larger the band, the less efficiently it will transfer from your gel to the membrane, so you do not want your "targeted" band to be much above 12kb if you can help it.

From the literature, the following enzymes work very well for mouse Southerns: BamHI, BglI, Bgl II, EcoRI, EcoRV, HindIII, KpnI, NcoI, NdeI, PstI, ScaI and SphI. Southern digestions often use 2 different enzymes to get distinct bands.  If you need to do this, make sure your two enzymes are efficient cutters in the same buffer.  The NEB buffer chart and double-digest chart can be found in the NEB catalog or at
http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/double_digests.asp.

Avoid enzymes being easily blocked by CpG methylation in or near the restriction site. Check the catalog of New England Biolabs, or you can use their web-cutter program (http://tools.neb.com/NEBcutter2/index.php) they include what modification might affect your cut). In spite of methylation problems, I have found papers that used NaeI, Not I, Sal I or XhoI successfully.  These enzymes might also work well for you.

Restriction sites that are included in the PGK Neo and may be useful for the Southern are: NaeI, NcoI, SpeI. Also, there is a BamHI site just downstream of the NotI site and another one 90bp later, in between the first LoxP and Frt sites just downstream of the Xma/Sma site.

## Example Southern Screening Plan



Your probe should be 150-500+ bp long. A length of 300-800bp seems to work the best, but smaller probes can be used if needed. The probe must hybridize to DNA that is outside of the sequence that is included in the targeting construct, but that is included in the fragment of DNA crated by your restriction enzyme digest. It can, in fact, start with the basepair that is immediately before the DNA that is used for the construct or with the basepairs just inside of the restriction cut. A probe can be generated by PCR, DNA synthesis, or restriction digest.

## Step 8: Test your Southern strategy with mouse genomic DNA to get a wild type band.

You can get wild type DNA from Aurora, from cells or even from mouse tail DNA. Use only 129Sv or BL/6J DNA (whichever matches your construct) as you will need the correct one for the Southern to work optimally.

For a Southern Blot protocol and a protocol to make radioactive probe, see the methods section of this Initiative (http://www.cellmigration.org/resource/komouse/komouse_resources.shtml). In 2005 there were

problems with the production of Amersham's Hybond N and N+ membranes. Some investigators have switched to Sigma's BioBond-Plus™ nylon membrane (alkalis transfer, like the N+) or Hybond-XL (this is used with neutral transfer) with better success.  I hope to have a neutral transfer protocol posted soon in the methods section.

If you are using BAC DNA as your genomic source, you can usually include BAC DNA as a + control that may have less background than the whole mouse genome does.  This is a nice control if you this is one of your first Southerns.  The lane with the BAC DNA should give a whopping, huge band since much of the DNA you load is your gene, as compated to a whole genome of other DNA.  Only load a tiny (1ug) amount onto your gel or this will overwhelm your results.

After some optimization of your Southern, you must be able to show a clean band using the entire mouse genome. It is alright to have background bands, as long as none of them are near the expected size of your targeted band.  A picture of your Southern Blot is standard for publication of a mouse, so you want a strategy that will enable you to produce a good figure.

If your Southern Blot does not result in a clean band, you must devise and test a new strategy with new enzymes.  If no other good sites exist, you may need to introduce sites by PCR/ cloning.  If this is the case, go back to Step 5 and develop a strategy that will enable other Southern possibilities.

## Step 9:  Build your construct

Do not start to build if you have not confirmed the screening strategy with a Southern band.  If your original Southern strategies do not give clean bands, you may need to introduce other sites in your construct through your cloning, which will require that you go back to **Step 5**

Use many steps of restriction digests and PCR to build your construct in small pieces.

Remember, if you try to introduce a NotI site on your PCR product, you may need 8 bp of flanking sequence to get the enzyme to cut the site efficiently. For other info like this, see "Cleavage Close to the End of DNA Fragments" in the New England Biolabs Catalog (and http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/cleavage_linearized_vector.asp). Another source is http://www.fermentas.com/techinfo/re/restrdigpcrii.htm

 All key pieces that are generated by PCR should be sequenced to confirm that no mutations were introduced during amplification.  Also, you must sequence across the lone LoxP site to ensure that it is in the correct orientation relative to the LoxP sites flanking the Neo.

Once again, here is the LoxP orientation (non-palindromic sequence is underlined) as it is drawn on the map for pPGK Neo.F2L2.DTA :

5' ATAACTTCGTATAATGTATGCTATACGAAGTTAT 3' is drawn ">"

5' ATAACTTCGTATAGCATACATTATACGAAGTTAT 3' is drawn "<"

Generally, you will be adding a long arm of genomic DNA (4-5kb) and a shorter arm of genomic DNA (2-3kb). Investigators have found that the second one you add is much more difficult than the first.  It can be very frustrating as you try to clone the second arm in. Many bacteria (especially DH5alpha) will not be

happy hosting a large plasmid, so using bugs that handle larger plasmids can help. I recommend Invitrogen's GeneHogs, (cat# C8080-10 ) or another strain of DH10B for your final cloning steps.  For other tips on cloning your second arm, see the end of this guide (after step 10 and the recommended guides).

Remember, you will need to linearize your construct plasmid for targeting in ES cells.  Make sure you have at least one restriction site that will cut in the backbone, but not in the sequence you have added (no cutting in mouse genomic sequence, loxP, Frt, PGK Neo or PGK DTA).

## Step 10:  Prepare your DNA for electroporation

See the methods section for a step-by-step protocol for preparing your DNA for electroporation, or follow the summary below.

Inoculate 250 mL media with the bacteria containing your construct, grow up overnight.  Use a Qiagen Maxi Kit to isolate a large quantity of your plasmid.  Linearize at least 120 micrograms of your construct, run it on an agarose gel side-by-side with an aliquot of uncut plasmid.  Then purify the cut DNA by QiagenGel Extraction Kit. DNA purified with CsCl is also excellent for targeting.  Place 50 micrograms of DNA in each of two tubes.  If the DNA is approximately 1 microgram/ml in TE, you may send it as is.  If the DNA is less concentrated, please ethanol precipitate and then store the DNA in 70% EtOH and submit it to the facility. Make sure you use a screw-cap eppendorf tube or use Parafilm to secure the top of a standard tube to avoid spillage during shipment.

Please note that the MIT Transgenic Facility is not able to offer electroporation/targeting services to investigators outside of the MIT/CMC community.

If you are making a mouse with the Cell Migration Consortium, the following information MUST be submitted to the Targeting Facility and approved before we will assign a date for electroporation of your construct.  The purpose of this requirement is to ensure that the ES cell facility operates efficiently, produces the maximum number of targeted clones, and keeps costs minimal.  This ensures that you have already verified all screening strategies, which will limit the amount of time your ES cells are frozen while you screen the DNA.  The longer the cells stay frozen, the less likely they are to contribute to the germline of a mouse.  If you have followed this guide, you should have all of this information readily available anyway. You may submit many of these items well in advance of preparing the actual DNA, or you may submit them all with the linear DNA.

An example of the format for submitted information is shown below, but here is a short summary:.

a. Restriction maps of the wild type, genomic locus (clearly labeled as 129Sv or BL/6) and the expected structure of the correctly targeted locus.  Maps should include the locations of relevant exons, selectable markers, LoxP and Frts, and the restriction sites making the boundaries of the targeting construct.

b. Schematic of your Southern strategy and the Southern blot image showing that you can get a clean wild-type band with your probe.  It is alright to have background bands, as long as none of them are near the expected size of your targeted band.

c. Detailed explanation of the PCR screening strategy, including a schematic showing the location of primers, expected product sizes with each step of recombination, and positive controls for the PCRs.  Include photos of gels showing PCR results.

d. Sequence information across the lone LoxP site, showing that it is in the correct orientation relative to the LoxP sites flanking the Neo (as determined by restriction digest or through orient-specific cloning). If the lone LoxP site is backwards, you will not get removal of your exons.

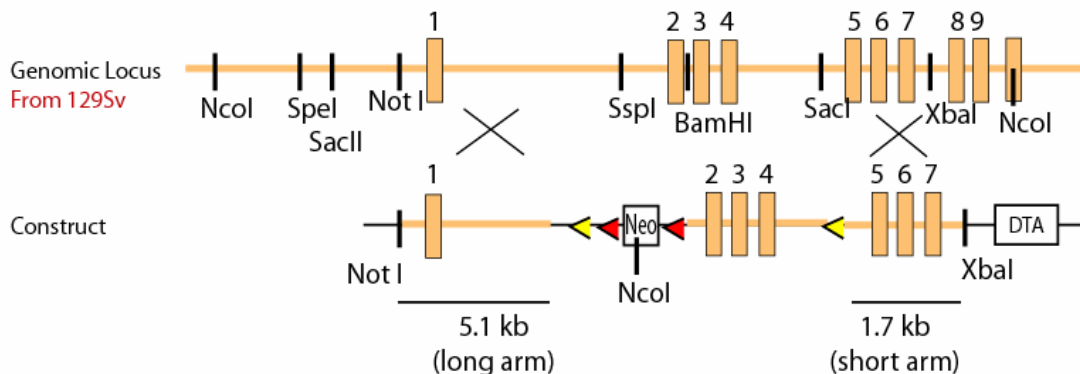e. Photo of the gel showing linearization of your construct.

A shipping address for the linear construct and the above info will be given to you when you are near completion of your construct.

We isolate 350-400 selection-resistant clones per electroporation and the investigator is then responsible for performing the PCR and Southern screens to determine which clones have undergone homologous recombination.
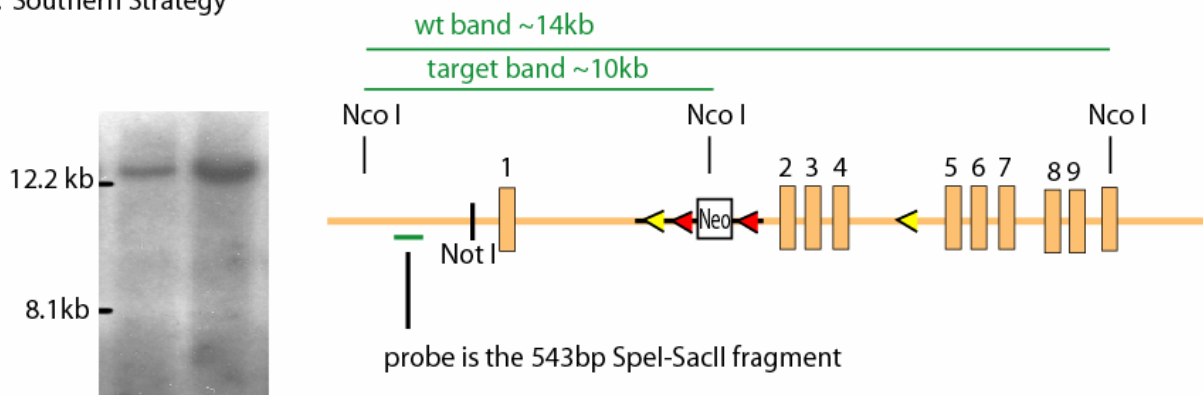
With a well designed targeting construct, we generally see that 0.5-10% of selection-resistant clones have undergone homologous recombination. Some loci tend to recombine very easily (30-50% for ROSA26 and KRAS) while others are extremely difficult to target.

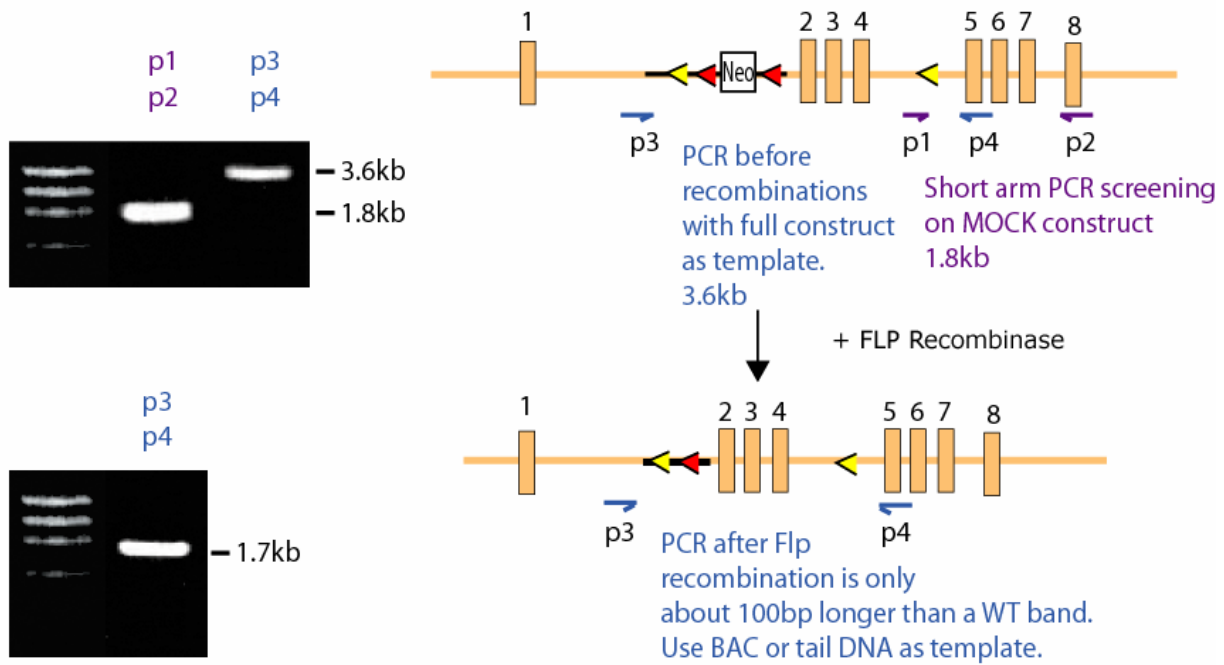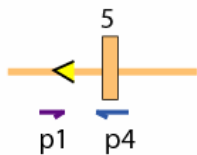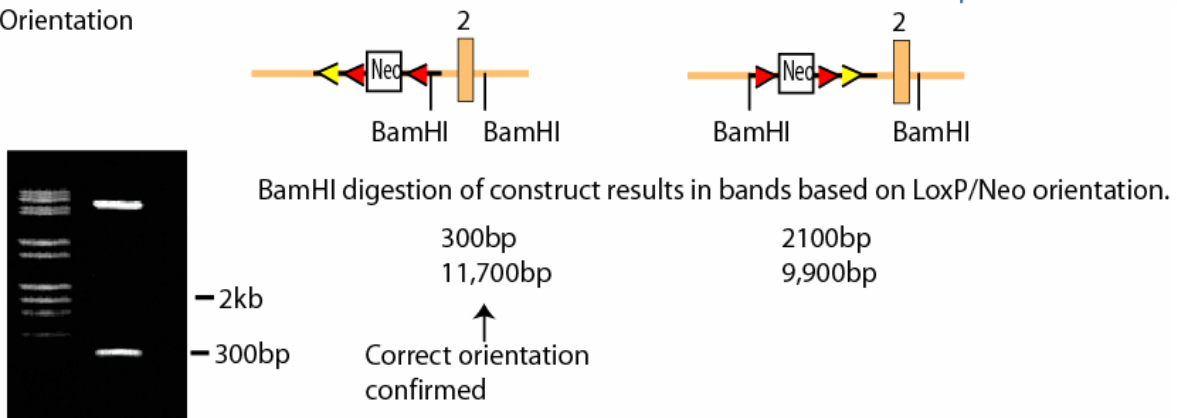## Example of Gene Targeting Information to be Submitted

## c. PCR Screening



p1
p2

p3
p4

— 3.6kb
— 1.8kb

1

2 3 4

5 6 7

8

Neo

p3

p1  p4  p2

**PCR before recombinations with full construct as template. 3.6kb**

**Short arm PCR screening on MOCK construct 1.8kb**

+ FLP Recombinase

p3
p4

— 1.7kb

1

2 3 4

5 6 7

8

p3

p4

**PCR after Flp recombination is only about 100bp longer than a WT band. Use BAC or tail DNA as template.**

## d. LoxP Orientation

2

Neo

BamHI    BamHI

2

Neo

BamHI    BamHI

BamHI digestion of construct results in bands based on LoxP/Neo orientation.

300bp
11,700bp

2100bp
9,900bp

— 2kb

— 300bp

↑
Correct orientation confirmed

5

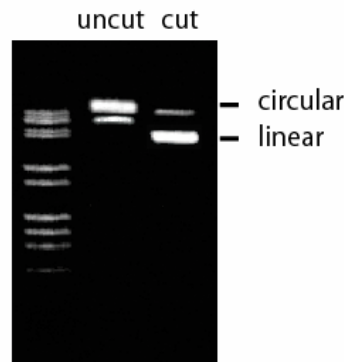p1    p4

p1 primer extends into the non-palindromic region of the LoxP site, so the presence of a PCR band from p1 and p4 (shown in c) confirms the LoxP orientation. Sequencing of this PCR band confirmed orientation and shows no mutations in the LoxP site.

## e. Linearization of construct

uncut  cut

— circular
— linear

**Other recommended guides:**

**(Please note these are already outdated, but they will provide further background information.)**

- Melton, DW. Gene-targeting strategies. Methods Mol Biol. 2002;180:151-73.
- Cheah SS, Behringer RR. Contemporary gene targeting strategies for the novice. Mol Biotechnol. 2001 Nov;19(3):297-304.
- Cheah SS, Behringer RR. Gene-targeting strategies. Methods Mol Biol. 2000; 36:455-63.
- Ramirez-Solis R, Davis AC, Bradley A. Gene targeting in embryonic stem cells. Methods Enzymol. 1993;225:855-78.

- Garcia-Otin AL, Guillou F. Mammalian genome targeting using site-specific recombinases. Front Biosci. 2006 Jan 1;11:1108-36.

- Claire M. Wade et al. The mosaic structure of variation in the laboratory mouse genome. Nature 2002, 420, 574 - 578.
- David Threadgill et al. Genealogy of the 129 inbred strains: 129/SvJ is a contaminated inbred strain. Mamm Genome. 1997 Jun;8(6):390-3.

**Tips for cloning in the second arm of your construct:**

Several people have had problems getting the second homology arm to go into the vectors.
Here are a few things that have helped –

**1-** When you do the ligation, make sure there is 6x more pieces of the insert (arm) than there is the vector. You can use the ability of Ethidium Bromide to stick to the DNA to quantitate how much DNA you have. If you run your fragments out on a gel and use 1ug of your ladder, you can use the brightness of your piece as compared to the brightness of a ladder fragment to determine how many ug are in your bands, then figure out the ratios for the ligation.

For example, if you have 1ug of a Lambda HindIII ladder, you add up the kb totals for each band (23.13 +9.42+.... 0.56+0.13 = 48.5) So, each kb has about 1ug/50 = .02ug or 20ng. So, the 23kb band has a brightness of about 462ng. Find the ladder band that whose brightness is most similar to your band, and you can now estimate how many ng you have of your piece. Then do the ratio - if you have 35ng of your 4.5kb insert, you want to use 1/6th as much vector. So if your vector is currently 9kb (twice as big as the insert) use 35/6= about 6 ng x2 (for twice the kb size) = 12 ng of the vector.

This is a trick I learned late in grad school and has been an excellent solution for a lot of cloning problems

**2-** Phosphatase your vector ends using CIP to prevent re-ligation of your vector on itself. Some people always do this for all ligations anyway...I only did this for hard stuff. CIP= Calf intestinal alkaline phoshatase

**3-** When you mini-prep up your DNA from the colonies, the plasmid ends up being in low copy number due to its size. Rather than 1-2 uL of DNA for your diagnostic restriction digests, use 6-10uL so that you can really see the band.