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Cre-lox design guide: conditional knockout models

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With a conditional knockout model you control where and when your gene of interest is inactivated.

This level of control opens up new experimental options such as tissue-specific gene knockout that can't be achieved with standard knockout models. A properly designed conditional knockout (cKO) model is a versatile tool for a wide variety of studies.

cKO models are created by introducing two specific genetic modifications into a gene of interest. In the most common design two copies of the 34bp loxP sequence are placed according to the guidelines listed below. To achieve knockout the new cKO line is crossed with a line expressing Cre recombinase, an enzyme that recognizes the loxP sequences and deletes the gene sequence between them. Hundreds of existing lines with tissue-specific Cre expression can be used with a new conditional knockout model enabling a wide variety of experiments.

The right design is <u>crucial</u> when creating a new conditional knockout model.
A successful design has two requirements:

1. **Conditional gene inactivation by Cre**
2. **Wild-type expression before Cre is active**

When modifying a gene to create a conditional knockout allele both requirements must be met. Here are the most important guidelines for designing a new cKO model:

Conditional gene inactivation by Cre

A) Target the earliest exon that will result in a frameshift and premature stop codons upon deletion.

B) If frameshift is not possible, target a crucial domain or delete as much sequence as possible.

Wild-type expression before Cre is active

C) Avoid making changes in regulatory regions such as the gene's promoter.

D) Avoid disruption of splice sites.

E) Make sequence changes in large introns when possible.

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STEP-BY-STEP GUIDE TO DESIGNING A CONDITIONAL KNOCKOUT MODEL

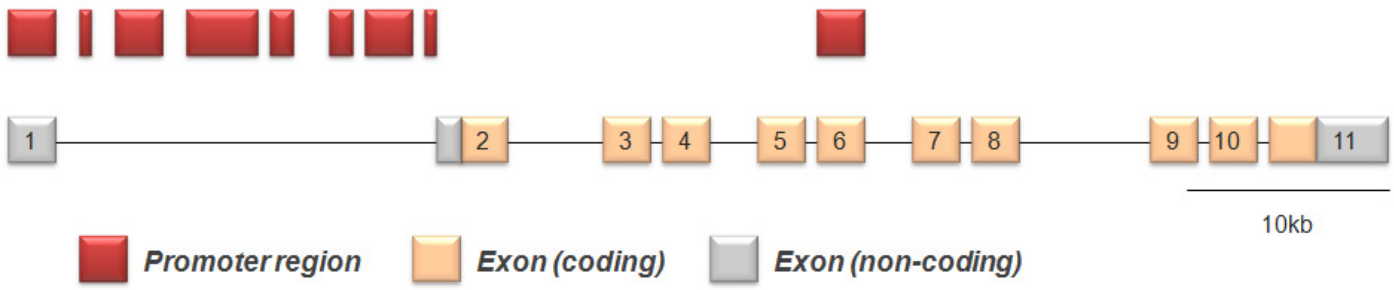
Example 1: cKO by frameshift

1. Analyze your gene of interest.

There's no one-size-fits-all strategy for making cKO models so every gene must be carefully analyzed to identify possible approaches. Factors to evaluate include genetic features (such as the sizes of exons and introns, the locations of promoter sequences, and possible splice variants) as well as any other available information about the gene and the protein(s) it encodes. Details on existing knockout models may inform the design of a cKO model but there are important differences in how the two model types are designed.

Ensembl is an invaluable resource for both rapid and in-depth analysis of genomic sequence. You can visualize the region of the genome where your target gene is located and easily note key genomic features.

Here's a simplified representation of a gene:



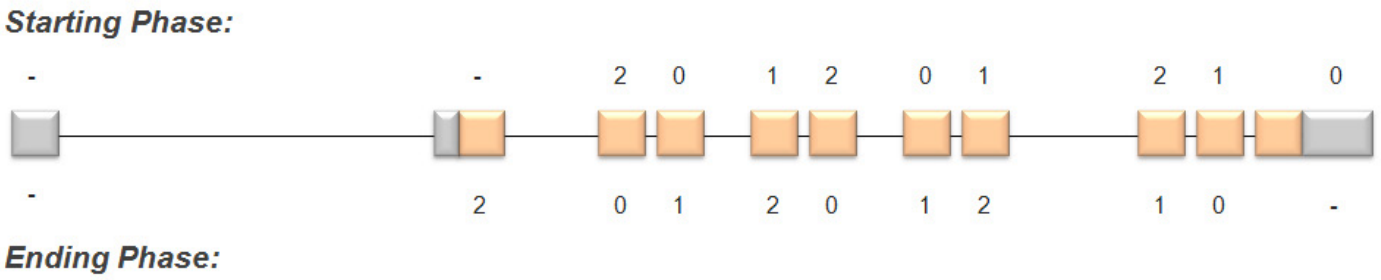
In this example the gene has 11 exons, with exon 1 consisting of only 5' untranslated region sequence. Multiple regions of regulatory sequence are present in intron 1 and the coding sequence begins in exon 2. Begin an analysis by noting the sizes of the introns and the starting and ending phases of the exons (more on this below). The goal at this stage is to gather information that will guide the placement of the loxP sequences.

2. Identify possible cKO strategies.

The most straightforward strategy for making a cKO model is to place loxP sites such that deleting the intervening region will create a frameshift in the coding sequence. Generally wild-type mRNAs are spliced so they contain a single open reading frame (ORF) encoding the correct peptide sequence. In this example the ORF contains 503 codons, corresponding to 502 amino acids and a termination codon at the end.

The 5′ and 3′ end of exons don't necessarily line up with the beginning or ends of codons. Each exon has what is referred to as a starting and ending phase that indicates how many nucleotides of the terminal codon are present.

Here's the example gene with Ensembl's notation used to indicate the phases:



Note in the example that the ending phase of one exon matches the starting phase of the subsequent exon. The phases of adjacent exons must be matched to each other to allow correct translation after splicing occurs. If the ending phase of one exon doesn't match the starting phase of the next exon a frameshift will occur and result in a nonsense mutation. Usually this will result in early stop codons being added to the transcript, leading to gene knockout. This fact is the basis of the standard cKO design.

In the example gene deleting any single exon from 3 to 10 would result in a frameshift since the starting and ending phases keep changing. Therefore the list of possible standard strategies to evaluate includes floxing exon 3, floxing exon 4, etc. It's also worth evaluating the possibility of floxing multiple closely-spaced exons such as the 3-4 pair and 5-6 pair. With the list of strategy options compiled it's time to compare and decide which is best.

3. Evaluate which cKO strategy best matches your experimental plans.

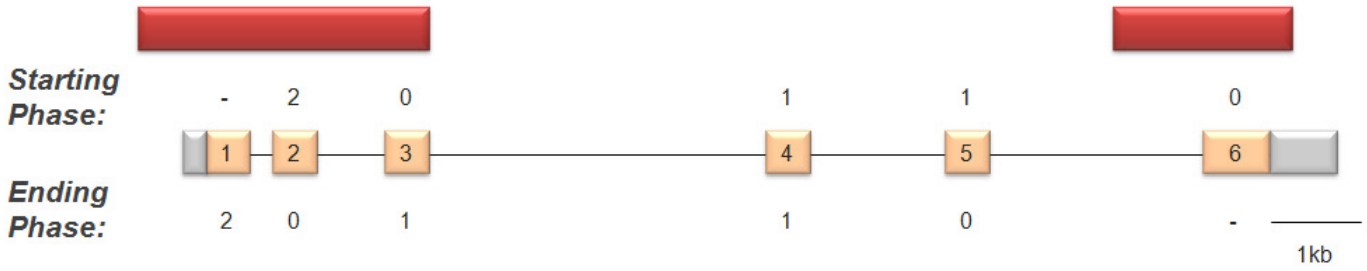
For most genes the guidelines at the start of this guide can be used to determine the best strategy. Guideline A - target the first exon whose deletion will result in a frameshift - suggests that exon 3 should be floxed in this case. However guidelines D and E suggest that an alternate strategy may be better because in this case intron 3 is less than 500bp. Although the loxP sequence is small it may disrupt splicing if placed too close to an intron/exon boundary. Therefore the option of floxing exons 3 and 4 together should also be evaluated. Either strategy (floxing exon 3 alone or floxing exons 3 and 4 together) is viable. There may be gene-specific reasons to favor one over the other, for example if a close examination of the genomic sequence identifies a repeat-rich region where placing the loxP sequence could be difficult.

Example 2: Other cKO strategies

1. Analyze your gene of interest.

In some cases a standard frameshift-inducing cKO design may not work for a specific gene.

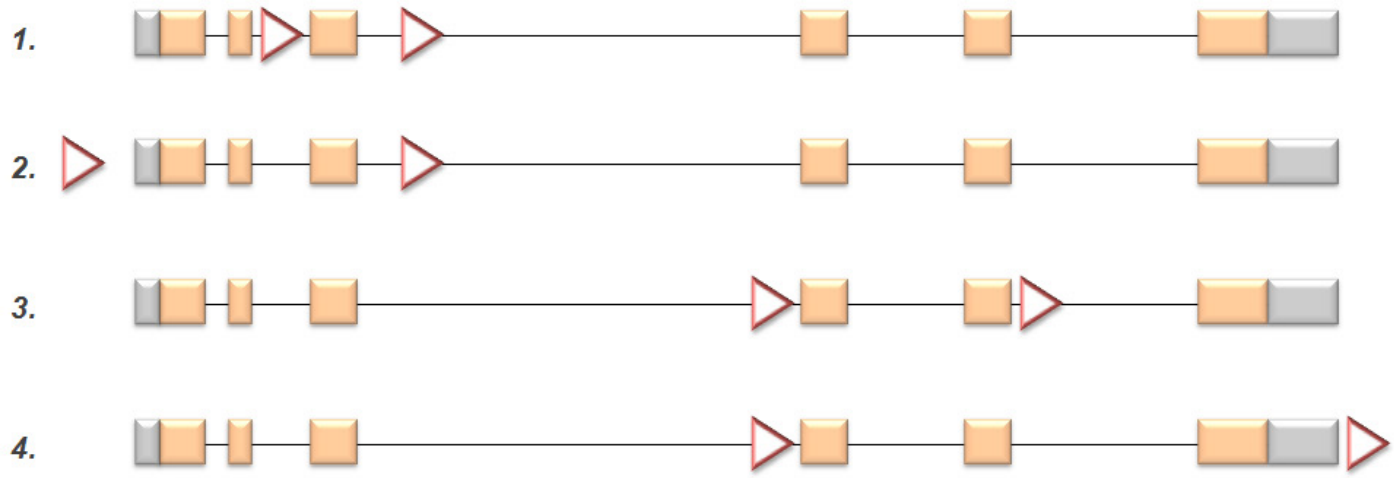
Here's an example:



Floxing exon 3 would normally be recommended as that will create a frameshift. However that region of the genome is annotated as having promoter activity (red boxes). It's possible that the small change of inserting a 34bp loxP site in that region will have no effect on the gene's expression pattern. However it's also possible that a key transcription factor binding site could be disrupted by even such a small change, possibly creating a hypomorphic or even null allele. In this case other factors besides the intron/exon placement will influence the choice of cKO strategy. One simple thing to look at is the protein sequence encoded by each exon. If a frameshift cKO is not possible, can a crucial protein domain be deleted?

2. Identify possible cKO strategies.

Here are four possible strategies:



Strategy 1 is a standard design: conditional deletion of exon 3 will lead to frameshift and early stop codons in the mRNA. However in this case it requires placing a loxP site in a regulatory region. The other 3 strategies work to avoid disrupting the promoter but each one makes different tradeoffs which are further discussed below.

3. Evaluate which cKO strategy best matches your experimental plans.

Each strategy option in this case has different advantages and potential drawbacks.

- **Strategy 1** risks disrupting the gene's promoter by placing a loxP site in it.
- **Strategy 2** spaces the loxP sites farther apart (2.3kb distance between sites) so they're outside the promoter and encompass exons 1-3. It's likely that deleting the promoter and early coding exons will completely inactivate the gene. However there is a chance that transcription could start from a cryptic site and make a partial gene product.
- **Strategy 3** is another option for creating a frameshift leading to early stop codons, resulting in a truncated protein product. The first 80 amino acids would be translated normally in this case, then about 30 residues would be added with incorrect sequence before an early stop codon was reached. It's possible for the truncated protein to cause unwanted effects if it's stable and active.
- **Strategy 4** allows for conditional deletion of over 75% of the coding sequence and all the 3' untranslated region. It's likely that this strategy will result in an RNA product that's quickly degraded. However the loxP sites would be separated by about 3.5kb which means it would be more challenging to create the model.

Information about the gene of interest should guide the final decision on strategy. For example, if the promoter has been well studied it may be possible to adopt strategy 1 and avoid disrupting important regulatory sequences. If N-terminal protein fragments are known to be active then strategy 3 should be ruled out. Any genetic modification comes with the chance of unexpected results so uncertainty can be reduced but never totally eliminated. Careful validation of any new line is essential to demonstrate that the gene switches from normal function to knockout in the desired manner.

In Conclusion

Your gene of interest must be carefully analyzed in order to identify possible design approaches prior to beginning your cKO model. Factors to remember include evaluating genetic features (sizes of exons and introns, the locations of promoter sequences, possible splice variants) and reviewing any other available information about the gene and the protein(s) it encodes via a database such as Ensembl.

With careful analysis along with using this guide, your ideal cKO design is within reach.

GLOSSARY

flox: Place lox sequences on either side of a genomic region. Often a gene is said to be floxed when loxP sites have been inserted into the gene's sequence to create a conditional knockout allele.

recombinase: Enzyme that cleaves DNA then religates the fragments together. Site-specific recombinases such as Cre will only catalyze the reaction when a specific DNA sequence is present.

regulatory regions/promoter sequences: Specific DNA sequences that affect gene transcription. Mutations in these regions may alter a gene's expression pattern.

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