

**TRANSGENIC TECHNOLOGY
CENTER**



General Guidelines and Useful Tips for the Design of Genome Editing Projects

We strongly encourage Investigators/ lab staff to contact the staff of the Transgenic Core to discuss the desired genome modification that they would like to engineer into the mouse genome before ordering any CRISPR reagents from vendors.

A. Define the project: What type of genetic mutation do you want to introduce into the mouse genome and what is the optimal way to accomplish this? The specific type of mutation you want to create will dictate the number of sgRNAs that need to be designed and the type of DNA donor template that will need to be used. These various DNA donor templates that can be used to drive homologous recombination (HR) include a ssODN (< 200bp), a long single stranded DNA (lssDNA up to 3kb) or a double stranded DNA (up to 15kb).

1. **Gene knockout** is created by non-homologous end joining (NHEJ) and is generated by an insertion/ deletion (indel) frame shift. This is accomplished by designing a sgRNA for a critical exon in the target gene.
2. **Multiple gene knockouts**, or a large genomic deletion. Multiple gene KOs are generally accomplished by designing sgRNAs to target critical exons in each of the targeted genes. A large chromosomal deletion is accomplished by designing a sgRNA for the beginning and one for the end of the genomic region you intend to delete.
3. **Simple knock in** is accomplished by introducing an oligonucleotide that harbors a point mutation or a fusion tag into the exon of choice. This is done by using one sgRNA.

4. **Complex knock in** is accomplished by using a large DNA template for HR. This is done by using a lssDNA (a *megamer* - IDT jargon) or dsDNA-based template). This is done using one sgRNAs.
5. **Conditional allele** can be created using 2 different approaches. The first involves introducing ssODNs harboring *loxP* sites into the 5' and 3' sites flanking the exon(s) of interest. This is done by using 2 sgRNAs. The second approach involves using a lssDNA template harboring 2 *loxP* sites that flank the DNA sequence of interest. This approach uses 2 sgRNAs.

B. Reagents provided to you by the UTSW Transgenic Core:

The fee you pay the Core for the CRISPR service includes the cost of the *Sp. Cas9* protein from IDT that we use to accomplish your project. If your project involves the use of a different editing enzyme such as *Cas12* or *Cpf*, please contact the Core staff to discuss the project in more detail.

C. Know the important details of your gene: Use a genome browser such as NCBI, UCSC, or ENSEMBL to gather the relevant information about your target gene. This includes such details as any alternative transcripts, the number and importance of exons, regulatory motifs that reside in particular introns, and coding sequences in order to successfully design sgRNAs and donor DNA templates.

It's important to use **MGI- Mouse Genome Informatics** to determine if there are any known phenotypes associated with a knockout of your target gene. It is particularly important to know if a KO of your gene will likely result in a lethal phenotype, either embryonic or early postnatal. Knowing and communicating this to the Core staff will allow us to modify the conditions we use to generate the mutant strain of mice so that we create predominantly mice heterozygous for the KO allele.

D. Design of the sgRNA(s): The efficiency of the genomic editing that occurs in the injected zygotes depends, in large part, on the correct design of the sgRNA for the target. Key components of this design include:

1. **Choose your target sites:** Identify the genomic region or exon you want to target. If your gene of interest expresses multiple transcripts, be sure your guide will

target the correct DNA sequence to ablate only the desired transcript.

- **For a KO project:** There are at least 2 options including choosing a sgRNA that targets just 3' of ATG or a DNA sequence in the first coding exon that is shared by all isoforms. The second approach involves designing a sgRNA to target a known DNA motif that is critical for its full expression. One of the more common repair outcomes of random NHEJ events is in-frame deletions. The existence of microhomologies near the cut site can be used to predict the repair outcome and to increase the chance of getting an out-of-frame INDEL. This homology does not affect the sgRNA cutting but can help to generate a knock-out allele. You can use any of the microhomology predictor tools to verify any microhomology of your gRNA.
- **For a KI project:** A priority in designing a gRNA for this purpose is to identify a sgRNA that cuts as close to the mutation as possible, ideally within 10bp. The larger the distance the guide is from the target, the lower the frequency of homology-directed repair will be.

- 2. Identify a good candidate sgRNA:** Use sgRNA algorithm online tools such as CRISPOR, Benchling, or the Broad to pick candidate sgRNAs that have:
- High on target activity
 - Minimal off-target effects: a putative gRNA should be perfectly complimentary to the region you want to target in the 8-12 bases closest to the PAM (Seed region).

Other considerations when choosing a guide RNA include:

- The GC content should be between 30-80%, higher is better but not too high.
- Avoid additional G's after the PAM
 - A purine in the most PAM-proximal position can enhance Cas9 cutting efficiency.

E. Guide RNA Validation: Putative sgRNAs do not have the same activity, therefore we recommend that you choose at least 3 sgRNAs per target and test gRNA activity *in vitro* before

proceeding with the experiment. You can use an array of methods to assess the activity of the putative guides. These methods include the Surveyor nuclease (CEL-1 assay), T7 endonuclease I, “Guide it sgRNA” screening kit (Takara). You can also test the putative sgRNA activity *in vivo* using transfections into established cell lines or a “blastocyst assay” involving an injection or electroporation of the RNP cocktail into zygotes. This assay can be conducted in-house.

F. Donor Template Design:

-Preventing re-cutting: An important design issue that must be taken into consideration when creating a donor DNA template is that re-cutting of the donor DNA template can occur after a successful genome modification. To prevent this event from happening, you should use one of the following approaches to modify the donor template:

1. Split the protospacer sequence (crRNA target sequence) or a portion of the protospacer and the PAM between the two homology arms.
2. Introduce silent nucleotide changes into the PAM donor template
3. Introduce silent SNPs in the crRNA target region most proximal to the PAM (Seed region) to prevent subsequent recognition by the gRNA or introduce a new restriction site near the PAM sequence.

-Homology arms polarity: The polarity of the ssODN should be adjusted (ssODN in sense or antisense orientation) such that base pairing between the 3' arm of the ssODN and the genomic strand is not interrupted by the intended modifications. Optimal ssODN polarity depends on the relative position of the generated double strand break (DSB) in relation to the integration site. If the DSB is generated downstream of the intended integration site, then a sense ssODN is preferred whereas cleavage upstream of the integration site favors an antisense ssODN.

-Asymmetry of the arms of homology: Asymmetric donor ssODN templates are optimal for annealing by overlapping the Cas9 cut site with 30-36bp of homology on the 3' end and with 67-91bp extension on the PAM-proximal side of the break. The use of asymmetric arms of homology in ssODNs templates has resulted in the highest efficiency of HDR in the UTSW Core's

experience regardless of which strand is used.

-Chemical modifications: The stability of the ssODN and thus HR editing efficiency is enhanced by a phosphorothioate (PS) modification of the last 2 nucleotide bonds at the 5' and 3' ends. This modification can be included during commercial synthesis of the ODN (IDT generated modification).

- Donor ssODN DNA templates for generating conditional alleles: There are a number of recent reviews that discuss all of the issues related to the use of ssODNs harboring *loxP* motifs to generate conditional alleles in rodents. The UTSW Transgenic Technology Center has predominantly used the ssODN approach to generate conditional alleles. One of the keys to making this approach successful is to generate a sufficient number of putative founder mice to obtain a small cadre of founders that harbor both the 5' and 3' *loxP* motifs on the same allele. There are a few things to consider when designing the placement of the *loxP* motifs into your gene of interest. Stay at least 200bp from the splice donor and acceptor sites and stay at least >500bp upstream of the transcription start site. Another consideration is to investigate if the placement of the *loxP* motif disrupts other regulatory motifs that have either not yet been defined or you are not aware of their existence. To help determine if this is a possibility you can search relevant data bases for areas of high conservation between species and to assess predicted motifs. These data bases include:

UCSC browser- <http://genome.ucsc.edu/>

Vista-Point- <http://genome.lbl.gov/vista/index.shtml>

ECRbrowser- <https://ecrbrowser.dcode.org/>

-Long ssDNAs/ Megamer DNA donor templates: Long ssDNA templates are available commercially from 200bp up to 8kb and even up to 10kb with a mandated consult. The use of these DNA donors allows efficient generation of modifications that previously required the generation of dsDNA templates. The optimal design parameters for lssDNA templates are somewhat different than ssODNs and are highlighted in current literature from IDT. Efficient editing is achieved with arms of homology of between 50-300nt with no clear preference for a particular polarity of the ssDNA. The UTSW Core recommends

long ssDNA donors for modifications that are too large for ssODNs. Commercial suppliers are limited in the size they can synthesize and will reject requests that involve areas of a high GC content. The current commercial suppliers are IDT (idtdna.com) and Genewiz (genewiz.com). A commercial kit is also available from Takara that allows the generation of a lssDNA template of up to 5kb.

<https://www.takarabio.com/products/gene-function/gene-editing/crispr-cas9/long-ssdna-for-knockins>

One of the more common uses of lssDNA donor templates is to create a conditional allele by designing a DNA template that contains *loxP* motifs flanking the DNA sequence to be deleted by Cre recombinase. A challenge using this approach is that if you use canonical *loxP* sequences and the motifs are too close together (<500bp) there is a high probability that the intervening DNA will recombine during the integration event. Strategies to circumvent this include using variant, non-canonical *loxP* sites in the targeting template and increasing the distance separating the 2 *loxP* sites by a minimum of 500bp in a >800bp template. More information on the use of lssDNA templates is available from IDT. Typically, the use of a lssDNA template to create a conditional allele requires the use of 2 sgRNAs that target sites just inside the arms of homology.

-Plasmid-based DNA donor templates: Modifications that involve large changes to the genome require the use of plasmid-based donor templates. Homology arm length in these vectors can typically range from 600-900bp if the flanking donor DNA template is flanked by nuclease target sites that are identical to the genomic target site. As a consequence, a linear donor template supply is liberated concurrent with genomic cleavage. This linearized template is believed to stimulate a novel HDR pathway. No negative consequences of *in vivo* donor excision have been reported and the construction can be accomplished by routine PCR amplification. An alternative strategy to improve the efficiency of HDR of large, plasmid-based donor templates is to introduce the RNP cocktail into two-cell mouse embryos. This strategy has proven to be a very effective technique to enhance HR events that simply failed when the cocktail was introduced into the mouse zygote. The

staff of the UTSW Transgenic Technology Center are just beginning to employ this strategy in our ongoing efforts to improve the frequency of HR of plasmid-based templates.

Useful links and Reading:

Review of CRISPR Tools:

<https://bioinfogp.cnb.csic.es/tools/wereview/crisprtools/>

Genome Browsers:

ENSEMBL: https://useast.ensembl.org/Mus_musculus/Info/Index

MGI: <http://www.informatics.jax.org>

NCBI: <https://www.ncbi.nlm.nih.gov>

UCSC: <https://genome.ucsc.edu>

Tools for sgRNA design:

BENCHLING: <https://benchling.com/signin/welcome#>

BROAD: <https://zlab.bio/guide-design-resources>

Cas-OFFinder: <http://www.rgenome.net/cas-offinder/>

CRISPOR: <http://crispor.tefor.net>

HORIZON: <https://horizondiscovery.com/en/products/tools/CRISPR-Design-Tool>

IDT: https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM

SIGMA: https://www.milliporesigmabioinfo.com/bioinfo_tools/?domain=STANDARD

SYNTHOGO: <https://www.synthego.com/products/bioinformatics/crispr-design-tool>

UTSW: <http://www.crispinator.com/ko/>

Tools for HDR design:

<https://www.benchling.com>

<https://www.idtdna.com/pages/tools/alt-r-crispr-hdr-design-tool>

https://www.milliporesigmabioinfo.com/bioinfo_tools/faces/login/login.xhtml

Tools for checking microhomology:

<http://www.rgenome.net/mich-calculator/>

<https://indelphi.giffordlab.mit.edu/guide>

Relevant Literature:

Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors -<https://www.nature.com/articles/nprot.2017.153>

Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA <https://www.nature.com/articles/nbt.3481>

How to create state-of-the-art genetic model systems: strategies for optimal CRISPR-mediated genome editing <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6061873/>