How to generate a CRISPR mouse

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In my case I generated a **Q128A mutation in Kirrel3 (K3) gene** but these steps could be applied for any other mutation

I'm outlining the steps below

Aim: To generate a Q128A mutation in Kirrel3 gene using CRISPR in a mouse

Note: Depending on what you're looking at, it might also be worth first testing for homology between mouse protein and Human protein

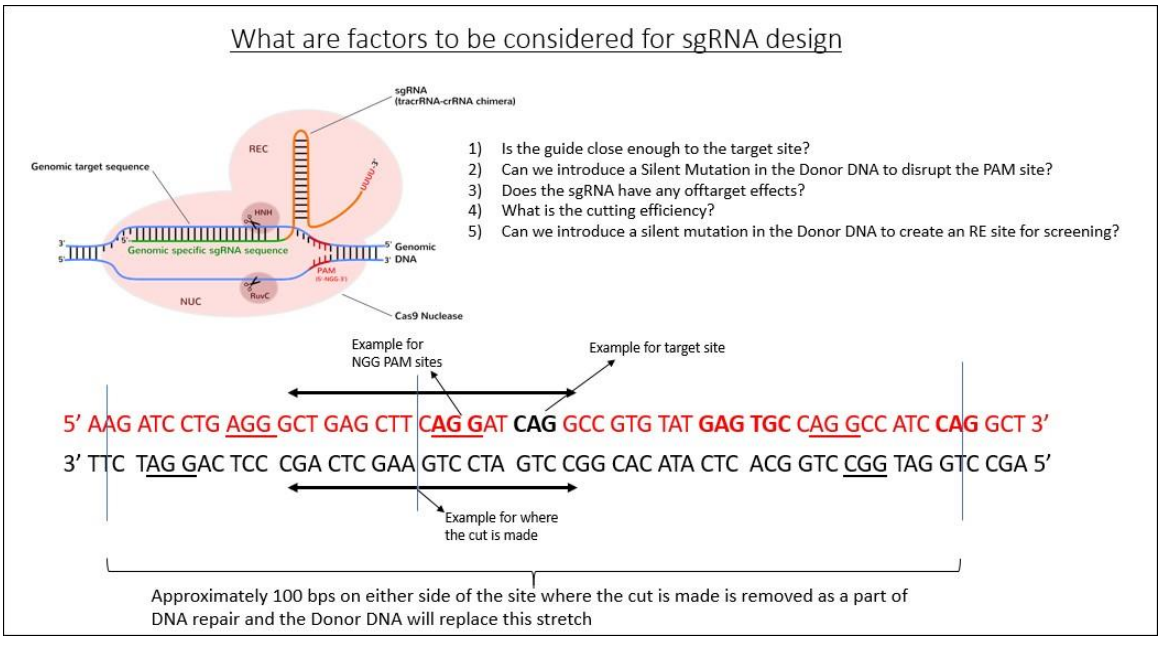
In my case between mouse K3 and Human K3 Similarity = 99% Identity = 98%

Also, worth testing if the target region that you want to mutate in mouse (in my case Q128A K3) is conserved between mouse and human

Designing sgRNAs

1) Check for FASTA DNA sequence mouse Kirrel3 protein
   *For finding FASTA DNA sequence you can use NCBI or e! ENSEMBL https://bit.ly/2FQ8vsU
2) FASTA protein sequence for mouse Kirrel3 protein
   *For finding FASTA protein sequence you can use NCBI or UniProt: https://www.uniprot.org/uniprot/Q8BR86
3) Match the two and identify the stretch of DNA surrounding Q128 (CAG) using
   ➔ https://blast.ncbi.nlm.nih.gov/Blast.cgi
   ➔ Choose the appropriate BLAST: If you put a selected protein sequence and want to align it with a DNA sequence it will be protein to nucleotide BLAST (options are available in the link)
4) Input a stretch of DNA 100bps on either side of CAG (203 bps total) to MIT sgRNA tool
5) Shortlist sgRNAs from the list of sgRNAs scored based on off target effects

Some criteria to consider when choosing sgRNA sequence



The above figure lists some important factors to consider when choosing a sgRNA design

Note in red is the example DNA sequence that is recognized by the sgRNA that I shortlisted

sgRNAs can recognize the 3' strand too! Depending on the factors below you can choose a forward or reverse guide.

Out of the list of sgRNAs on MIT tool top 4 were selected for screening

The top four that satisfy the 5 criteria mentioned in the above figure

The sgRNA needs to be such that:

1) The PAM site is close to the target site (closer the better)
2) You need to be able to introduce a silent mutation in the donor DNA to disrupt the PAM site so that once the donor DNA is inserted it's not recognized by the sgRNA again for another cut
3) There should minimal off target effects i.e. the chances of the guide recognizing other random regions in the genome should be low
4) The efficiency with which it cuts the genome should be high
5) * This is a note for later but a silent mutation needs to be introduced in the donor DNA so that we have a restriction enzyme site to screen for the incorporation of the donor DNA

So, based on this these factors we shortlisted some sgRNAs

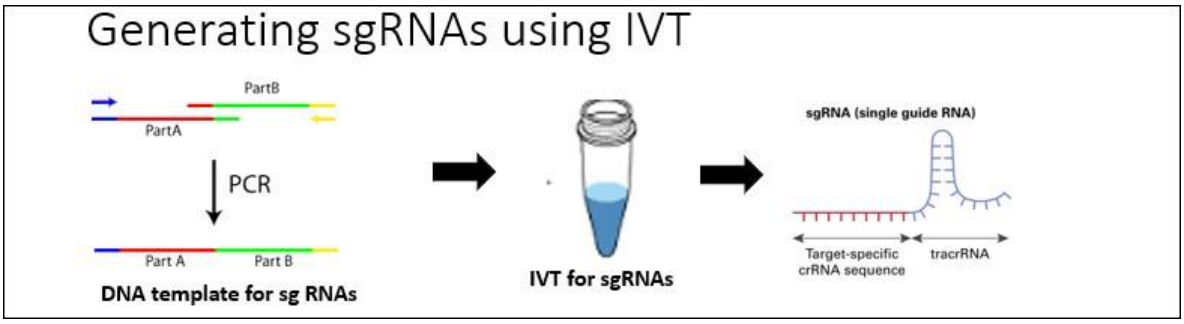
The sgRNAs will make a cut 3-4 nts away from its respective PAM site

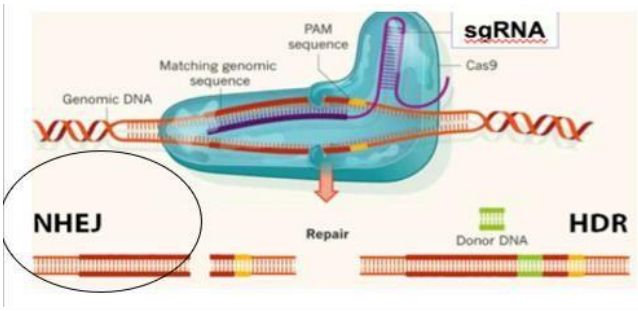
About 100bps on either side of the DNA are removed as a part of DNA repair

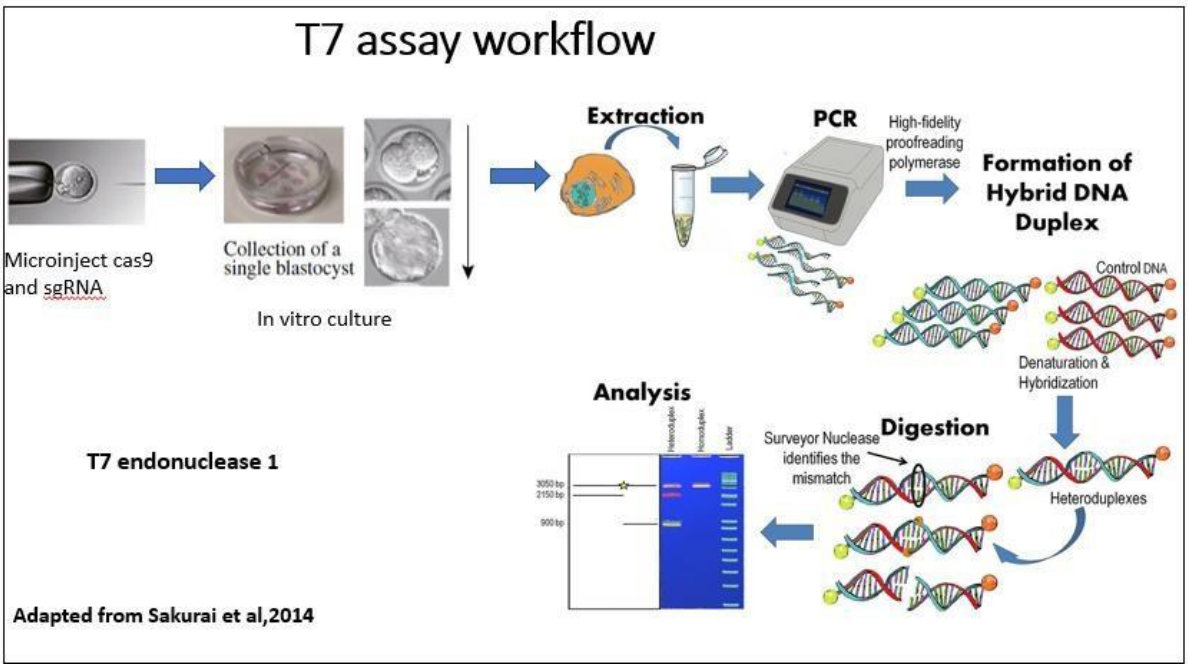
Once the design for sgRNAs are ready, we need to generate them and test them to pick the best one

Steps to generate and test sgRNAs

1) Use In vitro transcription as described in Engen sgRNA synthesis kit to generate sgRNAs
   Link: https://bit.ly/3c8OEn8 (make sure it's compatible with the cas9 being used)
2) Send it to a transgenic facility where your sgRNA and cas9 enzyme will be injected into a mouse embryo at a single celled stage (for all practical purposes it's a few more than one cell)
3) It's cultured till it reaches a blastocyst stage
4) In the absence of a donor DNA the system undergoes NHEJ repair I.e. non homologous end joining repair.
5) Taking advantage of this repair mechanism we use an assay called T7 assay to test for the cutting efficiency of sgRNAs
6) In this assay DNA extracted from the blastocyst cells is PCR amplified
7) The sample post PCR is subjected to a heat and cool cycle; If the DNA has undergone NHEJ repair it will form heteroduplexes after this cycle that can be recognized by the T7 endonuclease enzyme
8) **Note**: Heteroduplexes are formed because the sgRNA will likely cut DNA in some cells but not all. During the heat and cool down cycle DNA molecules will monomerize and dimerize, if the newly dimerized DNA is a heteroduplex it will be recognized and cut
9) **Follow the schematics below**







Using this assay we select guides that cut the best

Next we design the donor DNA using the following steps

How I designed my Donor DNA

I used to following protocol: https://bit.ly/35yPdEp

Some important points that I had to consider that may apply to you

1) My sgRNA was a forward guide
2) It actually recognized the reverse DNA strand
3) As a result when the DSB or double strand break is introduced, the forward strand is set free first

4) So, the Donor DNA oligo needs to be complementary to the forward strand that is set free
5) Thus the Donor DNA needs be the reverse strand sequence with the desired mutation at the target site with the RE site (silent mutation) for screening as mentioned earlier
6) The Donor DNA constitutes about 127nts 36 distal and 91 nts proximal to the PAM site

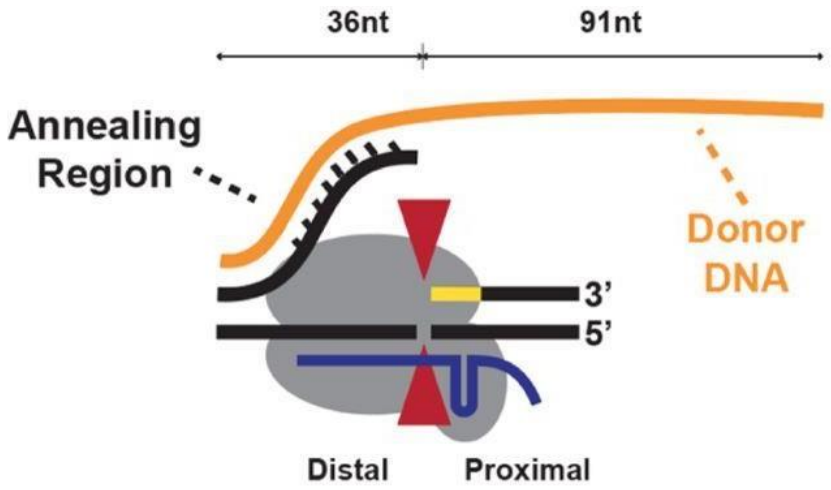


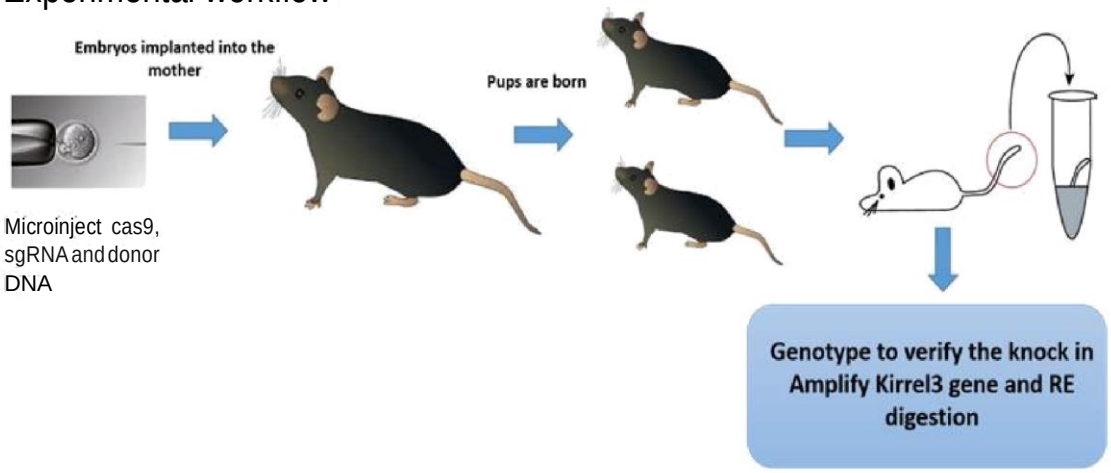
Figure 2: Single stranded donor DNA interaction with the Cas9-DNA duplex

Once the donor DNA and sgRNA are ready, we go ahead with the following plan

1) Microinject cas9, sgRNA and donor DNA into a mouse embryo 2-3 cell stage (done by transgenic facility)
2) Implant the embryo in a surrogate female mouse
3) Let pups be born
4) Screen the pups by taking a sample from their tail and testing for the presence of donor DNA by using the RE site
5) If positive for RE site -> sequence the DNA to test for presence of mutation of interest and no nonspecific mutations – insertions and/or deletions
6) Note: these pups- may or may not have the Q128A mutation in the germline
7) Full mutants generated from each mouse at this stage will be a separate mouse line
8) So we breed this with a WT mouse and if the pups are heterozygous for the Q128A mutation then we can be sure that mutation is present in all cells of the body
9) Once we have Hets for Q128A we breed 2 Hets from each mouse line to give us full Mutants for Q128A
10) We can then breed the F2 Full Mutants or Hets with WT to get F3 Q128A mutants and test for the defect of interest (so the likelihood of a non-specific off target mutation co-segregating with the mutation of interest at F3 is low)
11) If the same defects are observed in different mouse lines -> that also indicates that it is less likely due off target effects and more likely due to the specific mutation

The following schematics summarize this: -

Experimental workflow



Adapted from Sakurai et al.2014