

CRISPR-Cas is a system with a terrible name that contrary to popular belief does not actually do any genetic engineering or modification of DNA bases directly. Instead, the system uses a trick that has been well known in genetic design for many many years. It cuts DNA. Yep, that’s all Cas9 does is cut DNA. See when DNA is damaged or cut most all organisms start to do DNA repair and this can end in one of three ways 1) The DNA is repaired perfectly and everything is ok 2) The DNA is repaired but some mistakes happen leading to problems translating the gene into protein because of frame shifts or mutations 3) The DNA is repaired using a template artificially supplied that results in a completely new sequence. When using CRISPR people aim to make use of (2) or (3) but most of the time when people talk about CRISPR they usually just mean (3).

**This means that most all CRISPR systems are composed of 2-3 components**

1. The Cas9 protein which cuts the DNA
2. The tracrRNA and crRNA, which when synthetically combined are called a “guide RNA” but also called sgRNA(synthetic guide RNA) or gRNA
3. The template for repair

There are two unknowns then for every CRISPR experiment that you need to figure out

1. Where in the genome will this happen
2. What do I want to put into the genome or what base changes do I want to make

**Step 1:** Where do you want to make your change?

This usually comes down to two things

1. Do you just want to insert something in the genome and it doesn’t matter where?
2. Do you want to modify a specific gene?

When using CRISPR Cas9 mediated genome modification you will either modify an existing gene or insert something new. When modifying an existing gene the sequence of the gene can be obtained from one of the many databases that hold human genome information. Many companies now have programs that can automatically target most genes so all you really need to know is the name of the gene that you want to target.

If your goal is to insert something in the genome of the organism and it doesn’t matter where there are what you can consider standard places that people insert things in organism genomes. These regions have usually been tested to show little or no effect on the organism.

Humans Rosa26 or H11 ortholog locus or AASV1 http://www.bushmanlab.org/assets/pdf/publications/22129804.pdf

For px601

gRNA U6 Forward

Gagggcctatttcccatgat

gRNA Reverse

aaaaatctcgccaacaagtt

Px601 BsaI

For 426 Yeast

gRNA SNR52 Forward

tctttgaaaagataatgtat

gRNA Reverse

Agacataaaaaacaaaaaaa

or

taactaattacatgactcga

hRosa26

<https://www.ncbi.nlm.nih.gov/nucleotide/528476658?report=genbank&log$=nuclalign&blast_rank=21&RID=VSGJFKSN014&from=9388046&to=9388495>

ctcgacacca actctagtcc gtgggaagat aaactaatcg gagtcgcccc tcaaatctta cagctgctca ctcccctgca gggcaacgcc cagggaccaa gttagcccct taagcctagg caaaagaatc ccgcccataa tcgagaagcg actcgacatg gaggcgatga cgagatcacg cgaggaggaa aggagggagg gcttcttcca ggcccagggc ggtccttaca agacgggagg cagcaaagaa ctcccataaa ggtattgcgg cactcccctc cccctgccca gaagggtgcg gccttctctc cacctcctcc accgcagctc cctcaggatt gcagctcgcg ccggtttttg gagaacaagc gcctcccacc cacaaaccag ccggaccgac ccccgctcct cccccacccc cacgagtgcc tgtagcaggt cgggcttgtc

Depending on how serious your experiment is you might want to sequence these regions to look for organism specific mutations but in most cases you can probably just use the sequences available from NCBI.

This sequence allows us to do two things

1. Design a gRNA to insert something in the genome
2. Create homology arms for our template sequence so it is inserted into the genome

Designing gRNAs

Validated gRNAs

<https://www.addgene.org/crispr/reference/grna-sequence/>

Create your own gRNA

<https://www.atum.bio/eCommerce/cas9/>

$300 for gRNA in a plasmid that also expresses Cas9

Our gRNAfor hRosa26 insert (PAM in yellow)

TTGCAGCTCGCGCCGGTTTTTGG

AACGTCGAGCGCGGCCAAAAACC

GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTAACTGGTAC

When I say we are designing a guide RNA we are really only designing 20 base pairs of the guide RNA known as the spacer. The rest of the guide RNA will almost always stay the same. The spacer is also located in the crRNA for those working with crRNAs

The spacer should be 20 bases long and it should end in NCC(“N” means any nucleotide) or NGG. What you do is find **NGG** in the top strand(what we have) and use the 20 nucleotides preceding it as your spacer or find **CCN** on your top strand and use the reverse complement of 20 nucleotides after it as your spacer, which will make the CCN, NGG on the strand that the spacer is bound to.

CCA is the CCN and the next 20 bases we can find the reverse complement to make our spacer. An easy way to get a reverse complement is this website: <http://www.bioinformatics.org/sms/rev_comp.html>

Next we should pick the homology arms. Homology arms are regions in your template that are identical to the genome of the organism you are trying to insert something into. This makes it so that the organism actually thinks your template is a real template. These arms go on each side of the template DNA and the size depends on the size of what you are trying to insert.

Homology arms on each side of 800bp work pretty well if you are inserting a few kb of DNA. If you are only making single point mutations or changing one or two bases you can go as low as 50-100bp. The homology arms work best when there are within 100bp of the cut site but even better if they are within 10bp. If you include the spacer and NGG(CCN) beware because the CRISPR system will also cut your template if you are using double stranded DNA!!!

For us the upstream homology arm can be 40bp and have this sequence

And the downstream homology arm can be 40bp and have this sequence

Template DNA gene fragment encoding U6 and FE-modified sgRNA for increased stability once expressed AGTATTACGGCATGTGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC

G - <spacer>

GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTGCAGACAAATGGCTCTAGAGGTACGGCC GCTTCGAGCAGACATGATAAGATACATTGA

PCR - use proofreading polymerase pfu

30 cycles

94C - 30s

56C - 30s

68C - 1 minute

After the PCR do a DpnI digest to remove the old plasmid add 1uL to the PCR reaction and incubate at 37C for 1 hour.

Transform into DH10B and select with Amp

To create the template you need to do PCR

To amplify GFP with promoter and terminator from pJK prok1 eGFP plasmid

Forward Primer with homology arm

ATTTTGGGTGGCTGGCCGTTAAAAATTTTAACTGCATTTAGtttactggcatgcataaggc

Reverse Primer with homology arm

TTAGCTCAACGATTAATAACATTTTTTCATTAATTTAAGTcaaaaaacccctcaagaccc

If the strain is not ampicillin resistant i.e. HME 63 is a no go you can also amplify the Ampicillin cassette and select for ampicillin

The forward primer is the same

Reverse primer with homology arm

TTAGCTCAACGATTAATAACATTTTTTCATTAATTTAAGTgaagatcctttgatcttttc

PCR - use proofreading polymerase pfu

30 cycle

94C - 30s

56C - 30s

68C - 1 minute 30 seconds

Do a gel purification of the PCR product or

Do a DpnI digest by adding 1uL DpnI and then incubating at 37C for 1 hour and then do a PCR purification