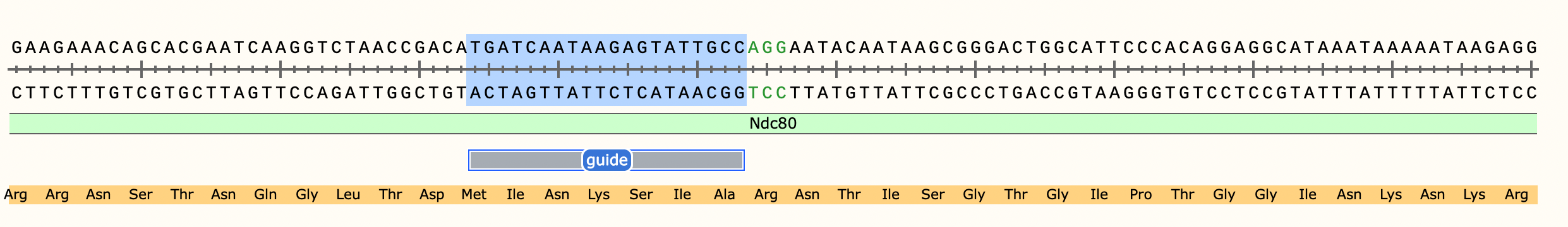
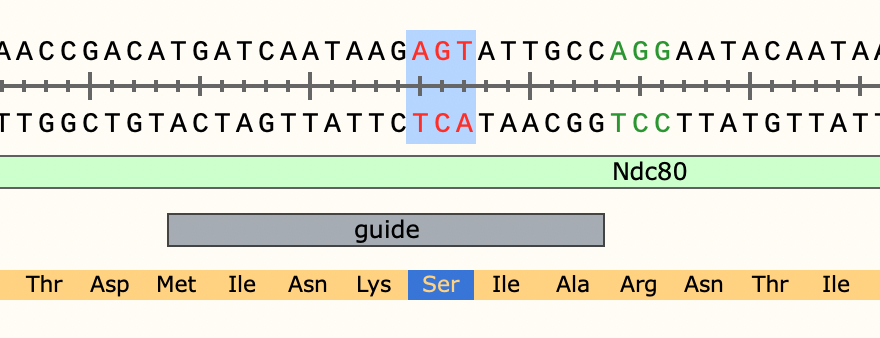
**Manual Design Guide + donor workflow:**

1. Choose the gene to mutate (ex. Ndc80) and the guide
   1. I open gene in snapgene or ape. This automatically fills in the bottom strand, or reverse complement, as well as detects the gene which I then annotate by creating a “feature”. I know where it starts and ends because of the way I downloaded the file to contain 1000 bases upstream and 1000 bases downstream.
   2. Search for NGG (where N is any base, A, T, C, or G) (aka the PAM)
   3. Take the 20 bases upstream of the NGG and that is the guide.



Guide: TGATCAATAAGAGTATTGCC

1. If I want to mutate the Ser (Serine 49) to alanine then it will be changing the AGT to GCT. I will also need to mutate the PAM (AGG in this case). Conveniently, the AGG is “in frame” meaning the entire PAM encodes for one amino acid, Arg, rather than being split between two different amino acids. At least one of the G’s needs to be mutated, and another codon for Arg is AGA which would mutate the second G without changing the amino acid. I will give a list of rules for mutating PAMs in another section below.



* 1. To make the donor, take 132 base pairs surrounding the mutations (either centered around both the main mutation and the PAM mutation, or if easier could just center all the donors for a given guide around the PAM).



* 1. Make the mutations in the donor.



Guide: TGATCAATAAGAGTATTGCC

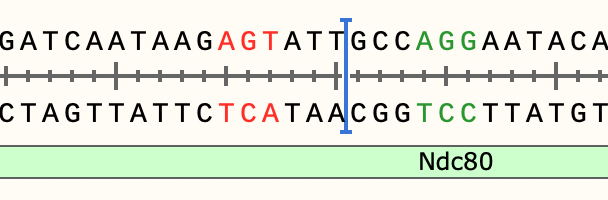
Donor: ACAATTGAGGAGAAGAAACAGCACGAATCAAGGTCTAACCGACATGATCAATAAGGCTATTGCCAGAAATACAATAAGCGGGACTGGCATTCCCACAGGAGGCATAAATAAAAATAAGAGGACAAGAAGCAC

(red is the targeted mutation and green is the PAM that gets mutated so it is no longer a PAM)

1. Add the extra sequence (in grey) around the guide and donor (this sequence will stay consistent among all guide+donor pairs)

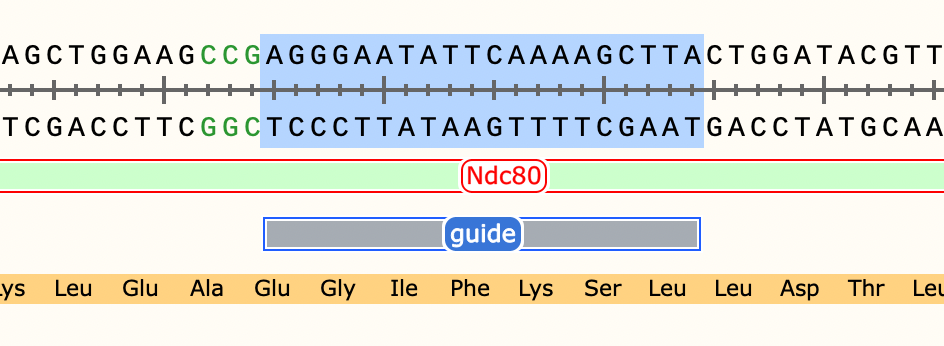
Final guide+donor to order: GACCGTGCGACTGGGCGTCTCGGATCTGATCAATAAGAGTATTGCCGTTTGAAGAGCATACGCTCTTCTTCTACAATTGAGGAGAAGAAACAGCACGAATCAAGGTCTAACCGACATGATCAATAAGGCTATTGCCAGAAATACAATAAGCGGGACTGGCATTCCCACAGGAGGCATAAATAAAAATAAGAGGACAAGAAGCACACATCGAGACGTGTCCCTGCCTTGCG

We will likely want to mutate 5 amino acids on either side of the cut site which is 3-4 bases upstream of the PAM (see cursor location in image). If it is easier to think about it in terms of the PAM, this will result in 6 amino acids upstream and 4 downstream. That may change slightly depending on if the PAM is split between amino acids or not but it won’t matter enough that you need to care about that technicality.



We will also want to do the PAMs on the reverse complement strand of DNA. I will give a brief example of how I do those:

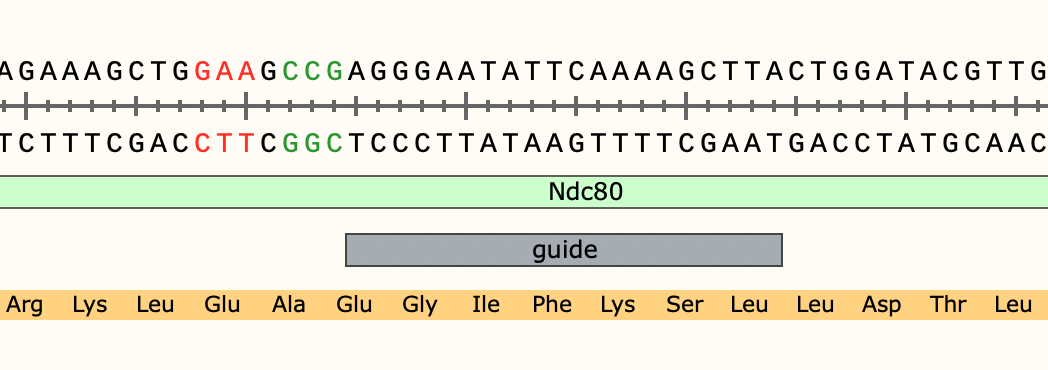
1. You can search for a CCN on the top strand or look for the NGG on the bottom strand (reverse complement strands are read backwards). The guide with be 20 bases **after** the CCN.



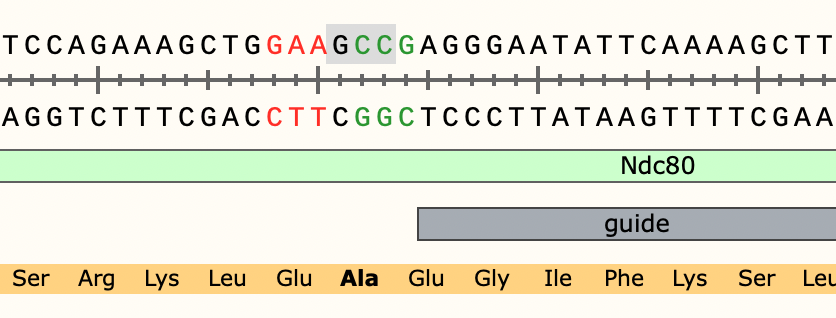
Guide: TAAGCTTTTGAATATTCCCT

The guide in this case needs to be entered as the reverse complement strand read from the left to right. Matt and I realized this is where it might get tricky since the files you have only contain the top strand and not the reverse complement. We may need to come up with a solution for this.

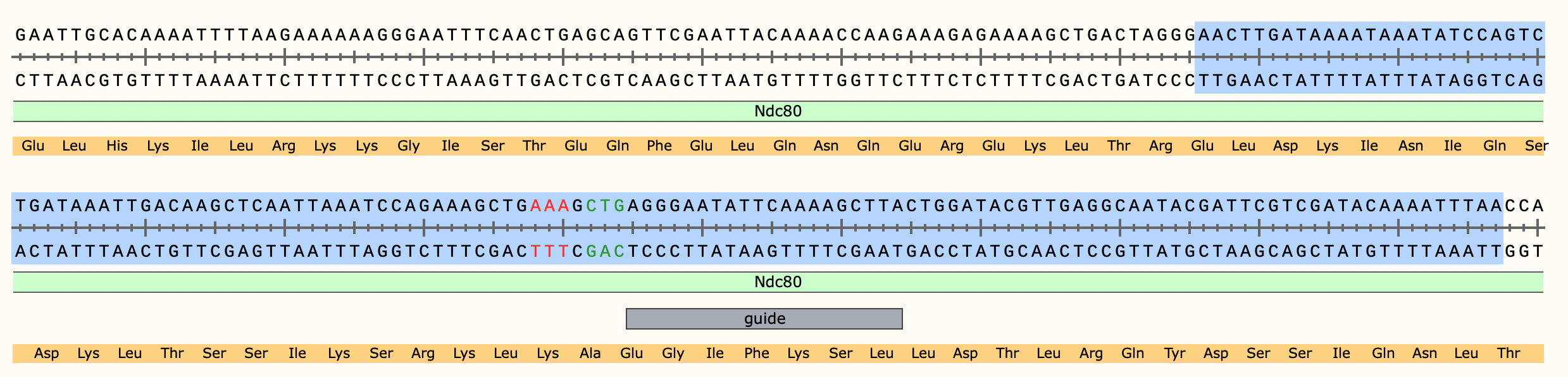
I have also been doing the donor as based on the reverse complement, but it doesn’t actually matter if it is based on the top or bottom strand.

1. Design the donor. Let’s mutate the Glu before the PAM to a Lys (GAA to AAA). 

In this case the PAM is split between the Ala and Glu, but both G’s are part of the Ala sequence. If we mutated the Ala to GCA, then the bottom strand (from right to left) would be CGT, effectively mutating one of the G’s in the PAM while still leaving it an Ala (called a silent mutation because the mutation doesn’t change the amino acid).



Mutations:

 Donor: AACTTGATAAAATAAATATCCAGTCTGATAAATTGACAAGCTCAATTAAATCCAGAAAGCTGAAAGCTGAGGGAATATTCAAAAGCTTACTGGATACGTTGAGGCAATACGATTCGTCGATACAAAATTTAA

Or if doing the reverse complement: TTAAATTTTGTATCGACGAATCGTATTGCCTCAACGTATCCAGTAAGCTTTTGAATATTCCCTCAGCTTTCAGCTTTCTGGATTTAATTGAGCTTGTCAATTTATCAGACTGGATATTTATTTTATCAAGTT

Again, I don’t think we need to do the reverse complement for the donor, but you could if that were easier for some reason.

Final:

GACCGTGCGACTGGGCGTCTCGGATCTAAGCTTTTGAATATTCCCTGTTTGAAGAGCATACGCTCTTCTTCTAACTTGATAAAATAAATATCCAGTCTGATAAATTGACAAGCTCAATTAAATCCAGAAAGCTGAAAGCTGAGGGAATATTCAAAAGCTTACTGGATACGTTGAGGCAATACGATTCGTCGATACAAAATTTAAACATCGAGACGTGTCCCTGCCTTGCG

**Preferred codons table:** (These are the preferred codons to mutate to)

|  |  |
| --- | --- |
| Phe | TTT |
| Leu | TTG |
| Ser | TCT |
| Tyr | TAT |
| Cys | TGT |
| Trp | TGG |
| Pro | CCA |
| His | CAT |
| Gln | CAA |
| Arg | AGA |
| Ile | ATT |
| Met | ATG |
| Thr | ACT |
| Asn | AAT |
| Lys | AAA |
| Val | GTT |
| Ala | GCT |
| Asp | GAT |
| Glu | GAA |
| Gly | GGT |

**PAM mutation guide so Cas9 doesn’t re-cut.**

\*We would like to always do silent mutations for this part (meaning the amino acid sequence doesn’t change).

1. Try to change the PAM (NGG) sequence. Mutating either G to anything else is fine. This might be too complicated but, if possible, I would like to avoid mutating the PAM to NAG.
2. If you can’t mutate the PAM then mutate at least one location in the “seed” region (10 bases upstream of the PAM). The closer the silent mutation is to the PAM the better it works. We may decide that we want to do two silent mutations if we find that one in the seed region isn’t enough to prevent re-cutting.
3. Oftentimes the mutation we intend to make will be in the seed.
   1. If it is still possible to make a silent PAM mutation then that would be good (Although we are currently testing this and this parameter might change).
   2. If there is no way to make a silent PAM mutation and the mutation is more than 5 bases away from the PAM then the next best thing would be to make a silent mutation within the 5 bases upstream of the PAM.
   3. If the mutation is within 5 bases from the PAM and you can’t make a silent PAM mutation, then I wouldn’t make any additional mutation.