HMB311H1: Laboratory in Fundamental Genetics & Its Applications
CRISPR I: gRNA Design Computer Lab

A Lab Developed by Alexander Sullivan & edited by Dr. Alistair Dias

SOFTWARE REQUIRED

- Text editor (Notepad/Notepad++, Sublime Text Editor, Atom, etc.…)
- Browser for web access (Google Chrome, Mozilla Firefox, Microsoft Edge, etc.…)

PLEASE COMPLETE COMPUTER PROTOCOL #1 (Benchling Registration) BEFORE COMING TO LAB (SEE PAGE 4)

OBJECTIVES

- ✓ To understand how to use online bioinformatic tools for CRISPR experimental design
- ✓ To understand how to target mutations causing disease for CRISPR experimentation
- ✓ To be able to create, design and customize gRNA and its appropriate primers

BACKGROUND

In this dry lab portion of your CRISPR module we will be looking at online tools that help you look through the sequence of a candidate gene and design a corresponding guide RNA (gRNA) along with specific primers to synthesize your own gRNA. You will need access to Benchling (account required, may require you to sign-up using a UofT/university associated email) for this protocol.

Both bioinformatics (creation of biological software's/applications and tools) and computational biology (the study of biology using computational techniques) are an ever-growing field with every new discovery in the field of science; whether it be computer science/programming, math, physics, biology, etc. With each discovery and innovative technique, new software and applications must be created with its own unique or modified algorithms to solve different problems that can be found accompanying the respective issues. One example of this comes with CRISPR.

When CRISPR became a keen interest of many labs, tools and software's had to be created to accompany the mass need for a tool to help determine and design the optimal CRISPR sequences. This included finding and creating optimal gRNA sites with corresponding and appropriate PAM (Protospacer Adjacent Motifs) sites based on certain parameters defined by the scientist that matched their specific experiment. Here, you will be using Benchling's CRISPR design tool[1] to find and create your gRNA sites based on specific PAM sites to learn how to target and select specific and appropriate gRNAs.

Supplementary Information Box #1: Many different tools!

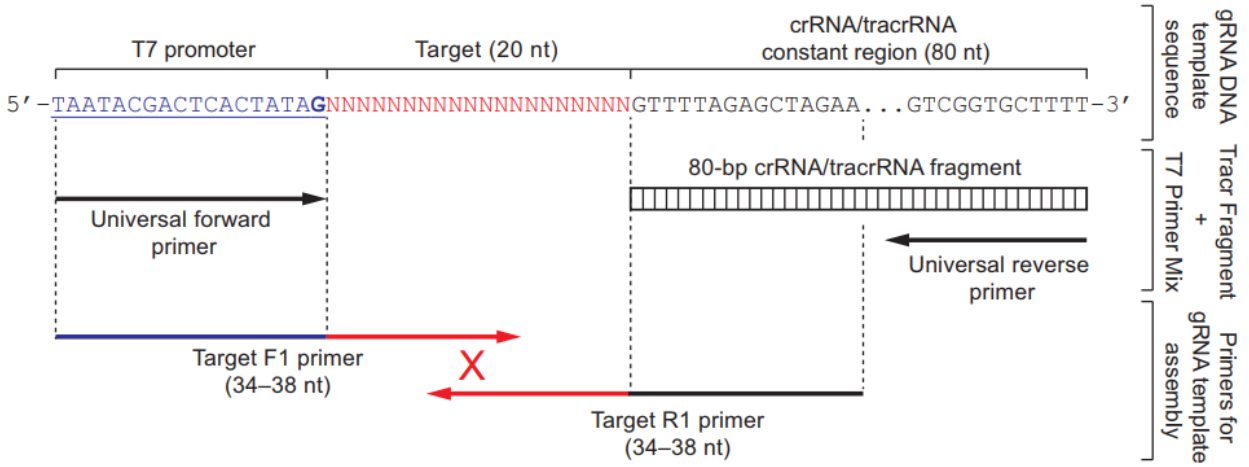
There are many different online web-based applications and local-client based software you can utilize to create your desired gRNA and PAM sites as well as your primers which all have their own benefits and disadvantages.

We are specifically looking at Benchling, a virtual lab notebook web-tool that began as a iGEM project by MIT in 2012[2]. Not only does Benchling provide the user with a clean, user-friendly interface, it provides the user, you, with many different tools such as plasmid design and cutting, genome visualization, primer design, band/digest prediction, sequence alignment, CRISPR design and many other tools with multiple in-depth customizable options specific to your options that utilize many different algorithms that have become commonly accepted in the field of bioinformatics.

Another tool that exists is the ThermoFisher GeneArt CRISPR Search and Design Tool[3]. This tool allows you to insert a gene or a gene name and search through it for an all possible CRISPR sites that work with their products. This tool also provides you with a table of recommended sequences and how many binding sites that specific CRISPR sequence has. The disadvantage to this web-tool is that it does not provide the user, you, with the options to customize the settings in depth to specify what regions, without you manually checking, you want the gRNA to bind to. This tool is optimal if you are planning on buying products directly from ThermoFisher as it tells the user if the users' sequence is compatible with their products and provides the user with Genomic Cleavage Detection (GCD) primers to be utilized in GCD assays (by GCD Kits, a technique used to rapidly determine if locus-specific double-stranded breaks occur in their genome edits[4]) to purchase with their CRISPR product.

There are many more options that exist out there, like MIT's own and crucial MIT CRISPR Design tool[5] or Galaxy's CRISPR Recognition Tool[6], that you can utilize to create your own gRNA with PAM sites and its appropriate primers. Once you understand what conditions your design preparations you require for your CRISPR project, you will understand which tool would be optimal for your design process.

You will also be learning to design your own target forward (F1) and reverse (R1) primers similar to how they were designed in your wet lab protocol; utilizing ThermoFisher's GeneArt Precision gRNA Synthesis' forward and reverse oligonucleotides for PCR assembly guide (as seen in the figure below). These primers allow for amplification of your desired target DNA sequence. The overlapping of the two primers you see within the target DNA sequence occurs because it allows the sequences to not only anneal later but act both as a primer and a template in PCR to generate a full-length DNA template.[11] These two primers are called nested PCRs primers because they are "nested" within the sequence. In general, nested PCRs role is to provide intrinsic product carryover to ensure all the target gene is covered as well as provide maximum concentration of PCR products[17].



To create an optimal gRNA, you must be able to understand what makes an optimal gRNA binding site. When segmented sequences align, there is always the possibility that it can be bind to multiple positions along the genome. If the sequence (gRNA) binds on your specified location, this is called on-target binding while if it binds to an undesired location, this is called off-target binding. To determine if your gRNA is optimal for your experiment, you want to minimize possible off-target binding sites and maximize on-target binding. The probability of efficient binding can be calculated manually by doing a sequence alignment and seeing where the gRNA binds to on the genome to determine off-target binding and how well it can encompass the on-target sequence. Thankfully, there are algorithms that exist that do these calculations for you (See Supplementary Information Box #2).

The algorithms we will be using, which are the default setting for Benchling, are the CFD on-target scoring algorithm[7] and the MIT Scoring Method for off-target scoring[8]. For both variables, you would want an output as close to 100 as possible as that would represent the best possible scoring and sequence for your CRISPR experiment. Usually, when looking at on-target sites, you want to choose a site that is within 20% of your max value. For example, if your highest guide sequence (gRNA) generated has an on-target score of 100 (this gRNA may not be in the right position on your gene) then overall you want to choose a gRNA (which does bind to your desired genome location) with an on-target score of 80. In general, it would not be optimal to choose something below 40[8]. When looking at program generated gRNA sequence lists, look at your max on-target score and try and stick to within 20% of this score when selecting your final gRNA (e.g. max on-target score = 62.9 x 0.2 = 50.3). When looking at off-target sites using the CFD algorithm, the scoring will depend on what PAM sequence you are looking for. Let's say you are looking for a common PAM site like NGG, in this case you want an off-target site that is relatively high: near or above 80. While, if you are looking at a less common PAM site, a lower off-target score can be considered acceptable relative to the other off-target scores that have been calculated for your specified gene[9]. Do beware, the lower the off-target score, the more likely the gRNA will bind to an undesired location. Overall, it is important to note that on-target scoring algorithms are less reliable than off-target scoring calculations. For the purposes of your online assignment, trust your off-target scoring (the closer the off-target score is to 100, the better the gRNA selection) over the on-target scoring.

Supplementary Information Box #2: Algorithms involved

Do not be scared off by the word "algorithm". Though it is commonly thrown around to make something sound fancy or complicated, all it means it a set of rules for an operation or calculation to follow to determine an output.

The MIT Scoring Method for off-target scoring is rather simple. Each mismatch would have a weighted value to it based on its position along its sequence ($W[e]$) and be accounted towards the score of a single hit (a single hit is defined by the whole sequence that contains mismatches, not a single mismatch) for each mismatch in that single hit (n_{mm}^2). This can be shown using the equation (don't worry too much about the equation, you will *not* be tested on it)[8, 14]:

$$s_{hit} = \prod_{e \in M} (1 - W[e]) * \frac{1}{\left(\frac{(19 - \overline{d})}{19} * 4 + 1\right)} * \frac{1}{n_{mm}^2}$$

From there, the scores would be aggregated using an assigned score where the more mismatches you have, the smaller the number would be. Therefor, the less the number of single hits you have (less mismatches), the score would be closer to a theoretical 100 therefore, that would be the best option for a guide sequence. This can be visualized using the equation[8, 14]:

$$S_{guide} = \frac{100}{100 + \sum_{i=1}^{n_{mm}} S_{hit}(h_i)}$$

/*** I cannot find the breakdown of the CFD equations to determine its output, going to need more time to understand "CFD Rule Sets" and "CFD Poolsets" to determine how they calculated the on-target scores ***/

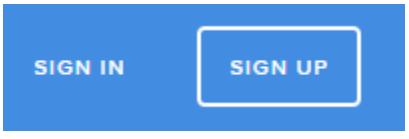
NOTE: *The software that you will be required to have installed onto your computer will be a text editor and a web browser. Your operating system does not matter but you may not be able to run certain applications on mobile devices.*

Computer Protocol #1: Signing up for Benchling (Complete Before Lab!)

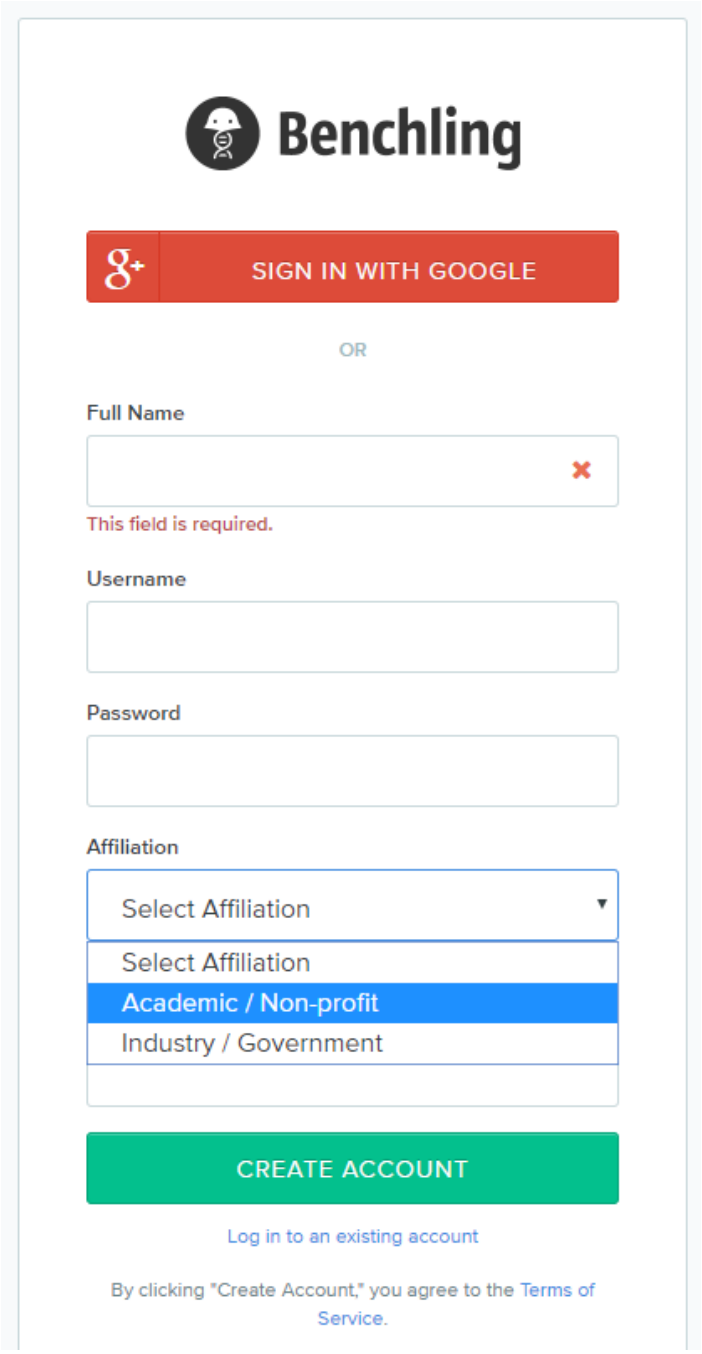
The web-tool that we will be using today is called Benchling. Benchling is a virtual lab notebook that is used by the academia and the industry to maintain their lab notes while providing easy-to-use and user-friendly bioinformatic tools[1]. In Computer Protocol #1, you will be brought through the basics of how-to sign-up and set up Benchling for the completion of your assignment.

1. Go to the following link: https://benchling.com/
2. On the top right of the page, you should see an option to "SIGN UP", click that button

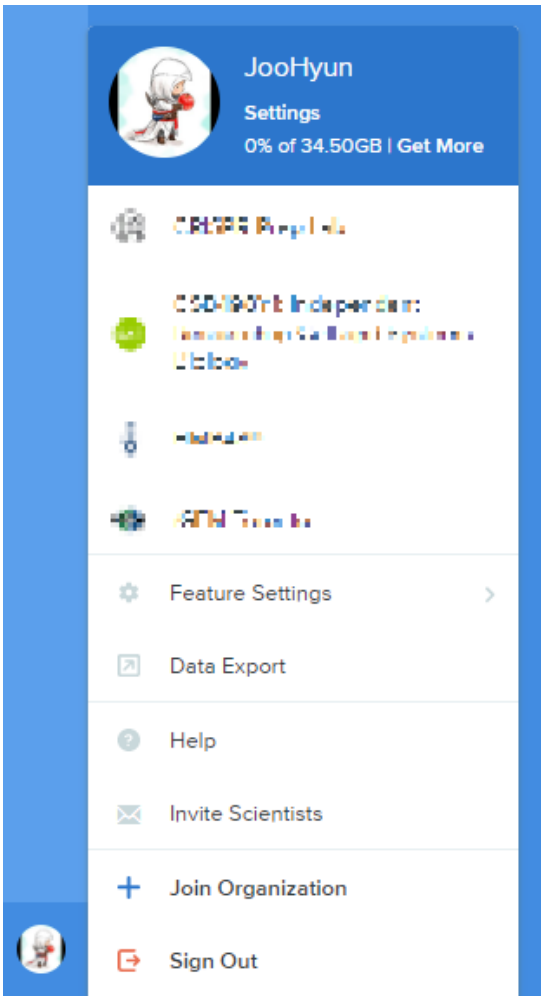
*****IT IS REQUIRED FOR YOU TO SIGN-UP TO USE BENCHLING*****



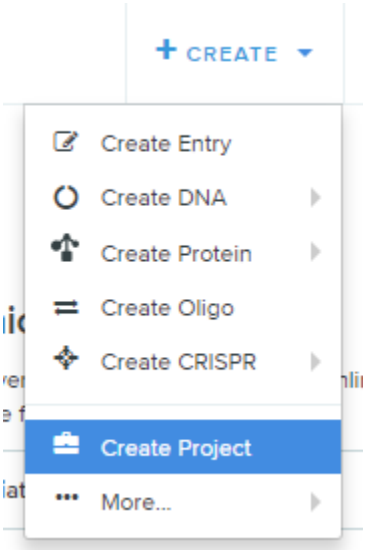
3. On the sign-up page, change your affiliation “Academic/Non-profit” and follow the rest of the on-screen instructions to complete your sign-up. If you are running into any issues during sign-up, please contact your course instructor



4. Once you have completed your sign-up onto Benchling, follow on-screen prompts to finish setting up your personal Benchling account
5. You are going to want to create a new project. Click on your profile icon on the bottom left of Benchling and go to “Settings” (not “Feature Settings” but “Settings”)



6. On the top right, click on the “Create” dropdown and choose the “Create Project” option



- It may be important to note that every time you work with Benchling, you do *NOT* need to create a new project. Later into this tutorial you will learn how to create folders within your project. If your work is still related to the same topic/field as your

project, it helps with your organization to create folders and sub-folders opposed to new projects each time.

- *Though*: This is dependent on you, the user, on your preferred method of organization as there is no formal way of keep your files organized relating to this type of work

7. From here, just follow the on-screen instructions on how to create your project. We recommend you call your project something recognizable like "HMB CRISPR Teaching Lab" or with your course code. Once you have completed this, please move onto Computer Protocol #2 where you will be designing your gRNA and primers.

Computer Protocol #2A: Creating gRNA and primers (will be demoed in lab)

This section will give you some practice using Benchling, designing gRNAs and creating F1 and R1 primers (using the Thermofisher method) before you complete your online assignment. We will practice with the sequence we are targeting for your wet lab CRISPR module, enhanced Blue Fluorescence Protein (eBFP). The full sequence for eBFP is shown below with start codon (yellow), chromophore region (green) and stop codon (red) highlighted:

TCCACCCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATT
GACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACC
GGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCC
ACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTG
CCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCCACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGA
CTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCG
AGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCAC
AAGCTGGAGTACAACTTCAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCG
CCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCG
ACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACC
GCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGG
TACCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAA
ACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTAC
AATAAAGCAATAGCATCACAATTTCACAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGG

1. In any text editor like Notepad/Notepad++, Sublime Text or Atom, type the following:
   `> eBFP Sequence`
2. Then, create two new lines (press Enter twice) and copy & paste the above sequence into the text editor, it should look as the following:
   `> eBFP Sequence`

   TCCACCCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCG
   CCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGAT
   CCGCTAGCGCTACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGC
   TGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCT
   GAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCCACGGCGTGCAGTGC
   TTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCA
   CCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCAT
   CGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTTCAACAGCCACAAC
   GTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCG
   TGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAG
   CACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGG
   ATCACTCTCGGCATGGACGAGCTGTACAAGTCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTA
   CCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTT
   AAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCT
   TATAATGGTTACAATAAAGCAATAGCATCACAATTTCACAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGG

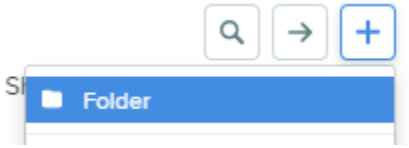
3. Choose “Save As” (typically found under the “File” dropdown option) and save your file as a text file (.txt). If you are using Notepad++, Sublime or Atom, please save your file as a fasta file (by adding a .fasta extension manually).

Supplementary Information Box #3: FASTA

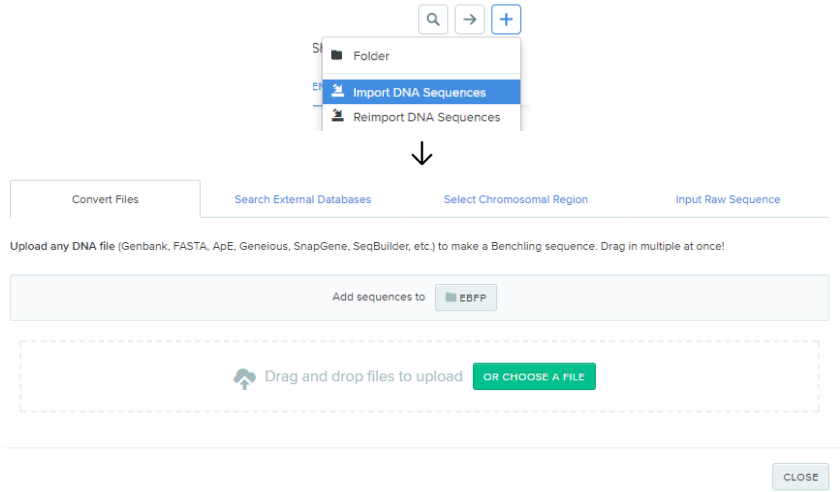
FASTA files are a common type of file format that is used in bioinformatics and computational biology. It is a sequence record file that consists of a single-line description (indicated by the “>”) followed by its sequence.

These file formats are extremely important and crucial as they are easy to use and easy to parse through with clear indications for many software and applications to read and understand. Many web-apps and software’s accept these types of file formats, so it is important that you understand how they are structured, how you can modify them and how you can create them for your own sequences/data.

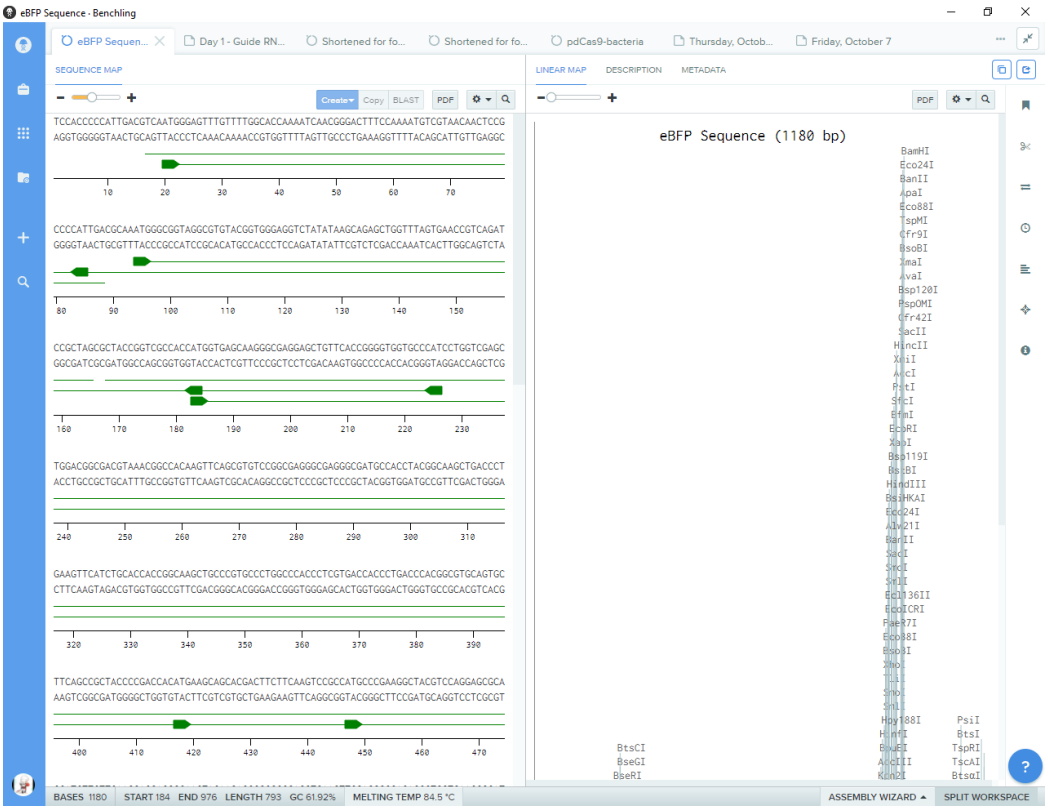
4. In Benchling, under your named main Project folder, click on the “+” symbol (see pic below) and create a sub folder (you do not have to create a folder, but it may help keep you organized)
    I) To create a folder, you will click on the plus symbol while the “Projects” viewer is opened



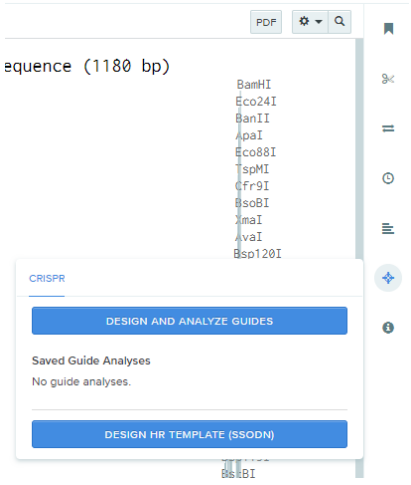
5. While in the new folder, click on the “+” again and click “Import DNA Sequence”. Under the “Convert Files” tab, either drag and drop your file or browse/choose your previously created eBFP (.fasta or .txt) file to upload and click close AFTER Benchling tells you the upload is complete



6. The sequence should appear as shown in the picture below in a split screen (restriction sites and a linear map). Note: If you have 3 windows open, you can close the project list window by clicking the home key at the top left-hand corner of the screen (the jellyfish looking icon!)

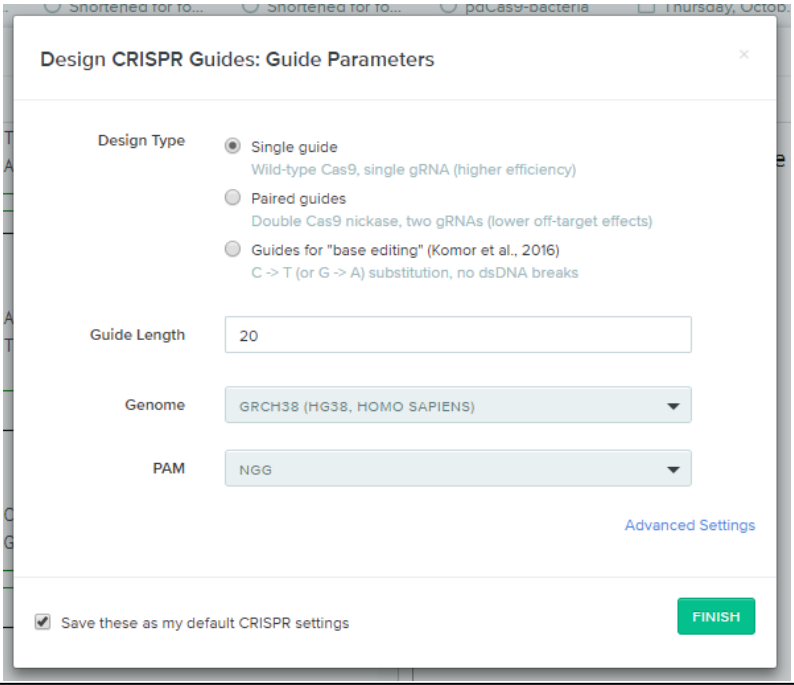


7. On the right side of Benchling while your sequence is open, you should see the following button with the glyph icon "✥". This button opens a CRISPR design tool which would allow you to create and design guide sequences. Open this and click on "Design and analyze guides"



8. For now, keep the default settings on the "Design CRISPR Guides: Guide Parameters" modal and click on the green "Finish" button. If you wish to learn more about the "Advance Settings", see the supplementary information box #1 and #4.

   ***** Default settings may not be the same for all people, please check if your default settings are the same as below. Specifically, check that the genome is "GRCH38 (HG38, HOMO SAPIENS)"*****



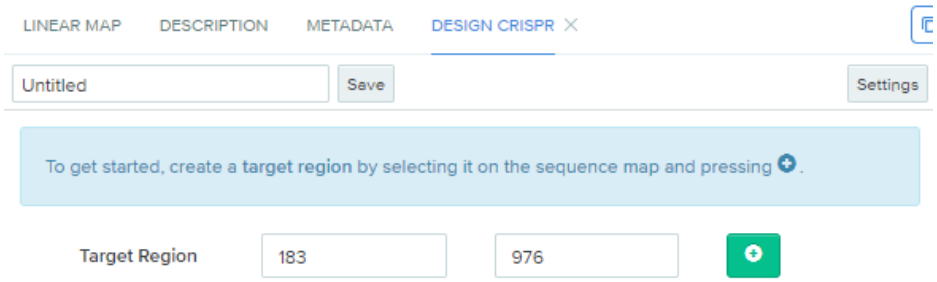
Supplementary Information Box #4: Advanced Settings

Though in your protocol today you will only be using the default settings designing your gRNA, in the future, you might want to utilize the "Advanced Settings" that Benchling has available for you to utilize. These include:

1. Masked Regions on or off (default off)
    - A masked region is a region of a sequence/gene that has skipping repeats or low complexity regions
2. Change off-target scoring algorithm
    - Change from MIT Scoring Method to CFD off-target scoring method
3. Turn off optimized scoring for on-target scores (by default this is on)
    - If you are utilizing SpCas9, a recent paper has determined how to optimize CFD off-target scoring to allow for maximum activity, you can turn this option off if you would want[15]
4. Display certain properties on the sequence
    - In this setting, there is a threshold setting this prevents any on-target below 10 and off-target below 25 from being shown, you can change these values here
5. Guide composition
    - Nucleotide proportion minimum and maximum for gRNA settings can be determined here (default is 0% min, 80% max)

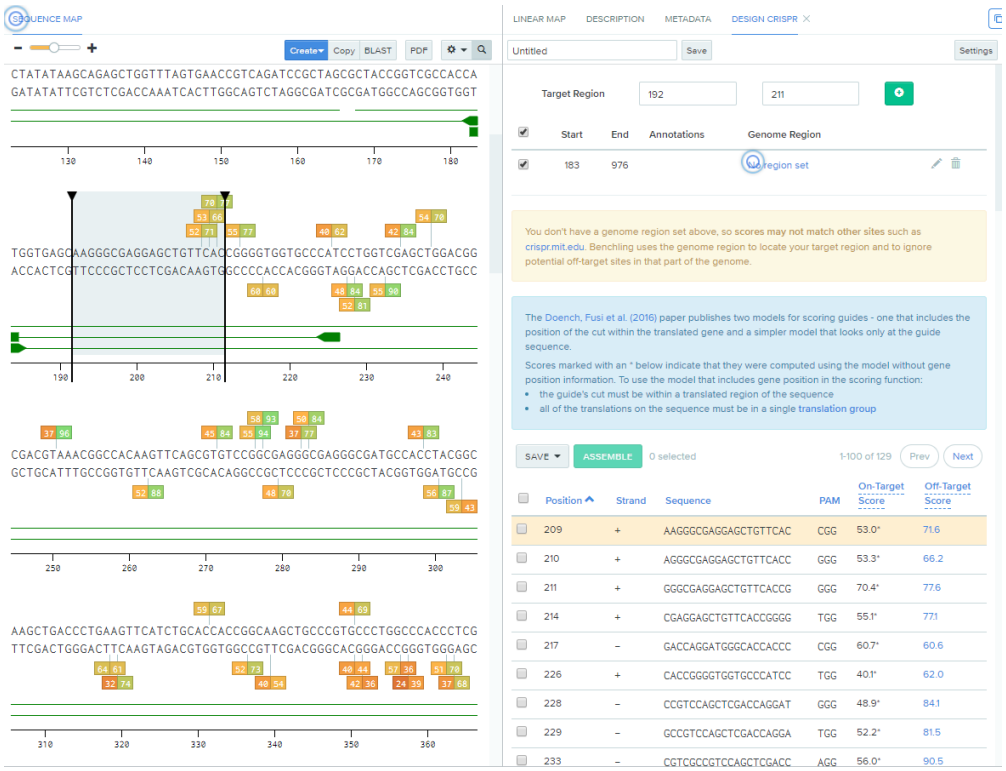
9. We now need to determine the sequence number range we want to scan to identify potential guides. Our target site (SNP in this case) that we wish to have our CRISPR gRNA modify is at sequence position 382, changing His66 to Tyr66 (eBFP to GFP). Therefore, we need our potential gRNA to contain the nucleotide at this position. For the purposes of this example and to

encompass as much of the sequence as possible we'll select a range from nucleotides 183 to 976. Enter this range as your Target Region and click the green button. For other examples you can generally select a range of plus or minus 60 nucleotides from desired cut site/nucleotide/area you want to genetically alter and then round to even number.

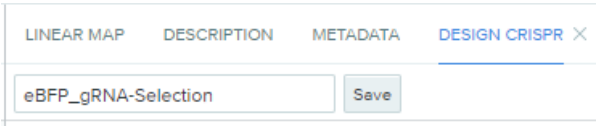


Question: Do you think the target region has to be the region that contains the sequence that encodes the protein product of the sequence? Would a shorter target region work (let's say 300-500)? How about a larger target region (let's say 1-1000)? Do you think one be more optimal over another and why?

10. Once the target region has finished loading (give it some time as more sequences will appear over time), you should see a list of potential guide sequences become available. Look through them and click on different ones (do *not* click on "Save" or "Assemble" yet), you will notice different regions of the sequence map will be selected, these correspond to the guide sequence.

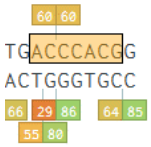


It is recommended that you save your "Design CRISPR" tab in-case you need to work on this at a later point in time or your browser/computer/network crashes. This can be done at the top of the "Design CRISPR" tab:



11. As mentioned before, the modification we wish to create a gRNA for is at position 382 (a cytosine), changing Tyr66 to His66. Therefore, you want to look for a sequence that contains this nucleotide position. We are going to choose a sequence based on strand direction, on- and off-target score and most importantly, position number. Normally you would have to know exactly what you are looking for before you start designing a gRNA and its primers but due to this being demo example, we are giving you this information. Overall, consider the following when finding your optimal gRNA:

    I) First, we need to look at position, and in this case, we want a sequence that contains our target position. Click through the options and see which gRNAs contain position 382. For an easier find (while nothing is highlighted in the sequence map tab) you can click also on the in the top right of this tab and find "ACCCACG" (the chromophore coding sequence) to have the sequence highlighted for easy location of the cytosine at position 382 ("ACC**C**ACG", see pic below).

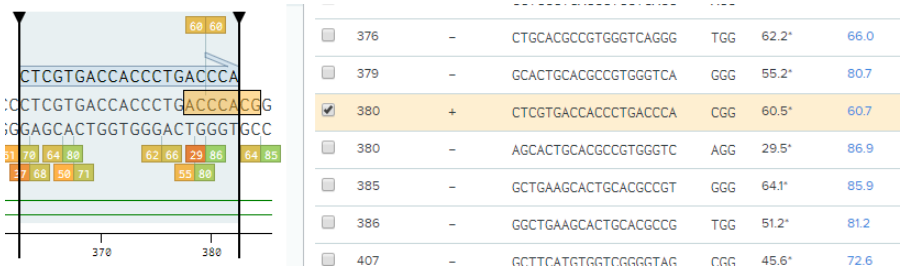


    II) If you are looking to use CRISPR to cut and modify/change a single (or few) nucleotides, the desired nucleotide should be within 3-8 nucleotides upstream of the PAM site.

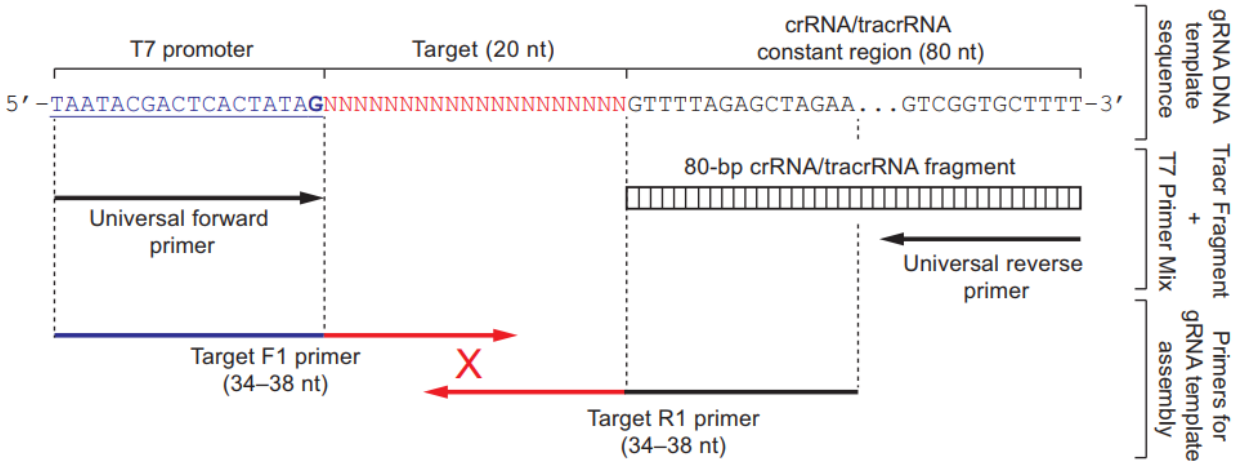
    III) Next, look at on-target and off-target scores. The higher the score, the better. Generally, you should look for an on-target score above 40 as anything lower would be advised against using. Optimally, you would want a score of 80 or higher for off-target. You can click on the off-target score to see other potential off-target binding sites in the genome for a selected species from Step 8.

    IV) Once you have found potential sequences, you can look at the strand direction (the top sequence indicates the sense-strand (+) while bottom is the antisense-strand (-)). You can use either strand depending on your on-target and off-target scores while also making sure your target nucleotide/region is part of your gRNA sequence. It will be a little easier to design primers for the sense strand but if you are only left with an antisense-strand you will just need to read your primers out in the right direction (5' to 3') based on the gRNA sequence.

12. From the sub-steps given in Step 11, you should only be left with one option available; a guide sequence that reads 5'-CTCGTGACCACCCTGACCCA-3' (PAM: 5'-CGG-3') on the sense-strand (+) starting at position 380 with an off-target score of 60.7. Select the checkbox AND MAKE SURE ROW IS HIGHLIGHTED by clicking on it.



13. Once you have found your desired CRISPR's target DNA sequence, you will begin designing your target forward (F1) and reverse (R1) primers, the nested PCR primers. The following is a reiteration of the format of how the primers will be designed. Try and understand it before moving on:



Supplementary Information Box #4: T7 Promoters

T7 promoters are commonly used in synthetic biology and in biological techniques as it a promoter that produces a high level of gene expression when T7 RNA Polymerase (T7-RNAP), a highly specific bacterial polymerase with a very low error rate, is present in its environment.

When used in a synthetic/artificial sequence, such as our CRISPR's gRNA DNA template sequence, it can enable a high level of expression of our target gene with its tracrRNA attached to it.

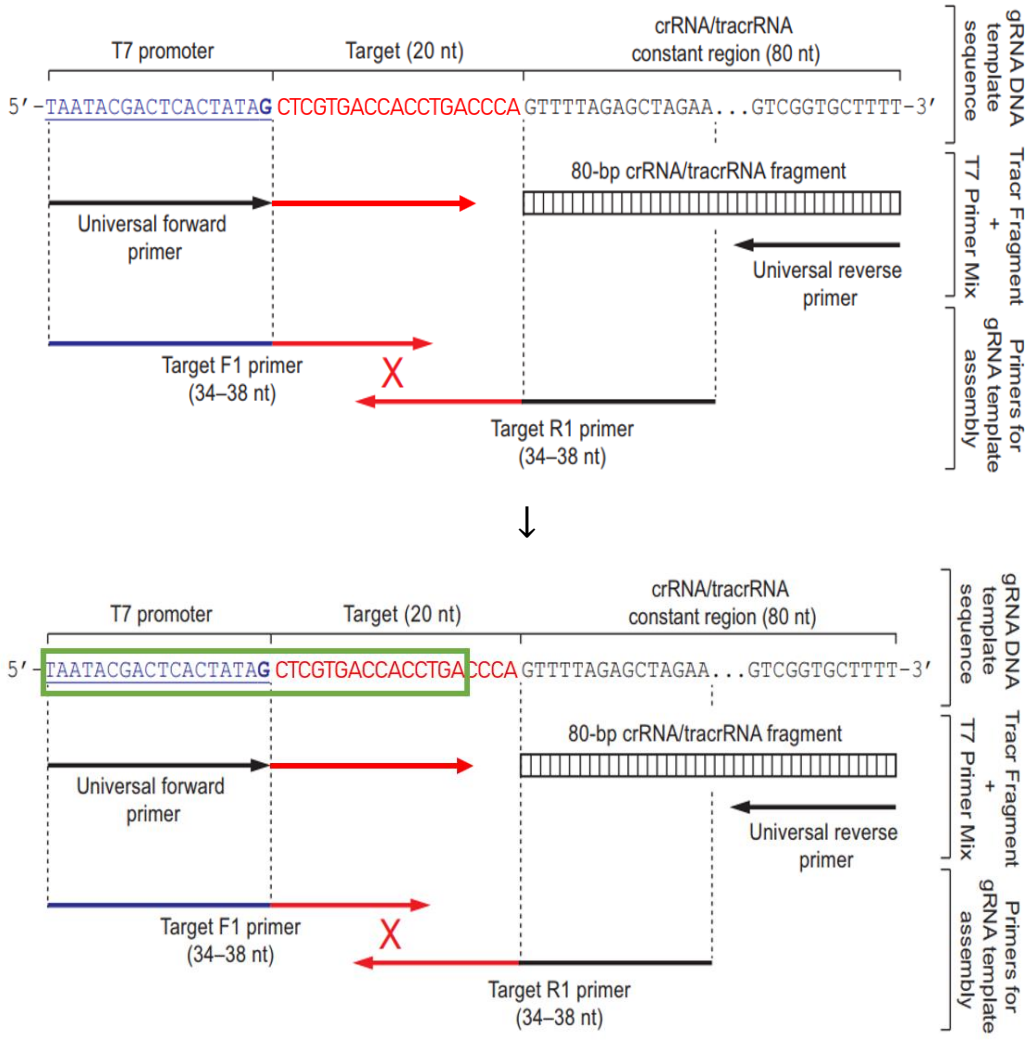
14. First, we are going to design the target forward primer (or the Target F1 primer). But it is important to first state some rules that come with designing Target F1 primers. First, the Target F1 primers should be between 34-38 nucleotides long where the first 18 are fixed from the T7 promoter[11]. Second, the Target F1 primers target sequence (everything after the first 18 nucleotides) *SHOULD*

(does not have to be) be equal to or less than 20 bases long as it has been found to lead to improved specificity and lower off-target cutting rates[12]. Lastly, if the target sequence contains a guanine at the 5' end, it is your choice to keep it in your target sequence BECAUSE the T7 promoter ends with a guanine.[11] The following is a guide when designing your F1 primer:

I) The Target F1 primer always begins with 5'-TAATACGACTCACTATAG-3' (the T7 promoter)

II) Then you will be isolating the first 16-20 nucleotides of your designated eBFP target sequence. For simplicity, we are just going to use the first 16 nucleotides of our designated eBFP target sequence, 5'-CTCGTGACCACCCTGACCCA-3'→5'-CTCGTGACCACCCTGA-3', this is the first 16 nucleotides.

III) Put those two sequences from I) and II) together to generate your Target F1 Primer: 5'-TAATACGACTCACTATAGCTCGTGACCACCCTGA-3' (see pic below circled in green).

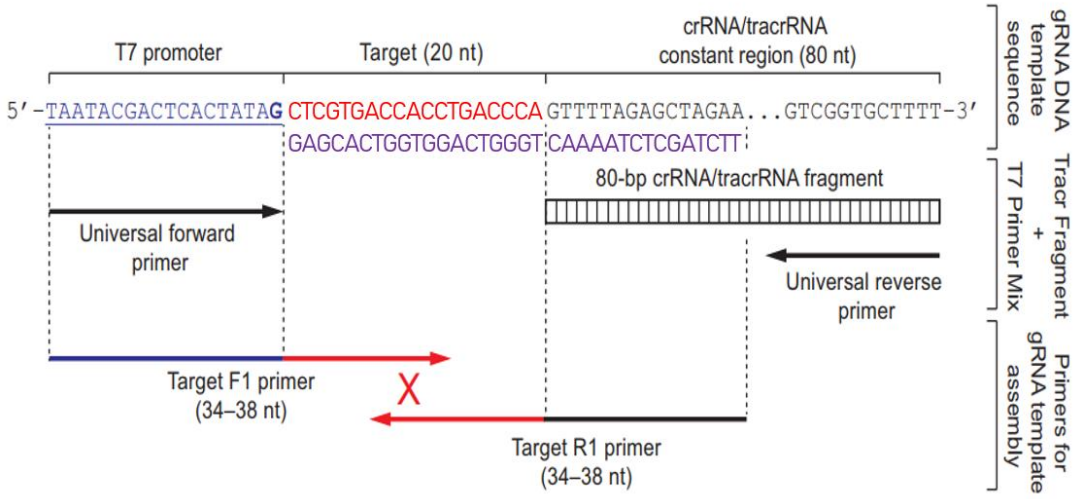


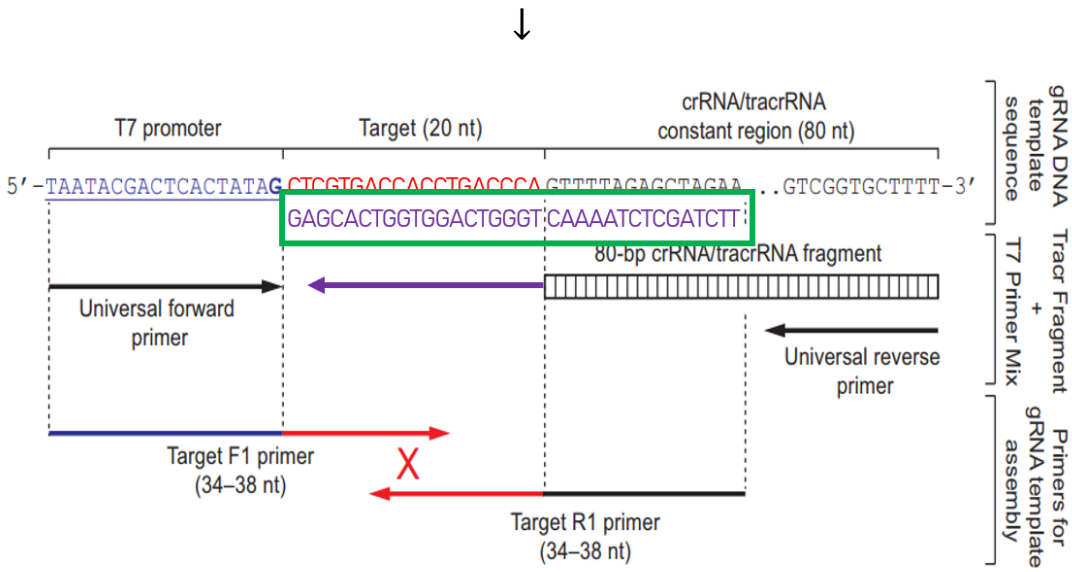
15. Next, we will be designing the target reverse primer (or the Target R1 primer). The following are some rules that come with designing Target R1 primers. First, the Target R1 primers should be

between 34-38 nucleotides long where the first 15 are fixed from the reverse compliment of the tracrRNA constant region[11]. Second, the Target R1 primers target sequence (everything after the first 15 nucleotides) *SHOULD* (does not have to be) be equal to or less than 20 bases long. This has been found to lead to improved specificity and lower off-target cutting rates[12]. There are two ways you can approach this:

I) Reversing complementing everything first then stitching the sequence together:
   i. The Target R1 primer always begins with 5'-TTCTAGCTCTAAAAC-3'
   ii. Then you will be isolating the first 19-20 nucleotides of the reverse compliment of your designated eBFP target sequence. You can write down the reverse compliment sequence yourself or use an online tool like Bioinformatics.org's Reverse Complement converter[13] to do it for you. Therefore, we will be using the whole reverse compliment sequence: 5'-TGGGTCAGGGTGGTCACGAG-3'
   iii. Put those two sequences from I.i) and I.ii) together to have your Target R1 Primer: 5'-TTCTAGCTCTAAAACTGGGTCAGGGTGGTCACGAG-3'

II) Putting the sequence together first then reverse complementing it:
   i. The Target R1 primer always ends with the 5'-GTTTTAGAGCTAGAA-3'
   ii. Then you will be isolating the first 19-20 nucleotides of your designated eBFP target sequence (5'-CTCGTGACCACCCTGACCCA-3') and adding the sequence from II.i) onto the end of that sequence: 5'-CTCGTGACCACCCTGACCCAGTTTTAGAGCTAGAA -3'
   iii. Next, you will be generating the reverse complement sequence from what you generated in II.ii) which you can do either by writing down the reverse compliment sequence yourself or use an online tool like Bioinformatics.org's Reverse Complement converter[13]. Therefore, you would get something like this for your Target R1 Primer: 5'-TTCTAGCTCTAAAACTGGGTCAGGGTGGTCACGAG-3'





Computer Protocol #2B: Steps 11-15 with ACTN3 (Optional)

The following alterative protocol consists of almost all the same steps with a few differences including what to do if Benchling provides you with multiple gRNA sequences for your desired site. You are not required to do this protocol but if you are struggling to understand or would like to get more practice with Benchling and creating gRNA, this alternative protocol can help you.

The following protocol is designed for the following gene with an alternative step 11: actinin alpha 3 (ACTN3). This gene is an alpha-actin binding protein that is involved in crosslinking actin in thin filaments. It functions as a structural/linking component of sarcomere Z lines in skeletal muscle. A mutation in ACTN3 leads to a "defective" gene which is found to cause elite athletic performance in sprinters and endurance runners[18]. Here, we are trying to find a gRNA (and associated F1 and R1 primers) to restore ACTN3 to its wild-type sequence. More specifically, we are changing a thymine in the mutant back to a cytosine (wild-type) at position 567. The sequence shown below is that of the mutant ACTN3 gene.

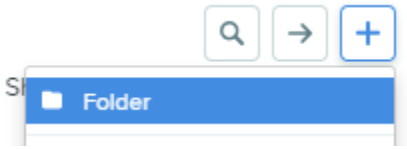
GGAAAAGGGCTATGAGGACTGGCTGCTCTCGGAGATCCGGCGCCTGCAGCGACTCCAGCACCTGGCTGAGAAGTTCCGGCAGAAGG
CCTCCCTGCACGAAGCCTGGACCCGGGGAAAGGAGGAGATGCTGAGCCAGCGCGACTACGATTCGGCTTTGCTACAGGAGGTGCGG
GCGTTGCTGCGGCGCCACGAGGCCTTTGAGAGCGACCTGGCGGCGCACCAGGACCGCGTGGAGCACATTGCCGCGCTGGCCCAGGA
GCTCAATGAGCTGGACTACCACGAGGCAGCCTCAGTGAATAGCCGCTGCCAGGCCATCTGCGATCAGTGGGACAACCTGGGCACCC
TGACCCAGAAGAGGCGGGATGCGCTAGAGCGGATGGAGAAGCTCCTGGAGACCATTGACCGGCTGCAACTGGAGTTTGCCCGGCGG
GCCGCGCCCTTCAACAACTGGCTGGATGGTGCCGTGGAGGACCTGCAGGACGTGTGGCTGGTACACTCTGTGGAGGAGACCCAGAG
CCTGCTGACAGCGCACGATCAGTTCAAGGCAACACTGCCCGAGGCTGACTGAGAGCGAGGTGCCATCATGGGCATCCAGGGTGAGA
TCCAGAAGATCTGCCAGACGTATGGGCTGCGGCCCTGCTCCACCAATCCCTACATCACCCTCAGCCCGCAGGACATCAACACCAAG
TGGGATATGGTCCGAAAGCTGGTGCCCAGCTGTGACCAGACACTGCAGGAGGAGCTGGCACGGCAGCAGGTAAACGAGAGGCTCCG
GCGACAGTTTGCGGCCCAGGCCAATGCCATTGGACCCTGGATCCAGGCGAAGGTGGAGGAAGTGGGGCGGCTGGCAGCAGGGCTAG
CTGGCTCTCTGGAGGAGCAGATGGCTGGGCTACGGCAGCAGGAGCAGAAC

1. In any text editor like Notepad/Notepad++, Sublime Text or Atom, write the following:
   `> ACTN3 Sequence`
2. Then, create two new lines (press Enter twice) and copy & paste the above sequence into the text editor, it should look as the following:
   `> ACTN3 Sequence`

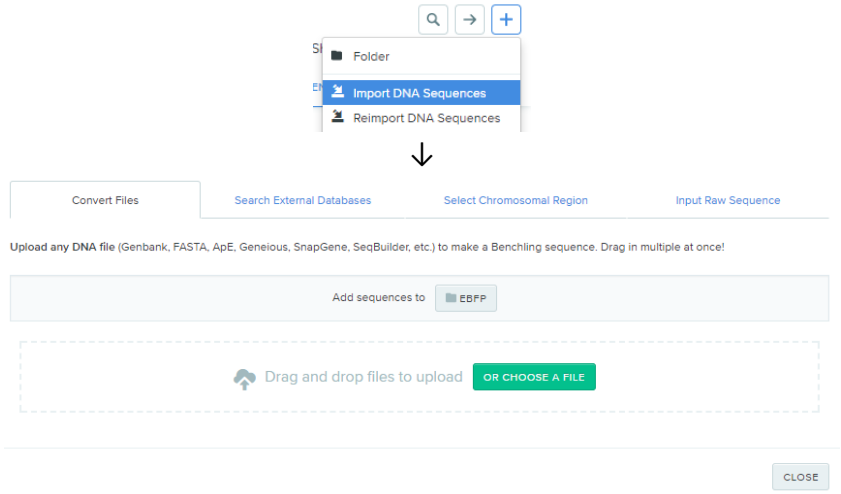
GGAAAAGGGCTATGAGGACTGGCTGCTCTCGGAGATCCGGCGCCTGCAGCGACTCCAGCACCTGGCTGAGAAGTTCCGG
CAGAAGGCCTCCCTGCACGAAGCCTGGACCCGGGGAAAGGAGGAGATGCTGAGCCAGCGCGACTACGATTCGGCTTTGC
TACAGGAGGTGCGGGCGTTGCTGCGGCGCCACGAGGCCTTTGAGAGCGACCTGGCGGCGCACCAGGACCGCGTGGAGCA
CATTGCCGCGCTGGCCCAGGAGCTCAATGAGCTGGACTACCACGAGGCAGCCTCAGTGAATAGCCGCTGCCAGGCCATC
TGCGATCAGTGGGACAACCTGGGCACCCTGACCCAGAAGAGGCGGGATGCGCTAGAGCGGATGGAGAAGCTCCTGGAGA
CCATTGACCGGCTGCAACTGGAGTTTGCCCGGCGGGCCGCGCCCTTCAACAACTGGCTGGATGGTGCCGTGGAGGACCT
GCAGGACGTGTGGCTGGTACACTCTGTGGAGGAGACCCAGAGCCTGCTGACAGCGCACGATCAGTTCAAGGCAACACTG
CCCGAGGCTGACTGAGAGCGAGGTGCCATCATGGGCATCCAGGGTGAGATCCAGAAGATCTGCCAGACGTATGGGCTGC
GGCCCTGCTCCACCAATCCCTACATCACCCTCAGCCCGCAGGACATCAACACCAAGTGGGATATGGTCCGAAAGCTGGT
GCCCAGCTGTGACCAGACACTGCAGGAGGAGCTGGCACGGCAGCAGGTAAACGAGAGGCTCCGGCGACAGTTTGCGGCC
CAGGCCAATGCCATTGGACCCTGGATCCAGGCGAAGGTGGAGGAAGTGGGGCGGCTGGCAGCAGGGCTAGCTGGCTCTC
TGGAGGAGCAGATGGCTGGGCTACGGCAGCAGGAGCAGAAC

3. Choose “Save As” (typically found under the “File” dropdown option) and save your file as a text file (.txt). If you are using Notepad++, Sublime or Atom, please save your file as a fasta file (by adding a .fasta extension manually).

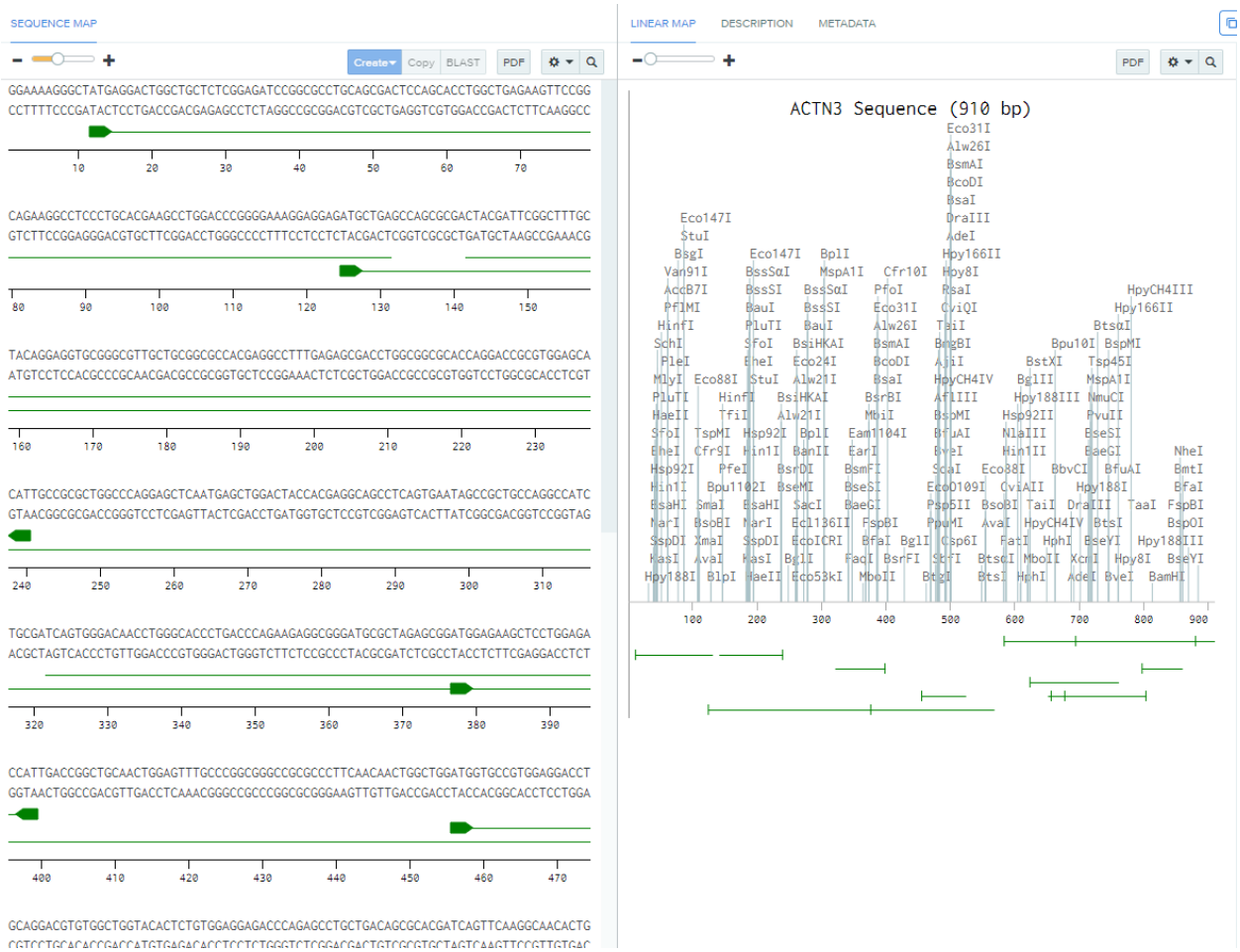
4. In Benchling, under the appropriate Project, click on the “Inventory” tab and create a folder (you do not have to create a folder, but it may help keep you organized)
    I) To create a folder, you will click on the plus symbol while the “Projects” viewer is opened



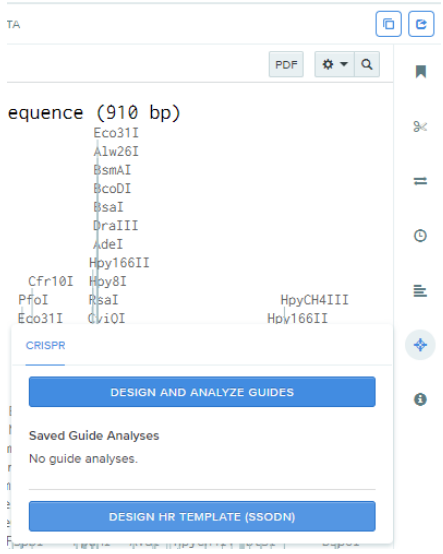
5. While in the new folder, click on the plus in the “Projects” viewer and “Import DNA Sequence”. When this modal opens, while in the “Convert Files” tab, either drag and drop your file or browse/choose your eBFP (.fasta or .txt) file to upload



6. When the file is finished uploading, open the sequence. It should appear in the inventory’s folder that you uploaded it to. The sequence screen should appear similar to the following:

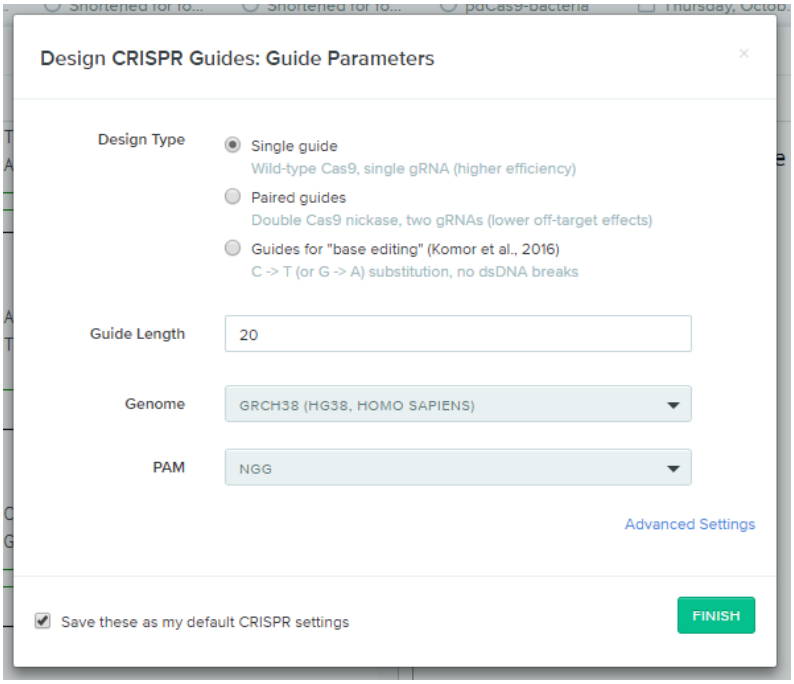


7. On the right side of Benchling while your sequence is open, you should see the following button with the glyph icon "✥". This button opens a CRISPR design tool which would allow you to create and design guide sequences. Open this and click on "Design and analyze guides"

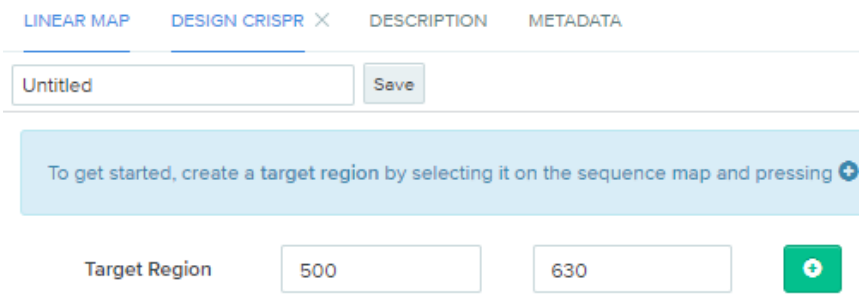


8. For now, keep the default settings on the “Design CRISPR Guides: Guide Parameters” modal and click on the green “Finish” button. If you wish to learn more about the “Advance Settings”, see the supplementary information box #1 and #4.

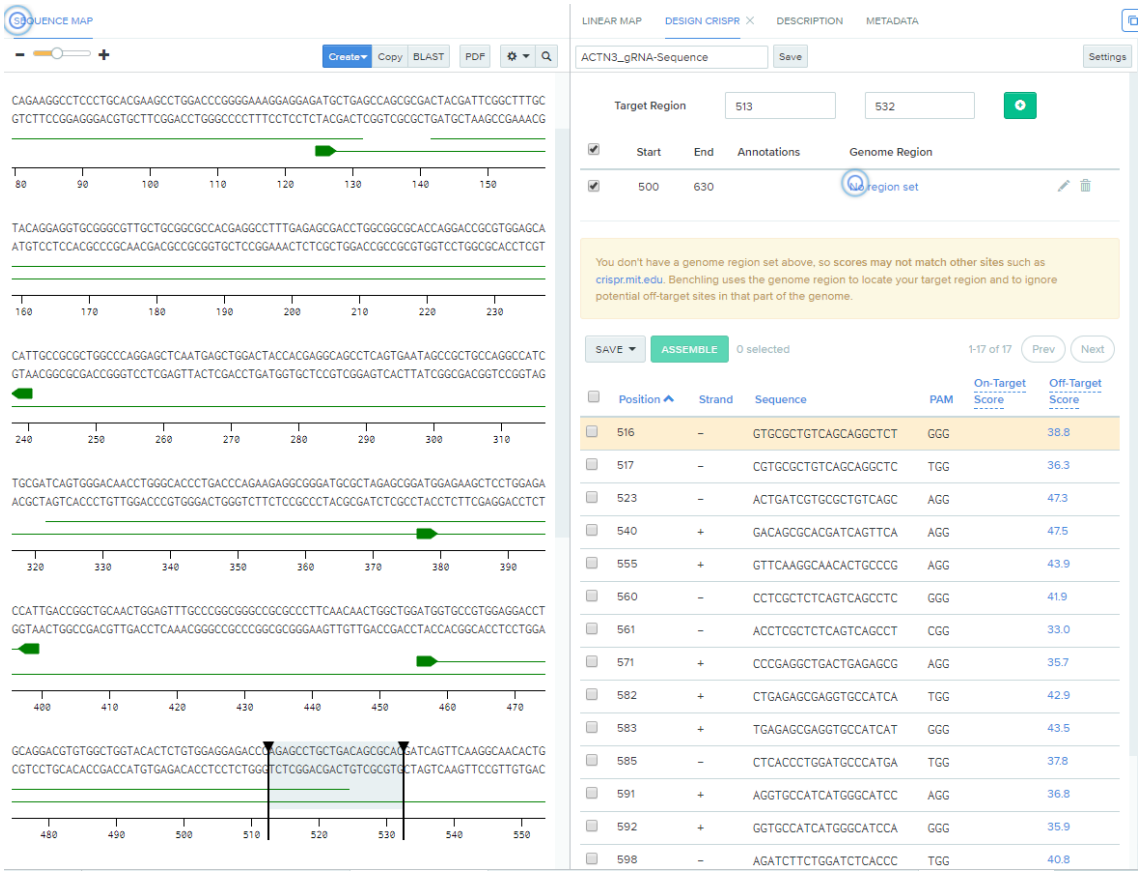
   ***** Default settings may not be the same for all people, please check if your default settings are the same as below. Specifically, check that the genome is “GRCH38 (HG38, HOMO SAPIENS)”*****



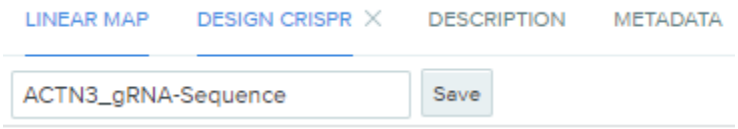
9. Our target site that we wish to have our CRISPR gRNA modify is at sequence position 567, which changes a STOP codon UGA (the mutation leading to a truncated protein) to a CODING CODON CGA (codes for arginine) changing Ter577 (Ter = Termination) to Arg577 in the protein sequence. Overall, we want our gRNA to contain position 567, therefore input a nucleotide range of 500 to 630 as your “Target Region”



10. Once the target region has finished loading you should see a list of potential guide sequences become available. Look through them and click on different ones (do *not* click on “Save” or “Assemble” yet), you will notice different regions of the sequence map will be selected, these correspond to the guide sequence.



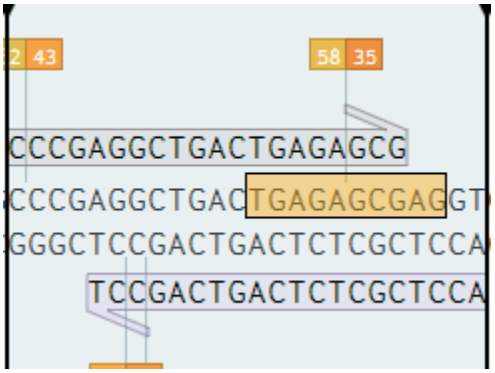
* Once all the guides load, it is <u>recommended</u> that you save your "Design CRISPR" tab in-case you need to work on this at a later point in time or your browser/computer/network crashes. This can be done at the top of the "Design CRISPR" tab:



11. As mentioned before, the modification we wish to create a gRNA for is at position 567 (a thymine) which changes Ter577 back to a Arg577 in the protein. Therefore, you want to look for a sequence that contains this nucleotide position. We are going to choose a sequence based on strand direction, on- and off-target score as well as, most importantly, position. Normally you would have to know exactly what you are looking for before you start designing a gRNA and its primers but due to this being a tutorial, we are giving you this information.

    I) Firstly, look at position. You want a sequence that contains our target position. Click through the options and see which gRNAs contain position 567 (you should have 5 options). While nothing is highlighted in the sequence map tab, you can click on the 🔍 in the top right of this tab and find "TGAGAGCGAG" to have the sequence you want to be looking for highlighted. The thymine in the <u>front</u> of the "**<u>T</u>**GAGAGCGAG" is the exact position you are looking for. See pictures below for clarification.

II) If you are looking to use CRISPR to cut and modify/change a single (or few) nucleotides, the desired nucleotide should be within 3-8 nucleotides upstream of the PAM site which is where the cut-site is located. To visually count this, you could count 3-8 nucleotides into the gRNA sequence from the start of the sequence (this is indicated by the arrow up on the gRNA sequence on Benchling). After this manual count you should have narrowed down your count to two possible gRNAs (see picture below).



III) Next, look at the on-target and off-target scores. The higher the score, the better. Generally, you should look for an on-target score above 40 as anything lower would be advised against using. Optimally, you would want a score of 80 or higher for off-target. You can click on the off-target score to see other potential off-target binding sites in the

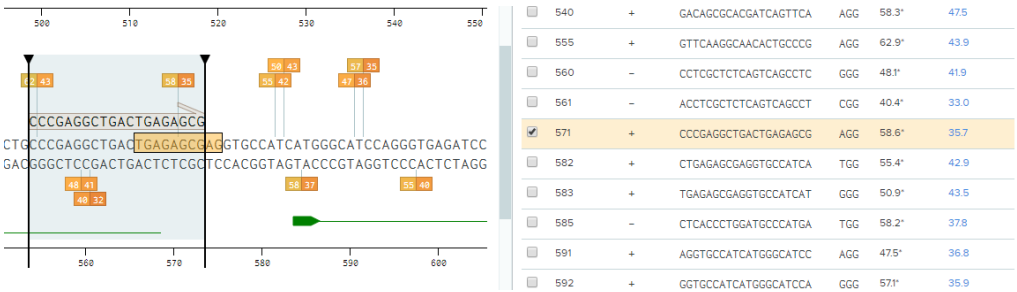
genome for a selected species from Step 8. Here are the on-target and off-target scores for the two-remaining gRNA's:

☑	561	–	ACCTCGCTCTCAGTCAGCCT	CGG	40.4*	33.0
☑	571	+	CCCGAGGCTGACTGAGAGCG	AGG	58.6*	35.7

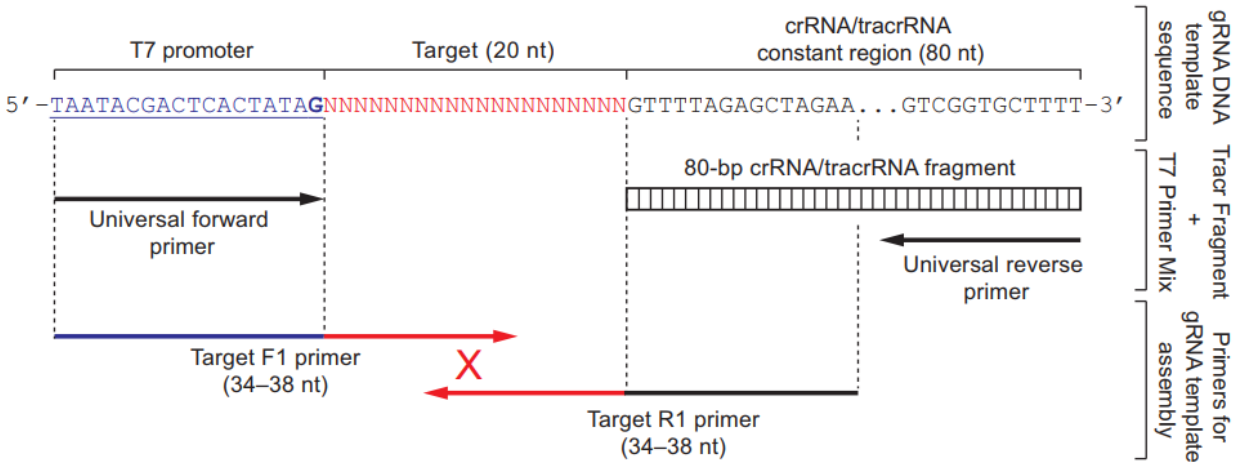
Both have an on-target score above 40, which means both are usable gRNAs, however, considering the max on-target score achievable is 62.9 (click on the on the on-target score heading tab to list the gRNAs with highest on-target score first), we must stick with a gRNA within 20% if this max score (62.9 x .20 = 50.3). This eliminates the anti-sense strand option and leaves us with the sense-strand gRNA which starts at position 571.

IV) Once you have found potential sequences, look at the strand direction (the top sequence indicates the sense-strand (+) while bottom is the antisense-strand (-)). We are looking for a sequence that matches the same side your target gene is on. Typically, on Benchling, this is the sense-strand (+), the top strand. Antisense-strands (-) still work and will still give you the same output but if you have the option between an antisense-strand (-) and a sense-strand (+), the sense-strand (+) is the more optimal option. In this case the off-targets scores are not optimal, but you are left with this gRNA as your only option because the on-target score is OK. From an experimental perspective this is not ideal which leads to possibility of looking at using OTHER PAM sites)

12. From the sub-steps given in Step 11, you should only be left with one option available; a guide sequence that reads 5'- CCCGAGGCTGACTGAGAGCG-3' (PAM: 5'-AGG-3') on the sense-strand starting at position 571. Select the checkbox and highlight the row.



13. Once you have found your desired CRISPR's target DNA sequence, you will begin designing your target forward (F1) and reverse (R1) primers, the nested PCR primers. Following is a reiteration of the format of how the primers will be designed. Try and understand it before moving on:



14. First, we are going to design the target forward primer (or the Target F1 primer). But it is important to first state some rules that come with designing Target F1 primers. First, the Target F1 primers should be between 34-38 nucleotides long where the first 18 are fixed from the T7 promoter[11]. Second, the Target F1 primers target sequence (everything after the first 18 nucleotides) *SHOULD* (does not have to be) be equal to or less than 20 bases long as it has been found to lead to improved specificity and lower off-target cutting rates[12]. Lastly, if the target sequence contains a guanine at the 5' end, it is your choice to keep it in your target sequence BECAUSE the T7 promoter ends with a guanine.[11] The following is a guide when designing your F1 primer:

    I) The Target F1 primer always begins with 5'-TAATACGACTCACTATAG-3' (T7 promoter).

    II) Then isolate the first 16-20 nucleotides of your designated ACTN3 gRNA target sequence. For simplicity, we are just going to use the first 16 nucleotides of our designated ACTN3 target sequence (5'-CCCGAGGCTGACTGAGAGCG-3'): 5'- CCCGAGGCTGACTGAG-3'

    III) Put those two sequences from I) and II) together to have your Target F1 Primer: 5'-TAATACGACTCACTATAGCCCGAGGCTGACTGAG -3'

15. Next, we will be designing the target reverse primer (or the Target R1 primer). The following are some rules that come with designing Target R1 primers. First, the Target R1 primers should be between 34-38 nucleotides long where the first 15 are fixed from the reverse compliment of the tracrRNA constant region[11]. Second, the Target R1 primers target sequence (everything after the first 15 nucleotides) *SHOULD* (does not have to be) be equal to or less than 20 bases long. This has been found to lead to improved specificity and lower off-target cutting rates[12]. Although there are two ways to approach the design of the R1 primer (as shown for eBFP), for simplicity, only one of these methods is shown below for ACTN3.

I) The Target R1 primer always ends with the tracrRNA fragment, 5'-GTTTTAGAGCTAGAA-3'

II) Then isolate the first 19-20 nucleotides of your designated ACTN3 target sequence (5'-CCCGAGGCTGACTGAGAGCG-3') and add this sequence to the 5' end of the tracrRNA fragment shown in I) to produce, 5'-CCCGAGGCTGACTGAGAGCGGTTTTAGAGCTAGAA -3'

III) Next, you will be generating the reverse complement sequence for what you generated in II). You can do this by writing down the reverse compliment sequence yourself or use an online tool like Bioinformatics.org's Reverse Complement converter[13]. The reverse compliment will produce the following sequence for your Target R1 Primer: 5'-TTCTAGCTCTAAAACCGCTCTCAGTCAGCCTCGGG-3' for ACTN3.

Conclusions

You have successfully found and created a gRNA for eBFP (and ACTN3 if you chose to do it) with its respective target forward (F1) and reverse (R1) primers using online tools. These primers can be used in conjunction the ThermoFisher GeneArt Precision gRNA Synthesis Kit (used in first CRISPR lab manual protocol) to synthesize your own gRNA for experimentation. With this knowledge, you should be able to apply this protocol to any gene you wish to target with CRISPR and where you wish to synthesize your own gRNAs. If you choose to use CRISPR kits from other companies, modifications to this protocol may be necessary but the general concept stays the same.

SEE NEXT PAGE FOR YOUR TAKE-HOME ONLINE ASSIGNMENT

Take Home Online gRNA/Primer Design Assignment Using SciGrade (due Oct.30/18 @ 1:10pm through SciGrade Online Submission:

You will now apply what you have learned from your in-lab demo. Below is a list of three genes where mutations in their sequences lead to certain diseases/conditions. Your objective is to find these mutations, design a gRNA, and figure out corresponding F1/R1 primers to synthesize your own gRNA. From a hypothetical experimental perspective, you are trying to revert the sequences to their original wild-type sequences using CRISPR. You will be using an online submission program call SciGrade to submit your answers.

SciGrade Account Registration & Usage Instructions

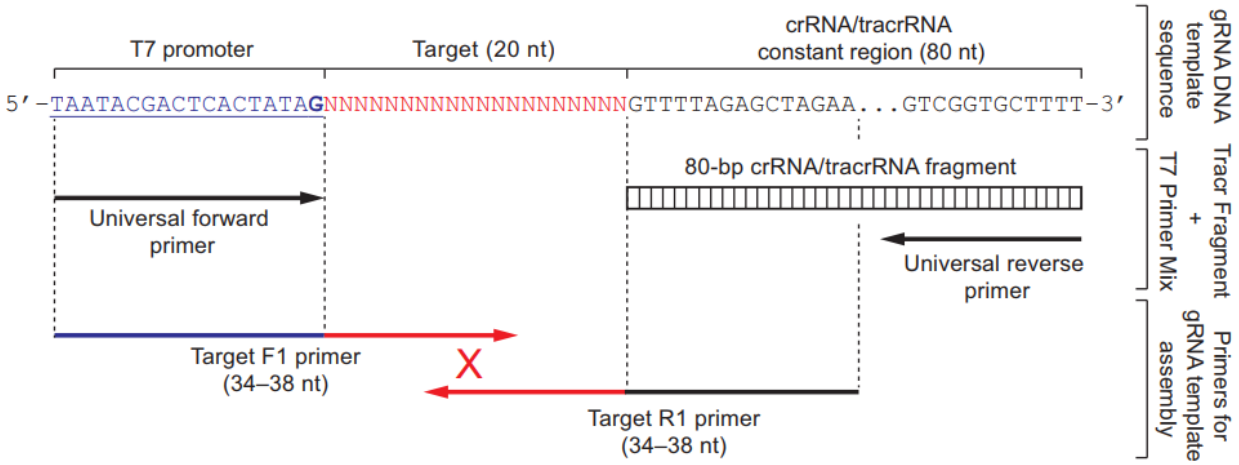
1. To use SciGrade you will need a google ID/Gmail account. If you do not have one, you can easily create one by going to www.gmail.com.
2. After you create your google account go to www.scigrade.com.
3. Click on Login in the top right-hand corner and then click Register.
4. Enter your student number followed by your University of Toronto email address (although the system recognizes you using your gmail account you will still need a University of Toronto email to use the online system).
5. Click on Check (the system will add you to the student server for SciGrade).
6. Go back and click Login at the top-right hand corner of the page, enter your student number and then click on "Signed in" next to the google icon.
7. This will bring you to the Dry Lab Assignment Page.
8. OPTIONAL - Here you can click on "Practice" to enter gRNA/Primer information for eBFP and ACTN3 (as you did for the demo) by selecting them from the drop-down list. For these practice genes, the system will provide you feedback on your entered answers (no real marks assigned).
9. If you click on "Assignment" you will be able to complete the assignment genes (HBB, CCR5 and APOE). Do not click on the Assignment genes until you are ready to complete the assignment. Select the gene you wish to work on from the drop-down list and click on LOAD GENE. Entered answers are FINAL and no feedback is provided if you enter incorrect answers. When completing the Assignment genes, make sure to click submit after you have entered ALL the information required (see below) for each gene. **After you select a new gene to work on, make sure to click on LOAD GENE again**.

Instructions for gRNA creation:

1. Using Benchling, you will find and select a gRNA for the assignment genes using the settings and instructions given in the demo protocol above.
2. When you select a gRNA, make sure to also write down its PAM sequence, on-target score, off-target score and its strand direction. You will need ALL this information to complete the assignment for each gene.

Instructions for forward/reverse (F1/R1) primers:

- You will ***NOT*** be using the primers given from Benchling but rather create your own. Using the illustration below as a guide and the instructions given in the demo protocol you will create forward (F1) and reverse (R1) primers for your assignment genes.



Assignment Genes

*****PLEASE NOTE THE FOLLOWING:**

- **THE SEQUENCES PROVIDED ARE CONDENSED VERSIONS OF THE GENE'S ACTUAL SEQUENCE WITH MUTATIONS ALREADY INDICATED IN GREEN.**
- **WHEN YOU COPY AND PASTE A GENE SEQUENCE BELOW INTO NOTEPAD (OR OTHER TEXT EDITOR) ENSURE THAT YOU DELETE THE BULLET POINT (THE SQUARE) OR BENCHLING MAY NOT RECOGNIZE THE SEQUENCE.**
- **EACH GENE NAME IS HYPERLINKED TO THEIR GENE INFORMATION PAGE BUT THE GENE MAY NOT EXACTLY MATCH WHAT YOU SEE. BLAST RESULTS, HOWEVER, WILL STILL SHOW THE SAME GENE AS DESCRIBED.**
- **FOR THE PURPOSES OF YOUR ASSIGNMENT/BENCHLING USE THE SEQUENCES AND TARGET SITES PROVIDED BELOW.**

- HBB – Sickle cell anemia
  - Sickle cell anemia is caused by a SNP in the human hemoglobin beta-chain which results in a GAG codon being replaced with a GTG codon (A → T). In the protein this results in a hydrophilic glutamic acid residue being replaced with a hydrophobic valine residue. This mutation causes red blood cells to adopt a sickle like shape. Find a suitable gRNA to target the SNP described and design accompanying F1 and R1 primers. The target site and sequence information are shown below. Submit your answers through Scigrade.
  - CRISPR Target site:
    - Nucleotide position 73 - You are trying to change a thymine (highlighted in green) back to an alanine (WILD-TYPE).
  - Sequence (MUTATED SEQUENCE):
    - ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCATCTGACTC
      CTGAGGTGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGG
      CCCTGGGCAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGT
      CCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCT
      TTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACT
      GTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGG
      CCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTG
      TGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGG
      TTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGAT
      TCTGCCTAATAAAAAACATTTATTTTCATTGC

- CCR5 – C-C motif chemokine receptor 5
    - The CCR gene encodes for the beta-chemokine receptor family which is expressed in T cells and macrophages, playing an important role as a co-receptor for macrophage-tropic viruses (like HIV) to enter host cells. An SNP in this protein results in a TAC codon being replaced by a TAA or TAG codon (C → A or G). In the protein this results in Tyrosine 184 being replaced by a termination end and subsequently a truncated protein which does not respond well to ligand binding (i.e. HIV infection). Therefore, this SNP is advantageous and results in increased resistance to HIV infection.

    - CRISPR Target site:
        - Nucleotide position 208 – You are trying to target a cytosine residue in the WILD TYPE sequence and change it to an adenine or guanine residue (resulting in a truncated protein and hence increased HIV resistance).

    - Sequence (WILD-TYPE SEQUENCE):
        - GAATCTTCTTCATCATCCTCCTGACAATCGATAGGTACCTGGCTGTCGTCCATGCTGTGTTTGCTT
          TAAAAGCCAGGACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTG
          CGTCTCTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGCTCTC
          ATTTTCCATACAGTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAAGATAGTCATCTTGGGGC
          TGGTCCTGCCGCTGCTTGTCATGGTCATCTGCTACTCGGGAATCCTAAAAACTCTGCTTCGGTGTC
          GAAATGAGAAGAAGAGGCACAGGGCTGTGAGGCTTATCTTCACCATCATGATTGTTTATTTTCTCT
          TCTGGGCTCCCTACAACATTGTCCTTCTCCTGAACACCTTCCAGGAATTCTTTGGCCTGAATAATT
          GCAGTAGCTCTAACAGGTTGGACCAAGC

- APOE – Apolipoprotein E
    - This gene encodes for a major fat-binding protein of the chylomicron (a lipid carrier in the body). It is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. Mutations in this gene generally result in increased plasma cholesterol and triglycerides and one specific SNP results in a CGC codon being replaced by a TGC codon (C→T). In the protein this results in an Arg176 to Cys176 substitution. It has been found that this mutation is associated with an increased risk of Alzheimer's disease[20]

    - CRISPR Target site:
        - Nucleotide position 222 – You are trying to target a thymine residue in the MUTANT sequence (highlighted in green) and change it to a cytosine (WILD-TYPE).

    - Sequence (MUTATED SEQUENCE):
        - CGGTGGCGGAGGAGACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCG
          CGGACATGGAGGACGTGTGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCC
          AGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCC
          GCGATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCGCCGAGC
          GCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTGCGGGCCGCCA
          CTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCAGGCCTGGGGCGAGCGGCTGCGCG
          CGCGGATGGAGGAGATGGGCAGCCGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGG
          AGGTGCGCGCCAAGCTGGAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCC
          GCCTCAAGAGCTGGTTCGAGCCCCTGGTGGAA

This is an ALL or NOTHING Assignment. If this assignment is not submitted by Oct.30/18 through SciGrade you will receive a mark of 0!

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