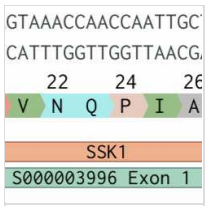

Jul 26, 2022

Designing Knockout Oligonucleotides

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Yeast ORFans CURE

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ABSTRACT

The Yeast ORFan CURE is a project to "knock out" (disable) a bunch of yeast genes of unknown function. Doing so requires small synthetic pieces of DNA called "oligonucleotides" or "oligos". This protocol walks you through selecting a gene of unknown function and designing the oligonucleotides you'll need.

PROTOCOL CITATION

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KEYWORDS

cas9, pcr, oligonucleotide, primer, design

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66552

PARENT PROTOCOLS

In steps of

Designing Knockout Oligonucleotides (Instructor Protocol)

GUIDELINES

Please follow these directions very carefully -- even a single-"letter" mistake can be the difference between an experiment working and its failure!

SAFETY WARNINGS

This is an entirely computational protocol - no hazards are anticipated.

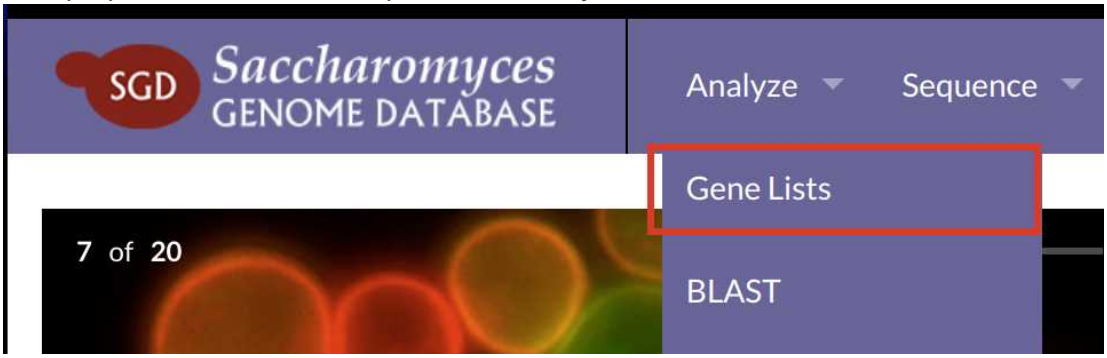
BEFORE STARTING

This protocol assumes you are using Benchling to manage your DNA and oligo sequences and to find Cas9 targets. Sign up for an account at https://benchling.com before getting started. (It's free for academic institutions!)

Choose a gene to knock out

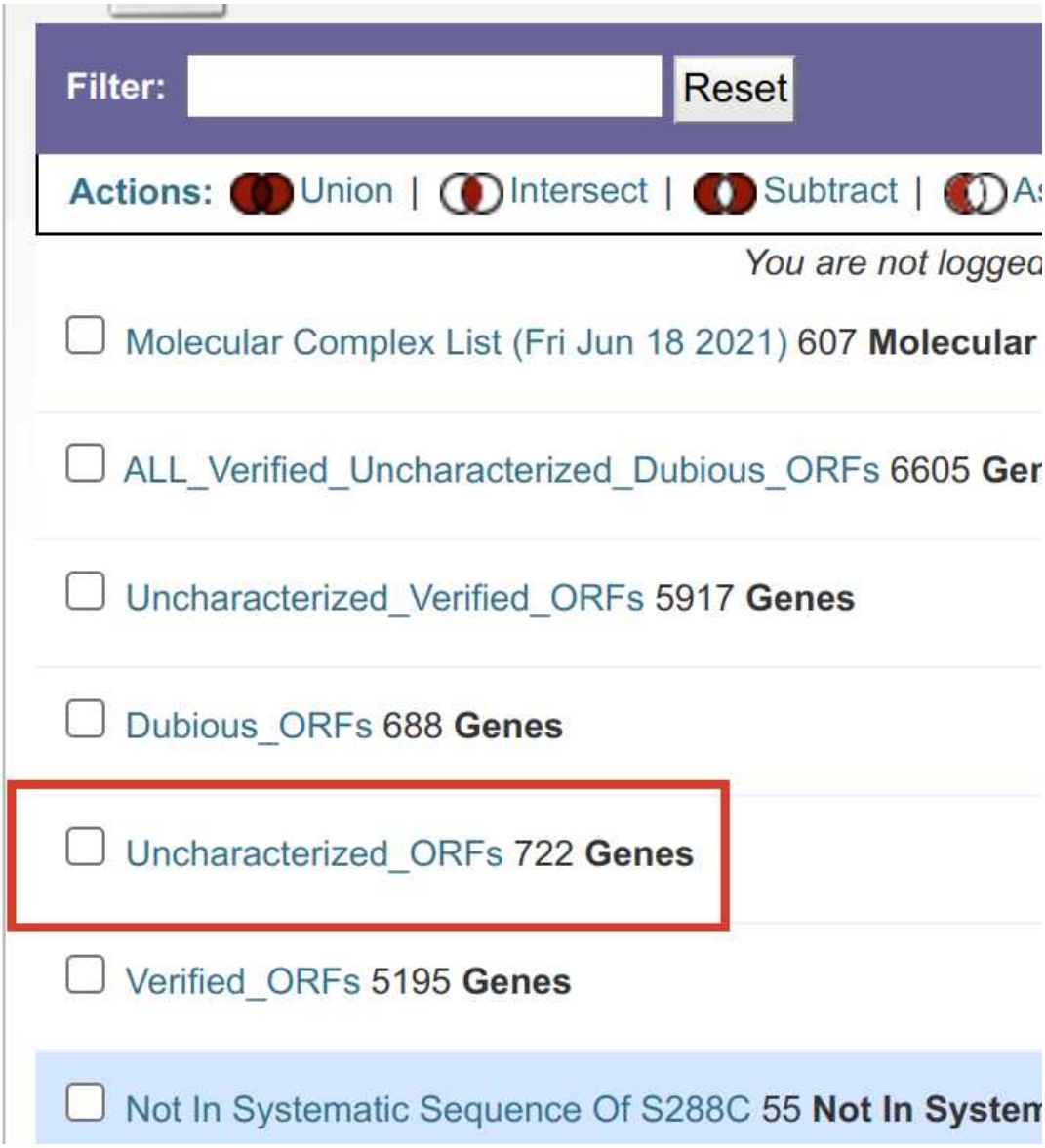
1 Point your web browser to the Saccharomyces Genome Database at https://www.yeastgenome.org/.

2 In the purple menu bar at the top, choose "Analyze", then "Gene Lists"

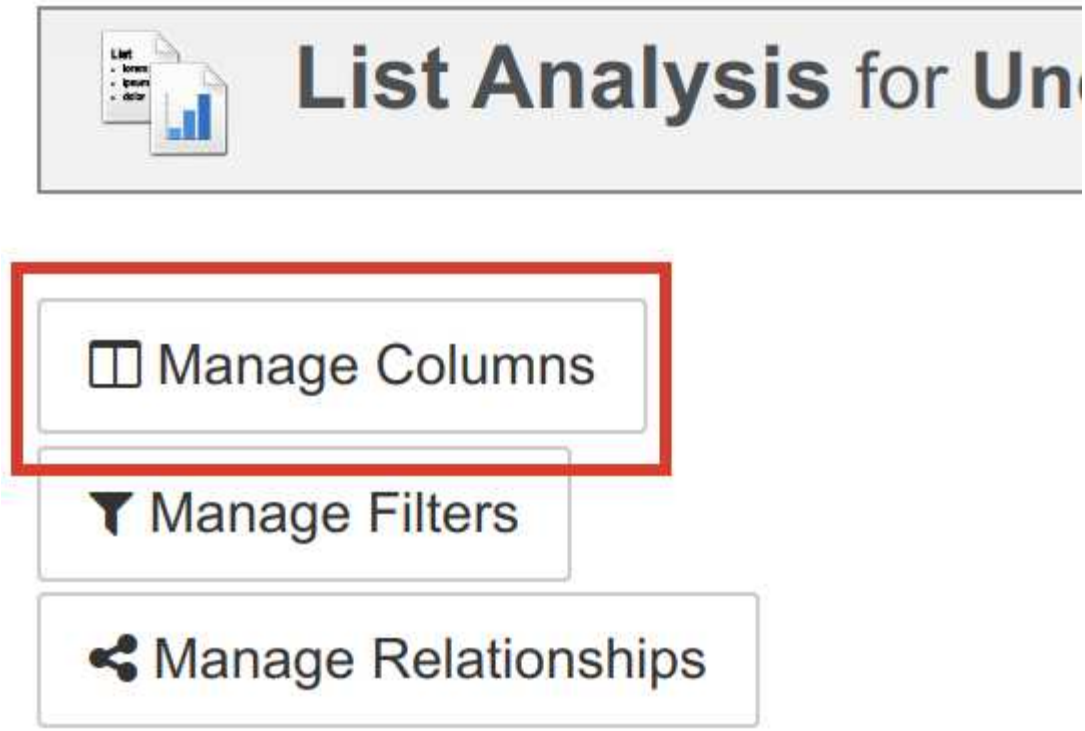


3 In the light-blue menu bar on the upper-left, choose "View"

4 Click the link to the "Uncharacterized_ORFs" list. (Do not check the checkbox, click the link.)



5 What you are presented with is a list of all the genes in yeast that do not have a known function. However, there's one piece of important data that's not yet shown -- the length of each gene. To show it, start by clicking the "Manage Columns" button at the top of the list.



6 Click the big green "Add a Column" button.

7 Click "Length", then "Add 1 new column" (in green), then "Apply Changes" (in blue).

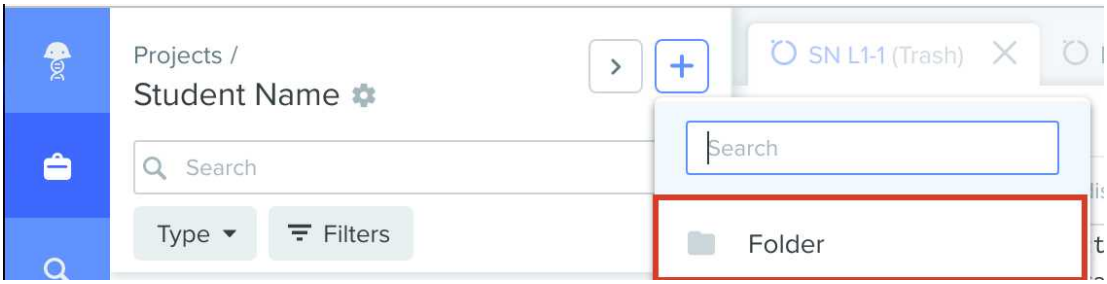
8 Now, choose any gene you want on the list. From the beginning or the middle or the end, it doesn't matter -- the list goes on for pages. The only constraint is that *it must have a "Gene Length" of more than 1000 bases*.

We choose genes that have more than 1000 bases because they're more likely than shorter genes to have an actual function in the genome, rather than being evolutionary "leftovers."

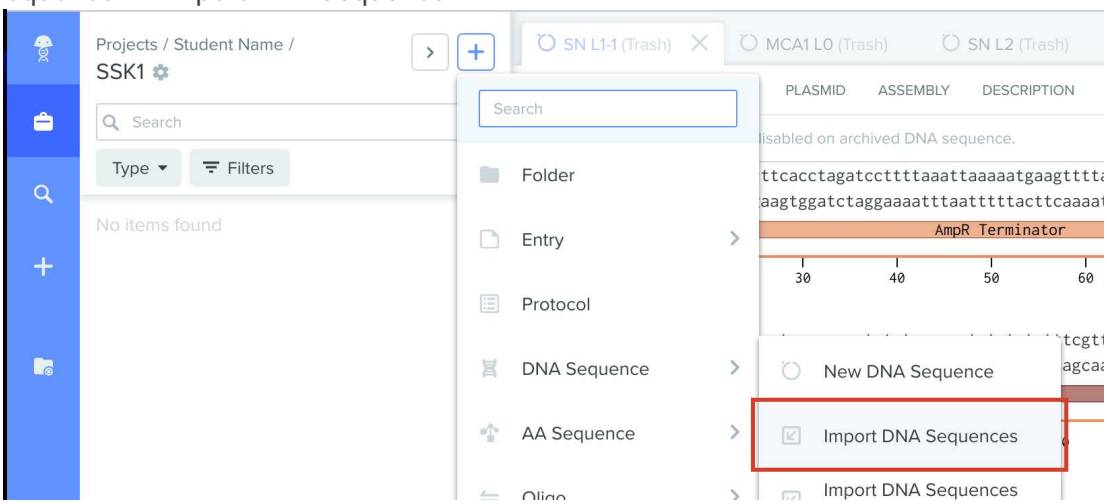
Download the gene sequence to Benchling

9 In the following directions, I will be using the abbreviation YFG to mean "your favorite gene." Anywhere you see YFG, substitute the name of the gene you and your partner will be knocking out. In the images, I will be demonstrating with a gene called SSK1.

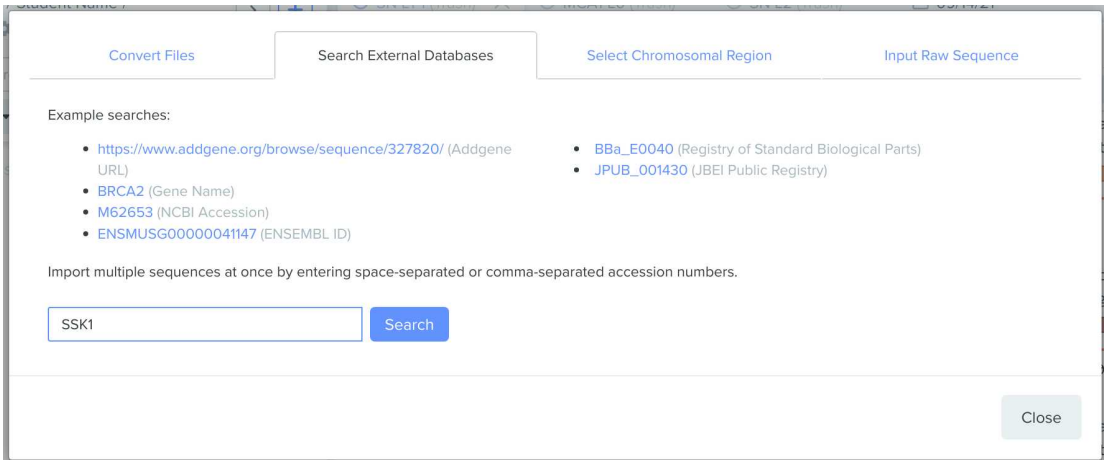
In your project folder on Benchling, click the "+" button, choose "Folder", and create a folder named "YFG".



10 In the new folder you just created, click the "+" button again. This time, choose "DNA Sequence" > "Import DNA Sequence"



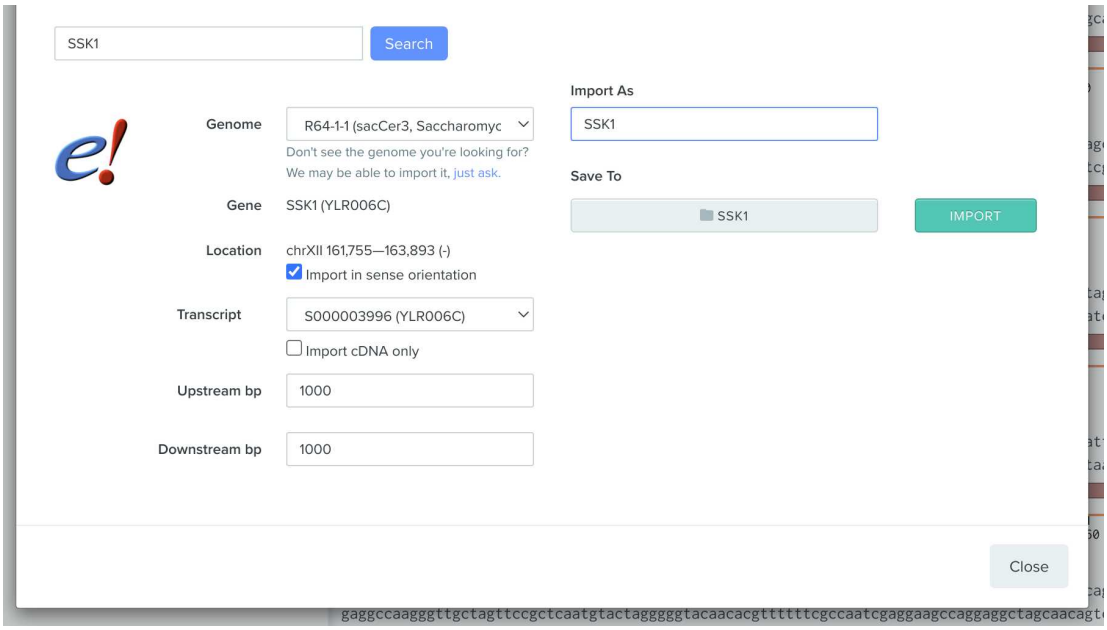
11 In the resulting modal dialog box, choose "Search External Databases" at the top and type in your gene name in the search box. Click "Search."



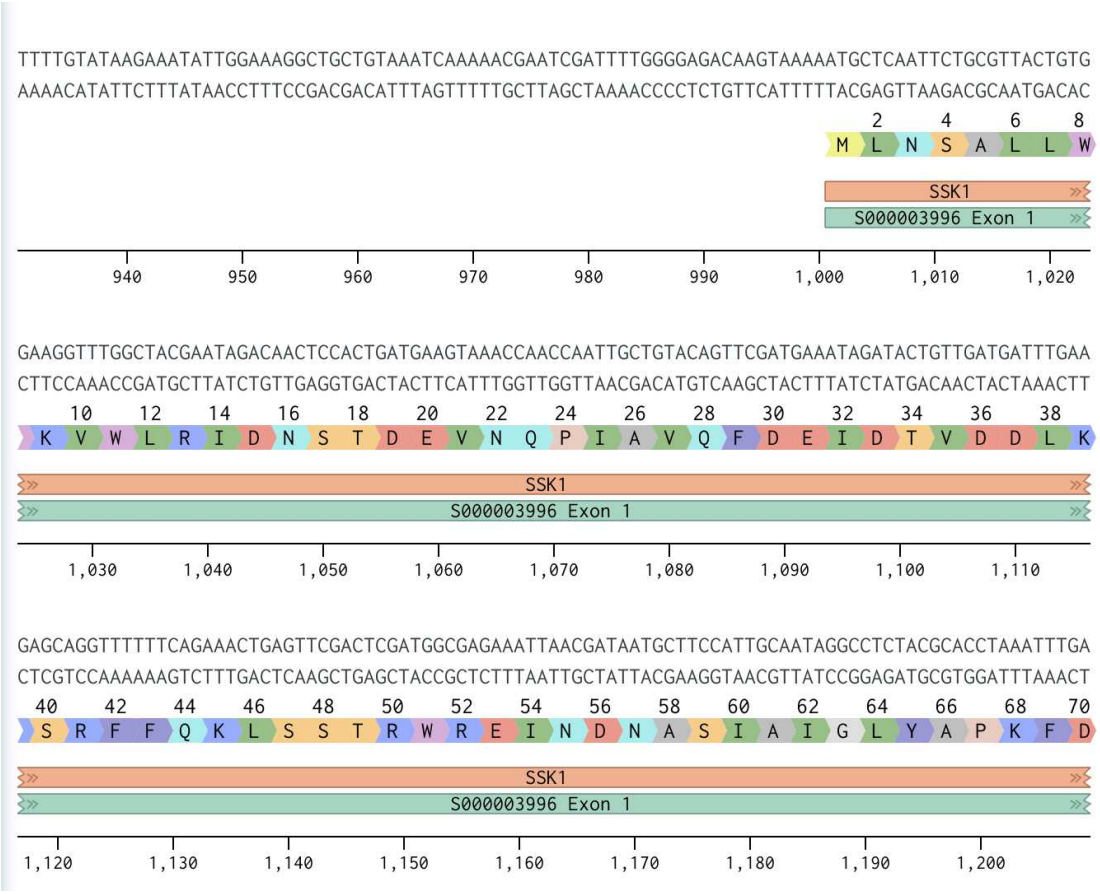
12 After a moment, the search result will appear. Make sure that the "Genome" box reads "R64-1-1 (sacCer3)". There are three things that you will need to change:

- Change **Upstream Bp** to 1000
- Change **Downstream Bp** to 1000
- Change **Import As** to your gene name

Then click "IMPORT".



13 You will see the newly created document in its folder -- open it by clicking on it. You'll see the DNA sequence that we just imported -- lots of As, Cs, Ts and Gs. If you scroll down, you will see a set of colored bars (annotations) under the DNA sequence. The region with the colored bars is the sequence of the gene you'll be knocking out. Next, we'll need to design a few pieces of synthetic DNA specifically for this sequence.

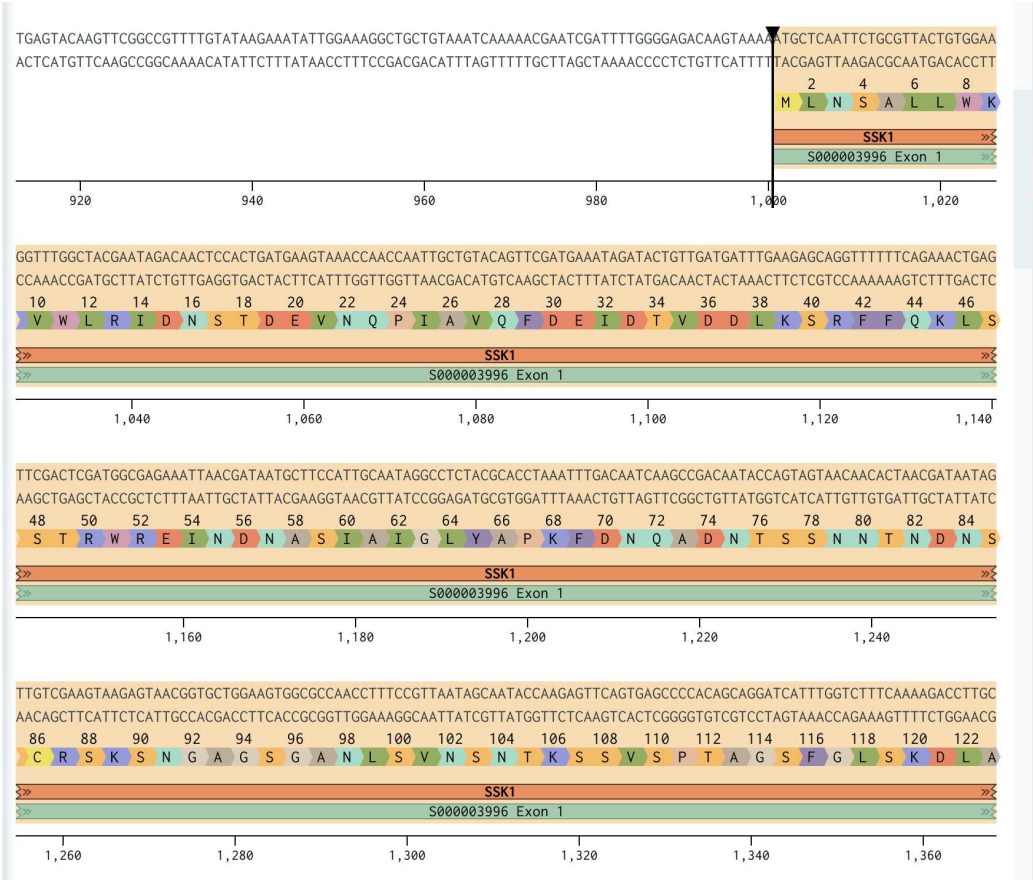


Choose a target sequence

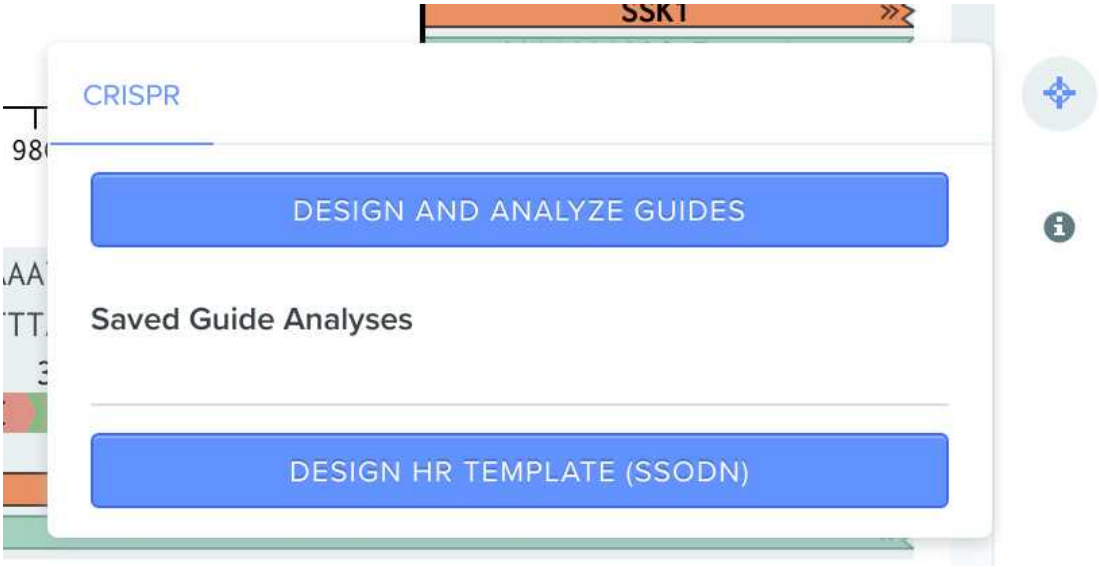
14

We'll be using an enzyme called Cas9 to disrupt the gene you chose. Unfortunately, we can only "target" Cas9 to particular sequences based on a fairly strict set of rules. Fortunately, Benchling will show us which sites match those rules.

Click on the annotation (the colored bar) under your gene sequence. This will cause Benchling to select the entire sequence. Then, click the little "target reticule" icon on the right-side toolbar (in the red box in the image below).

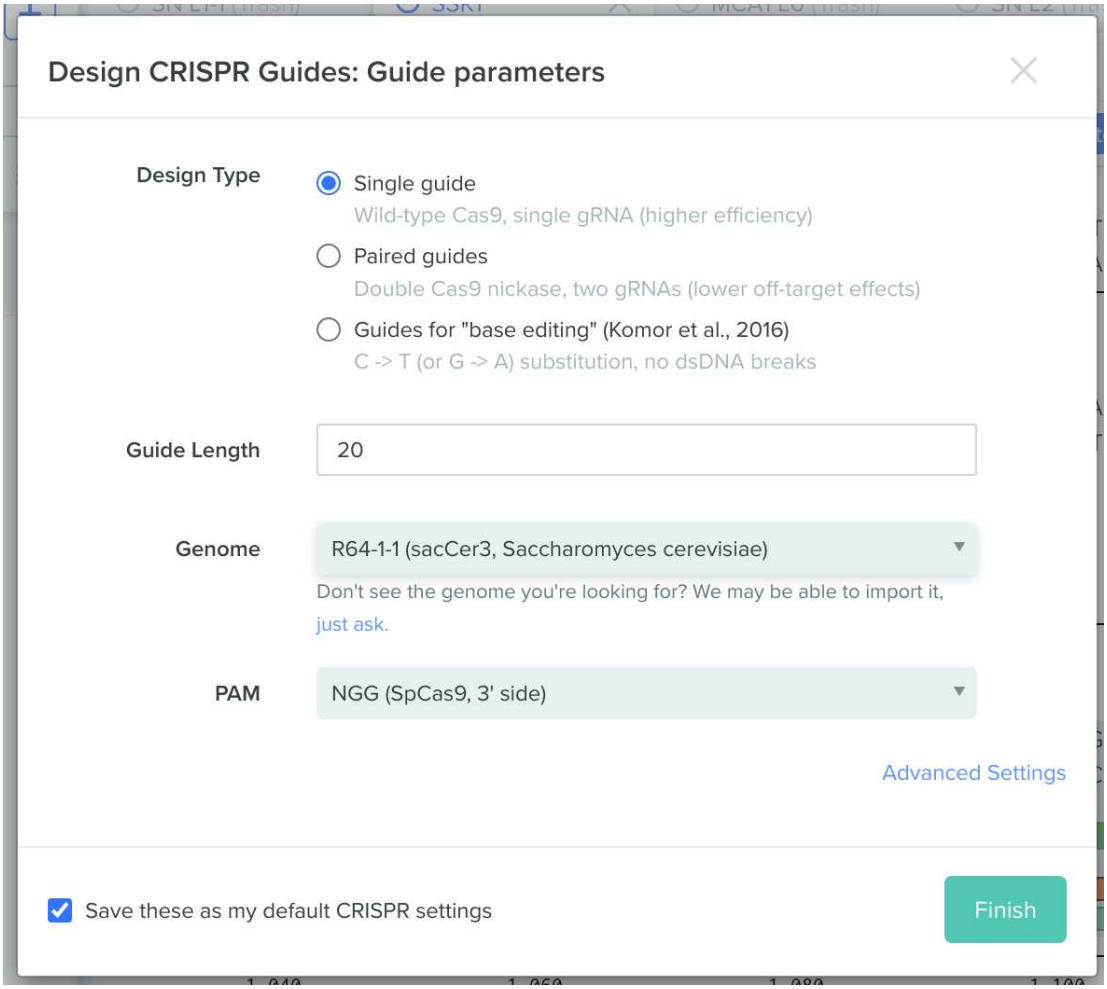


15 Click the "Design and Analyze Guides" button.

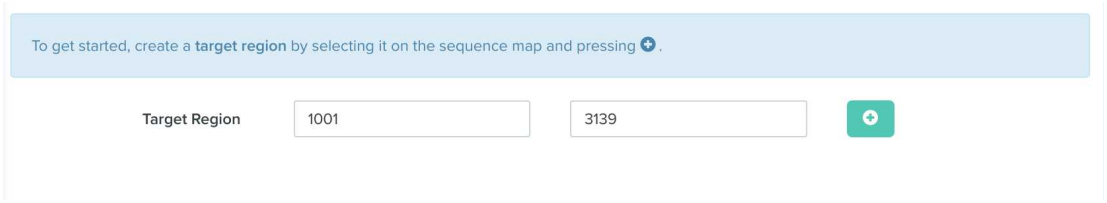


16 In the "Guide parameters" box, you should only have to make one change. In the **Genome** box, search for "R64" and choose the one result -- "R64-1-1 (sacCer3, Saccharomyces cerevisiae)".

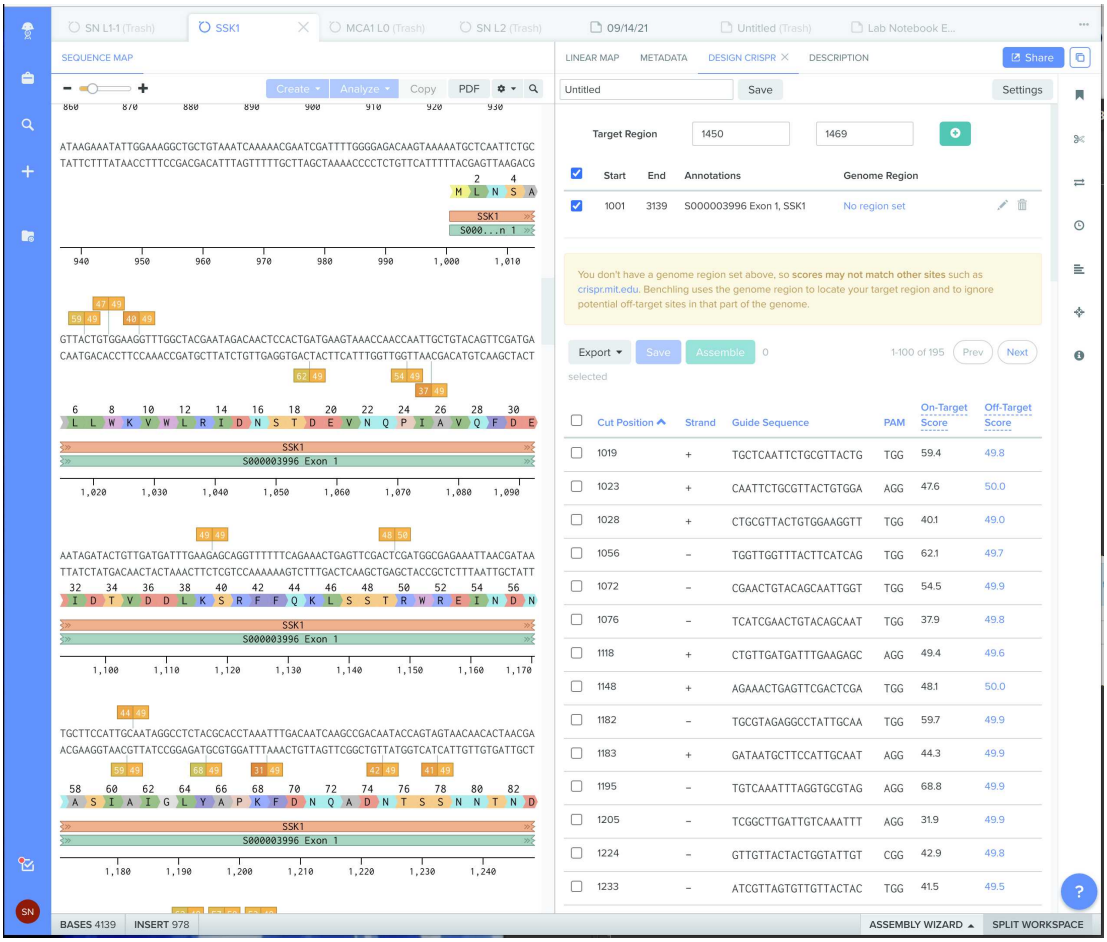
This is the same genome you downloaded your sequence from! Double check that the rest of the settings are correct, then, click "Finish".



17 Because you've already selected a region to find guide sequences in, there will already be a region set in **Target Region**. Click the green "+" next to it.



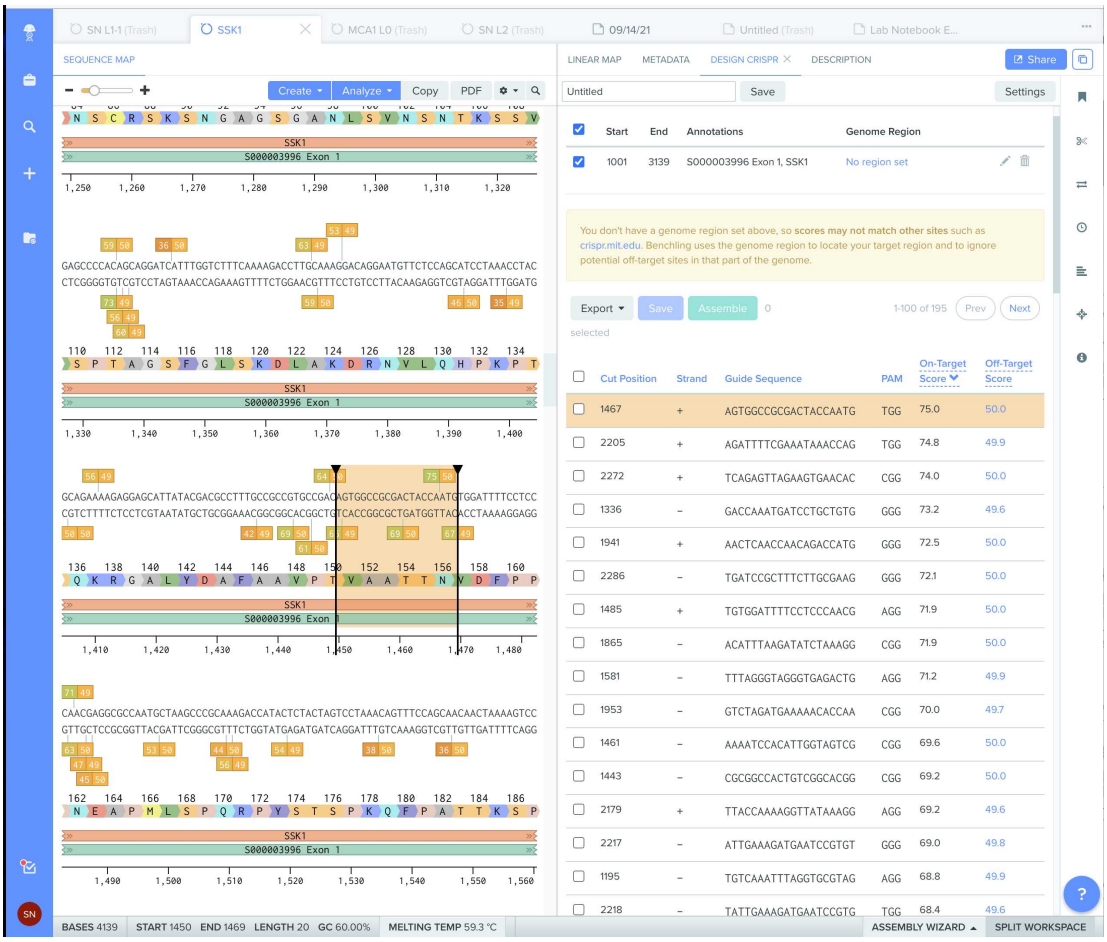
18 If you are only seeing a table of guide sequences and cut positions, click the "SPLIT WORKSPACE" button on the bottom-right corner of the window. You want to be looking at BOTH the guide sequences AND the little "flags" on the sequence that show you where they are, as below. If you're having trouble, flag down an instructor or TA.



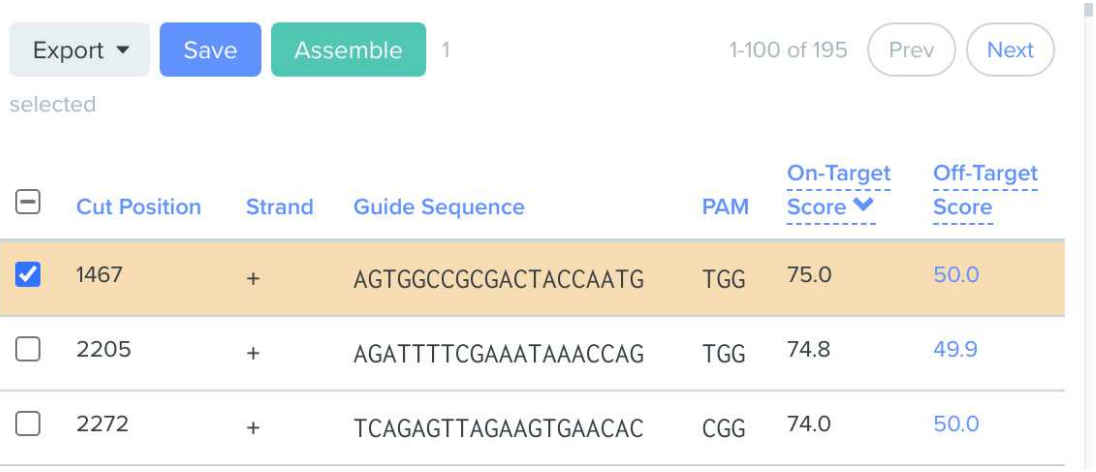
19 Now, it's time to choose exactly where you'll be disrupting your gene. Each "flag" has two colored numbers -- the left is the "on-target" score, and the right is the "off-target" score. We want to balance three things:

- We want the on-target score to be high -- over 70, if at all possible.
- We want the off-target score to be low -- though in this example, they're all very similar.
- We want the location to be as far towards the beginning of the gene as possible -- in the first 1/3 of the gene, if at all possible.

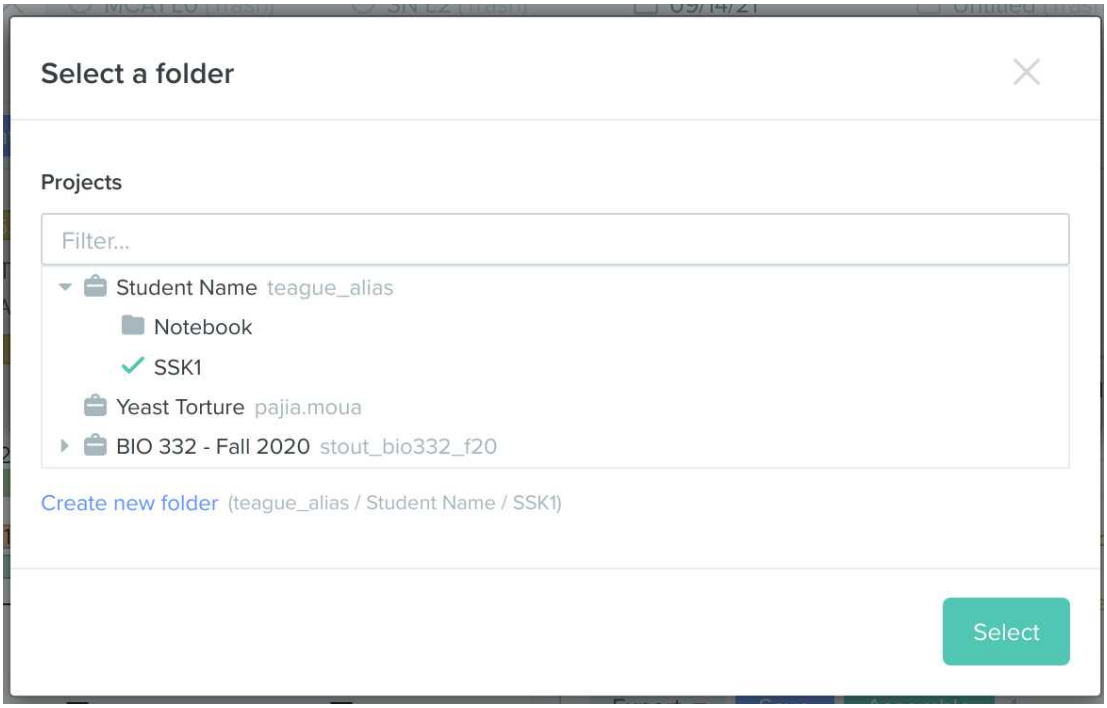
You can also sort by the on-target score by clicking the "On-Target Score" column header. Then, when you click on the guide sequence on the right side of the screen, the corresponding portion of the gene will show up on the left. In this case, the target with the highest on-target score is only 467 bases away from the start of the gene, well within the first 1/3 -- so we'll go with this one. If you have questions or are unsure which one to choose, flag down the instructor or TA.



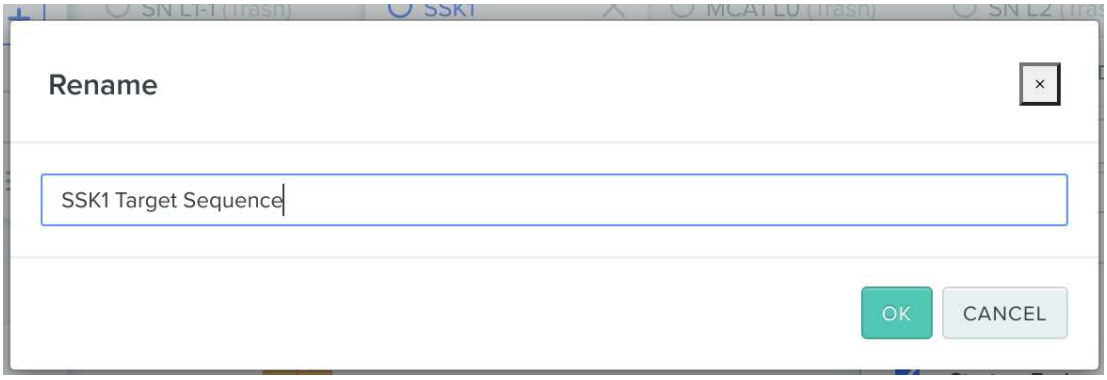
20 Once you have chosen your target sequence, click the check box next to it and click the blue "Save" button.



21 In the "Select a folder" modal dialog box, choose the folder that your gene sequence is in. Click "Select", then click "Save".

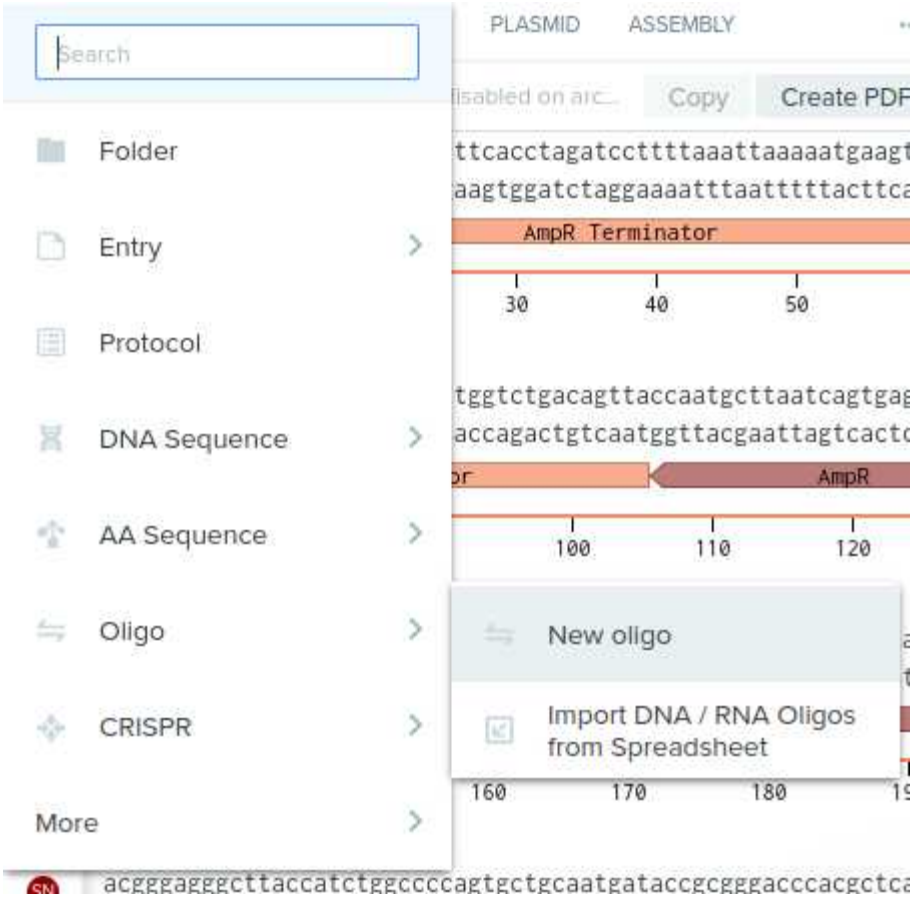


22 Return to the folder to find the 20-base sequence that you just saved there. Right-click on it, choose "Rename", and rename the document to "YFG Target Sequence".



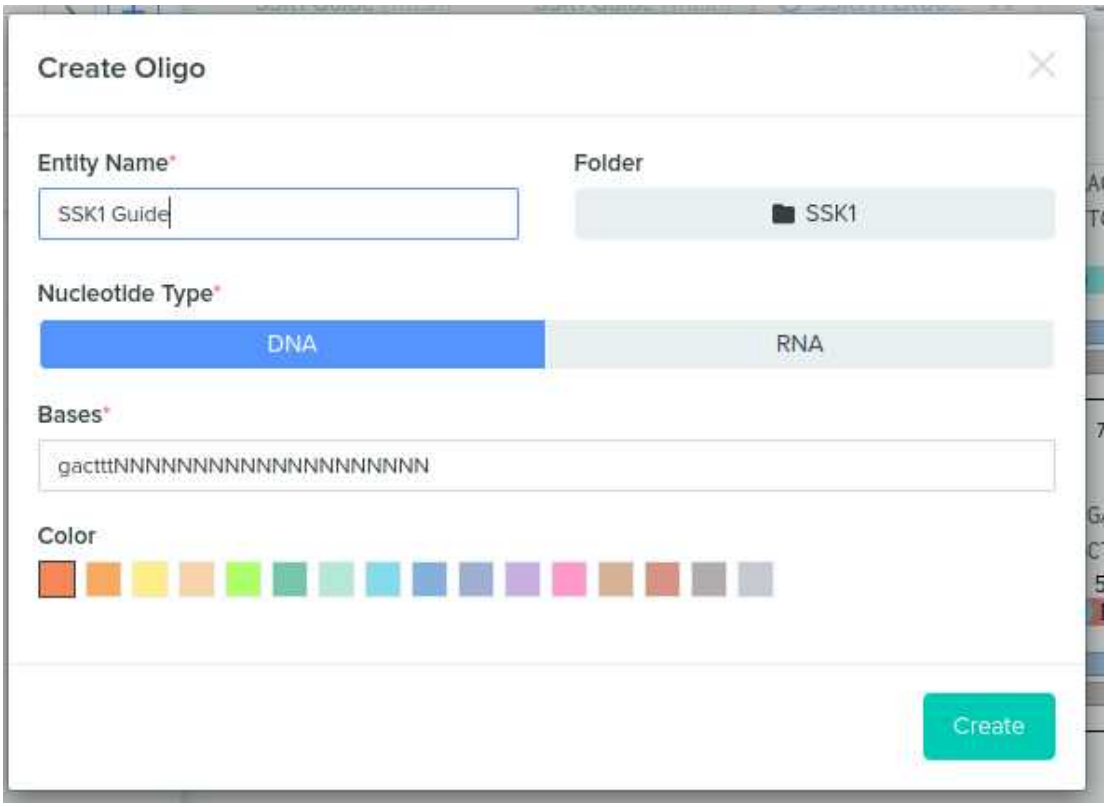
Design Cas9 Guide Oligos

23 Click the "+" button. Under 'Oligo", select "New Oligo".

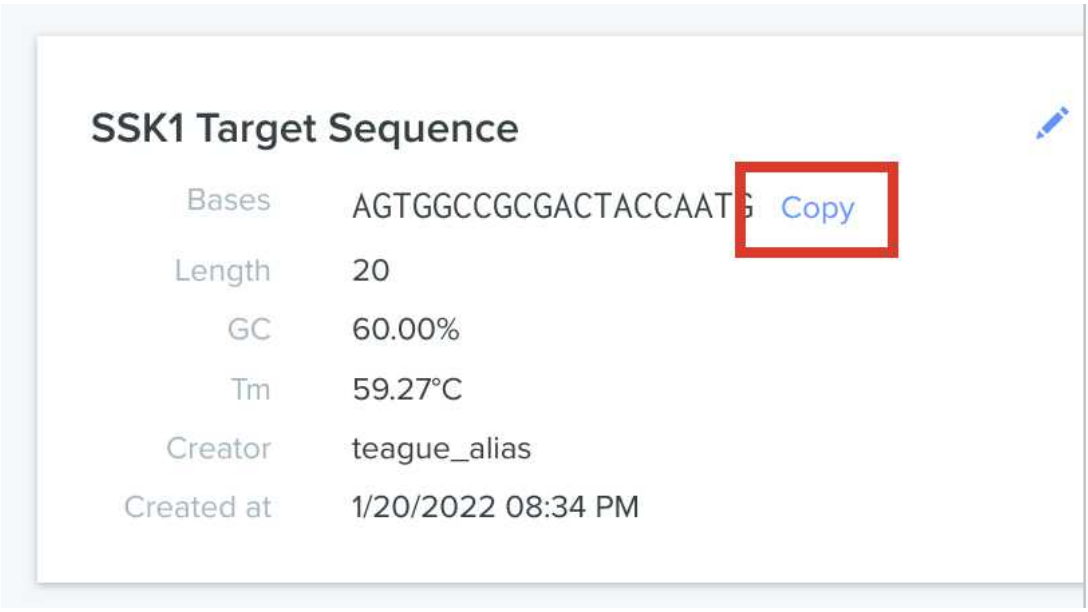


24 Set the name to "YFG Guide". Then, copy the sequence below and paste it into the "Bases" field and click "Create".

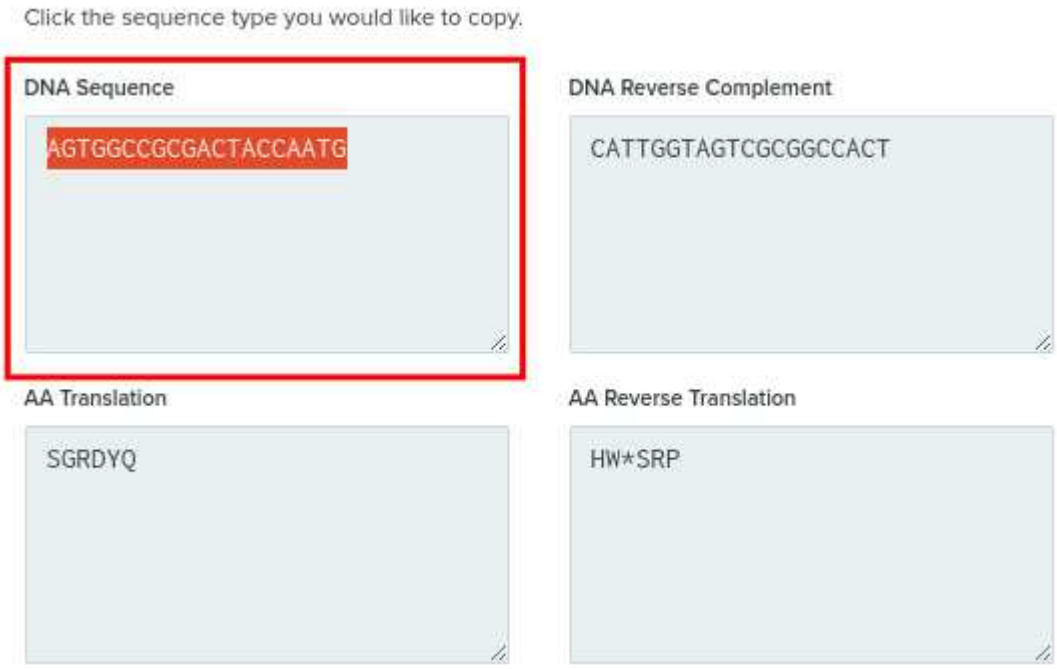
```
gactttNNNNNNNNNNNNNNNNNNN
```



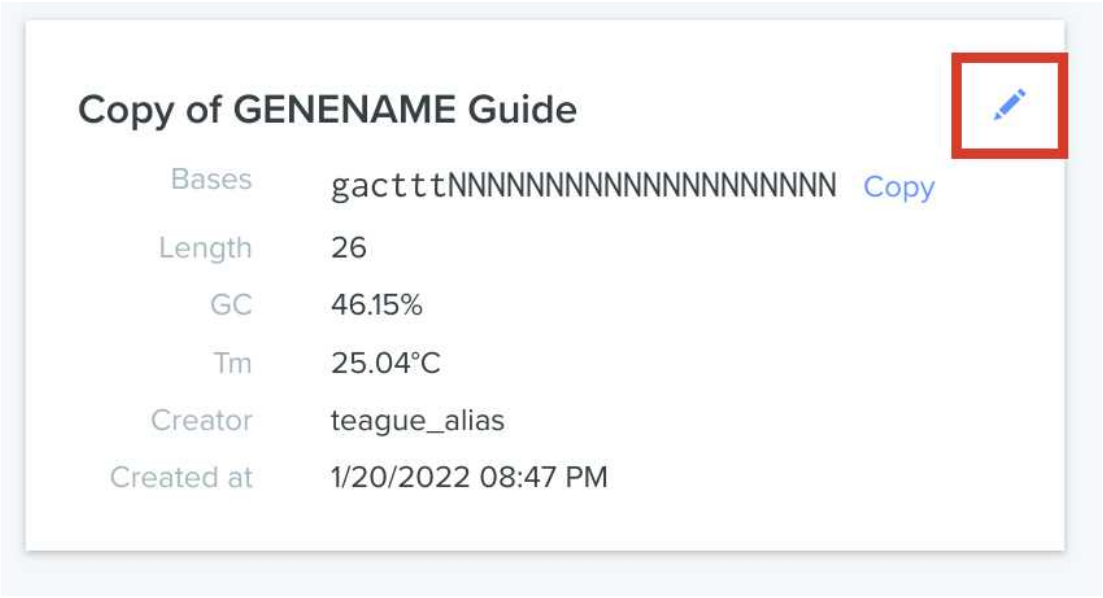
25 Open the "YFG Target Sequence" document. Click the blue "Copy" link next to the sequence.



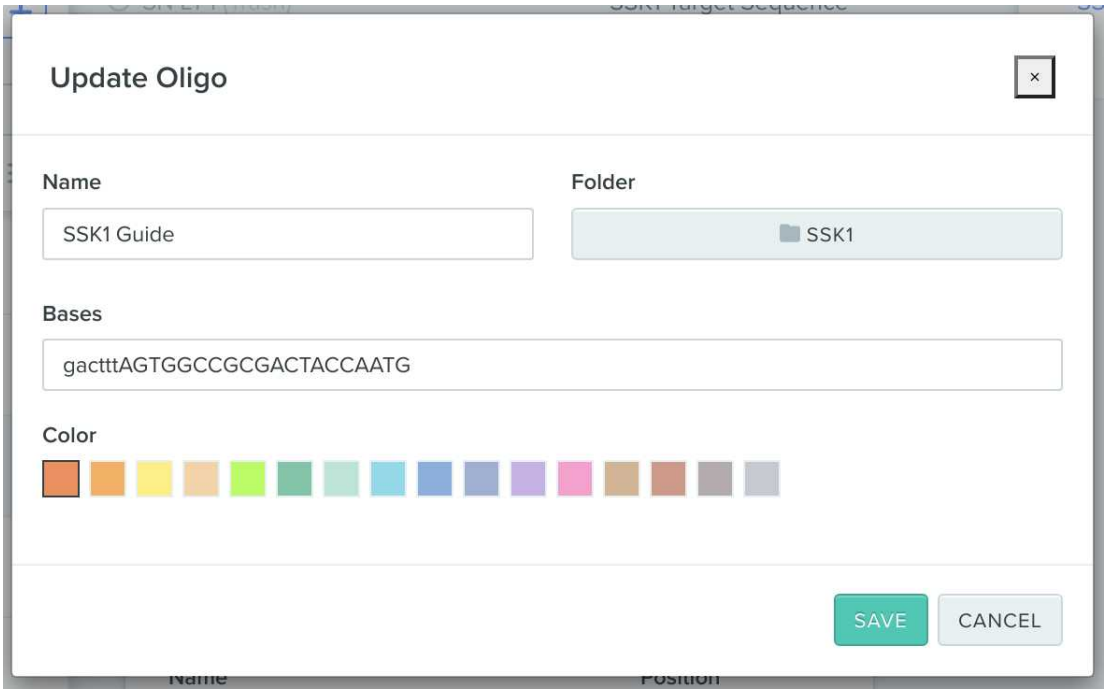
26 Click the "DNA Sequence" box.



27 Open the "YFG Guide" document again. Click the "pencil" icon to edit it.

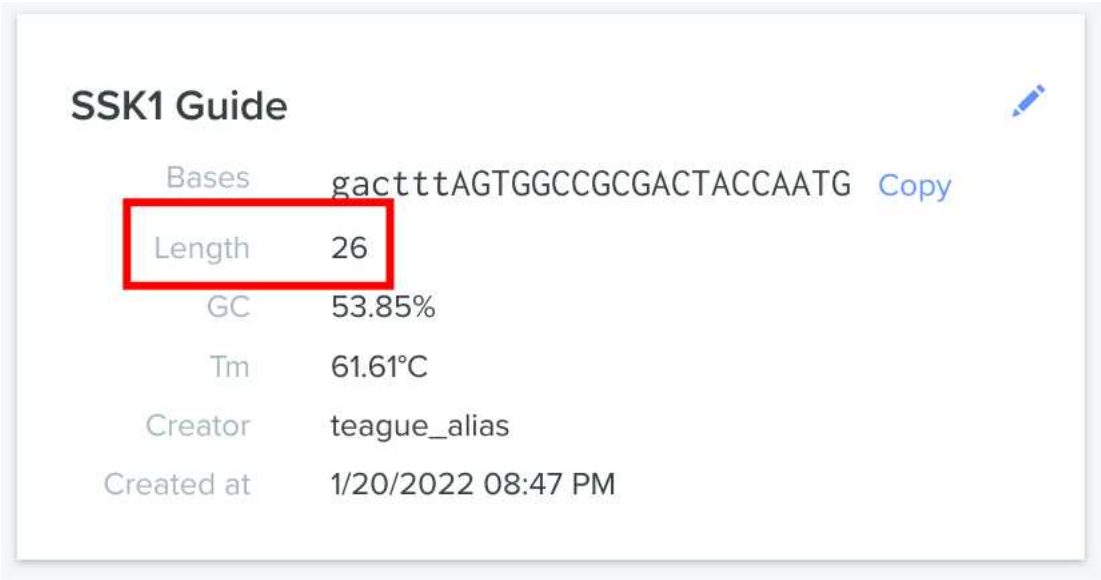


28 Select the 20 "N"s in the sequence, then right-click and choose "Paste" to replace those 20 Ns with the 20 bases of your target sequence.

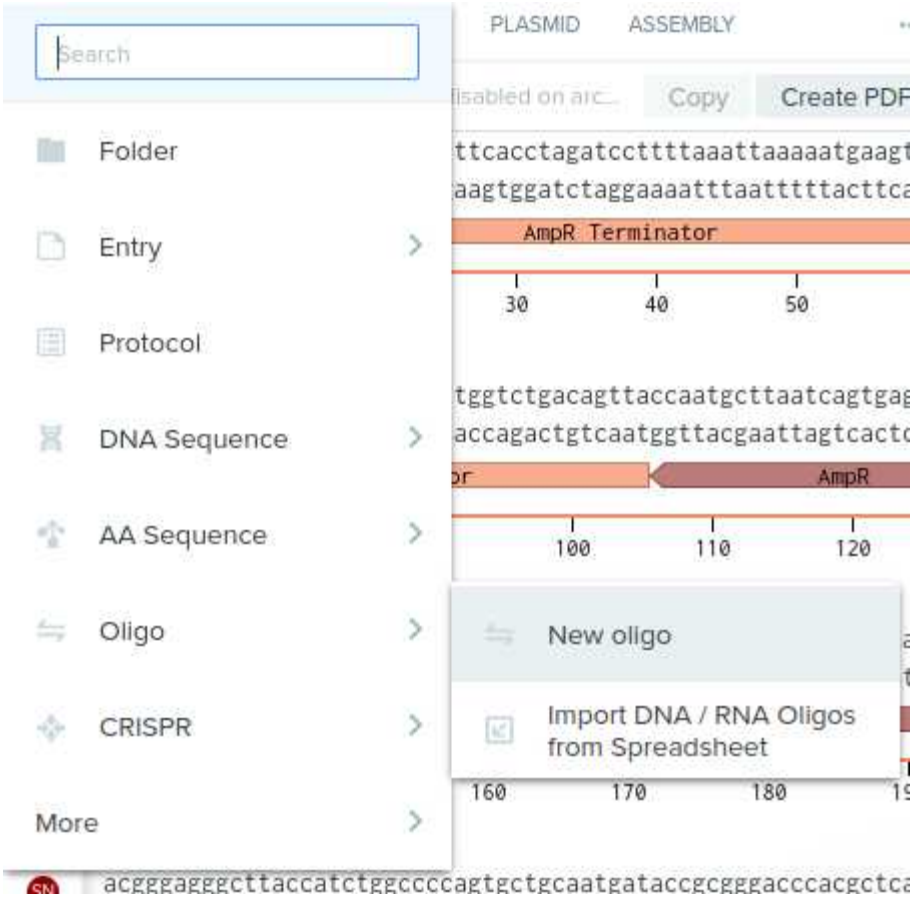


29 ⚠

Important: double-check that the DNA sequence is still 26 bases long. One base added or removed will screw up our experiments!

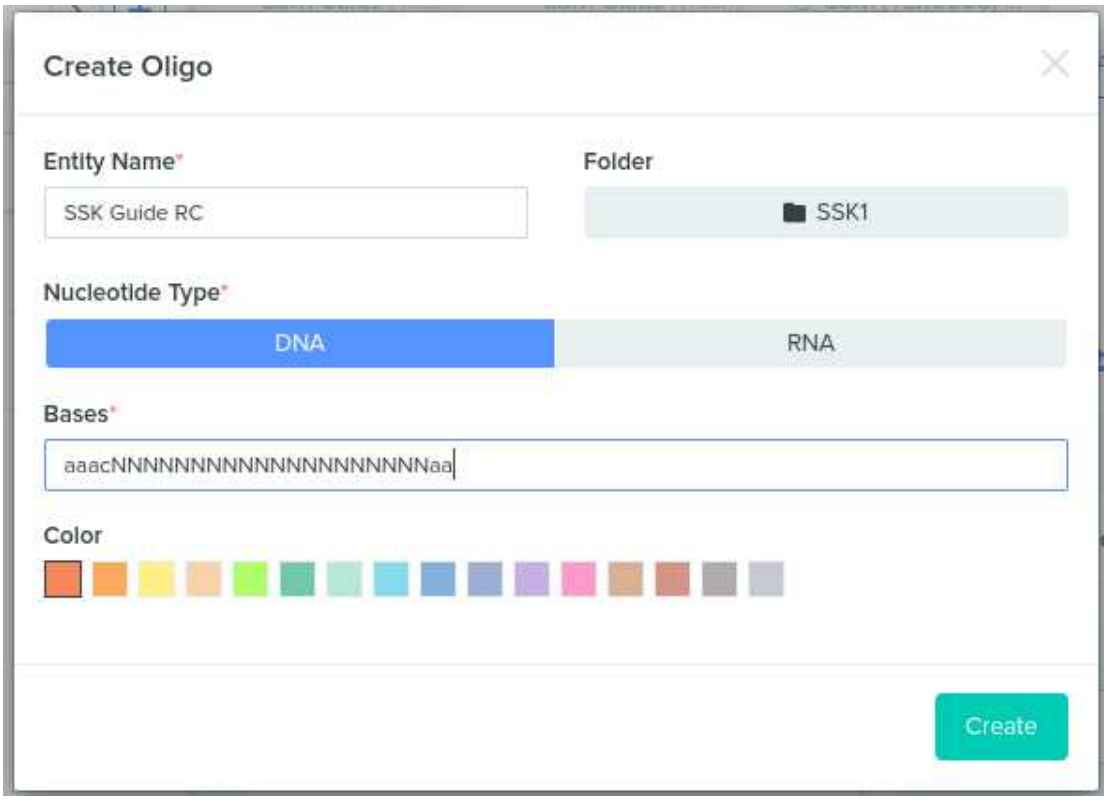


30 Again, click the "+" button. Under 'Oligo", select "New Oligo".



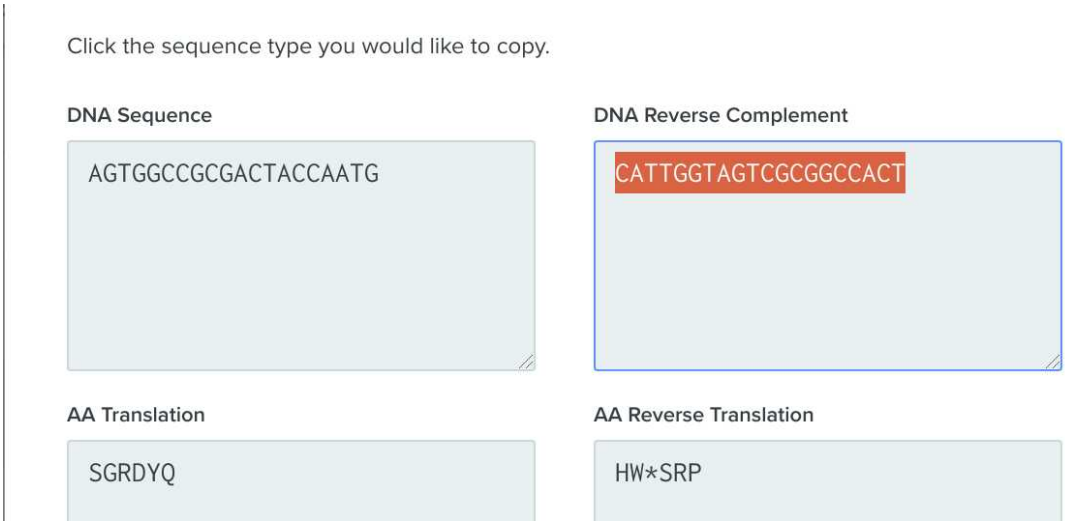
31 Set the name to "YFG Guide RC". ("RC" stands for "Reverse Complement.") Then, copy the sequence below and paste it into the "Bases" field and click "Create".

```
aaacNNNNNNNNNNNNNNNNNNNaa
```

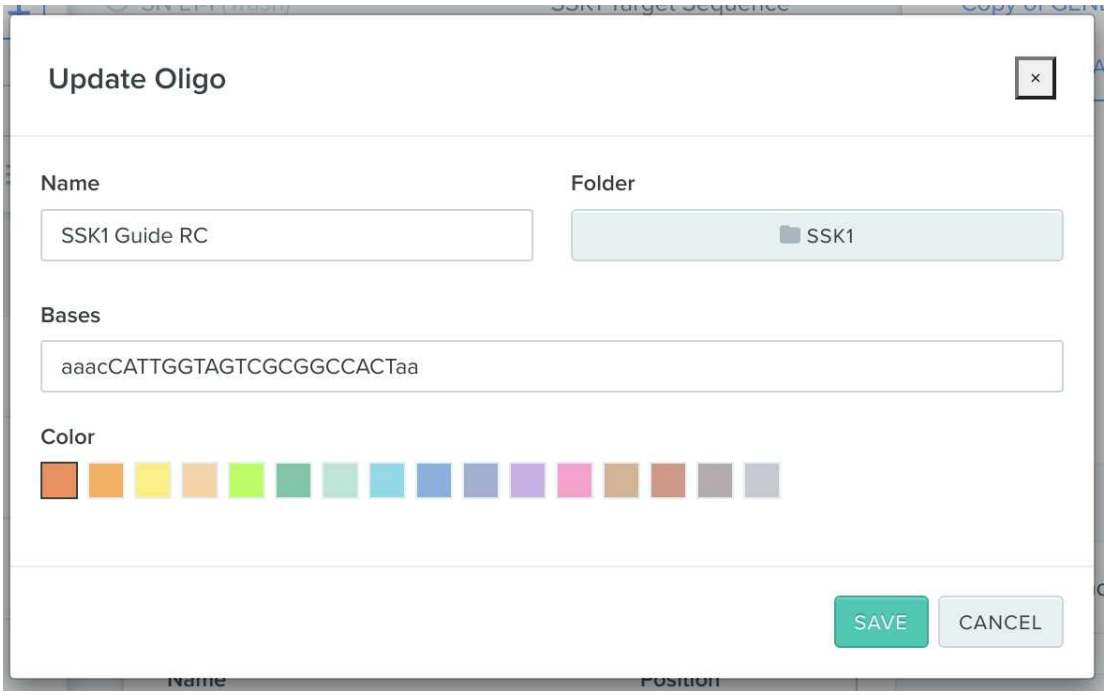


Note that this sequence is DIFFERENT than the previous one!

32 Open your target sequence again. Click "Copy".**This time, select "DNA Reverse Complement"**



33 Open the **YFG Guide RC.** Click the "pencil" icon to edit it. Same as last time, replace the "N"s with the 20 bases you just copied from the target sequence. **Be careful to select only the Ns, leaving the four bases at the beginning and the two at the end unchanged.** Click "Save".

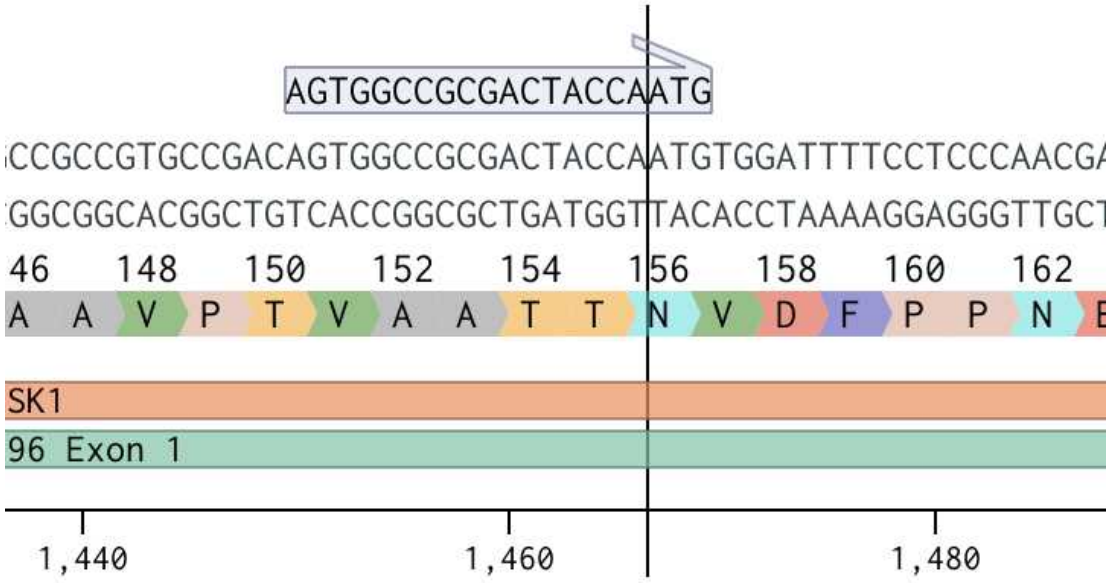


34 ⚠

Again, double-check to make sure that the saved document is 26 bases long. If it's not, go back and try it again. If you're having trouble, ask an instructor or TA!

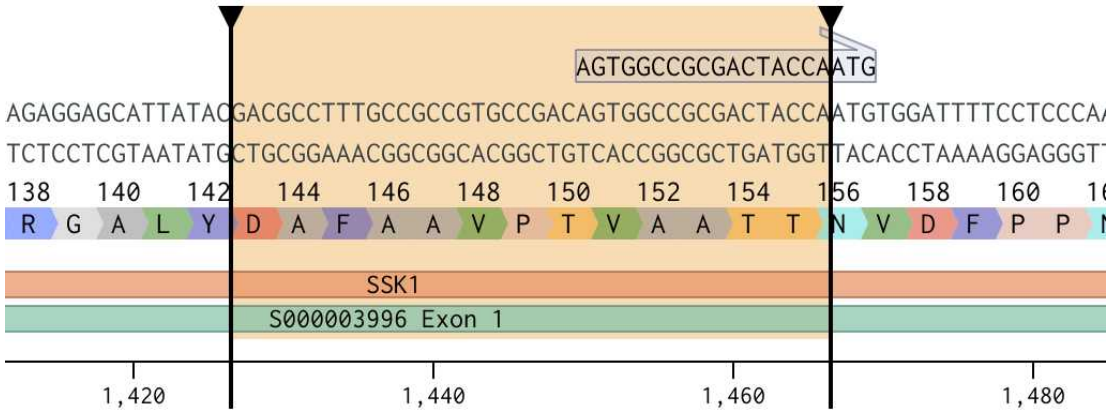
Design the URA3 PCR primers

35 Navigate back to your gene's sequence. Scroll down until you find an "arrow" annotation above the sequence -- that's the place you chose your "target sequence", earlier. **Three bases from the "tip" of the arrow is exactly where Cas9 will cut this DNA sequence.** See the location of the cursor in the image below.



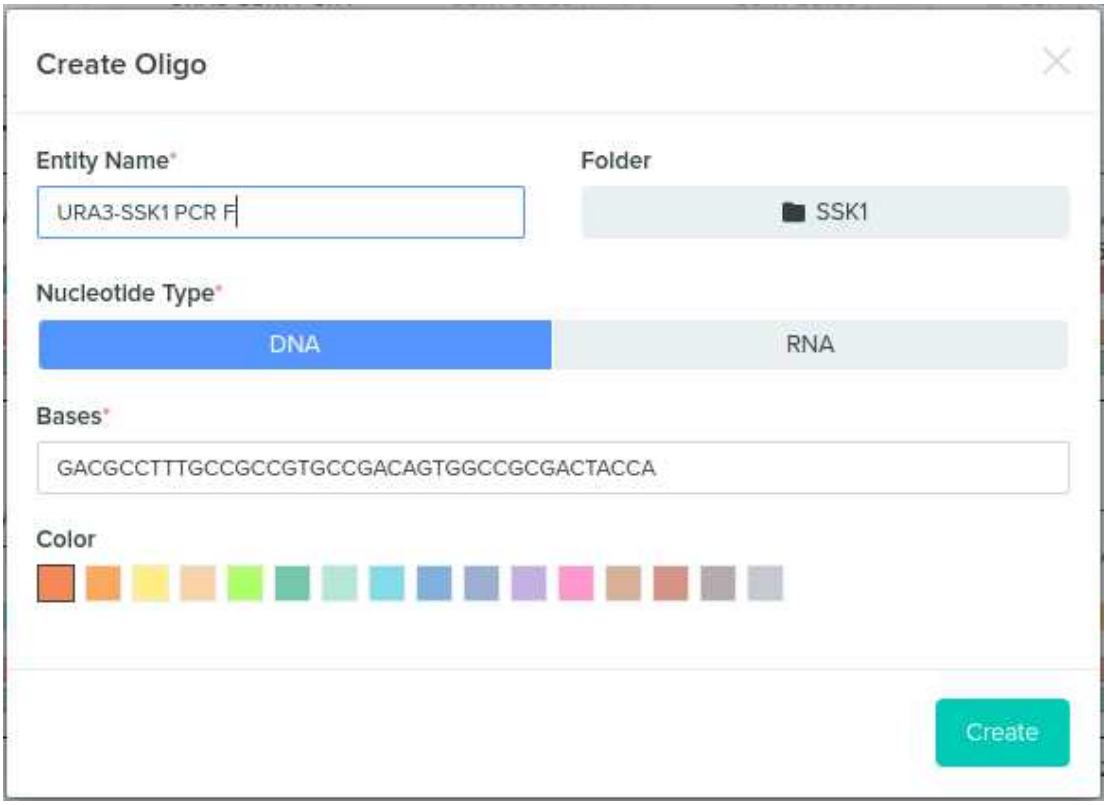
36 Starting at the cut site, use your mouse cursor to select the 4**0 bases before it.**

It doesn't matter which direction the arrow is facing -- make sure that you select from three bases after the "point" of the arrow, and go *backwards* for 40 bases.



37 Right-click the selected sequence, choose "Copy", and then choose "DNA sequence".

38 Again, click the "+" button. Under 'Oligo", select "New Oligo". Name it **URA3-YFG PCR F.** Paste the 40 bases you copied in the previous step into the Bases field.

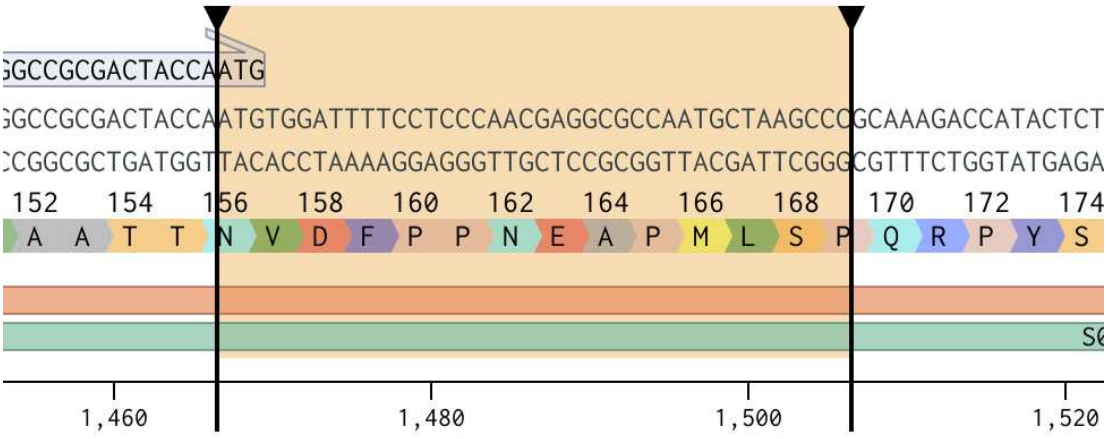


39 Copy the bases from the code block below and paste them at the END of the base sequence of your new oligo. Save the oligo.

```
tctgtggataaccgtagtc
```

40 Return to your gene's sequence. Set your cursor at the cut site, 3 bases from the "tip" of the arrow. Now, using your cursor, select the 40 bases **after the cut site.**

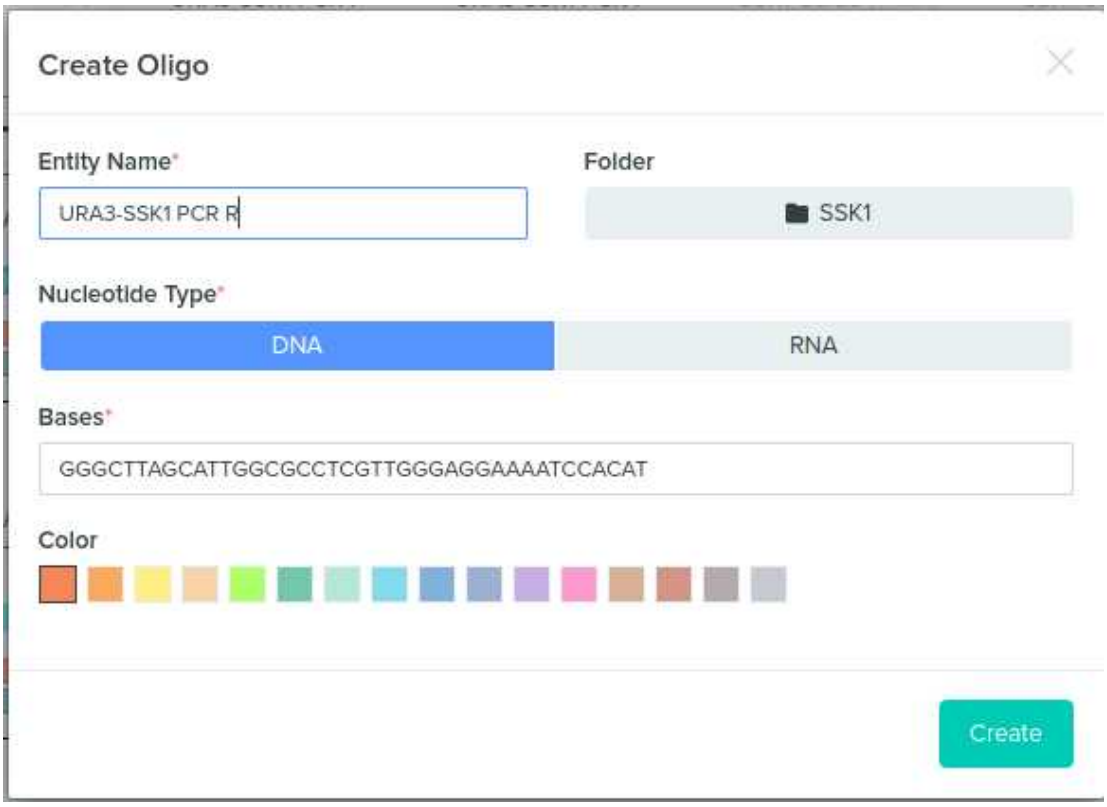
Again, it doesn't matter which direction the arrow is facing. Start your selection 3 bases from the "point" of the arrow, and select the 40 bases after it.



Right-click on the sequence and select "Copy". Choose **DNA Reverse Complement.**

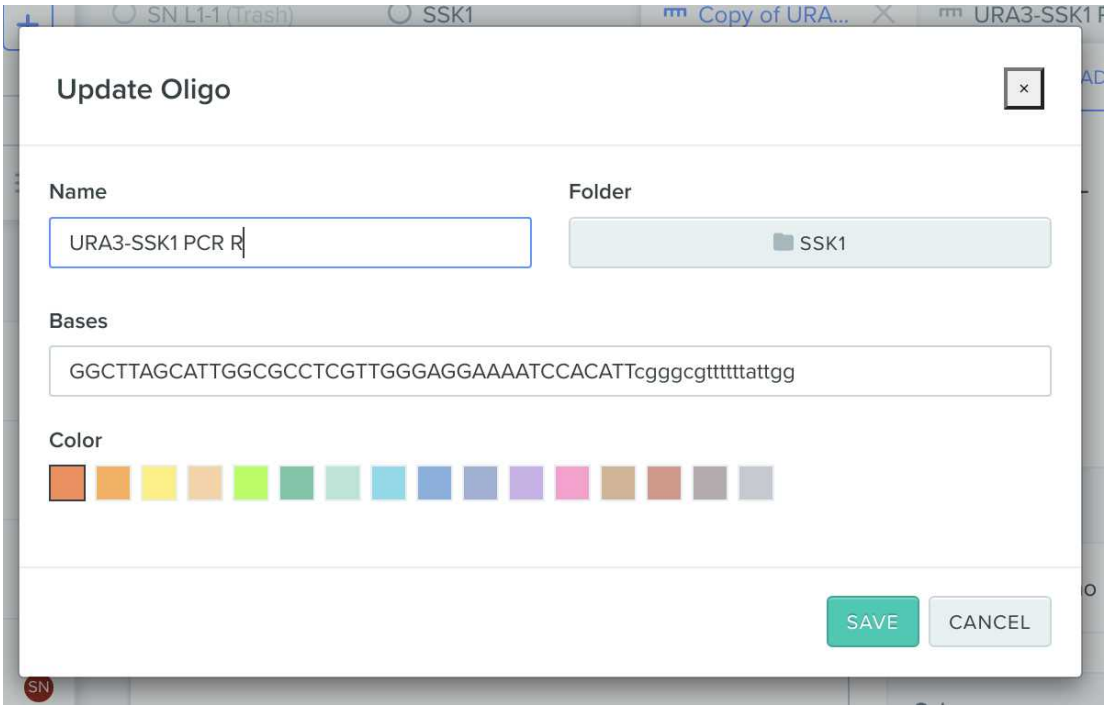
41

42 Again, click the "+" button. Under 'Oligo", select "New Oligo". Name it **URA3-YFG PCR R.** Paste the 40 bases you copied in the previous step into the Bases field.



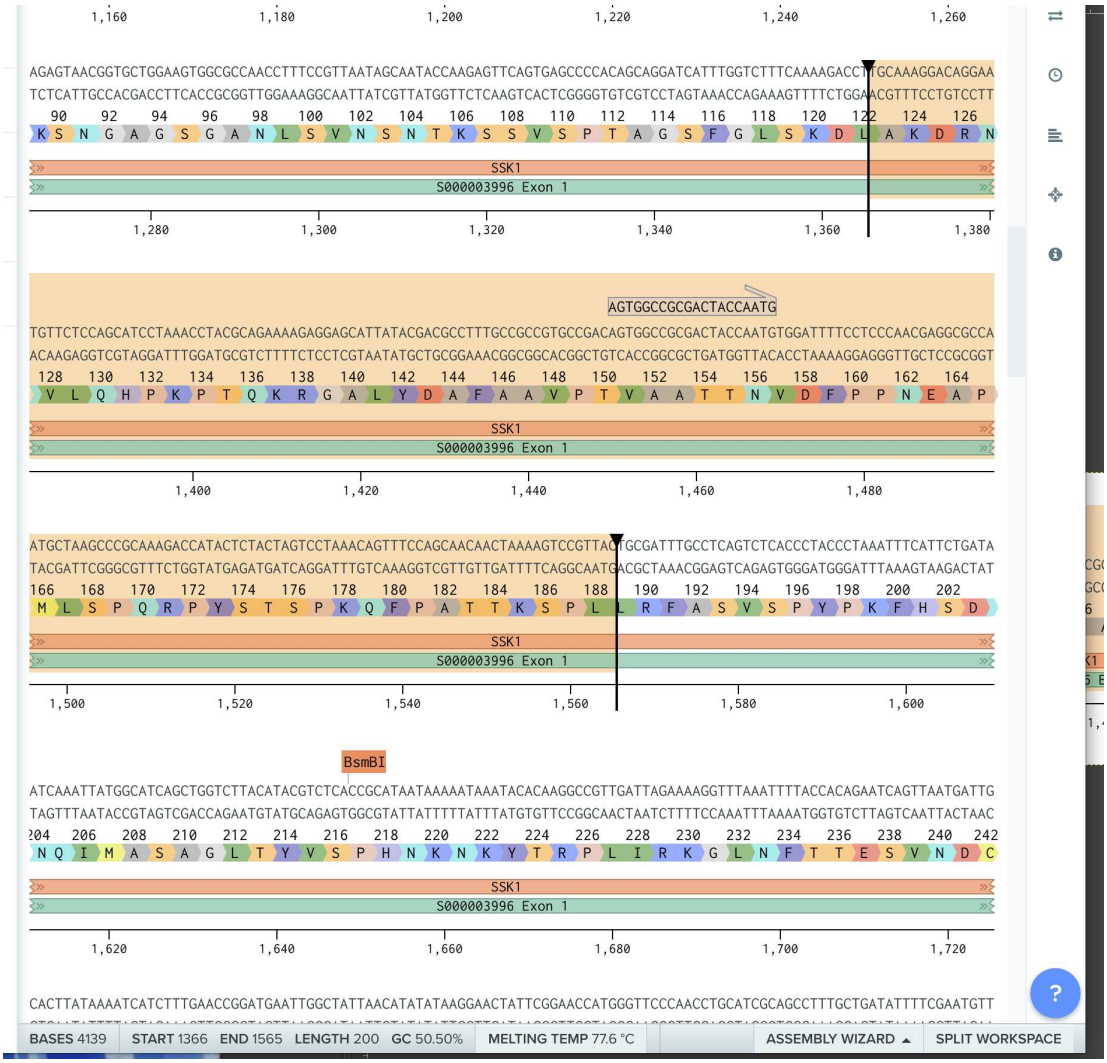
43 Copy the bases from the code block below and paste them at the END of the base sequence of your new oligo. Save the oligo.

```
cgggcgtttttattgg
```

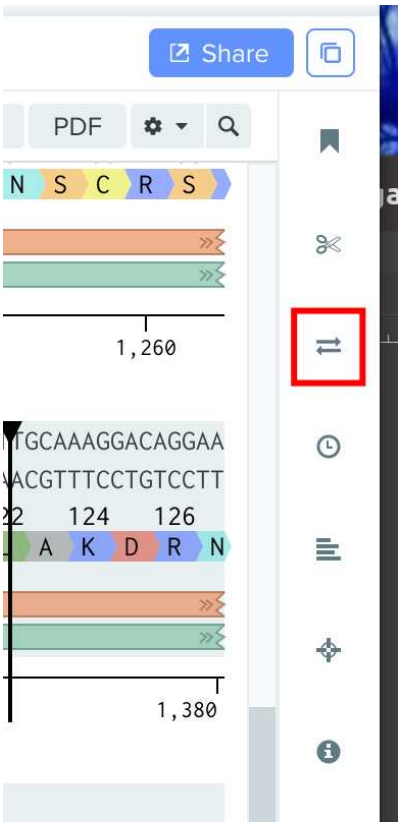


Design Knockout PCR Primers

44 Return to your gene's sequence and find the knockout target sequence again. Using your cursor, select 200 bases of DNA -- 100 bases before the target sequence to 100 bases after. (You can be a little sloppy here. To see how large your selection is, look at the LENGTH field on the status bar at the bottom of the browser window.)



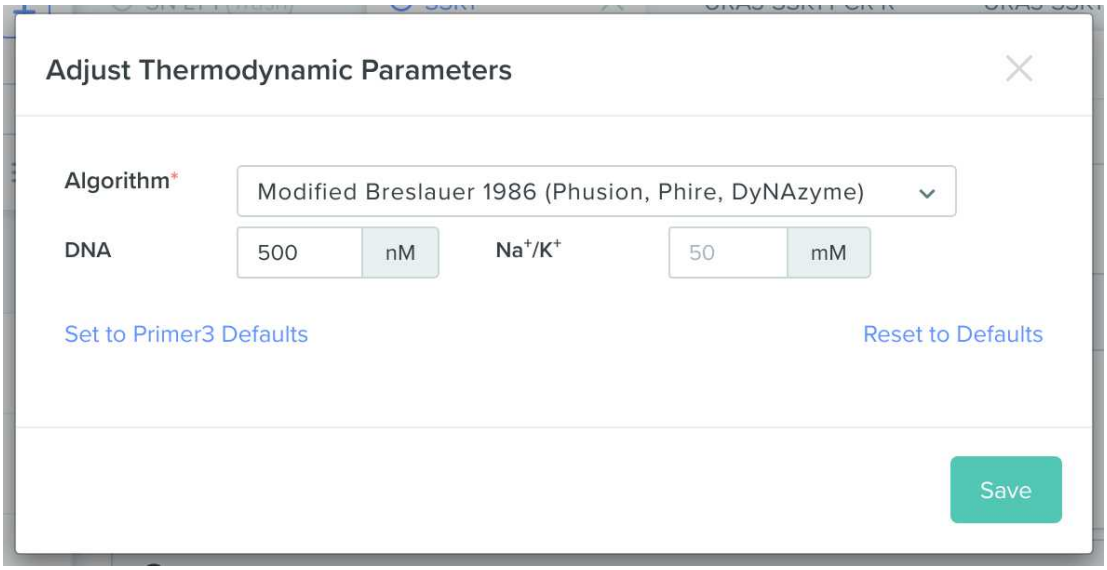
45 Select the PCR Primers tool on the right-side toolbar -- it's the two little arrows pointing in opposite directions:



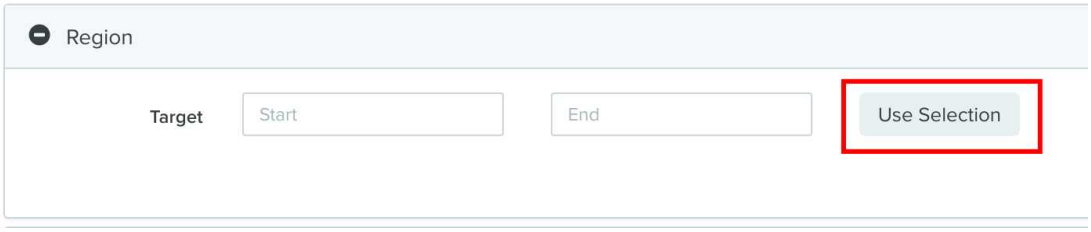
46 Click the blue "Create Primers" button, and choose "Wizard" from the drop-down box.

47 Leave "Task" set to PCR. Click the blue "Tm Params" link.

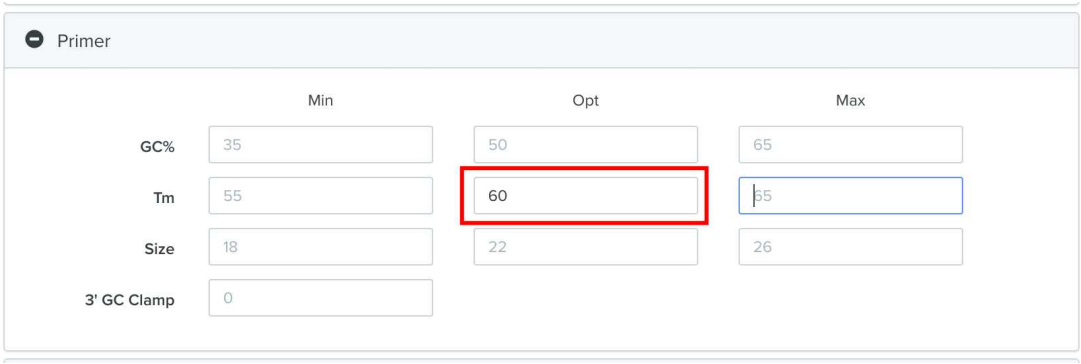
48 In the modal dialog box that comes up, under "Algorithm", choose "Modified Breslauer 1986" and set "DNA" to "500 nM". Click "Save".



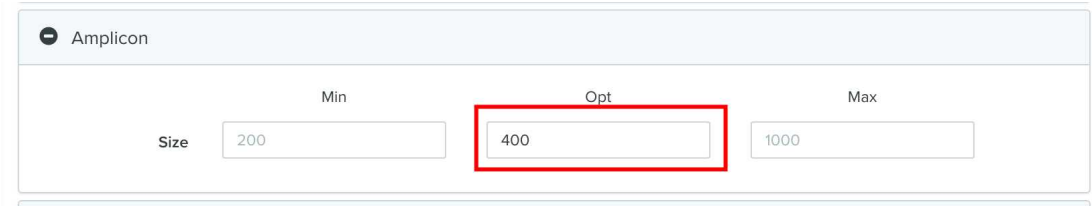
49 Under "Region", set the "Target" by clicking "Use Selection."



50 Under "Primer", change the "Opt" "Tm" to 60. The rest of the defaults are fine.

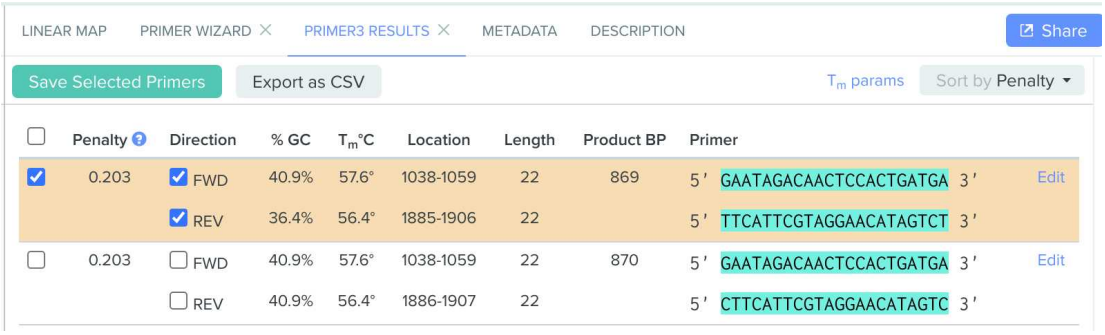


51 Under "Amplicon", set the "Opt" to 400. The rest of the defaults are fine.



52 Click "Generate Primers" (blue button in the upper-right-hand corner.)

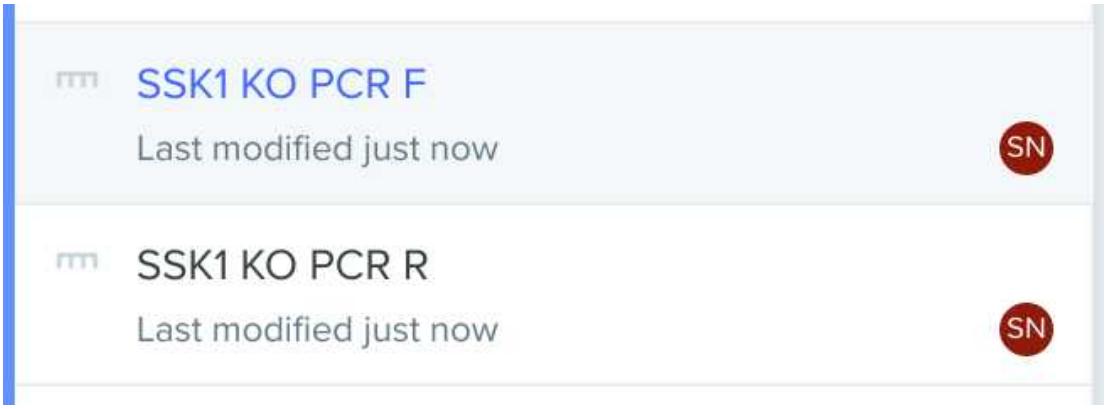
53 Choose the pair of primers whose "Product BP" is closest to 400 bp by clicking the checkbox on the left of the primer pair.



54 Click "Save Selected Primers". Save the primers to the folder with the rest of your sequences.

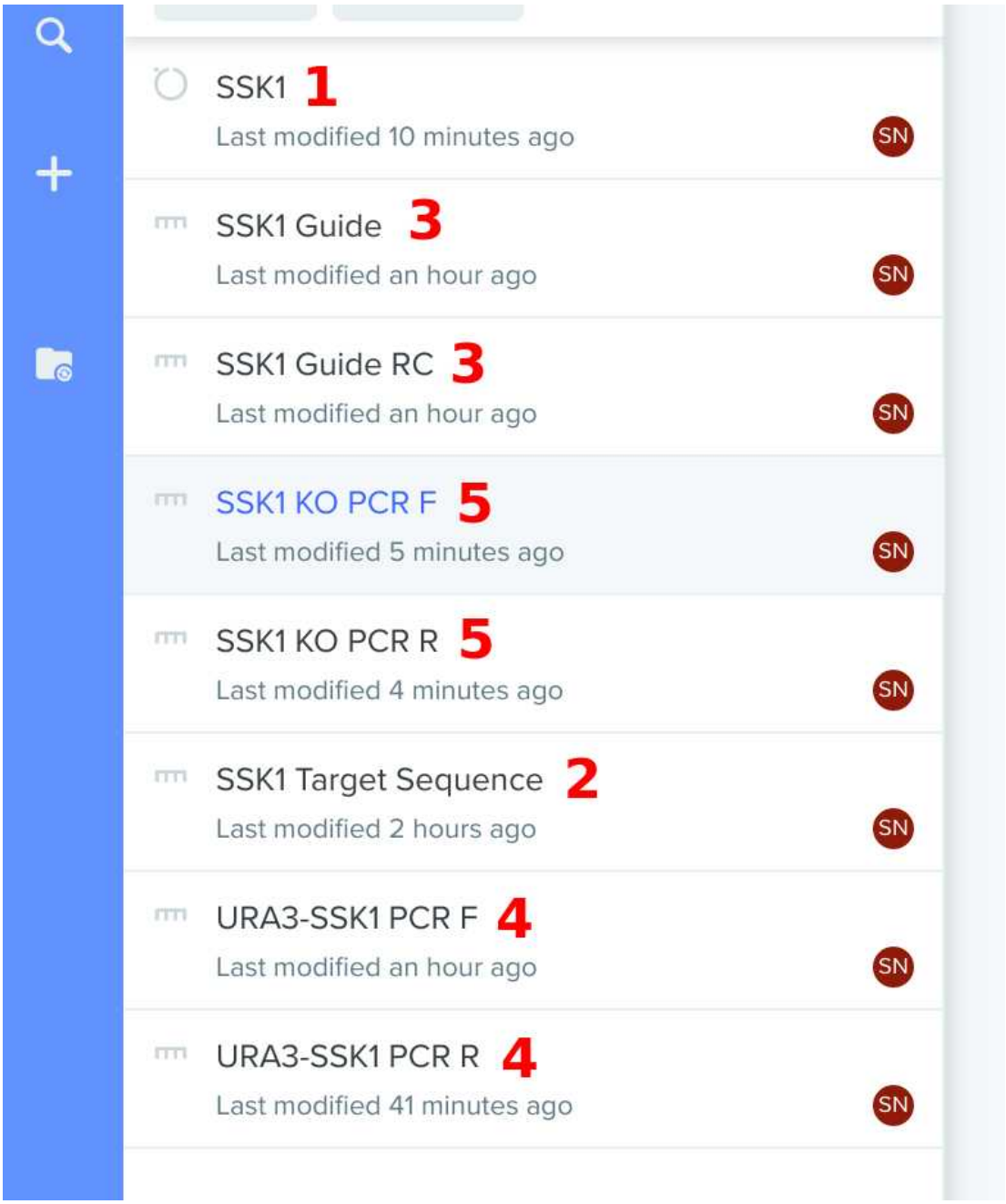
55 We're almost done! Navigate back to the folder to which you just saved those new sequences. If they don't show up immediately, you may need to "refresh" your web browser.

56 The two primer sequences will show up as "YFG ####-#### FWD 1" and "YFG ####-#### REV 1". Rename these documents to "YFG KO PCR F" and "YFG KO PCR R" by clicking the "pencil" icon and typing in a new name (replacing YFG with your gene name, as usual.) **Do not edit the primer sequence.**



57 Double check that your folder is complete before leaving. You should have:

1. A document containing the gene that you're going to knock out (named YFG)
2. A document containing the 20-bp target sequence (named YFG Target Sequence)
3. A guide oligo (YFG Guide) and its reverse-complement (YFG Guide RC). Both should be 26 bases long.
4. Two URA3 PCR oligos: URA3-SSK1 PCR F and URA3-SSK1 PCR R. The first should be 59 bases long; the second should be 57 bases long.
5. Two KO PCR oligos: YFG KO PCR F and YFG KO PCR R. Their length may vary, but both should be about 22 bases long.



You're done! Pat yourselves on the back, that was a slog. But -- in less than a week, we'll actually have these synthetic DNA sequences made and we'll be ready to get on with the science!