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Investigating butterfly wing color gene using CRISPR technology

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CRISPR technology is a transformative genomic revolution, thus, it is important that all citizens, scientists and non-scientists, are familiar with its molecular architecture and its impact on society. CRISPR-based technology is already impacting a broad societal landscape, because it is moving beyond basic science into the realms of medicine, agriculture, food, policy and business. This transformative technology offers potential for enriching undergraduate science education by challenging students to face real-world applications and ethical dilemmas associated with CRISPR-based technologies. Educating students about the fundamental concepts associated with gene editing technology so that they can apply and connect the ways in which this technology intersects the fields of biology, health, policy, ethics and engineering should be a priority given the rapid state and intricacies at which the technology is expanding. In this lab, we aimed to develop activities that teach the fundamentals of CRISPR through paper modeling and gene analysis of a butterfly gene using DNA sequence software, and then have students apply their knowledge to an experiment where they analyze wild-type and CRISPR mutants in butterflies. Specifically, students analyze the molecular and phenotypic CRISPR effects when a gene responsible for butterfly wing color is targeted by CRISPR. Thus, this lab promotes understanding of genotype-phenotype relationship using butterflies as the model system.

Keywords: CRISPR technology, gene knockout, molecular biology, butterflies

Link To Supplemental Materials: https://doi.org/10.37590/able.v43.sup11

Introduction

Undergraduate students are learning that gene editing is now possible with CRISPR technology to treat human genetic blood and retinal diseases (Ledford 2020; Frangoul et al. 2021). In recent news, biotech Verve Therapeutics company announced that it had edited a gene in an person with the genetic condition familial hypercholesterolemia that causes high cholesterol and predisposes them to heart disease, with the goal of lowering cholesterol level and heart disease risk (Eisenstein 2022). Despite the significant advances made with CRISPR technology in the last ten years since it was adapted as a genetic modifying tool, CRISPR technology and its current applications are not explained in much detail in many biology textbooks. To address this, we aimed to successfully integrate CRISPR technology into the undergraduate curriculum. Previously, we have used CRISPR technology to deactivate expression of GFP in bacteria cells, and most recently, students engaged in the full process of CRISPR gene targeting in *Vanessa cardui* (painted lady) butterflies (Pieczynski et al. 2019; Thulluru et al. 2022). Specifically, students targeted the *optix* gene with CRISPR technology, and analyzed how the loss-of-function of optix due to CRISPR modification affected butterfly wing color and pattern and used molecular biology tools to analyze the specific CRISPR modifications. *Vanessa cardui* butterflies and caterpillars are easy to rear in lab, and we can collect hundreds of butterfly eggs from a batch of 30 butterflies. The butterfly eggs are big enough at 1mm in size that they can be microinjected by undergraduate students using a coarse air microinjection system with CRISPR tools, a mixture of cas9 protein and guideRNAs that target the *optix* gene. Caterpillar hatchlings from injected eggs are then subjected to molecular analyses such as polymerase chain reaction (PCR) and DNA sequencing to determine the success of CRISPR targeting, or reared for three weeks until butterfly stage to visualize phenotypic outcomes of

increased black pigmentation and loss of orange pigmentation of butterfly wings. This demonstrates to students that optix is responsible for promoting orange pigmentation and/or repressing black pigmentation, as the loss-of-function of this gene results in increased black pigmentation and decreased orange pigmentation. We have conducted this lab experience two times in upper-level Genetics and Molecular Biology & Biotechnology lab. The first iteration of the lab we spent 8 weeks, with a three-hour lab that meets once a week. The second iteration of the lab we spent 5 weeks with a lab that met twice a week. We measured students' ownership of the lab using the Project Ownership Survey (Hanauer and Dolan 2014), and found that students developed a higher emotional and cognitive ownership than a non-CRISPR microbiology lab.

Using CRISPR technology in butterflies is engaging to students. However, not all institutions are able to set up a microinjection station to deliver CRISPR components into butterfly eggs. One microinjection station includes a stereomicroscope, microinjection system that can deliver pressure through a needle in a short pulse and a one-axis coarse micromanipulator. In a class of 18 students, we have six stations set up so that students can cycle through microinjection within a lab period.

Here we present a modified version of the CRISPR experience with *Vanessa cardui* that can be implemented at many institutions. Briefly, the first part involves guiding students through a kinesthetic paper modeling activity where students model how the CRISPR tools target and associate with the target gene. We developed this activity because we found that students struggled with the CRISPR targeting mechanism (Pieczynski et al. 2019). They follow this paper activity with the modeling and annotation of CRISPR targeting of the *optix* gene on the DNA software called Snapgene on the computer. The second part of the lab, students set up a polymerase chain of "wild-type" and "CRISPR-d" butterfly DNA to amplify the target *optix* gene, followed by DNA gel electrophoresis to analyze PCR amplicons. In the final part of the lab, students analyze phenotypic outcomes, and conclude with the analysis of DNA sequences of wild-type to CRISPR-d gene sequences.

Table 1: Student lab activities and learning outcomes

Class	Student Lab Activity	Student learning outcomes: Students will:	Tools needed
1	**Analyze *optix* gene sequence** **Analyze target sequence for cas9 and gRNA** **Design gRNA** **(2 hours)**	• use SnapGene software to annotate gene, design primers to amplify and sequence gene • be guided to determine target sequence and PAM sequence for cas9 • use SnapGene software to annotate these features • use paper model to model CRISPR targeting	Snapgene software/computer
1 **2**	**PCR amplification of *optix* from wild-type and "CRISPR-d" butterfly DNA (30 mins)** **Run PCR amplicons on gel (1 hour)**	• use PCR to amplify their region of interest from the gene using DNA from larvae • run DNA agarose gel	PCR Primers PCR kit Water for PCR PCR tubes DNA gel electrophoresis rigs 1xTAE (5L) Agarose (15g) Gel Green
3	***optix* sequence analysis of PCR product with reference gene from database (1 hour)** **Analyze images of wild-type and CRISPR-d knockout butterfly wings (30 mins – this can be done in class 2 instead while the DNA gel is running)**	• align DNA sequencing data with the reported sequence • determine nucleotide changes that have resulted from CRISPR targeting • predict how this would affect *optix* gene function in butterfly wing color	Snapgene/computer

We recommend that students work in pairs during the wet-lab component of the lab.

1) CRISPR design and targeting – kinesthetic activity on paper and on computer

First students will design CRISPR tools to target the butterfly gene *optix* on paper and on the computer using a molecular DNA software. Specifically, students will be given a paper model of part of the *optix* gene, and they will find a CRISPR target site and determine the appropriate gRNA sequence. Using scissors, they will make a CRISPR cut (mimicking cas9 nuclease activity). As two target sequences are being targeted by CRISPR, we will aim to cut at two target sites with our scissors and then tape together the two cut ends, mimicking a loss-of-function deletion mutation that is desired. As an alternate scenario, students will also model if only one guideRNA, and not both guideRNAs, has successfully targeted its target site, and then through non-homologous end-joining repaired the DNA lesion, resulting in insertions or deletions.

On the computer, students will use the DNA software SnapGene to analyze the butterfly *optix* gene. This gene is a single exon, and does not have any introns, which simplifies the analysis for students. They will determine the coding sequence (start, stop codons and reading frame) and determine how big of a protein optix is. Students will annotate two CRISPR target sites and the desired mutations. Students will also annotate two primers for Polymerase Chain Reaction (PCR) to amplify part of the *optix* gene for molecular analysis.

2) Molecular Analysis of Wild-type and CRISPR mutants

To determine the specific CRISPR edits that can be created by CRISPR targeting of the *optix* gene, students will use molecular biology techniques to assay wild-type and three CRISPR mutant DNA for the *V. cardui optix* gene. To do this, students will use PCR to amplify the gene from wild-type and CRISPR mutant DNA. The instructor will provide purified DNA to the students to use as their template DNA in their PCR reaction. Students will set up PCR reactions by pipetting reagents that include primers, 2x PCR master mix (containing dNTPs and Taq DNA polymerase) and water. PCR cycling conditions are listed in the student handout. After the PCR, part of the PCR products are run on a 1.2% w/v agarose DNA gel. The students should visualize the following on the DNA gel (Table 2, Figure 1).

Table 2. Expected PCR product sizes on DNA agarose gel

Reaction	Size	Observations/Notes
WT (wild-type)	444bp	The expected DNA fragment size
M1 (mutant 1)	192bp	One smaller DNA fragment, showing deletion between two gRNA target sites has occurred successfully (Figure 2).
M2 (mutant 2)	441bp	DNA fragment size similar to wild-type because it has only three nucleotides deleted. You can ask students why this may be. Students will discuss, and hopefully a student will suggest that the deletion between the two gRNA target sites may not have occurred but single base changes or a very small deletion of a few nucleotides may have occurred at only 1 or both gRNA sites (Figure 2).
M3 (mutant 3)	444bp	DNA fragment is same size as wild-type. This mutant has a substitution mutation (Figure 2).

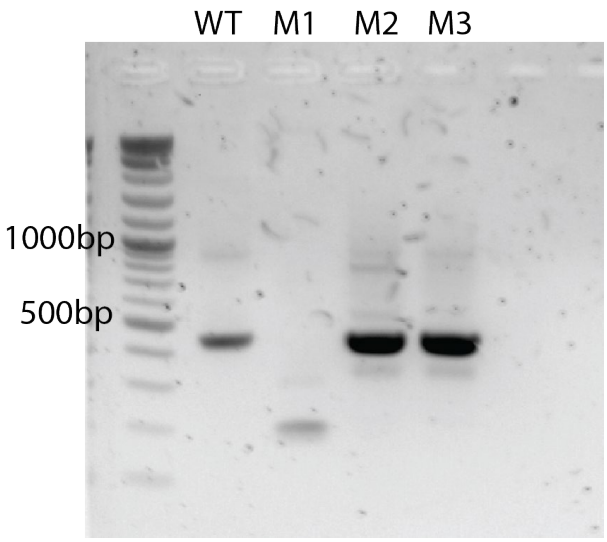


Figure 1. PCR products of wild-type and CRISPR mutant on agarose gel. Expected band sizes for PCR reactions amplifying wild-type (WT) and CRISPR mutants (M1, M2, M3). M1 band shows CRISPR modifications created a ~200 base pair deletion. M2 and M3 band is similar to WT in size due to small deletion and point mutation.

3) Phenotypic Analysis of Wild-type and CRISPR mutant butterfly wings

While students are running their DNA agarose gels, which typically takes about 30-45 minutes, the instructor will provide students with the images of what the wild-type and two CRISPR butterfly mutants look like. The instructor can ask students to chat amongst themselves as to what they observe when comparing mutant wings to wild-type wings, and come up with suggestions as to why they believe they see this phenotype. What does the data suggest about the normal function of *optix*? Since normally the butterfly wings are orange and brown, and the two CRISPR mutants have black wings or patches of black in their wings, this shows a loss of orange color. This suggests that the *optix* gene normally function to promote orange pigmentation on the butterfly wings because when the gene is deactivated, we see less orange on the butterfly wings.

The three mutants show different degrees of CRISPR knockout. The M1 mutant shows a complete knockout, there is no orange. The M2 and M3 mutants show a partial knockout, where the right wing has more black and the left wing has some orange scales on the butterfly wings. Students will make these observations, and you can probe them to discuss why there could be a difference in the degree of loss of orange. This is due to the way CRISPR is delivered to the butterfly during development, we deliver CRISPR into butterfly eggs, and depending on how many cells are targeted in a butterfly embryo will determine the extent of the knockout. This sometimes leads to the discussion of the bioethical implications of CRISPR gene modification.

4) DNA sequence analysis of WT and CRISPR mutants

Students will use the DNA sequencing files to conduct a DNA sequence alignment in the molecular software SnapGene. Students will use their original sequence file that they annotated from Part 1 to align the four different sequence files (Wild-type, Mutant 1, Mutant 2, and Mutant 3). The sequence alignment will show students how the wild-type sequence aligns with their reference sequence and the nucleotide changes that have occurred in CRISPR mutants. The DNA sequencing results are listed in Table 2. This activity will probe students into thinking about how a large deletion and substitution due to CRISPR activity can result in loss in gene function. Having the students refer back to the images of the butterfly wings from Part 3 will allow them to make the connections between genotype and phenotypes.

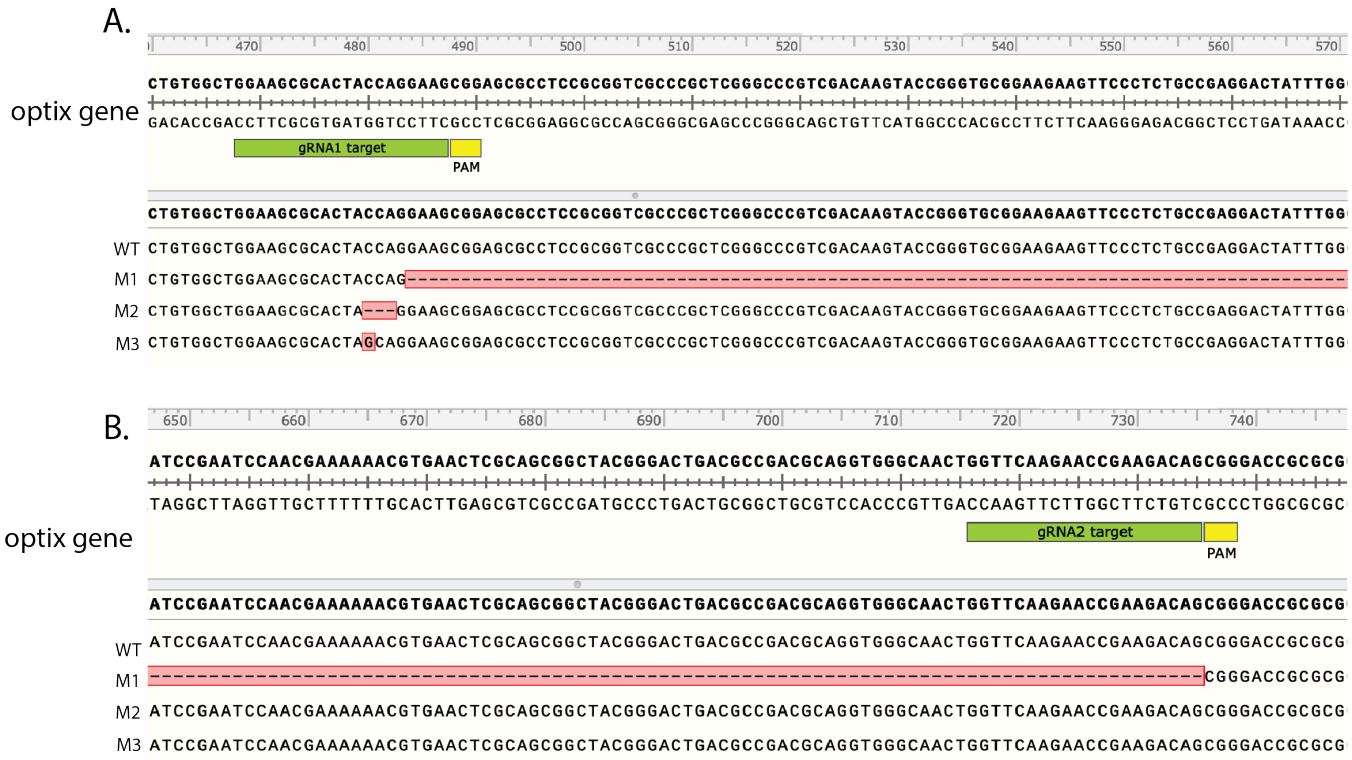


Figure 2. DNA sequence of alignment of wild-type and CRISPR mutants. Visualization of the sequence alignment of PCR-amplified *optix* gene from wild-type and CRISPR mutants. gRNA1 and gRNA2 target sites are labeled in green in the reference *optix* gene sequence that was created in Part 1. Dashed lines indicate deleted nucleotides and substitution of nucleotide is also indicated in red.

Table 2. CRISPR modifications in *optix* gene and result on protein length

Reaction	Notes
WT (wild-type)	Wild-type is identical to the reference. Wild-type has 274 amino acids
M1 (mutant 1) – the ~200bp DNA band	252 nucleotides have been deleted, resulting in loss of 84 amino acids in the middle of the protein.
M2 (mutant 2)	Deletion of CCA at the gRNA1 target site, which leads to formation of a TAG codon (early stop codon), and a truncated protein.
M3 (mutant 3)	Substitution of a C to a G at gRNA1 target site, which leads to early stop codon, and truncated protein.

Student Outline

Part 1: CRISPR Design

Background:

CRISPR is a revolutionary biotechnology tool used to either deactivate and "knockout" a gene, or make specific nucleotide changes to genes in cells. CRISPR technology is currently being used to alter DNA of specific genes in clinical trials for retinal disease and sickle cell anemia (Ledford 2020; Frangoul et al. 2021). Specifically, scientists are use CRISPR to knockout a specific gene or create nucleotides changes that will allow individuals who are affected with these diseases to be able to see (for retinal disease) and not suffer from pain associated with red blood cells not functioning properly to carry oxygen (for sickle cell disease).

Scientists are also using CRISPR technology to knockout different butterfly genes to study the function of these genes during the development of a butterfly. When scientists deactivate a gene in an organism, they look to see what phenotypic effect it has on the organism.

In today's lab, you will learn about the various components of CRISPR technology, and how CRISPR tools can be designed to target a gene of interest in painted lady butterflies called *Vanessa cardui.* Currently, scientists are studying how butterflies develop their wing color and pattern (Mazo-Vargas et al. 2017; Zhang et al. 2017). They can now use CRISPR technology to deactivate specific genes and determine which genes are responsible for butterfly wing color and pattern.

CRISPR has two main components that form a complex together (Figure 1):

1) an endonuclease **enzyme** called **cas9** that has the ability to cut DNA and make double-stranded cuts and

2) an RNA molecule called **guideRNA** (**gRNA**), which has 20 nucleotides of complementarity to the desired cas9 cut site in the gene of interest and a universal linker nucleotide sequence that binds **cas9** and allows the enzyme to target the desired location in the gene.

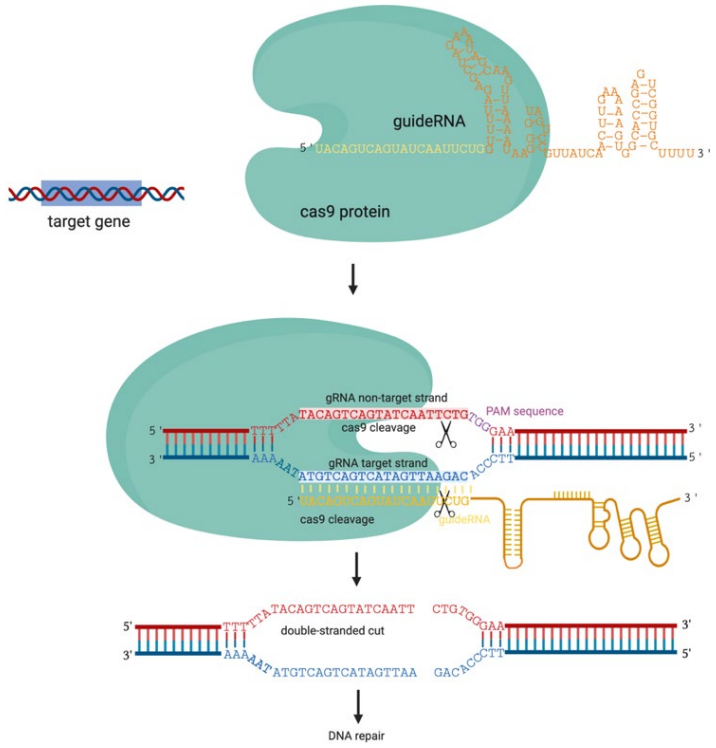


Figure 1. Structure of cas9 enzyme with bound gRNA and target DNA sequence.

cas9 forms a complex with gRNA. 20 nucleotides of the gRNA (in yellow) anneals to the complementary DNA strand. PAM sequence (in purple) aids cas9 binding. cas9 has two nuclease domains and cleaves 3-4 nucleotides from the PAM sequence on both DNA strands, leading to double-stranded break and the cell to activate a DNA repair mechanism. This figure is a modified version of a figure from Pieczynski and Kee, 2021.

After the cas9/gRNA complex targets and cuts at the desired location within DNA, the cell's DNA repair mechanism are activated and try to repair the lesion, as shown below (Figure 2). If changes to the DNA sequence are desired, the user provides a "repair template" DNA molecule containing the desired nucleotide changes. Providing a repair template triggers a double stranded DNA repair mechanism called Homology-Directed Repair (HDR). If a gene knockout is desired, the researcher simply omits the repair template. This will trigger Non-Homologous End-Joining (NHEJ), which essentially just stitches the ends of the DNA together, often resulting in insertion, deletion, frameshift or substitution mutations that lead to a "gene knockout".

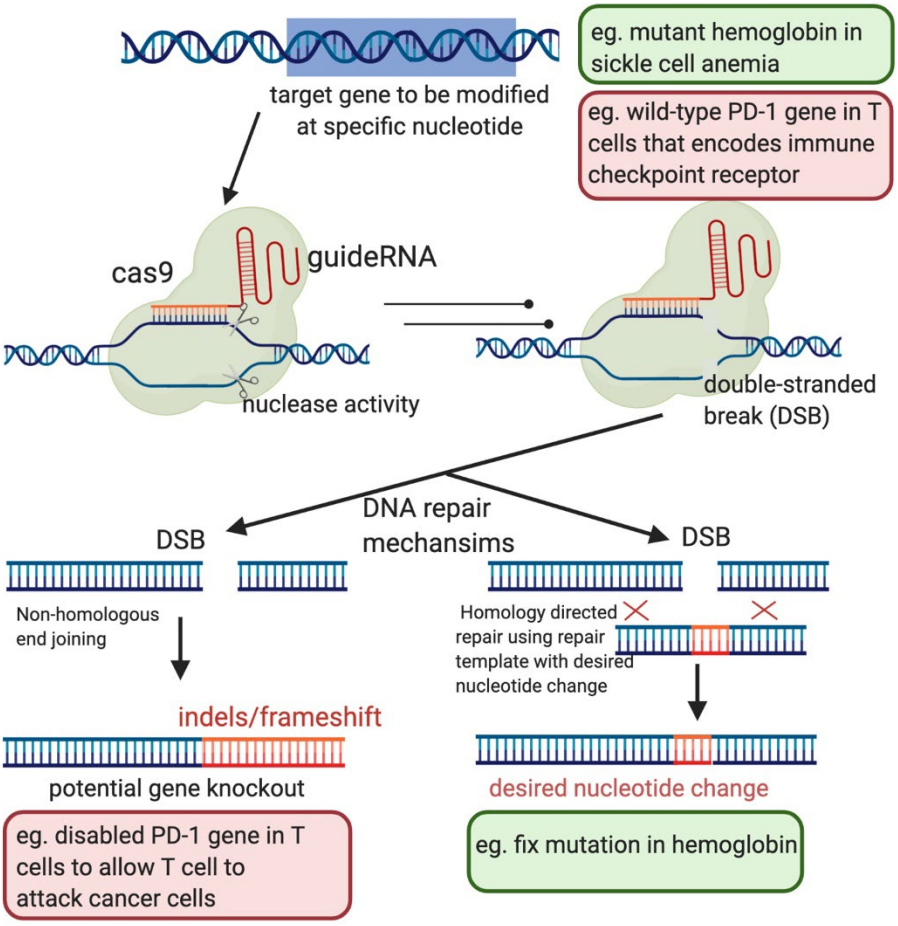


Figure 2. **CRISPR gene editing mechanism**. After gRNA/cas9 complex is targeted to target gene and creates a double-stranded break (DSB), one of two DNA repair mechanism is utilized.

Today we will first design two gRNAs to the target butterfly gene *optix*. Our strategy is schematized as shown below. By targeting the gene at two different locations, we can use CRISPR to delete about 200 base pairs or create insertion/deletions/substitutions at either of the gRNA target sites.

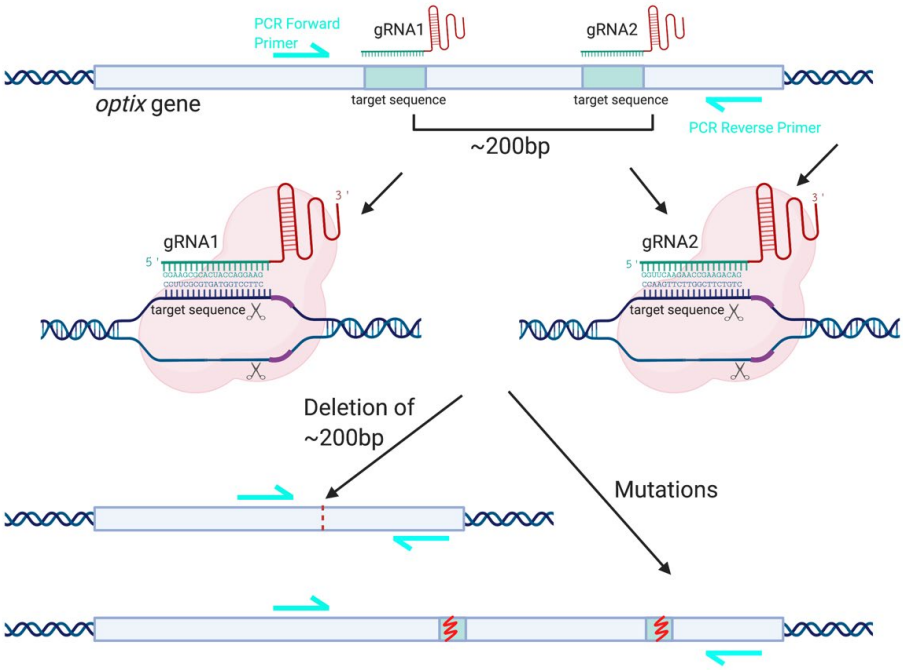


Figure 3. CRISPR strategy in *Vanessa cardui* painted ladies. CRISPR targeting with two gRNA/cas9 complexes. DNA repair can lead to a deletion of 200 base pairs between gRNA target sites or insertions or deletions at each target site. This figure is a modified version of a figure from Thulluru et al, 2022.

Student learning outcomes:

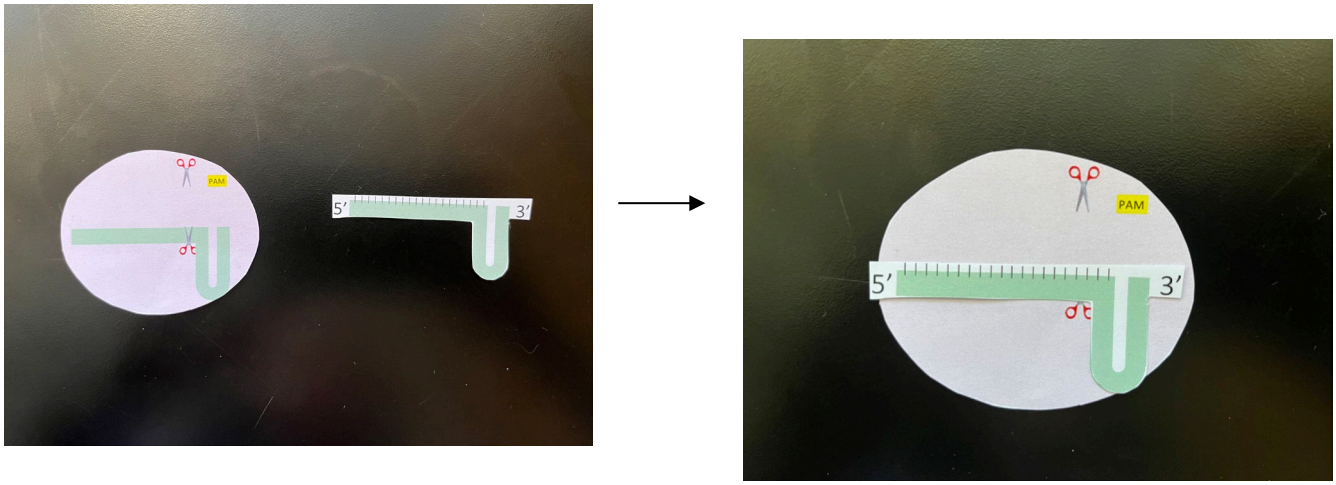
- use paper model to model CRISPR targeting with gRNA and cas9
- design two CRISPR target sequences in butterfly *optix* gene
- design PCR primers to amplify sequence in *optix* gene
- use SnapGene DNA software to annotate these features

Part A Instructions:

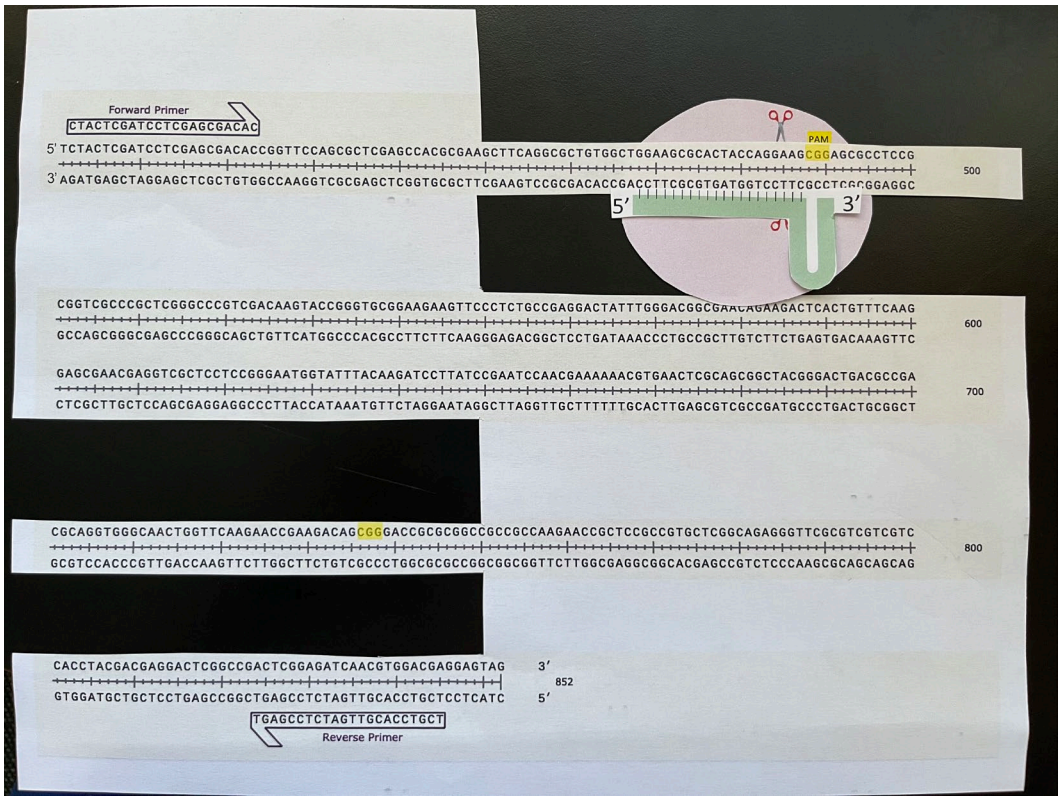
You are modeling what happens in a cell when CRISPR tools, the gRNAs and cas9, are delivered into butterfly cells. We will use a paper model to see what will happen in the cells of the butterfly egg.

What you have in front of you is a sheet of paper that shows the sequence of our gene of interest and a paper model of two guideRNA molecules and cas9 enzyme.

1. Each gRNA will bind to cas9 enzyme to form a complex. Let's model this by placing the gRNA (green) on the cas9 enzyme (pink), in the gRNA binding site (green matching shape in the pink circle).
Note: Normally, the gRNA (in green) will be labeled with the specific nucleotide sequence that has been designed to program and find its target DNA. However, here we have not labeled yet, because you will be designing and labeling it with the correct nucleotides. The gRNA has a specific structure that enables cas9 to bind to it.



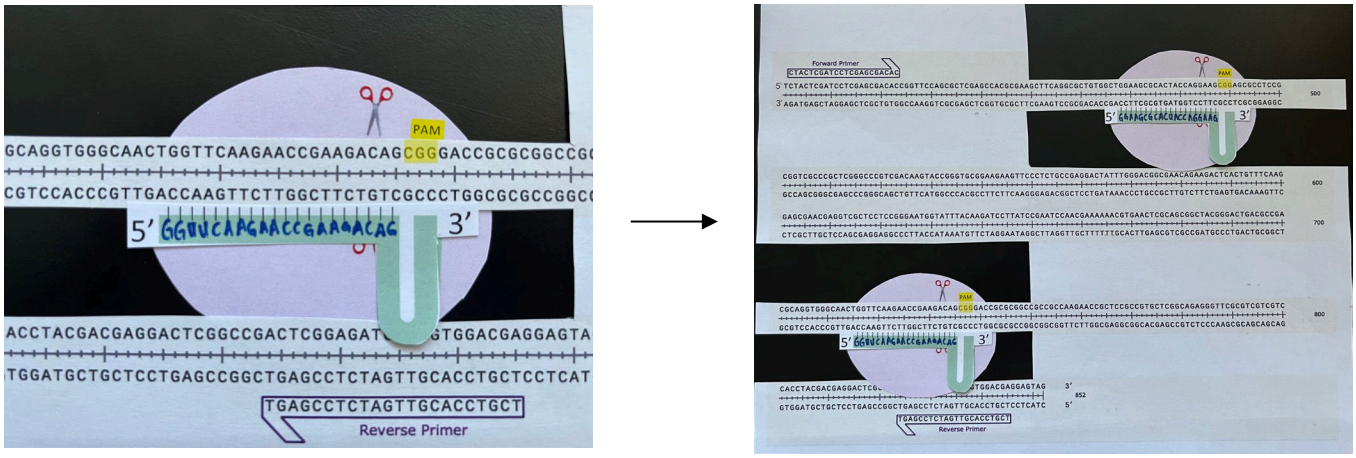
2. The gRNA/cas9 complex is now ready and programmed to scan the DNA for the complementary sequence to the gRNA and the appropriate PAM sequence that is adjacent to the target sequence. Place your gRNA as shown below, where the PAM binding site (in yellow) is on top of the CGG (PAM site) highlighted in yellow in the top DNA strand.



3. Now that gRNA has bound to PAM, cas9 will use its helicase enzymatic activity to unwind the double-stranded and determine if the gRNA sequence is complementary to the target DNA sequence adjacent to the PAM. In the gRNA molecule, write the complementary sequence to the bottom DNA strand. For example, the bottom strand above the gRNA molecule reads 3'CCAA…., therefore in your gRNA molecule you would write 5'GGUU….

If the gRNA sequence is not complementary to the DNA, then the double-stranded DNA will be zipped up and cas9 will look for another PAM and target DNA sequence.

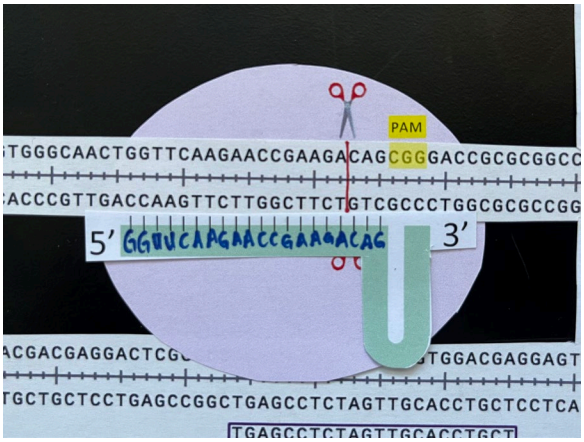
4. You have a second gRNA, gRNA2. Model gRNA and cas9 forming a complex and then binding to the appropriate target DNA sequence. The second PAM sequence is highlighted in yellow.



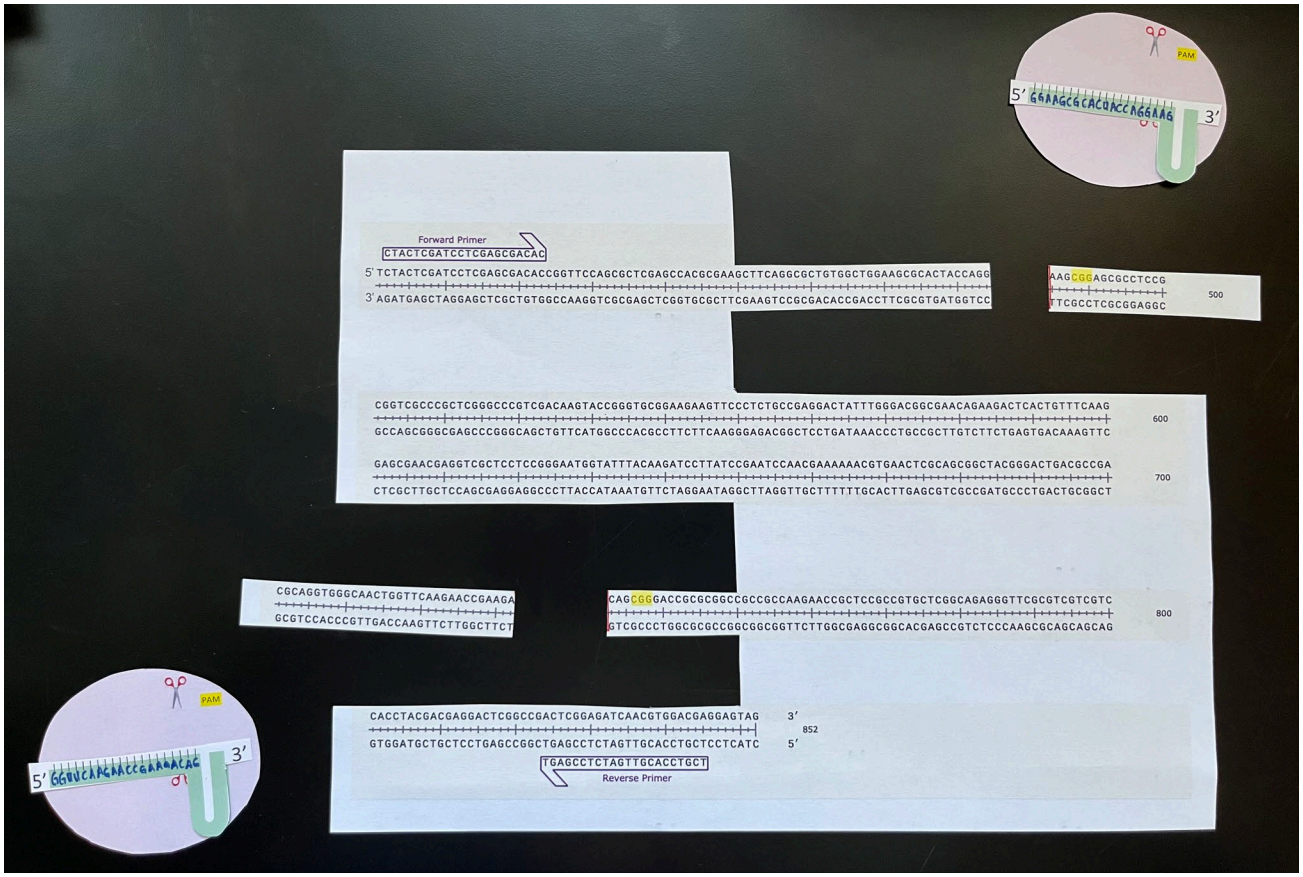
5. Now that both gRNAs are bound to the DNA, the nuclease activity of cas9 will be activated. The cas9 enzyme will cut about three nucleotides from the PAM sequence. Notice how there are two scissors in your cas9 paper model? This is to show where the enzyme will cut both DNA strands, top and bottom. Using a pair of scissors, model this cut by cutting three nucleotides from the PAM sequence where the scissors lines up with the DNA. Be sure not to cut the gRNA molecule.

gRNA 1:

gRNA2:



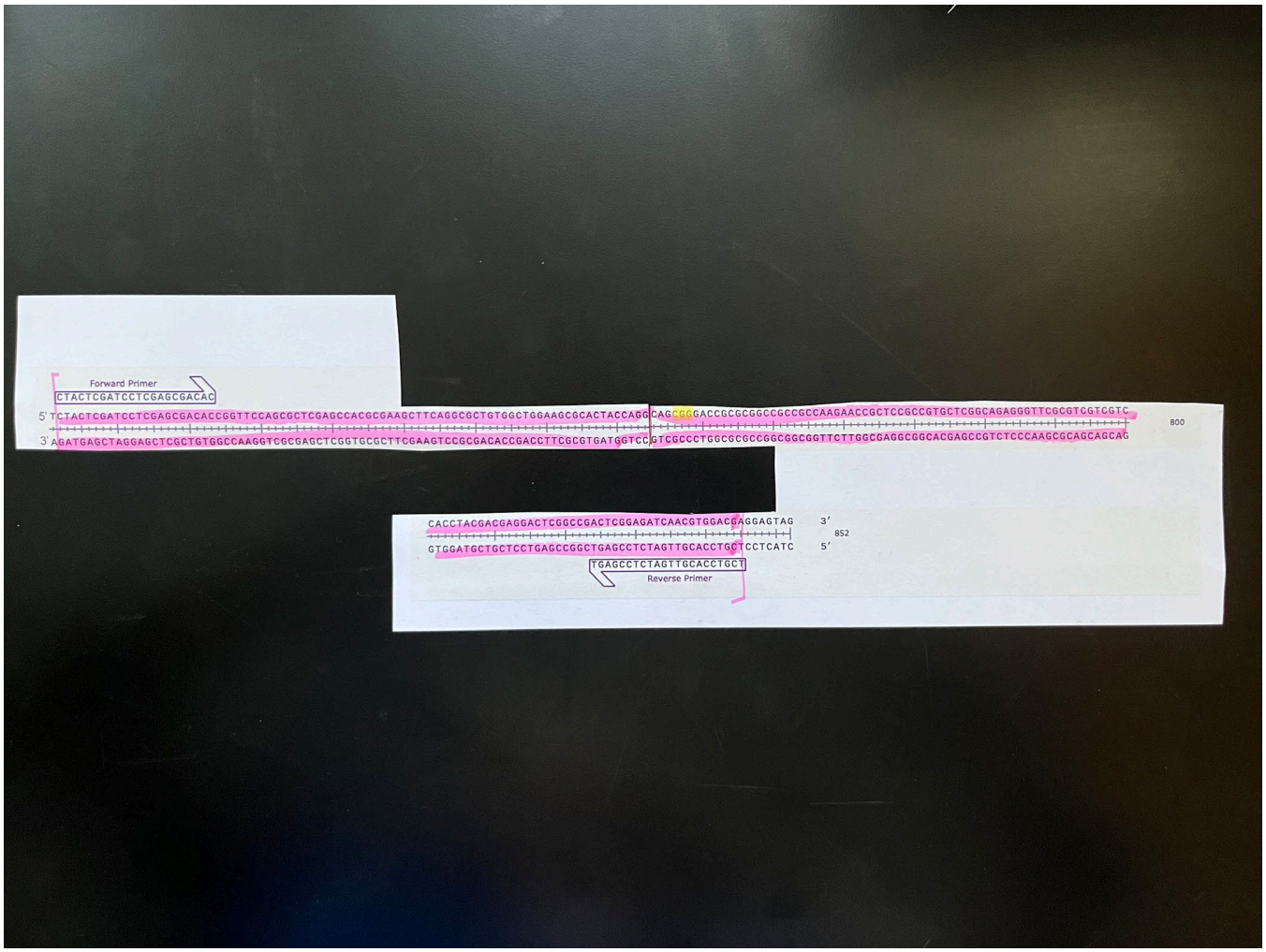
Double-stranded cuts in the gene after both gRNA/cas9 complexes cut.



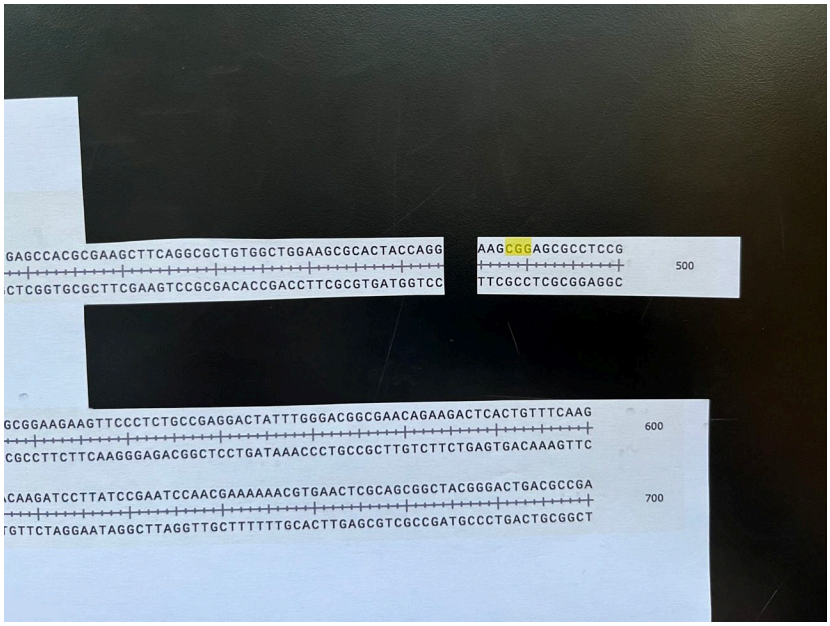
6. After cas9 cuts the DNA, there are enzymes in the cell that will try to repair the DNA double-stranded cut. This is how CRISPR technology takes advantage of the cell's ability to repair the DNA to make changes to the target DNA sequence.

If both gRNA/cas9 complexes cut at the two target sites then DNA sequence between the two DNA cuts will be lost and the flanking two ends of the DNA (highlighted in pink below) will be joined together by enzymes.

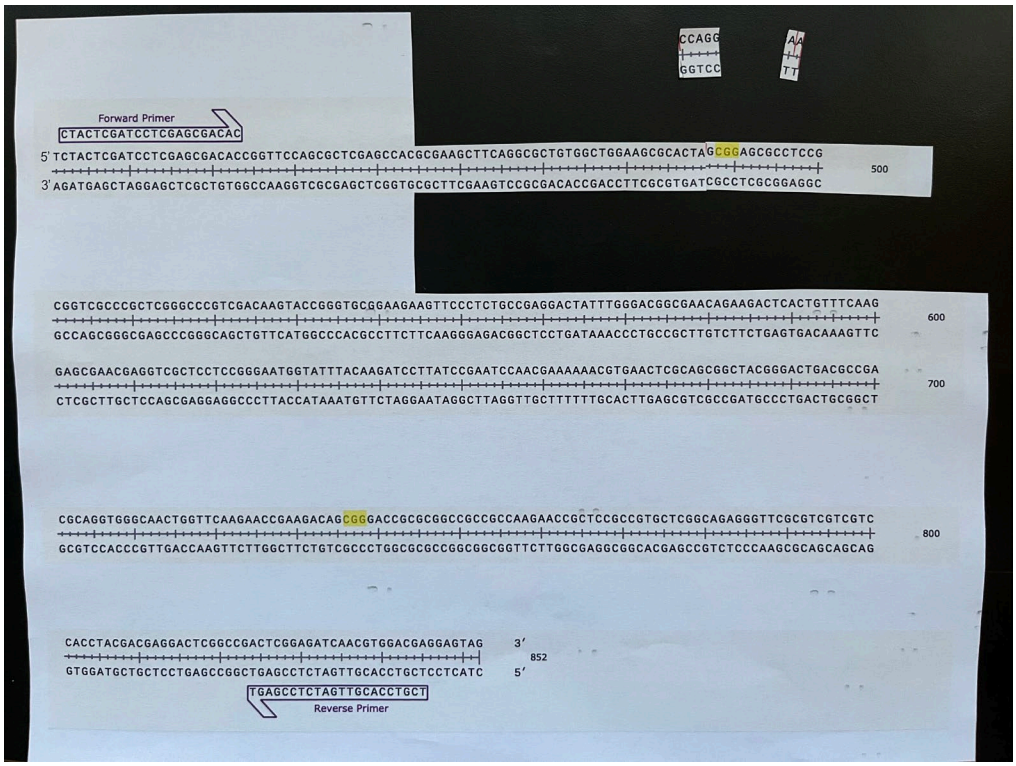
About how many nucleotides will be deleted if this happened? _____



7. If only one gRNA/cas9 targets its target sequence, what will occur? Using a second piece of paper that contains the *optix* gene, let's model this. Using your scissors, you will have a double-stranded DNA cut at the target site 1.



The cell will use a DNA repair process called non-homologous end joining to repair the DNA cut. Cellular enzymes can come in and, at the DNA break, will remove a few nucleotides and then stitch the DNA back together. Using a pair of scissors, cut a few nucleotides out and then join the DNA back together. How does the DNA sequence for the gene change due to CRISPR and DNA repair activity?



Part B Instructions:

Before proceeding to the first step, you must have SnapGene, the DNA software, downloaded onto your computer. Your instructor has provided the DNA sequence of optix for you as a .dna file.

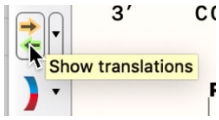
Gene analysis of the butterfly optix gene

1. Open the file Vc_optix in SnapGene.

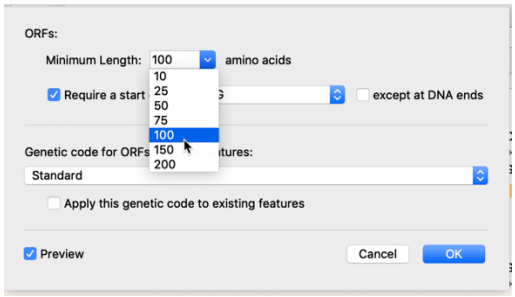
2. You will see a double-stranded DNA sequence, with the 5' and 3' end labeled. Note that nucleotide numbers are on the side.

3. You will see some icons on the left side, unclick the first one that looks like this:

4. To see the full coding sequence of the *optix* gene (from the Start codon until the Stop codon), click on "Show Translation" button, which looks like this, the 4th icon from the top on the left.



This will bring up a window that looks like this:



5. Change the minimum length to 150, click OK.

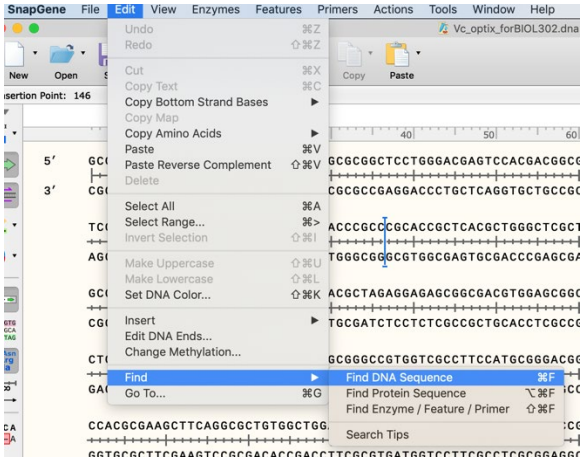
6. Now you should see the translated amino acid sequence for optix going from nucleotide 28 to 852. If you hover over the stop codon at the end of the sequence you will see a yellow box like this, which tells you how many amino acids the protein is.

CRISPR design:

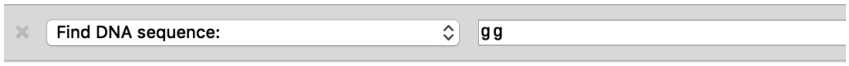
1. The CRISPR complex (gRNA/cas9) will bind to its target sequence primarily through two interactions:

1) the cas9 recognizes a three-nucleotide sequence called PAM (proto-spacer adjacent motif). PAM has the specific sequence 5'-NGG-3', where N is any DNA base (C, G, T, A). There are many PAM sequences in every gene. Let's see how this is possible with our sequence.
2) a gRNA molecule that binds to the complementary DNA.

2. Let's see what potential PAM sequences our gene has.
In your toolbar, click "Edit", then click "Find", "Find DNA sequence", like this:



3. In the bottom you will see "Find DNA sequence" box, type GG, and all the GGs will be highlighted in yellow



How many GGs are present? _____

4. In addition to cas9 finding an appropriate PAM sequence, the gRNA molecule has 20 nucleotides that will base pair with the target DNA. Cas9 has the ability to unwind the double-stranded DNA and allow for the gRNA to bind with the complementary DNA. This complementary DNA is actually NEXT to the PAM sequence.

5. Let's find the first target sequence.

Under find DNA, find the following sequence using the Find tool as done in step 3:

`5′ GGAAGCGCACTACCAGGAAG 3′` (copy the nucleotides only and not the 5' and 3')

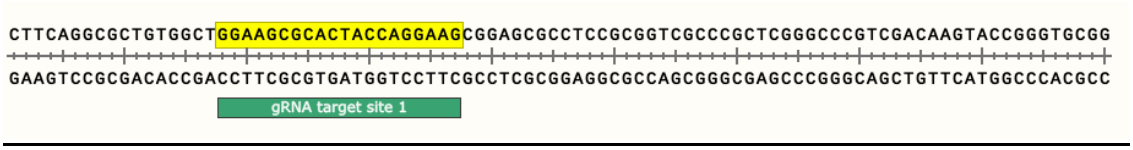
The sequence will be highlighted.

6. Let's label this sequence that gRNA will bind to.
Go to the toolbar, click Features, Add Features, and write "gRNA target 1".
You can change the color as well by clicking the color icon and changing it to your desired color.

7. The gRNA that will bind this sequence will have the sequence:

5' -GGAAGCGCACUACCAGGAAGNNNNNNNNNNNNNNNNNN-3' (where N is other nucleotides that form the hairpin shown in the schematic above)

Looking at this gRNA sequence and your DNA sequence that you just labeled, which DNA strand, top or bottom, would this RNA be complementary to? Remember that the top strand goes 5' (left) to 3' (right), and bottom strand goes 3' (left) to 5' (right).



If you said that the gRNA would base pair with the bottom strand, you are correct! Remember that in RNA we have Us instead of Ts as in DNA, and that U can base pair with A.

To help, below write the DNA sequence that is complementary to the gRNA sequence below, and write the 3' and 5' of the DNA strand

5' -GGAAGCGCACUACCAGGAAGNNNNNNNNNNNNNNNNNN-3'

8. Now look at the DNA sequence on your computer, is there a PAM (5'-NGG-3') sequence next to the gRNA target site?

9. Let's highlight it and make a new feature to label this. To do this highlight the three nucleotides:

click Features, "Add Features", and label it "PAM sequence".

It should look like this:

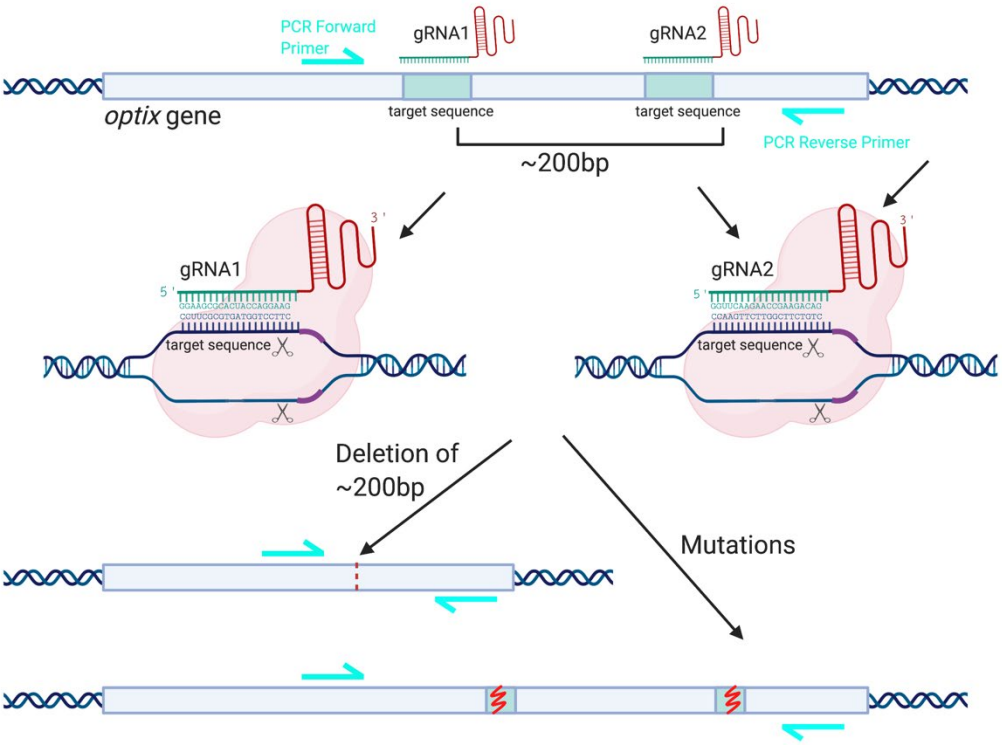
10. After gRNA binds to its target sequence and cas9 is bound to the PAM, the nuclease (DNA cutting) activity of Cas9 can now cut BOTH DNA strands. Cas9 typically cuts 3 nucleotides from the PAM sequence in the target sequence.

Below, draw a vertical line between the nucleotide 484 (G) and 485 (A).

11. Do steps 5-11 for a second target site.

5′ GGTTCAAGAACCGAAGACAG 3′

12. Now once the DNA is cut in the cell, DNA repair mechanisms will come in and try to repair the DNA cuts. Since we have tried to cut the DNA in two different locations, we will be attempting to have the cell delete the nucleotides between the two DNA cut sites as shown below. Another possible outcome is that mutations occur at each individual gRNA target site during the DNA repair process.



13. In looking at your two gRNA target sites, how many base pairs are between the two DNA cut sites?

_____

Primer Design:

1. You will conducting molecular biology experiments to characterize CRISPR mutants in the lab. The technique we will use is **PCR (Polymerase Chain Reaction)**, which is a technique used to make lots of copies of, or amplify, specific DNA sequences. There are several key components needed for a PCR reaction to work successfully and amplify your DNA sequence of interest. One of the components is a pair of DNA primers that are designed to the specific ends of the sequence we want to make copies of. Before we do PCR in lab, we will need to label our PCR primers in our gene. We will discuss in detail how PCR works in the next part.

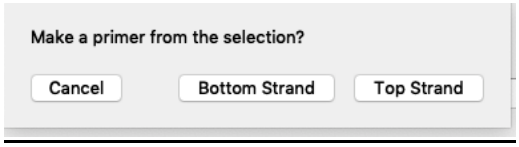
2. The two primer sequences are:

Primer OptixFW: `5′-CTACTCGATCCTCGAGCGACAC-3′`

Primer OptixRev: `5′-TCGTCCACGTTGATCTCCGAGT-3′`

Using the Find sequence tool, find the Forward (OptixFW) sequence, this will highlight the primer sequence.

Go to “Primers” in the toolbar, “Add Primer”, a window will pop up that says:



Click Top strand, and then re-label it as Primer “OptixFW” and it should look like this:

Do the same with reverse primer, but click “Bottom strand” in the window and label it as “OptixREV”.

PCR is a way to make lots of copies of DNA, specifically the DNA between the two primers in this experiment. By making lots of copies of this region, we can sequence our DNA.

If you highlight the nucleotides from the beginning of the forward primer to the end of the reverse primer, how long is this DNA sequence that we will PCR?

By highlighting these nucleotides, you will see in the top left corner: _____bp

3. Save the file to your computer as V_cardui_optix_wild-type. We will use this file for Part 3 of the lab.

Part 2: Molecular Analysis of Wild-type and CRISPR butterfly mutants.

Experimental Question: What are the nucleotide changes that result from CRISPR targeting of *optix* gene compared to control unmodified *optix* gene?

Part 2A: PCR Amplification of Wild-type and CRISPR butterfly mutants

Background:

PCR: PCR is a method to make many copies of a specific region of DNA by using primers to "bracket" the region intended to be amplified. This technique is very important for performing downstream analyses, such as determining the genotype of an organism or individual.

PCR is performed in a thermocycler. Just like the name implies, this machine cycles temperatures. The cycling of the temperatures in a PCR reaction is what allows the reaction to occur. A typical PCR reaction has 3 main steps, outlined below:

1. **Melting** – Heating the DNA to a high temperature (usually 95 degrees C). Melting causes the hydrogen bonds between complementary DNA pieces to break and the result of this is that the double strands of DNA to separate.
2. **Annealing** – Cooling the DNA to a temperature between 45 – 65 degrees C. This step is done rapidly and allows your primers to bind (or anneal) to their specific sites in the now single stranded DNA from the melting step above. Note that there is a range of temperatures that annealing occurs at. The exact temperature that primers anneal at is determined by the researcher.
3. **Extension** – Sometimes this step is called elongation. This is the step where DNA polymerase can utilize the annealed primers to start copying the DNA you would like to amplify using PCR. The extension temperature is either 68 or 72 degrees C depending on the type of DNA polymerase used.

If I perform the 3 steps of PCR once, I will be able to take 1 original piece of double stranded DNA and make 2 identical pieces. A typical PCR reaction repeats the 3 steps above between 25 and 30 times. This allows you to exponentially amplify a piece of DNA. It might not seem like much, however using this PCR technique, you can start with 1 piece of DNA and after 30 cycles end up with millions of copies of your specific target DNA.

The typical PCR reaction contains at least 5 components (and water), outlined below:

1. **Primers** – short, single stranded pieces of DNA or RNA that bind or anneal to a specific target in the genome. Primers work in pairs, one forward and one reverse, to bracket the region to be amplified during the reaction. Importantly, primers provide a place for the enzyme that copies DNA (DNA polymerase) to start from.

2. **A heat stable DNA polymerase** – DNA polymerase is the enzyme that builds DNA strands. A PCR reaction uses high heat to melt complementary DNA strands. High heat usually makes proteins like enzymes denature and become unusable. However, researchers have isolated heat stable DNA polymerases that can withstand the melting step without losing their activity.

3. **Template DNA** – DNA polymerase cannot just build DNA on its own. It needs to read a template in order to know the correct sequence of DNA to build. Primers anneal to their specific target on the template DNA and DNA polymerase uses the primers to know where to start the reaction.

4. **Free nucleotides** – these are called dNTPs for short. They are the different nucleotides (A, T, C, and G) that DNA polymerase utilizes to build the new DNA strand.

5. **Buffer** – For the PCR reaction to occur, everything must be in the right context. The buffer provides essential molecules/salts for the PCR reaction to occur.

Student Learning Objectives:

- Set up PCR reaction
- Load and run PCR products on a 1.5% agarose TAE gel
- Determine the genotype of DNA samples
- Analyze DNA sequencing samples and determine the nucleotide changes that have occurred

We will split this lab into two labs, by setting up our PCR reaction today, and then continuing the next steps in the following lab.

Part 2A Instructions for setting up your PCR reaction:

1. In your ice bucket you have the following PCR components in the table below.

We will make a **Master mix solution**, by pipetting all the components in the last column (in yellow) into one tube, except for the butterfly DNA that is unique to each reaction. This is because the three different reactions contain the same components except for the butterfly DNA, and the master mix will allow us to pipet less times and ensure each reaction has the same concentration of each component.
When making your master mix:

o Use a new pipet tip for each component

	Wild-type-WT (µl)	**Mutant 1 M1 (µl)**	**Mutant 2 M2 (µl)**	**Mutant 3**	**Master Mix X 5**
H_20	10.5	10.5	10.5	10.5	52.5
2x Master Mix (includes Taq Polymerase)	12.5	12.5	12.5	12.5	62.5
10µM Forward Primer	0.5	0.5	0.5	0.5	2.5
10µM Reverse Primer	0.5	0.5	0.5	0.5	2.5
Butterfly DNA (50ng/µL)	1	1	1	1	----------
Total volume	25	25	25	25	24 µl/tube

2. After making the master mix, use a new pipet tip and pipet up and down to mix the contents gently. Pulse-spin the tube for 5 seconds in a mini centrifuge to pull the contents to bottom of tube. Make sure to balance with another team during centrifugation.

3. Pipet 24 µl of the master mix into the three PCR tubes labeled WT, M1 and M2. These PCR tubes already contain 1µl of the butterfly DNA.

4. Give the PCR tubes to the instructor, and they will put it in the PCR machine.

5. The PCR cycle is:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	94°C	30 seconds	1
Denaturation Annealing Extension	94°C 55°C 68°C	30 seconds 30 seconds 1 minute	30
Final Extension	68°C	5 minutes	1
Hold	4°C	∞	

Part 2B: DNA gel electrophoresis of PCR products

Background:

Now that you have amplified your target gene with PCR, we will check whether your PCR was successful using a technique called **DNA gel electrophoresis**. DNA gel electrophoresis is a technique that separates DNA fragments by size and charge. Since DNA is negatively charged, it will move towards the positive charge. We can create a gel matrix that is made up of agarose, and place your DNA at the top of the agarose gel matrix, and run an electric current through the gel with the positive charge at the bottom of the gel. Larger fragments will migrate more slowly through the agarose matrix than shorter fragments, thus allowing us to separate different sized DNA fragments on the gel matrix. A dye is added that will bind to double-stranded DNA and fluoresce under specific light, thus enabling us to visualize the DNA fragments as "DNA bands" on the gel. We can compare the DNA bands to a "DNA ladder", which acts like a ruler and provides a reference for different sized DNA fragments such as 100 base pairs (bp), 200 bps, 300 bps, etc.

First you will prepare the DNA gel by microwaving agarose in a special buffer (either 1x TAE or 1x TBE, depending on the gel electrophoresis system) and adding the special fluorescent dye called GelGreen® that will bind to the DNA and fluoresce under blue light. You will pour the molten gel mixture into a gel cast with a comb, and then let the gel harden. You will prepare your PCR samples for loading onto the gel by adding appropriate amount of loading dye, which functions to add weight to your PCR samples so that it can be loaded into the gel, and also contains dye that allows us to track how far the samples have migrated on the gel.

Part 2B Instructions:

If using Owl Easy Cast (Thermofisher's) gel rigs/system:

1. Prepare a 1.5% agarose gel. Two pairs can share a gel.

- Place the gel chamber in gel rig with the 10-well comb in place
- Weigh 0.75g of agarose on a scale
- Add to flask
- Add 50mL of 1x TAE
- Add a Kimwipe™ to the top of flask
- Microwave for a minute, swirl, microwave again, until the agarose has melted completely
- Swirl for 5 minutes until solution cools slightly
- Add 5µl of GelGreen®
  - GelGreen® is a DNA intercalating agent that fluoresces upon exposure to blue/UV light.
- Pour into gel chamber
- Let gel harden

If using miniPCR's blue gel system (which uses TBE instead of TAE):

1. Prepare a 1.5% agarose gel. Two pairs can share a gel.

- Place the gel chamber in gel rig with the 9-well comb in place
- Weigh 0.45g of agarose on a scale
- Add to flask
- Add 30mL of 1x TBE
- Add a Kimwipe™ to the top of flask

- Microwave for a minute, swirl, microwave again, until the agarose has melted completely
- Swirl for 5 minutes until solution cools slightly
- Add 3µl of GelGreen®
  - GelGreen® is a DNA intercalating agent that fluoresces upon exposure to blue/UV light.
- Pour into gel chamber
- Let gel harden

2. While gel is hardening, prepare samples for running out on the gel

- Pipet 5µl of the PCR reactions into each tube
  - 5µl of WT PCR should go into tube WT
  - 5µl of M1 PCR should go into tube M1
  - 5µl of M2 PCR should go into tube M2
  - 5µl of M3 PCR should go into tube M3

- Pipet 1µl of the 6x loading dye into three tubes labeled WT, M1, M2, M3
- Pipet up and down gently to mix

3. Once gel has hardened, prepare gel for DNA loading

- Take the gel chamber out slowly
- Take comb out slowly
- Turn the gel chamber so that the side with the well is on the – (black) side and the bottom of the gel (with no wells) is by the + (red) side.
- Pour 1x TAE buffer until the gel is covered (to the “Fill line” on the gel rig).
- Using a fresh tip each time, load the following to each well:

(left)
Lane 1 – 10µl DNA ladder

Lane 2 – Team 1: WT

Lane 3 – Team 1: M1

Lane 4 – Team 1: M2

Lane 5 – Team 1: M3

Lane 6 – 10ul DNA ladder

Lane 7 – Team 2: WT

Lane 8 – Team 2: M1

Lane 9 – Team 2: M2

Lane 10 – Team 2: M3
(right)

4. Place the lid on the gel electrophoresis rig, connect the electrical leads to the power supply, red to red and black to black.

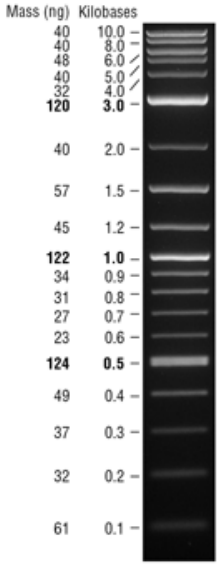
5. Turn on the power and run for 100V (constant volts) for 45 minutes. While the gel is running, go to **Part 3.**

6. When electrophoresis is complete, turn off power and remove the lid from the chamber. Very carefully remove the gel with gel tray from the chamber. Be careful as the gel is very slippery.

7. View the gel under the UV/blue light source. Take a photo with your phone. Your instructor will image the gel for you using a gel imager and upload the digital file online.

8. When you look at your gel, what do you see? Each line is what we call a DNA band, and it represents the length of double-stranded DNA, which in our experiment is our PCR product (called PCR amplicons). We use a “Ladder”, which is like a ruler to determine what size (length) our PCR products are.

9. The 1kb plus ladder we use looks like this:



Notice how the 0.5kb, which is the same as 500bp is more intense than the 0.4kb (400bp) band.

Compare your DNA bands to the ladder.

10. In the table below write down what size DNA fragments you see. In the right column, write your observations.

Reaction	Size	Observations
WT (wild-type)		
M1 (mutant 1)		
M2 (mutant 2)		
M3 (mutant 3)		

11. Is the WT DNA band the size we predicted to see (from Part 1 of the lab)?

12. For M1 – why do you think you see a DNA band that differs from the length of WT sample?

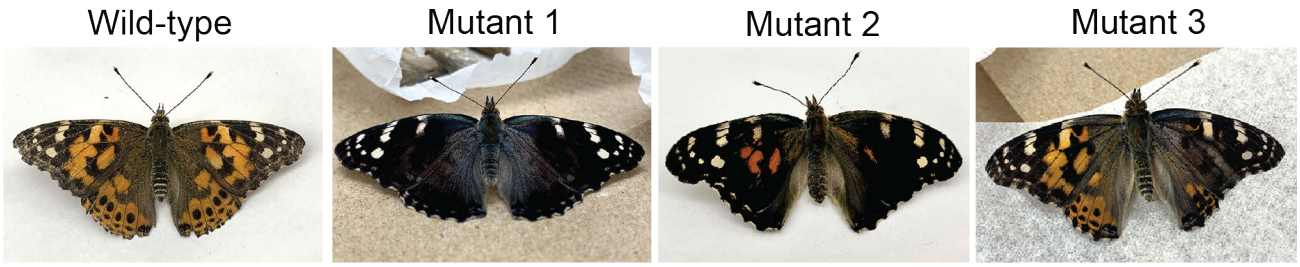
13. For M2 and M3 – why do you think you see this size DNA band? What is a possible explanation of what happened after CRISPR targeting and DNA cuts in the *optix* gene?

Part 3: Phenotypic Analysis of CRISPR mutants compared to wild-type.

Student Learning Outcome:

Experimental Question: What are the phenotypic changes that result from CRISPR targeting of *optix* gene in a painted lady butterfly? What do the changes tell us about the function of optix in a normal butterfly?

1. Below are images of the Wild-type and CRISPR mutant butterfly wings. You are analyzing the DNA from these butterflies in Parts 2 and 4.



2. Describe below how the butterfly wing colors in wild-type differ from CRISPR butterfly mutants.

3. What do you think is the function of optix in butterflies? Explain why you think so.

4. The three CRISPR butterfly knockout mutants look different from each other. Why do you think they look different?

Part 4: DNA sequence analysis of CRISPR mutants compared to Wildtype

The PCR products were sent for DNA sequencing by your instructor. Today we will analyze the DNA sequences of the wild-type and CRISPR knockout mutants. Your instructor has provided you the DNA sequencing files.

Experimental Question: What are the nucleotide changes that result from CRISPR targeting of *optix* gene compared to control unmodified or wild type *optix* gene? How do these nucleotide changes affect the optix protein?

Student Learning Objectives:

- align sequencing data with the reference sequence
- determine nucleotide changes that have resulted from CRISPR targeting
- predict how this would affect optix gene function in butterfly wing color

Instructions:

1. Open Snapgene on your computer.

2. Open the File.

3. We will align our DNA sequences from WT, M1, M2 and M3 to this reference sequence.

To align:

- Click Tools
- Align to Reference Sequence
- Choose the 4 files that you have downloaded

You will see on your screen your window split into two windows.
In the top window you see the reference *optix* gene. In the bottom window you will see the alignment of the other sequences. Look at each aligned sequence individually by clicking or unclicking the sequence in the “align with” box on the top-left corner.

4. You can use your mouse to scroll the sequences from left to right, or right to left.

Look to see where there are nucleotide differences. Any difference you see will be highlighted in Red. ***Do you see any nucleotide differences between:***

- ***WT and reference (original sequence)?***
- ***M1 and reference?***
- ***M2 and reference?***
- ***M3 and reference?***

a. How many amino acids long is the wild-type optix protein? _____

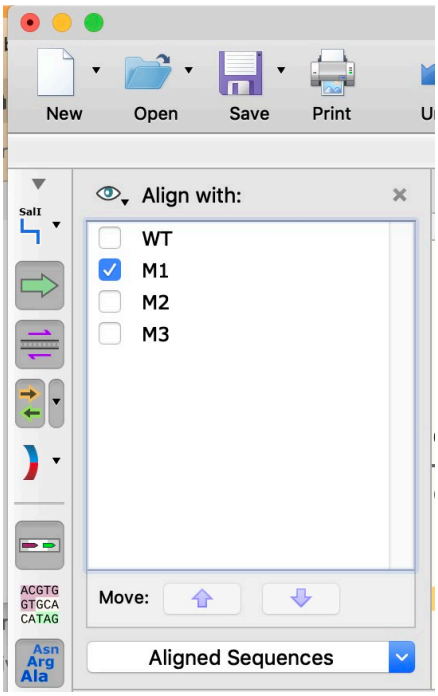
b. Which sequence shows deletion of nucleotides? _____

c. How does the deletion affect the protein length?_____

c. Which sequence shows a substitution mutation?_____

d. How does the substitution affect the encoded protein?_____

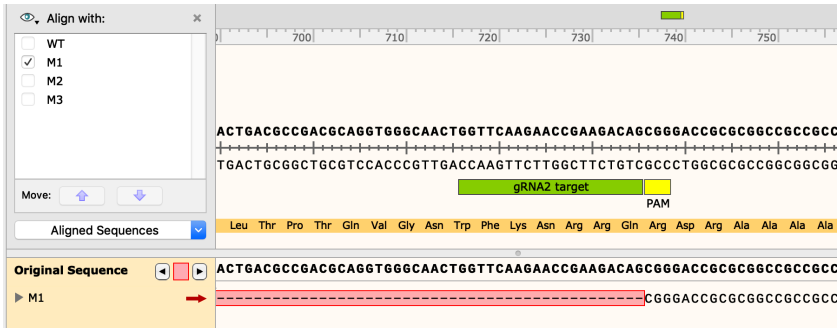
5. You can look at each mutant individually compared to the reference sequence. If you go to the top left corner you can click it such that only M1 is clicked.



For M1:

In the bottom window, you should see an alignment that looks like this for M1:

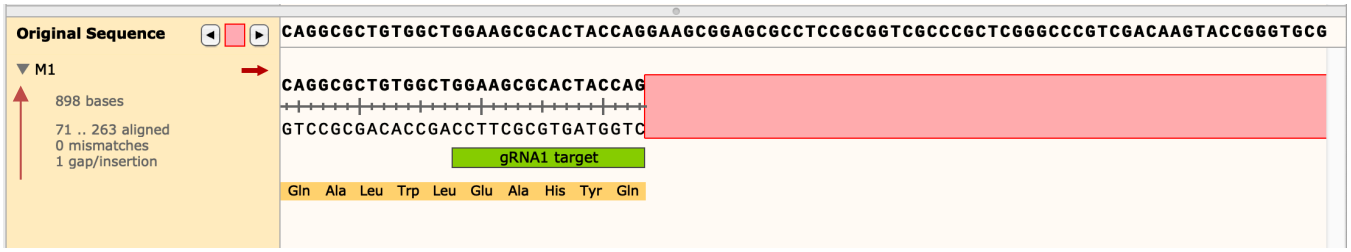
(keep scrolling to the right to see how it looks by gRNA target site 2)



Dashed red lines indicated nucleotides that are missing in M1.

Where is the deletion in relation to the CRISPR gRNA target sites? _____

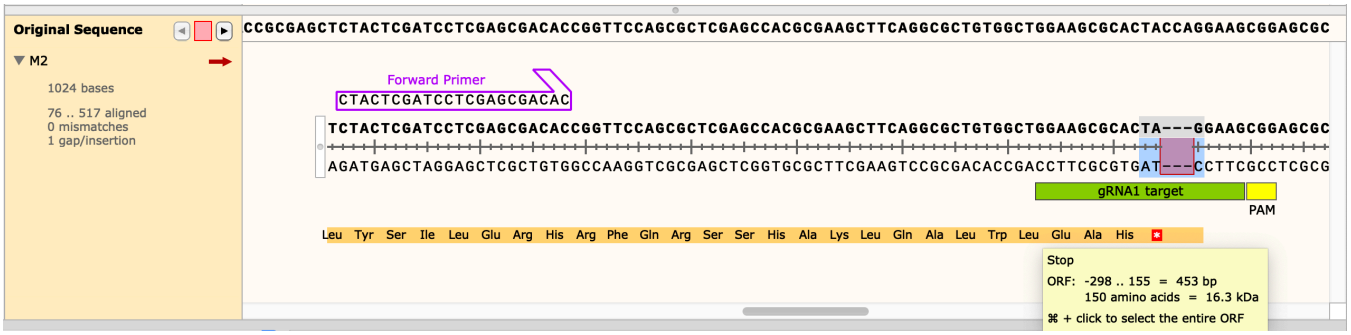
6. If you click on the gray triangle to the left of the sequence name in the yellow box (red arrow points to the gray triangle), it will allow you to expand the nucleotide view. You should now see the two strands of the double-stranded DNA molecule, and the change in amino acid sequence.



Determine how the nucleotide changes in the mutant gene affect the reading frame and amino acid sequence.

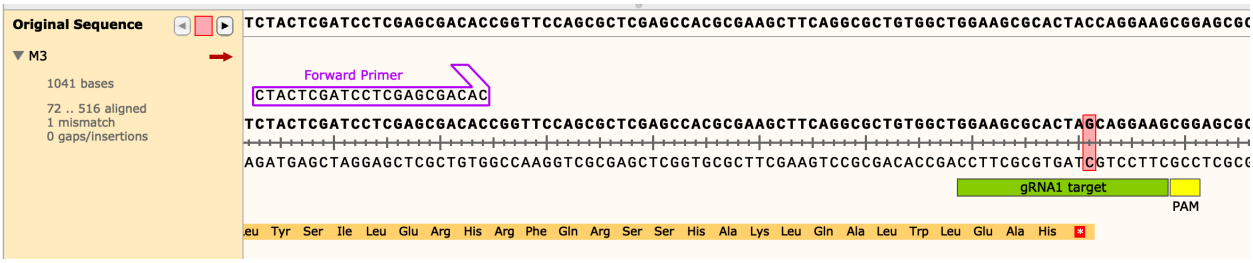
7. How do the nucleotide changes differ in M1 compared to M2? Explain why you think these changes lead to a "CRISPR gene knockout".

Tip: When you look at M2 aligned to the reference sequence you should see the following. What do you think the red asterisks indicates? Hovering over the red asterisks will allow you to see the length of the protein in the yellow box that pops up.

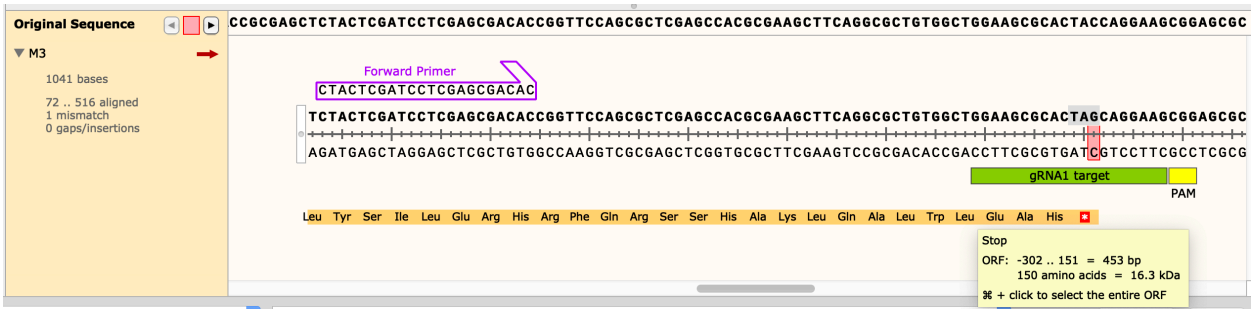


8. How do the nucleotide changes differ in M3 compared to M2 and M1? Explain why you think these changes lead to a “CRISPR gene knockout”.

Tip: When you look at M3 aligned to the reference sequence you should see the following. What do you see is highlighted in red?



If you hover your mouse over the asterisks, what do you see?



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Materials

List of Equipment and Tools Needed for Class of 20 students

- laptop/computer with SnapGene software https://www.snapgene.com

  - Students can download a full version with a free trial 30-day version at: https://www.snapgene.com/free-trial
  - Students can also download a free version called Snapgene Viewer that does not have all the features of the full version, for example, the free version does not allow for DNA sequence alignment (Part 3)
  - Instructor can purchase a 1 year license and Snapgene will provide free student licenses for the semester for the course

- PCR primers for *optix* gene, from IDT (10nm)

  Primer OptixFW: 5'-CTACTCGATCCTCGAGCGACAC-3'

  Primer OptixRev: 5'-TCGTCCACGTTGATCTCCGAGT-3'

  - When received from IDT, make 100µM stocks with nuclease-free water and dilute to 10µM for lab. What you receive is lyophilized primer, which you will dilute to 100µM (microMolar), and then from there making a working stock of 10µM. So for example, if you get a primer that on the tube says is 25.6nmoles, then add 256µl of water to get 100µM stock (multiple the number of nmoles by 10 and that is the volume of microliter/µl to get 100µM (microMolar) concentration. To make a 10µM stock for PCR, take for example 10µl of 100µM and 90µl of water (1:10 dilution).

- Taq DNA polymerase kit from NEB (OneTaq Hot Start 2x Master Mix with Standard Buffer, Cat number M0484S)

- Butterfly DNA – contact Lynn Kee for the DNA

- 1x TAE or 1x TBE (5L)

- agarose (10g)

- 100bp or 1kb or 1kb plus DNA ladder

- flasks for melting agarose (10)

- DNA gel electrophoresis rigs with at least 10 wells

  - 10 rigs, where two teams can run in 1 gel
  - 20 rigs, if each pair runs their own gel

- PCR thermocycler machine

- GelGreen® (to stain DNA in gel)

- UV/blue light light source to view DNA gel
*Note, we use miniPCR's blue gel system that utilizes agarose/TBE/GelGreen® and a blue light box that is fitted under the gel box, allowing students to visualize gel as it is running.

Notes for the Instructor

This lab was developed to scaffold in multiple exercises that promote student understanding and application of CRISPR technology in butterflies. We first build the foundational knowledge of how CRISPR works. In our experience, using the paper model first is key in demonstrating to students how the CRISPR components, gRNA and cas9 enzyme, are used to target the gene of interest at a specific location or locations. In previous labs where CRISPR was used, we noticed students struggled with conceptualizing how the gRNA/cas9 complex targets the gene of interest when we just used the computer software Snapgene to annotate the gRNA binding site and PAM sequence. As a result, we developed a paper model specific for the *optix* gene that incorporates key steps of gRNA design, polarity, complementary base pairing, nuclease activity and DNA repair. Following the paper model, the SnapGene exercise is a follow-up exercise that allows the students to reinforce what they just learned and visualize the gene and target sequences on the computer. Annotation of the key sequences and creation of potential CRISPR mutant sequences provides a foundation for the actual wet-lab component that follows. Additionally, annotating the PCR primers on the gene sequence before performing PCR helps to prepare the students for the PCR that follows. Students can predict PCR product sizes using the annotated PCR primers and gene sequence. They also can visualize which DNA sequence (top or bottom strand) the PCR primers must anneal to for a successful PCR to work.

Following the dry-lab exercises of Part 1, students set up the PCR with “purified butterfly DNA” in Part 2. To be able to do this lab, we cloned the butterfly *optix* gene sequences (WT and CRISPR mutants) into pGEM-T-easy vector. This is so that labs without the ability and resources to do the actual CRISPR delivery and rearing of CRISPR caterpillars and butterflies can still conduct molecular analysis on WT and CRISPR-d *optix* gene. The plasmid can be easily transformed into bacteria and selected for with Ampicillin antibiotics and a colony picked and grown in liquid culture, followed by a mini-prep to purify the plasmid for use in the class. Although in this lab, the students will not deliver the CRISPR tools into the butterfly eggs through microinjection, instructors can still show students the process of rearing butterflies and obtaining butterfly eggs. This is done by setting up butterfly cages with a set of 30 butterflies, by either rearing caterpillars or obtaining chrysalis that will hatch into butterflies. Butterfly eggs can be collected on mallow leaves after 3-4 days after butterfly emergence. After 4 days, butterfly eggs will hatch into caterpillar hatchlings, and students can collect the caterpillar hatchlings and conduct PCR on them to amplify wild-type *optix* gene. If this additional lab activity is to be done, then the Thermofisher Phire Kit should be used with the Dilution Buffer/DNA release to lyse the caterpillar to obtain caterpillar lysate with genomic DNA for use as the template DNA for the PCR reaction.

Together Part 2 and 4 allow students to visualize the effect of CRISPR targeting on the butterfly *optix* gene. We particularly like using SnapGene to analyze sequencing data. There are other ways to visualize alignment of sequencing data, but the overall layout of SnapGene is laid out well for undergraduates to comprehend. The top window shows the reference sequence that was annotated by the students, with the features for gRNA1 target site, gRNA 2 target site, PAM sequences and primers labeled. The bottom window shows the aligned sequences for wild-type and the mutants. Students can also see the effect the deletions and substitutions have on the reading frame and the amino acids coded by the codons.

The phenotypic analysis of the *optix* CRISPR mutants compared to wild-type is from images of wings generated by undergraduate students who have actually conducted the full CRISPR experiments in lab with microinjection of CRISPR into the butterfly eggs and rearing progeny to visualize phenotypic effects. The phenotype is striking, and students are usually in awe of the phenotypes produced by CRISPR modification. This phenotype allows students to make the connection between genotype and phenotype relationships. By discussing how a loss-of-function of a gene can lead to a specific phenotype, the instructor can encourage students to predict what the function of the normal gene is in butterfly color development.

This lab usually leads to the discussion of bioethical considerations in using CRISPR technology in animals, humans and agriculture. The instructor can leverage student interest and have student research specific topics of interest that use CRISPR technology and have debates or student presentations in follow-up classes.

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About the Authors

H. Lynn Kee has been an Associate Professor at Stetson University since 2017, where she teaches Introductory Biology, Genetics, and Molecular Biology & Biotechnology. She was recently awarded an NSF Improving Undergraduate STEM Education (IUSE) grant to develop and implement curricula focused on CRISPR technology in the undergraduate classroom and lab for biology majors and non-majors.

Appendix A

Instructor Key for Student Outline

Part 1 – Part A

Question 6.
About how many nucleotides will be deleted if this happened? _____248 bp_____

Question 7.

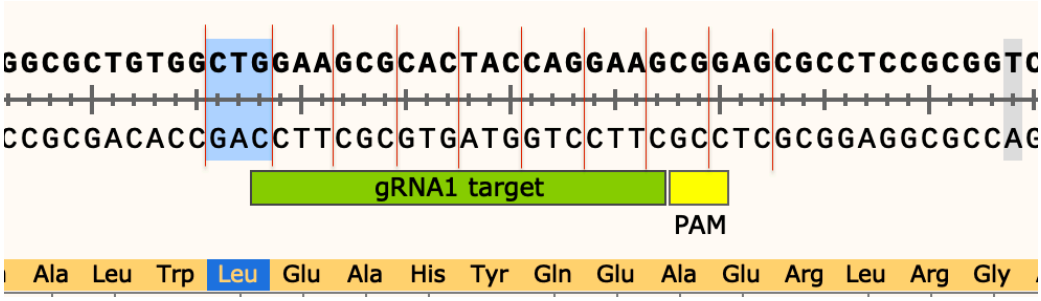
Students answers may vary depending on how many nucleotides they cut out.

Most students will say they have deleted several nucleotides. If 1, 2, 4, or 5 nucleotides are deleted, then a frameshift mutation will have occurred. If 3 nucleotides are deleted, then an in frame deletion of one codon may have occurred.

Have students compare with each other what they have created.

If you wanted to go deeper with students, and they have understand the concept of codons and reading frames, you can give them the reading frame (where to draw the lines between each codon) and a codon chart so that they can determine effect on amino acid sequence between wild-type sequence and CRISPR modified sequence.

Below you can see where the reading frame is based on the red lines.

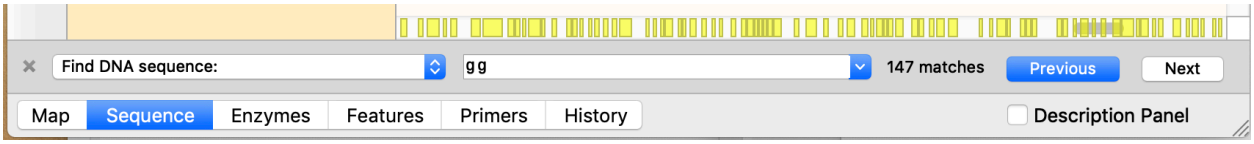


Part 1 - Part B

CRISPR design:

3. In the bottom you will see “Find DNA sequence” box, type GG, and all the GG’s will be highlighted in yellow

How many GG’s are present? _____147_____



11. Do steps 5-11 for a second target site.

5’ GGTTCAAGAACCGAAGACAG 3’

gRNA sequence is:

5’ -GGUUCAAGAACCGAAGACAGNNNNNNNNNNNNNNNNNNNN -3’

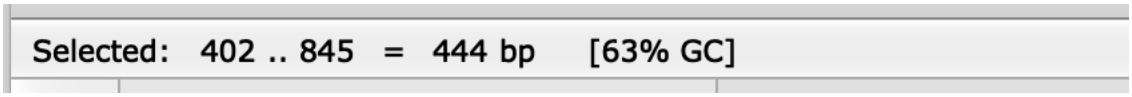
13. In looking at your two gRNA target sites, how many base pairs are between the two DNA cut sites?
__248bp_____

Primer Design:

If you highlight the nucleotides from the beginning of the forward primer to the end of the reverse primer, how long is this DNA sequence that we will PCR?

444bp

By highlighting these nucleotides, you will see in the top left corner : _444bp__bp



Part 2B: DNA gel electrophoresis of PCR products

10. In the table below write down what size DNA fragments you see. In the right column, write your observations.

Reaction	Size	Observations
WT (wild-type)	444bp (~400 bp)	
M1 (mutant 1)	192bp (~200bp)	About 200 bp smaller than wild-type PCR product
M2 (mutant 2)	441bp (~400bp)	Looks similar to wild-type PCR product
M3 (mutant 3)	444bp (~400bp)	Looks similar to wild-type PCR product

11. Is the WT DNA band the size we predicted to see (from Part 1 of the lab).

Yes

12. For M1 – why do you think you see a DNA band that differs from the length of WT sample?

If both complexes of gRNA1/cas9 and gRNA2/cas9 targeted both gRNA1 and gRNA2 target sites, and cas9 cut at these sites, then the cell repaired the DNA by joining the two flanking DNA strands together, a ~200bp DNA band could have been deleted. Thus, when PCR was used to amplify the gene, we observed about a 200bp band, where the DNA sequence between the two gRNA target sites have been lost during DNA repair.

13. For M2 and M3 – why do you think you see this size DNA band? What is a possible explanation of what happened after CRISPR targeting and DNA cuts in the *optix* gene?

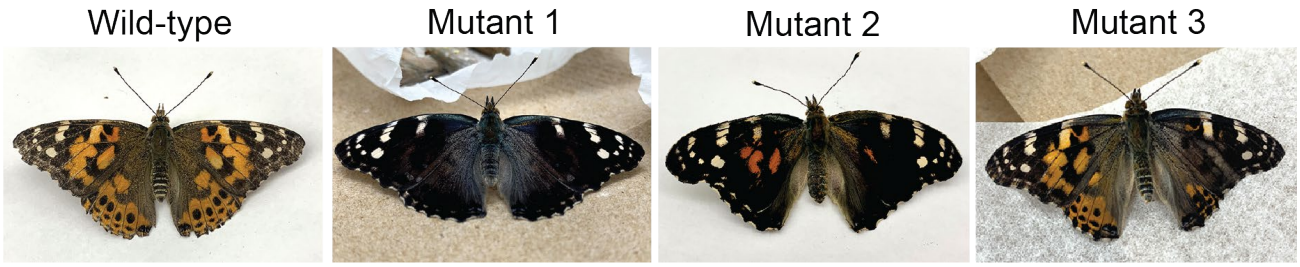
If only one gRNA/cas9 complex targeted one gRNA target site, such as just gRNA target site 1, when DNA repair occurred, a small insertion/deletion or substitution could have occurred. On a DNA agarose gel, the PCR product of this gene would appear to be similar to wild-type if the nucleotide change was very small. We cannot discern a single (or even a three) nucleotide difference on the DNA gel that we are running.

Part 3: Phenotypic Analysis of CRISPR mutants compared to wild-type.

Student Learning Outcome:

Experimental Question: What are the phenotypic changes that result from CRISPR targeting of *optix* gene in a painted lady butterfly? What do the changes tell us about the function of optix in a normal butterfly?

1. Below are images of the Wild-type and CRISPR mutant butterfly wings. You are analyzing the DNA from these butterflies in Parts 2 and 4.



2. Describe below how the butterfly wing colors in wild-type differ from CRISPR butterfly mutants.

Wild-type has patches of orange, black and white on its wing. Both wings looks similar to each other.

Mutant 1 is almost completely black. White patches look similar to wild-type.

Mutant 2 has a lot of black, with some orange and white patches. Right wing has more black than the left wing. White patches look similar to wild-type.

Mutant 3, there is more black on the right wing. The left wing looks very similar ot wild-type.

3. What do you think is the function of optix in butterflies? Explain why you think so.

Wild-type optix could either

1) function to promote orange pigmentation on the butterfly wings. This is because the loss of proper optix function in the CRISPR mutant results in the loss of orange pigmentation.

2) function to repress black pigmentation on the butterfly wings. This is because the loss of proper optix function in the CRISPR mutant results in more black pigmentation.

4. The three CRISPR butterfly knockout mutants look different from each other. Why do you think they look different?

We targeted the optix gene with CRISPR during embryonic development – butterfly eggs were injected with CRISPR (gRNA/cas9). If many cells that would become the wing were targeted and CRISPR modified, then we would see the phenotype of Mutant 1. If only some of the cells that would become the wing were targeted and CRISPR modified, then we would see phenotypes observed for Mutant 2 and 3. So depending on how many embryonic cells were CRISPR targeted and modified, we would see this variation in phenotypic changes caused by CRISPR. For example, in Mutant 3, the cells that would become the right wing were targeted and modified by CRISPR, but the cells that would become the left wing may not have been targeted and modified by CRISPR and retained wild-type optix gene.

Part 4: DNA sequence Analysis of CRISPR mutants compared to Wildtype

The PCR products were sent for DNA sequencing by your instructor. Today we will analyze the DNA sequences of the wild-type and CRISPR knockout mutants. Your instructor has provided you the DNA sequencing files.

Experimental Question: What are the nucleotide changes that result from CRISPR targeting of *optix* gene compared to control unmodified *optix* gene? How do these nucleotide changes affect the optix protein?

Student Learning Objectives:

- align sequencing data with the reference sequence
- determine nucleotide changes that have resulted from CRISPR targeting
- predict how this would affect optix gene function in butterfly wing color

Instructions:

1. Open Snapgene on your computer.

2. Open the File.

3. We will align our DNA sequences from WT, M1, M2 and M3 to this reference sequence.

To align:

- Click Tools
- Align to Reference Sequence
- Choose the 4 files that you have downloaded

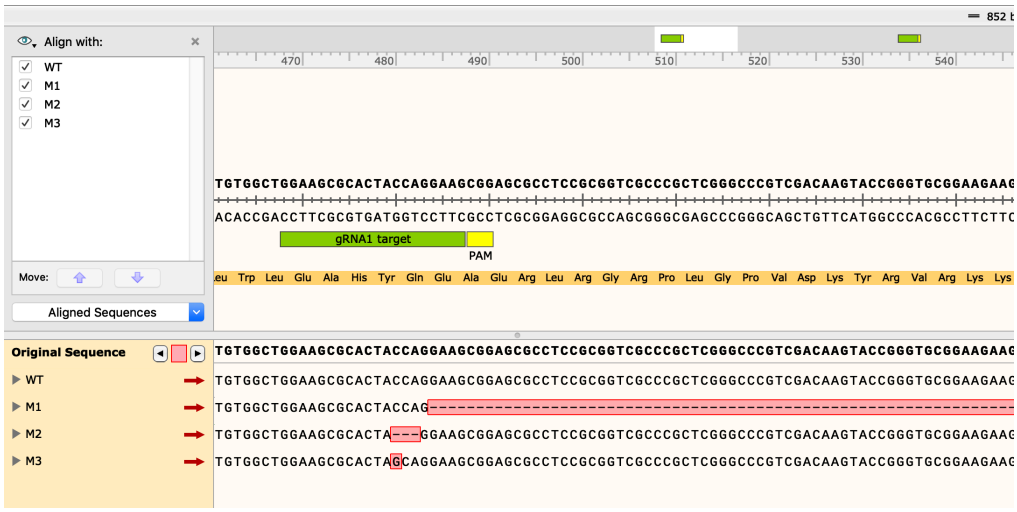
You will see on your screen your window split into two windows.
In the top window you see the reference *optix* gene. In the bottom window you will see, the alignment of the other sequences. Look at each aligned sequence individually by clicking or unclicking the sequence in the “align with” box on the top-left corner.

4. You can use your mouse to scroll the sequences from left to right, or right to left.

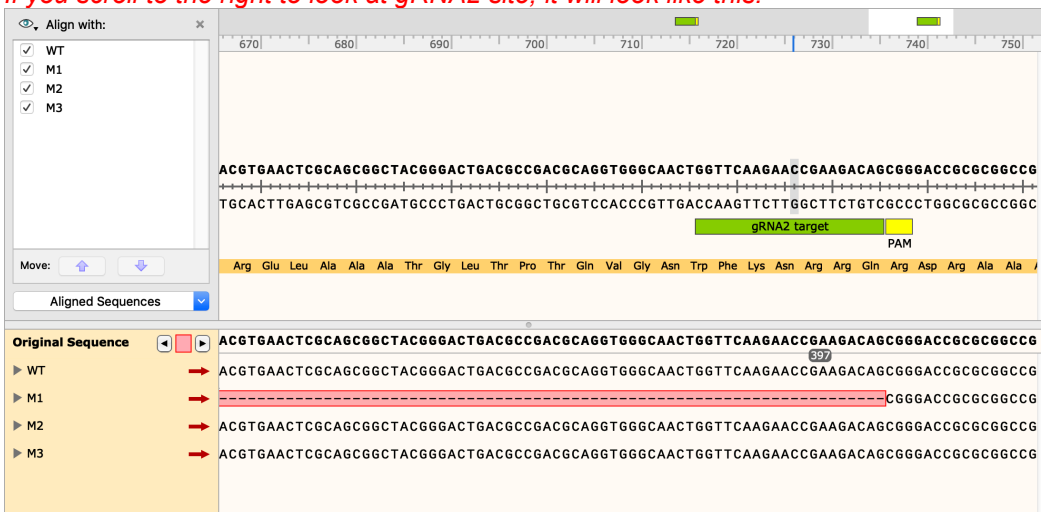
Look to see where there are nucleotide differences. Any difference you see will be highlighted in Red. ***Do you see any nucleotide differences between :***

- ***WT and reference (original sequence)?***
- ***M1 and reference?***
- ***M2 and reference?***
- ***M3 and reference?***

Students will see various changes highlighted in red. Their screen should look like this at the gRNA1 site. The bottom window shows the aligned sequences against their original reference sequence created in Part 1B of lab.



If you scroll to the right to look at gRNA2 site, it will look like this:



a. How many amino acids is wild-type optix protein? _____274 amino acids__

b. Which sequence shows deletion of nucleotides? ___Mutant 1 and Mutant 2_____

c. How does the deletion affect the protein length?_____

Deletion of nucleotides will result in shorter proteins. We will walk though this in more detail for each mutant in the next few questions.

In Mutant 1 – the protein is shorter and is now 190 amino acids long due to loss of 252 base pairs (question 5 and 6 help answer this question).

In Mutant 2 – CCA is deleted at gRNA target 1 site, which results in the creation of an early stop codon TAG, resulting in a truncated protein of length, 150 amino acids (question 7 help answer this question).

c. Which sequence shows a substitution mutation?_____

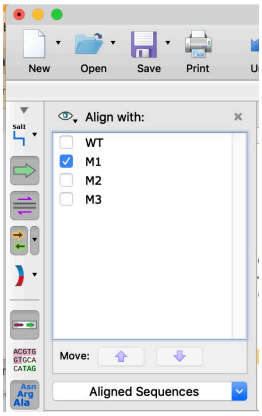
d. How does the substitution affect the encoded protein?

In Mutant 3 - a C is changed to G, which changes the codon and will affect translation and the amino acid that is supposed to be created.

This is shown in more detail in question 8.

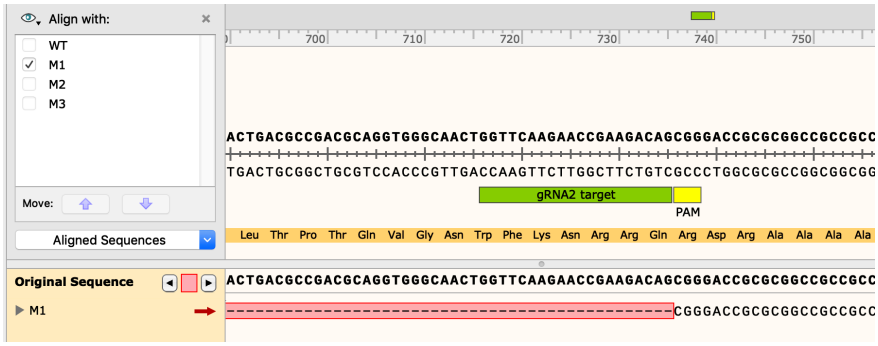
Specifically, this results in the TAC codon that codes for Tyr to be now TAG, a stop codon. This results in an early stop codon, and a truncated protein of length 150 amino acids.

5. You can look at each mutant individually compared to the reference sequence. If you go to the top left corner you can click it such that only M1 is clicked.



For M1: In the bottom window, you should see an alignment that looks like this for M1:

(keep scrolling to the right to see how it looks by gRNA target site 2)

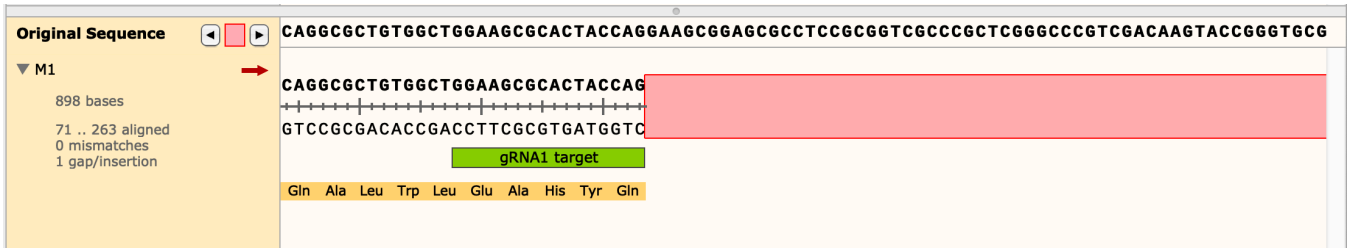


Dashed red lines indicated nucleotides that are missing in M1.

Where is the deletion in relation to the CRISPR gRNA target sites?

Deletion is between the two gRNA sites, before each of the PAM sites

6. If you click on the triangle to the left of the sequence name in the yellow box, it will allow you to expand the nucleotide view. You should now see the two strands of the double-stranded DNA molecule, and the change in amino acid sequence.



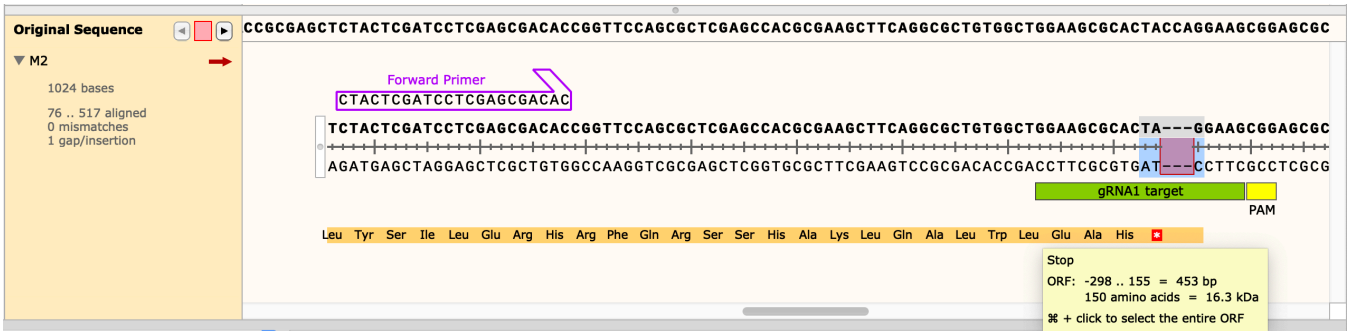
Determine how the nucleotide changes in the mutant gene affects the reading frame and amino acid sequence.

The loss of 252 base pairs results in the protein being shorter and is 190 amino acids long.

No frameshift of reading frame has occurred, as this seems to be an in-frame deletion..

7. How do the nucleotide changes differ in M1 compared to M2? Explain why you think these changes lead to a "CRISPR gene knockout".

Tip: When you look at M2 aligned to the reference sequence you should see the following. What do you think the red asterisks indicates? Hovering over the red asterisks will allow you to see the length of the protein in the yellow box that pops up.



The red asterisks indicate a stop codon in DNA resulting in no amino acid formed after His.

In Mutant 2 – CCA is deleted at gRNA target 1 site, shown with three --- dashed lines, which results in the creation of an early stop codon TAG, resulting in a truncated protein of length, 150 amino acids.

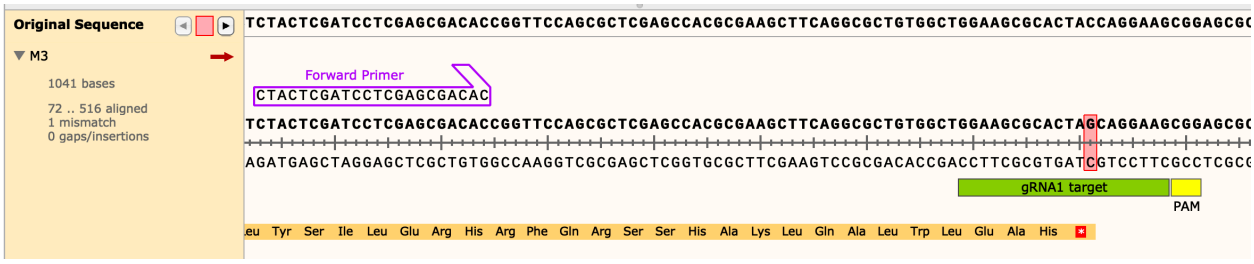
Both in Mutant 1 and Mutant 2, we create a shorter optix proteins, just in different ways, which causes the protein to not function properly. This results in a loss-of-function of the gene, which we would call a CRISPR knockout for the gene.

8. How do the nucleotide changes differ in M3 compared to M2 and M1? Explain why you think these changes lead to a “CRISPR gene knockout”.

Tip: When you look at M3 aligned to the reference sequence you should see the following. What do you see is highlighted in red?

***Click the gray triangle to the left of M3 so that you can see the double-stranded DNA and translation of the sequence*.**

You will see that a G is highlighted in red, where there used to be a C.



If you hover your mouse over the asterisks, what do you see?

In Mutant 3 - a C is changed to G, which results in the TAC codon that codes for Tyr to be now TAG, a stop codon (highlighted in gray). This results in an early stop codon, and a truncated protein of length 150 amino acids, which you can see when you hover over the red asterisks to allow the yellow box to pop up.

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