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Using Software Programs, SDM-Assist and SnapGene, to Maximize Learning Benefits of Performing Site Directed Mutagenesis while Minimizing Logistical Challenges

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Site Directed Mutagenesis (SDM) is a biological multi-step laboratory technique that introduces a point mutation in a gene, usually to mutate an individual amino acid. Implementing SDM in a Course Based Undergraduate Research Experience course comes with advantages and disadvantages. It is advantageous because it can easily accommodate research-backed educational guidelines including student ownership and decision-making while incorporating and connecting fundamental skills. For example, with SDM a student can decide the amino acid for mutagenesis. Initial steps require designing primers with one or more mismatches for the amino acid mutation as well as an additional mismatch to create/remove a restriction enzyme site in the plasmid, which can later be used to verify mutagenesis. The choice in amino acid influences which restriction enzymes sites will be available, thereby influence experimental results and create opportunities for a student to play a larger role in the process while using their "own" data. A disadvantage in using SDM is that it can be technically challenging to guide large classrooms of students through this process to arrive at their own decisions in a scientifically sound manner. For example, some amino acids are more technically challenging to mutate than others. In this workshop participants will learn how to use two freely available software programs, *SDM-assist* and *SnapGene*, to develop a student activity to maximize student decision-making while minimizing the technical challenges associated with the initial steps of this type of work. Participants will learn how each student can easily use SDM-assist to generate mutagenic primers and use SnapGene to predict the results of the mutagenesis. Participants will learn how these two programs have been incorporated in an upper-level Biology CURE. Real examples of student data will be shown and discussed.

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Keywords: Site Directed Mutagenesis (SDM), SDM-assist, SnapGene, mutagenic primers, protein structure and function

Introduction

Site Directed Mutagenesis (SDM) is a multi-step laboratory technique that introduces a point mutation in a gene, usually to mutate an individual amino acid. SDM is an ideal technique to use in an educational setting for a variety of reasons (Kowalski et al. 2016; Treacy et al. 2011). First, SDM requires multiple fundamental laboratory skills and techniques such as Polymerase Chain Reaction (PCR), bacterial transformation, and plasmid purification to name a few. Secondly, the required skills and techniques

build upon one another and allow the student to collect different types of data. Additionally, SDM affords an opportunity for a student to participate more in the decision-making process. Two areas in which a student can participate more in this process is during the experimental design stage for the Polymerase Chain Reaction (PCR) and mutagenesis.

Mutagenic Polymerase Chain Reaction

SDM is commonly done by Polymerase Chain Reaction (PCR). The Polymerase Chain Reaction (PCR) uses two different short synthetic oligonucleotides ("primers") to initiate DNA amplification. The primers are designed to bind sequence-specifically to a target region in a DNA strand. During the PCR reaction the polymerase extends from primers and they are incorporated into the PCR products. A variation of the traditional PCR is often referred to as Mutagenic PCR. Here, the primers are designed to contain one or more mismatches to the template DNA. See figure 1 below.

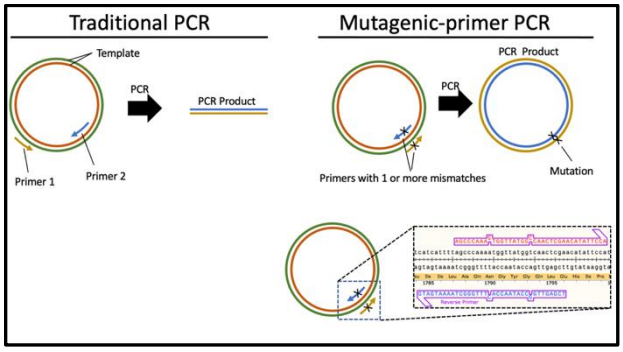


Figure 1) Overview of mutagenic PCR

Despite having one or a few mismatches against the template, the primers will still bind. Because primers are incorporated into the product, the mismatches will result in a nucleotide substitution in the amplified DNA. The mismatch is designed so that a DNA codon results in a different amino acid being incorporated into the protein (missense mutation).

Mutagenesis Verification

Following Mutagenic Primer PCR reaction, the product is transformed into competent *E. coli* cells, which are then grown on LB-agar plates for 1-2 days. After this time period, colonies should appear. In theory, the cells of each colony should contain the mutated plasmid generated in the PCR reaction but this should be verified first. One way to verify mutagenesis is through restriction enzyme digestion followed by agarose gel electrophoresis (Zhang et al. 2009). This is made possible during the primer design process. While designing primers to have a mismatch for a missense mutation, an additional mismatch can be created that will create or destroy a restriction enzyme site. In comparison to the template plasmid, the mutant plasmid will have one extra or one fewer restriction enzyme sites and this disparity can be exploited to verify mutagenesis.

Verifying mutagenesis is performed on plasmid samples purified from bacterial colonies. Here, a portion of the purified plasmid sample is used in a restriction enzyme digestion reaction. The enzyme used is the one associated with the primer design process. Alongside this reaction is a control reaction that uses the same restriction but with a nonmutated plasmid. The control and experimental reactions are subjected to agarose gel electrophoresis where a difference in banding pattern indicates a successful mutagenesis. A difference in the banding pattern is usually observed with a different number of bands and/or band sizes. An example is shown in figure 2

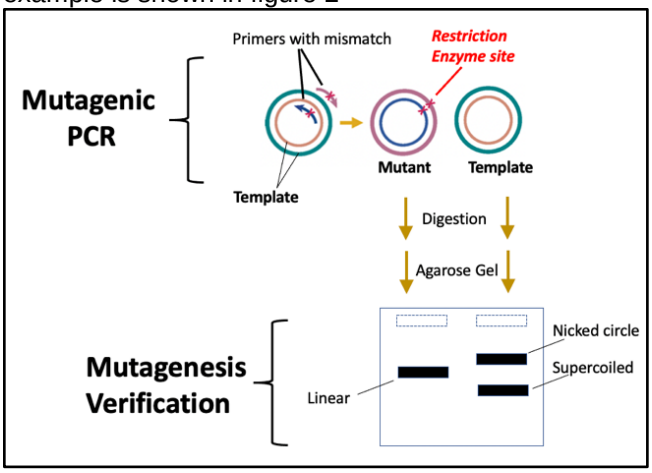


Figure 2) Mutagenesis verification. Mismatches in the primers are incorporated in the PCR product. A silent mutation can create a restriction enzyme site, which can be observed in an agarose gel.

Suppose a non-mutated plasmid (the template DNA that is used in a PCR reaction) does not contain a restriction site for the restriction enzyme *BssHII* and that the mutagenic primers contained a mismatch to create one restriction site for *BssHII*. The product of the PCR reaction will be a plasmid with one restriction site for *BssHII* (the template does not have a site). If the mutated plasmid and template plasmid were subjected to a digestion reaction using *BssHII*, the mutated plasmid will be cut once and the template will not. The results of these digestion reactions can be observed in an agarose gel with a different banding pattern.

PCR and Mutagenesis Verification

The SDM steps of PCR and mutagenesis verification have multiples steps in which a student can participate in the decision-making process such as deciding what amino acid to mutate and what to mutate it into, and designing primers to perform the missense mutation (to create an amino acid change) and nonsense mutations (to create/remove a restriction enzyme site. However, the primer design process is a difficult task. After obtaining the DNA sequence and locating the codon for the target amino acid, the surrounding bases are analyzed for candidate sequences. A candidate sequence is one in which a restriction enzyme site can be removed or created through one or two mismatches. Given the large number of restriction enzyme sites available, this type of analysis is extraordinarily challenging.

Software programs to aid primer design

The large number of commercially available restriction enzymes makes mutagenic primer design tremendously difficult. To make this process more manageable, the software programs, *SDM-assist* and *SnapGene,* can be used.

SDM-assist is a point-click software program that will analyze a DNA sequence and provide a list of potential restriction enzyme sites that could be created or removed (Karnik et al. 2013). See figure 3 for an overview.

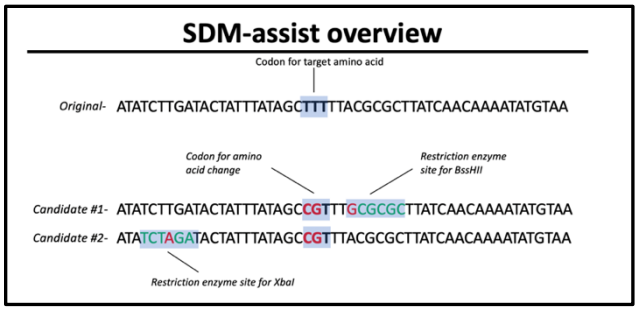


Figure 3) DNA analysis using SDM-assist. SDM-assist will analyze an input DNA sequence and generate a list of candidate DNA sequences that if replaced the original input sequence would generate a missense mutation and a silent mutation.

In essence, the user provides the original DNA sequence and identifies the DNA codon that must be changed for the desired amino acid change. In turn, SDM-assist will analyze the surrounding sequences for an additional mutation that creates or removes a restriction enzyme site. The program generates a list of candidate sequences that, if replaced the original, would mutate the amino acid and create/remove a restriction enzyme site. The user selects a candidate sequence and, in turn, the program provides a list of primers that could be used in a PCR reaction to create that particular sequence. The primers are scored on a scale of 0-100, providing an easy-to-use metric for comparison.

SnapGene

As previously mentioned, verification of PCR success (mutagenesis) is performed by digesting the PCR products with the corresponding enzyme and running the products through an agarose gel. Mutagenesis is verified if the banding pattern matches up with what is predicted. Because the product of the PCR reaction is an entire plasmid, predicting the agarose gel results requires analysis of the entire plasmid for presence of a candidate restriction enzyme suggested by SDM-assist. To do this analysis, we will use the software program, *SnapGene (from Insightful Science; available at snapgene.com.) SnapGene* is a DNA analysis software program and is freely available. We will use *SnapGene* to predict the results of a restriction enzyme digestion. The digestion reaction will be between the mutated plasmids and the corresponding restriction enzyme provided by *SDM-assist*. For example, suppose SDM-assist suggests that *BssHII* is a potential restriction enzyme site near the DNA codon for our target amino acid. Our mutant plasmid will now have this restriction enzyme site at this location. We will use *SnapGene* to analyze the rest of the plasmid for *BssHII* sites already present so as to predict the digestion results and determine if this restriction enzyme is appropriate for verification. An enzyme is appropriate if it generates a digestion pattern that unequivocally indicates the restriction enzyme site was created. See figure 4 for a flowchart.

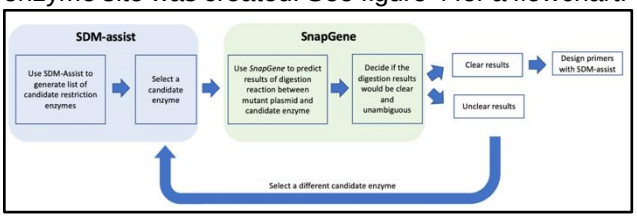


Figure 4) Overview of using SDM-assist and SnapGene to design primers. Candidate primers are generated with SDM-assist and analyzed using SnapGene to determine whether they are suitable for mutagenesis verification using restriction enzyme digestion.

Using SnapGene and SDM-assist in classroom

This activity is implemented in a laboratory classroom with 4 students per bench with a total of 6 benches. At each bench, there are two computers with both software programs available. Students work in small groups to design the primers. Generally speaking, the

majority of students finish the primer design process over the course of two 3-hour class periods. It is helpful to have a spreadsheet program (e.g., Microsoft Excel) available because the generated primer sequences can be outputted into an excel sheet where they can be formatted for ease of viewing.

Student Outline

Objectives

1. Use *SDM-assist* to design candidate primer sequences to create a missense mutation and nonsense mutation
2. Use *SnapGene* to evaluate primer sequences
3. Submit your primer sequences to your instructor

What are you doing in this activity?

You will design mutagenic primers with one or more mismatches to make the following mutations:

- A missense mutation in the Ketopantoate Reductase (KPR) enzyme to mutate the 279th amino acid from a phenylalanine to an arginine.
- A silent mutation to create a *BssHII* restriction enzyme site in the template.

How are you going to perform this activity?

In the activity, you will use two software programs, *SDM-assist* and *SnapGene.* Two DNA sequence files will be used in this activity. One file contains the sequence for the KPR gene and the other file contains the sequence for the PCR template DNA that we will use in our PCR reaction. The plasmid template contains the KPR gene. There are three major steps in this activity:

1. Use *SDM-assist* with the KPR gene to generate candidate mutagenic primers (primers with mismatches) that potentially could be used in a PCR reaction to generate a mutant plasmid containing the above mutations (see above "What are you doing in this activity")
2. Use *SnapGene* to predict the results of a restriction enzyme digestion reactions between 1) nonmutated + *BssHII* and 2) mutated plasmid + *BssHII*. You will decide whether or not there is a significant difference between the two, which will allow us to verify mutagenesis.
3. Submit your primer sequence to your instructor.

Learning objectives

By the end of this activity, a student should be familiar with the general features of the *SDM-assist* and *SnapGene* software programs and be able to use them to develop mutagenic primers to create a missense and silent mutation. A student should be able to predict the results of a restriction enzyme digestion reaction between a template plasmid molecule and mutant plasmid generated with mutagenic primers. If given a plasmid DNA sequence and the base sequences of a restriction site, a student should be able to design primers that will generate a mutant plasmid containing a new restriction enzyme site.

Introduction

The Polymerase Chain Reaction (PCR) uses two different short synthetic oligonucleotides ("primers") to initiate DNA amplification. In a traditional PCR, the primers bind to different regions of the template DNA (see figure 5, left side). The primers are synthetically designed and created to be fully complementary to a target site on opposite strands. During the PCR reaction the polymerase extends from primers and they are incorporated into the PCR products.

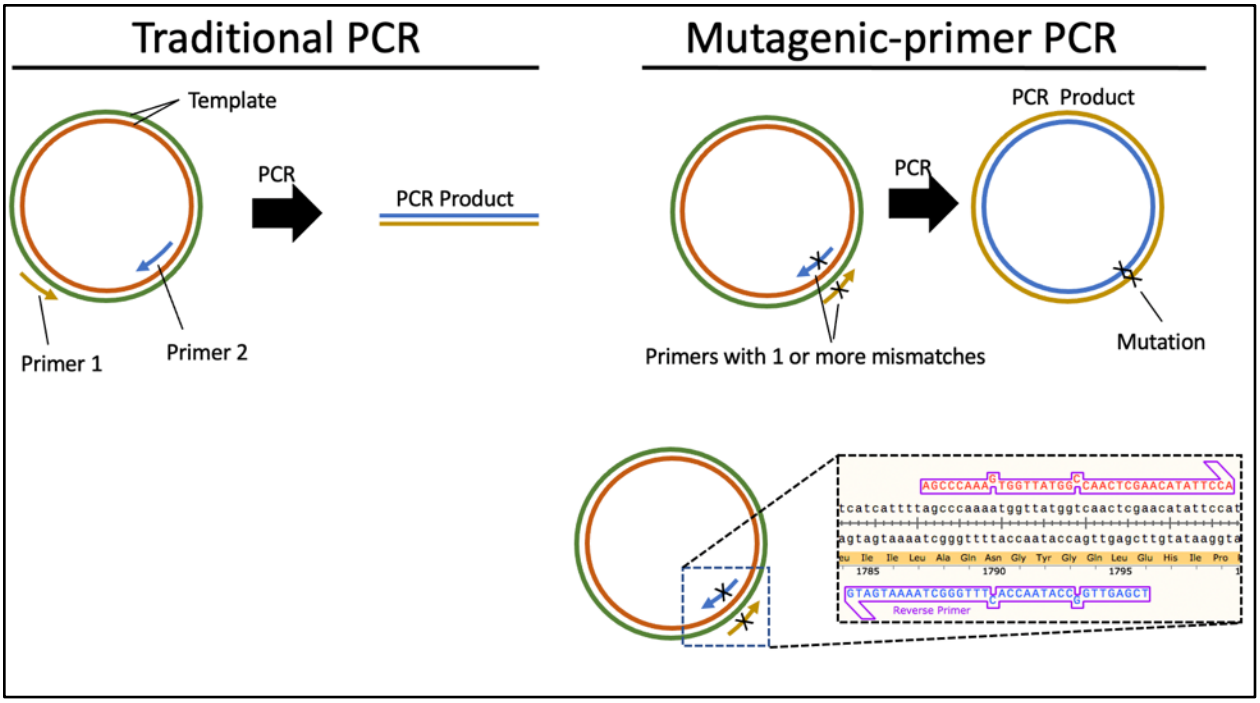


Figure 5) Overview of Mutagenic PCR. Primers contain one or more mismatches which are incorporated into the PCR product. The mismatches result in a mutation.

Variations in PCR. A variation of the traditional PCR amplifies the whole plasmid. The entire plasmid can be amplified because of primer-template binding location and direction. Primers bind to the same region of the template plasmid and point outward from each other resulting in the entire plasmid being amplified (see above image, right).

Another variation of the traditional PCR is often referred to as *Mutagenic Primer PCR* and is similar to whole plasmid PCR. A difference with whole plasmid PCR is with the primers, which contain one or more mismatches to the template DNA (see above image, right). Because primers are incorporated into the product, a mismatch will replace the corresponding base in the template and result in a mutation. This type of PCR is often used in Site-Directed Mutagenesis (SDM) which is a technique that involves mutating individual amino acids in a protein. Here, mismatches in the primers result in a nucleotide change in a DNA codon and in turn, this results in an amino acid mutation (missense mutation). Another mismatch can be incorporated to introduce a silent mutation for mutagenesis Verification (more information below).

Mutagenesis Verification. Following PCR reaction, the product is transformed into competent *E. coli* cell. The cells are then transferred onto LB-agar plates and in 1-2 days colonies should appear. In theory, the cells of each colony should contain the mutated plasmid but this should be verified. One way to verify mutagenesis is through restriction enzyme digestion followed by agarose gel electrophoresis. This is made possible during the primer design process. While designing primers to have a mismatch for an amino acid mutation, an additional mismatch can be created in the location of a restriction enzyme site. In comparison to the template plasmid, the mutant plasmid does not have that restriction enzyme site and this disparity can be verified by agarose gel electrophoresis. Alternatively, a mismatch can be added into the primers so that a restriction enzyme site is created. In comparison to the template plasmid, the mutant plasmid will have an additional restriction enzyme site, which can be verified by agarose gel electrophoresis. See figure 6 for an overview.

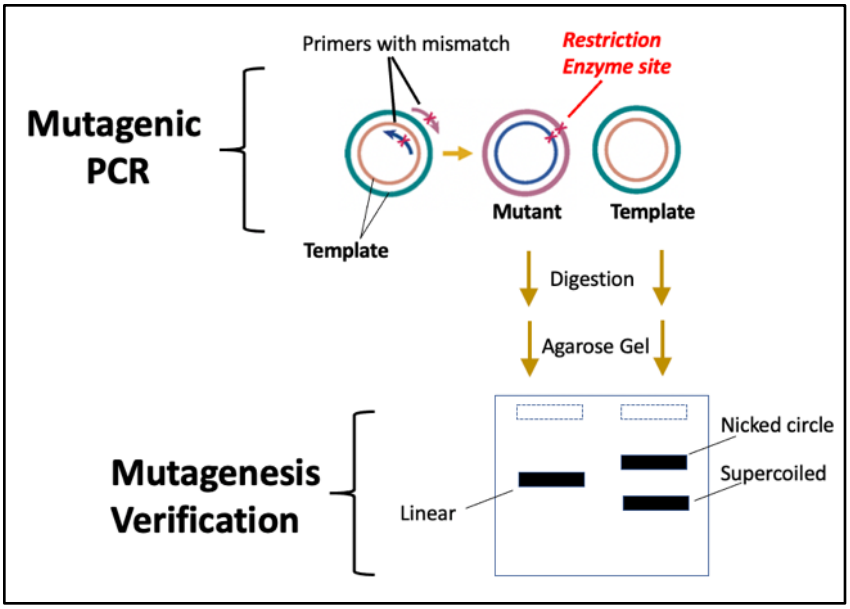


Figure 6) Mutagenesis verification using restriction enzyme digestion. Silent mutations are added to the plasmid during PCR. The silent mutations do not change an amino acid but will create or remove a restriction enzyme site.

Challenges in primer design: Designing mutagenic primers with mismatches for SDM and mutagenesis verification through restriction enzyme digestion is a difficult undertaking. After obtaining the DNA sequence and locating the codon for the target amino acid, the surrounding bases are analyzed for candidate sequences. A candidate sequence is one in which a restriction enzyme site can be removed or created through one or two mismatches. Given the large number of restriction enzyme sites available, this type of analysis is extraordinarily challenging.

Software programs to aid primer design. The large number of commercially available restriction enzymes makes mutagenic primer design tremendously difficult. To make this process more manageable, the software programs, *SDM-assist* and *SnapGene* can be used.

SDM-assist is a point-click software program that will analyze a DNA sequence and provide a list of potential restriction enzyme sites that could be created or removed. See figure 7 for an overview. In essence, the user provides the original DNA sequence and identifies the DNA codon that must be changed for the desired amino acid change. In turn, SDM-assist will analyze the surrounding sequences for an additional mutation that creates or removes a restriction enzyme site. The program generates a list of candidate sequences that, if replaced the original, would mutate the amino acid and create/remove a restriction enzyme site. The user selects a candidate sequence and, in turn, the program provides a list of primers that could be used in a PCR reaction to create that particular sequence. The primers are scored on a scale of 0-100, providing an easy-to-use metric for comparison.

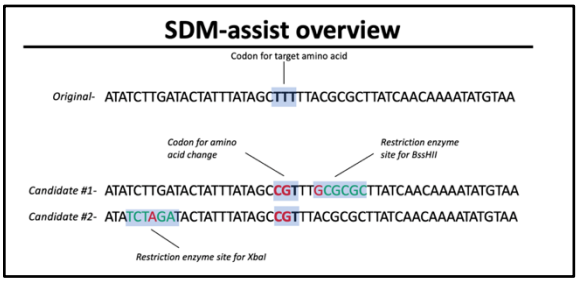


Figure 7) Overview of SDM-assist. The user inputs a gene sequence and specifies what amino acid to mutate. The program will automatically generate various candidate sequences that if replaced the original would result in an amino acid change.

Simultaneously, the program searches nearby sequences for possible restriction enzyme sites that already exist or sequences that are similar to a restriction enzyme site.

SnapGene. As previously mentioned, verification of PCR success (mutagenesis) is performed by digesting the PCR products with the corresponding enzyme and running the products through an agarose gel. Mutagenesis is verified if the banding pattern matches up with what is predicted. Because the product of our PCR reaction is an entire plasmid, predicting the agarose gel results requires analysis of the entire plasmid for presence of a candidate restriction enzyme suggested by SDM-assist. To do this analysis, we will use the software program, *SnapGene. SnapGene* is a DNA analysis software program and is freely available. We will use *SnapGene* to predict the results of a restriction enzyme digestion. The digestion reaction will be between the mutated plasmids and the corresponding restriction enzyme provided by *SDM-assist*. For example, suppose SDM-assist suggests that *BssHII* is a potential restriction enzyme site near the DNA codon for our target amino acid. Out mutant plasmid will now have this restriction enzyme site at this location. We will use *SnapGene* to analyze the rest of the plasmid for *BssHII* sites already present so as to predict the digestion results and determine if this restriction enzyme is appropriate for verification. An enzyme is appropriate if it generates a digestion pattern that unequivocally indicates the restriction enzyme site was created. See figure 8a for a flow chart.

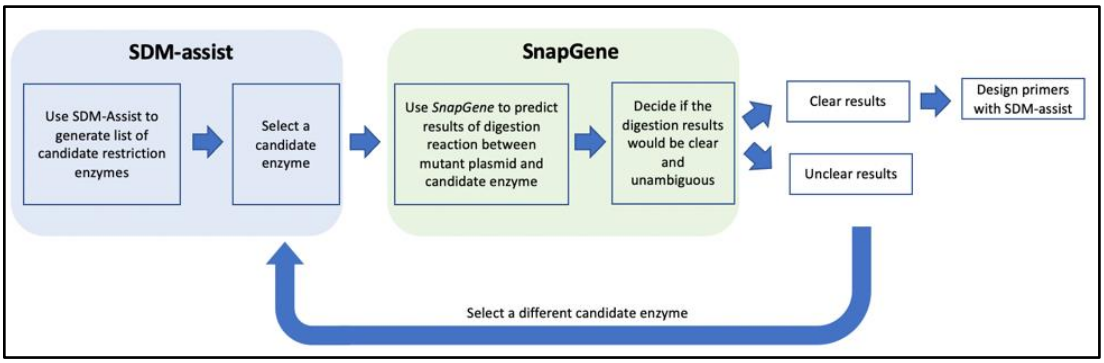


Figure 8) Overview of SDM-assist and SnapGene. Primers are generated using SDM-assist. The primers will contain a mismatch to create a silent mutation to exploit a restriction enzyme. The primers and corresponding plasmid DNA (used in PCR) are analyzed with SnapGene to determine if restriction enzyme digestion would yield a clear banding pattern in an agarose gel.

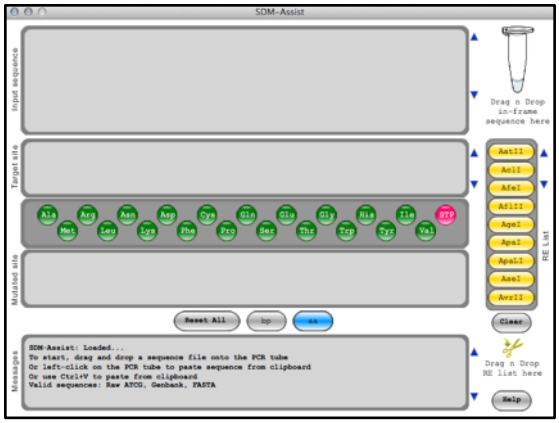
ACTIVITY

Primer Design

1. **Open the SDM-assist program.** Optional: The SDM-assist program may be downloaded from the following website.

http://www.psrg.org.uk/sdm-assist.html

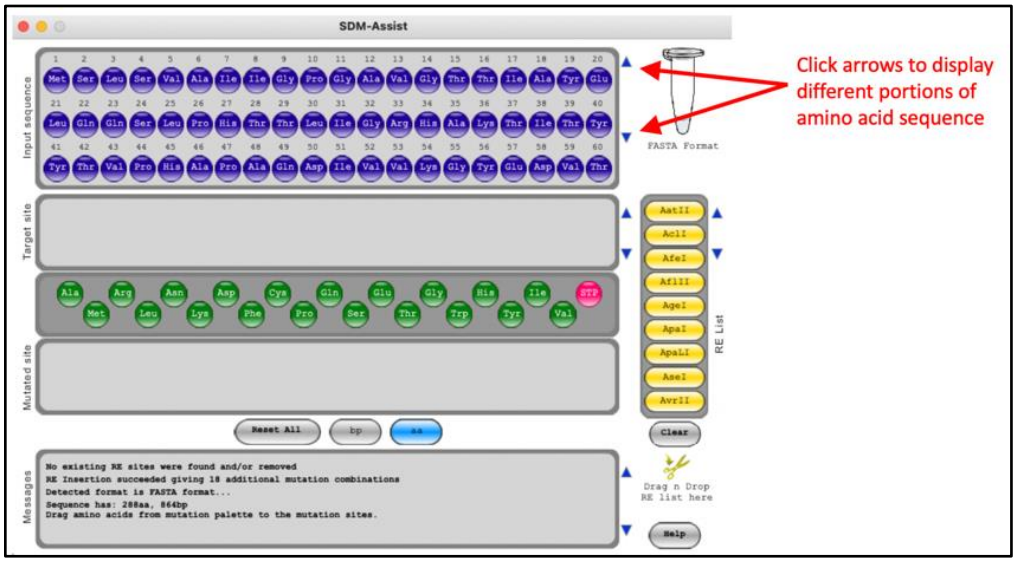
2. **Open SDM-assist.** The following terminal window will appear



3. Download and save (to computer desktop) the DNA sequence for your gene of interest. This file will be made available to you. Ask your instructor if the location has not been specified.

4. Load the DNA sequence for your gene of interest.

Find the file on your computer and left-click on it and drag it onto the microcentrifuge tube shown in the upper right corner of the image above. The terminal window will display the amino acid sequence corresponding to the DNA in-frame sequence. See blue circles below.



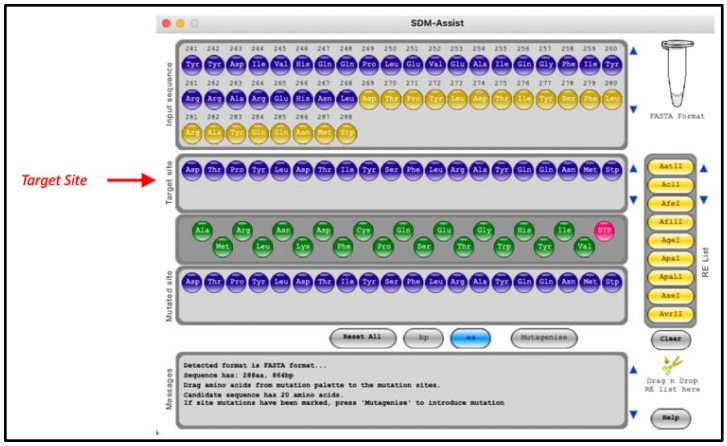
Concept check:

The upper left side of the program says "Input sequence" and the corresponding section has a lot of purple circles with associated letters and numbers. What do the purple circles and their numbers correspond to?

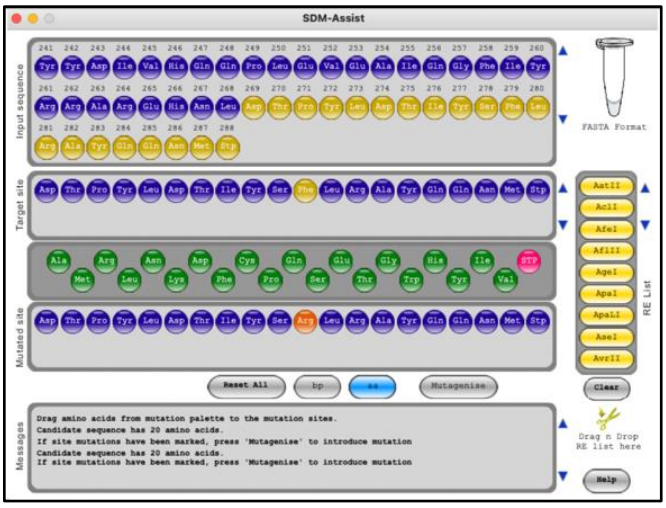
5. Click the down arrow next to the amino acid sequence (see red arrow in above image) to find the amino acid we will mutate. In this activity, we will mutate amino acid, Phe279.

6. Generate a *Target Site* by left clicking on amino acid 269 and then left clicking 288.

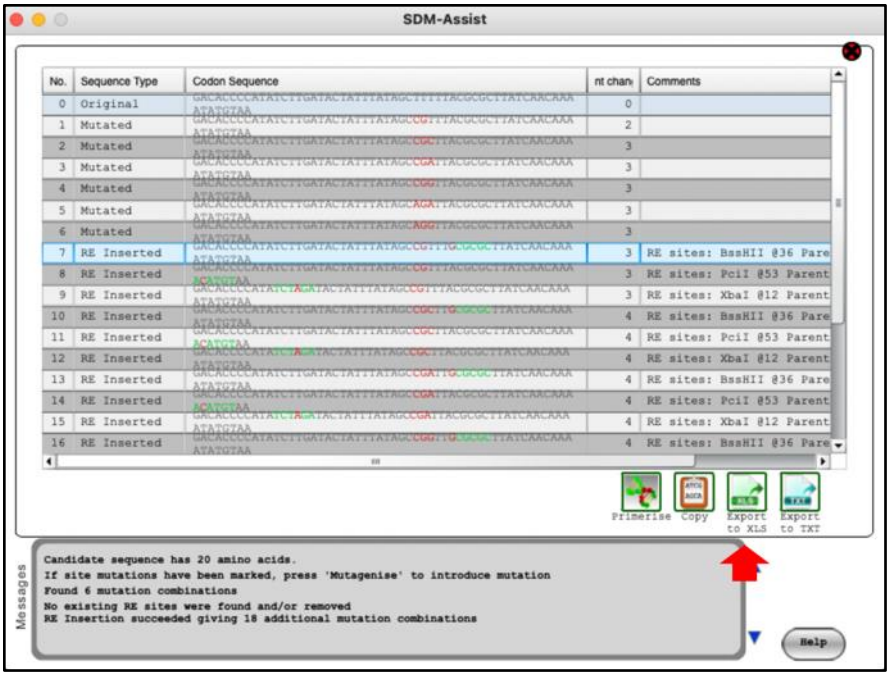
After doing this, the terminal window will look like the figure below where amino acids 269 - 288 in the Input sequence window have changed to a yellow color. The yellow amino acids are referred to as the "*Target Site"* and are displayed in the *Target Site* window (see below). The *Target Site* is approximately 20 amino acids with our amino acid of interest located in the middle.



7. **After creating the *Target Site,* we can specify an amino acid we would like to mutate. In this activity, we will mutate Phe279 into an arginine (Arg). Left-click on the green arginine (Arg) amino acid and drag it on top of the Phe amino acid located in your Target Site.**
After doing this step, your window should look similar to the image below. Comparing the *Target Site* and *Mutated Site*, we see that Arg has replaced Phe.



8. Click the *Mutagenize* button.
After clicking *Mutagenize, y*our screen will look something like the one shown below. *Recommended: You can view and save this data in excel by clicking Export to XLS (red arrow in image below). By viewing in excel, you can format the data for better viewing.*



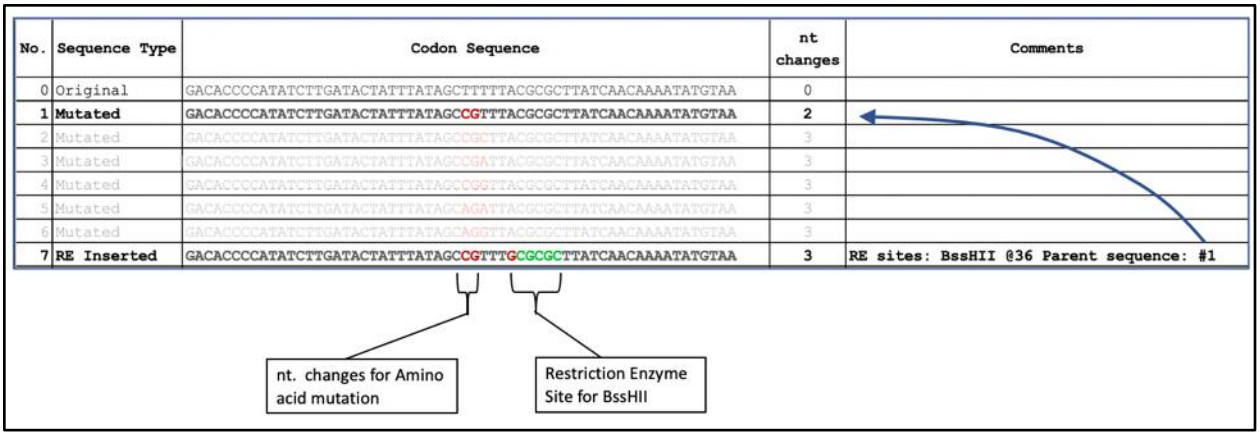
What are we looking at? There are 5 columns: 1) *Parent sequence number,* 2) *Sequence type*, 3) *Codon Sequence*, 4) *nt changes*, and 5) *Comments*. Generally speaking, this image is showing the original DNA sequences (Target Site codons) and mutated versions of it with nucleotide changes (nt. changes). The nucleotide changes can give you either **1)** an amino acid change or **2)** amino acid change and creation/removal of a restriction enzyme sequence.

- **Parent sequence number #0**: The original DNA codon sequence for the amino acids comprising the *Target Site*.

- **Parent sequence number #1 - 6:** For each row, the codons' sequence in column #3 is a "Mutated" type because there are bases that have been mutated compared to the original sequence (No. 0). The mutated bases are shown in red and the number of mutations is specified in the 4th column. If any of these sequences replaced the original sequence, we would mutate Phe to Arg.

- **Parent sequence numbers #7 - 16:** For each row, the type of codon sequence is an *"RE Inserted*", which means that in addition to an amino acid change, there are nt. changes (red letters) that will insert a restriction enzyme (RE) site into the DNA. The RE site is indicated by green and red letters. The *Comments* column indicates the name of the restriction enzyme and the specific location of the RE site as it relates to the entire sequence.

As an example, lets analyze parent sequence number #7 (see image below). A total of 3 nt. changes are present and indicated by red letters. The two nt. changes, "CG", create the amino acid change. The third nt. change, "G", creates a restriction enzyme site for BssHII. The third nt. change is located at the 36th nucleotide.

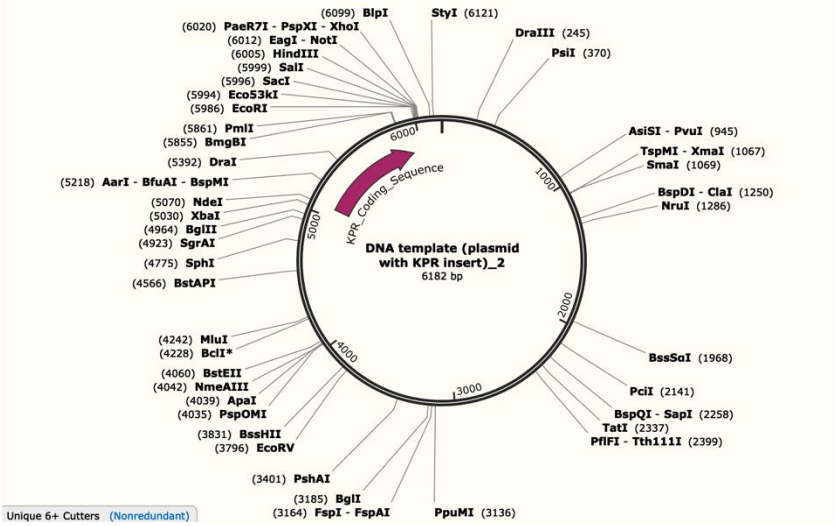


Primer creation and selection

At this point in the activity, SDM-assist has provided candidate DNA codon sequences to perform our missense mutation (Phe (279) is converted to an Arg) as well as a silent mutation to create a *BssHII* restriction enzyme site. If this sequence replaced the original, our mutant plasmid would have a new *BssHII* restriction enzyme site. *Is BssHII a good enzyme to use for verification?* To answer this question, we must know how many times our mutated plasmid will be digested and accurately predict digestion our agarose gel results. In order to determine this, we must analyze the rest of the DNA for other restriction sites for *BssHII*. To do this, we will use the SnapGene to predict the results of the restriction enzyme digestion verification between the mutant plasmid and this candidate restriction enzyme, *BssHII*. We need to make sure the results of the digestion reaction are clear and unequivocal.

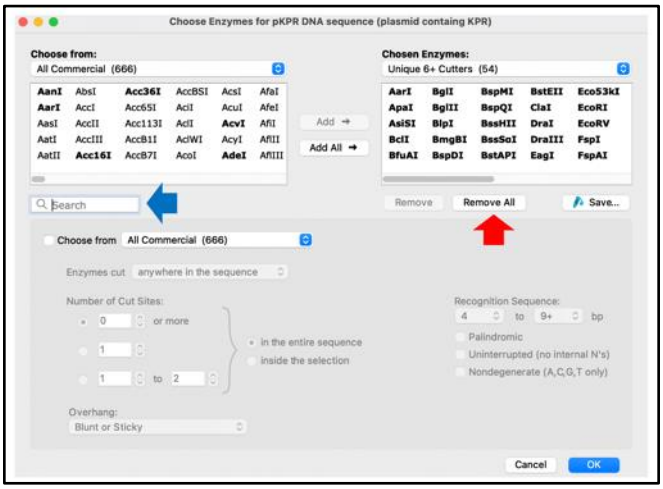
9. Open *SnapGene.*

10. Download the DNA sequence file corresponding to your DNA template (plasmid with KPR insert) and open it with the *SnapGene* program. This file will be made available to you. Ask your instructor if the location has not been specified. Upon opening it, the program will ask you whether it is a linear or circular molecule. Specify circular. Your window may look the image below

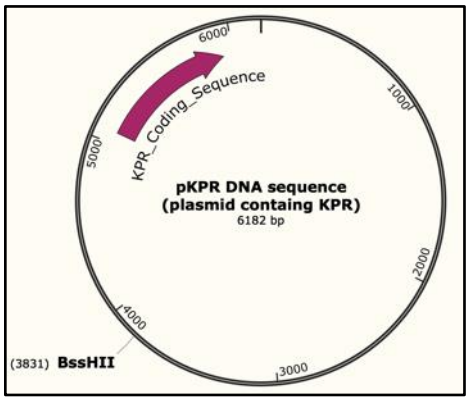


11. Display only the *BssHII* restriction enzyme site by doing the following:

- Click the *Enzymes* tab in the ribbon toolbar at the top of the screen.
- Click *Choose Enzymes.* A window should appear like the image below.



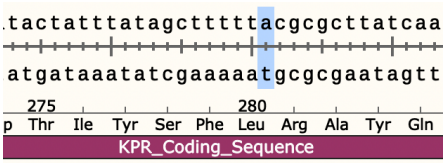
- Click *Remove All* (See Red arrow in above image).
- Type *"BssHII"* in the search area (See blue arrow in above image). The program should highlight this enzyme in the area above the search bar.
- Click "Add"
- Click "OK". Select the *Map* tab at the bottom. Your window will look similar to the image below. The program has found one restriction enzyme site at position 3831.



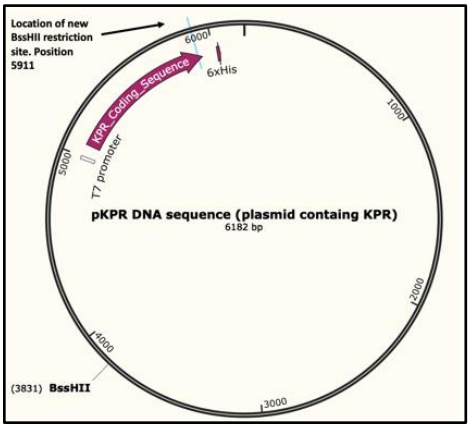
12. Locate the position of the silent mutation that will create another BssHII site. This is the mutation suggested by SDM-assist.

This site can be found by first locating the Phe279 amino acid. Once we do that, we can manually search the neighboring sequence.

- Click the *Sequence tab.*
- Scroll through and find Phe279
- Manually search the flanking sequences for the nucleotide that SDM-assist suggested to change to create the *BssHII* site.
- Highlight that nucleotide by left-click dragging across this nucleotide. The image below shows the nucleotide highlighted. Record this nucleotide's number.

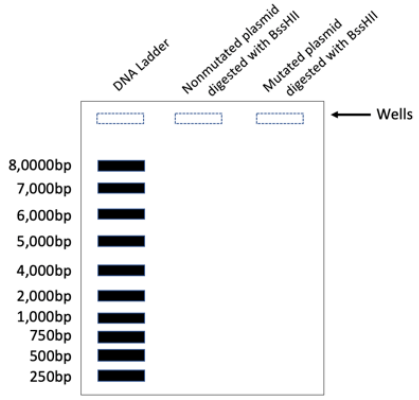


- Click the *Map* tab to see where this nucleotide is located in context to the entire plasmid. Your map should look similar to what is shown below.



13. Predict your agarose gel results

Calculate the products that would get generated if the **template plasmid** and mutated plasmid were separately digested with *BssHII*. How many bands would be generated? What sized bands would be generated in each reaction? Use the image below and draw your predicted results if each digested reaction were run in an agarose gel.

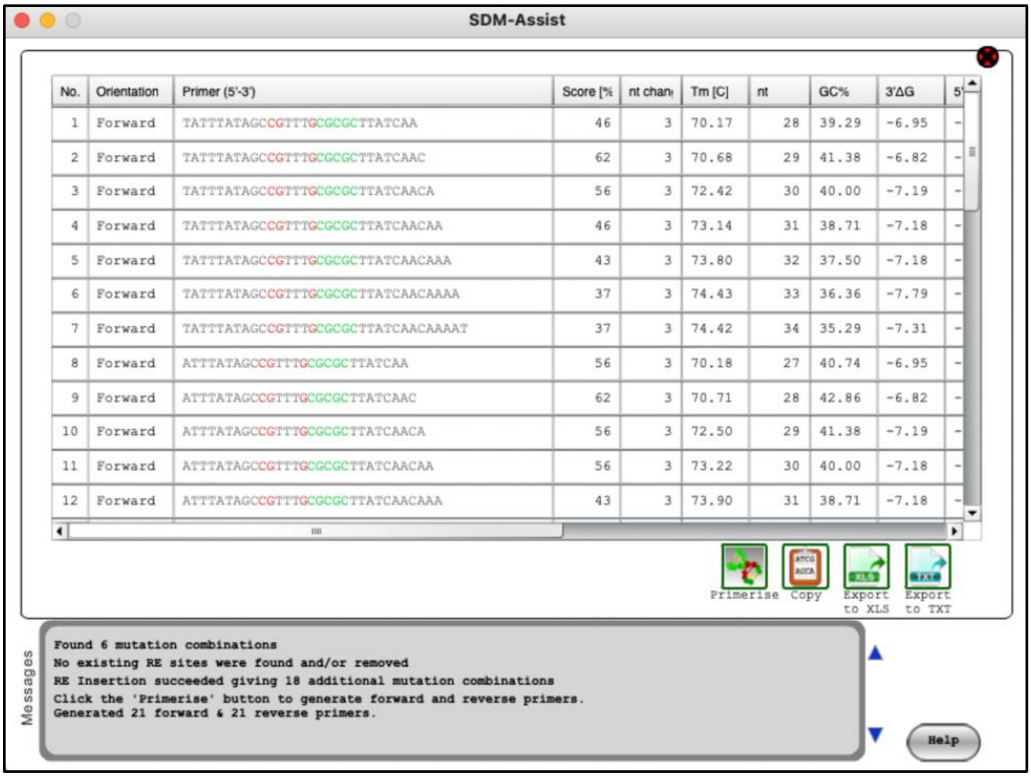


14. Generate forward and reverse primers to make this mutant plasmid.

Now that we see the agarose gel results would be clear and unambiguous. Let's go back to SDM-assist and have this program design primers for this.

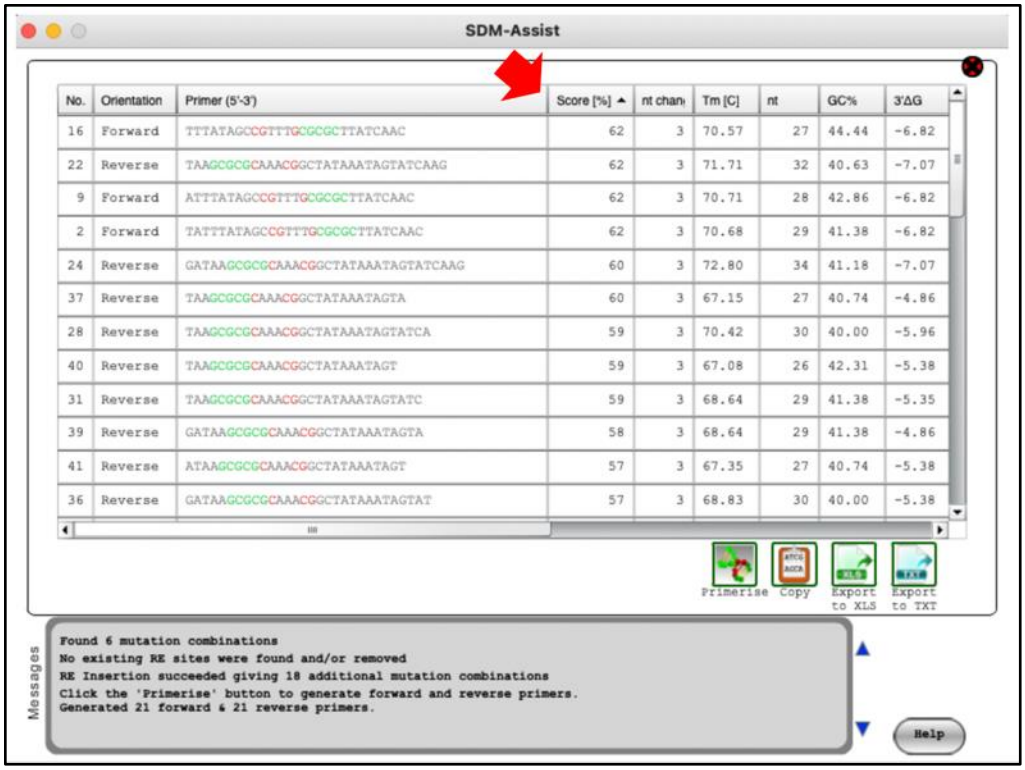
- Bring up SDM-assist and **click on parent sequence #7 in the SDM-assist program and click *Primerize.*** The Primerize button is located in the SDM-assist program window that shows your various mutated sequences. If you analyzed your information using excel, your program may have reverted back to the original terminal window (purple and green amino acids). If this is the case, you can repeat the earlier steps and click mutagenize to find the window.

After performing this step, a window should appear similar to what is shown below. As before, you can view and save this data in excel.



What are we looking at? The program has provided us many options to choose a pair of forward and reverse primers. Column #2 (Orientation) indicates whether the primer has forward or reverse orientation. The sequence of the primer is provided in the 3rd column. The "Score %" column indicates how well the primer should work. The score is based on a 0-100 scale. A higher number indicates a better primer. The score is influenced by columns to the right including primer length, nucleotide changes, melting temperature (Tm), GC %, etc.

15. Sort the primers based on score by clicking on the *Score [%]* tab (see red arrow in image below. Sort the rows so that the highest score is on top.



16. **Select a pair of primers.** SDM-assist has given us many options to choose from. This may seem daunting at first but previous students have successfully used primers that were selected two important parameters to keep in mind:

1. Primer score. Generally speaking, the higher the primer score, the higher the chances of the primers working. We have had success with primer scores in the 50s and above.

2. Melting temperature (Tm). The Tm is related to the temperature of the Annealing step in the PCR. The annealing temperature is chosen based on how well your primers bind. You want your forward and reverse primer to have a similar Tm. We have had success with a primer mix differing in 1-2^{o}C

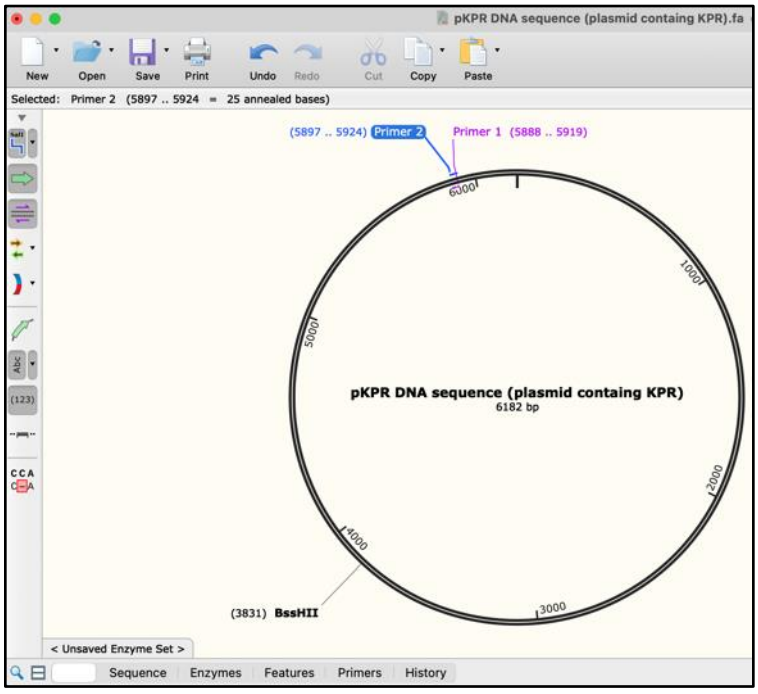
In the above image, we can see that there are 4 primers (3 forward and 1 reverse) with a score of 62 and similar Tm values. Primers #22 and 9 have the closest Tm values so let's choose them. Their primer sequences are below

No 22 (Reverse): TAAGCGCGCAAACGGCTATAAATAGTATCAAG
No 9 (Forward): ATTTATAGCCGTTTGCGCGCTTATCAAC

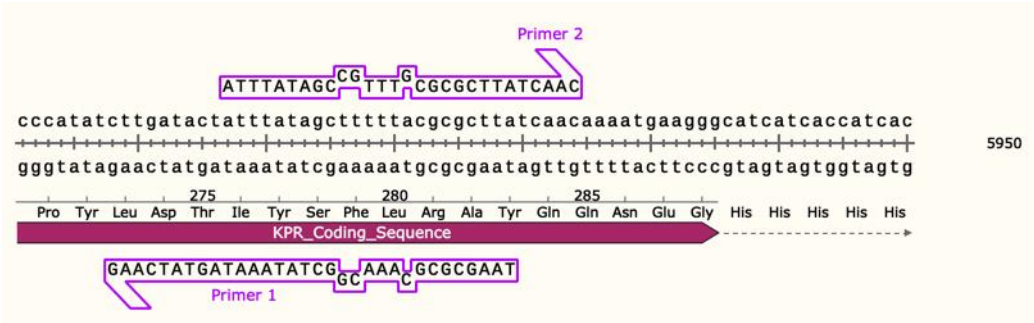
17. Double check with SnapGene that these primers contain the appropriate mismatches.

- If necessary, open *SnapGene* and load the DNA sequence file containing your template (plasmid with KPR insert). Repeat previous steps to remove restriction enzymes for ease of viewing
- Click the *Primers* tab in the ribbon toolbar at the top of the screen.
- Click *Add Primer.*
- Copy one of the above primers paste it next to the 5' tab.
- Click *Add Primer to Template.*

- Repeat this for the other primer. After you complete this, your window should be similar to the image below:



18. Click the *Sequence* tab and scroll to find the primers matched up with the template. The mutagenic mismatches are indicated by a bulge in the primer sequences. See image below.



19. Submit your primer sequences to your instructor by clicking on this link Google form.

REVIEW QUESTIONS

1. What are mutagenic primers (primers with a mismatch)?

2. Explain how mutagenesis is verified by drawing a schematic diagram.

3. Primers are incorporated into PCR products. In whole plasmid PCR, is the PCR product larger than the template?

4. Mutagenesis is verified by running an agarose gel of your restriction enzyme digestion reactions. In addition to digesting your mutated plasmid, it may be a good idea to also set up another reaction between nonmutated plasmid and enzyme. Why is this?

5. After clicking mutagenize in SDM-assist, a list of candidate sequences was provided. In this activity, we focused on sequence #7. However, assuming we could select any candidate sequence, what factors would you take into consideration in order to select a sequence for primer design?

6. How is the score for each primer calculated?

7. At which step of your project will you be verifying mutagenesis with restriction enzyme digestion?

8. Shop online for your enzyme and record below the company, price, and # of units you are receiving for that price. Find the product sheet for your enzyme and save it to your computer. What are the reaction conditions for its use? How long will it take to digest?

9. How much DNA sample will be available for you to perform the digestion/verification step. Explain whether or not you should use all of that sample for your digestion/verification step.

Materials

The following materials are required for this activity.

1. **DNA sequences.** The DNA sequence files are needed for 1) the gene of interest and 2) plasmid vector containing the gene of interest. The gene sequence is loaded into SDM-assist and the plasmid sequence is loaded into SnapGene.

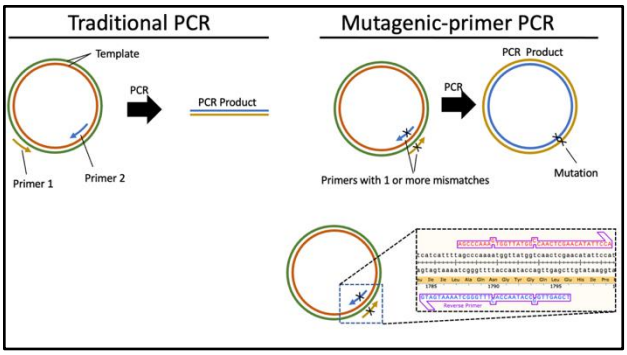
2. **Computer-** A Windows- or Mac-based computer is needed for each student or group of students. Groups of 2-3 students work well. Larger groups may have difficulty seeing the monitor and effectively contributing.

3. **Software downloaded on computers-** *SDM-assist* and *SnapGene Viewer*. These are freely available downloads. *SDM-assist* is available at http://www.psrg.org.uk/sdm-assist.html. SDM-assist requires Adobe Air to be installed as well. *Snap-gene* is available at https://www.snapgene.com/try-snapgene/

Notes for the Instructor

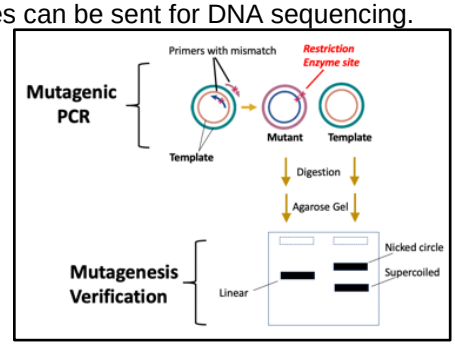
This activity was originally designed and performed as one step in a multi-step, course-based research project involving Site Directed Mutagenesis (SDM) on a protein. Students work together to mutate an amino acid and assay for altered function and structure. This activity is completed by students in a two 3-hour class session, on average. The fundamental techniques involved in this activity are PCR, restriction enzyme digestion, and agarose gel electrophoresis. By the time a student performs this activity, they will have practiced these techniques in lab and be familiar with fundamental concepts. This activity is implemented in a CURE-based laboratory course with 24 students in each section (4 sections total).

This type of PCR reaction is commonly referred to as mutagenic PCR or primer-based PCR mutagenesis and is the basis of the commercially available kit, Quickchange Lightning Site Directed Mutagenesis Kit (Agilent Catalog # 210518). In our class, we use this kit for our mutagenesis. The image below shows the differences between a traditional PCR and that performed with the kit.

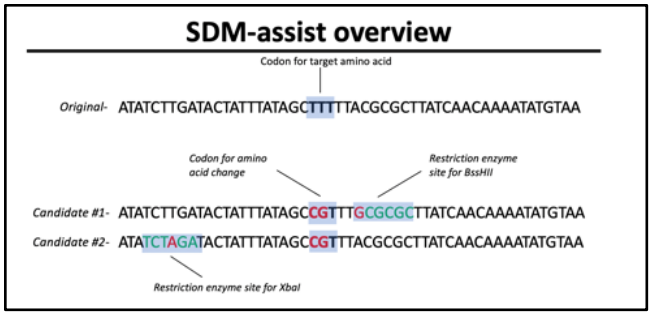


The Introduction section of the student's activity sheet (handout) provides an overview of the background information associated with the activity. Briefly, mutagenic PCR uses primers with mismatches. The mismatches create nucleotide substitutions in the template. The primers are designed so that the mismatch results in a DNA codon change, which in turn results in an amino acid change.

Mutagenesis Verification. Following PCR reaction, the product is transformed into *competent cells.* The cells are then transferred onto LB-agar plates and in 1-2 days colonies should appear. In theory, the cells of each colony should contain the mutated plasmid but this should be verified. One way to verify mutagenesis is through restriction enzyme digestion followed by agarose gel electrophoresis. This is made possible during the primer design process. While designing primers to have a mismatch for an amino acid mutation, an additional mismatch can be created in the location of a restriction enzyme site. In comparison to the template plasmid, the mutant plasmid does not have that restriction enzyme site and this disparity can be verified by agarose gel electrophoresis. Alternatively, a mismatch can be added into the primers so that a restriction enzyme site is created. In comparison to the template plasmid, the mutant plasmid will have an additional restriction enzyme site, which can be verified by agarose gel electrophoresis. At this point, samples can be sent for DNA sequencing.



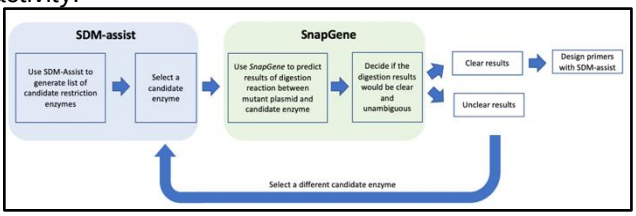
In this activity, a student will use a software program, *SDM-assist*, to design mutagenic primers. *SDM-assist* is a point-click software program that will analyze a DNA sequence and provide a list of potential restriction enzyme sites that could be created or removed through a nucleotide substitution. In essence, the user provides the original DNA sequence and identifies the DNA codon that must be changed for an amino acid change. In turn, SDM-assist will analyze the surrounding sequences for a potential silent mutation that could be made to create/remove a restriction enzyme site. The program provides a list of candidate sequences that, if replaced the original, would mutate the amino acid and create/remove a restriction enzyme site (See image below). The user selects a candidate sequence and then the program will provide a list of primers that could be used in a PCR reaction to create that particular sequence.



However, prior to the primer selection step, a student must figure out whether the newly created /removed restriction site is suitable for verification. As previously mentioned, verification of PCR success (mutagenesis) is performed by digesting the PCR products with the corresponding enzyme and running the products through an agarose gel. Mutagenesis is verified if the banding pattern matches up with what is predicted. The banding pattern should provide a clear and unequivocal result. Because the product of our PCR reaction is an entire plasmid, predicting the agarose gel results requires analysis of the entire plasmid for the presence of each candidate restriction enzyme listed by SDM-assist.

***SnapGene* is used to predict the results of the restriction enzyme digestion.** Initially, a student uses *SDM-assist* to analyze the DNA sequence near the target amino acid (Phe279). The program provides a list of candidate sequences that if replaced the original, would result in an amino acid change (Phe => Arg) and add a restriction enzyme site. In this activity, we focus on creating a *BssHII* restriction site (candidate sequence #7). The next step is for students to use this information in the *SnapGene* software and see if creating this particular *BssHII* site would generate a clear and unambiguous result in an agarose gel. When using *SnapGene*, the template plasmid is loaded and searched for *BssHII* sites that already exist. A goal for the students is to be able to draw the results of the digestion reaction as viewed in an agarose gel. The student should be able to draw the digestion results of the *template* + *BssHII* and *mutant plasmid* + *BssHI*. Should the students deem the results clear, the student goes back into the SDM-assist program and creates primers for the candidate sequence #7. The students load the primers into *SnapGene* to visualize primer-template binding and doublecheck the primers (mismatches are in correct locations). The primers are submitted to the instructor through a Google form.

The figure below summarizes the entire activity.

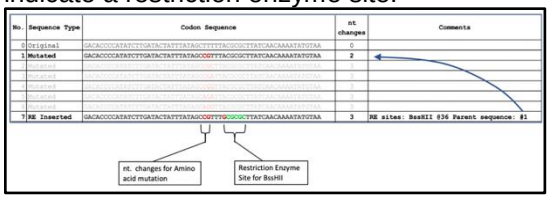


Opportunities for student ownership and unique data. This activity is implemented by having the instructor selecting a panel of amino acids from which the students can select one for mutagenesis. Depending on the location of the amino acids in relation to the protein, different restriction enzymes may be available for mutagenesis verification. Different restriction enzymes would yield different results, which creates an opportunity to obtain data that is truly unique to the student. For example, in the Fall semester of 2020, Glutamate 194 and Phe279 of Ketopantoate Reductase were mutated by students.

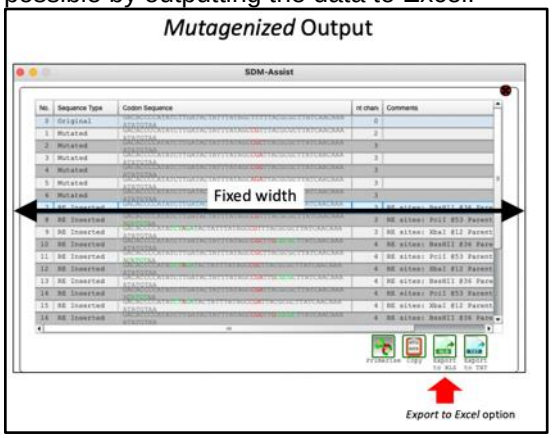
Points of confusion for students

- A general misconception by students learning about PCR is that they think primers cut DNA. Even if a student has been exposed to PCR, this misconception is exacerbated in this activity because the designed primers are associated with a restriction enzyme site. However, the primers do not cut the DNA.
- Some students may have difficulty interpreting the *Mutagenized* results provided by the SDM-assist program. An example result is shown in the image below. Row #0 is the DNA codon sequence corresponding to

the Target Site amino acids. Rows #1 and beyond are mutated versions of the original sequence. Red letters are nucleotide changes. Red and green letters combined indicate a restriction enzyme site.



- A student may be confused on where the "original" Codon Sequence (column #3) came from or what it corresponds to. Column #3 corresponds to the sequence of codons for the *Target Site* amino acids that were selected at the beginning of SDM-assist. Had the student selected a longer Target Site sequence, this Codon sequence in row #0 would be longer.
- A student may be confused with the Comments column (#5). As an example, the comments for Row #7 (image above) are indicating the corresponding Codon Sequence has a restriction enzyme (RE) site for *BssHII* inserted at nucleotide position #36 of parent sequence #1. In other words, the codon sequence for #7 is identical to that for #1 except a restriction enzyme site is being created with a 3rd nt. change.
- The *Mutagenized* output (see example image below) is shown in a window with fixed width and cannot be adjusted. There are scroll bars on the side and bottom of the window but our lives would be made easier if we could widen the window. Thankfully, the data can be outputted to excel for easier interpretation but students may need encouragement. Note: Additional learning opportunities are made possible by outputting the data to Excel.



- A major source of confusion is predicting the results of the restriction enzyme digestion reaction.
    - The restriction enzyme digestion reaction will be performed on the entire plasmid. Therefore, any restriction enzyme site modifications made with primers generated with SDM-assist will be made in the context of the entire plasmid. It is important to stress to students that the work they do with SDM-assist is only on a small fraction of the plasmid DNA. When using SDM-assist, the gene DNA sequence file is used. When using *SnapGene,* the plasmid DNA sequence file is used
    - The correct DNA sequence to analyze in *SnapGene* is the non-mutated template DNA (plasmid containing gene insert). A student may mistakenly load the sequence file for gene. This is easy to tell because of the bp size difference. The plasmid file is >6,000bp where the gene is ~800bp.
    - Even if the correct sequence file is loaded into *SnapGene,* a student may need to be reminded that they are looking at the non-mutated plasmid sequence, NOT the mutated plasmid sequence. While looking at the non-mutated plasmid sequence, a student may have difficulty applying the information learned from SDM-assist. For example, in this activity, a student uses SDM-assist to design primers to create a RE site for *BssHII*. The student uses *SnapGene* to predict the results of the entire mutated plasmid by searching for *BssHII* sites that already exist in the plasmid. The program will find a site at 3831bp. Here, the student may mistakenly think that this is the RE site they created in *SDM-assist* and when asked to predict the results of the mutant plasmid digestion reaction, the student incorrectly predicts it will be cut once. Misconceptions like these are not bad for the student to encounter but it is important for them to be exposed and for the student to work through them. The activity step,

"Predicting your agarose gel results", serves the purpose of exposing this type of misconception and provides an easy way for the student-instructor to work through them.

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Jason O'Donnell has been an Instructor at the University of Calgary since 2014, where he teaches an introductory-level Biology lecture course and an upper-level Biology laboratory course.

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