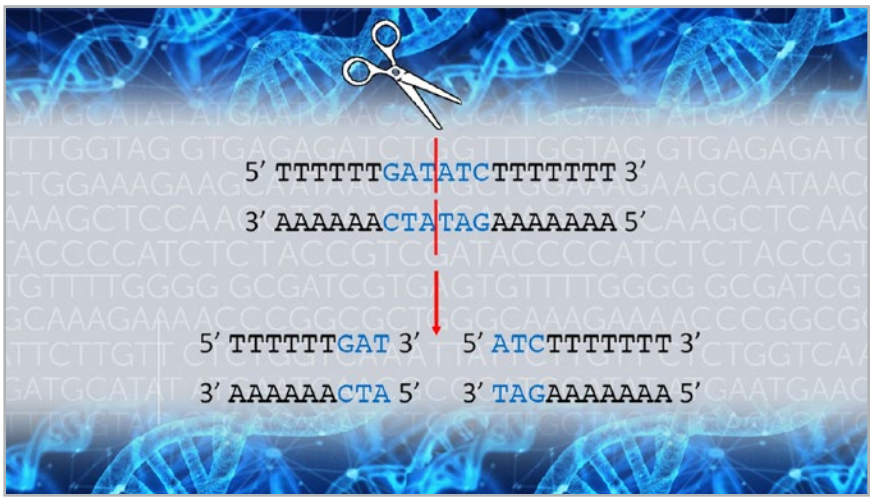
Restriction Analysis of DNA MiniLab

Student's Guide

Cat# M6053

Version 030619



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Objectives

This hands-on MiniLab kit provides pre-cut DNA samples, a DNA sample and restriction enzymes for students to perform a restriction digest to analyze the results on the MiniOne® Electrophoresis System. Students will explore the mechanism of restriction enzyme digestion and its use in biotechnology. Students predict the size of the restriction fragments from a DNA sequence and test their prediction by running the samples on an agarose gel and estimating fragment sizes by comparison to the bands in the DNA marker, using the pre-cut DNA samples as controls.

Laboratory Safety

1. Wear lab coats, gloves, and eye protection as required by district protocol.
2. Use caution with all electrical equipment such as PCR machines and electrophoresis units.
3. The PCR machine has surfaces that can be extremely **hot.** Use caution when opening and closing the lid and when placing and removing tubes.
4. Heating and pouring molten agarose is a splash hazard. Use caution when handling hot liquids. Wear eye protection and gloves to prevent burns.
5. Wash your hands thoroughly after handling biological materials and chemicals.

Introduction

If you needed a short piece of string but all you had was a long one, what would you do? You would probably grab a pair of scissors and cut it to the length you needed. In a somewhat similar fashion, cells have mechanisms for cutting long strands of nucleic acid into shorter strands—a type of molecular scissors called restriction enzymes (endonucleases). There are several reasons why cells need to cut their DNA or RNA. You may recall that when the mRNA is originally transcribed it has regions called introns that are removed. During meiosis when homologous chromosomes pair up, the process of crossing over or recombination, requires cutting and swapping pieces of DNA. Bacteria cells can recognize and destroy foreign DNA by cutting it into pieces. It is this last example that you will learn more about in this lab.

When bacteriophages (viruses) infect bacteria, they insert a strand of nucleic acid into the bacteria. When this is DNA, the bacteria have special enzymes called restriction enzymes that can protect the cell by cutting up the viral DNA and render it useless. These enzymes can also be called endonucleases because they cut inside a stand of DNA ("endo"), they act on nucleic acid ("nucle"), and they are enzymes ("ases").

Each restriction enzyme has a specific site or sequence of base pairs that it recognizes called the restriction site. This is where the enzyme will cleave the DNA. Restriction sites are usually about six base pairs in length. For example, the restriction enzyme EcoRI only binds to GAATTC while the restriction enzyme EcoRV binds to only binds to GAGATC. Another characteristic of restriction sites is that the double stranded DNA is palindromic. You probably know what a palindrome is—a word that reads the same forward and backwards such as "racecar." Recall that DNA is antiparallel, meaning one strand runs 5' to 3' and the other strand runs 3' to 5'. To make a sequence palindromic, both strands of DNA will have the same sequence when read in the 5' to 3' direction. For example, the restriction site for EcoRI is 5'-GAATTC-3' making the complementary strand 3'-CTTAAG-5'. When reversed to be read from 5' to 3', the complementary strand reads 5'-GAATTC-3', identical to the restriction site for EcoRI (Figure 1).

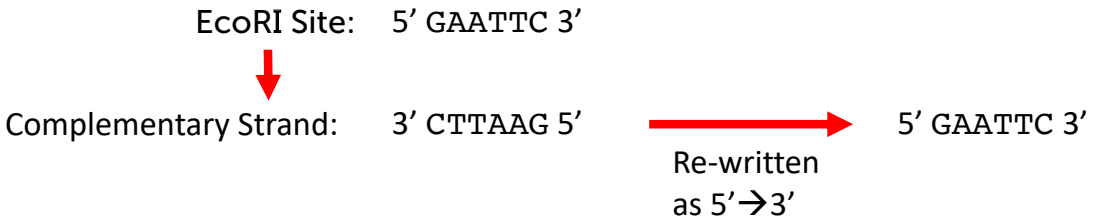


Figure 1. Palindromic restriction site for EcoRI.

CONSIDER THIS: Why do you think restriction sites are palindromic? How could it be beneficial for the cell?

To cleave the DNA backbone, a restriction enzyme must catalyze two reactions, it must break a phosphodiester bond on one strand and break a phosphodiester bond on the other strand. Because the sequence of a restriction site is palindromic, the location of the cleavage of the phosphodiester bond is the same for both strands. As a result, restriction enzymes can produce one of three fragment ends—it can produce a 3' overhang, a 5' overhang, or a blunt end (Figure 2). The overhangs generated by the restriction enzymes are known as "sticky ends" because the bases are exposed and free to complementary base pair (Figure 2). When there is no overhang it is called a blunt end.

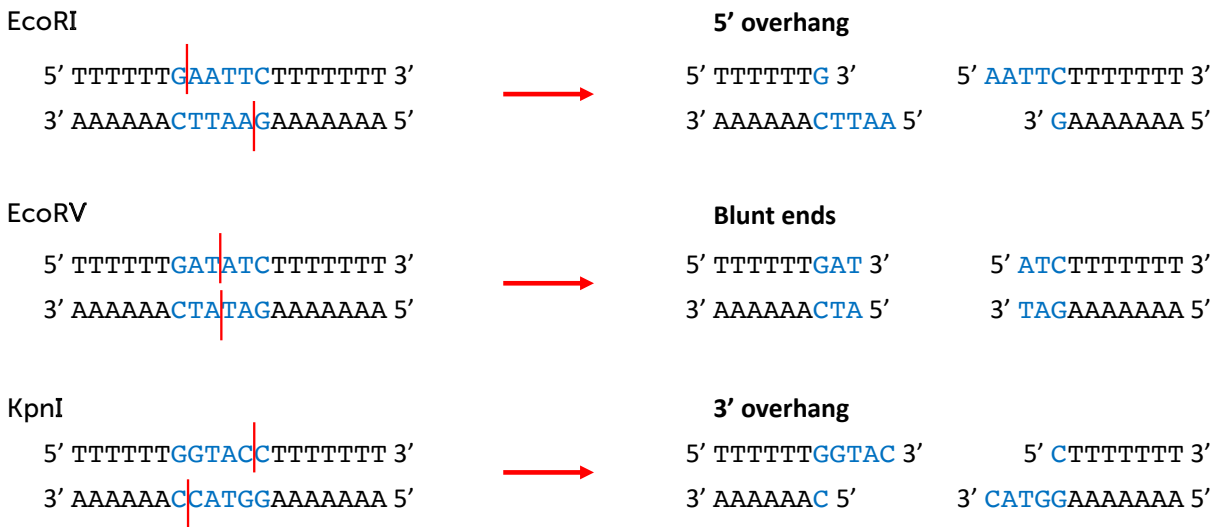


Figure 2. The restriction site for EcoRI is GAATTC and it cleaves right after the G on both strands (shown in red). The resulting fragment has a 5' overhang of 4 bases that are free to combine with complementary bases. The restriction site for EcoRV is GATATC and it cleaves between the TA on both strands leading to a cut straight across producing blunt ends. The restriction site for KpnI is GGTACC and it cleaves between CC on both strands giving 3' sticky ends.

The reaction of cleaving DNA with a restriction enzyme is called a **restriction digest** as the restriction enzyme is breaking the DNA into smaller parts. Because restriction enzymes will only recognize and cleave at a specific restriction site, a restriction digest will produce defined fragment lengths of DNA.

CONSIDER THIS: How can scientists use restriction enzymes as tools for genetic research?

The discovery of restriction enzymes has revolutionized molecular biology and genetics. Because restriction enzymes recognize specific sequences of DNA and cleave the DNA backbone, scientists can digest DNA into smaller, discrete fragments to study. Additionally, with the use of DNA ligase, scientists can recombine cut DNA to continue to investigate the function of sections of DNA. This ability to digest DNA into discrete fragments and potentially put those fragments back together in unique combinations has opened the door for new exciting experiments that allow scientists to learn more about a specific gene, to create DNA profiles of individuals, to sequence the human genome and much more.

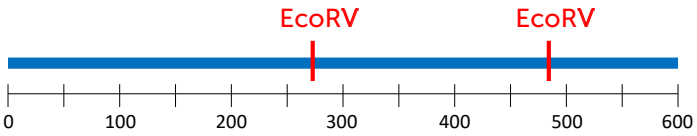
In this lab you will digest a known fragment of DNA with two different restriction enzymes individually and in combination. From the DNA sequence and the sequences of the restriction sites, you will be able to predict the outcomes of these three reactions. You will then run your restriction digests on an agarose gel to determine whether your prediction was correct. We have also provided you with DNA samples that have already been digested with the restriction enzyme combinations you will be using. When doing a scientific experiment, especially one that you have not done before, it is often helpful to compare your results to a set of controls, samples for which the results are already known. By comparing your digested samples to the pre-digested samples, you will be able to identify the source of any errors in your restriction digest.

Pre-Lab Questions

1. What are two functions of restriction enzymes?

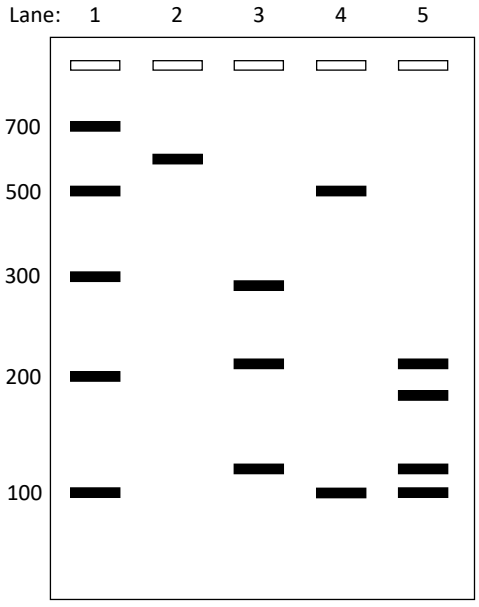
2. What are two characteristics of restriction sites?

3. Shown below is a map of a DNA fragment highlighting the location of two EcoRV restriction sites.



   a. If this DNA fragment is digested with EcoRV and the resulting digestion products analyzed with gel electrophoresis, what sizes of bands would you observe?

   b. You next set up two experiments: In Experiment 1, the DNA fragment is digested with KpnI, and in Experiment 2, the DNA fragment is digested with both EcoRV and KpnI. You run the digestion products on a gel and observe the following pattern of bands. Using the information in the gel, determine the location of the KpnI restriction site(s) on the original DNA fragment.



Lane Number	Sample
1	Ladder
2	Undigested
3	EcoRV digest
4	Experiment 1
5	Experiment 2

   c. In the data above, why is Experiment 1 alone not sufficient to identify the restriction site(s) for KpnI?

Day I: Set up and Run Restriction Digests, and Cast a MiniOne® Agarose Gel

Common workstations

- Benchtop microcentrifuge
- Microwave
- MiniOne® PCR System, or 2 water baths, one set to 37°C, and another set to 65°C
- Mobile device with MiniOne® PCR App
- Gloves, eye protection, lab coats

Student group workstations

- **0.2 mL PCR tubes** if using MiniOne® PCR System, or microcentrifuge tubes if using a water bath, four per student group
- 2-20 µL micropipette
- Micropipette tips, at least 12 tips per student group
- Aliquoted restriction enzymes, DNA sample, enzyme dilution buffer, and 5X loading dye
- MiniOne® Casting System and GreenGel™ Cup
- PCR tube rack (optional)
- Waste container for tips and tubes
- Microcentrifuge tube rack (optional)
- Fine point permanent marker

Experimental Procedures

Part I: Set Up and Run your Restriction Digests

1. Check your workstation to make sure you have all of the materials listed above.
2. Label four PCR tubes with your group name and the sample # according to Table 1.
3. Set up your restriction digests by adding the reagents to the four tubes according to Table 1. (Use PCR tubes if the incubation is going to be in a thermal cycler or use microcentrifuge tubes if the incubation is going to be in a water bath.)

Table 1: Experimental setup

	Tube 1	Tube 2	Tube 3	Tube 4
DNA Sample	10 µL	10 µL	10 µL	10 µL
Enzyme dilution buffer	10 µL	5 µL	5 µL	—
EcoRV	—	5 µL	—	5 µL
NcoI	—	—	5 µL	5 µL
Total volume	20 µL	20 µL	20 µL	20 µL

4. Gently flick the tubes with your finger to mix the reagents. Centrifuge for 15 seconds at 8,000 RPM to bring the reagents to the bottom of the tubes.
5. Place your tubes in the MiniOne® PCR System and close the lid. Incubate the restriction digest at 37°C for 900 seconds using constant temperature mode on your MiniOne® PCR mobile app. Enter 4°C for the final incubation temperature. If you do not have a PCR system, a water bath set to 37°C can be used, incubating for 15 minutes.
6. While you are waiting for your digest, prepare the MiniOne® Agarose Gel (Part II).
7. When the 15-minute incubation is complete, use the constant temperature mode again to heat your samples to 65°C for 300 seconds. Enter 4°C for the final incubation temperature. A water bath set to 65°C can be used for this step as well, incubating for 5 minutes.
8. When the 65°C heating step is complete, remove your samples from the MiniOne® PCR System or water bath and return to your bench.
9. Add 5 µL of 5X Loading Dye to each of your tubes. Use a new micropipette tip when adding to each sample. Gently flick each tube with your finger to mix the dye with the samples. Centrifuge for 15 seconds at 8,000 RPM to bring the reagents to the bottom of the tubes.
10. Give your samples to your teacher for storage until the next lesson.

Part II: Cast a MiniOne® Agarose Gel

1. Follow your teacher's instructions to microwave the GreenGel™ Cup and pour your melted gel into the casting system. Use the 9-well side of the comb to cast your gel. Do not disturb the gel until it is solidified (10-15 minutes).
2. Carefully remove the comb from the gel. Remove the gel tray with the solidified gel from the casting stand. Wipe off any excess agarose from the bottom of the tray. Now you may store your gel in a light-proof container for Day 2. Remember to add a few drops of running buffer on top of the gel to keep the gel moist during storage.

Day 2: Analyze Restriction Digests with Agarose Gel Electrophoresis

Set up common workstations

- Benchtop microcentrifuge
- Gloves, eye protection, lab coats

Set up student group workstations:

- 2-20 µL micropipette
- Universal fit micropipette tips, at least 5 tips per student group
- Aliquoted MiniOne® Universal Marker
- 8 DNA Samples (3 pre-digested DNA controls, 1 undigested DNA with loading dye, 4 experimental DNA samples)
- Aliquoted electrophoresis running buffer
- Gel tray with agarose gel in tray
- MiniOne® Electrophoresis System
- PCR tube rack (optional)
- Microcentrifuge tube rack (optional)
- Waste container for tips and tubes

Experimental Procedures

1. Connect the power supply to the back of the MiniOne® Electrophoresis carriage. Plug the power supply into the wall.
2. Ensure the black viewing platform is in the MiniOne® Gel Tank if it is not already installed. Place the gel and gel tray into the tank. **Make sure the wells of the agarose gel are aligned with the marks on the platform on the negative end.**
3. Place the tank with the gel into the carriage so the carbon electrodes are touching the gold rivets and the tank sits level with the carriage.
4. Measure 135 mL of TBE running buffer and pour into **one** side of the tank to push out the air, creating a nice even background without air bubbles or air trapped for imaging later. Pour the remaining buffer into the other side of the tank.
5. Turn on the low intensity blue light. Load 10 µL of each sample into one well of the gel. Make sure your group also loads 10 µL of the MiniOne® Universal DNA marker into one of the wells. Remember to use a new micropipette tip for each sample.

   Use Table 2 on the next page to keep track of which samples were loaded into each well and which well contains the marker.

Table 2: Load samples according to the following:

	Lane	Sample
	1	*MiniOne Universal Marker*
Control Samples	2	*Undigested DNA, untreated*
	3	*Pre-digested DNA, EcoRV cut*
	4	*Pre-digested DNA, NcoI cut*
	5	*Pre-digested DNA, double cut w/ EcoRV+NcoI*
Experimental Samples	6	*Undigested DNA, incubated w/o enzymes*
	7	*EcoRV cut*
	8	*NcoI cut*
	9	*Double cut w/EcoRV+NcoI*

6. Once the gel is loaded with all samples, do not move it. Place the orange photo hood on the carriage and press the power button once to start the run. The green light next to the power button will turn on to indicate that the gel is running. Allow the gel to run for 30 minutes or until the bands have clearly separated.
7. At the end of the run, turn off the power by pressing the power button again. The green light will turn off.
8 Turn on the high intensity blue light and use your phone or camera to take a picture of your gel. Sketch or paste a picture of your gel on the Gel Analysis Worksheet.
9. Draw the restriction map of the amplicon for EcoRV and NcoI.

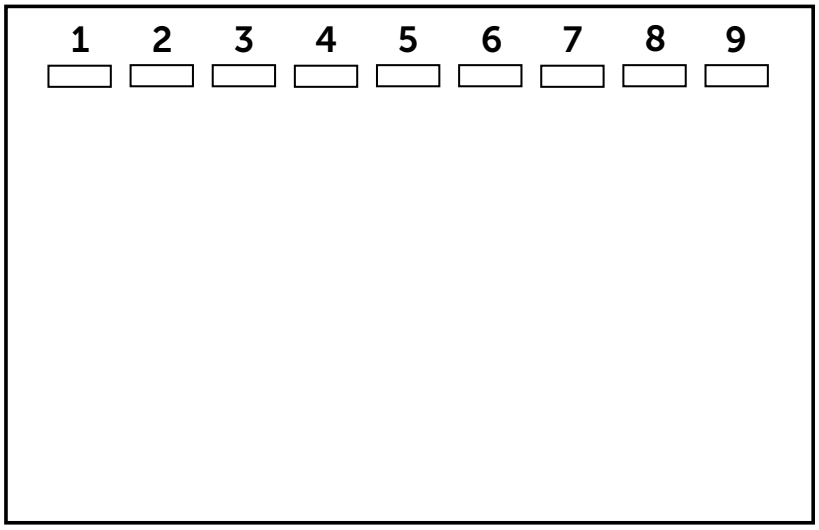
10. If you did not have the sequence and could not use character count how else might you estimate the number of base pairs in each fragment?

11. What is the purpose of the two undigested DNA controls?

12. Why is it beneficial to have precut DNA controls?

Gel Analysis Worksheet

Directions: After running the DNA samples on the gel, record an image of the gel and draw the results or tape the image on the template below:



Lane 1: _____

Lane 2: _____

Lane 3: _____

Lane 4: _____

Lane 5: _____

Lane 6: _____

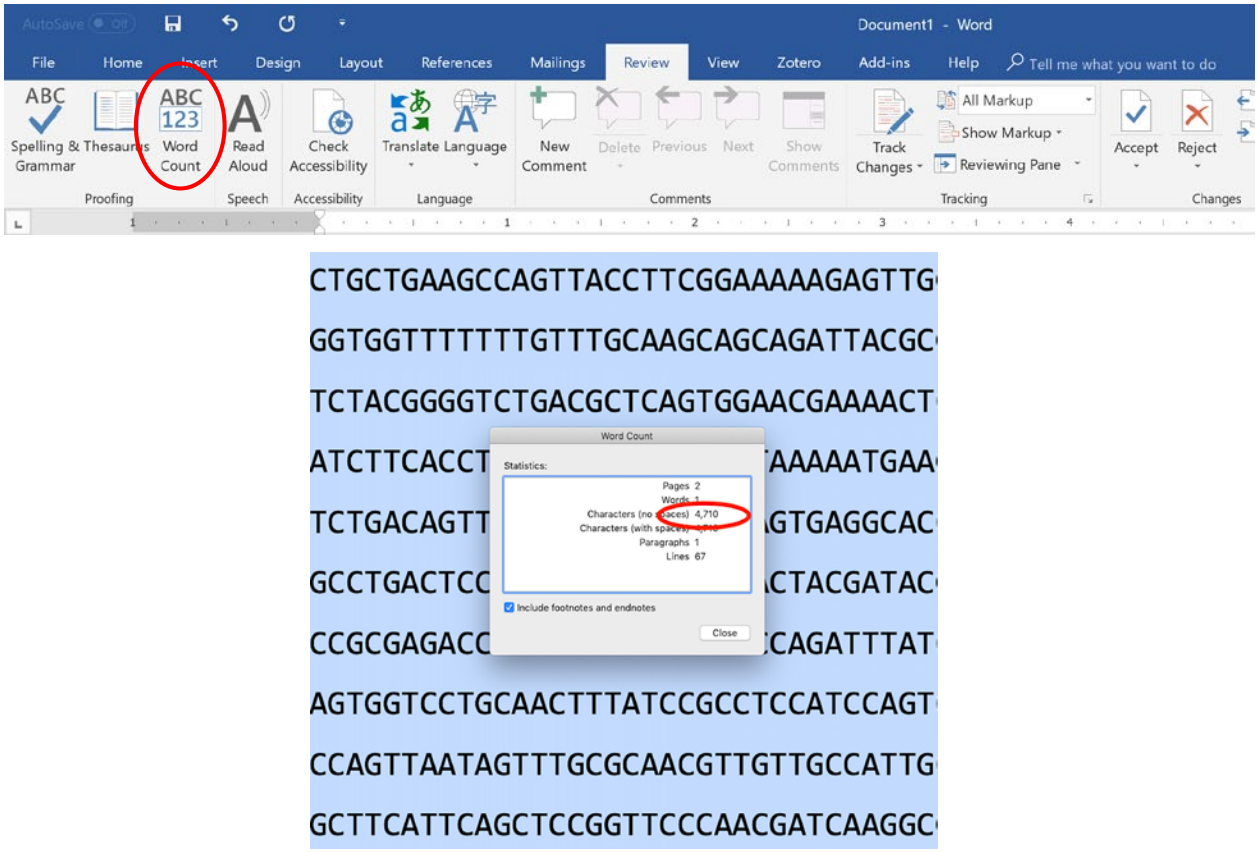
Lane 7: _____

Lane 8: _____

Lane 9: _____

Analysis Activity - Predicting restriction fragment sizes

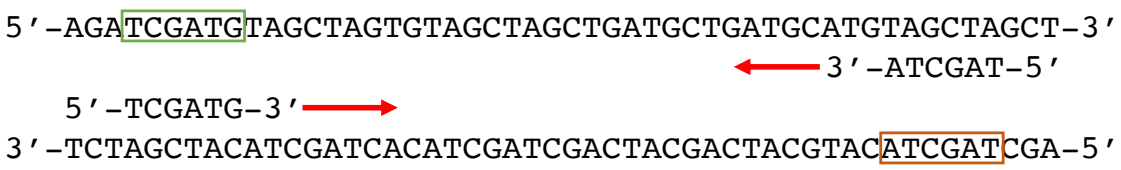
1. Open the accompanying document "M6051-plasmid-sequence.docx". This is the entire sequence of one strand of a plasmid containing the DNA sequence of interest in the 5'->3' direction. Copy and paste this sequence into a word document. Google Docs and other word processors will work just as well, but the screenshots below assume that you are using MS Word.
2. Under the **Review** tab, click the **Word Count** button to find the number of characters in the document. This is the number of base pairs in the plasmid.



3. Next, you will find the length of the DNA fragment supplied with this lab, that was cut with the restriction enzymes EcoRV and NcoI and its location in the plasmid. This DNA fragment was amplified with PCR from the above plasmid by using the following forward and reverse primers.

Forward primer	Reverse primer
5'- GCATTGTTTGGTAGGTGAGAG-3'	5'- CAGCTATGACCATGATTACGC-3'

4. We have only given you the sequence of one strand of DNA from the plasmid. Recall that primers bind to complementary sequences in both strands and orient in opposite directions:



5. The sequence of the forward primer is found within the strand we have given you. Use the **Find** feature in word to locate the binding site of the forward primer by typing **Ctrl+F** on your keyboard. Enter the sequence of the forward primer (just the nucleotides) into the dialog box and click find. The match for the primer sequence in the document should be highlighted. Mark this location by underlining the highlighted sequence.
6. We are now going to find the binding site for the reverse primer. This is a bit trickier. The sequence of the reverse primer is found within the complementary strand of the genome which we have not given you. Therefore, you will need to search for the reverse complement of the reverse primer sequence. Use the space below the reverse primer sequence above to write down its reverse complement.
7. Use the **Find** feature in word to find this sequence in the genome. Mark this location by underlining the highlighted sequence.
8. The region between the primer binding sites (including the primers themselves) is the region that is amplified by PCR to create the fragment we cut with restriction enzymes. Highlight this entire sequence and use the **Word Count** feature as above to find the number of characters in this sequence. Record the length of the PCR fragment below.
9. Copy and paste this sequence into a new document since we will only be looking for restriction sites within this region.
10. The recognition sequences (restriction sites) for the restriction enzymes we used to cut the fragment are listed below.

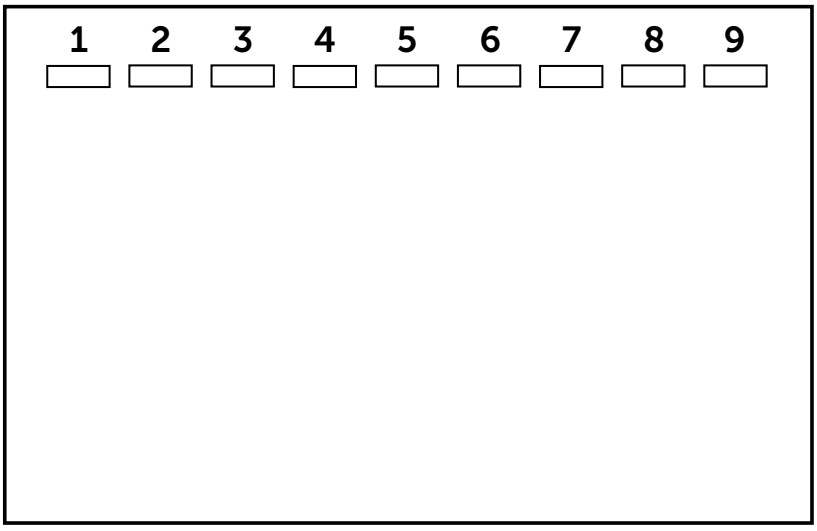
EcoRV	NcoI
GAT|ATC CTA|TAG	C|CATG G G GTAC|C

11. Use the **Find** function again to find the locations of all the EcoRV recognition sequences within the region. Mark the recognition sequence by highlighting or changing the text to a different color.
12. Use Table 2 below to record the number of fragments and their expected sizes produced by cutting with EcoRV.
13. Repeat steps 11 and 12 for NcoI and use a distinct color to mark the text.
14. Now that you have the restriction sites for both enzymes marked on your sequence, determine the sizes of fragments you would expect for the double digest and record on Table 2.

Table 2: Record which sample are loaded into each well.

Restriction Enzyme	# of fragments	Sizes of fragments
EcoRV		
NcoI		
EcoRV + NcoI		

15. Use the template below to sketch the expected results for both single digests and the double digest. Also include lanes on your gel for the undigested fragment and the MiniOne® Universal DNA Marker (200, 400, 600, 800, 1000, 2000, 3000, 6000, and 10000 bp).



Lane 1: _____
Lane 2: _____
Lane 3: _____
Lane 4: _____
Lane 5: _____
Lane 6: _____
Lane 7: _____
Lane 8: _____
Lane 9: _____

16. Compare the fragment sizes you calculated to the experimental results of your restriction digest. Was your prediction correct? If you have not run your gel yet, keep the above information to compare to your gel analysis results.

17. When your gel run is finished compare the results of your restriction digest to the lanes with the pre-cut controls. Are the sizes of the bands the same? If not, what do you think could account for the difference?

18. Save the word document with the fragment you marked up under a new name on your computer.

19. **Challenge Question:** If you had discovered a new restriction enzyme, describe the steps you would follow to determine the sequence of its restriction site.

Appendix A - Gel Electrophoresis

Gel electrophoresis is a technique used in many areas of science to analyze the components of complex chemical mixtures. Mixtures of DNA, RNA, proteins, or dyes can be separated into their individual components based on molecular size and electrical charge using a separation matrix within an electric field.

The gel used in gel electrophoresis is a tangle of polymers forming a three-dimensional matrix with water-filled pores through which molecules migrate. A higher density of polymers creates smaller pores. Like the holes in a sieve or colander, the size of the pores must be the appropriate size for the molecules being separated. Gels can be made from different substances depending on the application. One of the most commonly used and effective materials is agarose, a polymer extracted from seaweed. Agarose gels are formed (or cast) by pouring molten (melted) agarose into a tray where it solidifies into the desired shape as it cools. A comb is placed while the agarose is molten and then removed after it solidifies to create wells where the samples are loaded.

After the gel solidifies it is placed in an electrically conductive buffer between parallel positive (⊕ anode) and negative (⊖ cathode) electrodes. A voltage is applied between the electrodes, creating a uniform electric field within the gel. Molecules in the wells begin to move under the influence of the electric field: positively charged molecules migrate toward the ⊖ cathode and negatively charged molecules migrate toward the ⊕ anode.DNA carries a net negative charge and will migrate toward the anode.

The speed of a molecule's movement in an electric field is determined by the strength of its electric charge relative to its molecular weight. This is quantified as the charge to mass ratio. Speed of movement within a gel is also influenced by the size of the molecule relative to the pores in the gel. The polymers in the gel are like an obstacle course: smaller molecules maneuver easily through the pores, traveling faster and farther than large, bulky molecules. However, a large molecule can move faster through a gel than a smaller molecule when the strength of its charge relative to its mass is significantly higher. Shape can also affect how a molecule moves through the gel. Long spaghetti-like molecules will move slower than compact molecules, which slip easily through the pores. Molecules of the same size, shape, and charge will move together and form a distinct band. If multiple types of molecules are present in the sample, they will separate from each other and each will form a distinct band.

Appendix B - Recommended Reading

Scitable by Nature Education: Background reading about Restriction Enzymes
https://www.nature.com/scitable/topicpage/restriction-enzymes-545

Explanation of palindromic restriction sequences, from Science Primer
http://scienceprimer.com/palindromic-sequences

3D Animation of a restriction enzyme binding and cutting DNA from DNA Learning Center
https://www.dnalc.org/view/15488-Restriction-digest-3D-animation-with-no-audio.html

DNA electrophoresis sample loading: Video from Kirkwood Community College showing proper technique and some common mistakes:
https://www.youtube.com/watch?v=tTj8p05jAFM

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