Mission (Im)possible:
Plasmid Mapping
Student Materials

Mission (Im)possible

Introduction

Take a few seconds to answer the following question for yourself. *How are living things studied?*

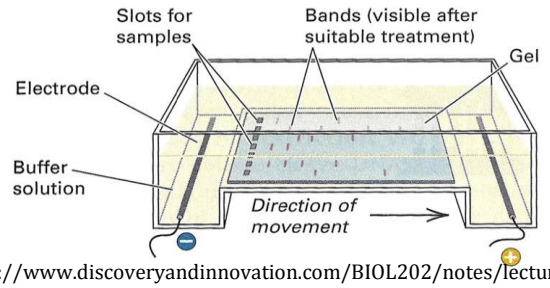
What came to your mind? Did you imagine Jane Goodall studying the chimps in Africa, an ecologist studying a fragile ecosystem or a doctor studying cancer? Did you imagine a microscope, a computer, or other technologies? Today you are going venture into the field of molecular biology by learning tools and techniques that allow the study of living things at the molecular level. Molecules like DNA, RNA and proteins are not living, but understanding these molecules informs us about living things. How can you study molecules of DNA, RNA or protein when they are too small to be seen, even with a microscope? A good first step may be to determine the size of a molecule.

A common way to measure molecular size is by **gel electrophoresis**, which is a big name for a simple concept. Here is an analogy to explain gel electrophoresis. Imagine a very dense forest of trees where each tree is only one foot away from every other tree. You and a mouse must each run through the forest from point A to point B. You and the mouse begin at the same time. Which will reach the other side first, you or the mouse?

In gel electrophoresis, the gel is analogous to the forest. The gel is a mesh formed of polymers, much like JELL-O®. JELL-O is a mesh of gelatin made from animal products like collagen whereas gels for electrophoresis are a mesh of agarose, a carbohydrate purified from seaweed. In gel electrophoresis the molecules are analogous to you and the mouse. Small molecules will move more quickly through the mesh than will large molecules, for the same reasons that the mouse moves more quickly through the forest than you do.

So far so good, but once a molecule is suspended in the gel, why does it move? The DNA, RNA and protein molecules often have a surface charge. This means that if an electric field is applied to the gel, molecules with a negative surface charge will move through the gel towards the positively charged anode and molecules with a positive surface charge will move through the gel towards the negatively charged cathode. When two (or more) molecules have the same charge, the molecules will move through the gel based upon their size; smaller molecules move faster through the gel then larger molecules (see **Figure 1**).

Figure 1. Agarose gel electrophoresis of DNA



http://www.discoveryandinnovation.com/BIOL202/notes/lecture23.html

Using gel electrophoresis to determine the size of DNA molecules is simple. Chemically speaking, every piece of DNA is nearly the same as every other piece of DNA. The exact order of the nucleotides (A, T, C, G) may differ, but the properties of each DNA molecule are the same. Remember that a nucleotide is composed of a ribose, a nitrogenous base, and a phosphate group.

Answer the following questions.

Is the surface charge of a DNA molecule positive or negative?

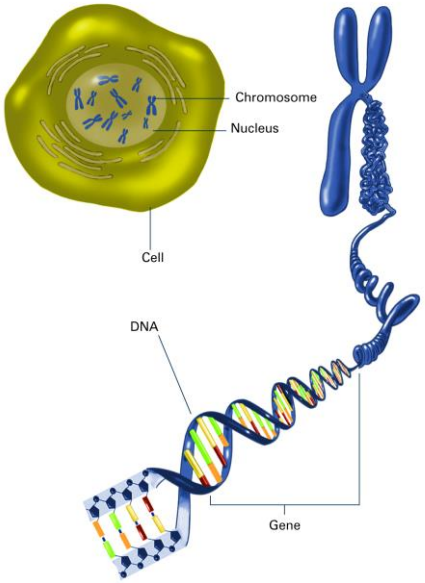
Which of the components (ribose, base, phosphate) determines that charge?

Toward which pole will DNA molecules migrate?

DNA is found in several different forms within cells. Much of the DNA is found in chromosomes. In eukaryotic cells the chromosomal DNA is found as long, linear double-stranded molecules. The chloroplasts and mitochondria of eukaryotic cells and prokaryotic cells contain DNA in the form of circular double stranded molecules. Some cells also contain small, circular, double-stranded DNA molecules that are NOT chromosomal DNA. These small circles of DNA are called **plasmids** (see **Figure 2**).

Figure 2. (a) Eukaryotic and (b) Prokaryotic DNA

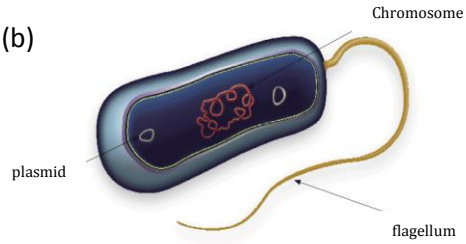
(a)



http://www.ncbi.nlm.nih.gov/pubmedhealth/PMHT0025047/

Please note that there is a vast difference in the size of a eukaryotic cell and a prokaryotic cell. If drawn to scale, the bacterial cell below would be about the same length as one of the chromosomes of the eukaryotic cell.

(b)



http://www.microbiologyonline.org.uk/about-microbiology/introducing-microbes/bacteria

Note: For a beautiful demonstration of cell size and scale see: http://learn.genetics.utah.edu/content/cells/scale/

Plasmids occur naturally in bacterial cells and in some eukaryotes. Often, the genes carried on plasmids provide bacteria (bacteria-plural, bacterium -singular) with genetic advantages, such as antibiotic resistance. When a bacterium divides, all of the plasmids contained within the cell are copied such that each daughter cell receives one or more copies of each plasmid. Bacteria can also transfer plasmids to one another. The sharing of plasmids between bacterial cells is often the means by which bacteria become resistant to antibiotics.

Scientists have taken advantage of plasmids and modified naturally occurring plasmids to use as tools to clone, transfer and make copies of genes. Plasmids that have been modified for experimental purposes are called **vectors**. By inserting a chosen DNA fragment or gene into a plasmid vector, researchers create recombinant DNA plasmids. Each plasmid can be introduced into a bacterium, and as the bacterium

divides and divides, more and more copies of the recombinant plasmid are made. In effect, bacteria can be used as factories to make millions of copies of any DNA fragment or gene that is contained on the recombinant plasmid.

When DNA is purified from cells, the larger DNA molecules, called chromosomes, are often broken into pieces or DNA fragments. These fragments are still larger than what the researcher would want to use. Fortunately, we have a large set of molecular biology tools including restriction endonucleases that are called **restriction enzymes**. Each different restriction enzyme can cut DNA at a different, defined sequence. The sequence that is recognized by a restriction enzyme is called a **restriction site**.

One example of a restriction enzyme is EcoRI. This enzyme recognizes and cuts at the DNA nucleotide sequence shown here after the first G.

5′ GAATTC 3′
3′ CTTAAG 5′

The restriction site for EcoRI can be shown as:

5′ G/AATTC 3′
3′ CTTAA/G 5′

If the 50 base pair (bp) DNA molecule shown below is cut with EcoRI, the result would be the two smaller DNA molecules shown.

```
5′ ATCGTAGGATCCTCGGAATATTCCGCGTATATCGGAATTCGGAACTCTCTC 3′
3′ TAGCATCCTAGGAGCCTTATAAGGCGCATATAGCCTTAAGCCTTGAGAGAG 5′
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↓

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5′ AATCGTAGGATCCTCGGAATATTCCGCGTATATCGG 3′                5′ AATTCGGAACTCTCTC 3′
3′ TTAGCATCCTAGGAGCCTTATAAGGCGCATATAGCCTTAA 5′               3′ GCCTTGAGAGAG 5′
```

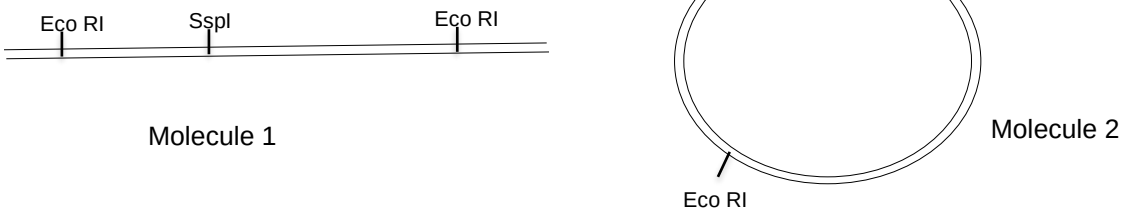
A different restriction enzyme called SspI recognizes the sequence:

5′ AAT/ATT 3′
3′ TTA/TAA 5′

Will SspI cut the 50 bp DNA sequence shown above? If so circle the resulting DNA fragments on the sequence above.

Drawings of two different DNA molecules are shown below and all the restriction enzyme sites for EcoRI and SspI are indicated in **Figure 3**.

Figure 3. Restriction enzyme sites on linear and circular DNA



*How many different **linear** fragments would you have if you…*

…cut Molecule 1 with EcoRI? _____. On the diagram, circle the resulting fragment that would move most quickly in an agarose gel during gel electrophoresis.

…cut Molecule 1 with SspI?_____.

…cut Molecule 2 with EcoRI? _____.

…cut Molecule 2 with SspI?_____.

In today's lab you will apply the concepts discussed above and use restriction enzymes and gel electrophoresis to identify two plasmids.

Mission (Im)possible:

Pre-Lab Questions

Directions: After reading through the introduction and protocol for the Mission (Im)possible lab, answer the questions below.

1. What is a plasmid, and where would it be found in nature?

2. You find that bacterial cells containing a specific plasmid live and reproduce in the presence of the antibiotic ampicillin, but bacterial cells without a plasmid are killed in the presence of ampicillin. What gene must be carried on this specific plasmid that allows cells to live and reproduce in the presence of the ampicillin? What is the function of the protein encoded by this gene?

3. Is DNA positively charged, negatively charged or uncharged?

4. Assume you make 1% agarose gel by melting 1 gram of agarose in 100 ml of buffer and use this gel to separate a large molecule of DNA from a small molecule of DNA. You run your gel for 30 minutes and find that the small DNA molecule has moved ½ way towards the positive electrode. If you were to repeat the experiment using a 2% agarose gel, do you expect the small molecule to move the same distance in 30 minutes or will it move a longer or shorter distance when compared to the 1% gel? Explain your reasoning.

5. You cut many molecules of the circular pSNAPf plasmid (size = 5849 base pairs) with restriction enzyme Q, and load the digested pSNAPf DNA into a well of an agarose gel. You separate the resulting DNA fragments using electrophoresis. You stain the gel and, when you view it, you see four different bands in the lane. Assume that enzyme Q cuts every molecule of pSNAPf at every possible restriction site.

   a. How many restriction sites for enzyme Q are there on the pSNAPf plasmid?

   b. Three of the four bands have the following sizes: 350 bp, 550 bp, and 1000 bp. What is the size (in base pairs) of the fourth band?

Mission (Im)possible

Lab Protocol

"Good Morning, Mr. Phelps. Your mission today is to determine the identity of the plasmid in each of the tubes labeled X and Z." The good news is that this is NOT an impossible mission. You know that one tube contains the plasmid pSNAPf and the other pMAL-5X. A drawing of each plasmid with relevant restriction enzyme sites (a **restriction map**) is shown below in **Figure 4.**

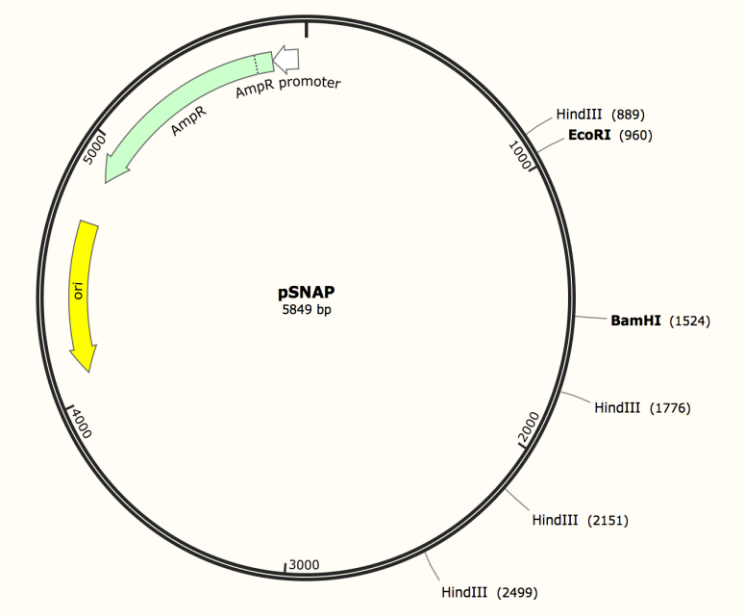
You have all needed buffers and the necessary reagents for gel electrophoresis. You and your partner will need to study the restriction maps of each plasmid and choose enzymes that will allow you to determine the identity of the plasmid in each of the tubes labeled plasmid X and plasmid Z.

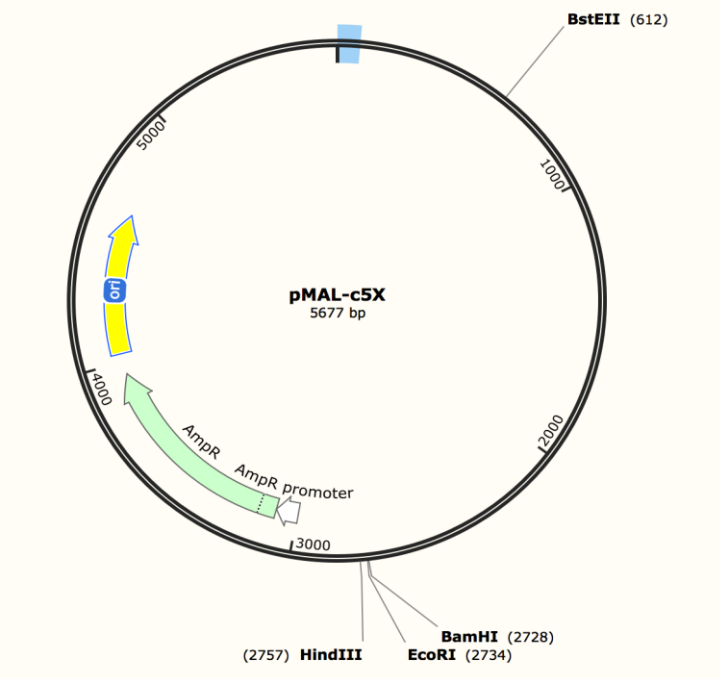
- *After considering the maps, which enzyme or enzymes will you use to determine the identity of unknown plasmids X and Z? (Please note that you can use two different enzymes in your reaction tube if need be.)*

- *What size fragments do you expect when pMAL-5X is cut with the restriction enzyme(s) you have listed above?*

- *What size fragments do you expect when pSNAPf is cut with the restriction enzyme(s) you have listed?*

Figure 4. Restriction maps of pSNAPf and pMAL-5X plasmids





Materials:

Check your workstations to make sure all supplies are present before beginning the lab.

Student Workstation:		Common Workstation:
1 tube	XE with 1 µL pMAL-c5X plasmid△	37°C water bath or incubator
1 tube	X with 1µ L pMAL-c5X plasmid△	UV or blue light source
1 tube	ZE with 1 µL pSNAPf plasmid△	microcentrifuge (optional)
1 tube	Z with 1 µL pSNAPf plasmid△	1X electrophoresis buffer
1 tube	dH_2O with 200 µL distilled water	ice buckets with restriction enzymes
1 tube	DNA Ladder with 12 µL Quick Load® Purple 1kb ladder	
1 tube	CutSmart® buffer with 30 µL buffer△	
1 tube	Loading Dye with 40 µL 6X loading dye	
1	agarose gel (1.0%) with DNA stain	
1	p20 micropipette and tips	
1	microcentrifuge tube rack	
1	microcentrifuge tube float	
1	ice bucket or Styrofoam cup with crushed ice	
1	ultrafine point permanent marker	
1	waste container	
1	gel electrophoresis unit with power supply	

△ **Caution: Enzymes, buffers and DNA are heat sensitive. Keep enzymes, buffers and DNA on ice at all times while working.**

Procedure:

1. Find the four tubes that are labeled as follows: **XE**, **X, ZE**, **and Z.** Write your initials on the tubes.

2. Using a p20 micropipette, set up the restriction digest you have planned for **plasmid X**, by adding the following reagants to the tube labeled **XE**. △**Caution: Make sure you use a clean tip for each reagent.**
   - ☑ 1 µL plasmid X DNA (added for you)
   - ☐ 40 or 42 µL* of sterile dH_2O
   - ☐ 5 µL of CutSmart Buffer
   - ☐ 2 µL of Enzyme 1 _____(write the name of enzyme here)
   - ☐ 2 µL of Enzyme 2* _____(write the name of enzyme here)

   *If you are using one enzyme, add 42 µL of dH_2O. If you are using two different enzymes, add 40 µL of dH_2O.

   *Using a second enzyme is opitional

   △ **Caution: Keep enzymes, buffers and DNA on ice.**

3. Using a p20 micropipette, set up control for **plasmid X**, by adding the following reagants to the tube labeled **X**. △ **Caution: Make sure you use a clean tip for each reagent.**
   - ☑ 1 µL plasmid X DNA (added for you)
   - ☐ 44 µL of sterile dH_2O
   - ☐ 5 µL of CutSmart Buffer

   △ **Caution: Keep enzymes, buffers and DNA on ice.**

4. Using a p20 micropipette, set up the restriction digest you have planned for **plasmid Z**, by adding the following reagants to the tube labeled **ZE**. ⚠ **Caution: Make sure you use a clean tip for each reagent.**
   - ☑ 1 µL plasmid Z DNA (added for you)
   - ☐ 40 or 42 µL* of sterile dH_2O
   - ☐ 5 µL of CutSmart Buffer
   - ☐ 2 µL of Enzyme 1 _____(write the name of enzyme here)
   - ☐ 2 µL of Enzyme 2* _____(write the name of enzyme here)

   ⚠ **Caution: Keep enzymes, buffers and DNA on ice.**

*If you are using one enzyme, add 42 µL of dH_2O. If you are using two different enzymes, add 40 µL of dH_2O.

*Using a second enzyme is opitional

5. Using a p20 micropipette, set up control for **plasmid Z**, by adding the following reagants to the tube labeled **Z**. ⚠ **Caution: Make sure you use a clean tip for each reagent.**
   - ☑ 1 µL plasmid Z DNA (added for you)
   - ☐ 44 µL of sterile dH_2O
   - ☐ 5 µL of CutSmart Buffer

   ⚠ **Caution: Keep enzymes, buffers and DNA on ice.**

6. Using the centrifuge, quickly spin the tubes to get all the reagents to the bottom. If you do not have a centrifuge, you can gently tap the tubes on the bench to collect the contents in the bottom.

7. Put the tubes in the 37°C water bath for 15-30 minutes or for the time it takes you to make your gel.
   ⓘ **Tip: The tube of ladder can remain in the rack on your bench.**

8. Prepare or get a 1.0% gel and 1X electrophoresis buffer as instructed by your teacher.

   🛑**Stopping Point – Check with your teacher before continuing with the protocol.**

9. After the tubes containing plasmid X and Z have incubated, remove them from the water bath.

10. Add 8 µL of loading dye to each of the four tubes containing plasmid DNA: **XE, X, ZE and Z.**
    ⚠ **Caution: Make sure you use a clean tip for each reaction tube.**

11. Mix by flicking the tubes and pool reagents at the bottom of the tubes by centrifuging or tapping on the bench.

12. Assemble the gel box, and position it so you can load it and run it without moving it.

    ⓘ **Tip: Check to make sure that the gel tray is in the correction orientation with the wells closest to the negative electrode.**

13. Load **10 µL** of the **ladder** into the gel. Record the location of the well in **Table 2** below.

14. △**Using a clean tip for each sample**, load **20 µL** of each sample into the gel (**XE, X, ZE and Z**). Record the location of the wells of each sample in **Table 2**

    ⓘ**Reminder: The solution in each of these tubes should be purple or dark blue.**

Table 2: Gel electrophoresis

Lane (from left to right)	1	2	3	4	5	6	7	8
Sample name								

15. Run your samples as instructed by your teachers until the front loading dye is two-thirds of the way down the gel.

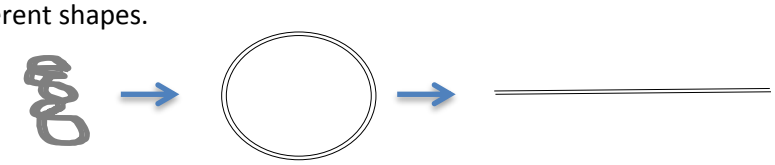
While the gel is running, discuss the following questions with a partner.

The DNA ladder tube contains different-sized DNA molecules each of a known size. What is the purpose of this tube in your experiment?

Remember that you did not add enzyme to the tubes X No enz control and the Z No enz control. What is the purpose of each of these controls, and why are they important?

Would you expect the DNA in the X- control and the Z- control tubes to be circular or linear?

Circular plasmid DNA within cells is twisted up into a compact, supercoiled shape. Often when plasmid DNA is extracted from bacterial cells, one of the DNA strands is broken which allows the plasmid to relax into a circular shape. Sometimes, both strands of the DNA are broken and this results in a linear molecule. All three forms of the molecule have the same number of base pairs (bp), but they have different shapes.



These three forms of a plasmid move through the agarose gel at different rates. Which of the three forms shown above would move the fastest through the agarose gel? Explain your reasoning.

You have learned that a tube of "uncut" plasmid is actually contains a population of molecules, some are supercoiled, some are relaxed circular, and some are linear. How are the linear DNA molecules found in the X-control tube different from the majority of linear DNA molecules found in the X + enzyme tube?

Mission (Im)possible:

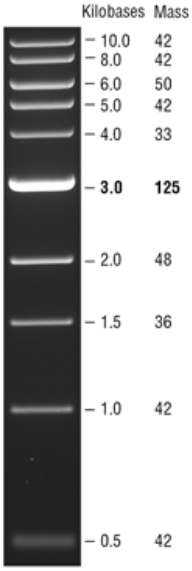
Data Collection Worksheet

On the image below draw what you see after gel electrophoresis. Refer to the ideal image of the 1Kb Ladder to the right when determining the size of the visible bands.

−electrode

+electrode

1 kb DNA Ladder *visualized on a 0.8% TAE agarose gel.*



- Do you see a single band or multiple bands in the X and Z control lanes?

- If you see multiple bands in the X and Z control lanes, what is your explanation? Your explanation should include a prediction of what molecules are represented in each band.

- Given your results, what is the identity of unknown plasmid X?

- Given your results, what is the identity of unknown plasmid Z?

Mission (Im)possible:

Post-Lab Questions and Analysis

Directions: After completing the Mission (Im)possible lab, answer the questions below.

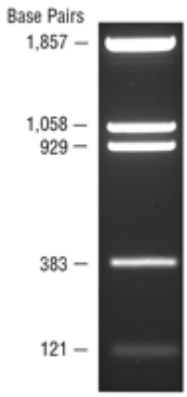
1. If you cut the two different plasmids with only EcoRI or BamHI, could you for distinguish pMAL-5X from pSNAPf? Explain why or why not.

2. Did your experiment allow you to identify which plasmid was in tube X and which was in tube Z? If your experiment did not allow you to identify the plasmids, explain what happened that prevented you from obtaining the needed data, and suggest changes you could make if you were to repeat the experiment.

3.The photo below shows the electrophoresis pattern of the plasmid pBR322 cut with a single restriction enzyme.

If prior to restriction digest you had a million molecules of the plasmid, how many molecules would be represented in each band?

What is the total size (in base pairs) of pBR322?



4. Explain why the brightness of the bands in the photo above decreases as the size of the fragments decreases. Hint: The stains used to visualize the DNA are called **intercalating stain.** The stain molecules bind to DNA by inserting between the nitrogenous bases of DNA.