DNA restriction analysis of pGLO plasmid DNA

a) Introduction

Restriction enzymes (also called restriction endonucleases) are enzymes that cut DNA. These enzymes occur naturally in bacteria, where they protect the bacteria by destroying DNA from virus that invade the bacteria.

Restriction enzymes are **not** able to cut the DNA just at any location. Each enzyme has a specific DNA sequence at which it binds to and cuts the DNA. This sequence is called the restriction site of the enzyme. For example, one restriction enzyme (called *Eco RI*) has the restriction site shown on the left. The enzyme cuts the DNA as shown under the arrow.

```
5' GAATTC  3'
3' CTTAAG  5'
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```
5' G      AATTC 3'
3' CTTAA      G 5'
```

Only at this sequence will *Eco RI* cut a piece of DNA, and it will cut the DNA at every location that has this sequence.

There are hundreds of different restriction enzymes. They are usually named after the bacteria from which they were first discovered. Different enzymes have different restriction sites. For example, another restriction enzyme (called *Xho I*) has the restriction site shown on the right. The enzyme cuts the DNA as shown under the arrow.

```
5' CTCGAG  3'
3' GAGCTC  5'
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```
5' C      TCGAG 3'
3' GAGCT      C 5'
```

Notice some things about the restriction sites. Both enzymes restriction sites are 6 base pairs long. Most restriction sites are between four to eight nucleotides in length, but six is the most common length. Also notice that both sites shown above are symmetrical, meaning that the sequence on both strands is the same if read in the 5' to 3' direction. This is

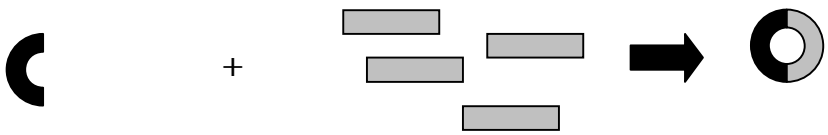
also a typical feature of restriction sites. Lastly, notice that the enzymes cut the DNA in a pattern that leaves complementary overhanging single-stranded regions. These single-stranded regions are called "sticky ends" because they tend to stick to each other by complementary base pairing.

If the sticky ends come together by complementary base pairing, does this restore the DNA back to its original uncut state? The answer is no. The nitrogenous bases of the sticky ends may stick together, but only weakly because only the hydrogen bonds between the complementary bases are keeping the DNA pieces together. To permanently rejoin the cut DNA strands, the backbone of the two DNA strands (the chain of phosphates and riboses) must be linked together again. An enzyme called DNA Ligase carries out this function. In a sense, you can think of DNA Ligase enzyme as being the opposite of a restriction enzyme. Restriction enzymes cut DNA and Ligase enzyme joins DNA.

b) Uses of restriction enzymes in molecular biology

Restriction enzymes and Ligase enzyme are two of the most widely used tools of molecular biologists (biologists who specialize in working with DNA). The main function that these enzymes are used for is making recombinant DNA molecules. A recombinant DNA molecule is a piece of DNA made by joining two DNA molecules from different sources, often different species.

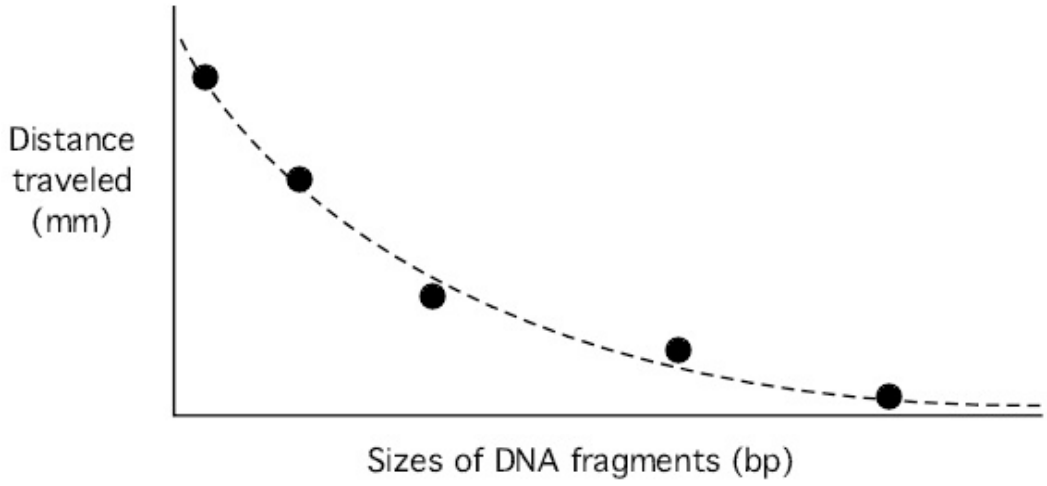
Imagine that a bacterial plasmid is cut open with a restriction enzyme like *Eco RI* (see diagram below). Imagine that the DNA of a human being is also cut with the same enzyme. Because the enzyme leaves the same sticky ends on all pieces of DNA, the human DNA pieces will stick to the plasmid DNA pieces. Adding Ligase enzyme will permanently join the human DNA to the plasmid DNA. The recombinant plasmid can be put into a bacteria where it will reproduce every time the bacteria divides. In this way many copies of the human DNA piece can be made.



Cut plasmid | Cut Human DNA pieces | Recombinant plasmid

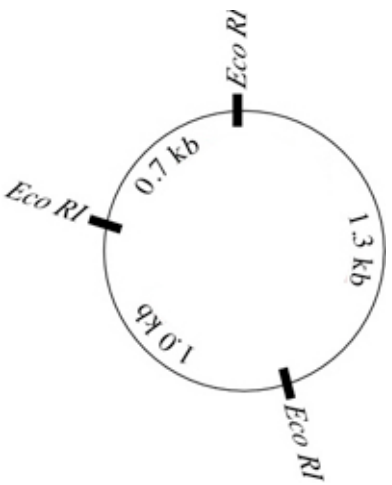
Another use of restriction enzymes in molecular biology is to make a "restriction map" of a piece of DNA. A restriction map is a drawing that shows the location of restriction sites on the piece of DNA and the distance between neighboring restriction sites. Such maps are extremely useful when planning how to construct a recombinant DNA molecule.

Restriction maps are made by first digesting a piece of DNA with a restriction enzyme, then separating the fragments using agarose gel electrophoresis. Recall that agarose gel electrophoresis separates DNA fragments of different sizes, but how can it be used to determine what those sizes actually are? To determine the sizes of the fragments, one lane of the gel is loaded with a "DNA size standard", which is a group of DNA fragments of known sizes. After the electrophoresis is completed, a graph is made of the distance traveled by each of the standard's DNA fragments (on the X axis) versus the size of each fragment (on the Y axis). The sizes of DNA fragments are usually measured in base pairs (bp) or kilobases (kb). The graph is a "standard curve", which is a graph that can be used to measure some unknown quantity (in this case, the size of the DNA fragments from the digestion).



The distances traveled by the DNA fragments of *unknown* size are measured on the gel. By finding where those distances match with the standard curve line, their sizes can be determined from the graph's Y-axis.

Once the sizes of the DNA fragments are known, a restriction map of the plasmid can be made. For example, suppose a plasmid 3 kb in length (remember, one kilobase = 1000 nucleotides) is digested with *Eco RI* and the fragments are run on an agarose gel to determine their sizes. If three fragments of lengths 0.7 kb, 1.0 kb, and 1.3 kb are found, the plasmid map must be as shown on the right. The numbers show the distances between each restriction site.



Take note of the proper format of a plasmid restriction map: (1) It shows each restriction site as a hash mark on the circle, (2) Each restriction site hash mark is labeled with the name of the restriction enzyme that cuts there, and (3) the distance (in kilobases) between each pair of neighboring restriction site hash marks is shown between the hash marks.

c) Overview of the restriction mapping experiment.

The restriction mapping of the pGLO plasmid will be divided into three laboratory periods.

Day 1 = Restriction digestion of the plasmid. On day one you will digest the pGLO plasmid DNA you purified last week with the restriction enzymes Eco RV and Pst I (alone and in combination). You will freeze the digested plasmid DNA until the next laboratory period.

Day 2 = Agarose gel electrophoresis of the digested plasmid DNA. The DNA will be loaded onto a 1% agarose gel, electrophoresed for about an hour, and then photographed.

Day 3 = Graphical analysis of the restriction digestions. Analysis of the gel will be used to construct a restriction map of the plasmid.

d) Procedure for day 1 (restriction digestions)

1) Obtain three empty microcentrifuge tubes, a p20 micropipette with disposable tips, and your purified plasmid DNA. Label the three microfuge tubes:
"Eco RV", "Pst I", and "Eco RV + Pst I".

Also, write your group name on each tube.

The buffer and the enzymes that you will add to these tubes are on the front desk. When working with very small volumes (such as 1 ul) it is very easy to pipette too much Therefore **show your instructor your pipette tip containing the 1 ul of buffer or enzyme before you add it to your digestion tubes.**

2) To the "Eco RV" tube, add the following:

8 ul plasmid DNA
1 ul 10X Buffer for Eco RV (this buffer is called buffer D)*
1 ul Eco RV restriction enzyme**

* Each restriction enzyme has its own buffer, which has a pH and ion concentration that is optimum for that enzyme. Be sure you are adding the buffer that matches the enzyme.

** The restriction enzymes are supplied to us dissolved in glycerol, which helps preserve the enzyme. But glycerol can cause the enzyme to cut the DNA at incorrect restriction sites if it is above 10% of the total volume. Therefore, the volume of a restriction enzyme should never be more than 10% of the total digestion volume. Notice that in all the digestions today, the restriction enzyme volume is never above 10% of the total volume.

To the "Pst I" tube, add the following:

8 ul plasmid DNA
1 ul 10X Buffer for Pst I (this buffer is called buffer H)
1 ul Pst I restriction enzyme

3) The "Eco RV + Pst I" tube is the double digest tube. It must contain both enzymes and a "universal buffer" that both restriction enzymes can function in. Use this mixture

16 ul plasmid DNA
2 ul 10X universal buffer
1 ul Eco RV
1 ul Pst I

Place all three of your tubes into the microcentrifuge and give them a brief spin to collect all the contents together at the bottom of the tube. Remember that all tubes must be in a balanced arrangement in a centrifuge. After centrifuging, do the following to each test tube: Put a fresh tip on the pipette, set it to half the volume in the test tube, and pipette up and down gently to thoroughly mix all the solutions in the test tube. Try not to introduce any bubbles into the digestion mixture (if you do, re-centrifuge the mixture to remove the bubbles). **Show your instructor your tubes before continuing.**

4) Place all three tubes in the 37ºC water bath for one hour. They can be incubated for up to 2 hours or so, but if they are left too long, the sticky ends of the DNA fragments may be degraded.

5) After one hour at 37ºC, remove your digestions from the water bath. Place them on the rack on the front desk. Your instructor will freeze them until the next laboratory period. This is the end of the day one activities.

e) Procedure for day 2 (agarose gel electrophoresis)

Today the DNA fragments from the restriction enzyme digestions will be analyzed on a 1.0% agarose gel. The fragments of DNA will migrate through the gel, with the smaller pieces migrating faster than the larger ones. How many pieces will there be? It depends on the number of restriction sites. There will be as many pieces of DNA as there are restriction sites.

The DNA from each digestion will be loaded into separate wells on the gel. The DNA size marker is also loaded onto the gel, in its own lane. Before loading, all DNA samples are mixed with a loading dye. It contains glycerol (a very dense substance to help the DNA sink into the wells) and a dye called bromophenol blue (to help see that the samples are loaded into the

wells). We cannot actually see the DNA while the gel runs, but the blue dye migrates ahead of the DNA. We stop the gel when the dye front approaches the edge of the gel.

The agarose gel was mixed with Nancy520. This is an orange dye that inserts itself between nucleotides. It fluoresces (glows) brightly under ultraviolet (UV) light. Its purpose is to make the DNA in the gel visible under UV light. Nancy520, however, must be handled with caution; since it reacts with DNA in a gel, it can react with DNA in your own cells, too. Exposure to large amounts can cause mutations and cancer. For that reason, **you must wear gloves when handling the agarose gels.**

After electrophoresis, the gels will be viewed under ultraviolet (UV) light to see the Nancy520 stained DNA bands. (Another DNA-staining dye, called Methylene blue, is sometimes used in teaching labs because it is safer. However, it is much less sensitive and the staining reaction takes much longer). The gels will be photographed to make a permanent record of the DNA bands. We will use the photograph of the gel (not the gel itself) when we analyze the DNA fragments on day 3.

1) Obtain your three digestion tubes and a 1% agarose gel. Remember that this gel contains the toxic compound Nancy520. Gloves must be worn when handling it. Also obtain a p20 micropipette and tips.

2) Carefully remove the comb and the rubber dams from the gel and place the gel (still in its casting tray) in the gel box. Add approximately 250 ml of the 1X TAE buffer to the box. Pour the solution into one end of the box, allowing it to wash over the gel and into the other end. The gel should be completely, but barely, submerged in the buffer. Be sure that the wells in the gel are filled with buffer.

3) Obtain a microcentrifuge tube and label it “DNA size standard”. Using your micropipette, transfer 10 ul of the DNA size standard on the front desk into the microcentrifuge tube.

4) Add 10X loading dye to all four tubes. What volume of loading dye do you add to each tube? The loading dye must be 1X concentration after it has been added to the DNA. Therefore the ul of 10X loading dye that you add must be 1/10 of the volume of each DNA sample. This ensures that the 10X loading dye becomes diluted by a factor of 10, meaning it becomes 1X

concentration. **Show your instructor your calculations (on the next page) before proceeding.**

The Eco RV digestion requires ____ ul of loading dye.
The Pst I digestion requires ____ ul of loading dye.
The Eco RV + Pst I digestion requires ____ ul of loading dye.
The DNA size standard requires ____ ul of loading dye.

As you add the loading dye, pipette gently up and down a few times to mix the dye with the contents of your tubes. Try not to introduce any bubbles into the mixture.

5) After the loading dye has been added to each tube, place all four of your tubes into the microcentrifuge and give them a brief spin to collect all the contents together at the bottom of the tube. Remember that all tubes must be in a balanced arrangement in a centrifuge.

6) Set a micropipette for the entire volume in each tube, then load each sample into its own well on the gel. Record which sample was loaded into each well in the table below. It is traditional to load the molecular weight marker in the first lane.

Lane 1 = _____	Lane 2 = _____
Lane 3 = _____	Lane 4 = _____
Lane 5 = _____	Lane 6 = _____

7) Set the power supply to run at a constant 100 volts. Refer to the *Electrophoresis* lab handout for directions in setting the power supply for a constant voltage. Stop the gel when the violet (darker blue) dye reaches the bottom of the gel. This should take between 30 minutes and one hour.

8) When the electrophoresis is done, turn the power supply settings to zero and turn off the power, and unplug it. Carefully remove the gel from the box (it might slide easily off the tray). Place the gel in the ultraviolet viewing box (which has a built-in digital camera). Your instructor will assist you in photographing your gel with a ruler at its side.

9) In data table 1, tape the photograph of your gel.

10) Clean up: All materials contain Nancy520, a toxic material. Therefore, nothing goes in the trash or down the sink. Place your gel in a special

disposal tray, place the clear plastic gel tray, the comb, and the dams in another special tray. Place the gel box (with the TAE buffer still in it) back on the cart where you obtained it. The laboratory technician will clean these materials. This is the end of the day two activities.

f) Procedure for day 3 (graphing DNA band distances)

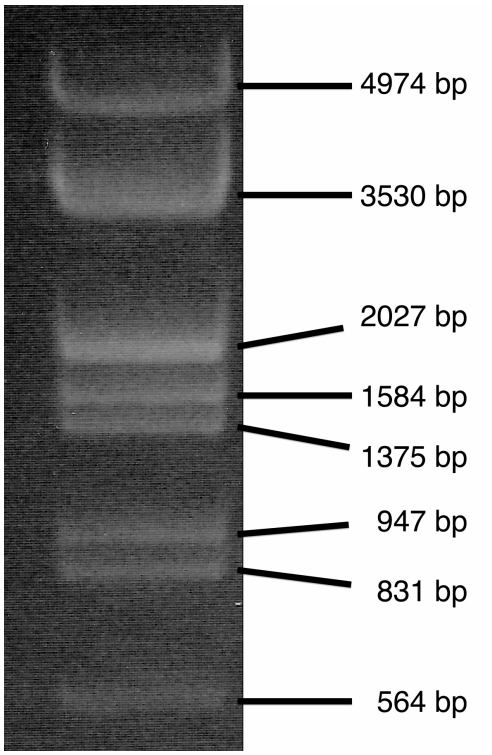
Overview: Using the photograph of the gel, the distance migrated by each band is measured. A standard curve of distance versus size is made using the bands in the DNA size standard lane. The sizes of the plasmid digest fragments can then be determined using the standard curve.

1) **Labeling the graph axes:** Obtain the graph paper from the last sheet of this handout. In the next step, you will use the graph paper to plot distance migrated versus fragment size. Note that it is "Semi-Log" graph paper. The Y-axis has normal spacing but the lines on the numbered X axis are at irregular "log" distances. The reason for using this type of graph paper is that it transforms the distance vs. size plot into a straight line. If you used normal graph paper, the plot would curve steeply downward for smaller pieces (see the illustration on page 3 of this handout) because small pieces travel faster by the *log* of their size difference compared to a larger piece.

Look at the X-axis again. Notice it has two "cycles", meaning it has two areas where the lines get closer and closer. Each cycle has 10 major lines. Label the 10 major lines in the first cycle 100, 200, 300, etc. all the way to 1000. Label the major lines of the second cycle 2000, 3000, etc. Each axis on a scientific graph must always have a label stating (a) what that axis is showing and, (b) what units it is being shown in. Write this label under the X-axis.

Mark the Y-axis in mm. The Y-axis should go from zero to at least your largest mm distance. You may have to experiment with how many mm per line on the Y-axis to fit the mm correctly. Write a label for the Y-axis. **Show your instructor when you are done.**

2) **Making a standard curve:**
The diagram on the right shows the same DNA size marker that you put in your agarose gel. Identify the these eight marker bands on your own gel, then use a ruler on your gel photograph to find the distance migrated by each marker band (in mm) from the well.



In data table 2, record the distance migrated by each marker band in your gel.

Next, plot the six marker bands on data table 2 onto the semi-log graph paper. In other words, make a standard curve of distance migrated (in mm on Y-axis) versus marker fragment size (in bp on X-axis). After the six size marker points have been plotted on the graph paper, use a ruler to draw the best-fit straight line for all six points on the graph. This is a single straight line that has equal numbers of points above and below the line. This graph is your "standard curve" for the agarose gel.

3) **Making a restriction map of the pGLO plasmid:** For each DNA fragment in your plasmid digestions, calculate its length in bases using the standard curve. To do this, first find the fragment's migration distance on the Y-axis, then move straight right until you intersect the best fit line. Then move straight downward to the X-axis. The point where you intersect the X-axis is the size of the fragment in bp. Record the distance migrated and the size of each restriction fragment in data table 3.

Using the sizes of each fragment, draw a Eco RV and Pst I restriction site map of the plasmid on data table 4. Use the figure on page 4 as an example. Be sure that your map shows each restriction site as a dash across the circle and that each restriction site dash is labeled with the restriction enzyme that cuts the plasmid at that site. Also be sure your map shows the distances between each restriction site and that it accurately corresponds to the fragments from all three of your digestions. Again, use the figure on page 4

as an example if you are not sure how the map is supposed to look. Show your instructor the map when you are finished.

e) Data tables

Data table 1: (Tape a photograph of the gel on back of this handout):

Data table 2: DNA size (bp) and distance migrated (mm) for the DNA size standard. After completing this data table, plot the data on the graph paper.

DNA size (bp)	Distance migrated (mm)
4974	_____
3530	_____
2027	_____
1584	_____
1375	_____
947	_____
831	_____
564	_____

Data table 3: For each of the three plasmid digestions, list the distance migrated (mm) and the DNA size (bp) for all bands.

Eco RV Digestion:

Distance Migrated (mm):	Band size (bp):

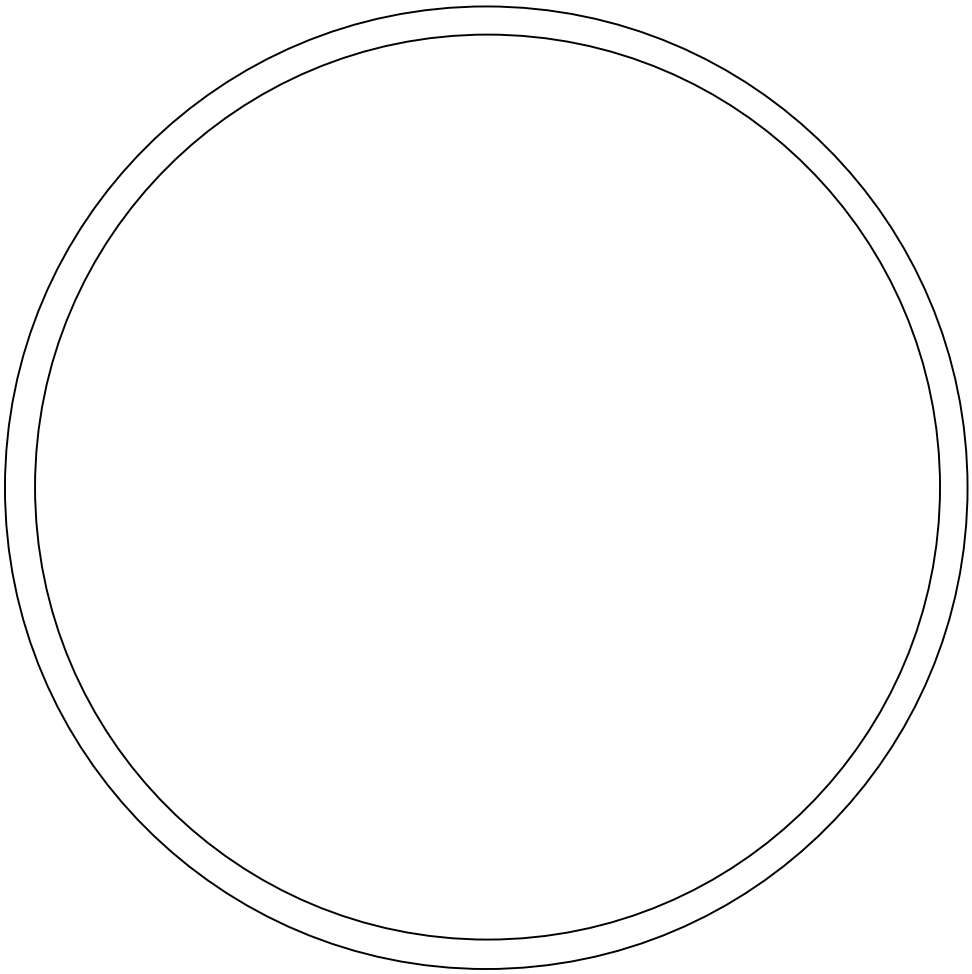
Pst I Digestion:

Distance Migrated (mm):	Band size (bp):

Eco RV + Pst I Digestion:

Distance Migrated (mm):	Band size (bp):

Data table 4:
Restriction map of pGLO plasmid



g) Review questions

1) How many Eco RI sites are in the following piece of DNA?

5' GTGTCACTCTATCAGAATTCACGTGAATTCACGTACGTGGTGAAA 3'
3' CACAGTGAGATAGTCTTAAGTGCACTTAAGTGCATGCACCACTTT 5'

2) Define each of the following terms:
a) Restriction enzyme:

b) Ligase enzyme:

c) Molecular biology:

d) Sticky end:

e) Recombinant DNA:

3) Why should the volume of a restriction enzyme in a digestion not exceed 10% of the total volume?

4) Suppose you need to do a triple digestion (Enzymes *Eco RI, Xho I, and Hind III* at the same time) and the wells in your gel can hold a maximum of 15 ul. Write the complete ingredients list of what you would mix into the tes tube to set up the digestion. The buffer is 10X buffer.

5) Restriction enzymes will digest DNA at room temperature. Why then is the digestion done at 37 C?

Why was the digestion not done at a temperature higher than 37 C?

6) Suppose you need to cast a 50 ml 0.7% agarose gel in TAE buffer. Your starting materials are 10X TAE and powdered agarose. State how much of each ingredient (and how much water) you would mix to prepare the gel.

7) Explain why semi-log graph paper is used for determining the size of DNA fragments.

8) A plasmid was digested with restriction enzymes *Hind III* and *Eco RV*. The band sizes are given below. Use the data to construct a restriction map of the plasmid.

Hind III: 4.8 kb
Eco RV: 3.0 kb and 1.8 kb
Hind III + Eco RV: 3.0 kb, 1.1 kb, and 0.7 kb

9) A plasmid was digested with restriction enzymes *Sac I* and *Xba I*. The fragment sizes are given below. Use the data to construct a restriction map of the plasmid.

Sac I: 1.7 kb and 3.5 kb

Xba I: 2 kb and 3.2 kb

Sac I + Xba I: 0.5 kb, 0.8 kb, 1.2 kb, and 2.7 kb

10) A plasmid was digested with restriction enzymes *Bam HI* and *Eco RV*. The band sizes are given below. Use the data to construct a restriction map of the plasmid.

Bam HI: 0.4 kb, 1.2 kb, 1.8 kb, and 2.2 kb

Eco RV: 1.4 kb and 4.2 kb

Bam H+ EcoRV: 0.3 kb, 0.4 kb, 0.5 kb, 1.2 kb, 1.4 kb, and 1.8 kb

11) All scientific graphs should have what next to each axis?

12) How many bases long is a typical restriction enzyme's restriction site? Give a range: _____

13) A restriction site is 6 bases long. The first three bases are 5' AGC. Write in all the other bases (the next 3 in the strand and the complementary bases on the opposite strand) using principles discussed in the introduction.