RESTRICTION ENZYME ANALYSIS – Methylene Blue stain

Protocol for Students

Background reading

Bacteriophage λ is a virus that attacks bacterial cells and is one of the most studied viruses. The information from the relatively simple virus genomes has been used to test theories and develop concepts that apply to the genetics of living organisms. The DNA of Bacteriophage λ is approximately 48,514 base pairs or 48.514 kilobase pairs in length while human genome is approximately 3 billion base pairs.

This experiment uses special "restriction" enzymes that act as chemical scissors to cut λ DNA into pieces. Each enzyme recognizes a unique sequence of 4-6 bases along the DNA strand and cuts the strand at these sites - the first step in a process called restriction mapping. These smaller, specific sections of an organism's DNA can then be studied in detail and an outline of the whole genome can be constructed. This procedure is one of the most important in modern biology.

The small fragments of DNA are separated by electrophoresis. The movement of the fragments will always be towards the positive electrode because DNA is a negatively charged molecule. The fragments move through the gel at a rate that is determined by their size and shape, with the smallest moving the fastest.

DNA cannot be seen as it moves through the gel. A loading dye must be added to each of the samples before it is pipetted into the wells. The progress of the dye can be seen in the gel. It will initially appear as a blue band, eventually resolving into two bands of colors.

The faster moving, purplish band is bromophenol blue dye that migrates at roughly the same rate as a 300 base pair fragment of DNA. The slower moving aqua band is xylene cyanol, nearly equivalent to a 9000 base pair fragment. The faster moving band must move at least 4-7cm from the wells to achieve the best separation of DNA for analysis. Care should be taken not to let the bromophenol blue band run off the end of the gel.

Following staining to locate the DNA the gel is observed and the fragments appear as a pattern of bands. In this protocol, we will compare our banding pattern with a predicted result shown in figure 1.

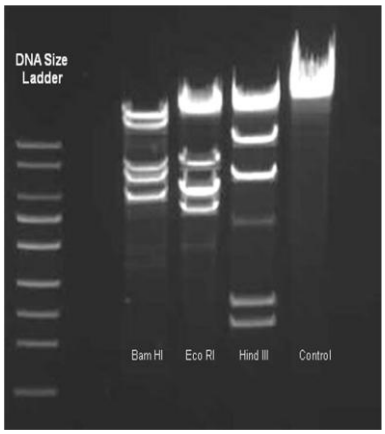

Figure 1: Lambda DNA restriction digest (Photo from J. Leach laboratory)

Information may be provided by your teacher that details the process of isolating and analyzing these bands to create a DNA fingerprint.

Objectives

1. Understand what a DNA restriction enzyme is and how it works.
2. Learn to use a micropipette.
3. Learn to separate DNA on an agarose gel using electrophoresis.
4. Understand how to use a restriction digestion map to identify a sample DNA.
5. Compare the λ DNA bands on a gel to the known λ DNA restriction map.

Materials

For each Lab Group

- Four microtubes
- Microtube rack
- 20 µl micropipette and sterile tips
- Waterproof pen
- 250 µl distilled water
- Gloves
- 100 µl 3X loading dye (day 2)
- 1.0% agarose gel (day 2)

Common Materials

- Beaker or foam cups with ice for each of the following:
  - 20 µl of 0.4 µg/µl λ DNA
  - 2.5 µl *Bam*HI restriction enzyme

    - 2.5 µl *Eco*RI restriction enzyme
    - 2.5 µl *Hind*III restriction enzyme
- 500 ml beaker (day 2
- Colored lab tape (day 2)
- Electrophoresis chamber (day 2)
- Power supply (day 2)
- Container with TBE buffer (1X)
- 37°C water bath w/floating rack
- 60°C water bath w/floating rack
- Cooler with crushed ice
- Freezer (non-frost-free, if possible)
- Distilled water
- 0.002% methylene blue stain (day 3)
- Stain container (day 3)

Precautions

The methylene blue dye will stain skin, clothes, and equipment. Always wear gloves and safety glasses. Do all staining in a central area near the sink.

Procedure

1. Put on gloves. **Keep all enzyme and DNA aliquots on ice through step 6.**
2. Label 4 microtubes, as indicated below, and place them in the tube rack:

    *Bam*HI

    *Hind*III

    *Eco*RI

    Control

Reagents	*Bam*HI	*Eco*RI	*Hind*III	Control
10X buffer	4 µl	4 µl	4 µl	4 µl
DNA	4 µl	4 µl	4 µl	4 µl
BamHI	2 µl	0	0	0
EcoRI	0	2 µl	0	0
HindIII	0	0	2 µl	0
Water	30 µl	30 µl	30 µl	32 µl

3. Set the micropipette to 4 µl and carefully add 4 µl of 10X restriction buffer to each tube. When adding the droplets of buffer to the restriction tube, touch the pipette tip to the bottom of the tube. Use a new tip for each buffer.
4. Set the micropipette to 4 µl and carefully add 4 µl of DNA to each tube, using a fresh tip each time.
5. Add 32 µl of distilled water to the control tube and 30 µl to the other reaction tubes.
6. Close the microtubes and heat in a 55°C waterbath for 10 minutes then immediately place on ice for 2 minutes.
7. Add 2 µl of the appropriate restriction enzyme to the reaction tubes as indicated on the grid. Use a fresh tip for each enzyme added.
8. Close the microtube caps and make sure that all the liquid is at the bottom of the tube. Give the tubes to the instructor. They will be incubated at 37°C overnight. The tubes will then be frozen until the next class (or up to 2 months). Each reaction will provide enough reaction product to load into two wells.

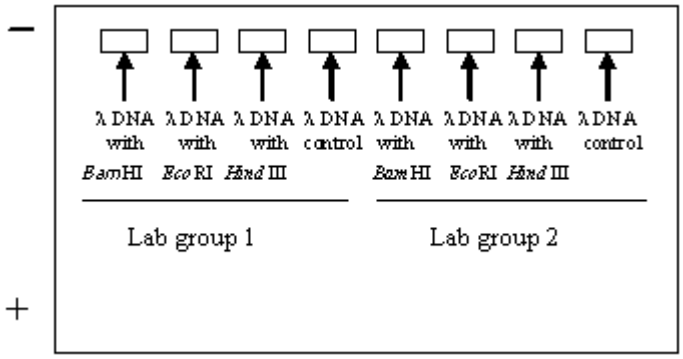

Figure 2. Sample loading into wells of gel

Day 2

1. Put on gloves. Fill a styrofoam cup with ice, collect your tubes and keep on ice until needed.
2. The 1.0% agarose gel will be placed into the gel box with the wells at the **negative (black)** end of the box.
3. Add approximately 150 ml of 1X TBE solution to the box so that the gel will be covered with about 2mm of buffer. Remove the comb by pulling straight up, making sure that the buffer covers the gel so that it will fill the wells and help them to retain their shape as the comb is removed.

4. **Heat the microtubes in a 60°C water bath for 3 minutes.** This will break any hydrogen bonds holding the ends of the linear DNA together in a circle.
5. Add 10 µl of loading dye to the bottom of each of the microtubes and eject the tip into the tube. Addition of the loading dye will also stop the restriction reaction taking place in each tube. (The reaction can be stored in the refrigerator at this point for use at a later date if necessary, in which case do not leave the tips in the tubes.)
6. Set up the electrophoresis apparatus as described in **Gel Electrophoresis of Dyes -** Activity 2**.**
7. Load 20 µl of each sample into a well as shown in figure 2 above. Use the tips that were left in each tube or make sure that you use a new tip for each sample if you stored the tubes overnight. Turn on and run the electrophoresis for about 30-45 minutes. When the purple dye from the loading dye is about 1 cm from the end of the gel, the power supply should be turned off and the gel box unplugged.
8. Place gel in a 0.002% methylene blue solution in 0.1X TBE and stain overnight at 4°C or for 2 hours at room temperature.

Day 3

1. Observe the gel over a white light. If destaining is needed to increase the visibility of the bands place the gel in 0.1X TBE with gentle agitation, changing the buffer every 30-60 minutes until you are satisfied with the degree of destaining. (from http://wheat.pw.usda.gov/zlazo/methods/lazo/metl.html.)
2. Photograph if desired.
3. Wash the area thoroughly to be sure that no stain solution is left in contact with surfaces. Wash your hands!
4. Complete the activity sheet and appropriate forensics activities from either website below.

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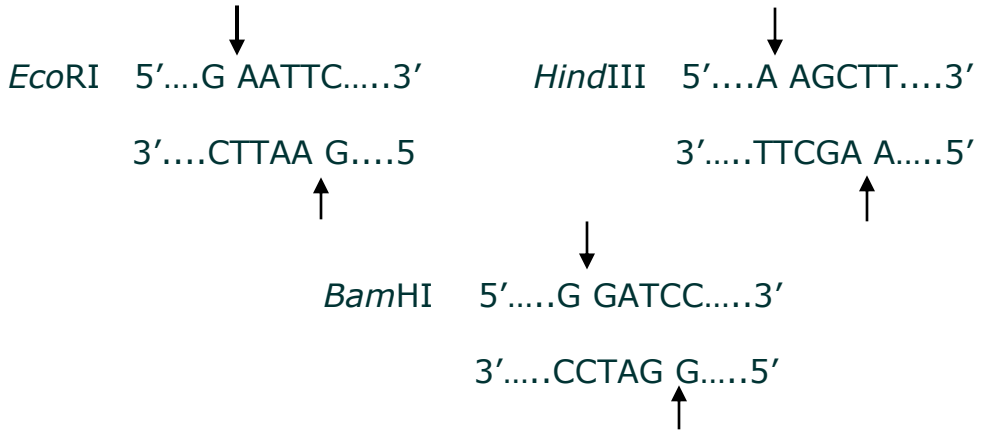
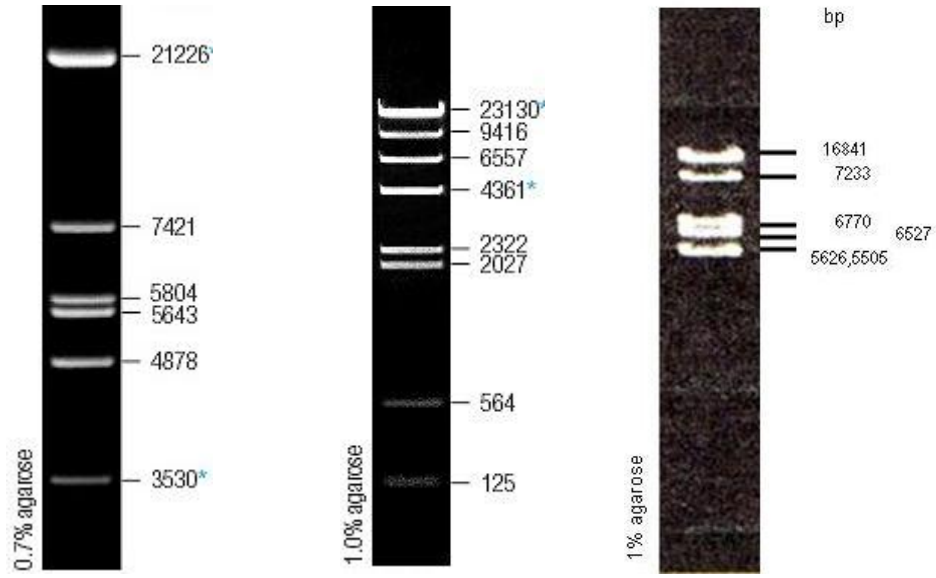
Student Activity

Restriction enzymes cut at specific sites along the DNA. These sites are determined by the sequence of bases which usually form ***palindromes***. Palindromes are groups of letters that read the same in both the forward and backwards orientation. In the case of DNA the letters are found on both the forward and the reverse strands of the DNA. For example, the 5' to 3' strand may have the sequence

GAATTC. The complimentary bases on the opposite strand will be CTTAAG which is of course the same as reading the first strand backwards! Many enzymes recognize these types of sequences and will attach to the DNA at this site along the DNA and then cut the strand between two of the bases. The restriction enzymes which we used in this laboratory are *Eco*RI, *Hind*III and *Bam*HI and their sequences are as follows, with the cut site indicated by the arrow.

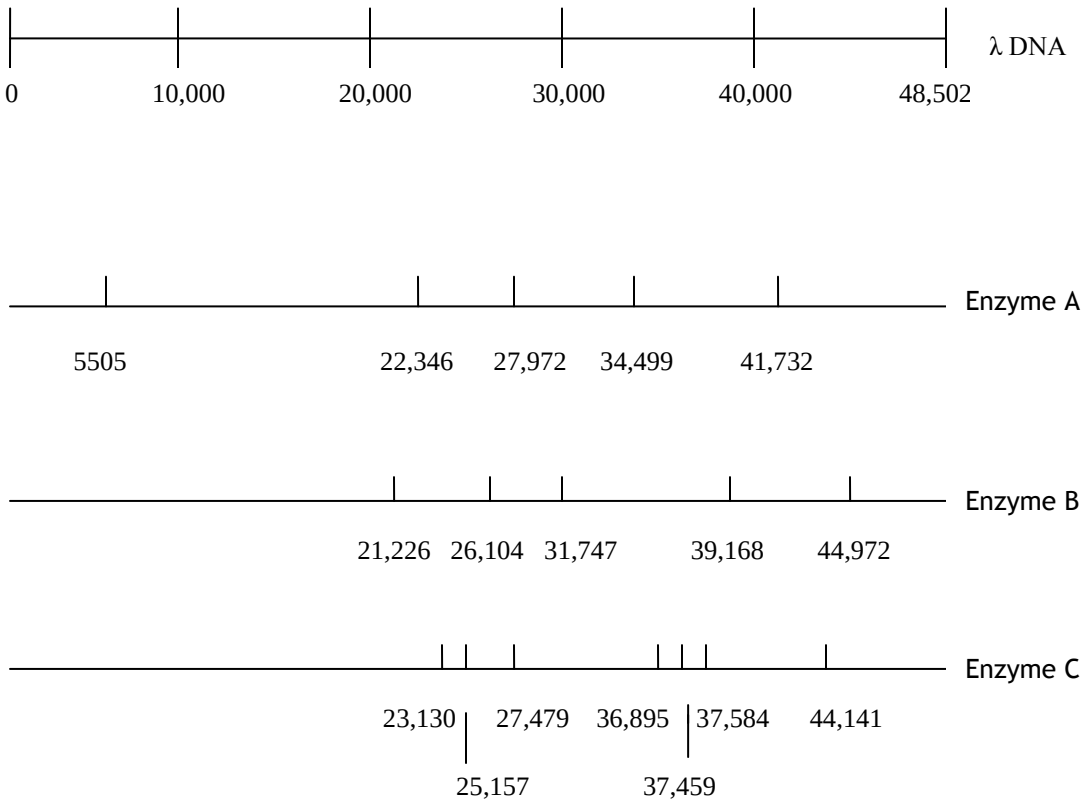




λ DNA cut with *Eco*RI λ DNA cut with *Hind*III λ DNA cut with *Bam*HI

This figure shows the size of each of the fragments/bands produced when λ DNA is cut with each of these restriction enzymes. The sizes were determined by comparison to a molecular ladder which has bands of known sizes when it is separated by electrophoresis at the same time as the digested λ DNA.

Restriction fragments of Lambda (λ) DNA (in base pairs)



The sites at which each of the 3 different enzymes will cut lambda DNA are shown in the maps Enzymes A, B and C above.

1. Calculate how big each of the fragments will be and write them on the maps.
2. How many fragments would you expect to see for each of the maps A, B and C?
3. Draw these fragments onto the graph below.
4. Now compare the size of the fragments that you have calculated with the bands shown in the photographs of the gels and determine which of the enzymes, *Bam*HI, *Eco*RI and *Hind*III were used to cut A, B and C.

5. How many times does the sequence GAATTC occur in the λ DNA sequence? What about AAGCTT and GGATCC?
6. Are there as many bands in your gel as you would expect to see based on the results of your calculations? If the number is different explain what you think has happened.