Lab 6: Restriction mapping of circular plasmid DNA

Objective: To map the placement of restriction endonuclease cleavage sites in a circular plasmid DNA by endonuclease digestion and gel electrophoresis.

Restriction enzymes were discovered about thirty years ago when the observation was made that bacterial cells “restricted” the growth of viruses within them and thus resisted infection. A class of enzymes was discovered (called restriction enzymes) that cleaved foreign (non-host organism) DNA at specific sites. It was soon realized that these enzymes could be used to cleave laboratory-purified DNA and, when those fragments were subjected to gel electrophoresis, that the fragments’ lengths could be determined.

It was also realized that, with clever use of different restriction enzymes, a length of DNA could be “mapped” – that is, the locations of these cleavage sites could be placed in order down the length of the DNA strand. Thus was born “restriction mapping”.

The enzymes that you will use in this lab are called restriction endonucleases because they cut within a strand of DNA. In the diagram above, the circular plasmid DNA is cut by two restriction endonucleases, B and H. Even though it appears that the fragments will be of the same length, do not be fooled; the two scenarios above represent different mapping results. Like proteins, DNA is “read” in only one direction; instead of N-terminus to C-terminus, DNA is “read” from the 5’ end to the 3’end. Thus, the DNA nucleotide at the 5’ end of the molecule (called “base 1”) attaches at its 3’ end to the 5’ end of the next nucleotide (“base 2”) and so on until the 3’ end of the molecule. Thus, if the circular plasmid DNA in the diagram is 900 bases long and is “read” clockwise starting at the B endonuclease cleavage site, then the left diagram shows the H endonuclease site at the 600th base and the right diagram shows the H endonuclease site at the 300th base. It is impossible from the results of obtaining a 600-base fragment and a 300-base fragment from the endonuclease digest to determine which scenario is correct.

Incidentally, restriction endonucleases are named as follows: The first italicized letter indicates the genus of the organism from which the enzyme was isolated.
The second and third italicized letters indicate the species. An additional letter indicates the particular strain used to produce the enzyme. The Roman numerals denote the sequences in which the restriction endonuclease enzyme from that particular genus, species, and strain of bacteria have been isolated.

Restriction endonucleases cut double-stranded DNA; most of these enzymes recognize a four-to-six base long sequence of DNA and cleave at a spot within that sequence. In fact, this type of restriction endonuclease recognize symmetric sequences (such as Hind III, which recognizes AAGCTT, which has a complementary strand TTCGAA, which is exactly the bases in reverse order of the first strand) and so they cleave asymmetrically (Hind III cleaves the bond between the A’s). Thus, the fragments of DNA after endonuclease cleavage have what are called “sticky ends” – short lengths of single-stranded DNA – that can be used to ligate (stick) this fragment onto lengths of other DNA. Thus, in the diagram on the previous page, I should have said that the circular DNA was cut into a 600-base pair (bp) fragment and a 300-bp fragment. Greater than 1000 bp fragments are measured in kilobase pairs or kb.

In this lab, you will use two different restriction endonucleases, Bgl I and Hind III, to develop a crude map of a circular plasmid DNA (a plasmid is a circular DNA with a cell that replicates independently of chromosomal DNA). Plasmid DNA may account for sudden changes in chromosomal DNA that some organisms exhibit.

For DNA, the gel electrophoresis will be done horizontally (that is, the gel is laid out flat) and the gel will be made of agarose, a polysaccharide. These two modifications are necessary to get good separation of the DNA fragments. In addition, as a calibration, Lambda bacteriophage DNA already cleaved by the Hind III restriction endonuclease, should give a variety of known fragment sizes (see exercise 8).

Materials:
- Five microcentrifuge tubes
- Micropipetter (5 to 50 μL range)
- Circular plasmid DNA
- Reaction buffer
- Bgl I endonuclease
- Gel loading solution
- 37°C water bath
- Gel photography unit (camera box)
- DNA gel electrophoresis setup including pre-poured agarose gel
- Gloves
- Appropriate micropipetter tips
- Standard “marker” DNA
- Electrophoresis running buffer
- Hind III endonuclease
- InstaStain Methylene Blue sheet
- 65°C water bath
- Power supply and leads

Procedure:
Part 1: Setting up the restriction digests

1. Label four 1.5-mL tubes (in which you will perform restriction reactions): “P” for native plasmid, “B” for plasmid cut with Bgl I, “H” for plasmid cut with Hind III, and “B/H” for plasmid cut with both Bgl I and Hind III.
2. Use the table below as a checklist while adding reagents to each reaction; go **left to right** to add the reagents in the correct order. **Use a fresh tip for each reagent.** All groups share the same enzyme vials, so it is vital not to cross-contaminate the reagents.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Reaction buffer</th>
<th>DNA</th>
<th>dH₂O</th>
<th>Bgl II</th>
<th>Hind III</th>
<th>Total volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>30 µL</td>
<td>10 µL</td>
<td>10 µL</td>
<td></td>
<td></td>
<td>50 µL</td>
</tr>
<tr>
<td>B</td>
<td>30 µL</td>
<td>10 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td></td>
<td>50 µL</td>
</tr>
<tr>
<td>H</td>
<td>30 µL</td>
<td>10 µL</td>
<td>5 µL</td>
<td></td>
<td>5 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>B/H</td>
<td>30 µL</td>
<td>10 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td></td>
<td>50 µL</td>
</tr>
</tbody>
</table>

3. Pool and mix the reagents by tapping the tube bottom on the lab bench (or with a short pulse in a microcentrifuge).

4. Incubate all reaction tubes for **60 minutes at 37°C**. Note: At this point, your instructor can remove your tubes at the end of the incubation and store them in the −20°C freezer until the next lab period.

**Part 2: Disrupt hydrogen bonding and load gel**

5. (If needed) Remove the tubes from the freezer and thaw in your hand for a minute.

6. Obtain 45 µL of the pre-digested Lambda bacteriophage DNA (labeled “Markers”) and put it in a clean microcentrifuge tube marked “M”.

7. Add 5 µL of gel loading solution to each of the five reaction tubes. Mix the solution with digested DNA by tapping the tube on the lab bench (or with a short pulse in a microcentrifuge).

8. Incubate all reaction tubes for 5 minutes at 65°C to disrupt the hydrogen bonding at the COS site of the lambda phage and plasmid DNA.

9. Obtain a gel electrophoresis unit with a freshly-poured agarose gel already in place. (Remember to sketch the setup at some point). Carefully remove the plastic comb (if present) to reveal the wells.

10. Fill the reservoirs in the gel apparatus with running (or electrophoresis) buffer. The gel should be fully under the surface of the buffer solution.
11. Use a micropipet to load the contents of each reaction tube into a separate well in the gel. **Use a fresh tip for each reaction tube.** When you are loading the gel:

- Steady the micropipet over the well using two hands.
- Be careful to expel any air in the micropipet tip end before loading the gel. If an air bubble forms a “cap” over a well, the DNA/loading dye will flow into the buffer around the edges of the well.
- Dip the micropipet tip through the surface of the buffer, position it over the well, and slowly expel the mixture. Sucrose in the loading dye weighs down the sample, causing it to sink into the well. Be careful not to punch the tip of the micropipet through the bottom of the gel!

**Part 3: Electrophoresis**

12. Close the top of the electrophoresis chamber and connect the electrical leads to the power supply, anode to anode (red to red) and cathode to cathode (black to black). Make sure both electrodes are connected to the same channel of the power supply.

13. Turn on the power supply and set the voltage at 125V. Bubbles should form on the exposed wire in the reservoirs of the gel apparatus. Shortly after the current is applied, the dye in the gel loading solution can be seen moving through the gel toward the positive pole of the electrophoresis apparatus.

14. The loading dye will eventually resolve into two bands of color. The faster-moving, purplish band is the dye bromophenol blue; the slower-moving, aqua band is xylene cyanol. Bromophenol blue migrates through the gel at the same rate as a DNA fragment approximately 300 base pairs long. Xylene cyanol migrates at a rate equivalent to approximately 2000 base pairs.

15. Allow the DNA to electrophorese until the xylene cyanol band has migrated 20 to 25 mm from the origin. At this distance, the bromophenol blue band has usually just fallen off the gel. This should take roughly 45 minutes.

16. Turn off the power supply, disconnect the leads from the inputs and remove the top of the electrophoresis chamber.

17. Carefully remove the gel from its casting tray onto a piece of plastic wrap. Make sure the gel is face-up.

18. Moisten the surface of the gel with several squirts of the buffer solution.

19. Wearing gloves, place the blue side of the InstaStain Methylene Blue sheet on the gel. Firmly run your fingers over the entire surface of the sheet to remove any air bubbles.

20. Place the gel casting tray on top of the gel + sheet, and place an empty 250-mL beaker on top of that.
21. Allow the staining to continue for 15 minutes. Make sure there is a supply of 37°C (or slightly cooler) distilled water.

22. Again wearing gloves, remove the beaker, casting tray, InstaStain sheet, and place the gel (face-up) in a large weigh boat. Add enough 37°C water to cover the gel and agitate gently for 15 minutes. Discard the water into the proper waste container. Some of the DNA bands on the gel should be visible.

23. Repeat step 22 with a fresh batch of 37°C water. After 15 minutes, the background of the gel should be reasonably non-blue.

24. Photograph the gel in the camera box under white (visible) light.

Data:

Attach a sketch or photograph of your gel.

Draft a table showing, for each lane, the migration distances in millimeters of every band. Leave an empty column (or row) so that you can transfer the numbers you will determine in exercise 8 (the Hind III fragment lengths of the Lambda bacteriophage DNA) to calibrate the lengths of your plasmid DNA fragments.

Analysis:

1. From exercise 8, you have the lengths of the Hind III fragments of λ phage DNA. Plot the migration distance of the bands in the Hind III track versus the fragment length in bp on normal, semi-log and log-log graph paper. Which graph gave you the most linear fit of the points? Include all three graphs.

2. Using the graph that gave you the most linear fit, determine the fragment lengths of your other tracks (make a neat table that shows each track separately, such as those in exercise 8).

Questions:

1. How many base pairs long is the native “uncut” circular plasmid DNA? What made this question difficult to answer, and what piece of information did you need in order to answer this question?

2. Restriction enzymes are sold in “units”, where 1 unit of the enzyme is defined as cleaving 1 μg of DNA in one hour. Assuming that you used 1 unit of enzyme and 1 μg of DNA in your reactions (the numbers are pretty close), determine the number of DNA molecules per second that are cleaved by the enzyme. You may also assume that the “average” molar mass of a nucleotide is 325 g/mol.

3. The number in question 2 may seem frightening, but consider that there are about 6 × 10¹¹ enzyme molecules in the centrifuge tube. What is the number of
circular plasmid DNA molecules per second by one enzyme molecule? It’s not so huge, after all. What factors don’t allow the enzyme to be more efficient?

**Conclusion**

Based on your data, the answers to the questions in this lab, and performing an analysis similar to the one you did in exercise 8, **draw the restriction map** of this circular plasmid DNA for Bgl I and Hind III sites; orient your drawing with the Bgl I site at the 12 o’clock position. Include the lengths in base pairs between cleavage sites.

**To turn in:**
A photocopy of the lab notebook page(s) showing:

- the sketch of the electrophoresis setup, including labels indicating the power supply, the leads to the proper polarity marked terminals, the gel itself, the buffer reservoir(s) and the wells.

- the data, analysis, questions and conclusion sections.