Restriction Mapping
of Plasmid DNA

TEACHER’S MANUAL WITH STUDENT GUIDE
Restriction Mapping of Plasmid DNA

Objectives

Students become molecular biologists investigating DNA of “uncertain origin” given to them by a top military official following a UFO sighting. Students perform electrophoresis with predigested pMAP plasmid DNA samples, and perform data analysis to construct a restriction enzyme map of the plasmid. Information is provided for the teacher to supply as hints to guide the students through this data analysis. Through these activities, students
• gain experience performing gel electrophoresis.
• learn how to do restriction enzyme mapping.
• practice analytical thinking.

Skills Required

Since the analysis part of this lab involves estimating DNA fragment sizes and restriction enzyme mapping, students must have a firm understanding of the action of restriction enzymes and the principles of gel electrophoresis. This laboratory exercise is a good follow-up to the Restriction Enzyme Cleavage of DNA Kit.

Materials

This kit contains all the necessary reagents needed to perform restriction mapping of pMAP. The materials are supplied for use with this kit only. Carolina Biological Supply Company disclaims all responsibility for any other use of any of these materials.

Materials included in the 4-station kit:
- 4 vials pMAP/PstI
- 4 vials pMAP/PstI/HpaI
- 4 vials pMAP/PstI/SspI
- 4 vials pMAP/PstI/HpaI/SspI
- 4 vials lambda DNA/PstI size markers
- 7.5 mL CarolinaBLU™ in a dropper bottle
- 4 staining trays

Materials included in the 8-station kit:
- 8 vials pMAP/PstI
- 8 vials pMAP/PstI/HpaI
- 8 vials pMAP/PstI/SspI
- 8 vials pMAP/PstI/HpaI/SspI
- 8 vials lambda DNA/PstI size markers
- 2 dropper bottles of CarolinaBLU™, 7.5 mL each
- 8 staining trays

Teacher’s Manual with masters for Student Guide and practice problems

2 g agarose*
150 mL 20× TBE*
24 needle-point pipets
250 mL final CarolinaBLU™ stain
8 gloves

*Quantities are sufficient for most “mini” gel systems.
Restriction Mapping of Plasmid DNA

Storage:
The DNA samples in this kit have been formulated to be stored at room temperature for short periods of time. This allows for shipment of the kit at room temperature and saves on shipping charges. However, for storage longer than 6 weeks, the DNA samples should be refrigerated or frozen. All the other materials in the kit can be stored at room temperature for extended periods of time.

Materials needed, but not provided:
- gel electrophoresis chambers with well-forming combs (at least 5-well)
- power supplies
- masking tape for sealing gel-casting trays (depending on style of tray)
- racks for vials of DNA
- flask or beaker for agarose
- container for 1× TBE electrophoresis buffer
- microwave, boiling water bath, or hot plate
- white-light box and/or overhead projector (desirable for viewing stained gels)
- camera (desirable for recording results)

Background

Restriction mapping is the first step in characterizing a novel DNA sequence. It is commonly used to confirm that a new fragment of DNA has been ligated into a plasmid. It is also used to verify the identity of a plasmid, since the pattern of DNA fragments produced by restriction digest can be like a fingerprint of a plasmid.

In this laboratory exercise, students use data analysis to determine the number and relative positions of cut sites for three restriction enzymes—PstI, HpaI, and SspI—on the plasmid pMAP. Samples of pMAP have been incubated with either PstI alone, PstI and HpaI, PstI and SspI, or PstI, HpaI, and SspI. These single, double, and triple digests are key to determining the number and relative positions of the restriction enzymes’ cut sites. The single restriction enzyme digest will help students deduce the number of cut sites present; the double and triple digests will allow the cut sites to be mapped relative to one another. The PstI digested lambda DNA gives a series of fragments of known size, to help students gauge the sizes of the pMAP fragments from the PstI, HpaI, and SspI digests by comparing them with the lambda DNA fragments.

The amount of information given to the students is up to the teacher. For example, the teacher could take any of the following approaches:

- Tell the students only that it is a circular plasmid.
- Tell the students that it is a circular plasmid of 5615 bp.
- Tell the students that it has two PstI sites.
- Tell the students that it has two PstI sites and one HpaI site.
- Tell the students that it has two PstI sites and one SspI site.

More information can be given when the students hesitate in their analysis.
Restriction Mapping of Plasmid DNA

Note: The sizes of the PstI digested lambda DNA fragments SHOULD be given to the student. Without this information, the student will be unable to estimate the sizes of the pMAP fragments.

DNA electrophoretic analysis requires several different activities. Plan your time as follows:

<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab Period 1</td>
<td>45 min–1 hr</td>
<td>Pre-lab: Mix TBE buffer, prepare agarose solution, set up workstations, pool small volumes of DNA</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>Practice pipetting, gel loading (optional)</td>
</tr>
<tr>
<td></td>
<td>10–15 min</td>
<td>Cast agarose gel</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>Load gel</td>
</tr>
<tr>
<td></td>
<td>40+ min</td>
<td>Post-lab: Electrophorese gel</td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>Stain gel</td>
</tr>
<tr>
<td></td>
<td>30+ min</td>
<td>Destain gel</td>
</tr>
<tr>
<td>Lab Period 2</td>
<td>40 min</td>
<td>Data Analysis of Restriction Digests (determine number and relative position of restriction enzyme cut sites)</td>
</tr>
</tbody>
</table>

Pre-Lab Preparation

Mix TBE Buffer
Because tris-borate-EDTA (TBE) buffer solution is stable, it can be made ahead of time and stored until ready to use. For the 4-station kit pour 75 mL of 20× TBE concentrate in a 2-L container. Add 1425 mL of distilled or deionized water. Mix. For the 8-station kit make twice this amount. Any TBE that has precipitated can be brought back into solution by microwaving. Heating it will not work.

Prepare Agarose Solution
Before class on Lab Day 1, prepare a 0.8% agarose solution. For the 4-station kit, add 2 g of agarose to 250 mL of 1× TBE. For the 8-station kit, add 4 g of agarose to 500 mL 1× TBE. Cover the agarose TBE mixture with aluminum foil and heat in a boiling water bath (or double boiler) for 7–10 min. The solution will become clear as the agarose dissolves. Swirl, and observe the bottom to ensure that no undissolved agarose remains. Alternatively, heat the solution at the high setting in a microwave oven for 4–7 min, without aluminum foil. Swirl the solution at the first sign of vigorous boiling to avoid boiling over. A hot plate may also be used. Cool the solution to approximately 60°C before use. Cover with aluminum foil and keep warm in a 60°C water bath until ready to use.
Addition of Stain to Agarose

Gels may be run with or without using the CarolinaBLU gel and buffer stain. Adding the stain to the gel and buffer lets you faintly visualize the bands during the electrophoresis. The concentration of stain added to the agarose/buffer is dependent on the voltage used for electrophoresis. If you are electrophoresing at voltages less than 50 V, a slightly lower concentration is used than if you are running at voltages greater than 50 V. The stain may be added to the entire volume of agarose and distributed, or the agarose may be distributed to each lab station and the stain added by the students at the rates listed below:

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Agarose Volume</th>
<th>Stain Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 50 V</td>
<td>30 mL</td>
<td>40 µL (1 drop)</td>
</tr>
<tr>
<td></td>
<td>60 mL</td>
<td>80 µL (2 drops)</td>
</tr>
<tr>
<td></td>
<td>400 mL</td>
<td>532 µL (14 drops)</td>
</tr>
<tr>
<td>&gt; 50 V</td>
<td>50 mL</td>
<td>80 µL (2 drops)</td>
</tr>
<tr>
<td></td>
<td>400 mL</td>
<td>640 µL (16 drops)</td>
</tr>
</tbody>
</table>

After the addition of the stain to the agarose, swirl to mix and immediately pour the gel. Gels may be prepared one day ahead of the lab day, if necessary. Gels stored longer tend to fade and lose their ability to stain the bands during electrophoresis. Store the gels covered with a small amount of buffer (leaving the masking tape in place) or store covered with buffer in the gel box with the lid on. Do not try using more stain in your gel than is recommended. This leads to precipitation of the DNA in the wells and can create aggregated DNA bands in the agarose gel.

Addition of Stain to Buffer

Use the chart below for addition of the stain to 1× TBE electrophoresis buffer:

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Buffer Volume</th>
<th>Stain Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 50 V</td>
<td>500 mL</td>
<td>500 µL (12 drops)</td>
</tr>
<tr>
<td></td>
<td>1.5 L</td>
<td>1.5 mL (36 drops)</td>
</tr>
<tr>
<td>&gt; 50 V</td>
<td>500 mL</td>
<td>960 µL (24 drops)</td>
</tr>
<tr>
<td></td>
<td>1.5 L</td>
<td>2.9 mL (72 drops)</td>
</tr>
</tbody>
</table>

The dropper bottle provided delivers 40–60 µL/drop. If a calibrated pipet is available, the dropper lid can be removed for quicker addition of larger volumes of stain. The volumes of buffer and agarose required for Carolina gel box options are listed below:

<table>
<thead>
<tr>
<th>Type Gel Box</th>
<th>Volume Buffer Required</th>
<th>Volume Agarose Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carolina Gel Box, 1 tray</td>
<td>250 mL</td>
<td>50 mL</td>
</tr>
<tr>
<td>Carolina Gel Box, 2 trays</td>
<td>450 mL</td>
<td>100 mL</td>
</tr>
</tbody>
</table>
While CarolinaBLU™ is not toxic, we recommend that the students wear gloves to prevent staining their skin. Buffer containing CarolinaBLU may be reused three times within 48 hours. If you need to store the buffer for longer than 48 hours, do not add CarolinaBLU™ to the buffer. Instead, use CarolinaBLU™ as final stain only.

Pool Small Volumes of DNA
DNA may become spread in a film around storage tube walls or caps during shipping. Prior to setting up workstations, pool samples at the bottom of their storage tubes, using one of three methods:

1. Spin tubes briefly in a microfuge.
2. Spin tubes briefly in a preparatory centrifuge, using adapter collars for 1.5-mL tubes. Alternatively, spin tubes within 15-mL tubes and remove carefully.
3. Tap tubes sharply on the benchtop.

Set Up Student Workstations
1. Prepare student stations, each with the following materials:
   vial pMAP/PstI
   masking tape (for casting gels)
   vial pMAP/PstI/HpaI
   permanent marker
   vial pMAP/PstI/SspI
   gel electrophoresis chamber
   vial pMAP/PstI/HpaI/SspI
   power supply (may be shared)
   rack for tubes
   staining tray
   4 needle-point pipets
   student guide

2. Groups must share the following materials: agarose solution, TBE electrophoresis buffer, and CarolinaBLU™ stain.

3. Hold agarose solution at 60°C in a water bath.

Student Lab Briefing
Principles of Restriction Enzymology and Gel Electrophoresis
Background information and dry labs for teaching about restriction enzymes and gel electrophoresis can be found in Molecular Biology and Biotechnology: A Guide for Teachers (Carolina catalog # RN-21-2240).

Practice Gel Loading (Optional)
You may wish to have students practice loading a gel. Practice gel-loading stations are available (Carolina catalog # RN-21-1145).

Fine Points of Laboratory Procedure
Be alert to the following cautions when performing the experiments. Where appropriate, discuss fine points with your students. For the detailed step-by-step lab procedure, see the Student Guide masters.
Storing Cast Agarose Gels
If needed, students may cast gels a day before use. Keep gels covered with TBE electrophoresis buffer to prevent drying.

Loading and Electrophoresing Gels
Make sure that your students record the order in which they loaded their samples. This is important! If students do not know the loading order, they will not know the sample identities and they will be unable to do the analysis.

If your students have difficulty using the pipets, suggest that instead of using the bulb to control the sample, they pinch the middle section (directly below the bulb) or lightly press the tapered section between the bulb and the middle section when manipulating their samples.

The migration of DNA through the agarose gel is dependent upon voltage—the higher the voltage, the faster the rate of migration. Best separation is achieved when the loading dye band (bromphenol blue) nears the end of the gel. Do not let the loading dye band run off the end of the gel. If it has been added to the gel and buffer, CarolinaBLU™ intercalates into the DNA during electrophoresis, allowing for immediate visualization of some bands. Times below are for “variable voltage” minigel systems with an 84-× 96-mm gel, using approximately 0.8% agarose. Exact run times will vary according to the apparatus.

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 V</td>
<td>50 min</td>
</tr>
<tr>
<td>120 V</td>
<td>1 h</td>
</tr>
<tr>
<td>100 V</td>
<td>1 h 15 min</td>
</tr>
<tr>
<td>75 V</td>
<td>1 h 45 min</td>
</tr>
<tr>
<td>50 V</td>
<td>3 h</td>
</tr>
<tr>
<td>30 V</td>
<td>5 h 45 min</td>
</tr>
<tr>
<td>20 V</td>
<td>10 h</td>
</tr>
</tbody>
</table>

The distance between the bands should increase as the loading dye nears the end of the gel.

CarolinaBLU™ Staining and Destaining
Although students may stain gels in class, it saves time to stain gels after class, as recommended in the scheduling section. Destaining gels overnight improves results.

Note: Wear disposable gloves during staining and cleanup.

1. Flood the gels with CarolinaBLU™ Final Stain, and allow them to stain for 15–20 min.

2. Following staining, decant as much CarolinaBLU™ solution as possible from the staining tray back into the original storage container. Place the stained gel on the light box. DNA bands should be visible. If the bands are faint, additional staining may be required.
3. Rinse the gel in distilled or deionized water. Chlorinated water tends to bleach bands with time. Let the gel soak for several minutes in several changes of fresh water. DNA bands will become increasingly distinct as the gel destains. For best results, continue to destain overnight in a small volume of water. (Gel may destain too much if left overnight in a large volume of water.) Cover staining tray to retard evaporation.

Viewing and Photographing Gels
Transillumination, where light passes up through the gel, gives superior viewing of gels stained with CarolinaBLU™. A digital or instant camera can be used to record the gels. In some systems, a plastic hood extending from the front of the camera forms a mini darkroom.

Data Analysis Answers and Discussion

1. Examine your stained gel on a light box or overhead projector.

2. Have your students assign sizes to their lambda DNA/PstI size marker bands. The marker band sizes are 514, 805, 1093, 1159, 1700, 1986, 2140, 2450, 2838, 4700, and 11,497 base pairs. 

   **Note:** This is not a complete list of lambda DNA/PstI fragments. It has been abbreviated in the interest of clarity. If you choose, you may show students the schematic of a gel lane in Figure 1, which has these size markers labeled. The 805-bp fragment beneath the 1093- and 1159-bp doublet is a handy reference point for getting started with assigning fragment sizes.

3. Have your students assign approximate sizes to the restriction-digested pMAP fragments by comparing them to the lambda DNA/PstI size marker. Their estimates will not be perfectly accurate.

4. Have your students determine the total size of the digested DNA by adding up the sizes of the fragments from each digest. They may take an average size from the four digests: pMAP/PstI, pMAP/PstI/HpaI, pMAP/PstI/SspI, and pMAP/PstI/HpaI/SspI. The students should understand that because the same DNA was digested in each sample, the fragment sizes should always add up to the same total.

Now the students will use the information they have to determine the number and relative positions of the restriction enzyme cut sites. The teacher may provide one of the additional hints below, if so desired. Advanced students may want to have some time on their own to analyze the data before the class as a whole goes through the data analysis.

**Hint:** You may tell the students that the DNA has two PstI sites and allow them to deduce that it is a circular piece of DNA since the pMAP/PstI digest produces two fragments instead of three, as a linear piece of DNA would.
Hint: You may tell the students that the DNA is circular and have them determine how many PstI sites are present.

5. Have your students draw pMAP with the PstI sites present. Since the sizes of the PstI fragments are known, the total size of the DNA is known, and the fact that the DNA is circular is known, the students should be able to produce the drawing below:

![Diagram of PstI sites on pMAP]

Note: Although this drawing accurately depicts the relative sizes of the fragments, the students do not have to determine the exact sizes of the fragments in order to determine the relative number and position of the restriction enzyme sites.

6. Have your students determine how many SspI sites are present by comparing the pMAP/PstI lane and the pMAP/PstI/SspI lane. Since the pMAP/PstI lane has two fragments, and the pMAP/PstI/SspI lane has three fragments, students should be able to tell that there is one SspI site.

7. Have your students determine approximately where the SspI site must be in relation to the PstI sites on the plasmid. Here, your students should be able to determine that the SspI site is within the 4693-bp PstI fragment, since the 923-bp PstI fragment does not change after SspI digestion.

8. Ask your students whether the SspI recognition site is close to the PstI recognition sites, or if it is more in the middle of the 4693-bp fragment. Given that the 4693-bp PstI fragment is cleaved into a 2106-bp fragment and a 2586-bp fragment, your students should be able to determine that the SspI site is in the middle of the 4693-bp fragment. They should now be able to draw the plasmid map shown below:

![Diagram of PstI and SspI sites on pMAP]

Note: Although this drawing depicts the exact sizes of the fragments, the students do not have to determine the exact sizes of the fragments in order to determine the relative number and position of the restriction enzyme sites.
9. Have your students determine how many *Hpa*I sites are present by comparing the pMAP/*Pst*I lane and the pMAP/*Pst*I/*Hpa*I lane. Since the pMAP/*Pst*I lane has two fragments, and the pMAP/*Pst*I/*Hpa*I lane has three fragments, students should be able to tell that there is one *Hpa*I site.

10. Now have your students determine approximately where the *Hpa*I site must be on the plasmid, relative to the *Pst*I sites. It might be best if this is done in a separate sketch from the *Ssp*I site sketch, since we have not yet determined where the *Hpa*I and *Ssp*I sites are relative to one another. Since the 923-bp *Pst*I fragment does not change after the *Hpa*I digest, they should be able to determine that the *Hpa*I site is not within that stretch of DNA, and therefore must be in the 4693-bp *Pst*I fragment. This means that both the *Hpa*I site and the *Ssp*I site are within the 4693-bp *Pst*I fragment.

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**Note:** Although this drawing contains the exact sizes of the fragments, the students do not have to determine the exact sizes of the fragments in order to determine the relative number and position of the restriction enzyme sites.

11. Have your students predict whether the 923-bp *Pst*I fragment will remain after pMAP is digested with both *Hpa*I and *Ssp*I. They can confirm that it does by examining the pMAP/*Pst*I/*Hpa*I/*Ssp*I digest on their gel. Ask them if they understand why this is so—they should respond that neither *Hpa*I or *Ssp*I cuts within that stretch of DNA.

12. Now we are ready to determine where the *Hpa*I and *Ssp*I sites are relative to one another. Have your students determine which fragments are unchanged from the pMAP/*Pst*I/*Hpa*I digest to the pMAP/*Pst*I/*Hpa*I/*Ssp*I digest. The answer is the 849-bp fragment and the 923-bp fragment that is between the two *Pst*I sites. The other fragment (3843 bp) present in the pMAP/*Pst*I/*Hpa*I digest is not present in the pMAP/*Pst*I/*Hpa*I/*Ssp*I digest.

13. Ask your students why the 3843-bp fragment disappeared. They should reply that it contains an *Ssp*I recognition site.

14. Now have your students determine which fragments are unchanged from the pMAP/*Pst*I/*Ssp*I digest to the pMAP/*Pst*I/*Hpa*I/*Ssp*I digest. The answer is the 923-bp *Pst*I fragment, and the 2586-bp (the larger) pMAP/*Pst*I/*Ssp*I fragment. The other fragment, which has changed, is the 2106-bp (the smaller) pMAP/*Pst*I/*Ssp*I fragment.

15. Ask your students why the 2106-bp (smaller) pMAP/*Pst*I/*Ssp*I fragment disappeared. They should reply that it contains an *Hpa*I recognition site.
Answers to Practice Problem 1

Note: Plasmid drawings are not to scale.

1. \( HpaI \)  \( HpaI/PstI \)  \( HpaI/SspI \)  \( HpaI/PstI/SspI \)
   
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3300</td>
<td>3000</td>
<td>2100</td>
<td>1800</td>
</tr>
<tr>
<td>2100</td>
<td>1200</td>
<td>1200</td>
<td></td>
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<tr>
<td>600</td>
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<tr>
<td>600</td>
<td>300</td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>

2. 3.9 kb (3900 base pairs)

3. This is circular DNA (plasmid). Restriction mapping can certainly be done on linear DNA; however, it is slightly more complex and would call for a different set of restriction enzyme digests.

4. Since there are two fragments after digestion with \( HpaI \) and three fragments after digestion with \( HpaI \) and \( PstI \), there is one \( PstI \) site.
5. The PstI site is within the 3300 bp-\textit{Hpa}I fragment.

6. Since there are two fragments after digestion with \textit{Hpa}I and three fragments after digestion with \textit{Hpa}I and \textit{Ssp}I, there is one \textit{Ssp}I site.

7. The \textit{Ssp}I site is in the 3300-bp \textit{Hpa}I fragment.

8. Yes. No \textit{Ssp}I or PstI sites are present within this fragment.

9. 600 and 1200 bp. The 2100-bp fragment disappeared because it contains a \textit{Ssp}I site.

10. 300 and 600 bp. The 3000-bp fragment disappeared, so it must contain a PstI site.

11. Yes. It means that there is a fragment with an \textit{Ssp}I site on one end and a PstI site on the other end.

12. Full plasmid map; you may draw either of the following two options (they are simply mirror images of one another):
Answers to Practice Problem 2

Note: Plasmid drawings are not to scale.

1. \[
\begin{array}{cccc}
\text{SspI} & \text{SspI/HpaI} & \text{SspI/PstI} & \text{SspI/HpaI/PstI} \\
2500 & 1600 & 1300 & 1200 \\
1000 & 1000 & 1000 & 900 \\
500 & 500 & 500 & 400 \\
\end{array}
\]

2. 4.0 kb or 4000 base pairs (bp)

3. There are three SspI sites.

4. Since the SspI digest has three fragments and the SspI/HpaI digest has four fragments, there is only one HpaI site.

5. Since the 500- and 1000-bp SspI fragments are unchanged, the HpaI site must be within the 2500-bp SspI fragment.

6. Since the SspI digest has three fragments and the SspI/PstI digest has four fragments, there is only one PstI site.
7. Since the 500- and 1000-bp SspI fragments are unchanged, the PstI site must be within the 2500-bp SspI fragment as well.

8. Yes. Both the PstI and HpaI sites are within the 2500-bp SspI fragment.

9. The 500-, 1000-, and 900-bp fragments remain. The 1600-bp fragment disappeared because it contains a PstI site.

10. The 500-, 1000-, and 1200-bp fragments remain. The 1300-bp fragment disappeared because it contains an HpaI site.

11. The 400-bp fragment. It appears only in the SspI/HpaI/PstI digest because it has an HpaI site on one end and a PstI site on the other end.

12. Full plasmid map; you may draw any of the following four options:
You are a molecular biologist working in a small university town. Things are normally very quiet around here, and that's how the residents like it.

Last night, however, something unusual happened. While driving home late from your research lab, you saw something in the sky over Hwy. 1175. A glowing red cloud surrounded a small black object in the northern part of the sky. As you watched, the glow became more intense, and the black object hurtled toward the ground. You braked your car and stared intently toward the black object. It passed behind the trees, and you could not see whether or not it struck the ground.

Suddenly, a military truck filled with soldiers carrying firearms zoomed past you, and came to a halt in front of you, forcing you to stop as well. A soldier jumped out of the cab of the truck and told you that they were cordoning off the road and that you would have to find another way home.

The next day, you are working at your lab bench when suddenly a top military officer enters the room. He is carrying a small tube filled with a tiny amount of clear liquid. The officer tells you that the tube contains DNA of uncertain origin and it must be characterized immediately. He posts a guard outside your lab door and leaves you to begin your work.

You know that restriction mapping is the first step in characterizing a novel DNA sequence. A restriction map of a piece of DNA is like a fingerprint of the DNA. You set up different restriction enzyme digests using three restriction enzymes that you already have on hand—PstI, HpaI, and SspI. Because you want to know not only the number of cut sites present in the DNA sequence for each restriction enzyme, but also the positions of those cut sites relative to one another, you set up a series of four single, double, and triple digests. First, you digest the unknown DNA with PstI alone. Then, you digest the unknown DNA with PstI and either HpaI or SspI. Finally, you digest the unknown DNA with all three restriction enzymes.

You also set up a restriction enzyme digest of lambda DNA with PstI, to make a size marker to compare to the unknown DNA.

Now you are ready to load the DNA onto the gel and begin gel electrophoresis. After the gel electrophoresis is finished, your real work will begin when you analyze the unknown DNA to determine the number of cut sites for each restriction enzyme and the positions of those cut sites relative to one another.

Procedure

A: Cast Agarose Gel

1. Seal the ends of the gel-casting tray with tape, and insert the well-forming comb. Place the gel-casting tray out of the way on the lab bench, so that the agarose poured in the next step can set without being disturbed.

2. Carefully pour enough agarose solution into the casting tray to fill to a depth of about 5 mm. The gel should cover only about one-third the height of the comb teeth. Use a pipet tip or toothpick to move large bubbles or solid debris to the sides or ends of the tray while the gel is still liquid.

3. The gel will become cloudy as it solidifies (about 10–15 min). Do not move or jar the casting tray while the agarose is solidifying.
4. When the agarose has set, unseal the ends of the casting tray. Place the tray in the gel box, so that the comb is at negative (black) end.

5. Fill the box with 1× tris-borate-EDTA (TBE) buffer to a level that just covers the entire surface of the gel.

6. Gently remove the comb, taking care not to rip the wells.

7. Make certain that the sample wells left by the comb are completely submerged. If dimples appear around the wells, slowly add buffer until they disappear.

8. The gel is now ready to load with DNA.

Note: If this will be your stopping point for the lab period, cover the electrophoresis chamber to prevent the gel from drying out.

B: Load Gel

Use a needlepoint pipet (or other gel-loading device) to load the contents of each reaction tube into a separate well in the gel. Use a fresh pipet for each reaction tube. Write down the order in which you load the samples. This is very important! If the loading order is lost, you will not be able to analyze your results.

1. Draw the sample into the pipet.

2. Steady the pipet over the well using two hands.

3. Be careful to expel any air in the pipet tip end before loading the sample. (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.)

4. Dip the pipet tip through the surface of the buffer, position it over the well, and slowly expel the sample. Sucrose in the loading dye weighs down the sample, causing it to sink to the bottom of the well. Be careful not to punch the pipet tip through the bottom of the well.

C: Electrophorese

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

2. Turn the power supply on and set the voltage as directed by your instructor. Shortly after the current is applied, the loading dye (bromphenol blue) should move through the gel toward the positive pole of the electrophoresis apparatus.

3. Bromphenol blue migrates through the gel at the same rate as a DNA fragment approximately 300 base pairs long.

4. Allow the DNA to electrophorese until the bromphenol blue band is about 2 cm from the end of the gel. Your instructor may monitor the progress of electrophoresis in your absence; in that case, omit Steps 5 and 6.

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

6. Carefully remove the casting tray, and slide the gel into the staining tray labeled with your group name. Take your gel to your instructor for staining.
Data Analysis

1. Examine your stained gel on a light box or overhead projector.

2. Assign sizes to the lambda DNA/PstI size marker bands on your gel. These marker bands are 514, 805, 1093, 1159, 1700, 1986, 2140, 2450, 2838, 4700, and 11,497 bp in size. Remember, small DNA fragments migrate more quickly than large ones.

3. Now, assign approximate sizes to the DNA fragments of unknown size by comparing them to the lambda DNA/PstI size marker. These approximations will not be perfectly accurate. That is all right since exact sizing is NOT required for determination of the number and relative positions of the cut sites of the restriction enzymes.

4. Determine the total size of the digested DNA by adding up the sizes of the fragments from each digest. You should take an average size from the four digests: pMAP/PstI, pMAP/PstI/HpaI, pMAP/PstI/SspI, and pMAP/PstI/HpaI/SspI. Remember, the same DNA was digested in each sample, so the fragment sizes should always add up to the same total.

Now you can begin the real data analysis to determine the number and relative positions of the restriction enzyme cut sites in the DNA of uncertain origin.

Number of PstI sites: Number of SspI sites: Number of HpaI sites:

Sketch the positions of these sites, relative to one another. Include the approximate distances between the sites in the DNA.
Restriction Mapping of Plasmid DNA

Problem 1: Digested with HpaI, HpaI/PstI, HpaI/SspI, and HpaI/PstI/SspI

1. Estimate the sizes of the DNA fragments (in base pairs) by comparing them with the lambda/PstI size marker. These estimated sizes do not have to be exact. Sizing of the smaller fragments will be more accurate than sizing of the larger fragments.

2. Determine the total size of the digested DNA by adding up the sizes of the fragments from each digest. You may take an average size from the four digests. The same DNA was digested in each sample, so the fragment sizes from the different digests should always add up to the same total.

3. There are two HpaI sites present. Based on the number of fragments obtained from the HpaI digest, is this DNA linear or circular? Draw the DNA with the HpaI sites present.
4. How many *Pst*I sites are present?

5. Where is the *Pst*I site? Draw the position of the *Pst*I site on the plasmid, relative to the *Hpa*I sites.

6. How many *Ssp*I sites are present?

7. Where is the *Ssp*I site? Draw the position of the *Ssp*I site on the plasmid, relative to the *Hpa*I sites. It might be best if this is done in a separate sketch from the *Pst*I site sketch since we have not yet determined where the *Ssp*I and *Pst*I sites are relative to one another.

8. Will the 600-bp *Hpa*I fragment remain unchanged after digestion with either *Pst*I or *Ssp*I? (Check the gel.)

9. Which fragments are unchanged from the *Hpa*I/*Pst*I digest to the *Hpa*I/*Pst*I/*Ssp*I digest? Which fragments disappeared? Why did those fragments disappear?

10. Which fragments are unchanged from the *Hpa*I/*Ssp*I digest to the *Hpa*I/*Pst*I/*Ssp*I digest? Which fragments disappeared? Why did those fragments disappear?

11. Is there a fragment that appears only in the *Hpa*I/*Pst*I/*Ssp*I digest? What does this mean?

12. Draw the full plasmid map, with all restriction enzyme recognition sites present in their relative locations.
Problem 2: Digested with SspI, SspI/HpaI, SspI/PstI, and SspI/HpaI/PstI

1. Estimate the sizes of the DNA fragments (in base pairs) by comparing them to the lambda/PstI size marker. These estimated sizes do not have to be exact. Sizing of the smaller fragments will be more accurate than sizing of the larger fragments.

2. Determine the total size of the digested DNA by adding up the sizes of the fragments from each digest. You may take an average size from the four digests. The same DNA was digested in each sample so the fragment sizes from the different digests should always add up to the same total.

3. This is plasmid DNA, which is circular. How many SspI sites are present? Draw the relative positions of the SspI restriction sites on the plasmid.
4. How many \textit{HpaI} sites are present?

5. Where is the \textit{HpaI} site? Draw the position of the \textit{HpaI} sites on the plasmid, relative to the \textit{SspI} sites.

6. How many \textit{PstI} sites are present?

7. Where is the \textit{PstI} site? Draw the position of the \textit{PstI} site on the plasmid, relative to the \textit{SspI} sites. It might be best if this is done in a separate sketch from the \textit{HpaI} site sketch, since we have not yet determined where the \textit{HpaI} and \textit{PstI} sites are relative to one another.

8. Will the 500- and 1000-bp \textit{SspI} fragments remain unchanged after digestion with either \textit{PstI} or \textit{HpaI}? (Check the gel.)

9. Which fragments are unchanged from the \textit{SspI}/\textit{HpaI} digest to the \textit{SspI}/\textit{PstI}/\textit{HpaI} digest? Which fragment disappeared? Why did that fragment disappear?

10. Which fragments are unchanged from the \textit{SspI}/\textit{PstI} digest to the \textit{SspI}/\textit{HpaI}/\textit{PstI} digest? Which fragment disappeared? Why did that fragment disappear?

11. Which fragment appears only in the \textit{SspI}/\textit{HpaI}/\textit{PstI} digest? Why is it present only in this digest?

12. Draw the full plasmid map with all restriction enzyme recognition sites present in their relative locations.