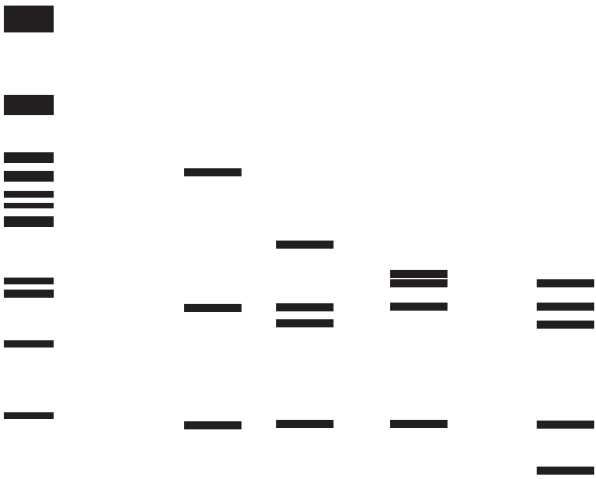
CAROLINA™
Teamed with Teachers

21-1175

Restriction Mapping of Plasmid DNA

Teacher's Manual



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Students become molecular biologists investigating mysterious 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.

Skills Required

This procedure is appropriate for the student who has a firm understanding of the action of restriction enzymes and the principles of gel electrophoresis, since it will be necessary for students to estimate pMAP DNA fragment size by comparison to the *Pst*I lambda DNA fragments. This laboratory exercise is a good follow-up to the Restriction Enzyme Cleavage of DNA Kit.

Materials

This reagent system contains all the necessary reagents needed to perform restriction mapping of pMAP. There are sufficient materials for 6 teams of students. 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 this kit:

- 6 Vials pMAP/*Pst*I
- 6 Vials pMAP/*Pst*I/*Hpa*I
- 6 Vials pMAP/*Pst*I/*Ssp*I
- 6 Vials pMAP/*Pst*I/*Hpa*I/*Ssp*I
- 6 Vials Lambda DNA/*Pst*I Size Markers
- 7.5 mL *Carolina BLU*™ in a Dropper
- 6 Staining Trays
- 3.2 g Agarose*
- 150 mL 20x TBE*
- 36 Needle-Point Pipets
- 12 1.5-mL Microfuge Tubes
- 250 mL Final *Carolina BLU*™ Stain
- 24 Sets of Student Instructions and Optional Practice Problems
- 8 Gloves

*Volumes are sufficient for most "mini" gel systems.
All materials may be stored at room temperature.

Materials needed, but not provided:

3 Gel Electrophoresis Chambers with 8 Well Combs

Power Supplies

Masking Tape for Sealing Gel Casting Trays

3 Racks for Vials of DNA

1-L Flask or Beaker for Agarose

2-L Container for 1x TBE Electrophoresis Buffer

Microwave or Boiling Water Bath

White-Light Box and/or Overhead Projector (desirable for viewing stained gels)

Polaroid® "Gun" Camera or other 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 3 restriction enzymes—*Pst*I, *Hpa*I, and *Ssp*I—on the plasmid pMAP. Samples of pMAP have been incubated with either *Pst*I alone, *Pst*I and *Hpa*I, *Pst*I and *Ssp*I, or *Pst*I, *Hpa*I, and *Ssp*I. 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 *Pst*I digested lambda DNA gives a series of fragments of known size, to help the student gauge the sizes of the pMAP fragments from the *Pst*I, *Hpa*I, and *Ssp*I digests by comparison with the lambda DNA fragments.

The amount of information given to the student is up to the discretion of the teacher.

For example, the teacher could take any of the following approaches:

- Tell the student only that it is a circular plasmid
- Tell the student that it is a circular plasmid of 5615 bp
- Tell the student that it has 2 *Pst*I sites

- Tell the student that it has 2 *Pst*I sites and 1 *Hpa*I site
- Tell the student that it has 2 *Pst*I sites and 1 *Ssp*I site

More information can be given when the students hesitate in their analysis.

Note: The sizes of the *Pst*I 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.

Time Requirements

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

Day	Time	Activity
Lab Period 1	30 min	**Pre-lab:** Mix TBE buffer
	30 min	**Pre-lab:** Prepare agarose solution, set up workstations, pool small volumes of DNA
	15 min	Practice pipetting, gel loading (optional)
	10 min	Cast agarose gel
	15 min	Load gel
	40+ min	**Post-lab:** Electrophorese gel
	20 min	Stain gel
	30 min	Destain gel
Lab Period 2	40 min	Data Analysis of Restriction Digests (determine number and relative position of restriction enzyme cut sites)

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. Pour 75 mL of 20x TBE concentrate in a 2-L container. Add 1425 mL of distilled or deionized water. If there is a precipitate in the bottle containing the TBE buffer concentrate, use a portion of the 1425 mL deionized water to rinse and add to total volume. Mix for 1–2 min.

Prepare Agarose Solution

Before class on Lab Day 1, prepare a 0.8% agarose solution. Add 3.2 g (entire bottle) of agarose to 400 mL 1x TBE electrophoresis buffer in a clean 1-L flask or beaker. Cover with aluminum foil and heat in a boiling water bath (or double boiler) for 7–10 min. Solution will become clear as agarose dissolves. Swirl and observe bottom to ensure that no undissolved agarose remains. Alternatively, heat solution at high setting of microwave oven for 4–7 min, without aluminum foil. Cool solution to approximately 60° C before use. Cover with aluminum foil and keep warm in 60° C water bath until ready to use.

Addition of Stain to Agarose

The concentration of stain added to the agarose/buffer is dependent on the voltage used for electrophoresis. If electrophoresing at voltages less than 50 V, a slightly lower concentration is used than if 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:

Voltage	Agarose Volume	Stain Volume
< 50 V	30 mL	40 µL (1 drop)
	60 mL	80 µL (2 drops)
	400 mL	532 µL (14 drops)
> 50 V	50 mL	80 µL (2 drops)
	400 mL	640 µL (16 drops)

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 bands during electrophoresis. Store gels covered with a small amount of buffer (leaving masking tape in place) or store covered in the gel box. 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 1x TBE electrophoresis buffer:

Voltage	Buffer Volume	Stain Volume
< 50 V	500 mL 1.5 L	500 µL (12 drops) 1.5 mL (36 drops)
> 50 V	500 mL 1.5 L	960 µL (24 drops) 2.9 mL (72 drops)

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

Type Gel Box	Volume Buffer Required	Volume Agarose Required
Mini Gel System Box	200 mL	30 mL
Carolina Gel Box, 1 tray	250 mL	50 mL
Carolina Gel Box, 2 trays	450 mL	100 mL

While *Carolina BLU*™ is not toxic, we recommend that the students wear gloves to prevent staining the skin. Buffer containing *Carolina BLU*™ may be reused 3 times within a 48-h period. If storing the buffer for a longer time is necessary, do not add *Carolina BLU*™ to the buffer. Instead, use *Carolina BLU*™ in the gel and/or as final stain only.

Pool Small Volumes of DNA

Because of the small volumes supplied, 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 3 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 1.5-mL tube and remove carefully.
3. Tap tubes sharply on benchtop.

Set Up Student Workstations

1. Prepare 6 student stations, each with the following materials:

Vial pMAP/*Pst*I	Masking Tape
Vial pMAP/*Pst*I/*Hpa*I	Permanent Marker
Vial pMAP/*Pst*I/*Ssp*I	Gel Electrophoresis Chamber
Vial pMAP/*Pst*I/*Hpa*I/*Ssp*I	Power Supply
Rack for Tubes	Staining Tray
4 Needle-Point Pipets	Student Guide

2. Groups must share the following materials: agarose solution, TBE electrophoresis buffer, and *Carolina BLU*™ 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 lab for teaching about restriction enzymes and gel electrophoresis can be found in *Recombinant DNA and Biotechnology: A Guide for Teachers* (Carolina catalog # 21-2214).

Practice Gel Loading (Optional)

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

Fine Points of Laboratory Procedure

Be alert to the following cautions when performing the experiments. Where appropriate, discuss fine points with students.

Storing Cast Agarose Gels

If needed, students may cast gels a day before use. Keep gels covered with TBE electrophoresis buffer to prevent drying.

Electrophoresing

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 (bromophenol blue) nears the end of the gel. Do not let the loading dye band run off the end of the gel. During electrophoresis, *Carolina BLU*™ intercalates into the DNA, allowing for its

immediate visualization. Times below are for "variable voltage" minigel systems with 84-x-96-mm gel, using approximately 0.8% agarose. Exact run times will vary according to apparatus.

Voltage	Time
150 V	50 min
120 V	1 h
100 V	1 h 15 min
75 V	1 h 45 min
50 V	3 h
30 V	5 h 45 min
20 V	10 h

Carolina BLU™ 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 gels with *Carolina BLU*™ Final Stain, and allow to stain for 15–20 min.
2. Following staining, decant as much *Carolina BLU*™ solution as possible from staining tray back into original storage container. Place stained gel on light box. DNA bands should be visible. If bands are faint, additional staining may be required.
3. Rinse gel in distilled or deionized water. Chlorinated water tends to bleach bands with time. Let gel soak for several minutes in several changes of fresh water. DNA bands will become increasingly distinct as gel destains. For best results, continue to destain overnight in a small volume of water. (Gel may destain too much if left overnight in large volume of water.) Cover staining tray to retard evaporation.

Viewing and Photographing Gels

Transillumination, where light passes up through gel, gives superior viewing of gels stained with *Carolina BLU*™. A Polaroid® "gun" camera, equipped with close-up diopter lens, can be used to photograph gels. A plastic hood extending from the front of the camera forms a mini darkroom and provides correct lens-to-subject distance. Alternatively, a close-focusing 35-mm camera can be used.

Laboratory Procedure

A: Cast Agarose Gel

1. Students seal ends of gel-casting tray with tape, and insert the well-forming comb. They should place gel-casting tray out of the way on the lab bench, so that agarose poured in next step can set undisturbed.
2. Students carefully pour enough agarose solution into casting tray to fill to a depth of about 6 mm. Gel should cover only about one-half the height of the comb teeth. The tip of a transfer pipet is used to move large bubbles or solid debris to sides or ends of tray, while gel is still liquid.
3. Gel will become cloudy as it solidifies (about 10 min). Do not move or jar casting tray while agarose is solidifying.
4. When agarose has set, students unseal ends of casting tray, and place tray in gel box, so that comb is at negative (black) end. DNA will move from the negative, towards the positive.
5. Students fill box with tris-borate-EDTA (TBE) buffer, to level that just covers entire surface of gel.
6. Students must gently remove comb, taking care not to rip the wells.
7. Make certain that sample wells left by comb are completely submerged. If dimples are noticed around wells, students slowly add buffer until dimples disappear.
8. The gel is now ready to load with DNA.

   Note: If students will be loading the gel during another period, instruct them to cover the electrophoresis tank to prevent drying of the gel.

B: Load gel

Students use needle-point pipet to load contents of each tube (pMAP/*Pst*I, pMAP/*Pst*I/*Hpa*I, pMAP/*Pst*I/*Ssp*I/, pMAP/*Pst*I/*Hpa*I/*Ssp*I and lambda DNA/*Pst*I size markers) into separate wells in the gel. A fresh pipet should be used for each tube.

1. Steady pipet over well using 2 hands.
2. Be careful to expel any air in pipet tip end before loading gel. (If an air bubble forms a cap over the well, DNA/loading dye will flow into buffer around edges of the well.)
3. Dip pipet tip through surface of buffer, position it over the well, and slowly expel the mixture. Sucrose in the loading dye weighs down the

sample, causing it to sink to to the bottom of the well. Students should be careful not to punch tip of pipet through bottom of gel.

4. Students should now record the order in which they loaded the DNA samples. This is very important! If the loading order is lost, the sample identities will be unknown, and the students will be unable to determine the number and relative positions of the restriction enzyme sites in pMAP.

If students have difficulty using the pipets, you may suggest the following technique: instead of using the bulb to control the sample, pinch together the second or middle section (directly below the bulb) or lightly press the tapered section between bulb and the middle section.

C: Electrophorese

1. Students close top of electrophoresis chamber and connect electrical leads to an approved power supply, anode to anode (red-red) and cathode to cathode (black-black). Make sure both electrodes are connected to the same channel of the power supply. If leads are reversed, DNA will run backwards, through the short part of the gel, and will then be lost in the buffer.
2. Students turn on power supply, and set voltage as directed by the teacher.
3. Shortly after current is applied, loading dye (bromophenol blue) can be seen moving through the gel towards the positive (red) pole of the electrophoresis apparatus. Bromophenol blue migrates through the gel at the same rate as a DNA fragment approximately 300 bp long.
4. Allow the DNA to electrophorese until the bromophenol blue band nears the end of the gel. The teacher may monitor the progress of electrophoresis in the students' absence. The DNA bands are lightly stained during electrophoresis, and the distance between the bands should increase as the loading dye nears the end of the gel.
5. The students turn off the power supply, disconnect the leads from the power supply, and remove the top of the electrophoresis chamber.
6. Students should carefully remove the casting tray and slide the gel into a staining tray labeled with their group name. The students bring their gels to the instructor for further staining.

Results and Discussion

(corresponds with Data Analysis on Student Instructions)

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

2. Have your students assign sizes to their lambda DNA/*Pst*I size markers. These markers are: 514, 805, 1093, 1159, 1700, 1986, 2140, 2450, 2838, 4700, and 11,497.

   Note: This is not a complete list of lambda DNA/*Pst*I fragments. It has been abbreviated in the interest of clarity. If you choose, you may show students the ideal gel 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.

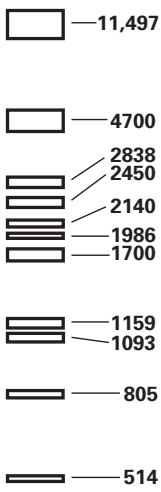


Figure 1 Ideal gel of lambda/*Pst*I size markers.

3. Have your students assign approximate sizes to the restriction-digested pMAP fragments by comparison to the lambda DNA/*Pst*I size markers. This will not be perfectly accurate.

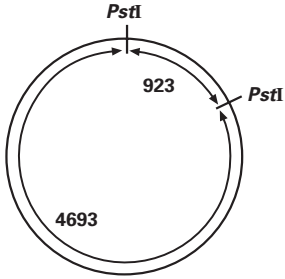
4. Have your students determine the total size of the digested DNA by adding up the sizes of the fragments from each digest. They may take an average size from the 4 digests: pMAP/*Pst*I, pMAP/*Pst*I/*Hpa*I, pMAP/*Pst*I/*Ssp*I, and pMAP/*Pst*I/*Hpa*I/*Ssp*I. The students should understand that the same DNA was digested in each sample so 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 2 *Pst*I sites and allow them to deduce that it is a circular piece of DNA since the pMAP/*Pst*I digest produces 2 fragments instead of 3, as a linear piece of DNA would.

   Hint: You may tell the students that the DNA is circular and have them determine how many *Pst*I sites are present.

5. Have your students draw pMAP with the *Pst*I sites present. Since the sizes of the *Pst*I 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:

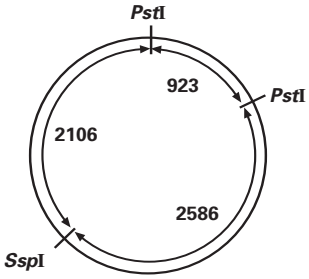


Note: Although this drawing accurately depicts the relative sizes of the fragments, the students do not have to determine the exact sizes of the fragments in order to determine the relative number and position of the restriction enzyme sites.

6. Have your students determine how many *Ssp*I sites are present by comparing the pMAP/*Pst*I lane and the pMAP/*Pst*I/*Ssp*I lane. Since the pMAP/*Pst*I lane has 2 fragments, and the pMAP/*Pst*I/*Ssp*I lane has 3 fragments, students should be able to tell that there is 1 *Ssp*I site.

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

8. Ask your students whether the *Ssp*I recognition site is close to the *Pst*I recognition sites, or if it is more in the middle of the 4693-bp fragment. Given that the 4693-bp *Pst*I fragment is cleaved into a 2106-bp fragment and a 2586-bp fragment, your students should be able to determine that the *Ssp*I site is in the middle of the 4693-bp fragment. They should now

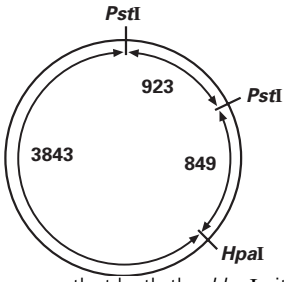


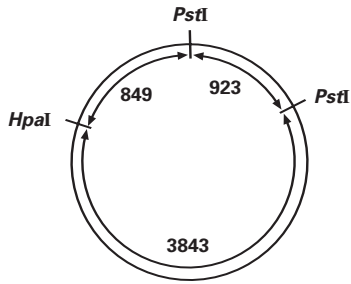
be able to draw the plasmid map shown below.

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 2 fragments, and the pMAP/*Pst*I/*Hpa*I lane has 3 fragments, students should be able to tell that there is 1 *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.

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

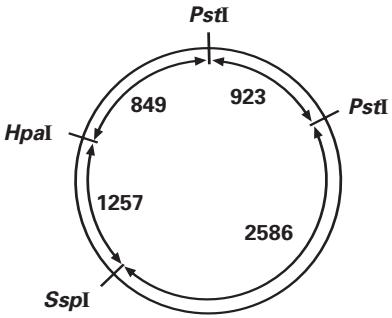
digest. The answer is the 849-bp fragment and the 923-bp fragment that is between the 2 *Pst*I sites. The other fragment (3843 bp) present in the pMAP/*Pst*I/*Hpa*I digest is not present in the pMAP/*Pst*I/*Hpa*I/*Ssp*I digest.

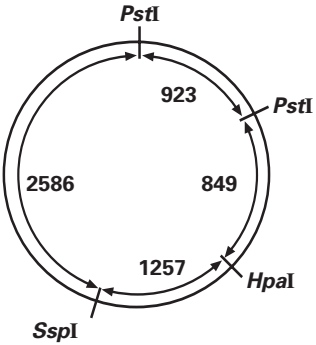
13. Ask your students why the 3843-bp fragment disappeared. They should reply that it contains an *Ssp*I recognition site.

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 a *Hpa*I recognition site.

16. Have your students draw the full plasmid map, with all restriction enzyme recognition sites present in their relative locations. Your students should draw either of the 2 following options. These are simply mirror-image representations of the same plasmid.





Note: Remember, student fragment sizes do not have to be exact.

Answers to Practice Problem 1

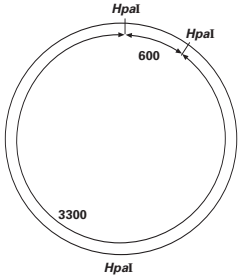
Note: Plasmid drawings are not to scale.

1.

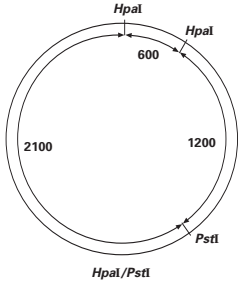
*Hpa*I	*Hpa*I/*Pst*I	*Hpa*I/*Ssp*I	*Hpa*I/*Pst*I/*Ssp*I
		300	300
600	600	600	600
	1200		1200
			1800
	2100		
		3000	
3300			

2. 3.9 kb (3900 base pairs)

3. This is circular DNA (plasmid). Restriction mapping can certainly be done

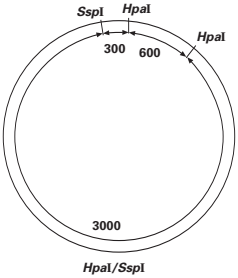


on linear DNA; however, it is slightly more complex and would call for a different set of restriction enzyme digests.



6. Since there are 2 fragments after digestion with *Hpa*I and 3 fragments after digestion with *Hpa*I and *Ssp*I, there is 1 *Ssp*I site.

7. The *Ssp*I site is in the 3300-bp *Hpa*I fragment.



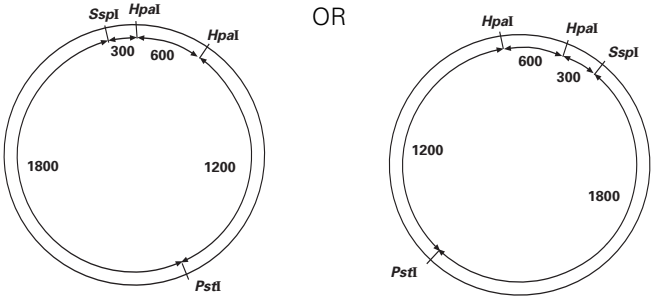
8. Yes. No *Ssp*I or *Pst*I sites are present within this fragment.

9. 600 and 1200 bp. The 2100-bp fragment disappeared because it contains a *Ssp*I site.

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

11. Yes. It means that there is a fragment with an *Ssp*I site on one end and a *Pst*I site on the other end.

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



Answers to Practice Problem 2

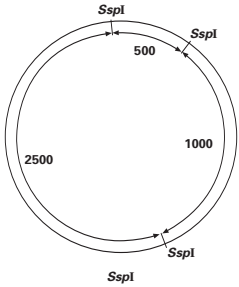
Note: Plasmid drawings are not to scale.

1.

*Ssp*I	*Ssp*I/*Hpa*I	*Ssp*I/*Pst*I	*Ssp*I/*Hpa*I/*Pst*I
			400
500	500	500	500
	900		900
1000	1000	1000	1000
		1200	1200
		1300	
	1600		
2500			

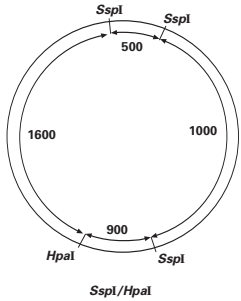
2. 4.0 kb or 4000 base pairs (bp)

3. There are 3 *Ssp*I sites.



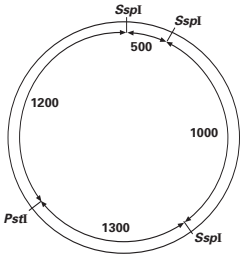
4. Since the *Ssp*I digest has 3 fragments and the *Ssp*I/*Hpa*I digest has 4 fragments, there is only 1 *Hpa*I site.

5. Since the 500- and 1000-bp *Ssp*I fragments are unchanged, the *Hpa*I site must be within the 2500-bp *Ssp*I fragment.



6. Since the *Ssp*I digest has 3 fragments and the *Ssp*I/*Pst*I digest has 4 fragments, there is only 1 *Pst*I site.

7. Since the 500- and 1000-bp *Ssp*I fragments are unchanged, the *Pst*I site must be within the 2500-bp *Ssp*I fragment as well.



*Ssp*I/*Pst*I

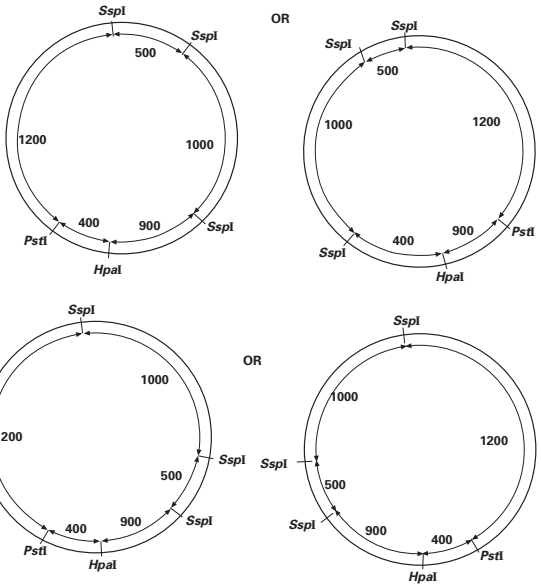
8. Yes. Both the *Pst*I and *Hpa*I sites are within the 2500-bp *Ssp*I fragment.

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

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

11. The 400-bp fragment. It only appears in the *Ssp*I/*Hpa*I/*Pst*I digest because it has an *Hpa*I site on one end and a *Pst*I site on the other end.

12. Full plasmid map; you may draw any of the following 4 options:



Student Instructions

21-1175

Name _____

Date _____

Restriction Mapping of Plasmid DNA

Background

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 towards 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 for the 3 restriction enzymes that you already have on hand—*Pst*I, *Hpa*I, and *Ssp*I. 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 4 single, double, and triple digests. First, you digest the unknown DNA with *Pst*I alone. Then, you digest the unknown DNA with *Pst*I and either *Hpa*I or *Ssp*I. Finally, you digest the unknown DNA with all 3 restriction enzymes.

You also set up a restriction enzyme digest with *Pst*I and lambda DNA, to make size markers 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 ends of gel-casting tray with tape, and insert well-forming comb. Place gel-casting tray out of the way on lab bench, so that agarose poured in next step can set without being disturbed.
2. Carefully pour enough agarose solution into casting tray to fill to depth of about 5 mm. Gel should cover only about one-third the height of comb teeth. Use a pipet tip or toothpick to move large bubbles or solid debris to sides or ends of tray, while gel is still liquid.
3. Gel will become cloudy as it solidifies (about 10 min). Do not move or jar casting tray while agarose is solidifying.
4. When agarose has set, unseal ends of casting tray. Place tray on platform of gel box, so that comb is at negative (black) end.
5. Fill box with tris-borate-EDTA (TBE) buffer to level that just covers entire surface of gel.
6. Gently remove comb, taking care not to rip wells.
7. Make certain that sample wells left by comb are completely submerged. If dimples are noticed around 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 gel from drying out.

B: Load Gel

Use micropipet to load contents of each reaction tube into a separate well in gel. Use a fresh tip for each reaction tube.

1. Draw sample into pipet or gel loading device.
2. Steady pipet over well using 2 hands.
3. Be careful to expel any air in micropipet tip end before loading gel. (If air bubble forms a cap over well, DNA/loading dye will flow into buffer around edges of well.)
4. Dip pipet tip through surface of buffer, position it over the well, and slowly expel the mixture. Sucrose in the loading dye weighs down the sample, causing it to sink to the bottom of the well. Be careful not to punch tip of pipet through the bottom of the gel.

C: Electrophorese

1. Close top of electrophoresis chamber, and connect electrical leads to an approved power supply, anode to anode (red-red) and cathode to cathode (black-black). Make sure both electrodes are connected to same channel of power supply.
2. Turn power supply on and set voltage as directed by your instructor. Shortly after current is applied, loading dye (bromophenol blue) can be seen moving through gel toward positive pole of electrophoresis apparatus.
3. Bromophenol blue migrates through gel at same rate as a DNA fragment approximately 300 base pairs long.
4. Allow the DNA to electrophorese until the bromophenol 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 power supply, disconnect leads from the inputs, and remove top of electrophoresis chamber.
6. Carefully remove casting tray, and slide gel into staining tray labeled with your group name. Take 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/*Pst*I size markers on your gel. These markers are 514, 805, 1093, 1159, 1700, 1986, 2140, 2450, 2838, 4700, and 11,497 bp. Remember, small DNA fragments migrate more quickly than large ones.
3. Now, assign approximate sizes to the fragments of unknown DNA by comparison to the lambda DNA/*Pst*I size markers. This will not be perfectly accurate. That's 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 4 digests: pMAP/*Pst*I, pMAP/*Pst*I/*Hpa*I, pMAP/*Pst*I/*Ssp*I/, and pMAP/*Pst*I/*Hpa*I/*Ssp*I. 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 *Pst*I sites: Number of *Ssp*I sites: Number of *Hpa*I sites:

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

Student Practice Problems

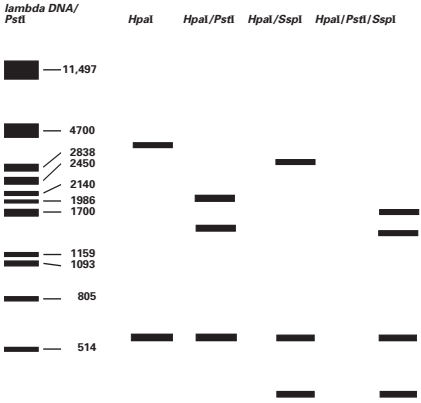
21-1175

Name _____

Date _____

Restriction Mapping of Plasmid DNA

Problem 1: Digested with *Hpa*I, *Hpa*I/*Pst*I, *Hpa*I/*Ssp*I, and *Hpa*I/*Pst*I/*Ssp*I



1. Estimate the sizes of the DNA fragments (in base pairs) by comparison to the lambda/*Pst*I size markers. These 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 4 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 2 *Hpa*I sites present. Based on the number of fragments obtained from the *Hpa*I digest, is this DNA linear or circular? Draw the DNA with the *Hpa*I 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.)

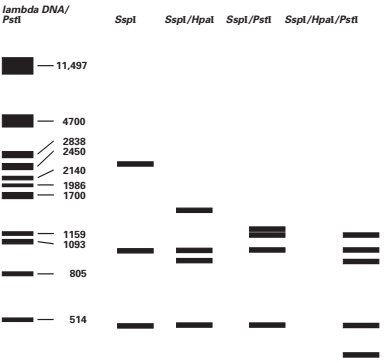
9. Which fragments are unchanged from the *Hpa*I/*Pst*I digest to the *Hpa*I/*Pst*I/*Ssp*I digest? Which fragments disappeared? Why did those fragments disappear?

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 *Ssp*I, *Ssp*I/*Hpa*I, *Ssp*I/*Pst*I, and *Ssp*I/*Hpa*I/*Pst*I



1. Estimate the sizes of the DNA fragments (in base pairs) by comparison to the lambda/*Pst*I size markers. These 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 4 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 *Ssp*I sites are present? Draw the relative positions of the *Ssp*I restriction sites on the plasmid.

4. How many *Hpa*I sites are present?

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

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

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

8. Will the 500- and 1000-bp *Ssp*I fragments remain unchanged after digestion with either *Pst*I or *Hpa*I? (Check the gel.)

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

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

11. Which fragment appears only in the *Ssp*I/*Hpa*I/*Pst*I 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.

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