

Extension Activity 1: Plasmid Mapping

Plasmids and Restriction Enzymes

This lesson will demonstrate the principles of plasmid mapping by examining restriction digestion patterns of plasmids used in the laboratory section of the kit and determining the position of restriction enzyme recognition sites in the plasmids by use of logic. Plasmid mapping has revolutionized molecular biology and paved the way for the biotechnology industry. This technique allows molecular biologists to quickly evaluate the success of cloning experiments as well as to easily identify plasmids and associated traits in different organisms. Although real-world DNA fingerprinting is performed on genomic DNA, this activity utilizes plasmid DNA to simulate how real DNA fingerprints are analyzed. Plasmids are circular, non-chromosomal pieces of DNA that can replicate in bacteria. Plasmids often carry genes encoding resistance to antibiotics which gives bacteria a selective advantage over their competitors.

Scientists routinely take advantage of plasmid DNA and a natural bacterial defense mechanism, the restriction enzyme, as the basis for much of biotechnology. Restriction enzymes allow bacteria to destroy DNA from invading bacteriophages (phages) which are viruses that infect and destroy bacteria. Restriction enzymes recognize specific DNA sequences within the phage DNA and then cut the DNA at that site. Fragmented DNA no longer poses a threat to bacterial survival. Purified restriction enzymes can be used in the laboratory to cut DNA isolated from any organism not just phage DNA.

After plasmids are cut with a restriction enzyme, they can be joined (or ligated) to a piece of DNA from any organism (foreign DNA) that has been cut with the same enzyme. The resulting hybrid DNA plasmid can be put into (transformed) bacterial cells. A hybrid plasmid can replicate itself in bacteria similar to the original plasmid, except that the foreign DNA that was incorporated is also perpetuated. Every hybrid plasmid now contains a copy of the piece of foreign DNA joined to it. We say that the foreign piece of DNA has been “cloned” and the plasmid DNA that carries it is called a “vector”.

The crime scene and suspect DNA samples in this kit were created by inserting lambda phage DNA that had been digested with the *Pst*I restriction enzyme into *Pst*I-digested plasmid pTZ18U. Recombinant plasmids were selected that gave distinct, striking banding patterns, or restriction fragment length polymorphisms (RFLP), when digested with restriction enzymes *Pst*I and *Eco*RI and analyzed on an agarose gel. Restriction maps of some of the crime scene and suspect plasmids are included on page 48.

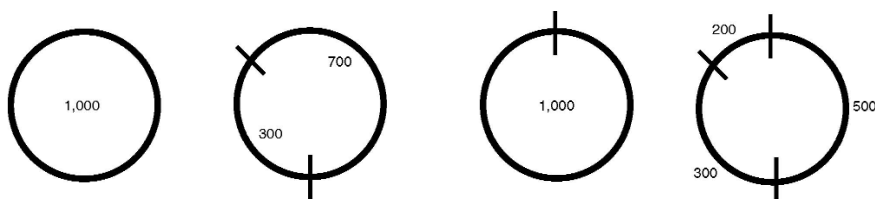
Plasmids can be mapped or described in terms of location of restriction sites using simple experiments and logic. The general procedure is to cut (digest) a plasmid with two restriction enzymes separately (two single digests) and the together (a double digest). Sizes of the resulting DNA fragments are determined then one uses logic to determine the relative location of the restriction sites. In the forensic DNA fingerprinting lab two restriction enzymes, *Pst*I and *Eco*RI, were used together in a double digest. The resulting fragments were run on a gel to solve the “who done it”. The background material can be used to construct a plasmid map

Since the plasmids are circular, the number of fragments represents the number of cuts or restriction sites. To visualize this, take a rubber band and cut it once. How many fragments are there? Cut the same rubber band again. How many fragments are there now? The most informative part of plasmid mapping comes from using logic to overlap the information from two single digests with information obtained from a double digest. How do the cuts from one restriction enzyme overlay with cuts from a second restriction enzyme? There are clues to see how to overlap them: Do any of the first fragments remain uncut

with the second enzyme? Do the sizes of any of fragments from the double digest add up the size of a fragment from a single digest? Do any of the fragments seem to remain the same size after being cut by the second enzyme? A simple example is shown below:

1000 bp plasmid example

DNA size marker	Undigested	Digested with Enzyme 1	Digested with Enzyme 2	Digested with Enzyme 1 & 2
1000 bp 700 bp 500 bp 300 bp 200 bp	1000bp	700 bp 300 bp	1000 bp	500 bp 300 bp 200 bp



Note that the two 1000 bp fragments (undigested sample and sample digested with enzyme 2) might not run at exactly the same distance on an agarose gel. There is a small difference in migration if a fragment of the same size is uncut (circular), cut (linear) or uncut and twisted (supercoiled). Also fragments that are very similar in size may migrate together in an agarose gel and it may not be possible to distinguish between them. In addition, fragments that are very small, may not be detected by the DNA stain or may run off the end of the gel.

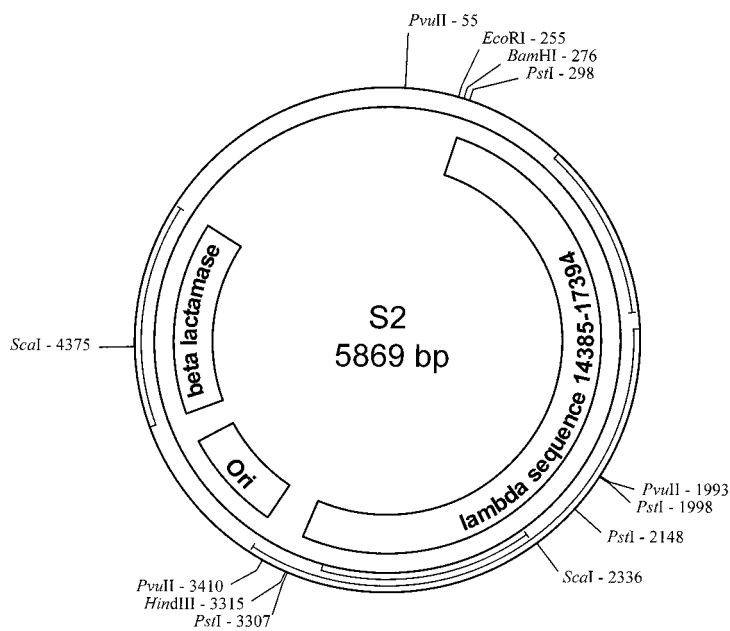
Reading a Plasmid Map

A plasmid map includes information on the size of the plasmid, the genes present, the origin of replication site, and restriction sites for restriction enzymes. All five of the plasmids used in the DNA fingerprinting activity were constructed from the same pTZ18U plasmid parent but had different foreign fragments of DNA inserted into them. In the DNA fingerprinting exercise, only two restriction enzymes were used, but other enzymes could also have been used to cut these plasmids. The restriction sites are marked on the map with a number that indicates the location of the site. Since the plasmid is circular, there is an arbitrary zero point. All of the restriction sites are indicated with a number between zero and the total base pairs in the plasmid. Fragment sizes can then be calculated by simple subtraction (and in some cases addition) between points on the plasmid.

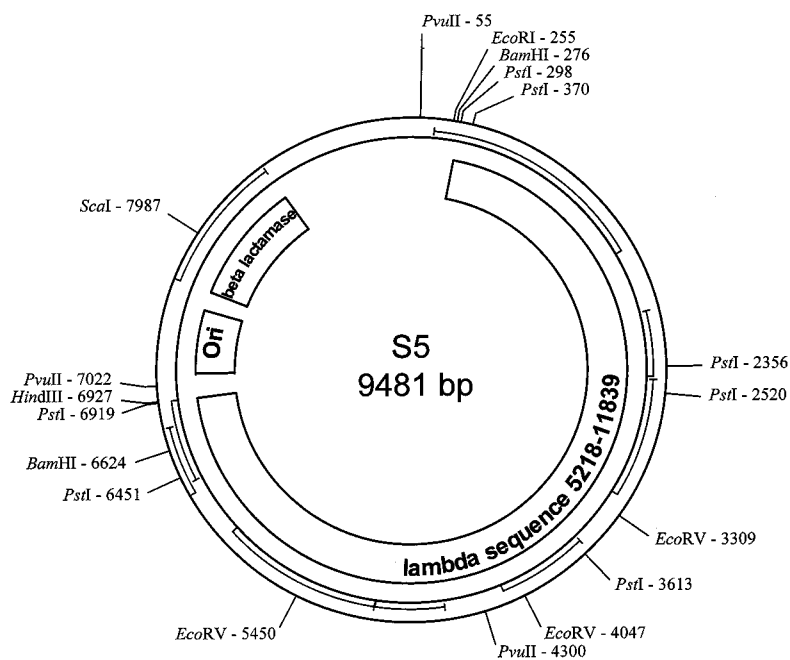
Plasmids Used In This Lesson

Plasmid maps show the positions (numbered by DNA base pairs) of sites where the plasmid may be cut by particular restriction enzymes. The name of the plasmid and its size of DNA in bp is shown inside the circle. In addition it shows the Origin of Replication (Ori), the gene encoding beta lactamase (the enzyme that gives the bacteria resistance to the antibiotic ampicillin) and the location where foreign DNA from the lambda bacteriophage was inserted. Refer to the plasmid maps on page 49 for more detail.

Sample Plasmid Maps



Plasmid S2



Plasmid S5

Reading a Plasmid Map Questions

1. From the map of plasmid S2 list all the restriction enzymes that would cut this plasmid.
2. Which plasmid, S2 or S5, is the biggest and what is its size?
3. Using plasmid S2 as an example, find the restriction sites for the enzyme *PvuII*. How many sites are there? What is their location? If *PvuII* was used to cut (digest) this plasmid, how many fragments would it make?
4. Next determine the size of the fragments created when plasmid S2 is cut by *PvuII*. DNA fragment size is calculated by subtracting the site locations from each other. (Note: if a fragment contains the 0 point of the plasmid, it is not just a simple subtraction!). How big are the fragments from plasmid S2 that is cut with *PvuII*? The fragment sizes should add up to the total for that plasmid (5869 bp).
5. If the fragments from the plasmid S2 digested with *PvuII* were run on an agarose gel, what would they look like? Draw the gel and label the fragments and their sizes.

6. Now you can determine the fragment sizes of the plasmids when cut with the two enzymes, *EcoRI* and *PstI*. Indicate the sizes of the fragments that would be generated if the plasmid were a digest by *PstI* alone, *EcoRI* alone or by both *PstI* and *EcoRI*.

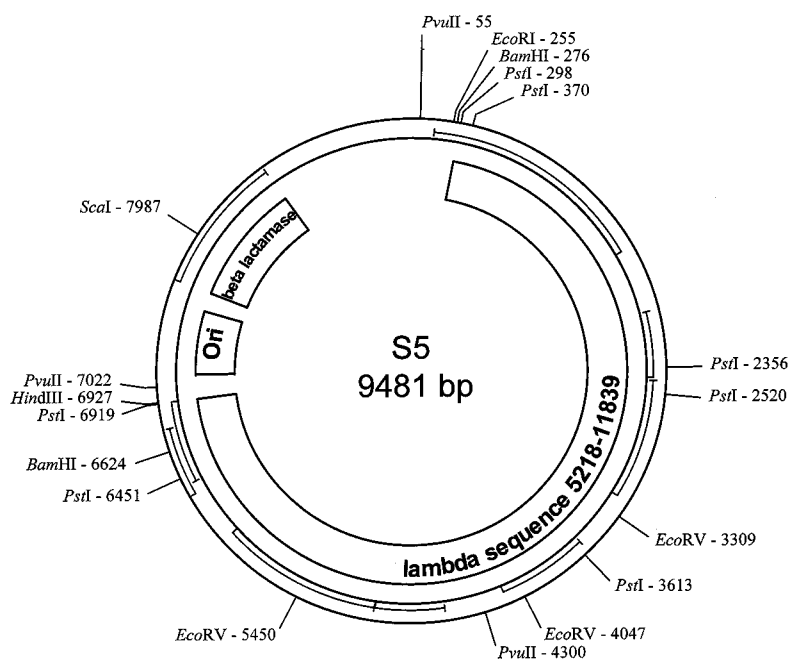
Plasmid S2 (5869 bp)			
Enzymes	<i>EcoRI</i>	<i>PstI</i>	Both
Fragments			
Total			

7. If plasmid S2 was digested and run on an agarose gel, what would the gel look like? Draw a gel and the fragment sizes if digested by *EcoRI* alone, *PstI* alone and by *EcoRI* and *PstI* together.
8. How does your diagram in question 7 compare to what was observed in your gel after the experiment? Indicate a reason for why your data in question 7 might be different from the actual experimental data seen from lesson 2.

Mapping the Plasmid

The first step in mapping a plasmid is to determine how many times a restriction site is found on that plasmid. First examine the results for plasmid S5 as an example. The data given in the following table are for the double digest using both *EcoI* and *PstI*. Also given are the data for single digests by the individual enzymes. The numbers in the columns under each enzyme represent the sizes of all of the fragments that are formed when each enzyme is used for a single digest.

The charts below show the sizes of fragments that will be generated when plasmid S5 has been digested with the indicated restriction enzyme.



Plasmid S5

Plasmid S5 (9481 bp)			
Enzymes	<i>EcoRI</i>	<i>PstI</i>	Both
Fragments	9481	2860	2838
		2838	2817
		1986	1986
		1093	1093
		468	468
		164	164
		72	72
			43
Total			

Mapping the Plasmid Questions

1. How big is plasmid S5? Add the fragments in each column. The total should add up to the size of the plasmid. Why?
2. Look at the data from the *EcoRI* digest of plasmid S5. How many fragments are there? Did the enzyme cut the plasmid, or did it remain as a circle? How could you tell?

3. Compare the data from the *Pst*I digest of plasmid S5 with that of the *Eco*RI digest. How many fragments are there? How many restriction sites are there for *Pst*I?
4. How many fragments are there when *Eco*RI and *Pst*I are used to digest plasmid S5? Does that answer the question of whether or not *Eco*RI cut the plasmid? Why?
5. Which fragment of *Pst*I digested plasmid S5 was shortened by a cut with *Eco*RI? How do you know this?
6. Draw the *Pst*I fragment that is cut with *Eco*RI in plasmid S5 to demonstrate how the fragment was cut with *Eco*RI.

Plasmid S3 (7367 bp)			
Enzymes	<i>Eco</i> RI	<i>Pst</i> I	Both
Fragments	6504	4507	3687
	863	2860	2817
			820
			43
Total			

7. Restriction mapping is an exercise in critical thinking and logic. Plasmid S5 is difficult to completely map because of the numerous *Pst*I restriction sites. With the data, it would be very difficult to place all the restriction sites in order. It is easier to map plasmid S3. Shown above is the data generated from digestion of plasmid S3 with *Eco*RI and *Pst*I. How many times did *Eco*RI cut plasmid S3? What are the fragment sizes?

8. The data from the *Eco*RI digest of plasmid S3 indicate that the fragments are not equal. Draw a possible map and label the *Eco*RI sites and the sizes of the fragments.
9. Now draw an approximate map of the *Pst*I sites on plasmid S3 and label the *Pst*I sites and the sizes of the fragments.
10. Draw a circular map of plasmid S3 digested with both *Pst*I and *Eco*RI. Mark sizes of each fragment and name the restriction sites on your figure.
11. Is there another possible order of restriction sites on plasmid S3 digested with both *Pst*I and *Eco*RI? How might you resolve these possibilities?
12. When the gels were run for this experiment, there were only three bands for plasmid S3. Which band is missing from your gel? Why?

Extension Activity 2: Constructing a Plasmid

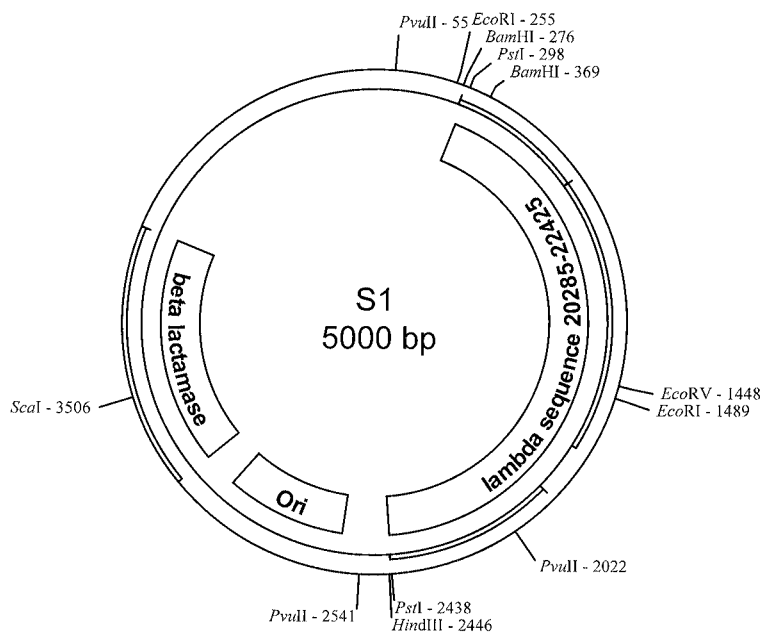
Some plasmids replicate when the bacterial genomic DNA replicates, but others replicate independently producing hundreds of copies of the plasmid within one cell. A self-replicating plasmid contains its own origin of replication (Ori). Many plasmids also contain genes that give antibiotic resistance to bacteria.

Plasmids are made of DNA just like the bacterial genome. Since DNA is universal in all living things, plasmids with inserted foreign DNA may be put into a bacteria (transforming them), causing the bacteria to transcribe and translate that message into a protein product. The biotech industry is in part based on this principle. Bacteria can be given human genes via a plasmid, and once transformed can produce a human protein product.

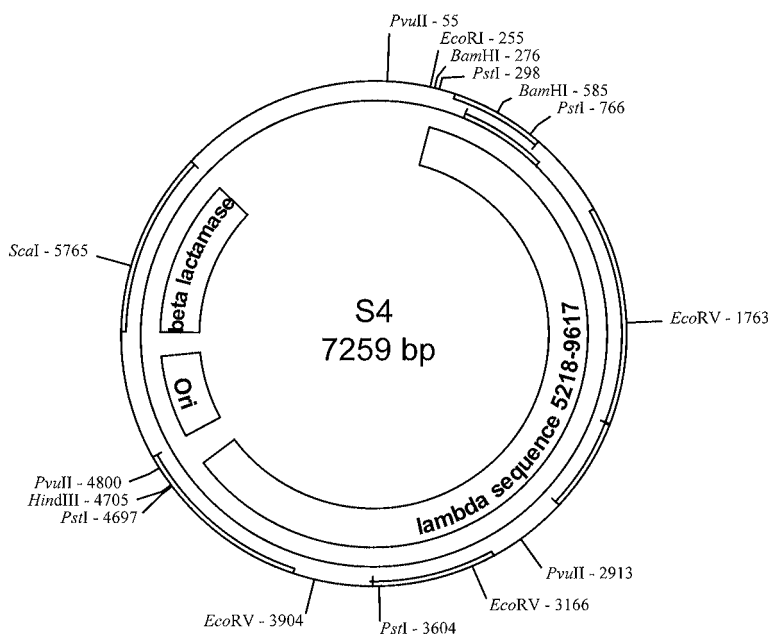
This exercise is based on plasmids used in the forensic DNA fingerprinting lab. Five plasmids were constructed from a parent plasmid, and then were cut (digested) to make fragments of different sizes for a gel analysis. How were the plasmids constructed? Could one have made the plasmids differently? Can one predict fragment sizes using plasmid maps constructed from the lambda bacteriophage genome? In this activity you will design a new plasmid.

The parent plasmid pTZ18U used for plasmid construction is 2860 bp in size. It has numerous restriction sites (see diagram on pages 56–57). The five plasmids constructed for the DNA fingerprinting lab were all based on this plasmid.

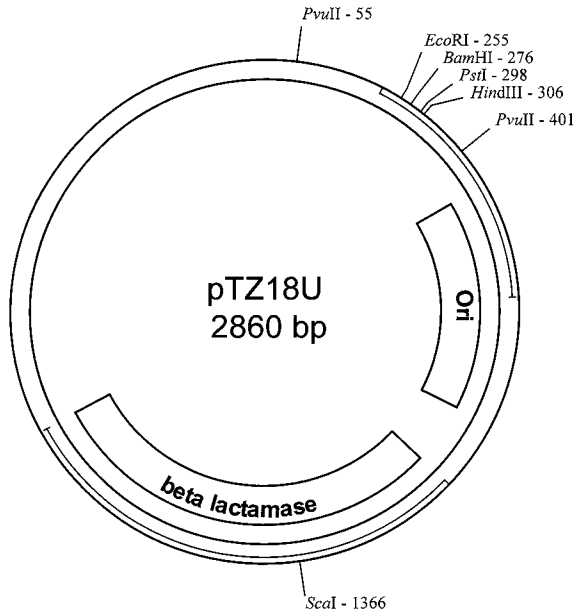
The pTZ18U plasmid was digested with the *Pst*I restriction enzyme. Lambda phage DNA was also digested with the *Pst*I restriction enzyme and the resulting fragments were then inserted into the *Pst*I – digested pTZ18U plasmid. As you look at the different plasmids, notice that each plasmid contains different fragments of lambda phage DNA. For example, plasmid S1 contains lambda sequence 20285–22425.



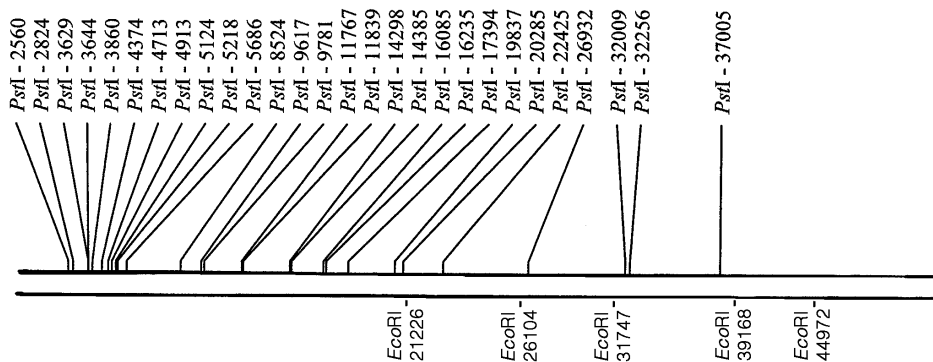
Plasmid S1



Plasmid S4



Parent Plasmid pTZ18U



lambda bacteriophage genome
48502 bp

Constructing a Plasmid Questions

1. Where is the *Pst*I site on the pTZ18U plasmid?
2. Look at plasmid S4. What segment of the lambda bacteriophage has been inserted?
3. After looking at the plasmid map and also the lambda phage map, can you determine how many *Pst*I restriction sites were added to the plasmid because of the inserted lambda phage DNA fragment? Note that it is possible for these extra *Pst*I sites to have been added if the original restriction digestion was done for a short time so that not all *Pst*I sites would have been completely cut in every piece of lambda phage DNA.
4. Look at plasmid S1. What segment of lambda was added to that plasmid? Were any *Pst*I restriction sites added to the plasmid with the inserted fragment of lambda DNA?
5. Now let us create a different plasmid from the parent plasmid. You will use *Eco*RI for the construction and need to refer to the lambda bacteriophage genome map that includes the *Eco*RI sites. You must make a plasmid that is at least 5,000 base pairs but not more than 10,000 base pairs in size. Remember that the parent plasmid is 2860 bp in size. Where is the *Eco*RI site on the parent pTZ18U plasmid?
6. Choose a segment of lambda bacteriophage genome that could be cut out by the *Eco*RI enzyme. Which segment will you use?

7. Draw your new plasmid with the insert of your choice. Be sure to include the restriction sites for *Pst*I and *Eco*RI in your drawing. How big is your new plasmid? Give the positions of the restriction sites in your new plasmid a number indicating the location. Remember that the first *Eco*RI site will still be position 255 as it is in the parent pTZ18U plasmid map.

8. How many restriction sites are there now for *Pst*I in your new plasmid? Predict what fragments you would generate if you were to digest your plasmid with:
 - i. *Eco*RI alone

 - ii. *Pst*I alone

 - iii. *Eco*RI and *Pst*I together (a double digest)

9. Draw an agarose gel for each of these digests and label the fragment sizes.

10. The lambda phage fragment can be inserted into the host plasmid in either orientation – forwards or backwards. How could you use plasmid mapping to determine in which orientation your fragment was inserted? Use a diagram in your explanation.