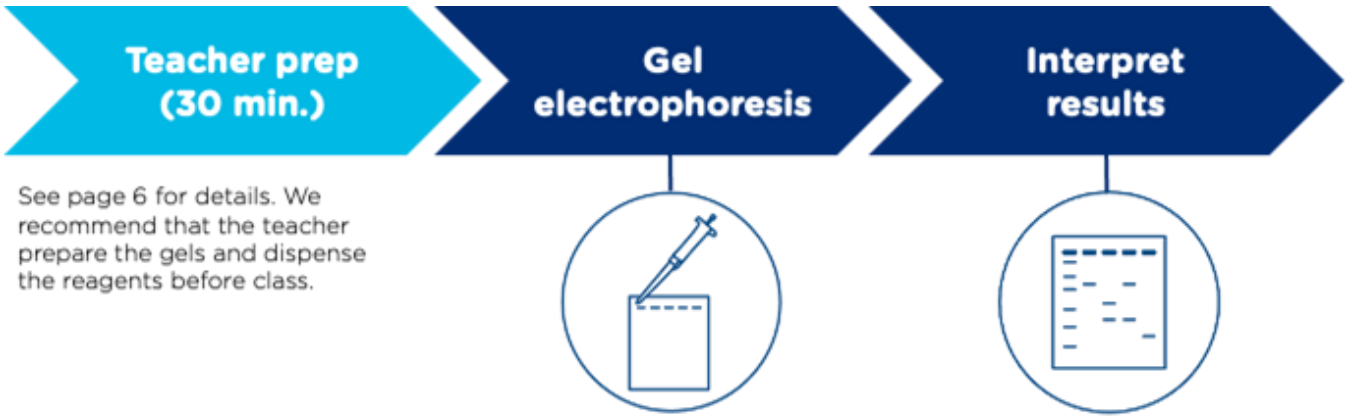
Plasmid DNA Restriction Mapping Lab

Contents

Experimental overview

Introduce students to the use of restriction enzymes for creating plasmid maps. The use of pre-digested plasmid DNA samples ready for gel electrophoresis analysis means this activity can be completed in a single class (see detailed class time requirements below).



Class time requirements

Steps		Time required
Prep	Make gels	We recommend the teacher prepare the gels outside of class (see page 8). Allot 30 minutes of class time if you opt to have students prepare the gels.
1	Load gel	10 minutes
2	Run gel	30-45 minutes The gel does not need to be actively monitored during this time.
3	Interpret results	5 minutes

Technical support

If you have any questions about implementing this activity, contact support@minipcr.com

Materials needed

Supplied in kit (KT-1507-02)

- The kit contains enough reagents for at least 8 lab groups.
- If kept in the freezer, reagents can be stored for 12 months after receipt.
- Reagents for preparing gels, plastic tubes for aliquoting reagents, and pipette tips are sold separately. See below for details.

Reagents and supplies	Provided	Required	Storage
Plasmid DNA	150 µl	15 µl per group	Freezer
BamHI Digested Plasmid	150 µl	15 µl per group	Freezer
ApaLI Digested Plasmid	150 µl	15 µl per group	Freezer
Double Digested Plasmid	150 µl	15 µl per group	Freezer
3K DNA Ladder	120 µl	10 µl per group	Freezer

Electrophoresis reagents and plastics sold separately

- This activity requires 2% agarose gels with a fluorescent DNA stain (*e.g.*, SeeGreen™ or GelGreen®) and 1.5 ml or 0.2 ml microtubes for dispensing and heating DNA samples.
- The Learning Lab Companion Kit (KT-1510-01) provides sufficient reagents to prepare and run 8 gels when using the blueGel™ or Bandit™ electrophoresis systems, as well as plastic tubes.
- Alternatively, bulk electrophoresis reagents and plastics (tubes, pipette tips) are available for purchase from miniPCR bio.
- Gel electrophoresis reagents and plastics can also be purchased from other suppliers.

Required equipment

- This lab is compatible with any horizontal gel electrophoresis system in combination with:
  - A fluorescent DNA stain (*e.g.*, SeeGreen™ or GelGreen®)
  - A transilluminator that is compatible with the DNA stain used. Fluorescent DNA stains typically require blue light (~470 nm) or UV (~260 nm) illumination.
- The table below outlines equipment from miniPCR bio that meets these requirements.

Item	Recommended quantity
Gel electrophoresis and visualization system	
Option 1: blueGel™ OR GELATO™ electrophoresis systems with integrated blue light transilluminator	1 blueGel can be shared by two groups 1 GELATO can be shared by four groups
Option 2: Bandit™ STEM Electrophoresis Kit paired with the Viewit™ Illumination Kit	1 Bandit + 1 Viewit per group
Option 3: Bandit™ STEM Electrophoresis Kit paired with a blueBox™ blue light transilluminator	1 Bandit per group + 1 blueBox for the class to share
Micropipettes and tips	
2-20 µl adjustable	1 pipette per group

Other materials supplied by user

- Distilled water
- Microwave or hot plate
- Disposable laboratory gloves
- Protective eyewear
- Fine-tipped permanent markers

Teacher prep

Protective gloves and eyewear should be worn for the entirety of this experiment.

Overview

The table below provides an overview of the teacher prep, and the subsequent pages provide detailed instructions.

Prep	Time required	Timeline
Dispense reagents	10 minutes	Can be completed up to one week before use.
Prepare electrophoresis buffer and agarose gels	20 minutes	Varies - If using gel reagents from miniPCR, gels can be prepared up to five days before use.

Dispense reagents

- DNA samples can be dispensed up to one week in advance and stored in the refrigerator.
- This kit provides sufficient reagents for at least 8 lab groups.

Materials needed

From the lab kit (stored in the freezer):

- Plasmid DNA
- BamHI digested plasmid
- ApaLI digested plasmid
- Double digested plasmid
- 3K DNA Ladder

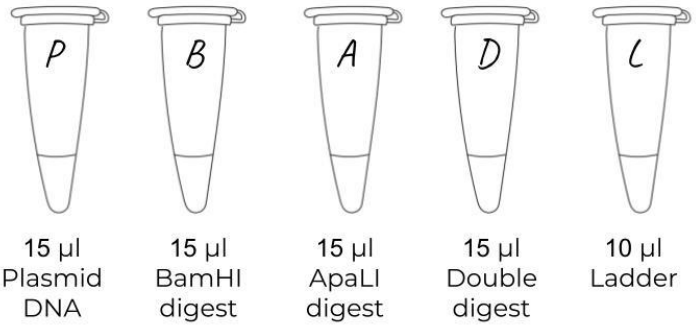
Supplied by user:

- Plastic tubes for dispensing reagents (1.5 ml or 0.2 ml tubes)
- 2-20 µl micropipette and tips
- Fine-tipped permanent marker

1. Thaw reagents by placing tubes at room temperature.
2. Collect the liquid at the bottom of each tube. Either spin briefly in a microcentrifuge or shake the liquid down with a flick of the wrist.
3. When you open each tube, check for liquid stuck inside the cap. If necessary, put the cap back on and repeat step 2.
4. For each lab group, dispense the following reagents into plastic tubes (1.5 ml or 0.2 ml capacity).
5. Label the upper sidewall of each tube with the corresponding sample ID.

   - Plasmid DNA (P) 15 µl
   - BamHI digest (B) 15 µl
   - ApaLI digest (A) 15 µl
   - Double digest (D) 15 µl
   - 3K DNA Ladder (L) 10 µl

6. Repeat steps 4 and 5 for as many lab groups as required by your class. This kit provides enough reagents for at least 8 groups.



7. If you are preparing the DNA samples more than 24 hours before class, store the tubes in the refrigerator until use. Dispensed DNA samples can be stored in the refrigerator for up to one week.

Prepare gel electrophoresis buffer and agarose gels

1. Prepare electrophoresis buffer.
   - Follow the manufacturer's instructions to prepare buffer solution.
   - The volume of buffer needed varies depending on the gel electrophoresis system.
     - For the blueGel and Bandit electrophoresis systems, 600 ml of TBE buffer is sufficient for at least eight gel runs.
     - For other systems, refer to the manufacturer's instructions for:
       (1) The buffer volume needed to prepare agarose gels.
       (2) The buffer volume needed for use as running buffer.

2. Prepare 2% agarose gels with fluorescent DNA stain.
   - Each group will need four lanes, plus one lane for ladder. If groups are sharing gels, a single lane for ladder per gel is sufficient.
   - This lab kit is compatible with any molecular grade agarose and fluorescent DNA stain (*e.g.*, SeeGreen™ or GelGreen®).
   - The volume of gel needed varies based on the gel electrophoresis system you are using. Refer to the manufacturer's instructions.
   - If using gel electrophoresis reagents from miniPCR bio, gels can be prepared up to five days in advance. Store prepared gels at room temperature in an airtight container protected from light. Do NOT soak the gels in buffer or wrap them in paper towels.

Student workstation setup

At the start of this experiment, every lab group should have:

DNA samples: • Plasmid DNA (tube P) • BamHI digest (tube B) • ApaLI digest (tube A) • Double digest (tube D)	15 µl each
3K DNA Ladder (tube L)	10 µl
2-20 µl micropipette and tips	1
Electrophoresis buffer *Volume depends on your electrophoresis system	30 ml TBE if using a blueGel or Bandit
5 wells* in a 2% agarose gel with fluorescent DNA stain	

*** Note:** If groups are sharing gels, they can also share the DNA ladder (*i.e.*, only 9 wells are needed per every two groups sharing a gel).

Student guide

Background information

Plasmids are powerful lab tools

Plasmids are small, circular pieces of DNA found in bacteria. They are independent of the bacterial chromosome, but typically plasmids are passed on to both daughter cells during cell division. Plasmids aren't always essential for survival, but they can give bacteria an advantage in the right situation. For example, some plasmids carry genes that help bacteria resist antibiotics or break down unusual food sources that other bacteria can't digest.

Scientists have taken these natural DNA carriers and turned them into powerful lab tools. In the lab, we use plasmids to store and multiply important bits of genetic information. With common genetic engineering techniques, we can cut and paste useful DNA directly into plasmids. These engineered plasmids can then be inserted into bacteria or other cells to replicate. The information encoded in plasmids can then enable these cells to perform valuable functions, such as producing human insulin for diabetics or enzymes that power laundry detergents.

Plasmid maps outline key DNA elements

To engineer a plasmid, researchers first need a clear picture of the DNA it contains and the location of its main elements. A **plasmid map** is a visual guide that shows the key features of a plasmid:

- A plasmid's total **length in base pairs** (bp)
- **Multiple cloning sites**, special regions where scientists can insert DNA of interest
- The **origin of replication,** necessary for the plasmid to be copied inside a cell
- **Antibiotic resistance genes** or other genes that help identify cells that carry the plasmid (broadly called **selectable markers**)

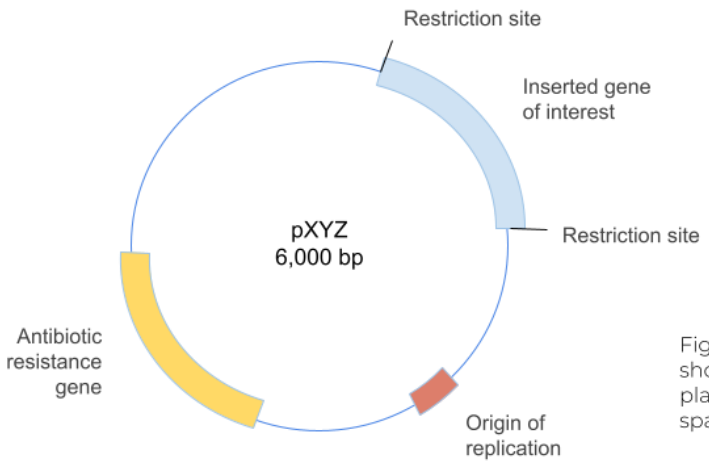


Fig. 1: a plasmid map showing typical features of plasmids as well as their spatial relationships.

Plasmid maps aid scientists in designing genetic engineering experiments, such as inserting DNA of interest into a plasmid. The information in the map is then used to help verify that newly engineered DNA pieces were built correctly. To understand how, you'll first need to become acquainted with restriction enzymes.

Restriction enzymes cut DNA at precise sites

Restriction enzymes evolved in bacteria as a defense system against invading viruses, but scientists have repurposed them as DNA-cutting tools used in the lab. Each restriction enzyme cuts DNA at a specific sequence, usually 4-8 base pairs (bp) long. These target sequences are called **recognition sites**.

Here are some common restriction enzymes and their recognition sites:

- The restriction enzyme **EcoRI** cuts within the **GAATTC** recognition site
- **BamHI** cuts within **GGATCC**
- **PvuII** cuts in the middle of **CAGCTG**

When you expose a plasmid to a restriction enzyme, the enzyme will cut the DNA wherever it finds its recognition site(s). As a result, the plasmid will go from a circular DNA molecule to linear fragments that scientists can separate and measure. Scientists use this process to verify their results when working with plasmids.

Genetic engineers use restriction mapping to check results

Imagine that you are preparing to use plasmid pXYZ for the first time. You have a tube that is labeled pXYZ, but before using the DNA, you must confirm its identity. The plasmid map shows two EcoRI recognition sites, spaced 1,200 bp apart. This means that cutting circular pXYZ with EcoRI should produce two linear DNA fragments:

- a 1,200 bp fragment – the "DNA of interest"
- a 4,800 bp fragment – the rest of the plasmid

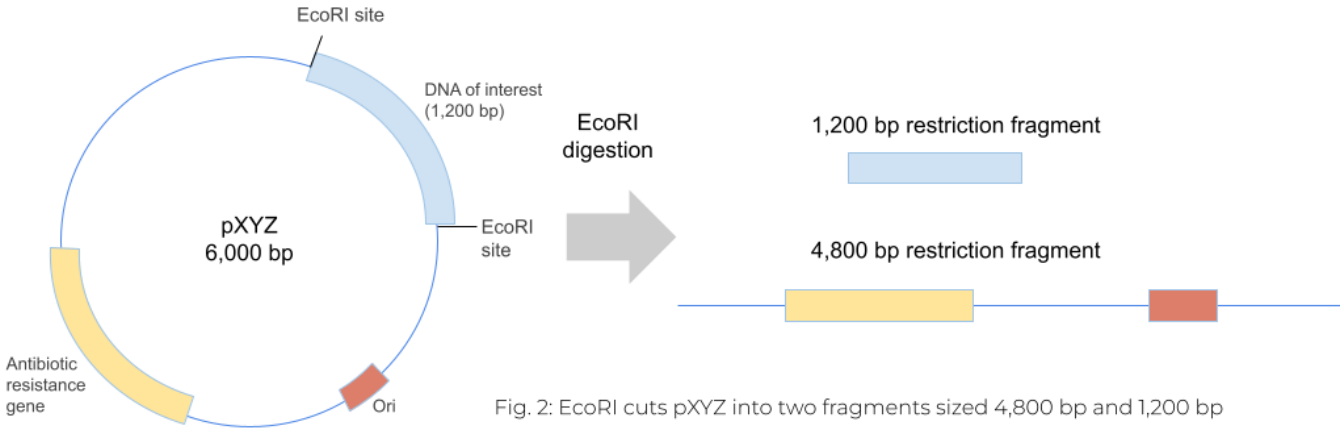


Fig. 2: EcoRI cuts pXYZ into two fragments sized 4,800 bp and 1,200 bp

In sum, exposing the plasmid DNA to restriction enzymes and checking the resulting DNA fragment sizes is a quick way to verify the identity of the DNA.

Pre-lab activity: Creating plasmid maps using gel electrophoresis analysis of restriction digests

Recall key features of the pXYZ plasmid introduced in the *Background information*:

- Circular DNA molecule
- 6,000 bp in size
- Two EcoRI restriction sites 1,200 bp apart

The plasmid map shows that cutting circular pXYZ with the enzyme EcoRI should produce two DNA pieces: 4,800 base pairs and 1,200 base pairs long. To confirm this, we can use gel electrophoresis. **Gel electrophoresis** separates DNA fragments by size. At the end of a gel electrophoresis experiment, smaller pieces of DNA will have traveled farther through an **agarose gel** than larger pieces of DNA. For more detailed information on electrophoresis, refer to https://www.minipcr.com/gel-electrophoresis/.

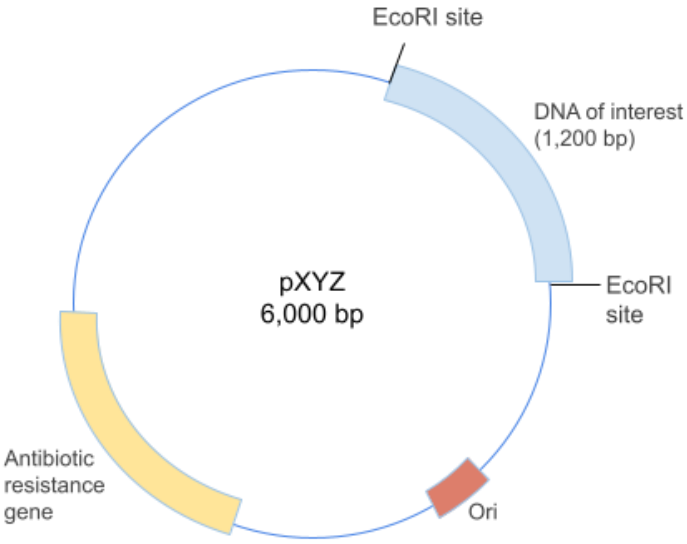
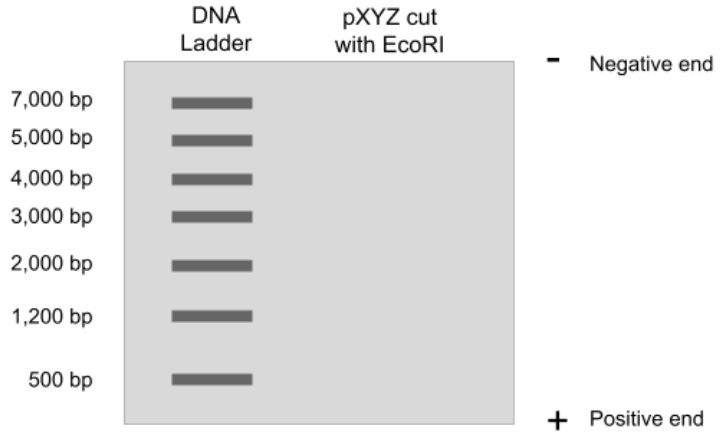


Fig. 3: Plasmid map of pXYZ showing EcoRI sites.

Questions

1. You have cut pXYZ with EcoRI. In the diagram below, draw the DNA bands corresponding to the resulting restriction fragments. Use the supplied DNA ladder as a reference.



2. A more complete plasmid map shows that pXYZ has additional restriction sites for the enzymes BamHI and XmnI, as well as their positions within the plasmid.

   The location (in bp) of each cut site is noted in parentheses after the name of the restriction enzyme. For example, the map indicates that one EcoRI site is at position 250 and another at 1,450.
   To calculate the distance between these sites, you can just subtract their positions: 1,450 *minus* 250 *equals* 1,200 (where the units are, of course, bp).

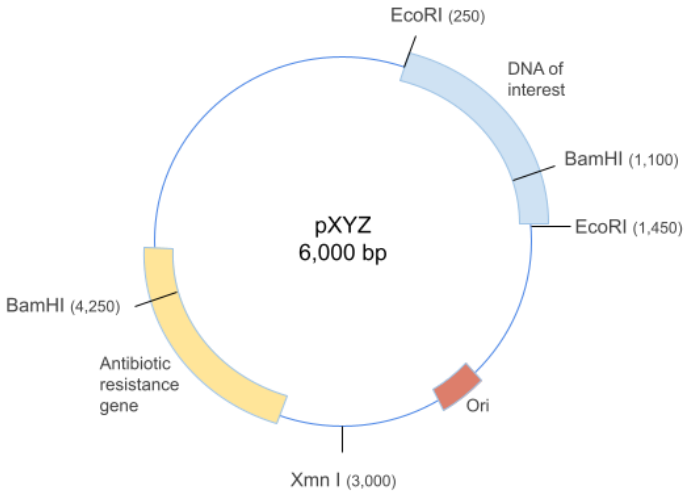
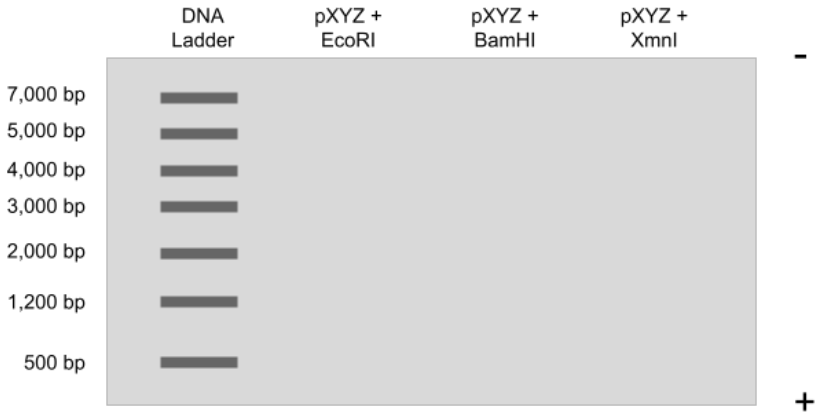


Fig. 4: Map of plasmid pXYZ showing additional restriction sites and their positions.

Complete the table below with the resulting restriction fragments and their sizes:

Restriction enzyme(s)	# DNA fragments	Restriction fragment sizes (bp)
None	1 (circular)	6,000
EcoRI	2	4,800 and 1,200
BamHI		
XmnI		

3. In the gel diagram below, draw the DNA bands for the resulting restriction fragments:



4. To create complete plasmid maps, scientists typically perform **double digests** that cut with two different restriction enzymes simultaneously.

   Complete the table below predicting the restriction fragment sizes resulting from the following double digests. Refer to Fig. 4 for the necessary information.

Restriction enzyme(s)	# DNA fragments	Restriction fragment sizes (bp)
None	1 (circular)	6,000
EcoRI + XmnI	3	1,200, 1,550 and 3,250
EcoRI + BamHI		
XmnI + BamHI		

5. In making these predictions, you may have noticed a useful feature of double digests: they can reveal where different recognition sites are in relation to each other. By comparing the single digest for restriction enzyme A to the double digest pattern of enzymes A and B, we can tell how close together or far apart their recognition sites are on the plasmid.

   pXYX cut with EcoRI produced two fragments: 1,200 bp and 4,800 bp. A double digest with EcoRI and XmnI yielded three fragments: 1,200 bp, 1,550 bp, and 3,250 bp. Based on these observations, which of the following statements are true? Select all that apply.

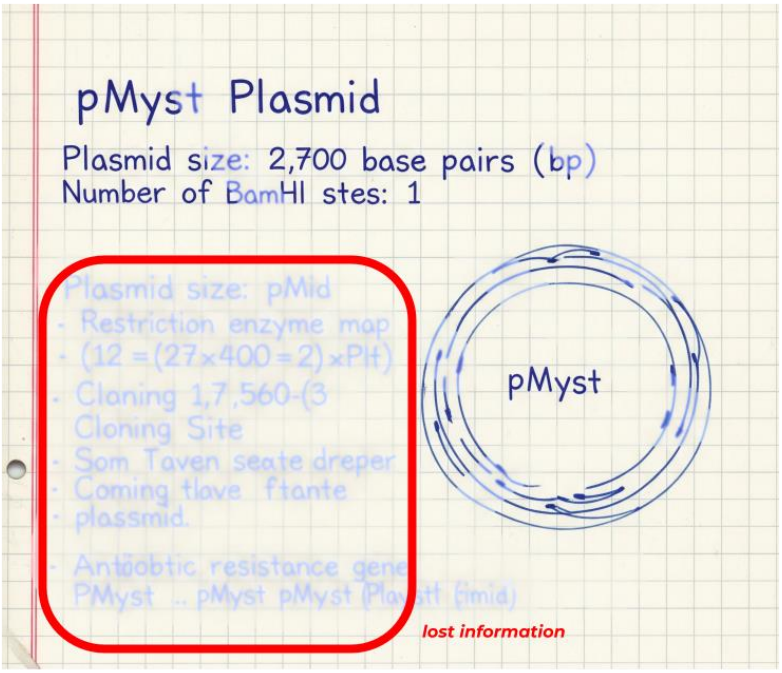
   A. XmnI cuts once in the pXYZ sequence
   B. XmnI cuts between two EcoRI sites spaced 1,200 bp apart
   C. XmnI cuts 3,250 bp from an EcoRI site
   D. XmnI cuts 1,550 bp from an EcoRI site

   Explain your reasoning with a diagram.

Restriction fragments are like puzzle pieces. Restriction mapping lets you figure out how these pieces connect, so you can draw the complete circular map showing exactly where each recognition site is located.

Today's lab

You're a molecular biologist working in a genetic engineering lab. You have just received a tube of DNA containing a plasmid made by a former colleague.



The tube is labeled "pMyst" (how mysterious!). The goal of your project is to use restriction enzymes to cut a piece of DNA out of pMyst – the "gene of interest" – and replace it with a new gene. But sadly, the plasmid map for pMyst has long been missing from the lab.

You find your old colleague's lab notebook, and their notes describe some basic features of pMyst. Unfortunately, it looks like someone spilled something on this page of the lab notebook, and only the information shown on the right is legible.

Luckily, you have just enough information to try to create a new plasmid map for pMyst. You have already set up restriction digests using BamHI and ApaLI. Now you will use gel electrophoresis to determine the fragment lengths for the following samples:

1. Uncut pMyst plasmid DNA
2. pMyst digested with BamHI
3. pMyst digested with ApaLI
4. pMyst digested with BamHI and ApaLI

You will use this information to create a plasmid map for pMyst.

Time to cut through this mystery!

Student lab protocol

1. Place the prepared gel into the electrophoresis chamber.
2. Add enough electrophoresis buffer to fill the chamber and just cover the gel.
   - You will need 30 ml of TBE buffer for a blueGel™ or Bandit™ electrophoresis system. Do not overfill the chamber.
   - If using another electrophoresis system, refer to the manufacturer's instructions for the recommended buffer type and volume.
3. Use a micropipette to load 10 µl of 3K DNA Ladder (Tube L) in the first well.
4. Changing pipette tips each time, load 10 µl of each DNA sample in its own well.
   - Plasmid DNA (Tube P)
   - BamHI digest (Tube B)
   - ApaLI digest (Tube A)
   - Double digest (Tube D)
5. Run the gel for 30-45 minutes.
   - The blueGel and Bandit electrophoresis systems run at a fixed voltage.
   - If using another gel electrophoresis system, set the voltage in the 70-90 V range. Visualize the DNA samples using a transilluminator.
6. To visualize the DNA samples, turn on the blue light in your electrophoresis system or move the gel to a transilluminator.
7. If necessary, continue running the gel until there is sufficient separation to interpret the results.

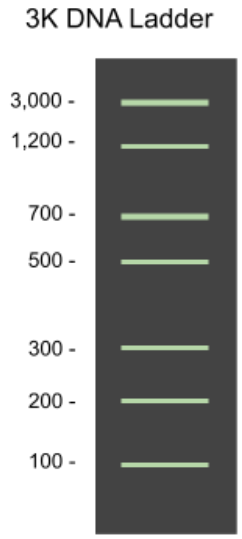
Detailed operating instructions for miniPCR electrophoresis systems

blueGel

https://links.minipcr.com/blueGelRun

Bandit

https://links.minipcr.com/BanditViewit



Pre-lab questions

Review

1. Which features of plasmids make them useful DNA carriers in the lab? Select all that apply.
    A. They can replicate inside bacterial cells
    B. They are easily manipulated using restriction enzymes
    C. They include selectable markers
    D. They are circular

2. What is a restriction enzyme?

3. In restriction mapping, scientists use restriction enzymes to create plasmid maps. Reorder the steps involved in restriction mapping so they follow a logical temporal sequence:
    a. Run DNA on an electrophoresis gel
    b. Cut DNA with restriction enzymes
    c. Make a prediction based on the plasmid map
    d. Compare prediction to gel electrophoresis results
    e. Measure linear DNA fragments using a DNA ladder

    Logical order: __ , __ , __ , __ , __ .

Critical thinking

4. A plasmid map displays some of the key DNA features that make plasmids able to thrive inside bacterial cells. When scientists further engineer, or modify, any given plasmid, they pay close attention to these important features in order to keep them intact.

    What issues would arise if you inserted a DNA sequence in the middle of a plasmid's feature such as:

    a. The plasmid's origin of replication

    b. The plasmid's selectable marker

Mathematical thinking

We're going to do some math to figure out how likely it is for a restriction enzyme to cut DNA. Let's use the letter **n** to refer to the length of a restriction enzyme's recognition site. For example:

- EcoRI recognizes a 6-base sequence: GAATTC. Here, n = 6
- Some enzymes recognize 4-base or 8-base sites. In these cases, n = 4, or n = 8

Because there are four possible bases: A, T, G, and C, at any one spot in a DNA sequence, the chance that a base matches what you want is 1 out of 4, or 0.25.

So for any restriction enzyme, to figure out the chance that a given stretch of DNA matches its recognition site, you multiply the probabilities together. That is: (0.25) × (0.25) × (0.25) … n times.

So the chance that all **n** bases match is **(0.25)^n**. This is one-quarter to the power of n.

5. In this lab, we use BamHI. The BamHI recognition sequence is six nucleotides long. What is the probability that BamHI cuts any randomly chosen group of six nucleotides? Show your work.

6. The pMyst plasmid is 2,700 base pairs long. Recall that BamHI cuts only once in the pMyst plasmid. Is this more or less than you would expect by chance alone? Justify your answer.

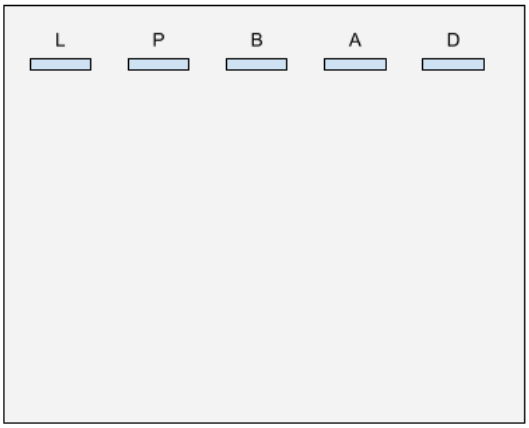
7. The recognition sequence for the restriction enzyme AluI is AGCT. How many times would you expect AluI to cut in the pMyst plasmid, based on probability alone? Show your work.

Post-lab questions

Data interpretation

1. Use the schematic gel on the right to draw what your results look like. For each sample, draw the bands that you see on your actual gel.

2. Next to each band, write approximately how long (in base pairs) the DNA in that band is. Use the image of the ladder on page 17 to help you.



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Critical thinking

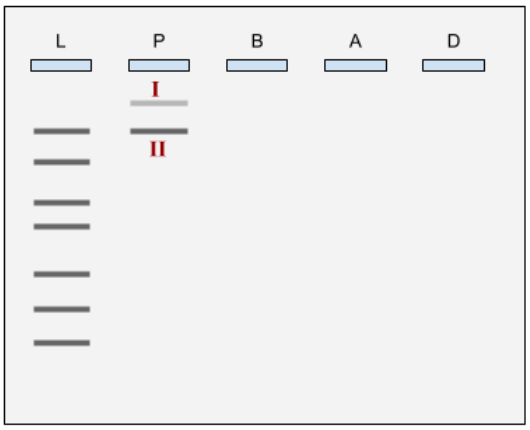
We're first going to analyze **Lane P** (uncut plasmid).

3. What overall shape is the uncut plasmid DNA?
    A. Circular
    B. Linear

4. How many fragments did you *expect* to see on **lane P** (uncut plasmid)? How many fragments do you *actually* see on **lane P**?
    A. Expected: _____
    B. Actual: _____

5. When circular plasmids are run on a gel, you may see more than one band. Plasmid DNA inside a cell is often tightly packed together, or **supercoiled**. Supercoiled DNA is tightly wound, helping it move easily through an agarose gel. When DNA is extracted from the cell, however, sometimes one strand of the DNA may get nicked. Nicked plasmid DNA stays circular, but becomes unpacked. Scientists call this unwound plasmid form **open circle** DNA.

    The bands have been numbered with Roman numerals. Next to the diagram, label each band shown on Lane P as either supercoiled or open circle DNA.



Band I: _____ DNA

Band II: _____ DNA

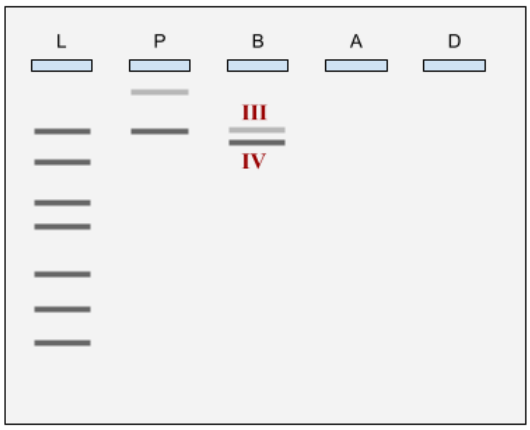
Let's now take a closer look at **Lane B**, containing plasmid DNA that was digested with BamHI.

6. Recall that BamHI cuts only once in the pMyst plasmid. What overall shape is the pMyst plasmid DNA after being cut by BamHI?
    A. Circular
    B. Linear

7. **Linear DNA** may run faster on a gel than circular DNA of the same length because it can easily get through agarose pores.

    In any restriction digestion reaction, scientists mix an enormous number of plasmid molecules with the restriction enzyme. As the restriction enzyme does its cutting job, individual plasmid copies go from circular to linear. If every last plasmid molecule were cut, all of the DNA in the sample would become linear. But at an earlier point in time, some of the plasmid molecules may remain uncut. This intermediate point, before every plasmid molecule is cut, is called an **incomplete digestion**.

    Next to the diagram below, label each band shown on Lane B as either cut or uncut plasmid.



Band III: _____ **DNA**

Band IV: _____ **DNA**

8. Based on the results in the diagram above, which of the following is true?
    A. BamHI digestion was complete
    B. BamHI digestion was incomplete
    C. I cannot tell with the information at hand

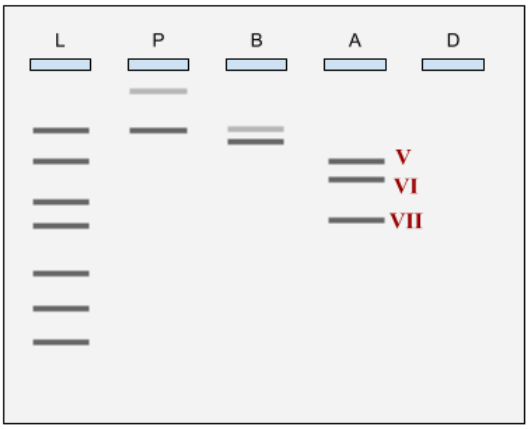
9. If you were a scientist tasked with increasing the efficiency of this restriction digestion, which of these experimental variables might you change? Select all that apply.
    A. Raise the incubation temperature to 37 °C
    B. Increase the volume of the reaction
    C. Extend the incubation time
    D. Add more restriction enzyme

Next, let's look at the ApaLI restriction fragments in **Lane A**.

10. Looking at the number of restriction fragments on this lane, how many times did ApaLI cut the pMyst plasmid?

11. Look at the middle fragment on lane A and compare it to the 3K DNA Ladder pattern (lane L). What is the most likely size of this middle fragment?
    A. 1,250 bp
    B. 950 bp
    C. 500 bp

12. Comparison to the 3K DNA Ladder also suggests that the lowest fragment is 500 bp in size. With this, and other information gathered so far about pMyst, complete the diagram below:



Band V: _____ bp

Band VI: _____ bp

Band VII: _____ bp

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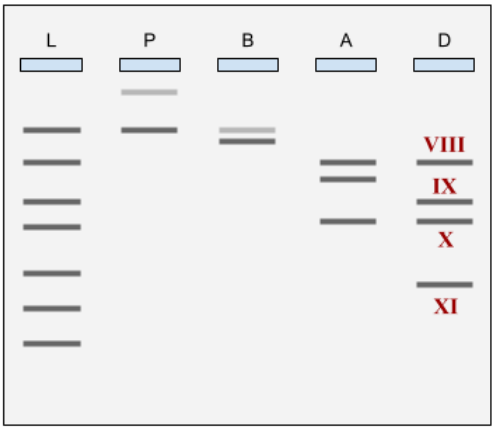
Finally, double digest **Lane D** shows the result of digesting pMyst with both restriction enzymes: BamHI and ApaLI.

13. How many DNA fragments do you observe in the double digest?

14. How does this compare to the number on lane A?

15. Based on these observations, which of the following ApaLI restriction fragments was cut in two by BamHI?
    A. 2,700 bp
    B. 1,250 bp
    C. 950 bp
    D. 500 bp

16. Complete the diagram below for the double digest:



Band VIII: _____ bp

Band IX: _____ bp

Band X: _____ bp

Band XI: _____ bp

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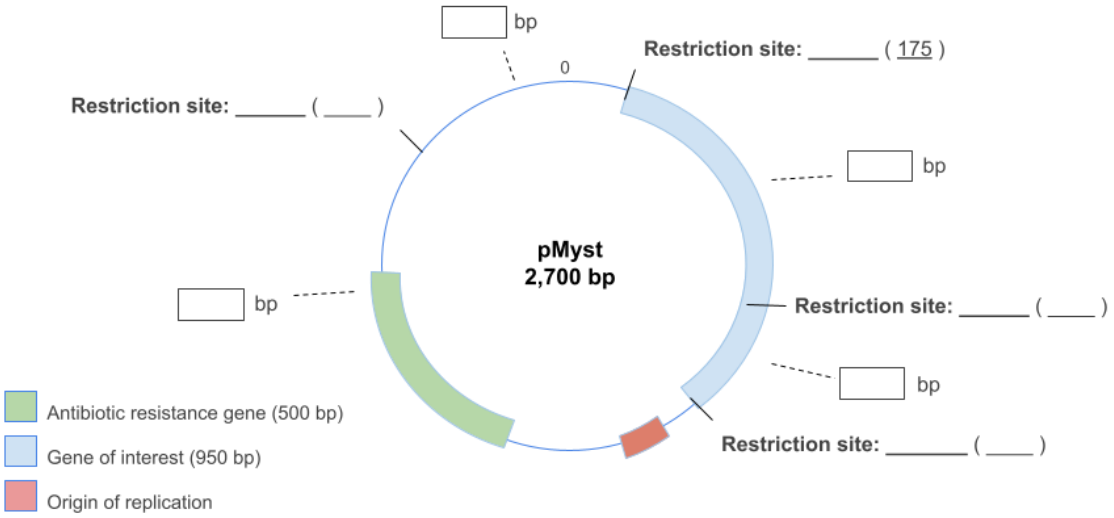
Creating a plasmid map

17. Let's put all this information together to update your lab notebook.

    Complete the plasmid map below:
    a. Name the restriction enzymes that correspond to each site.
    b. Label the sizes of the resulting restriction fragments.
    c. Fill inside the parentheses with each site's position in bp (position 0 has been marked for reference)

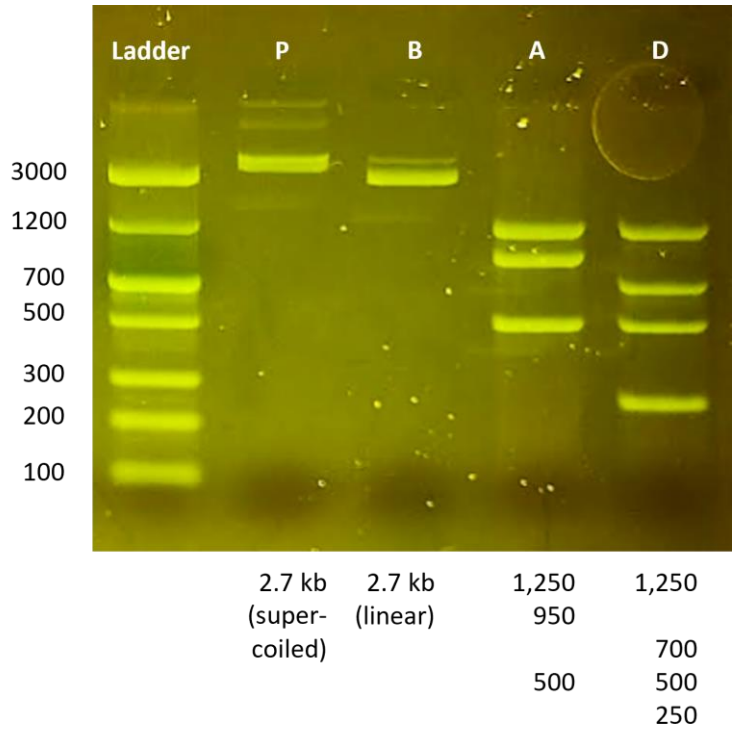


18. Now that you have created a complete plasmid map (congratulations!), your supervisor asks you to remove the "gene of interest" from the pMyst plasmid. What restriction enzyme(s) would you use for this genetic engineering task?
    A. BamHI
    B. ApaLI
    C. Both restriction enzymes
    D. I'd need a different restriction enzyme

    Explain your reasoning:

Expected results

- The uncut plasmid lane (P) may display several bands, corresponding to different structural forms:
    - Supercoiled: most compact and tightly wound (fast migrating)
    - Open circle: relaxed due to one DNA strand being nicked (slow migrating)
- The BamHI-cut plasmid lane (B) may display at least two bands:
    - Linear: both strands cut by the restriction digestion
    - Supercoiled: uncut DNA



Unexpected results and troubleshooting

Faint or missing bands. Insufficient DNA or a gel that is run too long may produce this result.

- Ensure that you are using the correct loading amount per well
- Check the gel during the run to monitor migration progress

Smearing of DNA bands. Overloaded wells or degraded DNA can produce smearing, as can the use of old or incorrect gel electrophoresis buffer.

- Reduce the amount of DNA per well
- Prepare fresh gel electrophoresis buffer

Insufficient band separation. Bands that are too close together or hard to distinguish may reflect an incorrect agarose pore size.

- Ensure that you are using a 2% agarose gel
- Run the gel longer

No bands appear at all. This may be the consequence of incorrect gel preparation, in particular if no fluorescent DNA stain is added.

- Ensure that you are using fluorescent DNA stain compatible with the wavelength of your illumination system (*e.g.,* 460 nm excitation for blueGel™ and Viewit™).
- If you are not using All-in-One Agarose Tabs, check that the correct amount of DNA stain was added to your agarose mixture.

Technical support

contact support@minipcr.com