



BIOTECHNOLOGY IN THE CLASSROOM
UNIVERSITY OF CALIFORNIA DAVIS

Biotechnology Laboratory

*Restriction Enzyme Analysis
(methylene blue stain)*

*Funded by a grant from the Genentech Foundation for
Biomedical Sciences*

University of California Davis • Department of Plant Pathology
One Shields Ave • Davis, California 95616 • (530) 752-6552

RESTRICTION ENZYME ANALYSIS

Lambda DNA (Methylene Blue Staining)



Introduction

Restriction mapping is a cornerstone of modern-day biotechnology. Restriction enzymes cleave DNA at known sites, making possible detailed analysis of specific sections of an organism's genome.

During this three-day exercise, students will digest samples of λ DNA with three different restriction enzymes. These DNA samples will be electrophoresed and the resulting fragments photographed under an ultraviolet transilluminator.

Attached readings and activities help explain the process of restriction enzyme analysis and how it is used in the DNA fingerprinting procedure.

Objectives

1. Understand the mechanics of a restriction digest.
2. Use a micropipette; operate a microcentrifuge; electrophorese an agarose gel.
3. Compare the λ DNA bands on a gel to a λ DNA restriction map.
4. Understand the importance of restriction enzymes.

Materials

For Each Lab Group

- four microtubes
- microtube rack
- 20 μ l micropipette (or 10 μ l micropipette) and sterile tips
- waterproof pen
- beaker or foam cup with crushed ice *
- 20 μ l of 0.4 μ g/ μ l λ DNA
- 20 μ l 2X restriction buffer
- 2.5 μ l *Bam*HI restriction enzyme
- 2.5 μ l *Eco*RI restriction enzyme
- 2.5 μ l *Hind*III restriction enzyme
- 10 μ l distilled water *
- gloves
- safety glasses *
- 500 ml beaker (*day 2*) *
- colored lab tape (*day 2*)
- electrophoresis chamber (*day 2*)
- power supply (*day 2*)
- 10 μ l loading dye (*day 2*)
- 0.7% agarose gel (*day 2*)

Common Materials

- microcentrifuge
- carboy with TBE solution (1X)
- 37°C water bath w/ floating rack
- 65°C water bath (*optional: day 2*)
- cooler with crushed ice *
- freezer (non frost-free, if possible) *
- Polaroid camera
- Polaroid Type 667 black and white film
- white light transilluminator
- distilled water *
- weigh boats (*day 3*)
- 0.1% methylene blue stain (*day 3*)

* *Materials provided by instructor.*



Advance Preparation

DAY 1:

1. Dilute the concentrated TBE (10X) solution to a final concentration of (1X) and store in a plastic carboy. If you saved the solution from the **Gel Electrophoresis with Dyes** activity, reuse it for this laboratory.

Figure on about 300 ml per group. For instance, if your class has 12 groups, prepare 360 ml 10X TBE + 3,240 ml distilled water, for a total of 3,600 ml 1X TBE.

2. Prepare aliquots of the following for each team: use .5 ml microtubes for enzymes
 - a) 20 μ l of 0.4 μ g/ μ l λ DNA
 - b) 20 μ l of 2X restriction buffer
 - c) 2.5 μ l of *Bam*HI restriction enzyme
 - d) 2.5 μ l of *Eco*RI restriction enzyme
 - e) 2.5 μ l of *Hind*III restriction enzyme
 - f) 10 μ l of distilled water
3. Obtain enough crushed ice and ice containers for each lab group. *Styrofoam cups work well.*
4. Fill a water bath with **distilled** water and adjust it to 37°C.

DAY 2:

1. *Optional:* fill a water bath with **distilled** water and adjust it to 65°C.
2. Pre pour enough agarose gels for each lab group.
 - a) Wear gloves.
 - b) Microwave or warm the agarose bottle in a hot waterbath until the gel liquefies. Be sure to use a microwave designated for science purposes (not food).
 - c) Cover your workspace with lab paper.
 - d) Make sure the sides of the gel tray are up. If necessary, use tape to seal the ends.
 - e) Place the plastic comb in the slots close to the end of the tray.

- f) With a disposable pipette run a thin line of gel around the periphery of each tray.
- g) Pour approximately 25 ml of agarose into each gel tray.
- h) Let cool until solidified (approximately 15 minutes).
- i) If storing overnight, place trays in a container with TBE solution or put in a zip-loc bag with TBE buffer so they do not dry out.

3. Aliquot 10 μ l of loading dye for each lab group.
4. Remove the microtubes from the freezer just before students are ready to use them. The small amounts will defrost quickly!

DAY 3:

1. Remove student gels from the refrigerator.
2. Set up weighboats for staining in a common area near a sink.

DAY 4:

1. Load the camera with film and set up the transilluminator.

Teaching Tips

USE OF MICROPIPETTORS:

See description in *Gel Electrophoresis of Dyes Laboratory*.

GEL DISPOSAL

Gels may be discarded in regular trash receptacle.



USE OF METHYLENE BLUE:

Methylene blue dye may be used to stain DNA. It is not as sensitive as ethidium bromide, so a higher concentration of DNA is required. Methylene blue will stain clothes, hands, and equipment so always wear gloves. Spills should be wiped up immediately and flushed with water. Staining should take place in a common area near a sink. Use distilled or deionized water to destain gels. Do NOT use tap water as high chlorine levels will damage the DNA. Methylene blue dye may be reused several times and disposed of down the sink.

USE OF POWER SUPPLIES:

SEE DESCRIPTION IN *GEL ELECTROPHORESIS OF DYES LABORATORY*

USE OF THE WHITE LIGHT TRANSILLUMINATOR:

When students are ready to analyze their results, place the stained gel on the white platform. Turn the power switch on to view the gel.

ENZYMES:

Restriction enzymes easily and rapidly degrade unless kept frozen. **Loss of activity will occur if the enzymes are subjected to warm temperatures for any length of time.**

When aliquoting student reagents, be careful to **keep them on ice**. Also, be sure to use good sterile technique when you prepare student aliquots.

It is best to store enzymes in a non-frost-free freezer since they maintain a more stable temperature. If you must use a frost-free freezer, store the enzymes in a foam container.

2X UNIVERSAL RESTRICTION BUFFER:

Restriction enzymes like the ones we use in this lab operate under different conditions of salt and pH. For optimal activity, a different buffer would be used for each enzyme. To simplify procedures, however, we use a universal buffer—a compromise between the conditions preferred by various enzymes.

LAMBDA (λ) DNA:

Because it is fairly inexpensive and readily available, purified DNA from bacteriophage λ is most suitable for demonstrating the concept of DNA restriction. Most λ DNA is 48,502 base pairs in length. The linear fragments migrate through an agarose gel at a distance that is inversely proportional to the \log_{10} value of their molecular weight. In this exercise, molecular weight is represented by the number of base pairs.

This type of DNA can exist as either a linear or circular molecule, creating some confusion when interpreting restriction digest results. To remedy this, the sample can be heated to 65°C for 5 minutes immediately prior to electrophoresis. This will break the hydrogen bonds holding the ends of the linear DNA together in a circle.

LOADING DYE:

This dye is added to the digest sample in each well. Although you cannot see the progress of the DNA down the gel, you can watch the dye move toward the positive electrode. It will appear as a blue band, eventually resolving into two bands of color.

The faster-moving, purplish band is bromophenol blue dye that migrates at roughly the same rate as a 300 base pair fragment of DNA. The slower-moving aqua band is xylene cyanol, nearly equivalent to a 9000 base pair fragment.



Best separation for analysis of DNA is achieved when the bromophenol blue band migrates 4-7 cm or more from the origin.

However, be careful not to let the bromophenol blue band run off the end of the gel.

USE OF THE MICROCENTRIFUGE:

During aliquoting and movement to and from freezer or refrigerator and ice bucket, reagent aliquots often become spread in a film around the sides or caps of the microtubes. To pool the reagents, spin them briefly in a microcentrifuge just prior to use.

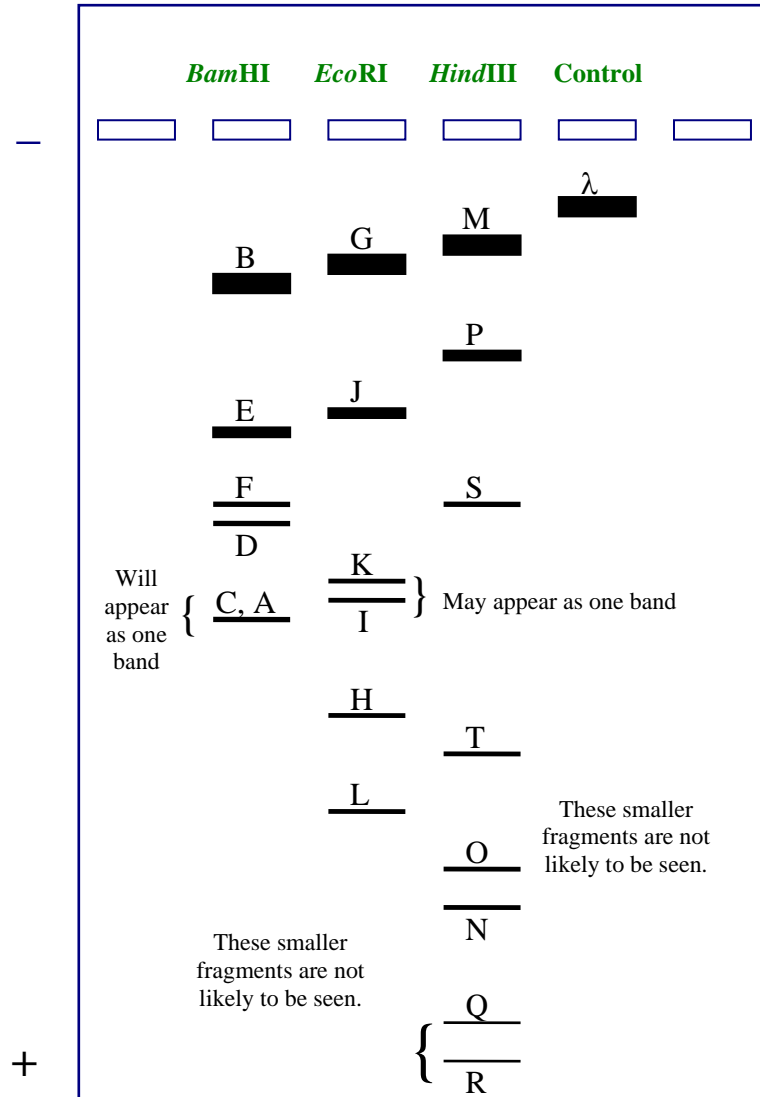
- ⊗ **NEVER run the centrifuge with the lid open.**
- ⊗ **NEVER run the centrifuge without the rotor.**
- ⊗ **NEVER put hands in the rotor area unless the rotor is completely stopped.**
- ⊗ **ALWAYS make sure the tubes are loaded in the rotor symmetrically; each tube should be balanced by another tube directly opposite it.**

USE OF THE CAMERA/TRANSILLUMINATOR

Follow these instructions when taking a picture of your gel electrophoresis results:

1. Load Type 667 black and white film:
 - a) Open the film compartment door.
 - b) Remove old film cartridge.
 - c) Insert a new film cartridge:
 - Hold the film pack by its edges.
 - Push the closed end of the pack in at an angle; then push it down into the camera.
 - Check that the white tabs are free, not caught between the pack and the camera.
 - d) Close the film door and push the latching bar up over the camera so that it clicks into position. The end of the film safety cover (black tab with silver arrows) should extend from the side of the closed unit.
- e) Grip the end of the black paper tab firmly and pull it straight out of the camera. A small white tab should stick out of the same slot. The film is now loaded.
2. Check the dials on the camera to make sure the aperture dial is set for **5.6** and the shutter speed dial reads **2**.
3. Place the stained gel directly on the platform.
4. Place the hooded camera over the white platform. The pistol grip should be facing you (towards the front of the machine). **Make sure that the hood is seated correctly.**
5. Turn **ON** the transilluminator and photograph the gel. Hold the camera steady, and simply squeeze the trigger on the pistol grip one time.
6. Turn **OFF** the transilluminator.
7. Remove the camera/hood from the transilluminator and place it on a solid surface. Holding the camera, pull the white tab out. Once the white tab is free, a black tab with yellow arrows should appear. Grip this tab in the center and pull it straight, at moderate speed, all the way out of the camera. This will start the film developing.
8. Allow the film to develop for 45 seconds.
9. When developing is complete, separate the finished print from the developing pod starting at the end nearest the striped tab. Discard the developing pod and print backing.
 - ⊗ **Take care not to get the caustic, greasy developing gel on your skin. If you do, immediately wash it off with lots of water.**

Answers to Student Activity (Lambda DNA)



Base Pair Length			
A	5,505	L	3,530
B	16,841	M	23,130
C	5,626	N	2,027
D	6,527	O	2,322
E	7,233	P	9,416
F	6,770	Q	564
G	21,226	R	125
H	4,878	S	6,557
I	5,643	T	4,336.1
J	7,421	lambda	48,502
K	5,804		

Answers to Student Activity (DNA Fingerprinting)

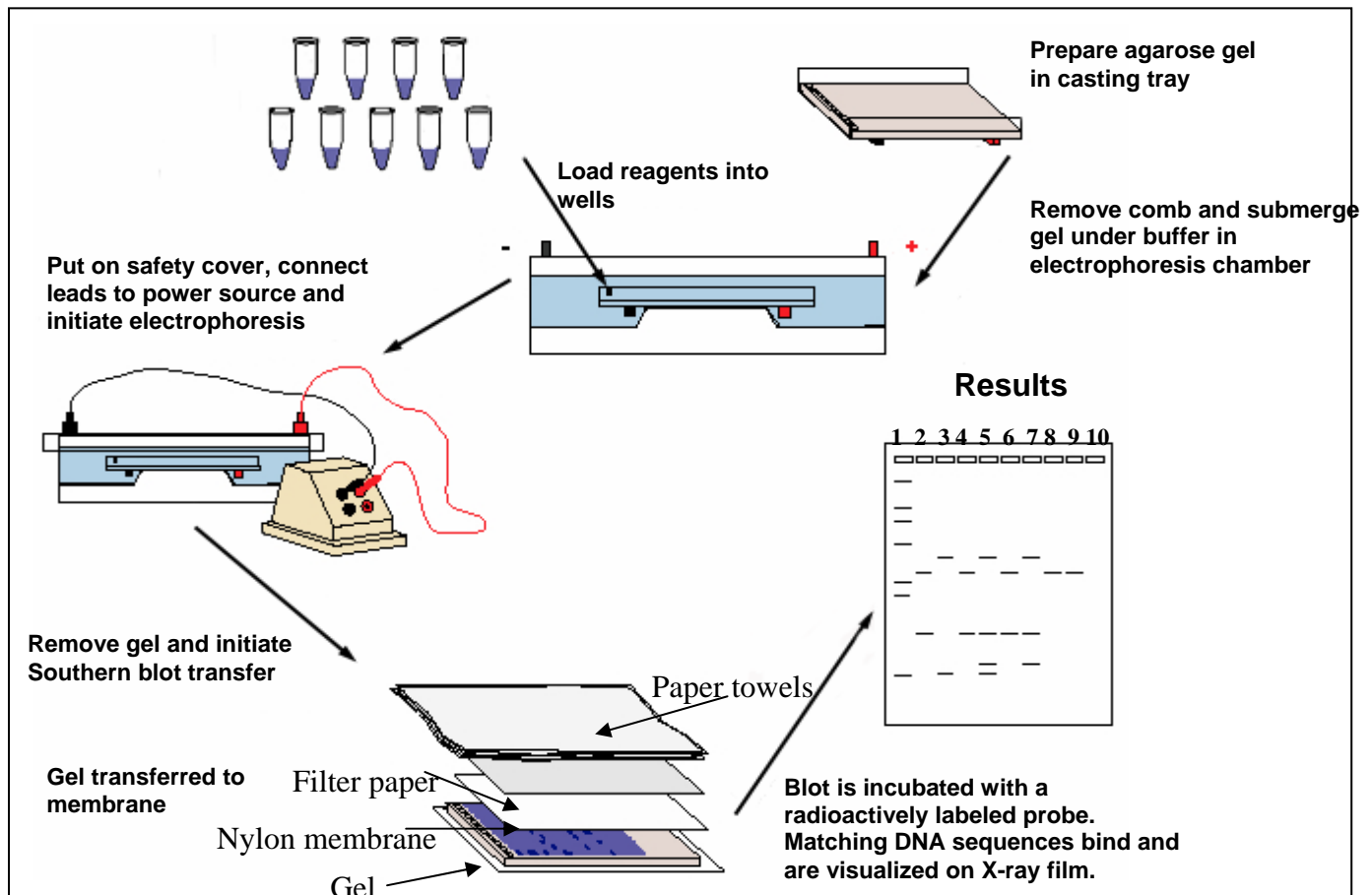
Part A: Forensic Science-A Paternity Case

1. Sample 5.

Part B: Forensic Science-A Rape Case

- The women were assaulted by different men.
 - Suspect X attacked victim A, and suspect Y attacked victim B.
- Since the bands in Well 3 match with the bands in Well 5, and the bands in Well 4 match with the bands in Well 6, we know that the semen samples came from the suspects. Wells 1 and 2 are important to determine that the samples in Wells 3 and 4 are not blood from the victims, because the bands in Wells 1 & 3 and Wells 2 & 4 do not match up, and are in fact semen from the suspects.

Southern Blotting



Edvotek

RESTRICTION ENZYME ANALYSIS

Lambda DNA (Methylene Blue Staining)



Background Reading

Since viruses have a relatively simple genome, scientists have studied their DNA and used this information to test theories and develop concepts that apply to the genetics of living organisms. One of the most studied viruses is called bacteriophage lambda (λ). Bacteriophage λ is a virus that preys on bacterial cells.

The size of DNA fragments is measured in base pairs or kilobase pairs (1000 base pairs). The DNA of Bacteriophage λ is approximately 48,514 base pairs in length. Compare this to the human genome of approximately 3 billion base pairs!

In this lab, you will use special enzymes that act as chemical scissors to cut λ DNA into pieces. This is the first step in a process called restriction mapping. The procedure is a cornerstone of modern day biology because it provides detailed information on specific sections of an organism's DNA.

Once the λ DNA has been cut, the pieces are electrophoresed. Because DNA is a negatively charged molecule, the fragments move toward the positive electrode. Fragments travel through the gel according to their molecular weight—with the smallest fragments moving the greatest distance.

When the gel is photographed under UV light, the fragments appear as a pattern of bands.

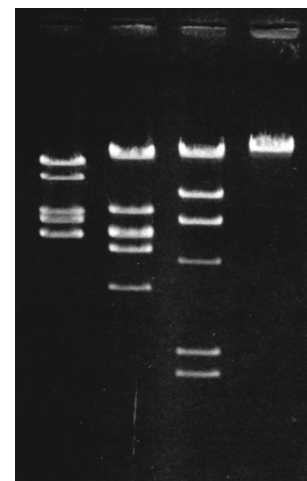
In this protocol, we will compare our banding pattern with a predicted result generated on the first day of the lab.

Readings that your teacher will hand out detail the process of isolating and analyzing these bands, creating a DNA fingerprint.

Objectives

1. Understand the mechanics of a restriction digest.
2. Use a micropipette; operate a microcentrifuge; electrophorese an agarose gel.
3. Compare the λ DNA bands on a gel to a λ DNA restriction map.
4. Understand the importance of restriction enzymes.

BamHI EcoRI HindIII Control



Ideal gel results from restriction digest.



Materials

For Each Lab Group

- four microtubes
- microtube rack
- 20 μl micropipette (or 10 μl micropipette) and sterile tips
- waterproof pen
- beaker or foam cup with ice
- 20 μl of 0.4 $\mu\text{g}/\mu\text{l}$ λ DNA
- 20 μl 2X restriction buffer
- 2.5 μl *Bam*HI restriction enzyme
- 2.5 μl *Eco*RI restriction enzyme
- 2.5 μl *Hind*III restriction enzyme
- 10 μl distilled water
- gloves
- safety glasses
- 500 ml beaker (*day 2*)
- colored lab tape (*day 2*)
- electrophoresis chamber (*day 2*)
- power supply (*day 2*)
- 10 μl loading dye (*day 2*)
- 0.7% agarose gel (*day 2*)

Common Materials

- microcentrifuge
- carboy with TBE buffer (1X)
- 37°C water bath w/floating rack
- 65°C water bath w/floating rack (*optional – day 2*)
- cooler with crushed ice
- freezer (non-frost-free, if possible)
- Polaroid camera
- Polaroid Type 667 black and white film
- white light transilluminator
- distilled water
- 0.1% methylene blue stain (*day 3*)
- weigh boats (*day 3*)

Precautions

- ⊗ **The methylene blue dye will stain skin, clothes, and equipment.** Always wear gloves and safety glasses. Do all staining in a central area near the sink.
- ⊗ **When using the microcentrifuge, always make sure the tubes are loaded in the rotor symmetrically;** each tube should be balanced by another tube directly opposite it. Never run the centrifuge with the lid open or the rotor missing. Never put hands in the rotor area unless the rotor is completely stopped.
- ⊗ **Take care not to get the caustic, greasy, developing gel on your skin.** If you do, immediately wash it off with lots of water.



Procedure

DAY 1: RESTRICTION DIGEST

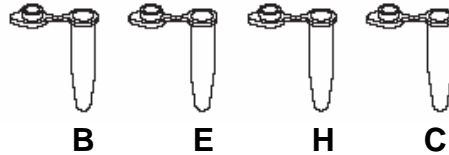
1. Put on gloves and safety glasses. Make sure your group has all the necessary materials before proceeding. **Keep all enzyme and DNA aliquots on ice through step 6.**
2. Label four microtubes, as indicated below, and place them in the tube rack:

B (BamHI & λ DNA)

E (EcoRI & λ DNA)

H (HindIII & λ DNA)

C (Control λ DNA only)



3. Using a fresh tip for **every** aliquot, pipet the reagents into each tube according to the chart below. *Be sure to touch the pipette tip to the side of the tube and make sure the reagent has been deposited.*

	Buffer	λ DNA	BamHI	EcoRI	HindIII	Sterile H ₂ O
B	5 μ l	4 μ l	2 μ l	-	-	-
E	5 μ l	4 μ l	-	2 μ l	-	-
H	5 μ l	4 μ l	-	-	2 μ l	-
C	5 μ l	4 μ l	-	-	-	2 μ l

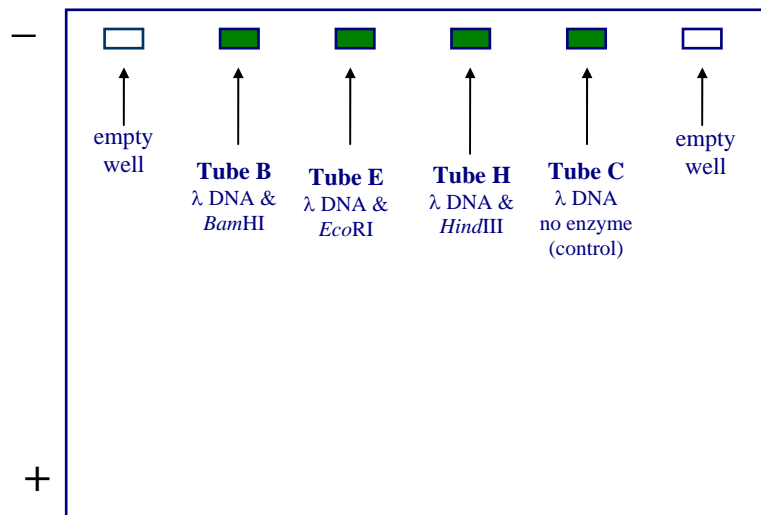
4. Close the microtube caps and mix the tubes by gently flicking with your finger.
5. Pool the reagents on the bottom of the tube by taping gently on the table or spinning in the microcentrifuge for about 3 seconds. **Be sure to place the tubes in a balanced configuration.**
6. Place the microtubes in a 37°C water bath. **Incubate for 20 minutes minimum.** Reactions can be incubated for a longer period of time. During this time, complete the lab activity sheet. If stopping here, wash hands and clean lab station prior to leaving class.

🕒 **STOP POINT:** Following incubation, freeze reaction tubes until day 2 of lab (reaction tubes can be frozen for up to one month).



DAY 2: ELECTROPHORESIS

1. Put on gloves and safety glasses. Make sure your group has all the necessary materials before proceeding. Collect your Day 1 reactant tubes from the freezer. **Keep tubes on ice until needed.**
2. Obtain a pre-poured gel from your instructor. If your tray has gates, move them to the down position. If tape is present, gently remove it.
3. Place the gel tray in the gel box with the wells at the **negative (black)** end of the box.
4. Obtain 250 ml of TBE (1X) solution from the plastic carboy and add it to the sides of the box until the wells are just barely submerged (about 2 mm under). **Do not add buffer solution directly on top of wells.** Return any leftover TBE solution to the carboy.
5. *Optional:* heat the microtubes in a 65°C water bath for 5 minutes. This will break any hydrogen bonds holding the ends of the linear DNA together in a circle.
6. Add 2 μl of loading dye to each of the microtubes. **Make sure to change pipet tips for each sample!**
7. Close the microtube caps and spin the tubes in the microcentrifuge for about 3 seconds or tap gently on the table to pool the reagents.
8. Load 10 μl of each sample into separate wells (*see diagram below*). **Make sure to change pipet tips for each sample!** Again, put the ejected tips and tubes into a separate waste container to give to your instructor for proper disposal.



9. Clean up spilled buffer or any other liquid surrounding the gel box.
10. **Make sure that the power supply is unplugged and switched off before proceeding.** Carefully place the lid on the gel box (first making sure it is clean and dry).



11. Connect the red (positive) patch cord to the red terminal on the power supply. Similarly, connect the black cord to the black terminal. Each power supply will run two gel boxes. Notice what channel you have plugged your box into.
12. Wait until the group that is sharing your power supply has completed steps 1-11 before proceeding.

IMPORTANT! Have your teacher look over all of your connections before plugging in the power supply.

13. Plug in the power supply and turn on the machine on. The run light will illuminate, signifying that power is running to the cell.
14. Turn the display select switch to the appropriate channel for your group. Turn the voltage indicator knob to 100V. **Make sure both groups set their voltage.**
15. Electrophorese for 30-45 minutes. When the purple dye from the loading dye (bromophenol blue) is about 2-3 centimeters from the end of the gel, the power supply should be turned off and the gel box unplugged.

⌚ **STOP POINT:** Following electrophoresis, leave the gel in the gel box or remove from gel tray and store in a weighboat filled with 1X TBE buffer solution until day 3 of lab.



DAY 3: STAINING GELS

1. Put on gloves and safety glasses.
2. Remove gel from gel tray and carefully place in a large weighboat. If gel was stored in a weighboat overnight, simply discard solution into the sink.
3. Using a disposable pipet, cover the surface of the gel with a 0.1% solution of Methylene Blue and stain for 1 min. (The MB solution should either be in distilled water or running buffer.)
4. Pour off the stain (it can be saved and reused many times) and destain the gel in successive changes of distilled water (The chlorine in tap water breaks down the DNA). Change the water about every 10 min. You can generally see bands in 20 min. Visualization is optimum in about 40 min. to 1 hr.

🕒 **STOP POINT:** You may leave the gels in distilled water to destain overnight.



DAY 4: PHOTOGRAPHING GELS

1. Put on gloves and safety glasses.
2. Retrieve your stained gel from the staining weighboat and discard the solution down the sink.
3. Place the gel on the white platform.
4. Turn the power **ON**. Briefly observe the number, placement and relative brightness of the DNA bands. Turn the transilluminator **OFF**.
5. With the transilluminator **OFF**, place the hooded camera over the white platform. The pistol grip should be facing you (towards the front of the machine). **Make sure that the hood is seated correctly.**
6. Turn **ON** the transilluminator and photograph the gel. Hold the camera steady, and simply squeeze the trigger on the pistol grip one time.
6. Turn **OFF** the transilluminator.
7. Remove the camera/hood from the transilluminator and place it on a solid surface. Holding the camera, pull the white tab out. Once the white tab is free, a black tab with yellow arrows should appear. Grip this tab in the center and pull it straight out of the camera. This will start the film developing.
8. Allow the film to develop for 45 seconds.
9. When developing is complete, separate the finished print from the developing paper starting at the end nearest the striped tab. Discard the developing paper. **Be careful to not get the developing gel on your skin. If you do, rinse with water.**
10. Follow your teacher's instructions for clean-up. Discard all gels into the trash. Gel boxes and plates must be rinsed in distilled or deionized water and air dried. Please take great care with the equipment so many more students may enjoy these experiments.
11. Wash hands and clean lab station.



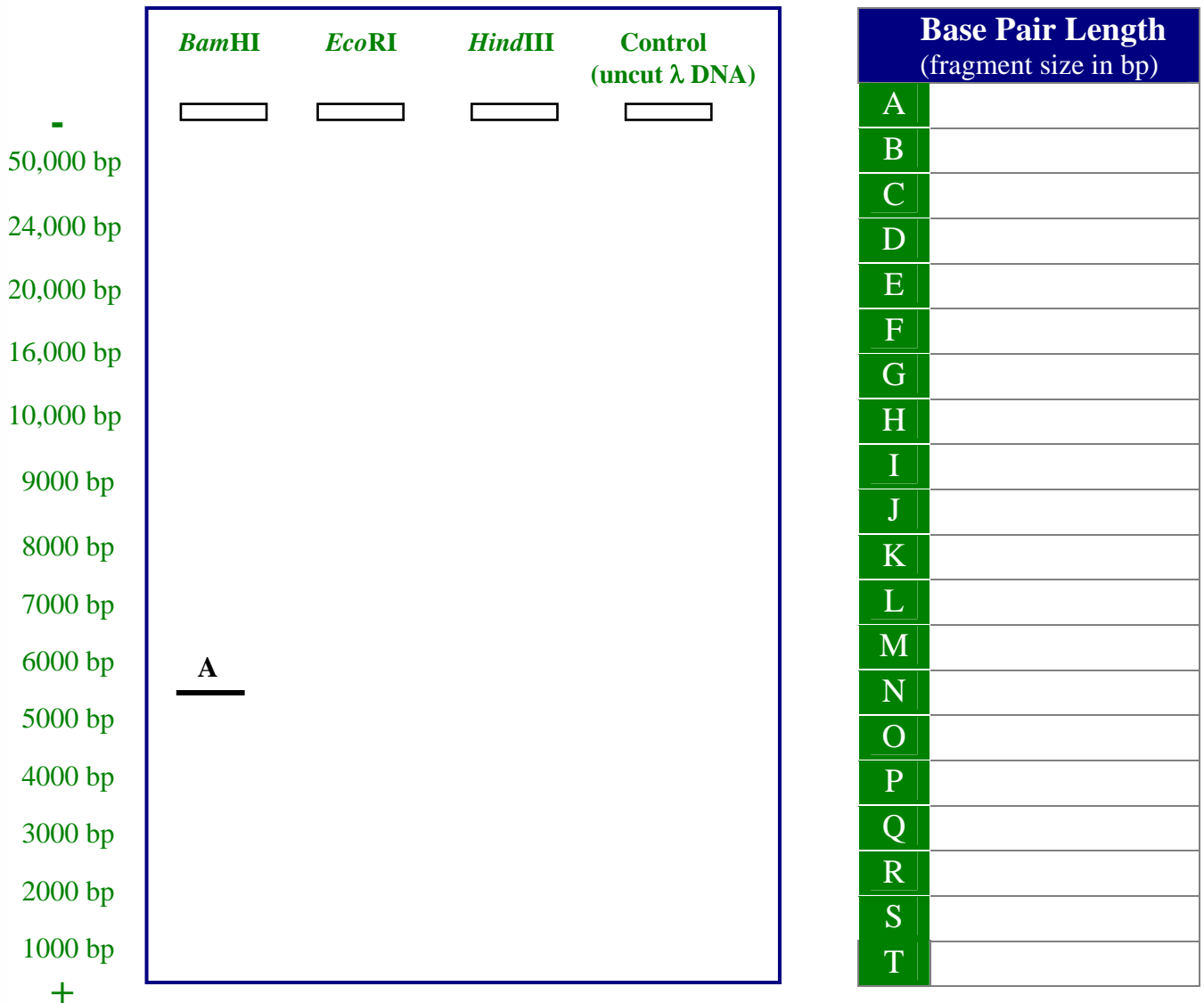
RESTRICTION ENZYME ANALYSIS

Lambda DNA



On the following page you will find a partial restriction map of the linear λ genome. The enzymes we are using in this laboratory cleave λ DNA in the areas noted on the map. Each fragment is assigned a letter.

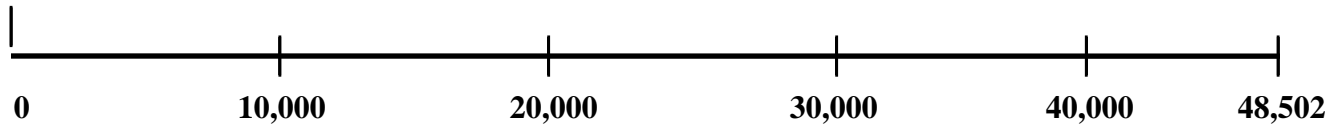
Calculate the base pair length of each fragment and write it down in the space provided below. Next, arrange your λ DNA fragment letters on the “paper gel” as you would expect them to appear after electrophoresis. Be careful to keep the fragments from each restriction enzyme in their proper lane. Also include where the uncut λ sample will be. The first fragment has been done for you.



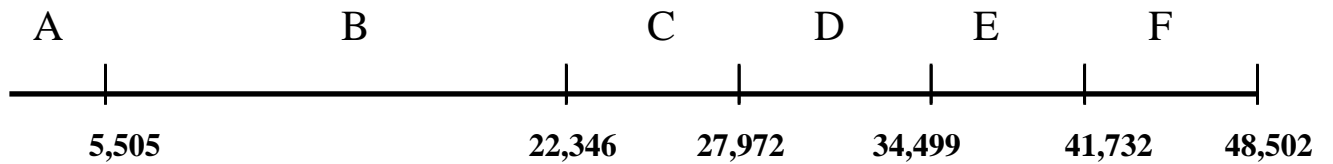


Restriction Maps of the Linear λ Genome (DNA Fragments in base pairs)

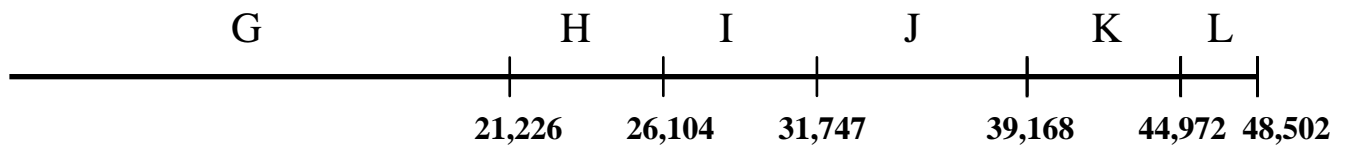
λ DNA



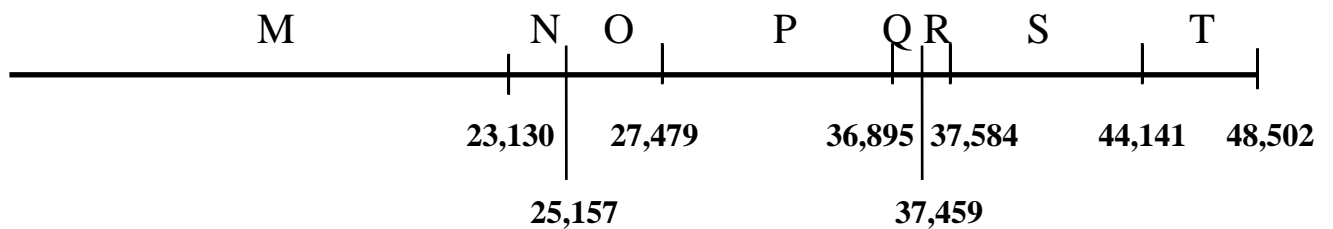
*BAM*HI CLEAVAGE SITES



*Eco*RI CLEAVAGE SITES



*HIND*III CLEAVAGE SITES



RESTRICTION ENZYME ANALYSIS

DNA Fingerprinting



Part A: Forensic Science— A Paternity Case

Assume that you are a molecular biologist involved in analyzing a paternity dispute. In order to determine with accuracy which of the men listed contributed genetic material to the child, you took blood samples from the mother, child and each potential father. Then you ran the samples on an agarose gel electrophoresis, adding an appropriate restriction enzyme to each of the samples. Results from the restriction enzyme digest are as follows:



1	2	3	4	5	
_____	_____	_____	_____	_____	Well 1: mother
_____	_____	_____	_____	_____	
_____	_____	_____	_____	_____	Well 2: child
_____	_____	_____	_____	_____	Well 3: father 1
_____	_____	_____	_____	_____	Well 4: father 2
_____	_____	_____	_____	_____	Well 5: father 3
_____	_____	_____	_____	_____	
_____	_____	_____	_____	_____	

1. Which sample contains blood from the child's actual father? _____





Part B: Forensic Science— A Rape Case

You are a molecular biologist involved in forensic medicine. Two young women have recently been raped within a span of two weeks and you have been given blood and semen samples from the crime scenes.

You ran an agarose gel electrophoresis with the samples in the following wells, applying the restriction enzyme, *EcoRI* to each sample.

Well 1: blood from victim A

Well 2: blood from victim B

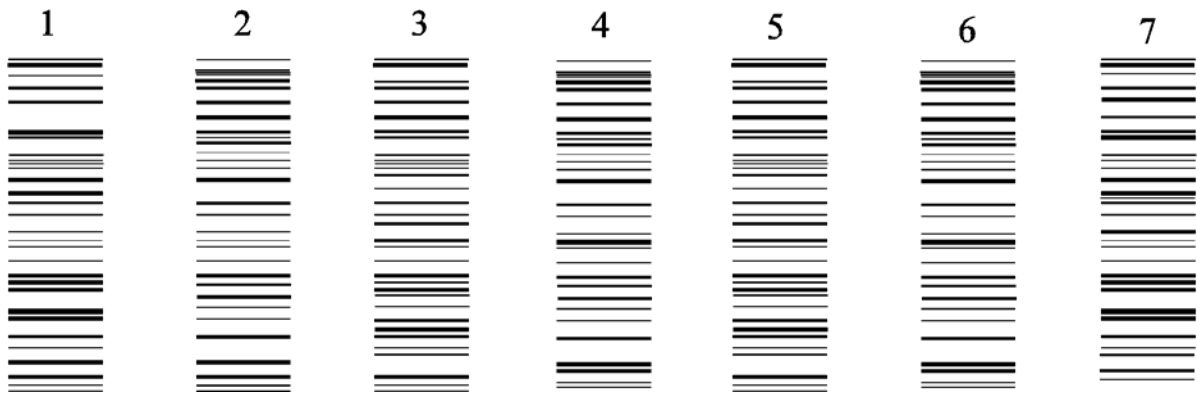
Well 3: semen collected on Victim A

Well 4: semen collected on Victim B

Well 5: blood from suspect X

Well 6: blood from suspect Y

Well 7: blood from suspect Z



1. Examine the results above and determine:
 - a) whether both women were, in fact, assaulted by the same man
 - b) which, if any, of the accused is involved in the crime
2. Write a brief summary of your findings.

Note:

In actuality, gel electrophoresis is combined with another technique called Southern Blotting in order to obtain a DNA fingerprint. Southern Blots involve the use of radioactively labeled DNA probes which attach to fragments of DNA which have been made single-stranded. *Your teacher can explain in greater detail.*