INVESTIGATION 9

BIOTECHNOLOGY: RESTRICTION ENZYME ANALYSIS OF DNA*

How can we use genetic information to identify and profile individuals?

THE SCENARIO

"OMG! Is that blood?" Laurel nearly broke Marcus's arm as she tried to push past him into the classroom.

Marcus grabbed the sleeve of her cardigan and yanked her back. "Don't! Can't you see the glass?" Laurel tried knocking his hand free, but the 6'4" varsity basketball captain held tight. He made her settle for looking from under his armpit.

Not that what she saw would make any sense. Their AP Biology lab looked like a riot scene. Four chairs and a potted plant were overturned in the center of the room, and broken pieces of glass were scattered across the floor along with several wet red drops. Plink ... plink ... plink. *Marcus's eyes were drawn to the teacher's desk where droplets of brownish liquid fell from a paper cup and collected in a puddle on the linoleum.*

"What happened?" Laurel asked. "Did somebody get hurt?" Laurel and her classmates had gathered in front of the door and strained to see inside Room 102.

Marcus inspected the scene and raised his right arm above his head, his fingers spread apart as if taking a shot from the free throw line. "Stay back!"

"Where's Ms. Mason?" Laurel said. "She told me I could meet her before class to review for the quiz."

"Okay, folks, keep it down." Mr. Gladson, the teacher in the classroom next door, came into the hall. His white lab coat was streaked with several rust-colored stains. The pungent odor of formaldehyde permeated the corridor. "In case you haven't noticed, the bell has rung." He wiped his nose with a tissue and then tossed it into a nearby trash can. A girl's fake shriek from inside the anatomy lab rose above the buzz of Marcus's classmates.

"What's going on?" Bobby's high-pitched whine was unmistakable — and so was the scent of his bubble gum.

"I think something might've happened to Ms. Mason," Marcus said. He dug around in his backpack and pulled out a magnifying glass. "We've got a crime scene to process."

* Transitioned from the *AP Biology Lab Manual* (2001)

"Go figure," Laurel said. "Sherlock Holmes in a varsity jacket."

For the next hour, Marcus and Laurel searched the classroom and discovered several pieces of "evidence" that Marcus described in his biology notebook:

- Ten small drops on floor confirmed by Kastle-Meyer test to be blood
- Shard of glass from a broken 500-mL Erlenmeyer flask, edge smeared with a reddish stain
- Paper cup with lipstick stains, presumed to be Ms. Mason's, found on her desk
- Wad of bubble gum stuck underneath overturned chair
- Mr. Gladson's discarded tissue recovered from trash can in hall outside Room 102
- Bobby's test on photosynthesis with large "F" scrawled in red ink on first page
- Copy of email from Mr. Gladson to Ms. Mason asking her to give up position as department chair

Marcus's new game was afoot!

BACKGROUND

Applications of DNA profiling extend beyond what we see on television crime shows. Are you sure that the hamburger you recently ate at the local fast-food restaurant was actually made from pure beef? DNA typing has revealed that often "hamburger" meat is a mixture of pork and other nonbeef meats, and some fast-food chains admit to adding soybeans to their "meat" products as protein fillers. In addition to confirming what you ate for lunch, DNA technology can be used to determine paternity, diagnose an inherited illness, and solve historical mysteries, such as the identity of the formerly anonymous individual buried at the Tomb of the Unknown Soldier in Washington, D.C.

DNA testing also makes it possible to profile ourselves genetically — which raises questions, including *Who owns your DNA and the information it carries?* This is not just a hypothetical question. The fate of dozens of companies, hundreds of patents, and billions of dollars' worth of research and development money depend on the answer. Biotechnology makes it possible for humans to engineer heritable changes in DNA, and this investigation provides an opportunity for you to explore the ethical, social, and medical issues surrounding the manipulation of genetic information.

Learning Objectives

In this investigation, you will learn how to use restriction enzymes and gel electrophoresis to create genetic profiles. You will use these profiles to help Marcus and Laurel narrow the list of suspects in the disappearance of Ms. Mason.

General Safety Precautions

Never handle gels with your bare hands. An electrophoresis apparatus can be dangerous because it is filled with a highly conductive salt solution and uses DC current at a voltage strong enough to cause a small shock. Always turn the power supply switch

"OFF" and wait 10 seconds before making any connection. Connect BOTH supply leads to the power supply (black to black and red to red, just like when you jump-start a car battery) BEFORE turning on the power supply. Your teacher will tell you for how long and at how many volts (usually 50 volts) you should run your gel. After use, turn off the power supply, and then disconnect BOTH leads from the power supply. *Remember, power supply on last … and off first.*

THE INVESTIGATIONS

Getting Started

Activity I: Restriction Enzymes

The DNA samples collected from the crime scene have been digested with restriction enzymes to generate smaller pieces of DNA, which will then be used to create DNA profiles of suspects.

Restriction enzymes are essential tools for analyzing DNA structure, and more than 200 enzymes are now available commercially. Each restriction enzyme is named for the bacterium in which it was first identified; for example, *Eco*RI was the first enzyme purified from *Escherichia coli,* and *Hin*dIII was the third enzyme isolated from *Haemophilus influenzae*. Scientists have hypothesized that bacteria use these enzymes during DNA repair and as a defense against their infection by bacteriophages. Molecular biologists use restriction enzymes to manipulate and analyze DNA sequences (Johnson 2009).

How do restriction enzymes work? These enzymes digest DNA by cutting the molecule at specific locations called restriction sites. Many restriction enzymes recognize a 4- to 10-nucleotide base pair (bp) palindrome, a sequence of DNA nucleotides that reads the same from either direction. Some restriction enzymes cut (or "cleave") DNA strands exactly in the center of the restriction site (or "cleavage site"), creating blunt ends, whereas others cut the backbone in two places, so that the pieces have single-stranded overhanging or "sticky" ends of unpaired nucleotides.

You have a piece of DNA with the following template strand:

5'-AAAGTCGCTGGAATTCACTGCATCGAATTCCCGGGGCTATATATGGAATTCGA-3'

1. What is the sequence of the complementary DNA strand? Draw it directly below the strand.
2. Assume you cut this fragment with the restriction enzyme *Eco*RI. The restriction site for *Eco*RI is 5'-GAATTC-3', and the enzyme makes a *staggered* ("sticky end") cut between G and A on both strands of the DNA molecule. Based on this information, draw an illustration showing how the DNA fragment is cut by *Eco*RI and the resulting products.

Two pieces of DNA that are cut with the same restriction enzyme, creating either sticky ends or blunt ends, can be "pasted" together using DNA ligase by reconnecting bonds, *even if the segments originated from different organisms.* An example of combining two "sticky end" sequences from different sources is shown in Figure 1. The ability of enzymes to "cut and paste" DNA fragments from different sources to make recombinant DNA molecules is the basis of biotechnology.

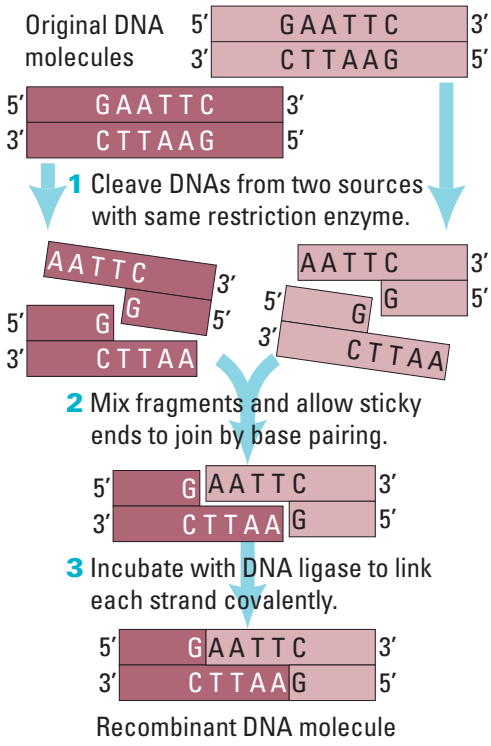

Figure 1. Recombinant DNA Using Restriction Enzymes

Activity II: DNA Mapping Using Restriction Enzymes

One application of restriction enzymes is restriction mapping. Restriction mapping is the process of cutting DNA at specific sequences with restriction enzymes, separating the fragments from each other by a process called gel electrophoresis (without pasting any fragments together), and then estimating the size of those fragments. The size and number of DNA fragments provide information about the structure of the original pieces of DNA from which they were cut.

Restriction mapping enables scientists to create a genetic signature or DNA "fingerprint" that is unique to each organism. The unique fragments, called restriction fragment length polymorphisms (RFLPs), can, for instance, be used to confirm that a mutation is present in one fragment of DNA but not in another, to determine the size of an unknown DNA fragment that was inserted into a plasmid, to compare the genomes of different species and determine evolutionary relationships, and to compare DNA

samples from different individuals within a population. This latter application is widely used in crime scene investigations.

Consider your classmates. More than 99% of your DNA is the same as their DNA. The small difference is attributed to differences in your genetic makeup, with each person having a genetic profile or "fingerprint" as unique as the ridges, arches, loops, and grooves at the ends of his or her fingers.

- Based on this information, can you make a prediction about the products of DNA from different sources cut with the same restriction enzymes? Will the RFLP patterns produced by gel electrophoresis produced by DNA mapping be the same or different if you use just one restriction enzyme? Do you have to use many restriction enzymes to find differences between individuals? Justify your prediction.
- Can you make a prediction about the RFLP patterns of identical twins cut with the same restriction enzymes? How about the RFLP patterns of fraternal twins or triplets?

Now that you understand the basic idea of genetic mapping by using restriction enzymes, let's explore how DNA fragments can be used to make a genetic profile.

Activity III: Basic Principles of Gel Electrophoresis

Creating DNA profiles depends on gel electrophoresis. Gel electrophoresis separates charged molecules, including nucleic acids and amino acids, by how fast they migrate through a porous gel under the influence of an electrical current. Your teacher will likely prepare the gel ahead of time by dissolving agarose powder (a gelatinlike substance purified from seaweed) in a current-carrying buffer. The gel solidifies around a comb placed at one end, forming wells into which you can load DNA fragments. When an electrical current is passed through the gel, the RFLPs (fragments) migrate from one pole to the other. Gel electrophoresis can separate DNA fragments from about 200 to 50,000 base pairs (bp).

- Why do DNA fragments migrate through the gel from the *negatively* charged pole to the *positively* charged pole?

The general process of gel electrophoresis is illustrated in Figure 2.

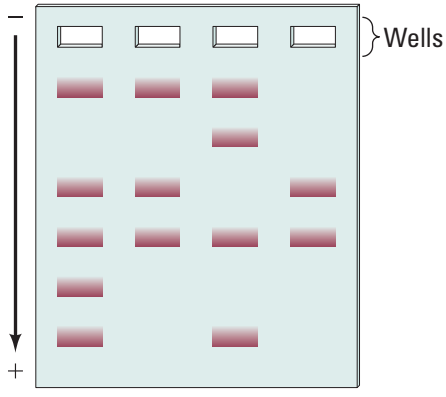

Figure 2. General Process of Gel Electrophoresis

Procedures

Learning to Use Gel Electrophoresis

To determine whose blood was on the classroom floor crime scene, you will need to be familiar with the techniques involved in creating genetic profiles using gel electrophoresis. The steps in the general procedure are described below. After you familiarize yourself with the procedure, you will analyze DNA profiles resulting from an "ideal" or mock gel before using what you have learned to conduct an independent investigation. In Designing and Conducting Your Investigation, you will use these skills to narrow the list of suspects in the disappearance of Ms. Mason based on DNA evidence collected at the crime scene.

Materials

Your Workstation

- 20 µL vials of DNA fragments prepared using restriction enzymes
- Rack for holding samples
- 3 plastic bulb transfer pipettes (or similar devices)
- Permanent marker
- Gel electrophoresis chamber
- Power supply
- Staining tray
- Semi-log graph paper
- Ruler

Common Workstation

- 0.8% agarose solution (or gel, if prepared by teacher)
- 1 X TAE (tris-acetate-EDTA) buffer
- Methylene blue stain

Record data and any answers to questions in your lab notebook.

Casting the Agarose Gel

Before proceeding, your teacher will direct you to short online videos that show how to prepare an agarose gel, load DNA samples into the wells in the gel, and run an electrophoresis.

Step 1 Seal the ends of the gel-casting tray with tape, dams, or any other method appropriate for the gel box that you are using. Insert the well-forming comb. Place the gel-casting tray out of the way on the lab bench so that the agarose poured in the next step can set undisturbed. (Your teacher might cast the gel for you ahead of time.)

Step 2 Carefully pour the liquid gel into the casting tray to a depth of 5–6 mm. The gel should cover only about one-half the height of the comb teeth (Figure 3). While the gel is still liquid, use the tip of a pipette to remove any bubbles.

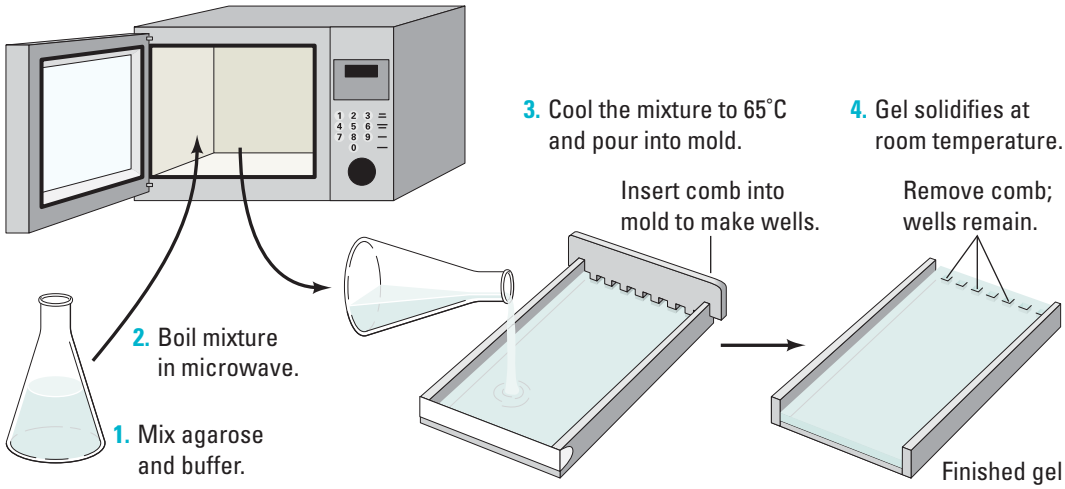

Figure 3. Casting an Agarose Gel

Step 3 The gel will become cloudy as it solidifies (15–20 minutes). Do not disturb or touch the gel while it is solidifying!

Step 4 When the agarose has set, carefully remove the ends of the casting tray and place the tray in the electrophoresis gel box so that the comb is at the negative (black) end.

- Why do you place the wells at the negative end of the gel box?
- What is the chemical nature of DNA? Will the DNA fragments migrate toward the positive end of the gel box or toward the negative end?

Step 5 Fill the box with 1x TAE buffer, to a level that just covers the entire surface of the gel.

Step 6 Gently remove the comb, taking care not to rip the wells. Make sure that the sample wells left by the comb are completely submerged in the buffer.

Step 7 The gel is now ready to be loaded with your DNA samples. (If your teacher says that you will load the gel on another lab day, close the electrophoresis box to prevent drying of the gel.)

Loading the Gel

Before loading your gel with samples of DNA, you should practice using the pipette or other loading device. One easy way to do this is to slowly aspire a sample of buffer and expel it into a "pretend well" on a paper towel ("pretend gel"). Your teacher might suggest another method for practicing how to load gels. Keep practicing until you feel comfortable loading and expelling a sample.

Make sure you record the order in which you load the samples. Be sure to use a fresh loading device (either plastic micropipette or other type of pipette) for each sample. Be sure you know how to use the pipette properly. When in doubt, ask your teacher. Take care not to puncture the bottom of the well with the pipette tip when you load your samples.

Step 1 Load 15–20 µL of each sample of DNA into a separate well in the gel, as shown in Figure 4.

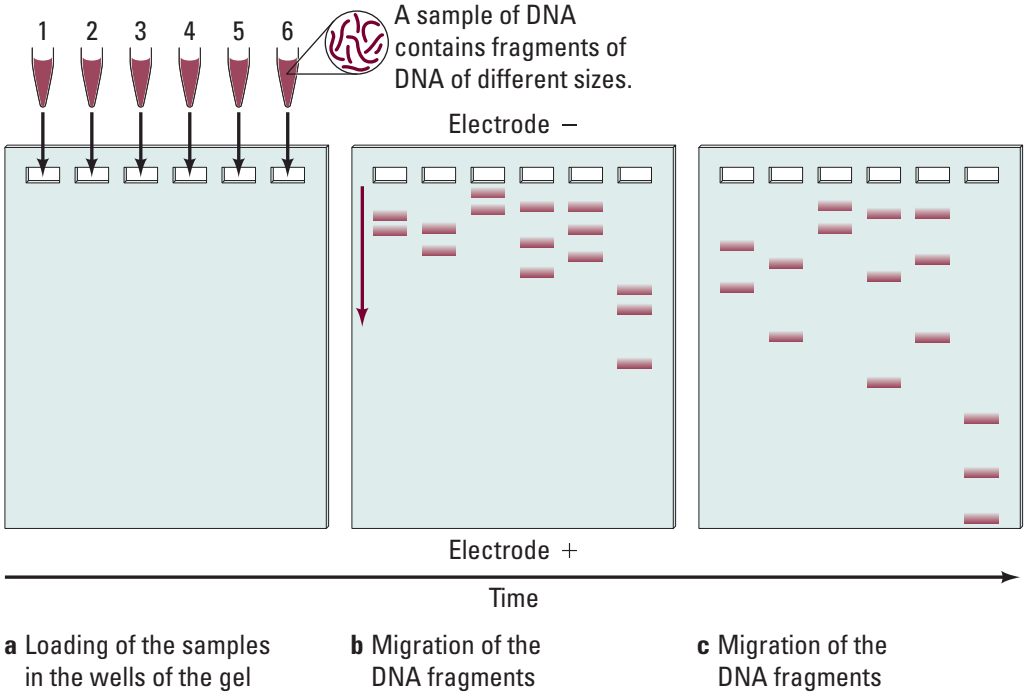

Figure 4. Loading an Agarose Gel and Migrating DNA Fragments Through Time

Step 2 Slowly draw up the contents of the first sample tube into the pipette.

Step 3 Using two hands, steady the pipette over the well you are going to load.

Step 4 Expel any air in the end of the pipette *before* loading the DNA sample.

Step 5 Dip the pipette tip through the surface of the buffer, position it just inside 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 puncture the bottom of the well with the pipette tip or reaspirate your sample up into the pipette.*

Step 6 Draw the pipette tip out of the buffer.

Step 7 Using a clean loading device for each sample, load the remaining samples into their wells.

Electrophoresis

Step 1 Close the top of the electrophoresis chamber and then connect the electrical leads to an appropriate power supply, positive (+) electrode to positive (+) electrode (black to black) and negative (-) electrode to negative (-) electrode (red to red). Make sure both electrodes are connected to the same channel of the power supply, just as you would connect leads to jump-start a car battery — black to black and red to red.

CAUTION: Be sure to keep the power OFF until you connect all leads!

Step 2 Turn on the power supply and set the voltage as directed by your teacher. (It is recommended that you "run the gel" at 50 volts for approximately 2 hours. If you run the gel at a higher voltage for less time, the fragments migrate too quickly through the gel with less separation. Again, ask your teacher for assistance if needed.)

Step 3 Shortly after the current is applied, you should see loading dye moving through the gel toward the positive pole of the electrophoresis apparatus. (**Note:** The purplish-blue band in the loading dye migrates through the gel at the same rate as a DNA fragment approximately 300 base pairs long.)

Step 4 Allow the DNA to electrophorese until the loading dye band is about 1 cm from the end of the gel. Your teacher may monitor the progress of the electrophoresis in your absence if you have to attend another class.

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

Step 6 Carefully remove the casting tray and slide the gel into a staining tray labeled with the name of your group.

- Measure in centimeters the distance that the purplish-blue loading dye has migrated into the gel. Measure from the front edge of the well to the front edge of the dye band (also called the dye front).
- Be sure to record your data (in centimeters).

Step 7 Take your gel to your teacher for further staining instructions. Again, your teacher might monitor the staining procedure.

Analyzing Results

Calculating the Sizes of Restriction Fragment Length Polymorphisms

Mathematical formulas have been developed for describing the relationship between the molecular weight of a DNA fragment and its mobility (i.e., how far it migrates in the gel). In general, DNA fragments, like the ones in your evidence samples, migrate at rates inversely proportional to the $\log_{10}$ of their molecular weights. **For simplicity's sake, base pair length (bp) is substituted for molecular weight when determining the size of DNA fragments.** Thus, the size in base pair length of a DNA fragment can be calculated using the distance the fragment travels through the gel. To calculate the base pair length, a DNA standard, composed of DNA fragments of *known* base pair length, is run on the same gel as the unknown fragments and is then used to create a standard curve. The standard curve, in this case a straight line, is created by graphing the distance each fragment traveled through the gel versus the $\log_{10}$ of its base pair length.

Creating the Standard Curve

As explained above, base pair (bp) length is substituted for molecular weight. Note that in plotting the standard curve, calculating the $\log_{10}$ of the base pair length of each fragment is unnecessary because the base pair size is plotted on the logarithmic axis of semi-log paper. Examine your stained gel on a light box or other surface that helps visualize the bands.

- What observations can you make?
- What quantitative measurements can you make?

1. Examine the "ideal" or mock gel shown in Figure 5 that includes DNA samples that have been cut with three restriction enzymes, *Bam*HI, *Eco*RI, and *Hin*dIII, to produce RFLPs (fragments). Sample D is DNA that has not been cut with enzyme(s). DNA cut with *Hin*dIII provides a set of fragments of known size and serves as a standard for comparison.

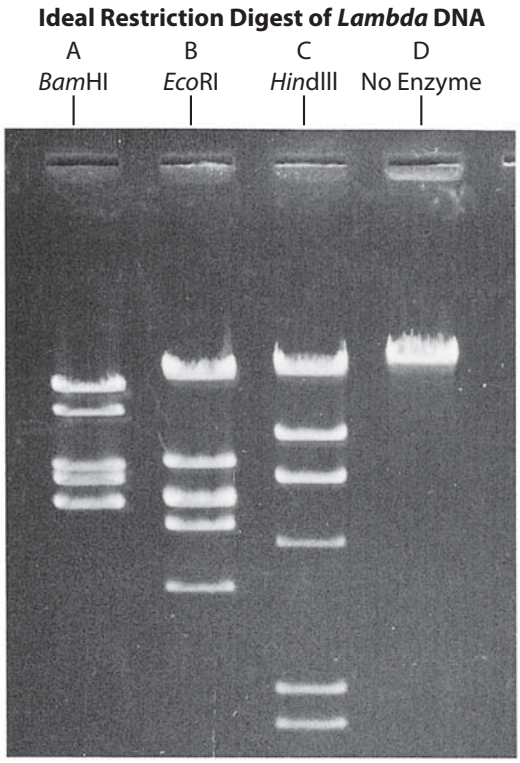

Figure 5. Ideal Gel

2. Using the ideal gel shown in Figure 5, measure the distance (in cm) that each fragment migrated *from* the origin (the well). (**Hint:** For consistency, measure from the front end of each well to the front edge of each band, i.e., the edge farthest from the well.). Enter the measured distances into Table 1. (See * and ** notes below the table for an explanation for why there are only six bands seen but more fragments.)

Table 1. DNA Fragment Migration Distance

*HIN*DIII		*BAM*HI		*ECO*RI	
Distance Traveled	BP Length	Distance Traveled	BP Length	Distance Traveled	BP Length
	*27,491				
	*23,130				
	9,416				
	6,557				
	4,361				
	2,322				
	2,027				
	**564				
	**125				

*For this "ideal" gel, assume that these two bands appear as a single band instead of resolving into separate bands.

** These bands do not appear on the ideal gel and likely will not be seen.

3. Plot the standard curve using the data from the DNA sample cut with *Hin*dIII. To do this, your teacher might ask you to graph the data directly using Excel with distance traveled as the (arithmetic) x-axis and the base pair (bp) length as the (logarithmic) y-axis. Based on this graph, why must the data be plotted using the log scale? You might want to plot the data again using semi-log paper.

   Connect the data point with a best-fit line. However, you should ignore the point plotted for the 27,491bp/23,130 doublet. When using 0.8% agarose gel, these fragments appear as one. Congratulations! Your best-fit line is the standard curve.

4. Now use the standard curve to calculate the approximate sizes of the *Eco*RI and *Bam*HI fragments. Using a ruler, how can you use the standard curve to calculate the sizes of unknown fragments?

Designing and Conducting Your Investigation

Now that you've learned about the techniques used to create DNA profiles or "fingerprints," it's time to apply the techniques as you investigate the disappearance of Ms. Mason. Your task is to design and conduct a procedure *based on DNA evidence* to determine whose blood is spattered on the classroom floor. The chief investigator (your teacher) will provide you with DNA evidence collected at the crime scene from the blood, Ms. Mason (saliva on her coffee cup), Mr. Gladson (tissue with which he wiped his nose), and Bobby (bubble gum). In addition, you will be given a sample of DNA cut with *Hin*dIII. Remember from your analysis of the "ideal" or mock gel that DNA cut with *Hin*dIII serves as a marker, providing a set of RFLPs of known sizes (standard).

Analyzing Results

Evaluate your crime scene samples to determine whose blood was on the classroom floor. Because this case likely will go to trial, visual analysis (qualitative data) of the DNA profiles is not sufficient to identify a perpetrator. Based on your results, write the conclusion to the scenario to reveal "whodunit" based on motive, means, opportunity, and DNA evidence.

Evaluating Results

1. What are some possible challenges you had in performing your investigation?
2. What are some possible sources of error in the electrophoresis procedure? How can you minimize any potential sources of error?

Thinking About Your Results

1. There are important social and ethical implications of DNA analysis. Already, DNA testing can reveal the presence of markers of certain genetic diseases, such as Huntington's. So, who should have access to your genetic profile? Health insurance companies? College admissions offices? Employers? What issues about confidentiality are raised by genetic testing? Who owns your DNA and its information?

2. Suppose a DNA test that predicted your chances of getting a disease, such as cancer, were available. You take the test for cancer, and the results say you have a two in three chance of getting cancer sometime in the next 20 years. Who should have access to this information? Your doctor? Health insurance companies? Employers? Would *you* want to know this information?

3. The Innocence Project (IP) is an international litigation and public policy organization dedicated to exonerating wrongfully convicted individuals through DNA testing. Three-quarters of DNA exoneration cases involve misidentification by witnesses. To date, nearly 300 people previously convicted of serious crimes in the U.S. have been exonerated by DNA testing. However, not everyone is in favor of the IP. One United States Supreme Court justice expressed concern that DNA testing poses risks to the criminal justice system, in which a person is judged by a jury of peers. What social and ethical issues are raised by using DNA evidence to re-examine old court decisions? What other arguments can you make (or find) against using DNA evidence for court cases?

4. With genetic engineering, biotechnicians can clip out beneficial genes from native plants in foreign countries and insert them into their crop plant relatives here in the United States, with great benefits to the latter — to prevent attack by insects, to increase productivity, or to allow the crops to be grown in colder climates. These benefits can be worth billions of dollars, but if the crops are grown in the United States, should countries where the native plants are located benefit from the bioengineering? Who owns the information in DNA? Who can profit from that information? Investigate this controversy on the Internet with examples drawn from different crops grown here in the U.S.

Where Can You Go from Here?

The following are suggestions for expanding your study of biotechnology.

1. Do you remember earlier when you read that more than 99% of your DNA is the same as another person's DNA, and that the 1% difference is attributed to small differences in genetic code? Conduct independent research on how these small differences can be detected by molecular biologists. Begin by investigating unique repeat DNA sequences called variable tandem repeats (VNTRs), short tandem repeats (STRs), and single nucleotide polymorphisms (SNPs). Prepare a mini-poster presentation for your classmates illustrating how these small differences can be used to individualize DNA from different organisms, including humans. Are the differences between you and other individuals in the genes themselves? If so, how do you account for the fact that everyone needs the same genes to produce your cell components and your organs, such as your liver and lungs?

2. Often scientists have only a small amount of DNA available for analysis. The polymerase chain reaction (PCR) is another key technique that molecular biologists use to amplify a specific sequence of DNA. Developed by Kary Mullis in 1983, PCR produces millions of copies of a DNA sequence in a few hours, with the original sequence serving as the template for replication. PCR has a variety of applications, including DNA cloning, determining DNA-based phylogeny, diagnosing hereditary diseases, and identifying genetic fingerprints. Ask your teacher if you can learn to perform PCR. PCR usually requires a relatively expensive piece of equipment, a DNA thermocycler; however, you can investigate less expensive methods of PCR.

3. Select an episode of one of your favorite TV crime investigation shows that focuses on DNA as evidence. Compare TV science with *real* science.

20 to master inquiry Questions DNA Fingerprinting Kit

Level 1 Questions

1. How important is enzyme concentration for a DNA digest?
2. How important is DNA concentration (substrate) for DNA digest?
3. How important is digest time?
4. How important is digest temperature?
5. How important is thoroughly mixing the sample prior to digest?
6. How important is agarose concentration in the gel?
7. How important is buffer concentration in the chamber?
8. How important is voltage at which the gel is run?
9. How much DNA is needed to be able to stain a gel with FAST Blast or other 'safe' stains?

Level 2 Questions

11. How important is restriction enzyme concentration when adding more than one enzyme to the same tube?
12. How important is overall reaction volume?
13. How important is restriction buffer concentration when doing a restriction digest?
14. Are enzymes as effective after exposure to UV light?
15. Can I mutate DNA using UV light? Does this change restriction sites?

Level 3 Questions

16. If I cut the DNA sample and then ligate together followed by another restriction digest will I get the same restriction pattern?
17. Can I ligate precut samples together to make a plasmid?
18. Can I cut a band out of the gel and ligate it into a plasmid?
19. Can I make a restriction map of a known plasmid using multiple restriction enzymes?
20. Some enzymes exhibit star activity when reaction conditions are not optimal. How can I determine if or when an enzyme exhibits star activity?