**Case Number 612700 The Case of the Basement Lab Revisited**

A few weeks ago you were instrumental in discovering a basement lab that was used to make rat poison to poison tenants’ pets. The victims were tenants that had cheap, rent controlled apartments. The identity of the poison was discovered by your careful testing. Further investigation of this lab and the suspect led to discovery of even more sinister deeds. The perpetrator also wanted to poison the tenants themselves and may have even succeeded because one of the tenants who had a rent-controlled apartment died six months ago under suspicious circumstances. While the evidence you gathered for the first experiment with this name was helpful, many felt it did not give conclusive information about the identity of the individual involved in the lab. Now that a potential murder is implicated, the budget to solve this case has gone up and you may do DNA fingerprinting on the blood sample that was discovered in the lab.

**SAFETY**

This forensic investigation will involve the use of Fast Blast DNA stain available from Bio-Rad, refer to the safety information in the MSDSs for safe handling and disposal of this material.

***NOTE: This lab requires answers to pre-lab questions found on the next page in addition to the normal requirements for a pre-lab!***

**Introduction to DNA Fingerprinting**

 You are about to perform a procedure known as DNA fingerprinting. The data obtained may allow you to determine if the samples of DNA that you will be provided with are from the same individual or from different individuals.

 DNA consists of a series of nitrogenous base molecules held together by weak hydrogen bonds. These base pairs are in turn bonded to a sugar-phosphate backbone. The four nitrogenous bases are adenine, thymine, guanine, and cytosine (A, T, G, and C). Remember the base-paring rule is A – T and G – C. Refer to the figure below of a DNA molecule.

 The schematics above represent a very small section of DNA from three different individuals. In this representation of DNA the symbol system is as follows:

**Backbone:**

S = Five carbon sugar molecule known as deoxyribose

P = Phosphate group

**DNA Nucleotide Bases:**

A = adenine C = cytosine G = guanine T = thymine

 Analysis of the three DNA samples above might help us detect similarities and differences in samples of DNA from different people.

**Pre-lab:** Please answer the following questions after reading the background information for this lab.

1.Compare the “backbone” of the sugar-phosphate arrangement in the side chains of all three figures. Are there any differences?

2.In the above figure, do all three samples contain the same bases? Describe your observations.

3.Are the bases paired in an identical manner in all three samples? Describe the pattern of the base pair bonding.

4.In your attempt to analyze DNA samples from three different individuals, what conclusions can you make about the similarities and differences of the DNA samples?

5.What will you need to compare between these DNA samples to determine if they are identical or non-identical?

In addition to answering the above five questions for your pre-lab, clearly state the purpose of the lab and the basic outline of the steps that you will take to achieve that purpose.

**Lab Exploration Part 1: Restriction Digestion of DNA Samples**

***How can we detect differences in base sequences?***

 At first sight, your task might seem rather difficult. You need to determine if the linear base pair sequence in the DNA samples is identical or not. An understanding of some relatively recent developments in recombinant DNA technology might help you to develop a plan.

 In 1968, Dr. Werner Arber at the University of Basel, Switzerland and Dr. Hamilton Smith at Johns Hopkins University, Baltimore, discovered a group of enzymes in bacteria, which when added to any DNA will result in the breakage [hydrolysis] of the sugar-phosphate bond between certain specific nucleotide bases [recognition sites]. This causes the double strand of DNA to break along the recognition site and the DNA molecule becomes fractured into two pieces. These molecular scissors or “cutting” enzymes are restriction endonucleases.

 [Can you figure out why they are called restriction endonucleases?]

 Two common restriction enzymes (endonucleases) are EcoRI and PstI which will be provided to you in this lab procedure. To better understand how EcoRI and PstI may help you in performing your DNA fingerprinting test, first you must understand and visualize the nature of the “cutting” effect of a restriction endonuclease on DNA:

 The line through the base pairs represents the sites where bonds will break if a restriction endonuclease recognizes the site GAATIC. The following analysis questions refer to how a piece of DNA would be affected if a restriction endonuclease were to “cut” the DNA molecule in the manner shown above.

***In your notebook answer the following questions. The answers to which should also appear in your lab report.***

1. How many pieces of DNA would result from this cut? \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

2. Write the base sequence of the DNA fragments on both the left and right side of the “cut”.

 Left: Right:

3. What differences are there in the two pieces?

4. DNA fragment size can be expressed as the number of base pairs in the fragment. Indicate the size of the fragments [mention any discrepancy you may detect].

 a. The smaller fragment is \_\_\_\_\_\_\_\_\_\_\_\_ base pairs (bp).

 b. What is the length of the longer fragment? \_\_\_\_\_\_\_\_\_\_\_\_

5. Consider the two samples of DNA shown below – single strands are shown for simplicity:

Sample #1

C A G T G A T C T C G A A T T C G C T A G T A A C G T T \

Sample #2

T C A T G A A T T C C T G G A A T C A G C A A A T G C A

 If both samples are treated with a restriction enzyme [recognition sequence GAATTC] then indicate the number of fragments and the size of each fragment from each sample of DNA.

 Sample #1 Sample #2

 # of fragments: \_\_\_\_\_\_\_\_\_\_\_\_ # of fragments: \_\_\_\_\_\_\_\_\_\_\_\_

List fragment size in order: Largest 🡪 smallest

 Sample #1 Sample #2

**Practice pipetting:** Before you start processing the DNA samples, it is necessary to get familiar with the equipment that will be used. This experiment will involve the use of micropipetters. Watch the instructor demonstrate the use of them and then practice yourself by performing the following procedure.

1. Pour approximately 5-10mL of red dyed sample out of the reagent bottle.
2. Obtain 5 - 50mL volumetric flasks from your instructor. Label them 100, 200, 300, 400, and 500.
3. Into the flask marked 100, pipet 100L of the red dyed solution.
4. Into the flask marked 200, pipet 200L of the red dyed solution, repeat for the 300, 400, and 500 flasks with 300 400, and 500L respectively of red dyed solution.
5. Fill each flask up about half way with deionized water.
6. Thoroughly mix each flask with the water.
7. Fill each flask up to the line on the neck of the flask with water, invert at least three times.
8. Once you have all the solutions made, measure their absorbance on the Spec 20 instruments. Your instructor will show you how to operate these.
9. With your absorbance data, make a calibration curve as shown in class with EXCEL on your laptop. When you are making the graph and you choose to display the equation on the chart under options, also choose to display the R-squared value on the chart.
10. Your R-squared value should be 0.95 or greater if it is not, see the instructor.

**Restriction Digestion of DNA Samples**

 Upon careful observation, it is apparent that the only difference between the DNA of different individuals is the linear sequence of their base pairs. In the lab, your team will be given 6 DNA samples. These correspond with the 6 suspects. Recall that your task is to determine if any of them came from the same individual or if they came from different individuals.

 Thus far you have learned the following:

* The similarities and differences between the DNA from different individuals.
* How restriction endonucleases cut (hydrolyze) DNA molecules.
* How adding the same restriction endonuclease to two samples of DNA might provide some clues about differences in their linear base pair sequence.

 Now that you have a fairly clear understanding of these three items you are ready to proceed to the first phase of the DNA fingerprinting procedure—performing a restriction digest of your DNA samples.

**Restriction Digestion of DNA Samples (Laboratory Procedure)**

Digest the DNA Samples

1. Label reaction tubes.

A. Obtain one each of the following colored micro test tubes. Label the 6 colored micro test tubes as follows: (Your instructor will give you the identity of each of the suspects.)

 Green tube: CS (crime scene)

 Blue tube: S1 (suspect 1)

 Orange tube: S2 (suspect 2)

 Violet tube: S3 (suspect 3)

 Red tube: S4 (suspect 4)

 Yellow tube: S5 (suspect 5)

 Put your name on the tubes! The restriction digests will take place in these tubes. These tubes may now be kept in your foam micro test tube holder.

2. Locate the clear micro test tube that contains the restriction enzyme mix labeled “ENZ”.

3. Obtain your DNA samples.

 Using a fresh tip for each sample, transfer 10 μl of each DNA sample from the colored stock tubes located at the common workstation into each of the corresponding labeled colored tubes.In your notebook record your observations including:

1. A description of the samples of DNA (physical properties).

2. Is there any observable difference between the samples of DNA?

3. Describe the appearance of the restriction endonuclease mix.

4. Combine and react.

 Using a new pipet tip for each sample, pipet μL of the enzyme mix “ENZ” to each reaction tube as shown below.

 Note: Change tips whenever you switch reagents, or, if the tip touches any of the liquid in one of the test tubes accidentally. When in doubt, change the tip! DNA goes in the tube before the enzyme. Always add the enzyme last.

Now your DNA samples should contain:

 Total

 DNA Samples EcoRI/PstI Reaction

 (10 μL each) Enzyme Mix Volume

Crime Scene [CS] 10 μL 20 μL

Suspect 1 [S1] 10 μL 20 μL

Suspect 2 [S2] 10 μL 20 μL

Suspect 3 [S3] 10 μL 20 μL

Suspect 4 [S4] 10 μL 20 μL

Suspect 5 [S5] 10 μL 20 μL

5. Mix the tube contents.

 Tightly cap on each tube. Mix the components by gently flicking the tubes with you finger. If there is a centrifuge available, pulse the tubes for two seconds to force the liquid into the bottom of the tube to mix and combine reactants. (Be sure the tubes are in a BALANCED arrangement in the rotor.) If your lab is not equipped with a centrifuge, briskly shake the tube (once in sufficient) like a thermometer. Tapping the tubes on the lab bench will also help to combine and mix the contents.

6. Incubate the samples.

 Place the tubes in the foam micro tube holder and incubate them at 37° C for 45 minutes. Alternatively, the tubes can be incubated in a large volume of water heated to 37° C and allowed to slowly reach room temperature overnight. After the incubation, store the DNA digests in the refrigerator until the next lab period.

 While you are waiting for your samples to digest, answer the following questions in your notebook.

1. Before you incubated your samples, describe any visible signs of change in the contents of the tubes containing the DNA after it was combined with the restriction enzymes.

2. Can you see any evidence to indicate that your samples of DNA were fragmented or altered in any way by the addition of EcoRI/PstI?

3. In the absence of any visible evidence of change, is it still possible that the DNA samples were fragmented? Explain your reasoning.

4. At the start of the next lab period, answer this question in your notebook. Are there any visible clues that the restriction enzymes may have in some way changed the DNA in any of the tubes? Explain your reasoning.

**Part 2: Electrophoresis and Staining of DNA Samples**

*Consideration: How can we detect the position of EcoRI and PstI restriction sites on our DNA samples?*

 Since we are attempting to detect changes at the molecular level, and there are not visible clues for us to analyze, this task might seem beyond our capabilities and impossible to do. Let’s see if we can figure this out. One way to determine the location of restriction sites might be to determine the following:

1. How many different sizes of DNA fragments are in each sample?

2. What are the relative sizes of each fragment?

 Therefore, you must somehow get evidence to answer the following question: Do the EcoRI and PstI restriction sites occur at the same locations in any of the DNA samples?

 The following facts will be helpful to you in your attempt to determine the actual range of DNA fragment sizes in your samples.

**Electrophoretic Analysis of Restriction Fragments**

 A restriction enzyme acts like molecular scissors, making cuts at the specific sequence of base pairs that it recognizes. The three-dimensional structure or shape of a restriction enzyme allows it to fit perfectly in the groove formed by the two strands of a DNA molecule. When attached to the DNA, the enzyme sides along the double helix until it recognizes a specific sequence of base pairs which signals the enzyme to stop sliding. The enzyme then chemically separates, or cuts, the DNA molecule at that site—called a restriction site.

 If a specific restriction site occurs in more than one location on a DNA molecule, a restriction enzyme will make a cut at each of those sites, resulting in multiple fragments of DNA. Therefore, if a given piece of linear DNA is cut with a restriction enzyme whose specific recognition sequence is found at five different locations on the DNA molecule, the result will be six fragments of different lengths. The length of each fragment will depend upon the location of restriction sites on the DNA molecule.

 A DNA fragment that has been cut with restriction enzymes can be separated using a process known as agarose gel electrophoresis. The term electrophoresis means to carry with electricity. Agarose gel electrophoresis separates DNA fragments by size. DNA fragments are loaded into an agarose gel slab, which is placed into a chamber filled with a conductive buffer solution. A direct current is passed between wire electrodes at each end of the chamber. Since DNA fragments are negatively charged, they will be drawn toward the positive pole (anode) when placed in an electric field. The matrix of the agarose gel acts as a molecular sieve through which smaller DNA fragments can move more easily than larger ones. Therefore, the rate at which a DNA fragment migrates through the gel is inversely proportional to its size in base pairs. Over a period of time, smaller DNA fragments will travel farther than larger ones. Fragments of the same size stay together and migrate in single bands of DNA. These bands will be seen in the gel after the DNA is stained.

 An analogous situation is one where all the desks and chairs in the classroom have been randomly pushed together. An individual student can wind his/her way through the maze quickly and with little difficulty, whereas a string of 4 students would require more time and have difficulty working their way through the maze.

Part 2: Agarose Gel Electrophoresis (Laboratory Procedure)

1. Obtain a pre-poured agarose gel from your teacher.

2. Remove your digested samples from the refrigerator. Using a new tip for each sample add 5 μL of sample loading dye “LD” to each tube:

DNA Samples Loading dye

 Crime Scene [CS] 5 μL

 Suspect 1 [S1] 5 μL

 Suspect 2 [S2] 5 μL

 Suspect 3 [S3] 5 μL

 Suspect 4 [S4] 5 μL

 Suspect 5 [S5] 5 μL

 Tightly cap each tube. Mix the components by gently flicking the tubes with your finger. If a centrifuge is available, pulse spin the tubes to bring the contents to the bottom of the tube. Otherwise, gently tap the tubes on the table top.

3. Place the casting tray with the solidified gel in it, into the platform in the gel box. The wells should be at the (-) cathode end of the box, where the black lead is connected. Very carefully, remove the comb from the gel by pulling it straight up.

4. Pour ~ 275 mL of electrophoresis buffer into the electrophoresis chamber. Pour buffer in the gel box until it just covers the wells of the gel by 1-2 mm.

5. Obtain the tube of HindIII lambda digest (DNA marker).

6. Using a separate pipet tip for each sample, load your digested DNA samples into the gel. Gels are read from left to right. The first sample is loaded in the well at the left hand corner of the gel.

 Lane 1: HindIII DNA size marker, clear, 10 μL

Lane 2: CS, green, 20 μL

Lane 3: S1, blue, 20 μL

Lane 4: S2, orange, 20 μL

Lane 5: S3, violet, 20 μL

Lane 6: S4, red, 20 μL

Lane 7: S5, yellow, 20 μL

7. Secure the lid on the gel box. The lid will attach to the base in only one orientation: red to red and black to black. Connect electrical leads to the power supply.

8. Turn on the power supply. Set it for 100 V and electrophorese the samples for 30-40 minutes.

While you are waiting for the gel to run, answer the questions below in your notebook.

9. When the electrophoresis is complete, turn off the power supply and remove the lid from the gel box. Carefully remove the gel tray and the gel from the electrophoresis chamber. Be careful, the gel is very slippery! Refer to the section below review questions for detailed instructions on staining your gel.

Questions to be answered in your notebook while gel is running:

1. The electrophoresis apparatus creates an electrical field with positive and negative poles at the ends of the gel. DNA molecules are negatively charged. To which electrode pole of the electrophoresis field would you expect DNA to migrate? (+ or -)? Explain.

2. What color represents the negative pole?

3. After DNA samples are loaded into the sample wells, they are “forced” to move through the gel matrix. What size fragments (large vs. small) would you expect to move toward the opposite end of the gel most quickly? Explain.

4. Which fragments (large vs. small) are expected to travel the shortest distance from the well? Explain.

**Staining DNA with Fast Blast DNA Stain (Laboratory Procedure)**

Consideration: Are any of the DNA samples from the suspects the same as an individual at the crime scene?

 Take a moment to think about how you will perform the analysis of your gel. In the final two steps, you will:

A. Visualize DNA fragments in your gel.

B. Analyze the number and positions of visible DNA bands on your gel.

Making DNA Fragments Visible

 Since DNA is naturally colorless, it is not immediately visible in the gel. Unaided visual examination of the gel after electrophoresis indicates only the positions of the loading dyes and not the positions of the DNA fragments. DNA fragments are visualized by staining the gel with a blue stain called Fast Blast DNA stain. The blue stain molecules are positively charged and have a high affinity for the DNA. These blue stain molecules strongly bind to the DNA fragments and allow DNA to become visible. These visible bands of DNA may then be traced, photographed, sketched, or retained as a permanently dried gel for analysis.

 The drawing below represents an example of a stained DNA gel after electrophoresis. For fingerprinting analysis, the following information is important to remember:

* Each lane has a different sample of DNA
* Each DNA sample was treated with the same restriction endonucleases.



Protocol: Overnight staining of Agarose Gels in 1x Fast Blast DNA Stain

 For overnight staining, Fast Blast DNA stain (500x) should be diluted to a 1x concentration. We recommend using 120 mL of 1x Fast Blast to stain two 7 x 7 cm or 7 x 10 cm agarose gels in individual staining trays provided in Bio-Rad’s education kits. If alternative staining trays are used, add a sufficient volume of staining solution to completely submerge the gels.

 Following DNA electrophoresis agarose gels must be removed from their gel trays before being placed in the staining solution. This is easily accomplished by holding the base of the gel tray in one hand and gently pushing out the gel with the thumb of the other hand. Because the gel is fragile, special attention must be given when handling it.

1. Label the staining tray with your initials and class period. You will stain 2 gels per tray.

2. Stain gels (overnight)[[1]](#footnote-2)

Pour 1x stain into a gel staining tray. Remove the gel from the gel tray and carefully slide it into the staining tray containing the stain. If necessary, add more 1x staining solution to completely submerge the gels. Place the staining tray on a rocking platform and agitate overnight. If no

rocking platform is available, agitate the gels staining tray a few times during the staining period. You should begin to see DNA bands after 2 hours, but at least 8 hours of staining is recommended for complete visibility of stained bands.

3. Record results

No destaining is required after staining with 1x Fast Blast. The gels can be analyzed immediately after staining.

a. Place your gel on a light background and record your results by making a diagram as follows. Place a clear sheet of plastic sheet or acetate over the gel. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica picture of your gel. Remove the plastic sheet for later analysis. Alternatively, gels can be photocopied on a yellow piece of transparent film for optimal contrast.

b. Dry the agarose gel as a permanent record of the experiment.

i. Trim away any unloaded lanes with a knife or razor blade. Cut your gel from top to bottom to remove the lanes that you did not load samples into, leaving only lanes 1-4.

ii. Place the gel directly upon the hydrophilic side of a piece of gel support film. (Water will form beads on the hydrophobic side of a piece of gel support film.) Center the gel on the film on a paper towel and let the gel dry in a well-ventilated area, making sure to avoid direct exposure to light. As the gel dries it will bond to the film but will not shrink. If left undisturbed on the support film, the gel will dry completely at room temperature after 2-3 days. The result will be a flat, transparent, and durable record for the experiment.

Note: Avoid extended exposure of dried gels to direct light to prevent band fading. However, DNA bands will reappear if the dried gels are stored in the dark for 2-3 weeks after fading.

**Part 4: Analysis of Results**

If the overnight staining protocol was used to stain gels, record you results and dry gels as instructed.

In your notebook trace a picture of your gel.

Answer the following questions in your notebook.

1. What can you assume is contained within each band?

2. If this were a fingerprinting gel, how many samples of DNA can you assume were placed in each separate well.

3. What would be a logical explanation as to why there is more than one band of DNA for each of the samples.

4. What caused the DNA to become fragmented?

5. Which of the DNA samples have the same number of restriction sites for the restriction endonucleases used? Write the lane numbers.

6. Which sample has the smallest DNA fragment?

7. Assuming a circular piece of DNA (plasmid) was used as starting material, how many restriction sites were there in lane three?

8. Which DNA samples appear to have been “cut” into the same number and size of fragments?

9. Based on your analysis of the gel, what is your conclusion about the DNA samples in the drawing? Do any of the samples seem to be from the same source? If so, which ones? Describe the evidence that supports your conclusion.

**Quantitative Analysis of DNA Fragment Sizes**

 If you were on trial, would you want to rely on a technician’s eyeball estimate of a match, or would you want some more accurate measurement?

 In order to make the most accurate comparison between the crime scene DNA and the suspect DNA, other than just a visual match, a quantitative measurement of the fragment sizes needs to be created. The procedure for doing this is described below. Record results in your notebook.

1. Using the ruler, measure the distance (in mm) that each of your DNA fragments or bands traveled from the well. Measure the distance from the bottom of the well to the center of each DNA band and record you numbers in the table. The data in the table will be used to construct a standard curve and to estimate the sizes of the crime scene and suspect restriction fragments.

2. To make an accurate estimate the fragment sizes for either the crime scene to suspect DNA samples, a standard curve is created using the distance (x-axis) and fragment size (y-axis) data from the known HindIII lambda digest (DNA marker). Using both linear and semilog graph paper, plot distance versus size for bands 2-6. On each graph, use a ruler and draw a line joining the points. Extend the line all the way to the right hand edge of the graph.

 Which graph provides the straightest line that you could use to estimate the crime scene or the suspects’ fragment sizes? Why do you think one graph is straighter than the other?

3. Decide which graph, linear or semilog, should be used to estimate the DNA fragment sizes of the crime scene and suspects. Justify your selection.

4. To estimate the size of an unknown crime scene or suspect fragment, find the distance that fragment traveled. Locate that distance on the x-axis of your standard graph. From that position on the x-axis, read up to the standard line, and then follow the graph line to over the y-axis. You might want to draw a light pencil mark from the x-axis up to the standard curve and over to the y-axis showing what you’ve done. Where the graph line meets the y-axis, this is the approximate size of your unknown DNA fragment. Do this for all crime scene and suspect fragments.

5. Compare the fragment sizes of the suspects and the crime scene.

 Is there a suspect that matches the crime scene?

 How sure are you that this is a match?

In your lab report, be sure to answer the questions that were posed during the lab. Give your results and conclusion about who is responsible for the basement lab including supporting evidence.

**Background:** This lab involves the use of electrophoresis. Explain how electrophoresis works for the background section of this report. Use references!

1. It is crucial that you shake gels gently and intermittently while performing the overnight staining in 1x Fast Blast stain since smaller fragments tend to diffuse without shaking.

 [↑](#footnote-ref-2)