DNA FINGERPRINTING

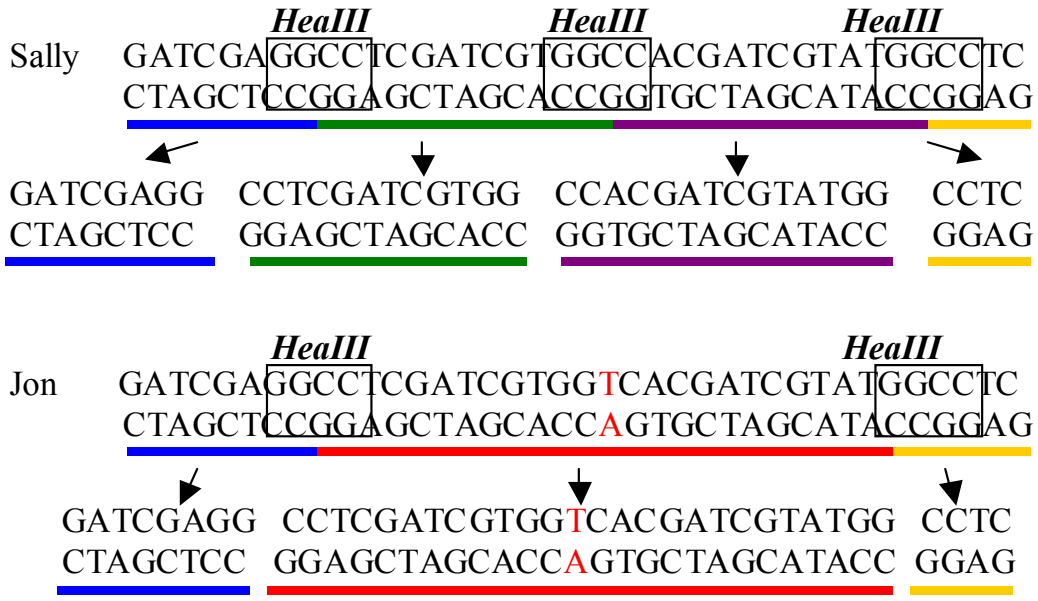
LAB EPH 2

Adapted from "Biotechnology Explorer DNA Fingerprinting Kit Instruction Manual". (Catalog No. 166-0007-EDU) BIO-RAD Laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547.

INTRODUCTION

DNA profiling or "fingerprinting" is a recombinant DNA technique used in many field of study. It involves restricting (cleaving) DNA using enzymes called Type II restriction endonucleases. Restriction endonucleases are isolated from bacteria. These enzymes act as a primitive immune system, chopping up the DNA of viruses that try to infect the bacterial cell. Type II endonucleases are enzymes that act as "molecular scissors" and are capable of cutting the phosphate backbone of DNA in a sequence-specific manner, producing smaller fragments of DNA (see Figure 1). To date, thousands of these enzymes have been identified!

Fig. 1 Restriction fragment length polymorphisms (RFLP's)

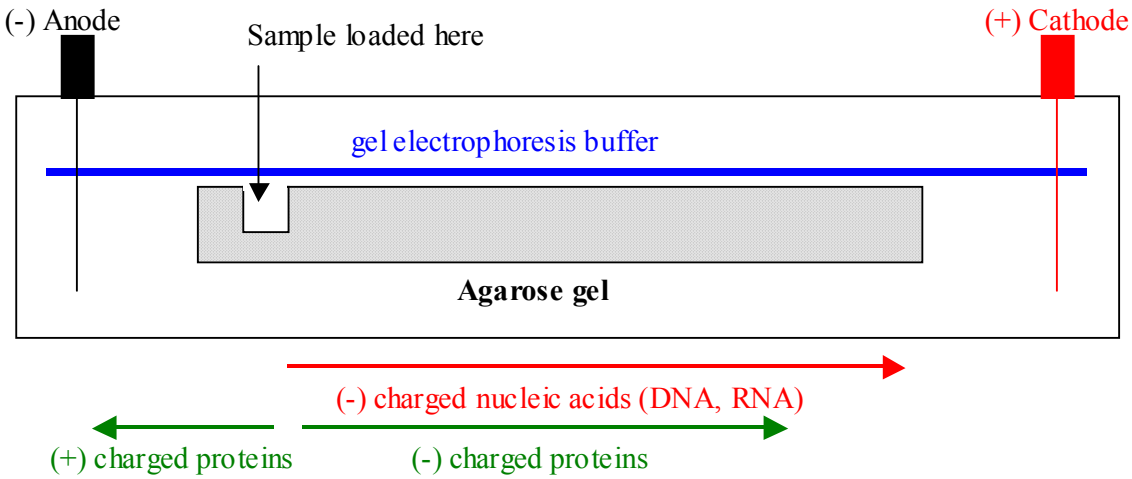


Restriction fragment length polymorphisms (RFLP's) are one way by which individuals can have their DNA "fingerprinted". Although people have the same basic genetic make-up, within each individual there are variations in the DNA (see Figure 1). While a single base change may not result in a noticeable mutation, it may cause a difference in the size

of certain restriction fragments. In the example shown above, Sally and Jon have an almost identical DNA sequence, with the exception of a single base pair. When this region of DNA is cut with the enzyme *HealII* (which recognizes the DNA sequence ...GGCC...), different DNA fragment sizes are produced as a result of the mutation. These fragment size differences can be detected by agarose gel electrophoresis.

Electrophoresis means "carrying with electricity". Biological molecules can be separated on an agarose gel based on their charge, size and conformation (see Figure 2.). Molecules that have a negative (-) charge, like the nucleic acids DNA and RNA, will migrate from the negative pole (anode) to the positive pole (cathode). Positively charged molecules, like some proteins, will migrate from the positive pole to the negative pole. The gel must be prepared and run while submerged in an electrophoresis buffer. This buffer contains salts for conducting the electrical current from one electrode to the other. In addition, the electrophoresis buffer helps maintain the pH during electrophoretic separation. If the pH of the buffer changes, then the charge of the molecules may change and alter their separation. This is especially true for proteins. As the electrical current carries the molecules, the type of gel matrix being used will determine whether the molecules are separated by size, conformation or both.

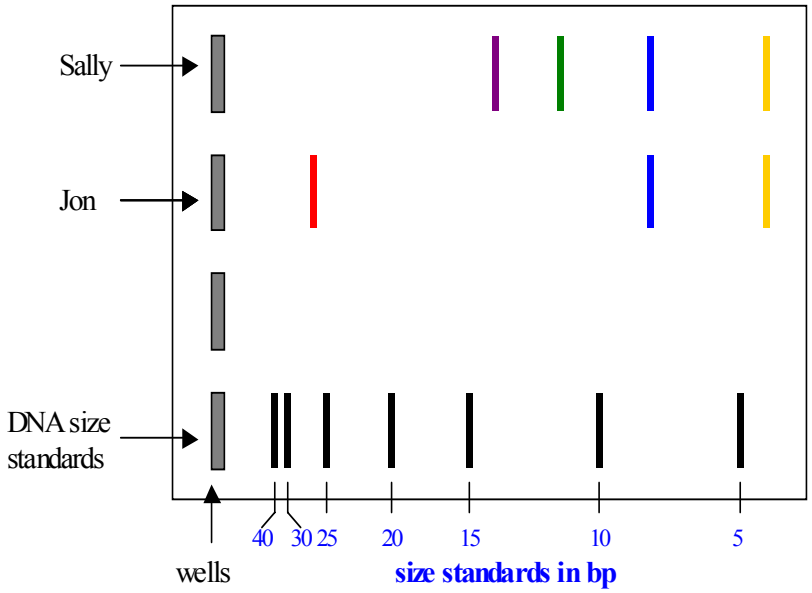
Fig. 2. Visualization of Electrophoretic Separation



Agarose gel electrophoresis is a procedure by which DNA fragments are separated on the basis of size. Agarose is a material derived from red seaweed (Phylum Rhodophyta). When agarose is melted and then cooled, it contains pores that act like a sieve. The size of the pores is determined by the concentration of the agarose in the gel. Increasing the agarose concentration decreases the pore size and limits the size of the DNA molecules

that can fit through the pores. The agarose concentration therefore determines the range of DNA fragment sizes that can be effectively separated on a gel. On a standard 1% agarose gel, DNA fragments from approximately 500 – 10,000 base pairs (bp) can be effectively separated. Small molecules will travel more quickly through the agarose matrix, thus migrate the furthest from the gel well. Larger fragments will take longer to move through the gel matrix, therefore they will migrate more slowly and will be closer to the gel well.

Fig. 3. Example of Electrophoretic Separation of RFLP's



Using the example of Jon and Sally's DNA, the following is a representation of how their DNA fragments would be separated on an agarose gel (Fig 3). Restriction analysis of Sally's DNA, which has three *HealII* sites, results in four fragments which are 14 bp, 12 bp, 8 bp and 4 bp in size. Jon's DNA, which has lost an *HealII* site due to a single base mutation, only produces 3 fragments. Two of these fragments are identical to Sally's (the 8 and 4 bp), but the third is 26 bp, the sum of Sally's 12 and 14 bp fragments. Although Sally and Jon have essentially the same DNA, this type of restriction analysis can reveal individual differences in the sequence, creating a unique DNA "fingerprint" for each individual. This example also shows how the smaller fragments (4 bp and 8 bp) pass through the agarose matrix more easily and move further from the wells. The largest DNA fragment (26 bp) moves more slowly during electrophoresis and is closest to the gel well.

The analysis of RFLPs is utilized for mapping genomes and in forensic science. Speciation, or the determination of species is possible, from either living organisms or

specimens from which DNA can be extracted. Medical uses of this technique include human gene therapy, pharmacogenetics and organ transplants.

For this exercise, you will be asked to analyze DNA from a mock crime scene. You should be able to identify which suspect committed the crime based on the results of an RFLP analysis of all the suspects. To do this, you will perform a restriction digest on a sample of DNA from the crime scene as well as on a DNA sample from each of the suspects. The DNA fragments produced from this digest will then be separated using agarose gel electrophoresis, as seen in Figure 3. Each suspect will have a unique DNA fragment pattern. This fragment pattern is then compared with the DNA fragment pattern from the crime scene. Remember, the fragment pattern must be an exact match before a suspect can be placed at the scene of a crime. With this in mind, it is very important to follow proper protocols to ensure that none of the DNA samples become contaminated!

PURPOSE

The goal of this exercise is to use electrophoresis to analyze RFLPs produced by digesting different DNA samples with a restriction endonuclease. The students will be able to:

- pour an agarose gel.
- digest DNA samples using a restriction endonuclease mix.
- determine the sizes of different restriction fragments.
- analyze the fragment pattern of several "suspects" and compare it to the DNA found at the "crime scene".
- determine which "suspect", if any, was at the scene of the crime.

EQUIPMENT/MATERIALS (for three days)

Day 1

agarose powder
1x TAE buffer
hot pad or mitt
clean beaker or flask
microwave (or hot plate & stir bar)
gel casting tray and gel dams or masking
ice in foam containers
tape
enzyme mix
DNA from "crime scene" and "suspects"
microfuge tubes
hot water bath, set at 37°C
micropipettor and tips

Day 2

electrophoresis chamber & power supply
digested DNA samples (from Day 1)
DNA loading dye
DNA size standards for electrophoresis
micropipettor and tips
paper towels

Day 3
plastic wrap
light box or overhead projector
Bio-Safe DNA stain & large weigh boats or staining dishes
rulers (in mm)

SAFETY PROCEDURES & PRECAUTIONS:

- Always wear an apron and goggles in the lab.

Day 1

1. To dissolve the agarose properly, the agarose and buffer solution must come to a boil. It is important to have a hot mitt to handle the flask containing the agarose solution. If you are using a microwave to heat the agarose solution, be aware of superboiling. It may look as though the solution is not boiling, but when you touch the flask, the liquid gushes up and out of the flask. To avoid this, let the solution microwave for 30 – 45 sec, then take the flask out with a hot mitt and swirl the solution (keeping it away from your face or body). Finally, if the solution has not cooled enough, there is a possibility of steam burns when actually pouring the solution into the casting tray. While some protocols advise cooling the melted agarose to 55ºC before pouring it into the casting tray, this often leads to premature solidification and formation of clumps in the gel. One solution to this problem would be to cool the gel in a 60ºC water bath for 5 minutes. An easier solution is to simply let the flask cool at room temperature for 4-5 minutes, swirl it to re-mix the solution, and then pour it into the casting tray. Use of a hot mitt is still strongly advised.

2. Gloves should be worn at all times to prevent contamination of the samples with DNA and/or nucleases from the students' hands. Care should be taken not to touch the glassware or utensils with bare hands as contamination can occur here as well. Particularly in this example, it is extremely important not to contaminate the DNA acquired from the "crime scene" with DNA from the people performing the investigation.

3. When performing the restriction endonuclease digests, it is necessary to change the tips on the micropipettor between each sample. This also holds true for adding the loading dye before electrophoresis. It is very easy to cross-contaminate the suspects' DNA samples during these procedures. Contamination between samples can result in mixed fragment patterns, thus keeping the guilty party from being definitively identified.

Day 2

Exercise caution when using the power supply. The area around the power supply and the electrophoresis chamber should be dry. Be sure the lead is sitting on the electrophoresis chamber properly and all the connections are in place before turning on the power. Likewise, the power supply should be shut off before disconnecting any of the electrical leads.

PROCEDURE

Day 1

AGAROSE GEL PREPARATION

1. Prepare the gel casting tray by taping the open ends of the tray **firmly** with masking tape. Use your nails to press down on the edges making sure that the ends are sealed and no leaking will occur.

2. Determine the volume of 1X running buffer required for the casting tray. For example, a 7 X 7 cm Bio-Rad casting tray requires 40 mL of 1X buffer. Measure out the required amount of buffer using a graduated cylinder and then pour it into a 125 mL or 250 mL Erlenmeyer flask. (If making up a large batch of agarose for casting numerous gels all at once, multiply the required volume of buffer for one gel by the number of gels being made plus two extra gels.)

3. Use a scale and small weigh boat to measure the amount of agarose required for a 0.8% agarose gel. For example, 0.32 g of agarose needs to be measured if using 40 mL of buffer and a 7 X 7 cm casting tray. (If making up a large batch of agarose for casting numerous gels all at once, multiply the required amount of agarose for one gel by the number of gels being made plus two extra gels.)

4. Heat the agarose solution in a microwave for one minute on “high”. The solution should just begin to boil. (If using a hot plate, heat with intermittent stirring until the solution begins to boil.) Carefully remove flask and gently swirl at arms length. Reheat the agarose for another 20-25 seconds in the microwave and then swirl again. Repeat this process (20-25 seconds) as needed until agarose grains are completely melted (2-3 more times should suffice). Then let cool at room temperature for 4-5 minutes.

5. Pour the hot-warm liquid agarose into the casting tray. Immediately place the gel comb into the end slot. How the comb is aligned depends on the apparatus being used. The most important factor is that the comb does not touch the bottom of the casting tray or the wells will not hold the sample.

6. Allow the gel to cool undisturbed for about 15 minutes. The agarose solution will become cloudy and firm to the touch when it is completely hardened. Placing the casting tray on a cool surface will decrease the gelling time.

7. The gel, still in the casting tray, can be stored for several days in a refrigerator if it is wrapped in plastic wrap or placed in a sealable bag. The combs may be removed prior to storage. Remove the comb slowly and carefully so that the bottoms of the wells do not rip.

DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASE

1. While the agarose gel is hardening, the students can perform the restriction digests. Obtain one each of the color coded microtubes (green, blue, orange, violet, red, yellow). Label the tubes as follows:

   Green = CS (crime scene)
   Blue = S1 (suspect 1)
   Orange = S2 (suspect 2)
   Violet = S3 (suspect 3)
   Red = S4 (suspect 4)
   Yellow = S5 (suspect 5)

   Be sure to put your initials and class period number on the tubes so that they can be identified. Place these tubes in your microtube rack.

2. A clear tube labeled "ENZ", for enzyme, will be provided. This tube will contain a mix of the restriction enzymes *EcoRI* and *PstI*. Keep this tube on ice until you are ready to start the digestion.

3. You will also need to obtain the DNA samples from a stock DNA. There will be a separate tube of DNA for the crime scene and each of the suspects; a total of six DNA samples. Add 10 µL of each stock solution into the appropriate tube using a micropipettor. (Ex. 10 µL of the crime scene stock DNA into the prelabeled CS microtube in your rack; 10 µL of the suspect 1 stock DNA into the S1 microtube; etc.) **YOU MUST CHANGE TIPS BETWEEN EACH SAMPLE**! If you do not change tips, you will contaminate both the stock solutions and your samples. Anyone using the stock solutions after you will also have contamination, making it impossible to perform the experiment successfully.

4. When all the DNA samples are placed in the appropriate tube, the restriction enzyme mix is added. Each tube will receive 10 µL of the enzyme mix. **AGAIN, YOU MUST CHANGE TIPS BETWEEN EACH SAMPLE**! This is another step of the procedure where contamination of the samples can occur. Each tube should now have a total reaction volume of 20 µL (10 µL DNA + 10 µL restriction enzyme mix).

5. To mix the enzyme and DNA, gently tap the side of each microtube. The solution may fly onto the sides of the microtube. To force the liquid back to the bottom of the tube, you can shake the tube (like a thermometer) or tap it on the benchtop. The restriction digest will not proceed evenly if the majority of the mix is not at the bottom of the microtube.
6. A floating microtube rack will be provided. Put all 6 of your microtubes in this rack and place the rack in a water bath set at 37°C. Incubate the samples for 30 – 45 min.
7. A tube with a blue solution labeled "LD" for loading dye will be provided. This can be added now that the restriction digest is complete. Carefully pipet 5 µL of the loading dye into each tube. **YOU MUST CHANGE TIPS BETWEEN EACH SAMPLE**! This is another place in the procedure where you must be careful to not contaminate the samples.
8. The digested DNA samples may be stored at 4°C (or the refrigerator) until the next class period.

Day 2
ELECTROPHORESIS

1. Carefully remove the tape from the ends of the casting tray and place the gel into the electrophoresis chamber. Orient the gel so that the wells are closer to the negative (black) terminal.
2. Fill the electrophoresis chamber with 1X TAE. Be sure that there is <u>just</u> enough buffer to <u>completely</u> submerge the agarose gel.
3. The teacher will demonstrate the proper way to hold a micropipette, fill it with sample and dispense the sample into a well.
4. Fill the pipet with the entire 25 µL of DNA sample. Place the tip <u>over</u> the top of one of the wells. The tip should be submerged in the buffer at this point. Holding the pipet steady, gently dispense the sample into the well. The loading dye in the sample will allow the sample to sink into the well. <u>Do not</u> place the pipet tip directly into the well or you will risk poking a hole in the side or bottom of the well, and your sample may leak out of the gel. **A NEW PIPET TIP SHOULD BE USED FOR EACH SAMPLE!** If possible, each student should have a turn loading a well. Remember to load 10 µL of the DNA size standards, which will be provided by your instructor.
5. Record the order in which the samples are loaded, either left to right, or top to bottom.

6. Make sure that the area around the electrophoresis chamber and power supply is dry. There is an ON button on the back of the power supply. This is on when a red "0" is displayed on the LED at the front of the machine. There are several buttons on the front of the power source. To set the supply to show volts, press the two " ■ " buttons until a green light comes on next to the "V" (for volts). Set the power source to 100V by pushing the "▲" to the left of the LED display. Check to be sure that the leads from the gel are plugged into the power supply. When the leads are connected properly (red to red, black to black), press the "run" button (a picture of a man running). A green light should come on next to the run button to indicate the power supply is working. There should be bubbles forming on the electrodes in the electrophoresis chamber if the current is flowing properly. More than 125 V is not recommended, as the agarose gel can melt. Check the gel after approximately 5 min. to make sure the sample is migrating through the gel in the correct direction. Allow the gel to run for a total of 30-45 minutes. The dye in the loading solution can be used to gauge the progress of the sample in the gel.

7. Electrophoresis is complete when the dark blue dye has run ⅔ to ¾ of the way down the gel. Turn off the power and remove the lid from the gel box. It is recommended that the gel be stained overnight at room temperature.

Day 3
GEL STAINING & ANALYSIS

1. Carefully remove the gel, still in its casting tray, from the electrophoresis chamber. Gently slide the gel into a staining tray (or large weigh boat) and cover the gel with approximately 60 mL Bio-Safe DNA stain. Although this stain is non-toxic, gloves are still recommended, as it will stain your hands! Cover the staining tray with plastic wrap. This solution will stain both the gel and the DNA. For best results, place the staining tray on a rocking platform and stain the gel overnight at room temperature.

2. The gel will need to be destained to visualize the DNA bands. Pour the Bio-Safe stain into another bottle. Then pour approximately 60 mL distilled or tap water into the staining tray. Let the gel destain for 15 minutes, or until the DNA becomes visible against the clear background of the gel. Again, this step works best if a rocking platform is available.

3. Place the gel on a piece of plastic wrap. View the gel on a white background with the light shining from beneath. Either a light box or overhead projector can be used for this purpose. If these are not available, placing the gel over a white paper with a bright light above will work as well.

4. The most accurate way to analyze the size of each DNA fragment is to measure the distance each fragment has migrated on the gel. Measuring the distances (in mm) from the bottom of the gel "well" to the band on the gel can do this. You will need to do this for each band in a lane. Do not forget to measure the distance of the bands for the DNA size standards as well. Record the number of bands in each lane, and the distance, in mm, in the Data Sheet.
5. The DNA size standards are fragments that are 23,130 bp, 9,416 bp, 6,557 bp, 4,361 bp, 2,322 bp and 2027 bp in size. This information can be used to plot a standard curve on a graph using semi-log graph paper, which will be provided by the instructor. The x-axis will be the distance each band has migrated in the gel (in mm) vs. the size in base pairs (bp). This information will be used to determine the size of the fragments in the other lanes. After mapping a point for each band, connect the points with a line. This is your standard curve.
6. To determine the size of a band in the crime scene lane, find the number of mm the band traveled, on the x-axis of the graph. From this point, move straight up (vertically) the graph until you intersect the line of the standard curve. Follow the intersect point to the y-axis (horizontally). The approximate size of the DNA fragment is where this point meets the y-axis. Repeat this procedure for all the bands in the crime scene sample, and for suspects 1-5. Record the sizes of each DNA fragment in the table above.
7. Compare the DNA fragment sizes from the crime scene to that of each suspect. Determine whether any of the suspects is an exact match.

References

"Biotechnology Explorer DNA Fingerprinting Kit Instruction Manual". (Catalog No. 166-0007-EDU) BIO-RAD Laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547. www.explorer.bio-rad.com

Introductory material adapted from: Corrette-Bennett, J.C. "Concepts of DNA Fingerprinting". Science in Motion Workshop. June, 2002. Westminster College, New Wilmington, PA.

DATA SHEET

Name _____
Name _____
Period _____ Class _____
Date _____

DNA FINGERPRINTING

DATA TABLE

Lane	Number of Bands	Distance (mm)	Size (bp)
M			
CS			
S1			
S2			
S3			
S4			
S5			

ANALYSIS

1. What is a Type II restriction endonuclease and what does it do?

2. What is produced when an individual's DNA is cleaved with a restriction endonuclease?

3. What is the cause of differences in DNA fragment patterns when an individual's DNA is cleaved with restriction enzymes?

4. What is the charge on a DNA molecule (positive or negative)? Toward which pole does the DNA migrate during electrophoresis on an agarose gel?

5. Which size DNA fragments move furthest from the gel well, small or large? Why?

6. Why is preventing contamination of your DNA samples so important? In what ways could contamination affect your analysis of the DNA from the crime scene and suspects?

7. Did any of the suspects have an exact DNA match to that found at the crime scene? Can you charge a person with a crime based on DNA evidence alone?