Electrophoresis and DNA Forensics | 36

Introduction

The world of forensic science was revolutionized with the discovery of scientific techniques for identifying humans—and, indeed, all living things—using DNA. DNA fingerprinting can be used to identify the source of DNA in forensic investigations and has also been used to diagnose genetic diseases, identify disaster victims, and study evolutionary relationships among organisms.

Concepts

- Gel electrophoresis
- Genes
- Polymerase chain reaction (PCR)
- Restriction fragment length polymorphism (RFLP)
- Variable number of tandem repeats (VNTR)
- Restriction enzymes

Background

Gel electrophoresis is an analytical method for the separation, identification and analysis of biological molecules, including DNA, RNA and proteins, in an electric field. In 1955, the British-born American scientist Oliver Smithies determined that when a colloidal gel made of starch was positioned between positive and negative electrodes, it acted like a molecular "sieve" for the macromolecules. Proteins with different sizes, shapes and molecular charge moved through the gel at different rates, with smaller molecules or fragments moving faster through the maze of microscopic pores. Charged proteins always moved toward the electrode with the opposite charge. For example, a negatively charged protein migrates through a gel toward the positive electrode, which is called the anode. DNA sequencing methods have been built upon the original electrophoresis principles developed by Smithies, who received the Nobel Prize for medicine in 2007 for innovations, including specific gene modifications in mice, that revolutionized genetic research.

In 1984, Sir Alec Jeffreys of the University of Leicester in England published the first account of the basic principles, techniques and applications of DNA fingerprinting. Combining his interest in human genetics with research experience in molecular biology probing mammalian genes, Sir Jeffreys had been studying the production and separation of DNA fragments to detect variations between individuals and identify the causes of inherited diseases. He had earlier found that when DNA was hydrolyzed or cut into fragments using so-called restriction enzymes and separated using electrophoresis, the results yielded distinctive banding patterns due to the formation of different-size DNA fragments. This result, called restriction fragment length polymorphism, or RFLP, arose due to variations in DNA sequences between individuals. Although RFLP showed differences in the pattern of enzyme recognition sites, it produced far too many bands and was therefore too crude or blunt a technique to be useful for the unique identification of individuals. By focusing on one gene of interest, and hybridizing part of it to DNA fragments lifted from electrophoresis analysis of DNA of different individuals, Jeffreys found more subtle variations in the number of repeating DNA base-pair sequences between genes on a chromosome. Every living thing, except identical twins, has a unique number of tandem DNA repeats called **variable number of tandem repeats** (VNTR). Since these tandem repeats are not part of a gene, they do not affect the viability of an organism. The basic procedures involved in DNA fingerprinting are described below.

In order to analyze DNA, it must first be extracted from the cells of an organism—any cell that contains a nucleus can be used to identify DNA. The sample of cells is homogenized in a blender or crushed in a mortar with salt and a detergent to break the cell membranes and expose the DNA. An enzyme is added to the mixture to facilitate the uncoupling of the DNA from its histones (DNA proteins). When alcohol is added on top of the treated cell mixture, the DNA becomes insoluble and moves to the alcohol layer, where it can be easily retrieved.

At this point, the DNA molecules are too long to be analyzed by electrophoresis. The DNA must first be fragmented at very specific base-pair locations using one or more **restriction enzymes**. Enzymes that break DNA molecules at internal positions are called **restriction endonucleases**. Enzymes that degrade DNA by digesting the molecule from the ends of the DNA strand are termed **exonucleases**. More than 2500 different restriction enzymes are available to molecular biologists to analyze DNA. Each restriction enzyme recognizes a specific nucleotide sequence. The enzyme "scans" the length of a DNA molecule and then digests it (breaks it apart) at or near a particular recognition sequence. The specific sequence may be five to sixteen base-pairs long. For example, the EcoRI endonuclease has the following six–base-pair recognition sequence:

5′ – G|A A T T C – 3′
3′ – C T T A A|G – 5′

EcoRI breaks the double-stranded DNA at the locations indicated by the dotted line and produces "ragged-ended" sequences, often called sticky ends. Other endonucleases cut the DNA cleanly at one specific base-pair, producing what are called blunt ends.

Restriction endonucleases are named using the following convention:

EcoRI

E = genus **Escherichia**

co = species **coli**

R = strain RY 13

I = first restriction enzyme to be isolated from this species

The first letter (capitalized) indicates the genus of the organism from which the enzyme was isolated. The lower case letters that follow indicate the species. Additional letters indicate the particular strain used to produce the enzyme. The Roman numeral denotes the sequence in which enzymes from a particular species and strain of bacteria were isolated.

Since the average human DNA sequence is more than 3.2 billion base-pairs long (about 20,000 genes), there may be as many as 750,000 fragments of DNA after a single restriction enzyme completes the fragmentation of a single cell's DNA. RFLP fragments created by the use of one or more restriction enzymes are loaded into an agarose gel in an electrophoresis chamber. Agarose is a refined form of agar that is made from seaweed. The agarose gel is positioned between two electrodes with the wells located by the cathode (negative electrode). This allows the negatively charged DNA to move toward the anode (positive electrode). The electrophoresis chamber is filled with a buffer solution, bathing the gel in a solution that shields the system from changes in pH.

DNA fragments are white to colorless and appear invisible in the gel. Colored tracking dyes are added to the DNA sample to monitor the progress of the sample as it moves through the gel. Typically, two dyes are added, one that migrates at a rate similar to the smaller DNA fragments and another that migrates at a rate similar to the largest DNA fragments. Once the first dye migrates to within 1 cm of the end of the gel, the power is shut off to the electrophoresis unit. The DNA stops migrating since the electromotive force stops.

The agarose gel is then removed from the electrophoresis chamber and transferred to a staining tray. The stain binds to the DNA fragments revealing a banding pattern. Molecular biologists use a radioactive stain and X-ray film to visualize the banding pattern. In this experiment a colored dye that may be viewed with visible light will be used. The banding pattern is unique since the DNA sample is unique to each individual or organism, except identical twins or asexual offspring of less complex organisms. The banding pattern is measured against a series of known DNA standards and samples prepared and analyzed with the unknown DNA sample. For example, paternity test runs include a known standard of human DNA plus samples of the mother, likely fathers, and that of the child. A match is determined by calculating the probability of an individual having a particular combination of bands in a population. The more matching bands that are observed, the more likely it is that the DNA comes from the same person.

A second sample may be run using a different restriction enzyme. A different banding pattern will be revealed for the same DNA samples because the DNA sequence is cleaved at different base-pair locations. With the exception of identical twins who share identical genotypes, the probability of two individuals having identical banding patterns for two series of DNA cuts is less than the current human population. The theoretical risk of a coincidental match has been estimated at 1 in 100 billion.

The mobility of negatively charged DNA fragments in an electrophoresis experiment may be standardized by running the fragments repeatedly under identical conditions, i.e., pH, voltage, time, gel type, gel concentration, etc. Under identical conditions, identical-length DNA fragments will move the same distance in a gel. Thus, the size of an unknown DNA fragment can be determined by comparing distance on an agarose gel with that of DNA marker samples of known size. The size of the DNA fragment is usually given in nucleotide base-pairs (bp). The smaller the DNA fragment, the faster it will move through the gel during electrophoresis.

Good scientific protocol is critical to the outcome of any laboratory work, especially in forensic analysis. Sloppy work might convict the wrong person or let a guilty suspect go free. Consequently, analysts must carefully document which restriction enzyme was used, the conditions and chemicals that were used, and the names of all known standards and controls that were prepared with the DNA sample.

RFLP is limited by the quantity of DNA available and the degree of degradation of the sample. Modern techniques in DNA forensic analysis utilize the polymerase chain reaction (PCR). This technology can be used to amplify (make numerous exact duplicates of) as little as a single molecule of DNA. The amplified sample may then be digested by restriction enzymes, electrophoresed, stained, and analyzed. PCR technology is able to analyze any detectable DNA sample, no matter how small or degraded the original sample.

Experiment Overview

The purpose of this activity is to demonstrate the basic principles of DNA forensics using gel electrophoresis. The process will be used to identify matching DNA profiles from a collection of DNA samples.

Materials

- Agarose gel*
- DNA samples:
  - DNA Sample 1
  - DNA Sample 2
  - DNA Sample 3
  - DNA Sample 4
  - DNA Reference Ladder†
- Methylene blue staining solution, 50 mL
- TAE electrophoresis buffer, 200 mL
- Water, distilled
- Beakers, 600-mL, 2
- Digital micropipet (shared)
- Electrophoresis chamber with power supply
- Light box or other light source (optional)
- Marker
- Paper towels
- Paper, white
- Pipet tips (disposable)
- Resealable bag
- Ruler, metric
- Staining tray
- Thermometer

*See the *Supplemetary Information* section for preparation instructions, if needed.
†The DNA reference sample contains a set or "ladder" of DNA fragments of known size.

Safety Precautions

Be sure all connecting wires, terminals and work surfaces are dry before using the electrophoresis units. Electrical Hazard: Treat these units like any other electrical source—very carefully! Do not try to open the lid of the unit while the power is on. Use heat protective gloves and eye protection when handling hot liquids. Methylene blue will stain skin and clothing. Wear chemical splash goggles, chemical-resistant gloves, and a lab coat or chemical-resistant apron. Wash hands thoroughly with soap and water before leaving the laboratory.

Procedure

Loading a Gel

1. Assemble the electrophoresis unit according to the manufacturer's instructions.
2. Make sure the electrophoresis chamber is on a level surface and do not move the unit after loading the samples.
3. **Gently** slide a prepared gel from a resealable bag into the casting tray with the wells toward the cathode (–) end of the unit.
4. Carefully position the gel and tray in the electrophoresis chamber. **Caution:** Be careful not to break or crack the gel. If the gel is damaged, it should not be used as cracks will affect the results.
5. Pour enough electrophoresis buffer into the unit to submerge the entire gel surface to a depth of 2–5 mm.
6. By convention, DNA gels are read from left to right, with the wells located at the top of the gel. With the gel lined up in the chamber and the wells located to the left, load the contents of DNA Sample 1 into the well closest to you. When the gel is turned so that the wells are at the top, "1" will be in the upper left corner.
7. Shake the microcentrifuge tubes containing DNA samples and lightly tap the bottom of each tube to mix the contents.
8. Withdraw 10 µL of DNA Sample 1 from the microcentrifuge tube using a digital micropipet.
9. Dispense the sample into the first well by holding the pipet tip just inside the well. The sample will sink to the bottom of the well. **Caution:** Do not puncture the bottom or sides of the well. Do not draw liquid back into the pipet after dispensing the sample (see Figure 1).
10. Using a fresh pipet tip, withdraw 10 µL of DNA Sample 2 and load it into well 2, adjacent to DNA Sample 1.
11. Repeat step 10 for the remaining DNA samples. Use a clean pipet tip for each sample. The DNA reference ladder should go into well 5.

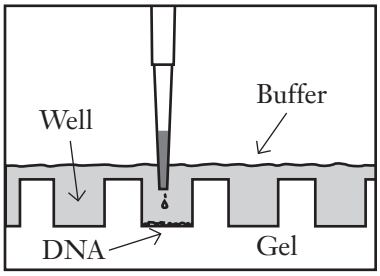


Figure 1.

Running the Gel

1. Place the lid on the electrophoresis chamber and connect the unit to the power supply.
2. Run the gel as directed by the instructor. Bubbles will be visible along the electrodes while the sample is running. The bubbles are due to the electrolytic decomposition of water—hydrogen at the cathode and oxygen at the anode.
3. Turn off the power when the first tracking dye is 1 cm from the positive end of the gel. (This may take 30 minutes to 2 hours.) The time necessary to run a gel depends on the apparatus and the applied voltage.
4. When the power is off, remove the cover and carefully remove the gel tray from the chamber. Place the gel tray on a paper towel, being careful not to break or crack the gel.

Staining and Analyzing the Gel

For best results, stain the gel immediately and then destain.

1. Slide the gel off the tray and into the staining tray. **Note:** Do not stain the gel tray.
2. **Gently** pour 40 mL of the methylene blue staining solution into the staining tray. Allow the gel to stain for at least 5–10 minutes.
3. Pour off the stain into a glass beaker. The stain may be reused by other groups.
4. To destain the gel, gently pour room temperature distilled water into the staining container. **Note:** Do not exceed 37 °C; warmer water may soften the gel. Occasionally agitate the water and destain for 10 minutes.
5. Pour off the water into a waste beaker and repeat step 4 until DNA bands are visible.
6. Record the relative locations of each band in the *Laboratory Report*. The gel may be placed on a light box to visualize faint bands.

Pre-Laboratory Assignment

36

Name:_____ Section:_____ Date:_____

Electrophoresis and DNA Forensics

1. List the safety precautions that must be followed when performing electrophoresis.

2. Explain the function of each of the following components of gel electrophoresis:
   a. Agarose gel

   b. Electrophoresis buffer

   c. Wells in the gel

   d. Electric current

3. Briefly summarize how gel electrophoresis is used to separate molecules.

4. Lambda phage DNA is 48,502 base-pairs (bp) long and has five recognition sites for the EcoRI restriction enzyme. Starting from the 5′-end, the sites occur at 21,226 bp; 26,104 bp; 31,747 bp; 39,168 bp; and 44,972 bp. How many fragments will be obtained when lambda phage DNA is digested with EcoRI? List the fragment size for each. **Hint:** To visualize the fragmentation process, draw a line to represent a DNA strand and then mark off the restriction sites.

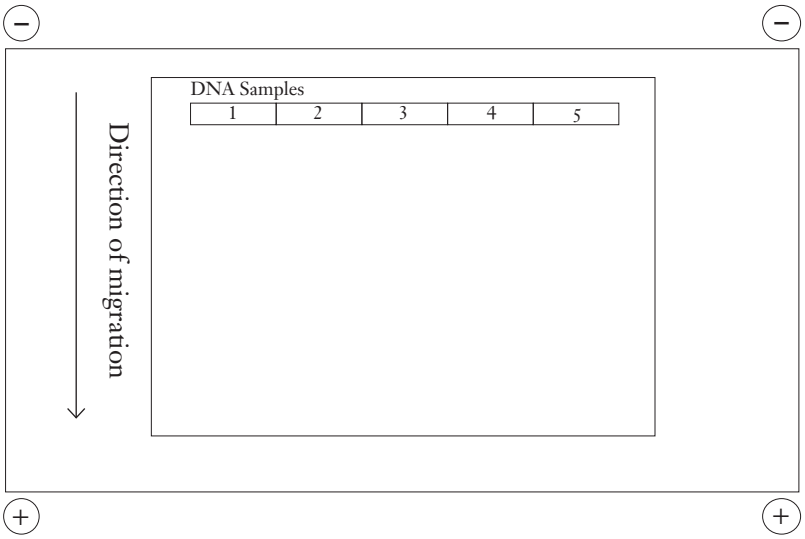
Laboratory Report

36

Name:_____ Section:_____ Date:_____

Electrophoresis and DNA Forensics

1. Sketch the approximate locations of the DNA bands in the graphic below.



2. Using a metric ruler, measure the migration distance in millimeters for each major band and enter the results in the following table.

DNA Sample 1		DNA Sample 2		DNA Sample 3		DNA Sample 4		Reference Ladder*	
DNA Fragment No.	**Migration Distance (mm)**	**DNA Fragment No.**	**Distance (mm)**	**DNA Fragment No.**	**Distance (mm)**	**DNA Fragment No.**	**Distance (mm)**	**DNA Fragment No.**	**Distance (mm)**
1		1		1		1		1	
2		2		2		2		2	
3		3		3		3		3	
4		4		4		4		4*	

*The DNA ladder will contain 10–14 visible bands. List the migration distances separately for the additional bands in the DNA ladder. For the DNA 1-kb ladder, additional bands should appear at 75, 81, 86, 92, 97 and 103 mm. There may also be four closely spaced fragments at 108–116 mm.

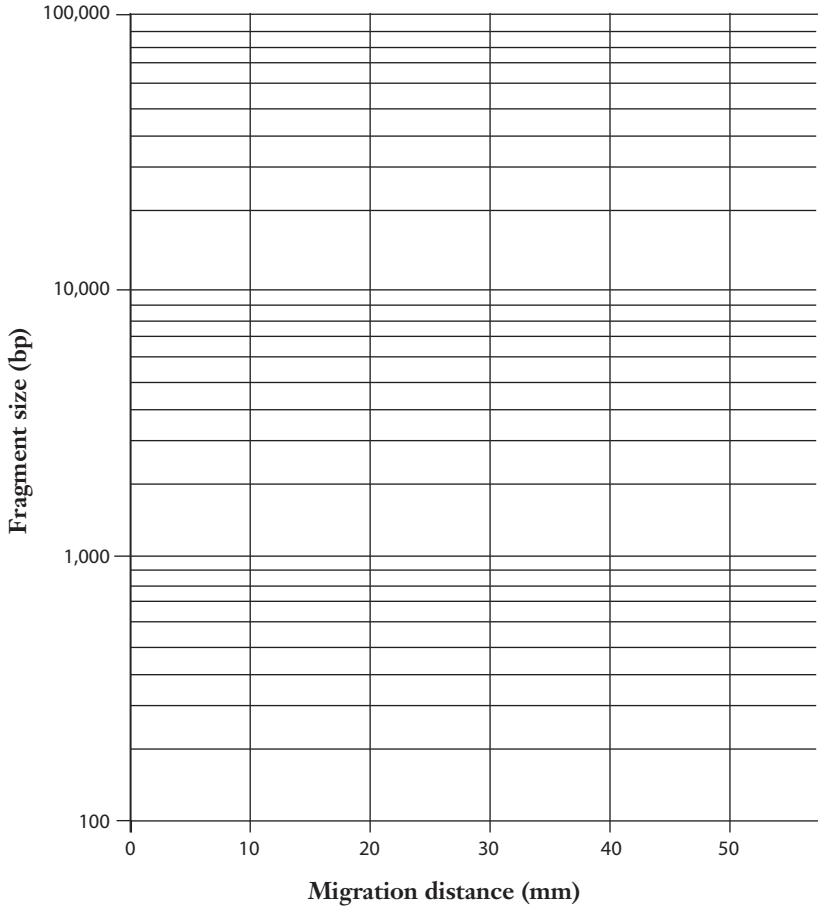
3. Evaluate the banding patterns in the electrophoresis experiment and identify any matching DNA samples.

4. List three errors that could affect the outcome of any gel electrophoresis procedure.

5. Why would a forensic scientist use the polymerase chain reaction technique to prepare DNA samples for analysis?

6. The table below lists the migration distance and fragment size for Lambda DNA cut by the restriction enzyme HindIII and analyzed on a 0.8% agarose gel at 70 V. Plot the data on the semi-log graph shown below. Draw a smooth curve through the points and explain how the graph could be used to determine the fragment size for an unknown band. Estimate the base-pair fragment size for a band that appears at 30 mm in the same gel.

Migration Distance (mm)	Fragment Size (bp)
14	23,130
19	9,416
22	6,557
26	4,361
37	2,322
40	2,027



Supplementary Information—Preparation of 0.8% Agarose Gel

Materials

Agarose
Balance, 0.01-g precision
Casting trays with well combs
Cotton, non-absorbent or foam plug
Erlenmeyer flask, borosilicate, 250 mL
Gloves, heat-protective
Marker or wax pencil
Microwave, hot water bath or stirring hot plate
Stirring rod
TAE electrophoresis buffer
Weighing dishes, small or weighing paper

Safety Precautions

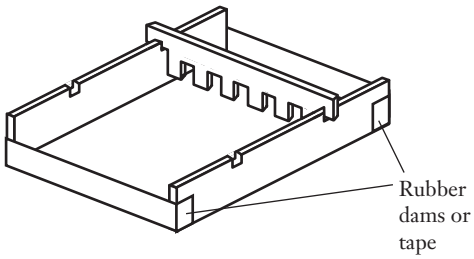
Wear chemical splash goggles and heat protective gloves when handling hot liquids. Be careful not to superheat the solution because it will NOT boil until stirred, whereupon it will boil over.

Preparation of one 0.8% agarose minigel

1. Stir 0.48 g of agarose into 60 mL of the electrophoresis buffer in a borosilicate Erlenmeyer flask. Stopper with a non-absorbent cotton or foam plug.
2. Mark the height of the solution on the Erlenmeyer flask.
3. Dissolve agarose by heating in a microwave, hot water bath, or on a hot plate. **Caution:** Be careful not to superheat the solution because it will NOT boil until you disturb or disrupt it, whereupon it may spontaneously boil out.
   a. Microwave—30–40 seconds, stir, repeat.
   b. Hot water bath—do not boil the water.
   c. Hot plate—do not boil or scorch the agarose solution.
4. Heat until the solution is clear and agarose appears to be fully dissolved. **Stir frequently,** and do not allow solution to boil.
5. Use heat protective gloves to remove the flask from the heat source.
6. Check the liquid level of the solution. Add distilled water to the original liquid level, if needed.
7. To prevent damage to the casting trays, allow the agarose to cool to 55 °C before pouring.

Prepare the casting trays while waiting for the agarose to cool.

1. Attach the rubber dams to the ends of the casting tray.
2. Place the well-forming comb in the groove at one end of the gel box.
3. Ensure the casting tray is on a level surface. Slowly pour the melted agarose into the assembled casting tray being careful not to create bubbles in the gel. Use a stirring rod or pipet tip to push any bubbles to the edge of the casting tray. Add only enough agarose to equal the height of the indentations in the well-forming comb—do not fill the tray to the top.



4. Allow the gel to sit undisturbed for at least 20 minutes until the gel is firm to the touch. The set gel will appear opaque and somewhat white. 60 minutes is optimal.
5. Once the gel is thoroughly set, carefully remove the well-forming comb by rocking it gently from side to side and then pulling it upward. Remove the end dams and carefully slip the gel out of the form.
6. Slide each gel into a separate resealable bag, add 5 mL of buffer, and refrigerate. **Note:** A solidified gel can be stored under buffer in a laboratory refrigerator for up to two weeks.