Molecular Ladder to Freedom - DNA Exoneration

Student Guide for Fragment Length Analysis

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Based on the original activity developed by

Fred Hutch Cancer Center

Science Education Partnership

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Laboratory Safety

1. Wear lab coats, gloves, and eye protection as required by district/college protocol.
2. Use caution with all electrical equipment such as PCR machines and electrophoresis units.
3. Heating and pouring molten agarose is a splash hazard. Use caution when handling hot liquids. Wear eye protection and gloves to prevent burns.
4. Wash your hands thoroughly after handling biological materials and chemicals.

Introduction

In this lab activity you will examine scenarios in which an incarcerated individual is appealing their conviction using post-conviction DNA testing to support their claim of innocence, the types of evidence and factors leading to wrongful convictions. You are encouraged to explore other exoneration cases through the National Registry of Exonerations, the Innocence Project, and the Equal Justice Initiative.

Lab Overview

The purpose of the lab is to take a fresh look at prior convictions that led to incarcerations, this time including DNA evidence as part of the entire body of evidence.

Teachers are provided with five different prepared DNA samples, representing an appellant, crime scene samples and new suspect(s). Case studies provided by The Fred Hutchinson Cancer Center's SEP program can be used to provide the scenarios students will be reviewing. By providing different scenarios to different workstations students can investigate various situations and have a class wide discussion about what led to the wrongful convictions.

Background information

Humans are biologically very similar to each other. We share 99.9% of our genomic sequence, which guides our development and all physiological functions. Despite our deep similarities, it's obvious that we all differ slightly from each other in a variety of observable characteristics: height, the shape of our faces, the color of our skin, hair and eyes all help distinguish us visibly as individuals. When an eyewitness points to a suspect in a line up and says, "that's the person who did it", they are relying on their human ability to individuate, or tell the difference, between two people based on observable physical characteristics.

Alphonse Bertillon faced an individuation problem as a clerk for the Paris Prefecture de Police in the 1880s: how to keep track of repeat offenders and distinguish them from first time offenders, especially when an experienced criminal might give a false identity. Dissatisfied with the haphazard system of photographs and descriptions in place at the time, Bertillon devised a set of eleven anatomical measurements that could be made on any arrestee. Measurements of the head, arms, hands, and feet were taken with specialized instruments and recorded on a card along with other identifying features. The reasoning behind Bertillon's system was that the probability must be incredibly small that any two people (except identical twins) would measure exactly the same for all eleven features, as long as the measurements were accurate enough. Bertillon also invented a systematic method of photographing arrestees, what we now call the mug shot.

Bertillon was meticulous in applying his system. Cards were filed according to anatomical categories based on the measurements, so repeat offenders could be quickly found in this physical database. However, the system was not as successful as it spread to the rest of Europe and the United States. Applying the Bertillon system required precision and rigorous attention to

detail. If their measurements were made in slightly different ways, two officers could get different numbers, leading to errors in identification. Measurements could also change throughout a person's lifetime. Since the set of features were selected to distinguish adult men, they were not as useful when applied to women and children.

The limitations of the Bertillon system are illustrated by the unusual case of Will West, a convict brought to the US penitentiary in Leavenworth, Kansas in 1903. As usual, prison officials photographed him and took Bertillon measurements. Searching their database, they found that the new prisoner's measurements matched William West, a man previously convicted of murder. The second William West though was still incarcerated at Leavenworth! By a strange coincidence, two men with identical Bertillon measurements and nearly the same name had been taken to the same prison. Their fingerprints however, were clearly distinct.

The idea that fingerprints are unique to individuals has been around for centuries, but it wasn't until 1892 when Sir Francis Galton published a systematic study of fingerprint features that law enforcement began to use fingerprints as a means for identification. Galton showed that no two people he examined had the same patterns of ridges and folds in their fingerprints and that a person's fingerprints do not change over a lifetime. Although fingerprinting, being faster and easier to apply, would eventually replace the Bertillon system, it was based on a similar concept: because of person-to-person variability, precise measurement of physical characteristics can uniquely identify an individual. Since folding of the skin on the fingers during development is not genetically determined even identical twins have distinct fingerprints. Fingerprinting had another advantage over physical measurements: fingerprints left on objects at the crime scene can be compared to suspects' fingerprints to either include or exclude them as the potential culprit.

Fingerprint matching relies on experience-based expert pattern analysis. Fingerprints from a crime scene and from known suspects are first classified according to general type (loop, whorl, arch), then fine details of the ridge patterns called minutiae are examined. The process is deliberately kept subjective so that experts can evaluate which set of features is most useful for analyzing a given print. The examiner then compares the known prints to the crime scene print and evaluates whether they could have come from the same person based on similarity of features. This information is then used to exclude or include an individual suspect as the source of the crime scene print.

Even though fingerprinting was an improvement over the Bertillon system, it comes with its own set of challenges. Fingerprints at crime scenes can be smudged or incomplete and their quality is impacted by properties of the surface. Depending on how the finger contacts the surface, ridges can be compressed or separated, which changes their appearance. Moreover, there is no universal agreement among experts as to how many characteristics must be identical for two fingerprints to be considered a "match" and little data about the probability of finding a match by chance. Studies have shown that fingerprint experts frequently disagree with each other when examining the same fingerprint, and even disagree with themselves when examining a fingerprint they have analyzed in the past!

An ideal system for individuation would remove as many opportunities for bias as possible, produce identical results every time it is applied to the same sample, and be backed by statistics showing what percentage of the population would match just by chance. Forensic DNA analysis

is currently the closest we have to that ideal. It gives reliable results, involves little subjective interpretation, and is supported by a large body of scientific research originating from outside of law enforcement. Although only 0.1% of the genome differs between individuals, in a genome that is 3 billion base pairs long, the 3 million base pairs that are unique give ample material for distinguishing two individuals within the population. Like fingerprints, DNA can be left behind at a crime scene. Blood, saliva, hair, and even the skin cells we shed everywhere we go, contain DNA that can be used to show that a suspect had been at a crime scene, or to rule out a suspect as a possible contributor of DNA.

DNA profiling, also known as DNA fingerprinting, relies on the fact that variation is not distributed uniformly throughout the genome. The sequences that code for proteins tend to be highly conserved, or similar between human individuals and often similar between species. Regions called minisatellites contain repetitive sequences, with the number of repeats being highly variable within the population. These regions are non-coding regions and do not cause any phenotypic differences between individuals. In 1984, Sir Alec Jeffreys, a researcher at University of Leicester studying inherited variation in genes, discovered that by analyzing minisatellites he could identify individuals and trace family relationships. The first application of DNA profiling was for verifying family relationships in immigration cases. The law enforcement applications of his discovery were also immediately apparent. The first case to use DNA testing involved the murders of two teenagers in Leicestershire. A suspect, Richard Buckland, was in custody and had confessed to one of the murders. Police thought that if they used DNA to link Buckland to both of the murders he would confess to the second. To their surprise, the DNA found on the victims did not match Buckland, and he was released. To find the real killer, the police took the unusual step of collecting DNA samples from every man in the neighborhood. No matches were found among the 4,000 samples they tested, until a man named Ian Kelly was overheard in a pub confessing to having taken the DNA test in place of another man, Colin Pitchfork. In 1987, Kelly and Pitchfork were both arrested and Pitchfork confessed to both murders.

DNA profiling was rapidly adopted by law enforcement. In the late 1980, many US states began requiring DNA collection from individuals convicted of certain violent crimes, whose profiles were then uploaded onto state DNA databases. In 1998, the FBI launched the National DNA Index System (NDIS), a database for DNA profiles generated by federal laboratories and certain DNA profiles collected at the state level. The FBI also established the Combined DNA Index System (CODIS) software which allows laboratories to search a sample against profiles stored in local, state, and federal databases to find potential matches.

Although it is used in less than 1% of criminal cases, DNA profiling stands out among forensic disciplines for its scientific rigor and its ability to accurately connect an individual suspect to physical evidence. It has also become a powerful tool for exonerating the innocent. Post-conviction DNA testing is typically performed at the request of the appellant appealing a guilty verdict. Organizations like the Innocence Project provide assistance and legal representation to those seeking DNA testing to prove their innocence. Of the 3,266 exoneration cases listed in the National Registry of Exonerations, post-conviction DNA testing resulted in 820 exonerations, including the exoneration of 21 death row inmates. Many of the individuals being exonerated

today based on DNA evidence have been in prison for over 20 years, typically on a murder conviction. DNA evidence was not presented in these original trials, whereas today DNA testing has become more common practice to include right away.

The increased use of DNA forensics has been aided by improvements in the technology since Jeffreys' pioneering work. In the first methods of DNA profiling, DNA isolated from the sample was directly treated with restriction enzymes. Since restriction enzymes recognize and cut specific sequences of DNA, due to different sequences in different people's genomes, different fragment lengths will be produced. Cutting DNA with restriction enzymes produces hundreds of thousands of fragments, each fragment with a distinct sequence and length. These fragments are then run on an agarose gel to separate them by size, then transferred to a membrane. The membrane is treated with a single-stranded nucleotide probe labeled with radioactive phosphorus that will bind to complementary sequences in the sample. The sequence of the nucleotide probe is developed to bind specifically to the minisatellite region being analyzed.

This process was time-consuming and required a relatively large starting quantity of sample, such as a bloodstain the size of a dime. PCR (polymerase chain reaction, see Appendix B) enables DNA amplification from a much smaller sample, even just a few cells. Starting from a few copies of the genome, millions of copies of the desired sequence can be produced in a couple hours. PCR also enables amplifying a specific sequence of interest which avoids the laborious process of running thousands of fragments, transferring them, and labeling them with a probe. In modern forensic laboratories, capillary electrophoresis is used instead of gel electrophoresis, allowing the detection and size determination of DNA fragments to be automated.

The PCR protocol used for forensic DNA profiling today targets regions of repetitive DNA called short tandem repeats (STRs). The repeated sequences in these regions are typically 2-6 bp long, which is much shorter than the repeats originally used by Jeffreys. The number of repeats at an STR locus varies from person to person, therefore so does the length of the fragment amplified by PCR. For example, consider an STR locus with a variable number of repeats of a 4 bp sequence such as CATG. If suspect A had 40 repeats at this locus and suspect B had 25 repeats at this locus, you would see a band at 160 bp (40 repeats x 4 bp each) in suspect A's profile and a band at 100 bp (25 repeats x 4 bp each) in suspect B's profile.

Simply knowing that the sizes of DNA fragments in a suspect's profile are the same as the DNA profile extracted from crime scene evidence means little without information about how common that profile is in the population. The probability of two people having the same number of repeats at one STR locus is quite high, meaning that an innocent person could be connected to a crime scene purely by chance if a single locus was used. However, when multiple loci are used, the probability of having the same number of repeats at all loci decreases dramatically. This is the same logic of individuation used in fingerprint analysis and the Bertillon system, but with the advantages that the probabilities of each trait in the population can be accurately calculated and the construction of each profile is not dependent on subjective evaluation. Since all STR loci used for forensic identification are inherited independently, the probabilities for each locus can be multiplied together to obtain a random match probability, the chance of a

randomly selected individual matching a given STR profile. The probabilities of finding each STR variant within different ethnic groups and geographic regions must also be taken into account.

Random match probabilities are usually extremely low, often in the range of one in a billion or trillion. This may be sufficient for estimating the probability of a match between a crime scene sample and a suspect if a single suspect has already been identified. However, these numbers greatly underestimate the probability of finding a match purely by chance if a crime scene profile is searched against a database to find suspects, since a database may include millions of individuals. In 2017, the FBI switched from 13 to 20 STR loci as the standard for CODIS to reduce the probability of finding a match in the database by chance.

As DNA analysis technologies have advanced, we are able to amplify DNA from ever smaller samples, which can complicate the interpretation of results. For example, we may know that a thief touched a doorknob to enter an apartment, but there may be other individuals who touched the doorknob, such as the residents of the apartment. A swab of the doorknob would contain the DNA profiles of multiple individuals. Distinguishing individual profiles within such a complex mixture, or even determining whether a known suspect is present, is extremely difficult and relies on computer programs that apply probabilistic models that may be obscure to those conducting the analysis. Furthermore, DNA found at crime scenes may be degraded or present in very small quantities, meaning that only incomplete profiles can be generated. Having incomplete profiles complicates the analysis and increases the probability that a match will be obtained by chance.

The sensitivity of the latest DNA analysis technology also opens the possibility that an individual's DNA might end up on an object they never touched. We leave minute quantities of DNA behind on everything we touch, and that DNA can be transferred when one object touches another. If the thief in the previous scenario had touched a taxi door handle with the same glove that they used to open the door to the apartment they were robbing, other sources of DNA would end up on the doorknob, such as the DNA from another person who touched the same taxi door handle before the thief. Forensic analysis of DNA will continue to be an important tool for identifying perpetrators of crimes and in exonerating the innocent, but complexities related to increased sensitivity of the tests must be taken into account by the judge and jury in criminal trials to avoid wrongful convictions. DNA evidence must also be interpreted in the context of all other evidence presented. If a man is accused of killing his roommate, it means little that his DNA was found in the apartment he shared with the victim. It would be a different story if his DNA was found in blood under the victim's fingernails. It is important to understand that DNA evidence can only inform that an individual's DNA was present at a crime scene at some point but not necessarily that they committed the crime. Failing to understand this is considered the Prosecutor's Fallacy.

We hope that what you learn about exonerations in this activity inspires you to see science as a force for advancing justice in our society. If you are motivated to take action, please see Lesson 7 of Fred Hutchinson Cancer Center SEPs DNA Exoneration curriculum to develop a student action plan for change.

Pre-lab Questions

1. What role can DNA play in reversing a wrongful conviction?

2. Have you heard of an exoneration case in the news? What was the crime, the initial evidence used in trial, which of the factors described in the introduction contributed to their accusation or conviction?

3. This graph shows that the total number of exonerations per year appears to be increasing. What do you think is contributing to this trend? https://www.law.umich.edu/special/exoneration/Pages/Exoneration-by-Year.aspx

4. False confession is a factor in 12% of cases for which the convicted person is later exonerated. Can you think of a situation where you may have confessed to something you did not do?

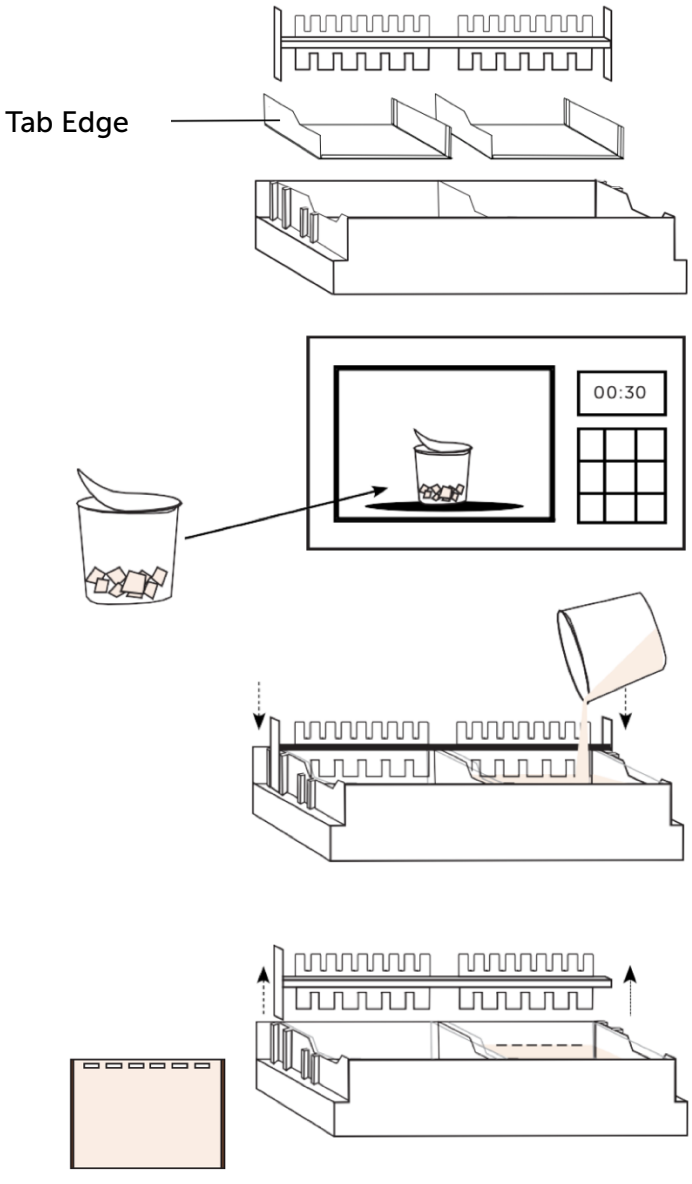
Part I: Electrophoresis

Materials

1 MiniOne® Casting System
1 MiniOne® Electrophoresis System
1 agarose GreenGel™ cup (1 %)
5 DNA samples
1 MiniOne® 500 bp Molecular Weight Marker
TAE running buffer (135 mL)
1 micropipette (2-20µL)
6 pipette tips

Lane #	Sample Name	Volume
1	M	10 µL
2	A	10 µL
3	S1	10 µL
4	S2	10 µL
5	S3	10 µL
6	S4	10 µL

How to Cast a Gel



1. Place the MiniOne® Casting Stand on a level surface and place gel trays in the two cavities. For proper tray orientation place the tab edge of the tray on the left side. Insert the comb into the slots at the top of the casting stand with the 6-well side facing down.

2. **Partially** peel the film off a GreenGel™ cup and microwave for 25-30 seconds. Allow to cool for 15 seconds. DO NOT microwave more than 5 gel cups at a time.

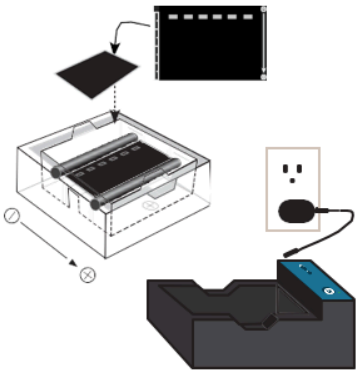
3. One gel cup is for making one agarose gel! Slowly pour the hot agarose solution into a gel tray. Make sure there are no air bubbles in the agarose solution. Let the agarose gel solidify for 10 minutes or until opaque.

   DO NOT disturb the gel until time is up.

4. Carefully remove comb when gel is ready. Remove gel tray with solidified gel from Casting Stand and wipe off any excess agarose from the bottom of the tray.

How to Load a Gel

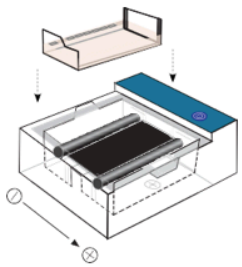
1. Ensure the black viewing platform is in the gel tank. **Make sure the wells are aligned with the marks on the platform on the negative end.**



2. Plug the power supply into the wall and carefully insert the other end into the back of the MiniOne® Carriage.

3. Place the gel tank into the carriage so the carbon electrodes are touching the gold rivets and the tank sits level with the carriage.

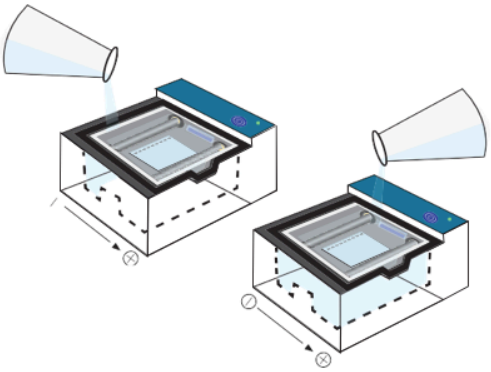
4. Place the gel tray with the gel into the gel tank. The gel tank should not have any buffer in it when putting the gel tray with gel into it.



Note: Place gel tank with the gel on the gel tray into carriage **before** pouring buffer in.

5. Turn the low intensity blue LED on by pressing the button on the carriage.

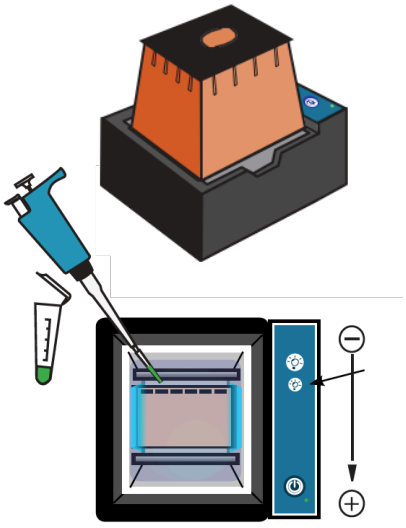
6. Measure 135 mL of TAE running buffer and pour into **one side** of the gel tank. Watch the air push out between the gel tray and viewing platform. Once air has been removed from under the gel tray, pour remaining buffer into the **other side of the gel tank.**



7. Place photo hood on the carriage.

8. Press the power button which should now be a solid green light. If **green light is solid,** turn off the unit and proceed to loading gels.

9. Turn the low intensity blue light on by pressing the button on the carriage to help visualize the wells when loading.

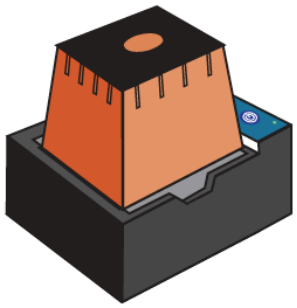


10. Load 10 µL per well. Remember to change pipette tips for each sample. **Load your samples according to the order given in the sample chart.**

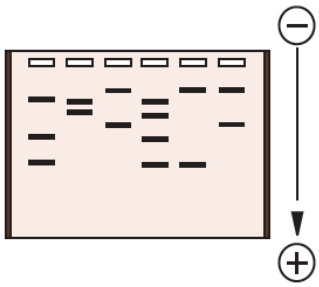
Run, Visualize and Capture Image

1. Once the gel is loaded, do not move it. Make sure the power supply is plugged in and place the photo hood on the carriage. Turn on the unit by pressing the button. The green LED next to the button will turn on.

   The green power LED will not turn on if:
   - **The tank is not properly placed inside the carriage.**
   - **There is no buffer in the tank.**
   - **The buffer is too diluted.**
   - **The photo hood is not on the carriage.**
   - **There is too little running buffer.**
   - **The power supply is not plugged in. Check by turning on the blue LEDs.**
   - **If the green power LED is blinking, please refer to the troubleshooting steps in the MiniOne® Electrophoresis Instruction Manual**

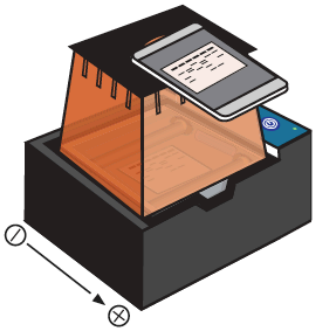


2. Have students periodically check the migration of the bands (~every five minutes).



3. Allow the gel to run **25 - 30 minutes** or until DNA separation is sufficient. Keep in mind small DNA samples run faster so it's important to periodically check where your bands are. After your run is complete, turn off the power by pressing the button. Use the low intensity for viewing during the run. Light will weaken the fluorescent DNA signal.
4. Document your results.

   Wipe off the condensation from the inside of the hood with a soft cloth if necessary, then place the hood back on the carriage. **Turn on** the high intensity light. Place your cell phone or camera directly on the photo hood to take a picture of the DNA. **DO NOT** zoom in as this will result in blurry pictures. (The photo hood is already at the optimal focal length for a smart device.



5. Clean up. Follow teacher's instructions on disposal and clean up.

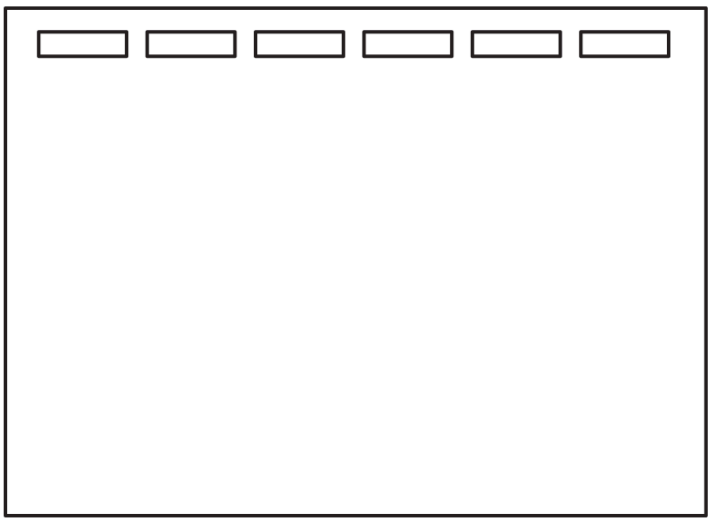
Clean Up

Note: All reagents in this lab can be disposed of as non-hazardous waste.

1. After collecting data and documenting results, remove the photo hood and unplug the power supply from the wall and from the back of the MiniOne® Carriage. Remove the clear running tank from the carriage and remove the gel and tray from the running tank.
2. Pour the used running buffer down the drain or into a waste beaker. Throw the gel away AND SAVE THE GEL TRAYS. Rinse the clear plastic running tank, gel tray, comb, and casting system with DI or distilled water. Allow the tanks to fully air dry before storing.
3. Use a paper towel or Kimwipe™ to gently wipe the gold rivets in the carriage (where the electrodes connect) to ensure all moisture is removed. Wipe up any buffer that may have spilled into the black carriage. Follow any additional directions the instructor gives for clean up and storage.

Part II: Results

What does your gel look like? Record images of the gel in the gel below



Lane 1: _____
Lane 2: _____
Lane 3: _____
Lane 4: _____
Lane 5: _____
Lane 6: _____

Part III : Fragment Length Analysis Worksheet

1. **Estimate fragment sizes:** Using the Molecular Weight Marker 500 bp as a reference, estimate the sizes of the bands of your samples. The MiniOne® 500 bp DNA Ladder has fragment sizes of 10kb, 5kb, 2.5kb, 2kb, 1.5kb, 1kb, and 500 base pairs (bp). Write your **estimated** fragment sizes below for each of your samples.

Step	Sample number	Appellant _____	Sample 1 _____	Sample 2 _____	Sample 3 _____	Sample 4 _____
1	Fragment size 1					
	Fragment size 2					
	Fragment size 3					
	Fragment size 4					
2	STR 1 genotype					
	STR 2 genotype					

2. **Genotype -** The genotype is written as the fragment size on each allele for the 2 different STR's, for example (4004, 2024).

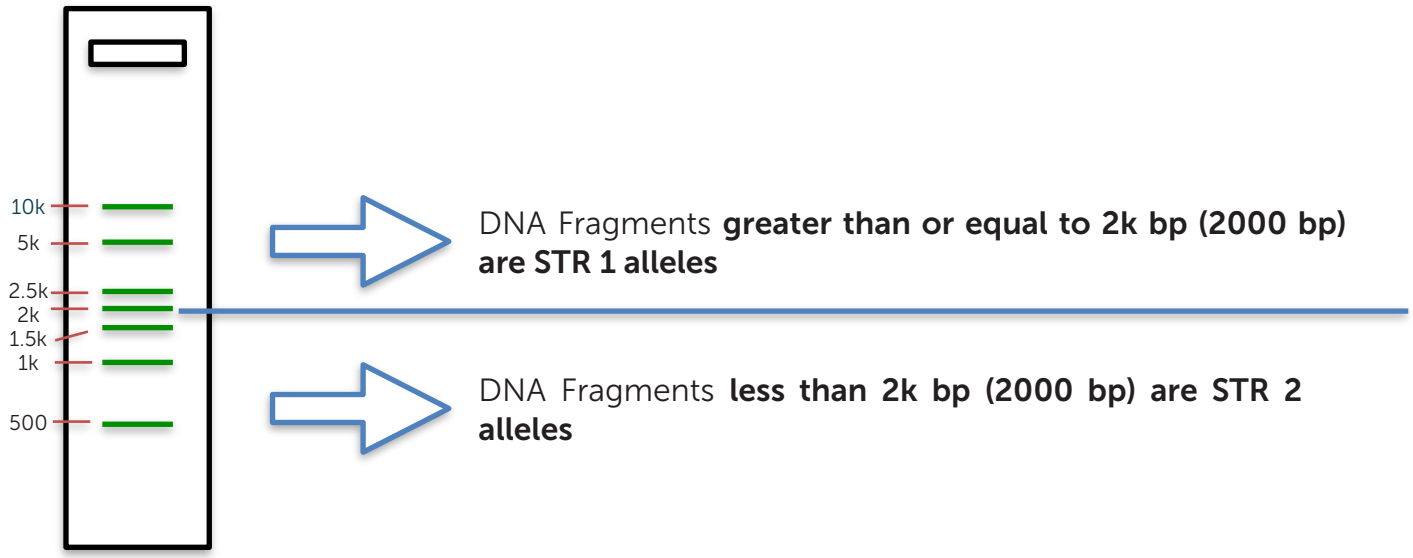


Fig 1. Molecular Weight Marker 500 bp band profile and sizes

3. Based on the DNA evidence, can you determine who was or was not likely to be at the scene of the crime? In combination with the prior evidence of case, what recommendation would you make to appeal the previous conviction?

Post lab analysis questions

1. In the case you examined were you able to determine which individual committed the crime? If so, which pieces of evidence did you find most convincing?

2. In the case you examined, were you able to rule out any individual as the perpetrator? If so, which pieces of evidence did you find most convincing?

3. For the case that you examined, which of the factors mentioned in the introduction may have contributed to wrongful conviction? Provide specific examples from the scenario that you read.

4. After discussing the cases with your classmates, choose one of the cases analyzed by another group. What similarities and differences do you see between the cases?

5. Choose one factor that contributed to a wrongful conviction in either your case or another discussed in class. What policies or reforms related to this factor might reduce the chances of wrongful convictions?

Appendix A - What is Gel Electrophoresis?

Gel electrophoresis is a technique used in many areas of science to analyze the components of complex chemical mixtures. Mixtures of DNA, RNA, proteins, or dyes can be separated into their individual components based on molecular size and electrical charge using a separation matrix within an electric field.

The gel used in gel electrophoresis is a tangle of polymers forming a three-dimensional matrix with water-filled pores through which molecules migrate. A higher density of polymers creates smaller pores. Like the holes in a sieve or colander, the size of the pores has to be the appropriate size for the molecules being separated. Gels can be made from different substances depending on the application. One of the most commonly used and effective materials is agarose, a polymer extracted from seaweed. Agarose gels are formed (or cast) by pouring molten (melted) agarose into a tray where it solidifies into the desired shape as it cools. A comb is placed while the agarose is molten and then removed after it solidifies to create wells where the samples are loaded.

After the gel solidifies it is placed in an electrically conductive buffer between parallel positive ((+) anode) and negative ((-) cathode) electrodes

A voltage is applied between the electrodes, creating a uniform electric field within the gel. Molecules in the wells begin to move under the influence of the electric field: positively charged molecules migrate toward the (-) cathode and negatively charged molecules migrate toward the (+) anode.

The speed of a molecule's movement in an electric field is determined by the strength of its electric charge relative to its molecular weight. This is quantified as the charge to mass ratio. Speed of movement within a gel is also influenced by the size of the molecule relative to the pores in the gel. The polymers in the gel are like an obstacle course: smaller molecules maneuver easily through the pores, traveling faster and farther than large, bulky molecules. However, a large molecule can move faster through a gel than a smaller molecule when the strength of its charge relative to its mass is significantly higher. Shape can also affect how a molecule moves through the gel. Long spaghetti-like molecules will move slower than compact molecules, which slip easily through the pores. Molecules of the same size, shape, and charge will move together and form a distinct band. If multiple types or sizes of molecules are present in the sample, they will separate from each other and each will form a distinct band.

Appendix B - Polymerase Chain Reaction

Polymerase chain reaction (PCR) addresses two major challenges in molecular biology producing usable quantities of DNA for analysis and isolating a specific region of the genome. First, the DNA that scientists want to analyze is often collected in extremely small quantities (for example, a single drop of blood from a crime scene). To have usable quantities of DNA we must make many copies using the original DNA as a template. Second, among the whole genome we must find only the part we want to analyze, whether it is a gene that causes a disease or a sequence that can help identify a species. This is no small matter since the human genome is over 3 billion base pairs (bp) long and we are often interested in regions less than 300 bp long.

The History of PCR

As with many ideas in biotechnology, nature provides us with most of the tools we need. Every time a cell divides it makes two replicates of its entire genome with the help of specialized enzymes. The phenomenon of complementary base pairing should give you a hint as to how a specific region can be targeted. In the late 1970s Frederick Sanger developed a method for copying DNA in vitro that used short pieces of DNA, called primers to initiate replication by a DNA polymerase, similar to the RNA primers in your model of cellular DNA replication. An artificial primer used in the Sanger method has a sequence that allows it to bind at only one location in the target DNA sequence.

Using only one DNA primer, the Sanger method can only produce one copy of the target at a time. In 1983 Kary Mullis proposed a modification to this method where a second primer is used to initiate replication using the first copy as a template. Recall that DNA has an antiparallel structure where one strand runs in the opposite direction relative to the second strand. To use the first copy as a target for replication, the second primer that Mullis added needed to initiate replication in the opposite direction, thus one primer is called the forward primer and the other is called the reverse. With the original sequence and its copy serving as templates, two copies are produced instead of one. Further, these two copies can each be used as templates in a second round of copying, which produces four copies. After each round, or cycle, the number of copies has doubled. Over multiple cycles, billions of copies of the DNA sequence between the two primers are generated. This method is called Polymerase Chain Reaction (PCR) – *polymerase* because of the enzyme that is used to copy DNA and *chain reaction* because the products of one cycle serve as templates for the next round of copying. This rapid and efficient technology for generating usable quantities of DNA won Mullis the Nobel Prize in Chemistry. The first application of PCR was a test for sickle cell anemia.

How PCR Works

Instead of trying to recreate the cell's intricate biochemical machinery in a test tube, scientists rely on heat to control the various steps of the PCR reaction. For DNA to be copied the nucleotide bases must be exposed, which means double stranded DNA must be separated into single strands. Just as heat applied to an ice cube weakens the hydrogen bonds between water molecules and causes a phase transition to liquid water, heat applied to double-stranded DNA weakens hydrogen bonds between bases causing separation of the strands into single-stranded DNA. As with ice, this is sometimes called melting, but is commonly referred to as denaturation.

In a PCR cycle, denaturation is performed at 90-98°C for 5-30 seconds. This temperature is just below the boiling point of water.

Once the template DNA has been separated into single strands, the temperature is lowered to encourage primers to bind to their target sequences. Just as in the water analogy, lower temperature favors the formation of hydrogen bonds between molecules, in this case the primer and the template DNA. This step, called annealing, is typically between 45 and 65°C for 5-30 seconds. The ideal temperature and duration of the annealing step depends on the sequences of the primers being used. It must be low enough that hydrogen bonds are able to form between the primer and specific complementary sequence, but not so low that nonspecific, or random, binding between primer and template occurs.

DNA polymerase binds to the primer-template complex and begins to add nucleotides (dNTPs) onto the 3' end of the primer. This step, called extension, results in a new copy attached to the template as double-stranded DNA. The duration of the extension step depends on the length of the DNA segment being copied and can be anywhere from 5 seconds to 5 minutes.

At this point you may have noticed a problem – DNA polymerase, which the entire PCR process relies on, is a protein enzyme that needs a very specific three-dimensional structure to copy DNA. Before getting to the extension step, the entire PCR mixture, including the polymerase, has already been through the denaturation step where the reaction was heated almost to boiling. Anyone who has cracked an egg into a frying pan will know what high heat does to proteins!

In the early days of PCR, fresh polymerase was added to the reaction tube every cycle to replace the polymerase that had been destroyed by the denaturation step. Having to open the tube and add new enzyme for up to 30 cycles was expensive, inefficient, and increased the chances of contaminating the reaction. The innovation that would remove these obstacles came from an unusual source – Yellowstone National Park.

In the 1970s, scientists had isolated a species of bacteria called Thermus aquaticus from a hot spring in Yellowstone. T. aquaticus thrives at 75- 80°C and can survive much higher temperatures. Its enzymes are similarly heat-tolerant. E. coli, the original source for the polymerase used in PCR, thrives at 37.5°C, the same temperature as the gut of mammals.

Since the biochemistry of DNA is similar across the tree of life, polymerase from T. aquaticus (called Taq Polymerase) can replace E. coli polymerase in PCR, with the modification that the extension step is performed at 70-75°C.

This innovation led to PCR becoming the ubiquitous, inexpensive, and efficient tool that it is today. Reactions can be set up once, sealed inside a tube, and placed in an automated thermal cycler. The thermal cycler (or PCR machine) is a specialized instrument that accurately and rapidly changes the temperature of the tube between denaturation, annealing, and extension. After 20-40 cycles of these three temperatures, billions of copies of the desired DNA product can be produced. The amplified DNA can be analyzed with gel electrophoresis at the end of the experiment or detected as they are being formed using more advanced equipment.

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