# Lab #6 Analysis of PCR Results, The Microscope, Microbe Viewing

Adapted from BIO187, BIO188, CPSC450, and BIO343

In forensics, DNA fingerprinting is used to distinguish between individuals. In Labs 3-4, we learned how to use Restriction Fragment Length Polymorphism [RFLP] analysis. In this lab, you will use the PCR products from Lab 3 in an analysis similar to RFLP and similar to the actual PCR process used in forensics: STR or Short Tandem Repeat sequences.

The human genome contains about 3000 million base pairs, which are distributed among 23 pairs of chromosomes: 22 pairs of homologous chromosomes (= 44 autosomes,) and a pair of 2 sex chromosomes. Each chromosome contains a series of specific genes, and each of a homologous chromosome pair contains alleles for the same genes. These alleles may or may not be the same. However, only 90 million base pairs, about 5%, of the human genome are genes (including introns: non-coding DNA sections within gene sequences.) The other 95% is called non-coding DNA [although it was once called "junk DNA."]

This non-coding DNA is interspersed on chromosomes between genes and within genes. The exact function of the non-coding DNA is not known, although it is thought that non-coding DNA allows for the accumulation of mutations and variations in genomes. These differences and/or mutations in our non-coding DNA are silently passed on to our descendants; we do not notice them because they do not usually affect our phenotypes [although some can cause genetic diseases.] Comparing human genomes, the similarities are striking: less than 0.1 % (about 3 million bases) differs from one person to the other. Non-coding DNA can be useful for DNA fingerprinting in forensics because non-coding DNA often contains differences that can be used to distinguish an individual's DNA. Forensic scientists use these variable regions of DNA as genetic markers to generate a DNA profile of an individual. Because most of your cells contain all of your DNA, samples can come from blood, bones, skin, saliva, or any other body tissues or products.

The human genome contains various repetitive DNA elements located in various spots. One such repetitive element is called the "Alu sequence". This is a DNA sequence, about 300 base pairs long, that is repeated almost 500,000 times throughout the human genome. It is easy to use for our beginner PCR analysis. Other repetitive elements include <u>tandem repeats</u> and <u>short tandem repeats,</u> which are often used in forensics. In this lab you will look at an Alu element in the PV92 region of chromosome 16. This particular Alu element is dimorphic, meaning the element is present in some alleles and not others. The presence or absence of this insert can be detected using PCR (what you did in Lab #3,) followed by gel electrophoresis (today.)

The primers used in the previous lab are designed to bracket a sequence within the PV92 region that is 415 base pairs [bp] long if the non-coding portion does not contain the Alu insertion, or 715 base pairs long if Alu is present. Because each person has 2 homologous chromosomes, each with an allele of the gene, there are three possible patterns for each person on the gel:

what looks like 1 band at 715 bp [because there are two identical 715 bp alleles present,]

what looks like 1 band at 415 bp [because there are two identical 415 bp alleles present,]

OR

2 distinguishable bands: one at 715 bp & the other at 415 bp [because there is one of each allele type present]

If we were using the actual type of PCR used to identify DNA for crime scene purposes, we would be using primers around Short Tandem Repeat [STR] sequences. As with Restriction Fragment Length Polymorphism [RFLP] analysis, any given person may have 1 or 2 bands per "probe type" [or, in the case of PCR, "primer type"] depending on if the person has 2 of the same allele types or 2 different alleles with a given number of repeats of the sequence in question. This is similar to the possible pattern of our Alu results above, except several different primer sets are used so that a combination of several of these patterns results. This presents a much more accurate match pattern than our single Alu pattern, and the STR results are similar to those obtained using several different probes for RFLP in much less time! See the technical information in the Appendix [there may be a quiz question on the Appendix, so be sure to read it!]

<u>More background information from the lecture modified from your Criminalistics text book:</u>

PCR Advantages over RFLP:

- One advantage in moving to shorter DNA strands is that they would be expected to be more stable and less subject to degradation brought about by adverse environmental conditions.
- The long RFLP strands in restriction enzyme analysis tend to readily break apart under the adverse conditions not uncommon at crime scenes.
- PCR also offers the advantage in that it can amplify minute quantities of DNA, thus overcoming the limited sample size problem often associated with crime scene evidence.

STRs:

- The latest method of DNA typing, <u>short tandem repeat</u> (STR) analysis, has emerged as the most successful and widely used DNA profiling procedure.
- STRs are locations on the chromosome that contain short sequences that repeat themselves within the DNA molecule.
- They serve as useful markers for identification because they are found in great abundance throughout the human genome.

STR advantages:

- STRs normally consist of repeating sequences of 3 to 7 bases in length, and the entire strand of an STR is also very short, less than 450 bases in length.
- This means that STRs are much less susceptible to degradation and may often be recovered from bodies or stains that have been subjected to extreme decomposition.
- Also, because of their shortness, STRs are ideal candidates for multiplication by PCR, thus overcoming the previously mentioned limited-sample-size problem often associated with crime-scene evidence.

# Purpose/Objectives:

- To learn a PCR analysis similar to STR analysis
- To perform for yourself some of the procedures and analyses necessary for STR analysis
- To compare "group members" vs. "crime scene" PCR data
- To determine which group members' DNA, if any, matches the "crime scene" DNAs
- Identify and explain the functions of the primary parts of a compound and dissecting microsope
- Use a compound and a dissecting microscope to look at differences between microbes

# Materials

6x loading dye
PCR reactions from lab #3
DNA Molecular Mass Marker "ladder"
Microcentrifuge
Agarose
balance
Graduated cylinder
Compound microscope
Incubated microbe plates from last week
Prepared slides of microbes
GelRed
gel tray
light box
micropipettors
weigh boat
1x TAE buffer
gel boxes & power supply
Dissecting microscope
colored pencils

# Procedures: Wear gloves and goggles for today's exercises

## Part 1. PCR results

### A. Preparing an agarose gel:

1. You gel boxes should have a "built-in" casting stand for pouring the liquid gel into the tray– turn your gel tray in the box so that the open ends are flush against the sides of the box making sure the rubber band is not folded. Add your 10-well comb to the slots on one side (not the middle slots) of the casting tray.

2. To make a standard 1% agarose (1g/100mL), 1X TAE gel in our class:

**a.** Weigh out 0.5 g agarose
**b.** Measure out 50 mL of 1X TAE
**c.** Add both to a 250 mL flask. You can add the powder first, and then rinse the inside of the neck as you pour the liquid in or add the liquid first, your preference. **– Swirl to mix**

3. Heat gel solution for 45-60 sec in the microwave, swirl to mix, and then heat again multiple times for 7-20 sec. until the agarose is completely dissolved.

*Note: FLASK WILL BE HOT – use protection!

**Do NOT let your solution boil over.** You'll have too little volume left for your gel and will need to start over. However, the solution should be pretty homogenous, without any white grains or "slimy-looking" spots. You can microwave a few seconds longer if all the agarose has not melted.

**And remember:** Microwaving solutions can be dangerous! Solutions can "superheat" such that they do not boil over until you bump or swirl the flask. At this point your hand and arm will be prime targets for a **serious burn**. Wear insulated gloves or a "hothand" grippy mitt.

**Wear safety glasses, too.**

4. Cool the flask for a few minutes on your bench, swirling the flask and solution once in a while to prevent the gel from solidifying in the flask. THIS IS AN IMPORTANT STEP. Pouring the gel solution without cooling can crack the gel tray. It is cool enough when you can grasp the flask with your hand and you can hold it there.

5. **Before you pour your gel,** ask your instructor to add the 5µl of GelRed stain to your liquid agarose. Swirl to mix. DNA fragments are visualized by staining the gel with a dye. The dye molecules of the stain we use strongly bind to the DNA fragments and allow DNA to become visible. These visible bands of DNA may then be photographed or sketched. There are two ways to stain your DNA: either post-stain your gel (such as the protocol from the Instructors' Procedures Appendix from last week) or you can add the stain to your precast agarose gel which is what we are doing today.

Note: Post-staining of gels is recommended for superior sensitivity and to eliminate the possibility of dye interference with DNA migration but precasting saves time and waste.

6. Pour the somewhat hot (liquid) gel into the casting tray – make sure your 10-well comb is in place. The liquid gel should be hot, but NOT TOO HOT – you can crack the tray if the gel is too hot when you pour it. That's why the cooling step is important.

Remember not to bump or move your assembly until the gel has solidified and turned an opaque silvery-gray throughout. This will take about 30 minutes.

**7.** While you are waiting for your gel to solidify, your instructor will talk about STRs, the microscopes, microbes, and analyzing your gel results.

## B. Loading and Running an Agarose Gel

1. Remove your PCR reactions from the Lab class rack and put them in a rack to bring them back to your group. Make sure they have thawed completely. If not, warm the tube(s) with your hand and flick gently until no ice is visible.

2. Before you spin your PCR tubes, you must put some PCR tube adapters in each slot of the microfuge. YOU MUST ADD THE ADAPTERS OR THE PCR TUBES WILL GET STUCK IN THE SLOTS. Carefully and briefly spin the PCR reaction tubes in a balanced configuration in the microfuge – you may need to spin them in batches.

3. Prepare your samples for the gel: add 10 µL of loading dye to each PCR reaction and mix gently. BE SURE TO USE THE CORRECT MICROPIPETTOR SET WITHIN ITS LIMITS.

4. Repeat Step 2 with the newly mixed samples.

5. Place the gel, tray and all, into the gel box with the wells at the negative [=black] post end..] Very gently remove the comb if it is still in place (do not damage your wells.)

6. Add 1X TAE electrophoresis buffer to the gel box. You should add enough buffer to fill the box so the gel is covered by the buffer. The boxes may also have "fill line" to indicate where to fill to. You are now ready to load your DNA samples.

7. Using a clean pipet tip on your micropipettor for each sample, load the samples into the wells of the gel provided in the order listed in the chart below. BE SURE TO USE THE CORRECT MICROPIPETTOR SET WITHIN ITS LIMITS. Note in your notebook what you load in your lanes! (cut out the chart below and tape it in your notebook in the data section, noting the name of each GM)

Note: Gently pipet each sample into the well. This solution is heavier than the electrophoresis buffer and will sink to the bottom of the well. You may see a little "mushroom cloud" effects. Try to pipet into the bottom of the well without piercing the bottom with the pipet tip.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Sample | DNA | Tube 1: | Tube 2: | Tube 3: | Tube 4: | Tube 5: | Tube 6: | Tube 7 | Tube 8 |
| Molecular Mass Marker | | GM 1 PCR | GM 2 PCR | GM 3 PCR | GM 4 PCR | CS1 PCR | CS2 PCR | CS3 PCR | Water PCR |
| Load Volume | 10 µL | 20 µL | 20 µL | 20 µL | 20 µL | 10 µL | 10 µL | 10 µL | 20 µL |

Carefully move your power supply and gel box to an out of the way spot on your bench. 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. Make sure the DNA wells are at the – end so they can move toward the + pole. Connect the electrical leads to the power supply in the proper slots. Make sure power supply is plugged in to the outlet.

8. Turn on the power supply. Make sure you are looking at the Volts setting, then set it to 100 V. Electrophorese the samples for at least 45 minutes, or until your instructor says they are finished.

9. While your gel is running, continue with Part 2.

## Part 2. The Microscope

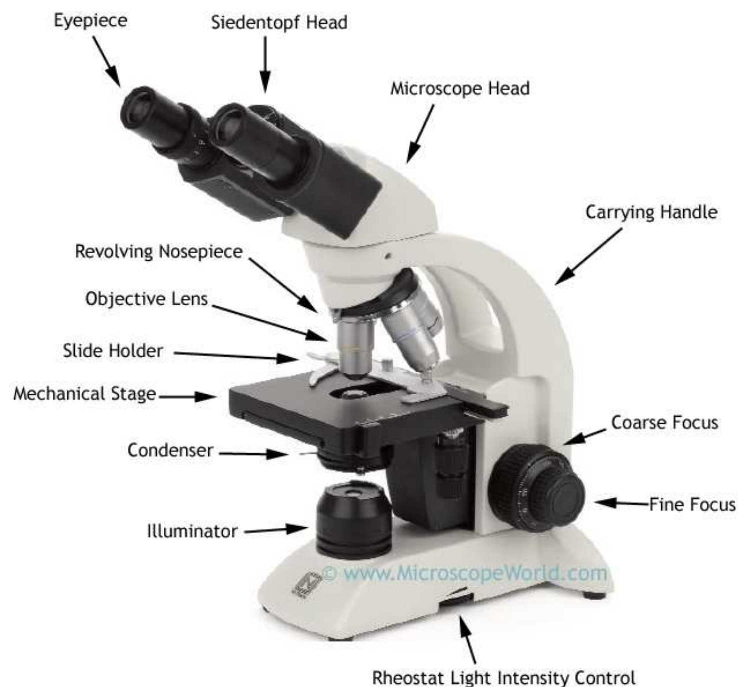
**Many** organisms and biological structures are too small to be seen with the unaided eye. Biologists often use a light microscope to observe such specimens. A light microscope is a coordinated system of lenses arranged to produce an enlarged, focusable image of a specimen. A light microscope magnifies a specimen, meaning that it increases it's apparent size. Magnification with a light microscope is usually accompanied by improved resolution, the ability to distinguish two points as separate points. Thus, the better the resolution, the sharper the image appears. The resolving power of the unaided eye is approximately 0.1 nm (1 in = 25.4 mm), meaning that our eye can distinguish two points 0.1 nm apart. A light microscope, used properly, can improve resolution as much as 1000-fold (i.e., to 0.1 µm).

The ability to discern detail also depends on contrast, the amount of difference between the lightest and darkest parts of an image. Therefore, many specimens examined with a light microscope are stained with artificial dyes that increase contrast and make the specimen more visible.

The invention of the light microscope was profoundly important to biology, because it was used to formulate the cell theory and study biological structure at the cellular level. Light microscopy has revealed a vast new world to the human eye and mind. Today the light microscope is the most fundamental tool of many biologists.

### THE COMPOUND LIGHT MICROSCOPE

Study and learn the parts of the typical compound light microscope shown. A light microscope has two, sometimes three systems: an illuminating system, an imaging system and possibly and viewing and recording system.



### *Illuminating System*

The illuminating system, which concentrates light on the specimen, usually consists of a light source, condenser lens and light diaphragm. The light source is a light bulb located at the base of the microscope. The light source illuminates the specimen by passing light through a thin, almost transparent part of the specimen. The condenser lens, located immediately below the specimen, focuses light from the light source onto the specimen. Just below the condenser is the condenser iris diaphragm, a knurled ring or lever that be opened and closed to regulate the amount of light reaching the specimen. When the condenser iris diaphragm is open, the image will be bright; when closed, the image will be dim.

### *Imaging System*

The imaging system improves resolution and magnifies the image. It consists of the objective and ocular (eyepiece) lenses and a body tube. The objectives are three or four lenses mounted on a revolving nosepiece. Each objective is a series of several lenses that magnify the image, improve resolution, and correct aberrations on the image. The most common configuration for student microscopes includes four objectives: low magnification (4x), medium magnification (10x), high magnification (40x), and oil immersion (100x). Using oil immersion requires special instructions…. **To avoid damaging your microscope, do not use the oil immersion objective for this exercise.**

The magnifying power of each objective is etched on the side of the lens. The ocular is the lens that you look through. Microscopes with one ocular are monocular microscopes and those with two are binocular microscopes. Oculars usually magnify the image ten times. The body tube is a metal casing through which light passes to the oculars. In microscopes with bent body tubes and inclined oculars, the body tube contains mirrors and a prism that redirects light to the oculars. The stage secures the glass slide on which the specimen is mounted.

### *Viewing and Recording System*

The viewing and recording system, if present, converts radiation to a viewable and/or permanent image. The viewing and recording system usually consists of a camera or video screen. Most student microscopes do not have viewing and recording systems.

### *Using a Compound Microscope*

Although the maximum magnification of light microscopes has not increased significantly during the last century, the construction and design of light microscopes have improved the resolution of newer models. For example, built-in light sources have replaced adjustable mirrors in the illuminating system and lenses are made of better glass than they were in the past.

Your lab instructor will review with you the parts of the microscopes, and their functions, you will use in the lab. After familiarizing yourself with the parts of a microscope, you are now ready for some hands-on experience with the instrument.

**PRECAUTIONS IN MICROSCOPE USE:**

1. Carry the microscope by the arm AND the base.
2. Always start your observations with the microscope set at the lowest power.
3. If you use eyeglasses you will need to fold down or remove the rubber eyecups.
4. Use the fine focus knob ONLY once you are on an objective of 20X or higher or you risk cracking the slide.
5. **Do not** spill liquids on the microscope (Avoid this by removing excess liquid from slides you prepare using the paper provided)
6. **Do NOT** use the oil immersion lens [this is usually the highest magnification objective and will look slightly different to the others.] The oil immersion lens often has oil left on it from past classes and will get this oil all over your slide and/or will crack the slide.
7. Always place the microscope on the lowest power when you are finished.
8. Wrap up the cord when you are finished using the microscope
9. Always replace dust cover when finished

**Steps to use in getting your microscope ready for use:**

1. Place your microscope on the lab bench and plug it in
2. Turn on the microscope with the switch located at the back near the power cord
3. Set the light setting (Red Knob) to the lowest setting
4. Fully open the aperture diaphragm of the condenser by sliding the control to the extreme left (this is located just under the mechanical stage in the front)
5. Make sure the shortest [lowest magnification (usually 4X or 10X)] objective is in place
6. Using the sub-stage condenser focusing knob, raise the condenser to the top of its excursion
7. **Pull out the slider with your thumb, carefully place the specimen slide against the back of the slide space, and carefully ease back the slider with your thumb**
8. Use the black knobs under the stage to move the slide around until you think the specimen will be in the middle of your view
9. Using the coarse adjustment knob bring the specimen into view. Then using the fine adjustment brings the specimen into sharp focus

10. You may need to adjust the condenser and or the illumination controls to get a better look at the specimen

11. Move the object of interest to the center of your view using the black knobs under the stage

**12.** Carefully change objectives to the next higher magnification, being careful not to crack the slide or damage the specimen. **DO NOT use the oil immersion objective.**

**Procedure: Determine magnification:**

1. Calculate and record in a Table the total magnification for each objective using the following formula:

$$Mag_{Tot} = Mag_{Obj} \times Mag_{Ocu}$$

$Mag_{Tot}$ = total magnification of the image

$Mag_{Obj}$ = magnification of the objective lens

$Mag_{Ocu}$ = magnification of the ocular lens

For example, if you are viewing the specimen with a 4x objective and a 10x ocular, the total magnification of the image is 4 x 10 = 40x. The specimen appears 40 times larger than it actually is.

**Procedure: View prepared slides of microbes**

1. One at a time, obtain **at least 3** prepared slides of DIFFERENT microbes from the slides in your blue box. Use the compound microscope to observe the slides. **Always start with the lowest objective (also called the scanning objective) to find the specimen and center it because the lowest objective has the largest field of view, then change to a higher magnification to see more detail.** Once you get close to the slide with coarse focus, you should only move the fine focus knob [the inner knob of the side focus knobs].

2. Sketch in your notebook what you see under a higher magnification for each of the 3 slides.

3. Label the sketches with what's on the slide label and with the magnification level. NOTE – you don't need to be an artist, but you must observe the microbes FIRST HAND at a microscope. You need to SIGN EACH SKETCH to affirm that the sketch is your original work from your own observations of the subject.

**THE DISSECTING MICROSCOPE**

The dissecting microscope offers some advantages over a compound microscope. Although a compound microscope can produce high magnifications and excellent resolution, it has a small working distance, the distance between the objective and the specimen. Therefore, it is difficult to manipulate a specimen while

observing it with a compound microscope. Specimens that can be observed with a compound microscope are limited to those thin enough for light to pass through them. In contrast, a dissecting microscope is used to view objects that are opaque or too large to see with a compound microscope.

A dissecting microscope provides a much larger working distance than does a compound microscope. This distance is usually several centimeters, making it possible to dissect and manipulate most specimens. Also, most specimens for dissection are too thick to observe with transmitted light from a light source below the specimen. Therefore, many dissecting microscopes use a light source above the specimen; the image is formed from reflective light.

Dissecting microscopes are always binocular. Each ocular views the specimen at different angles through one or more objective lenses. This arrangement provides a three-dimensional image within a large depth of field. This is in contrast to the image in a compound microscope, which is basically two-dimensional. However, the advantages of a stereoscopic microscope are often offset by lower resolution and magnification than a compound microscope. Most dissecting microscopes have magnifications of 4x to 50x.

**Procedure: Using the dissecting microscope**:

1. Use your dissecting microscope to examine the microbes on your own plates. DO NOT OPEN YOUR PLATES – we have no idea what you grew!! Sketch 3 **different** colonies from the microbes on yours or one of your group member's plate. Different colonies will look differently – their shape, size, color, etc all indicates a different type of microbe. Sketch on the best magnification for what you are drawing.

2. Make sure to label your drawing and include the magnification. Don't forget to sign your drawings.

3. When you are finished, place your microbe plates in the biohazard bag.

**Question 1:** Explain why the specimen must be centered in the field of view on low power before going to high power.

**Question 2:** A microscope has a 20x ocular and two objectives of 10x and 43x respectively: SHOW your formula AND ALL OF YOUR WORK!

a. Calculate the low power magnification of this microscope.

b. Calculate the high power magnification of this microscope.

**Question 3:** Indicate and describe a major way the stereomicroscope (dissecting) differs from the compound light microscope in terms of its use.

**Continuation of Part 1:**
**C. Viewing your gel**

1. When electrophoresis is complete, turn off the power and remove the lid.

2. Carefully remove the gel tray and the gel from the box. Be careful, the gel is very slippery.

3. Carefully nudge the gel off the gel tray with your thumb or the spatula and carefully slide it into your small plastic staining tray.

4. Once you have removed the gel from the electrophoresis chamber, you will examine the gel for expected DNA bands. The bands may appear fuzzy immediately at first, but will begin to develop into sharper bands within 5–15 min after removal from the chamber.

5. Viewing your gel: You can place your gel directly in the light box (lift the lid, put the gel on the box then close the lid to view). Record your observations by making a sketch of your gel in your notebook and noting which group member matches which crime scene.

6. You will also receive a picture of an "ideal gel" from your instructor. Make sure to tape it in your notebook and analyze the results of this one as well.

7. When you are done with your gel, place it in the Ziploc bag for waste located in the hood and pour your buffer from the electrophoresis box into the waste jug also located in the hood.

**Question 4:** Which group member matched which crime scene DNA profile?

**Question 5:** How does having only 1 set of band patterns affect your ability to narrow down a suspect?

**Part 3. Clean Up**
Remember to dispose of waste in the proper containers. Remember to have your instructor sign your notebook. Did you answer all the questions?