Name_____Period_____Assignment #_____

DNA Fingerprinting Using Enzymes (Kit #225)

See Lecture Questions 129, and 131-133

Prelab: Annotate all paragraphs and answer questions 1-11

Annotating Text
☐ **UNDERLINE** concepts you think might be useful for understanding or solving the problem
☐ **Box** information you think might be helpful for designing your investigation
☐ ← Write **notes** in the left margin
☐ → Write **questions and answers** in the right margin
Each paragraph (including each step of the procedures) must have something underlined or boxed, **AND** have something written in the margins (a question and/or note).

Restriction Enzymes

One of the most significant discoveries of molecular biology is a class of enzymes know as restriction endonucleases. These endonucleases (also known as restriction enzymes) are produced by many species of bacteria to protect themselves from invading viral DNA. Restriction enzymes act like molecular scissors, cutting double stranded DNA at specific sequences. The utility of restriction enzymes has made molecular cloning, DNA mapping, sequencing and various genome-wide studies possible, launching the era of biotechnology

Restriction Enzyme	Genus	Species	Strain	Recognition Site
Ava I	*Anabaena*	*variablis*	*n/a*	*C^YCGUG*
Bgl I	*Bacillus*	*globigii*	*n/a*	*GCCNNNN^NGGC*
EcoRI	*Escherichia*	*coli*	*RY 13*	*G^AATTC*
HaeIII	*Haemophilus*	*aegyptius*	*n/a*	*GG^CC*
HindIII	*Haemophilus*	*influenzae*	R_d	*A^AGCTT*
Sac I	*Streptomyces*	*achromogenes*	*n/a*	*GAGCT^C*

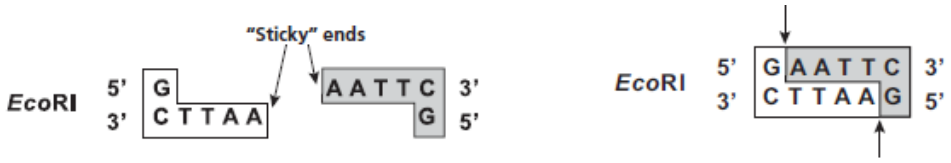
Table 1: Common Restriction Enzymes with Recognition Sites

Since they were first discovered in the 1970s, over 3,000 restriction enzymes have been identified, each one given a unique acronym describing the organism from which it was first isolated. The first letter of the acronym is the first letter of the genus, the next two letters are the first two letters of the species name of the organism, and additional letters and numerals indicate specific strains and order of discovery. For

example, *EcoRI* was the first restriction enzyme isolated from the *RY13* strain of the bacterium *Escherichia coli*.

Many restriction enzymes require Mg^{2+} for activity and recognize palindromic stretches of DNA, generally 4-8 base pairs in length. The probability that a given enzyme will cut, or "digest", a piece of DNA is directly proportional to the length of its recognitions site. Statistically, an enzyme will average one cut for every 4^{th} base pairs, where *n* is the length of the recognition site. For instance, an enzyme that recognizes a four base pairs long sequence (e.g., *HaeIII*) will cut DNA once every 256 (or 4^4) base pairs, while an enzyme that recognizes a six base pairs long site (e.g., *EcoRI*) will cut one every 4096 (or 4^6) base pairs. Therefore, the longer a DNA molecule is, the greater the probability is that it contains one of more restriction sites. For example, if *EcoRI* is used to digest human chromosomal DNA containing 3 Billion base pairs and a plasmid containing 5,000 base pairs, it will cut the chromosomal DNA over 700,000 times (3 billion base pairs, cut every 4096 base pairs), but may only cut the plasmid once (5,000 base pairs, cut every 4096 base pairs).

Digestion by a restriction enzyme generates DNA fragments with one of two types of DNA ends: "sticky" or "blunt". To illustrate this, first consider the recognition site and cleavage pattern of *EcoRI*.



EcoRI cleaves between the G and neighboring A, as indicated by the arrows in the left side of the figure. It is important to note that the positions of the cleavage are staggered, so the resulting fragments project short overhangs of single-stranded DNA with complementary sequences. Such overhangs are referred to as "sticky" ends because the single-strands can interact with –or stick to-other overhangs with a complementary sequence (Figure 1). Digestion of the same piece of DNA using different enzymes can produce sticky ends of different lengths and strand orientation (5' vs 3').

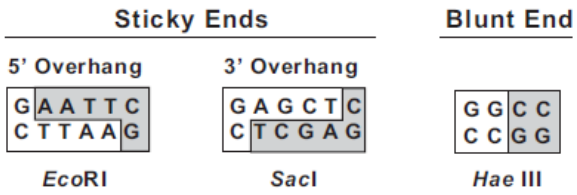
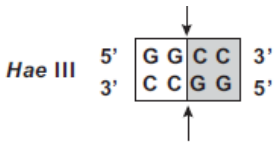
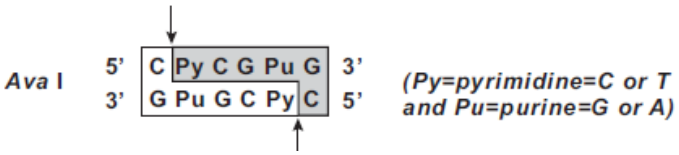


Figure 1: Different types of DNA ends produced by Restriction Enzymes.

In contrast to *EcoRI*, *HaeIII* cuts both DNA strands at the same position, which generates fragments without an overhang. These so-called 'blunt' ends can be joined with any other blunt end without regard for complementarity.



Some restriction enzymes, such as *AvaI*, recognize "degenerate" sites, which contain one or more variable positions.



Consequently, there are four possible sites that *AvaI* will recognize and cut: CCCGGG, CCCGAG, CTCGGG, and CTCGAG.

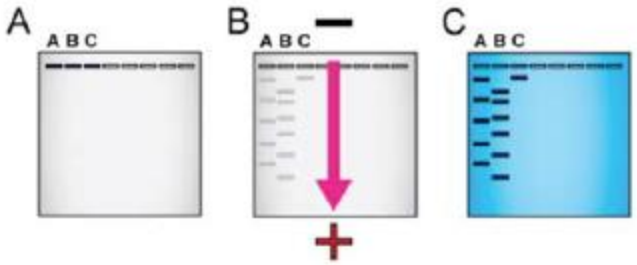
Depending on the distances between recognition sites, digestion of DNA by a restriction enzyme will produce DNA fragments of varying lengths. In order to analyze such a mixture of DNA fragments, scientists use a technique called agarose gel electrophoresis.

Agarose Gel Electrophoresis

Agarose gel electrophoresis separates DNA fragments according to size. First, DNA molecules are added into depressions (or 'wells') within a gel (Figure A), and then an electrical current is passed through the gel. Because the sugar-phosphate backbone of DNA has a strong negative charge, the current drives the restriction fragments through the gel toward the positive electrode (Figure B).

At first glance, an agarose gel appears to be a solid at room temperature, but on the molecular level, the gel contains small channels through which the DNA can pass. Small DNA fragments move through these holes easily, but large DNA fragments have a more difficult time squeezing through the tunnels. Because molecules with dissimilar sizes travel at different speeds, they become separated and form discrete "bans" within the gel. After the current is stopped, the bands can be visualized using a stain that sticks to DNA (Figure C).

While electrophoresis is a powerful separation technique, it is not without its technical limitations. Most significantly, if two different fragments share a similar size, they will migrate together through the gel and may appear as a single band. In addition, if digestion results in a broad distribution of DNA sizes, the fragments may stain as a smear. Lastly, DNA with a streamlined secondary structure (such as supercoiled DNA) can pass though the gel more quickly than similarly sized linear DNA, which prevents an accurate comparison of size.



Calculating the sizes of Restriction Fragment Length Polymorphisms

Mathematical formulas have been developed for describing the relationship between the molecular weight of a DNA fragment and its mobility (i.e., how far it migrates in the gel). In general, DNA fragments, like the ones in your evidence samples, migrate at rates inversely proportional to the $\log_{10}$ of their molecular weights. For simplicity's sake, base pair length (bp) is substituted for molecular weight when determining the size of DNA fragments. Thus, the size in base pair length of a DNA fragment can be calculated using the distance the fragment travels through the gel. To calculate the base pair length, a DNA standard, composed of DNA fragments of known base pair length, is run on the same gel as the unknown fragments and is then used to create a standard curve. The standard curve, in this case a straight line, is created by graphing the distance each fragment traveled though the gel versus the $\log_{10}$ of its base pair length.

Southern Blot Analysis

Restriction fragment length polymorphism (RFLP) analysis of genomic DNA is facilitated by Southern blot analysis. After electrophoresis, DNA fragments in the gel are denatured by soaking in an alkali solution. This causes double-stranded fragments to be converted into single-strands (no longer base-paired in a double helix). A replica of the electrophoretic pattern of DNA fragments in the gel is made by transferring (blotting) them to a sheet of nitrocellulose or nylon membrane (Figure 3). This is done by placing the membrane on the gel after electrophoresis and transferring DNA fragments to the membrane by capillary action or electrotransfer. DNA, which is not visible, becomes permanently absorbed to the membrane, which can then be manipulated easier than gels.

Analysis of the blot DNA is done by hybridization with a labeled oligonucleotide DNA probe. The probe is a DNA fragment that contains base sequences that are complementary to the variable arrays of tandemly repeated sequences found in the human chromosomes. Probes can be labeled with reporter molecules that are used for detection. A solution continuing the singe-stranded probe is incubated with the membrane containing the blotted, single-stranded (denatured) DNA fragments. Under the proper conditions, the probe will only base pair (hybridize) to those fragments containing the complementary sequences. The membrane is then washed to remove excess probe. Only DNA fragments that are hybridized to the probe will reveal their positions on the membrane. If the probes are isotopically labeled, the hybridized fragments will appear as discrete bands (fingerprint) on the film, and are in the same relative positions as they were in the agarose gel after electrophoresis. Only specific DNA fragments of the hundreds of thousands of fragments present, will hybridize with the probe because of the selective nature of the hybridization process.

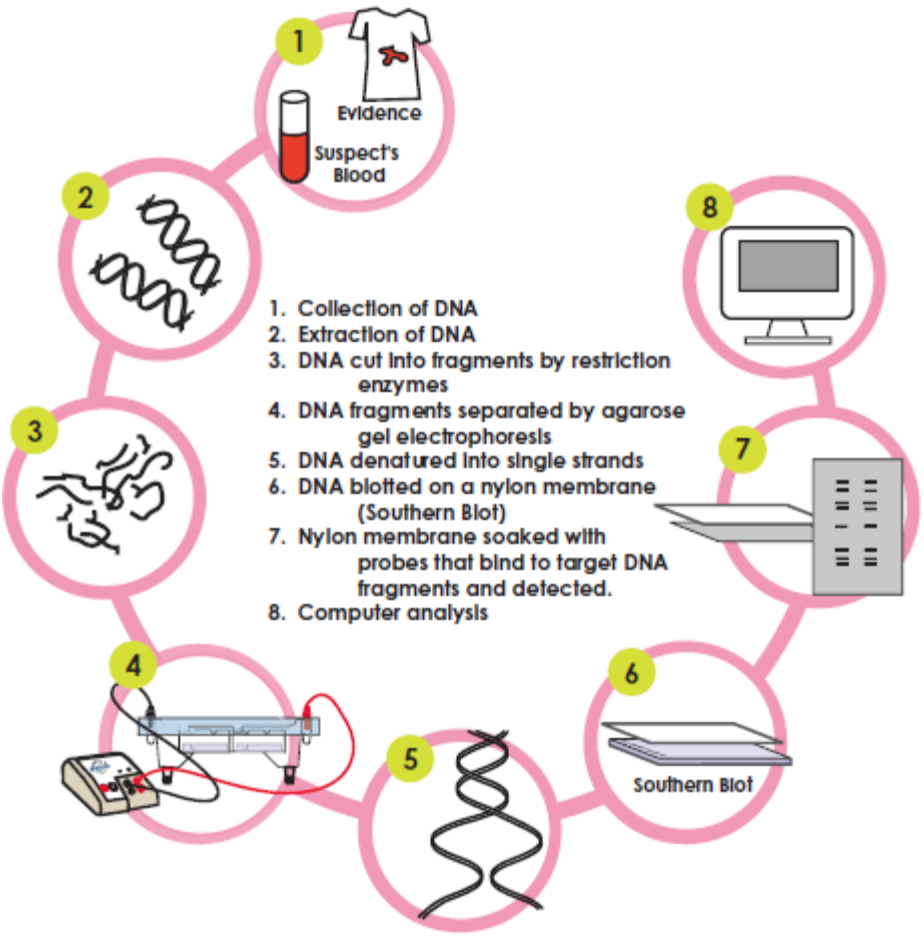


Figure 3: DNA Fingerprinting using RFLP and Southern blot analysis.

In forensic analysis, DNA samples can be extracted and purified from specimens of skin, blood stains, semen, or hair roots collected at the crime scene. RFLP analysis performed on these samples is then compared to those performed on samples obtained from the suspect. If RFLP analyses performs on these samples is then compared to those performed on samples obtained from the suspect. If RFLP patterns match, it is beyond reasonable doubt that the suspect (or biological material from the suspect, such as blood) was at the crime scene. In forensic DNA fingerprinting, different sets of probes hybridized to different types of repetitious sequences are used in DNA profile analysis in order to satisfy certain statistical criteria for positive identification.

DNA Fingerprinting Using Polymerase Chain Reaction (PCR)

RFLP-based DNA fingerprinting analysis has been overtaken by the Polymerase Chain Reaction (PCR) because of two important advantages. The first is the sensitivity of PCR, which allows for DNA fingerprinting identification using much smaller amounts of DNA since PCR amplifies DNA. A second advantage is the speed of PCR analysis, which allows critical questions to be answered more quickly as compared to Southern Blot analysis.

PCR amplification requires the use of thermostable DNA polymerase, such as *Taq* polymerase, Purified form a bacterium known as *Thermus aquaticus* that inhabits hot springs. *Taq* polymerase is commonly used in PCR because it remains stable at near-

boiling temperatures. Also included in the PCR reaction are the four deoxynucleotides (dATP, dCTP, dGTP, and dTTP) and two synthetic oligonucleotides, typically 15-30 base pairs in length, known as "primers". These components, together with the DNA to be amplified are incubated in an appropriate buffer that contains Mg^{2+}. The primers are designed to correspond to the start and end of the DNA to be amplified, known as the "target".

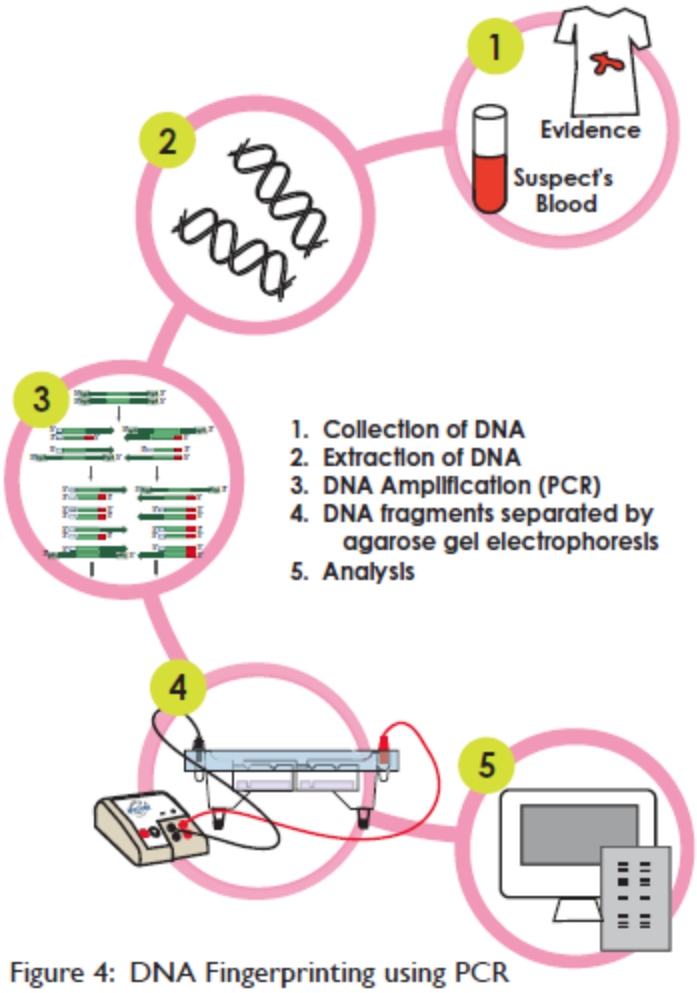


Figure 4: DNA Fingerprinting using PCR

The PCR reaction mixture (which contains the DNA polymerase, buffer, deoxynucleotide, primers, and templet) is subjected to sequential heating/cooling cycles at three different temperatures (Figure 5).

- In the first step, the template is heated to near boiling (92°-96°C) to denature or "melt" the DNA. This step, known as "denaturation" disrupts the hydrogen bonds between the two complimentary DNA strands and causes their separation.
- In the second PCR step, the mixture is cooled to a temperature that is typically in the range of 45°-65°C. In this step, known as "annealing", the primers (present in great excess to the template) bind to the separated DNA strands.
- In the PCR step, known as "extension", the temperature is raised to an intermediate value, usually 72°C. At this temperature the *Taq* polymerase is maximally active and rapidly adds nucleotides to the primers to complete the synthesis of the new complimentary strands.

DNA fingerprinting analysis has become increasingly significant in court cases involving murder, rape, physical battery, and other types of crimes. Jurors are often asked to determine the validity of DNA evidence, resulting in both acquittals and convictions of suspected criminals. To ensure greater accuracy, scientists have incorporated standardization procedures in DNA analysis. DNA Standard Markers are used to determine the exact size of individual DNA fragments in a DNA fingerprint. It is generally accepted that DNA fingerprints are identical only in the case of identical twins.

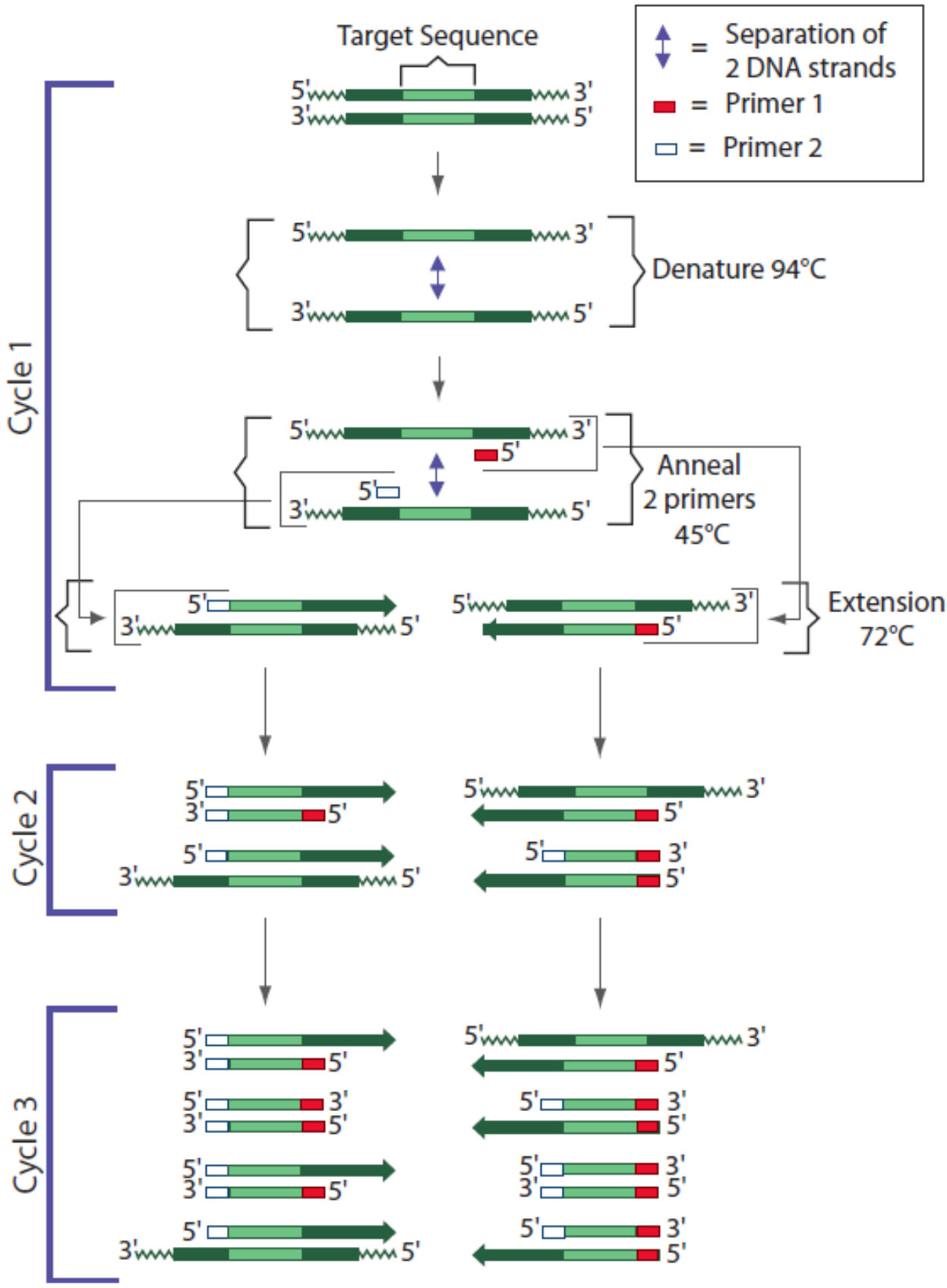


Figure 5: The Polymerase Chain Reaction

1) What is the sequence of the complementary DNA strand? Draw it directly below the strand.

5'-AAAGTCGCTGGAATTCACTGCATCGAATTCCCGGGGCTATATATGGAATTCGA-3'

2) Assume you cut the fragment above with the restriction enzyme *EcoRI*. The restriction site for *EcoRI* is 5'-GAATTC-3', and the enzyme makes a staggered ("sticky end") cut between G and A on both strands of the DNA molecule. Based on this information, show how the DNA fragment is cut by *EcoRI* on the DNA strand in the previous question, and draw the resulting products below.

3) How would the RFLP patterns of identical twins cut with the same restriction enzymes compare to fraternal twins and to complete strangers?

4) Why do DNA fragments migrate through the gel from the negatively charged pole to the positively charged pole?

5) Using the ideal gel shown in Figure 5, measure the distance (in mm) that each fragment migrated from the origin (the well). For consistency, measure from the front end of each well to the front edge of each band (the edge farthest from the well).

   Enter the measured distances into the table below

6) Use the standard curve below to determine the base pair length of each band and record in the table below.

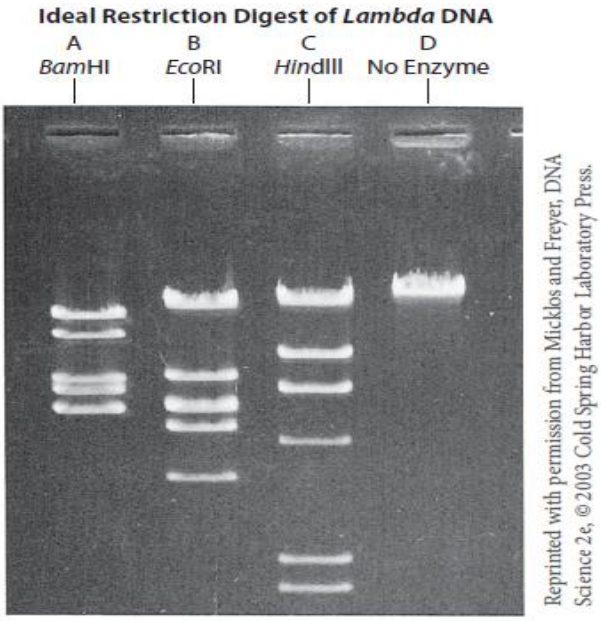
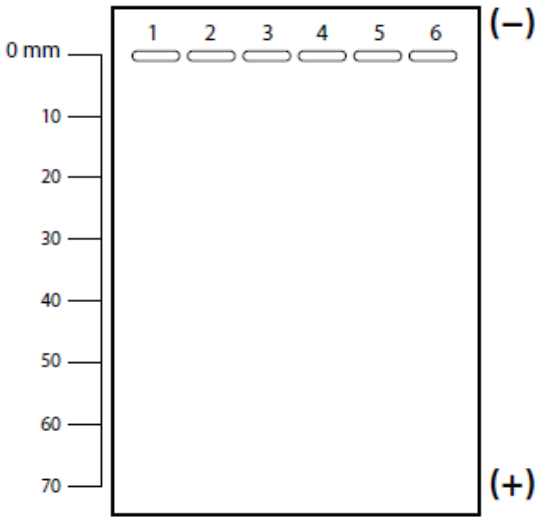


Figure 5. Ideal Gel

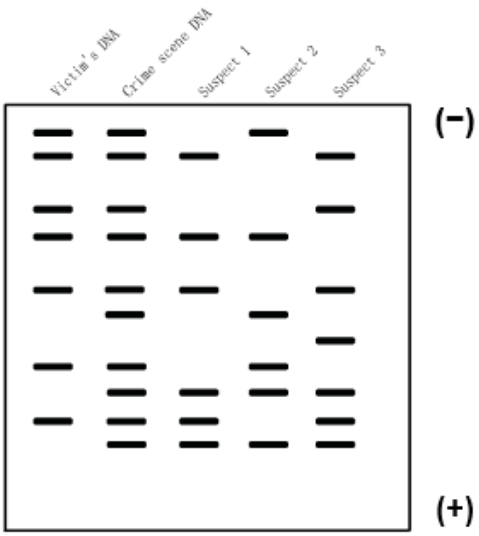
HindIII		BamHi		EcoRI	
Distance Traveled	**Base Pair Length**	**Distance Traveled**	**Base Pair Length**	**Distance Traveled**	**Base Pair Length**

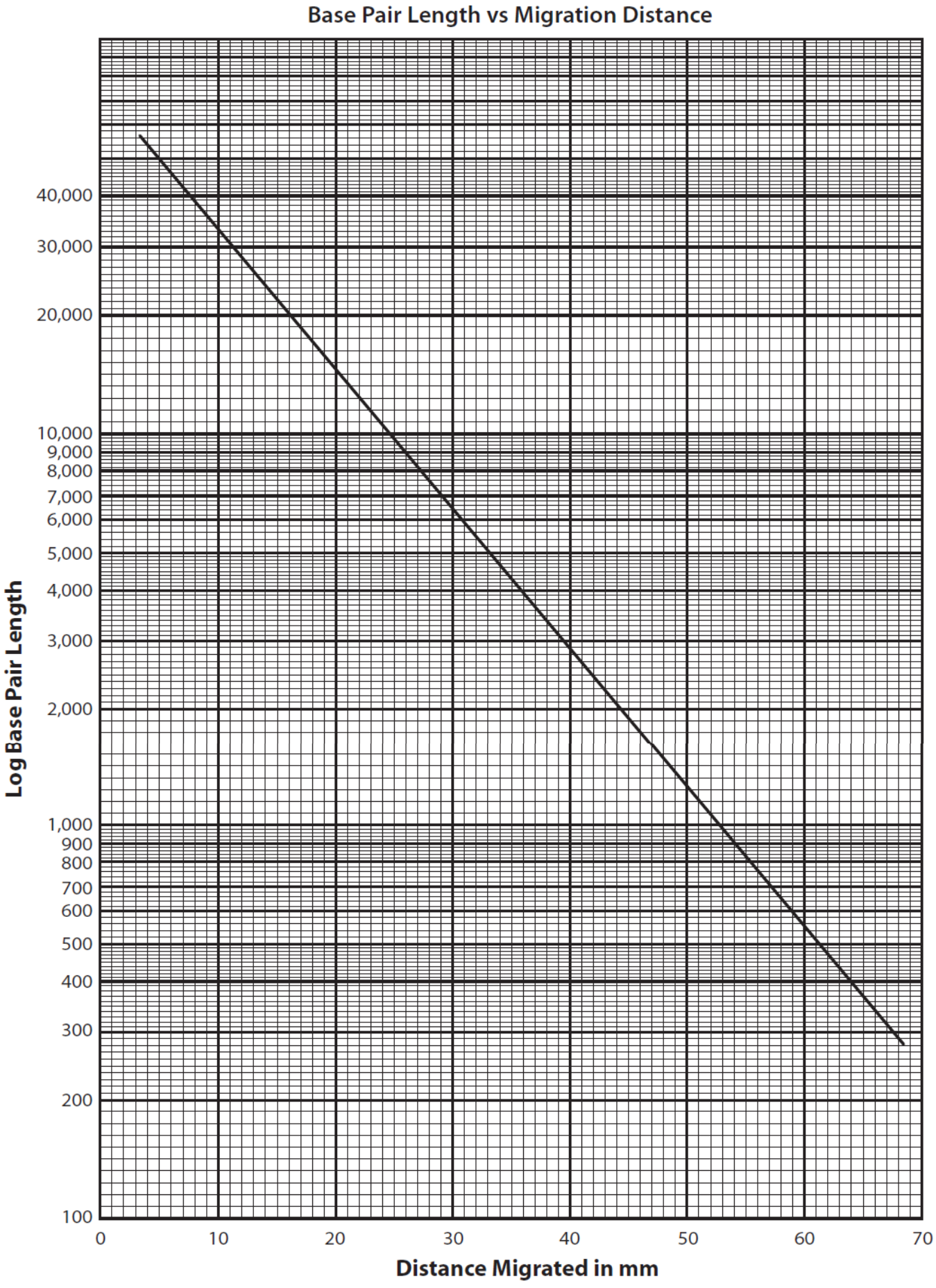
7) Suppose a restriction enzyme digest resulted in the following sized fragments: 8,700 bp; 9,500 bp; 10,400 bp, 4,500 bp; and 6,700 bp. Illustrate the resulting banding pattern on the gel to the right. Use the standard curve below to determine the migration distance.



8) Below is an illustration of the DNA fingerprint of a victim of murder, blood found at the scene of the crime, and the DNA fingerprints of 2 suspects. Was either of the suspects at the scene of the crime? If so, which suspect?

9) Justify your answer to the last question. A justification has 3 component: 1) Scientific knowledge and/or theory; 2) specific data from your analysis related to the knowledge; and 3) and explanation of HOW the data supports the knowledge.




Base Pair Length vs Migration Distance
Log Base Pair Length
40,000
30,000
20,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
900
800
700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
Distance Migrated in mm

10) What is the function of each of the following in gel electrophoresis?
    a. Agarose gel

    b. Running buffer

    c. Wells in the gel

    d. Electric current

11) How does electrophoresis sort different sized DNA fragments?

LABORATORY SAFETY GUIDELINES:

1. Wear gloves and goggles while working in the laboratory.
2. Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Always wash hands thoroughly with soap and water after working in the laboratory.
5. If you are unsure of something, ASK YOUR INSTRUCTOR!

Day One: Pour gels and practice loading wells

1. Tape the ends of 2 casting trays like the model
2. Place the casting tray on a level surface. Position a well template (or "comb") into one set of notches, near the end of the tray. Make sure the comb is secure and rests evenly across the tray.
3. Pour gel so that it covers most of the teeth of the comb (see model).

*****IMPORTANT*****

Agarose gel solidifies quickly! Return unused gel to the hot water bath ASAP

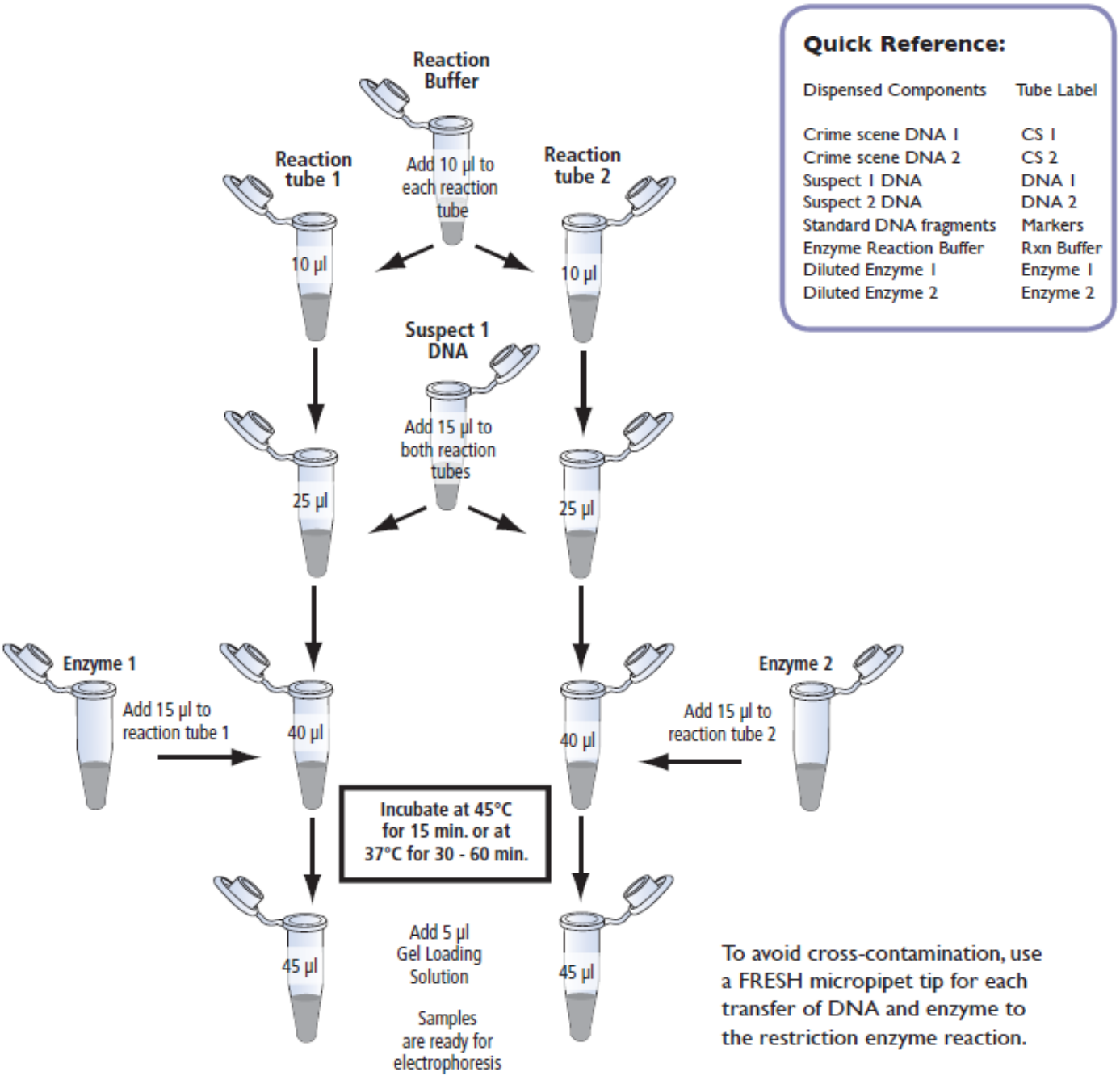
4. Wait 15-20 minutes. The gel will be less transparent when it has solidified.
5. Remove comb and tape, place in electrophoresis chamber, and cover with buffering solution. Re-watch demonstration video if necessary (**http://www.youtube.com/watch?v=tTj8p05jAFM**).
6. Practice loading wells with practice loading dye.
7. Close the lid of the electrophoresis chamber and connect the electrodes. Run for a few minutes to ensure equipment is functioning.
8. Remove lid and discard gel. Save the buffering solution.

9. Place the other casting tray with solidified gel in the electrophoresis chamber. Ensure the gel is covered with buffering solution and cover with tin foil for storage in refrigerator overnight.
10. Do not replace the lid. Leave it on your lab station

Crime Scene Investigation – Restriction Enzyme Digestion

In this experiment, emphasis is placed on concepts related to RFLP analysis. The experiment activities will focus on the identification of DNA by analyzing restriction fragmentation patterns separated by agarose gel electrophoresis. The DNA from two suspects are each digested with two restriction enzymes in separate reactions and compared to crime scene samples after agarose gel electrophoresis. This flow chart outlines the procedure used for the restriction enzyme digestion of DNA obtained from Suspect 1. The DNA from Suspect 2 is digested in the same manner, using reaction tubes 3 and 4 (not shown).

Day 2: Crime scene Investigation – Restriction Enzyme Digestion



1. Label microtest tubes 1 through 4 for four restriction enzyme digestion reactions. Put your initials or group number on the tubes.

2. Tap all the tubes on the lab bench to collect all the contents at the bottom of the tube.

3. Use an automatic micropipet to dispense 10 µl of Enzyme Reaction Buffer (Rxn Buffer) to each of four reaction tubes labeled 1 through 4.

4. Add DNA and enzyme to the reaction tubes as summarized in Chart 1. Use a FRESH micropipet tip for each transfer of DNA and enzyme.

Chart I: Summary of Restriction Enzyme Digestion Reactions

	Reaction Tube	Reaction Buffer	DNA 1 (µl)	DNA 2 (µl)	Enzyme 1 (µl)	Enzyme 2 (µl)	Final Volume (µl)
Crime Scene Samples	Crime Scene DNA, cut with enzyme 1 ready for electrophoresis				X	--	45 *
	Crime Scene DNA, cut with enzyme 2 ready for electrophoresis				--	X	45 *
Suspect 1	1	10	15	--	15	--	40
	2	10	15	--	--	15	40
Suspect 2	3	10	--	15	15	--	40
	4	10	--	15	--	15	40

* 10x Gel loading solution has already been added to the crime scene samples.

5. Cap the reaction tubes and tap gently to mix. Then tap each tube on the lab bench to collect contents at the bottom.

6. Incubate reaction tubes in a 45°C waterbath for 15 minutes; or in a 37°C waterbath for 30 - 60 minutes.

Chart II: Options for Restriction Enzyme Incubation

Waterbath Temperature	Incubation Time
45°C	15 min.
37°C	30-60 min.

After the incubation is completed:

7. Add 5 µl of 10x gel loading solution to reaction tubes 1 - 4 to stop the reactions. Cap and mix by tapping.

OPTIONAL STOPPING POINT

After addition of 10x gel loading solution to stop the reaction, samples are ready for electrophoresis. Samples may be stored in the refrigerator for electrophoresis.

Day 3: Electrophoresis

Load the Samples:

1. Load 40 µL of each of the DNA samples
2. Keep track of what sample is in each well in the results section below

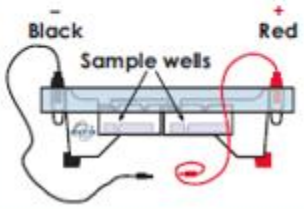
Run the Gel:

3. Close the lid of the electrophoresis chamber and connect the electrodes. Set the power source to 100v and run gel for approximately 30 minutes
4. Check that the current is flowing correctly. The DNA samples will migrate towards the positive (red) electrode during electrophoresis

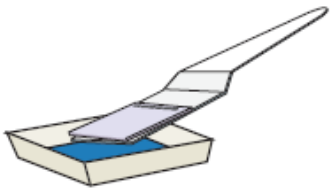
5. Turn off the power supply as soon as the gel has finished running.

Reminders:

During electrophoresis, the DNA samples migrate through the agarose gel towards the positive electrode. Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

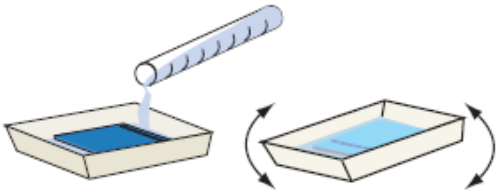


Staining and Destaining



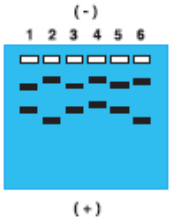
1. Remove the agarose gel from its bed and completely submerse the gel in a small, clean weigh boat or lid from pipet tip rack containing 75 ml of 1x FlashBlue™ stain. Add additional stain if needed to completely submerge the gel.
2. Stain the gel for 5 minutes.

   Note: Staining the gel for longer than 5 minutes will necessitate an extended destaining time. Frequent changes of distilled water will expedite the process.



3. Transfer the gel to another small tray and fill it with 250 - 300 ml of distilled water.
4. Gently agitate the tray every few minutes. Alternatively, place it on a shaking platform.
5. Destain the gel for 20 minutes.

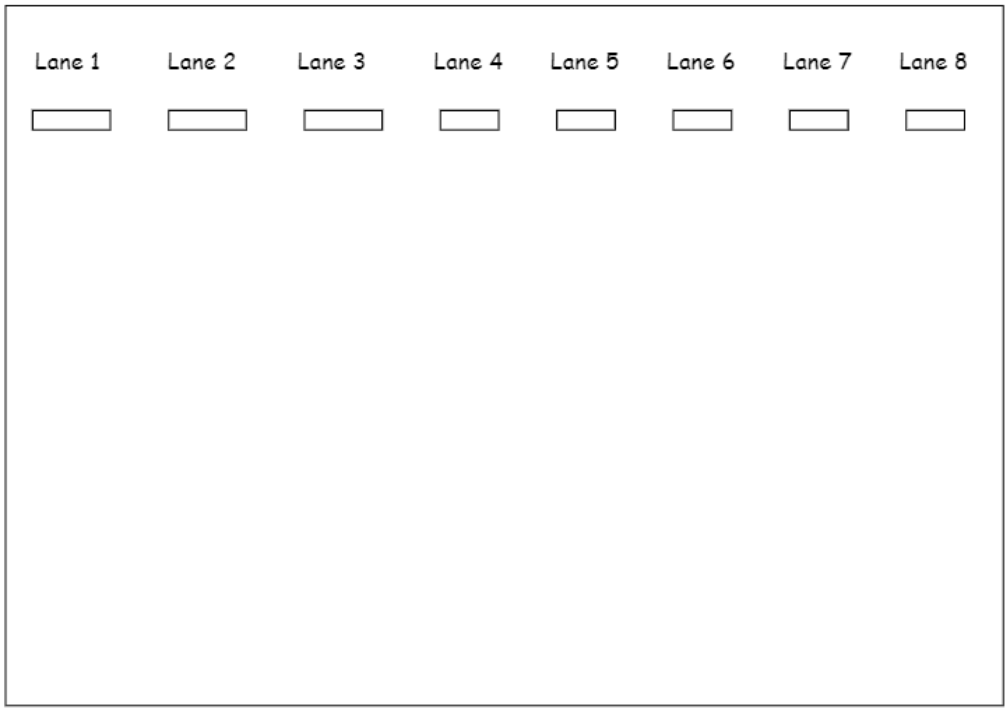
   Dark blue bands will become visible against a light blue background. Additional destaining may yield optimal results.



6. Carefully remove the gel from the destaining liquid and examine the gel on a Visible Light Gel Visualization System.

- Use of warmed distilled water at 37°C will accelerate destaining. Destaining will take longer with room temperature water.
- DO NOT EXCEED 37°C! Warmer temperatures will soften the gel and may cause it to break.
- The volume of distilled water for destaining depends upon the size of the tray. Use the smallest tray available that will accommodate the gel. The gel should be completely submerged during destaining.
- Do not exceed 3 changes of water for destaining. Excessive destaining will cause the bands to be very light.

Results: Record the distance (in mm) each sample traveled and write the number of base pairs (bp) each blotch represents. To determine bp, use the standard curve you made below.



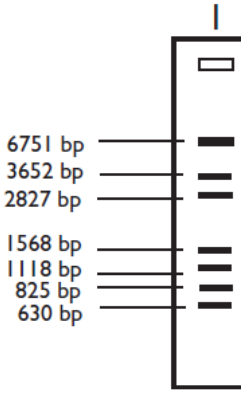
Analysis:

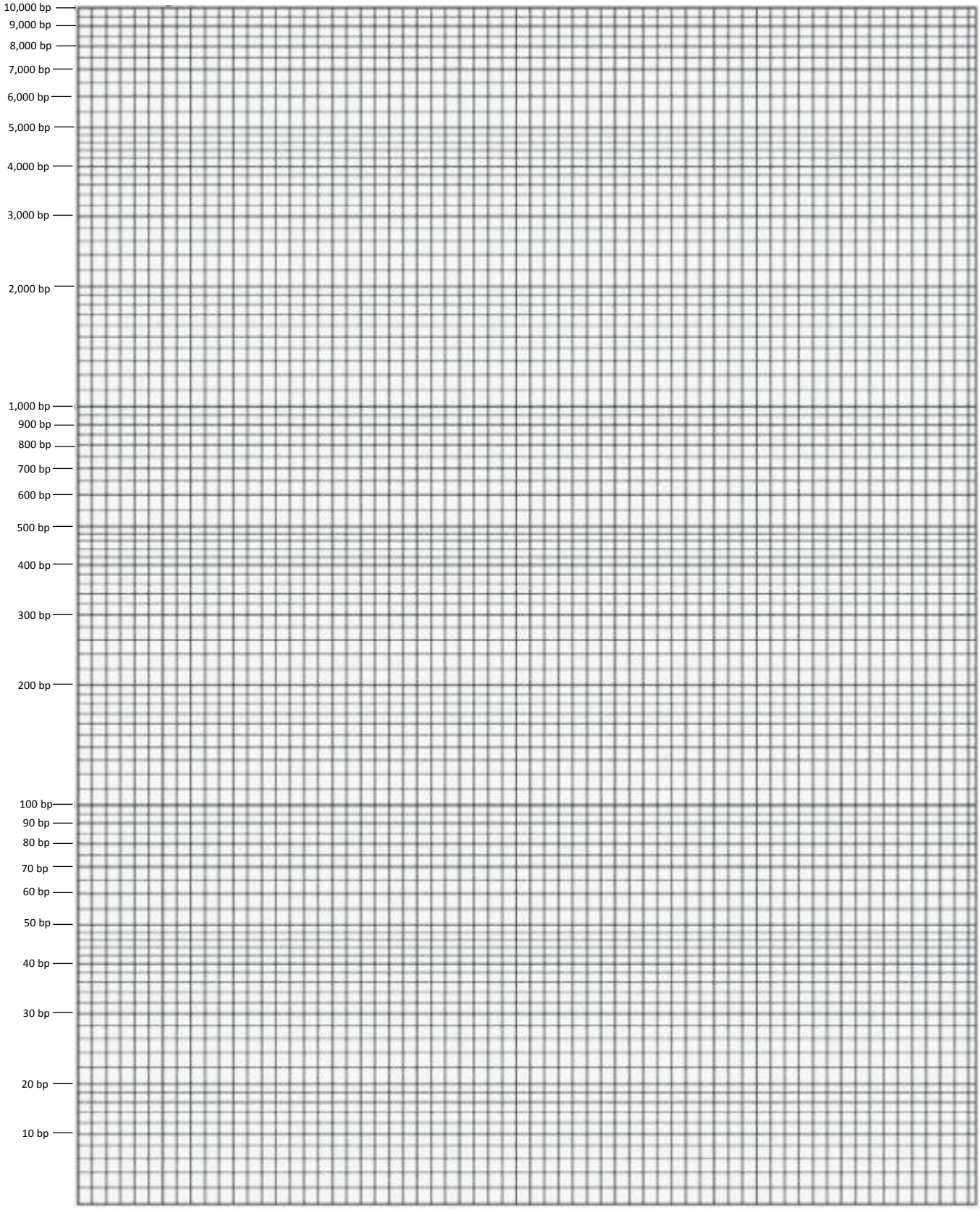
12) Which suspect's DNA matches that found at the crime scene?

13) Does this mean the suspect is guilty?

14) Justify your answer to the last question. A justification has 3 component: 1) Scientific knowledge and/or theory; 2) specific data from your analysis related to the knowledge; and 3) and explanation of HOW the data supports the knowledge.

15) Make a standard curve using the standard DNA marker. The fragment lengths are shown to the right.




10,000 bp
9,000 bp
8,000 bp
7,000 bp
6,000 bp
5,000 bp
4,000 bp
3,000 bp
2,000 bp
1,000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
90 bp
80 bp
70 bp
60 bp
50 bp
40 bp
30 bp
20 bp
10 bp

16) In order to avoid the cumbersome process of Southern Blotting, plasmid DNA was used for each suspect's DNA. How many restriction sites are there for restriction enzymes 1 and 2 for each suspect?

17) Draw the plasmids for suspects 1 and 2. Include the restriction sites, total number of base pairs the plasmid was composed of, and the number of base pairs between restriction sites.

18) Suppose a DNA test that predicted your chances of getting a disease, such as cancer, were available. You take the test for cancer, and the results say you have a two in three chance of getting cancer sometime in the next 20 years. Who should have access to this information? Your doctor? Health insurance companies? Employers? Explain your reasoning.

19) Would you want to know your probability of getting a disease based on a DNA test? Explain your reasoning.

20) The innocence Project is an international litigation and public policy organization dedicated to exonerating wrongfully convicted individuals through DNA testing. Three-quarters of DNA exoneration cases involve misidentification by witnesses. To date, over 300 people previously convicted of serious crimes in the U.S. have been exonerated by DNA testing. However, not everyone is in favor of the Innocence Project. One United States Supreme Court justice expressed concern that DNA testing poses risks to the criminal justice system, in which a person is judged by a jury of peers.

    What social and ethical issues are raised by using DNA evidence to reexamine old court decisions?

What other arguments can you make (or find) against using DNA evidence for court cases?

21) With genetic engineering, biotechnicians can clip out beneficial genes from native plants in foreign countries and insert them into their crop plant relatives here in the United States, with great benefits to the latter – to prevent attack by insects, to increase productivity, or to allow the crops to be grown in different climates. These benefits can be worth billions of dollars, but if the crops are grown in the United States, should countries where the native plants are located be compensated? Explain your reasoning.

22) Who owns or should own the information in DNA? Explain your reasoning.

23) Who profits from the information in DNA?

24) About 99% of your DNA is the same as every other human's DNA. Explain in detail how it is possible to look at a few regions of DNA and differentiate between individuals. You must address SNPs and STRs.

25) Everyone needs the same genes to produce cell components, and organs such as your liver and lungs. Are the differences between you and other individuals in the genes themselves?

26) Justify your answer to the last question. A justification has 3 component: 1) Scientific knowledge and/or theory; 2) specific data from your analysis related to the knowledge; and 3) and explanation of HOW the data supports the knowledge.