EDVOTEK® • The Biotechnology Education Company®

Edvo-Kit #
225

Edvo-Kit #225

DNA Fingerprinting Using Restriction Enzymes

Experiment Objective:

The objective of this simulated forensic analysis is to develop an understanding of the use of restriction enzymes as applied to RFLP-based DNA fingerprinting.

See page 3 for storage instructions.

Version 225.220526

PROTOCOL HAS BEEN UPDATED!
Please review before beginning experiment!

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Table of Contents

Safety Data Sheets can be found on our website: **www.edvotek.com/safety-data-sheets**

Experiment Components

COMPONENTS		Storage	Check (✓)
(Samples A and B are ready for electrophoresis)			
A	DNA Standard Marker	-20° C Freezer	❑
B	Crime scene DNA sample, pre-cut with Restriction Enzyme 1	-20° C Freezer	❑
C	Crime scene DNA sample, pre-cut with Restriction Enzyme 2	-20° C Freezer	❑
D	Suspect #1 DNA sample	-20° C Freezer	❑
E	Suspect #2 DNA sample	-20° C Freezer	❑
F	Enzyme Reaction Buffer	-20° C Freezer	❑
G	Dryzymes™ Restriction Enzyme 1 (*Eco*RI)	-20° C Freezer	❑
H	Dryzymes™ Restriction Enzyme 2 (*Hind*III)	-20° C Freezer	❑
I	Reconstitution buffer	-20° C Freezer	❑

This experiment is designed for 8 gels.

NOTE:
This kit contains two staining options - SYBR® Safe and FlashBlue™. Only one of these dyes should be used at a time. Refer to the Instructor's Guide for more information.

REAGENTS & SUPPLIES		
•	UltraSpec-Agarose™	Room Temp. ❑
•	Electrophoresis Buffer (50x)	Room Temp. ❑
•	SYBR® Safe Stain	Room Temp. ❑
•	FlashBlue™ Gel Stain	Room Temp. ❑
•	10X Gel Loading Solution	Room Temp. ❑
•	Practice Gel Loading Solution	Room Temp. ❑
•	1 mL pipet	Room Temp. ❑
•	Microtipped Transfer Pipets	Room Temp. ❑
•	Microcentrifuge tubes	Room Temp. ❑

Requirements *(not included with the experiment)*

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipettes with tips
- Balance
- Microwave, hot plate or burner
- Water bath (37°C)
- Pipet pump
- 250 mL flasks or beakers
- Small plastic trays or large weigh boats (for gel staining/destaining)
- UV Transilluminator or Blue Light visualization system (use if staining with SYBR® Safe)
- UV safety goggles (use if staining with SYBR® Safe)
- White light visualization system (use if staining with FlashBlue™)
- Distilled or deionized water
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Laboratory journal

Background Information

RESTRICTION ENZYMES

One of the most significant discoveries of molecular biology is a class of enzymes known 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.

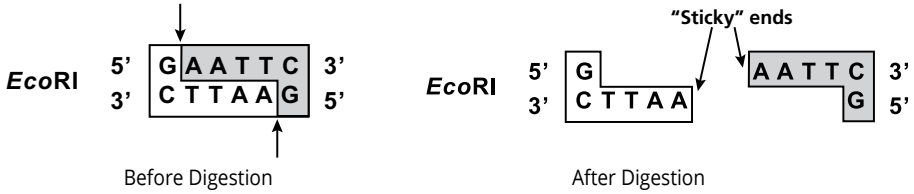
Restriction Enzyme	Organism	Species	Strain	Recognition Site
*Ava*I	*Anabaena*	*variabilis*	N/A	C^YCGUG
*Bgl*I	*Bactillus*	*globigii*	N/A	GCCNNNN^NGGC
*Eco*RI	*Escherichia*	*coli*	RY13	G^AATTC
*Hae*III	*Haemophilus*	*aegyptius*	N/A	GG^CC
*Hin*dIII	*Haemophilus*	*influenzae*	R_4	A^AGCTT
*Sac*I	*Streptomyces*	*achromogenes*	N/A	GAGCT^C

Table 1: Common Restriction Enzymes

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 *Escherchia coli*. (More examples are shown in Table 1.)

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 recognition site. Statistically, an enzyme will average one cut for every 4^n 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., *Hae*III) will cut DNA once every 256 (or 4^4) base pairs, while an enzyme that recognizes a six base pairs long site (e.g., *Eco*RI) will cut once every 4096 (or 4^6) base pairs. Therefore, the longer a DNA molecule is, the greater the probability is that it contains one or more restriction sites. For example, if *Eco*RI 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 *Eco*RI.



*Eco*RI 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. Digestion of the same piece of DNA using different enzymes can produce sticky ends of different lengths and strand orientation (5′ vs. 3′).

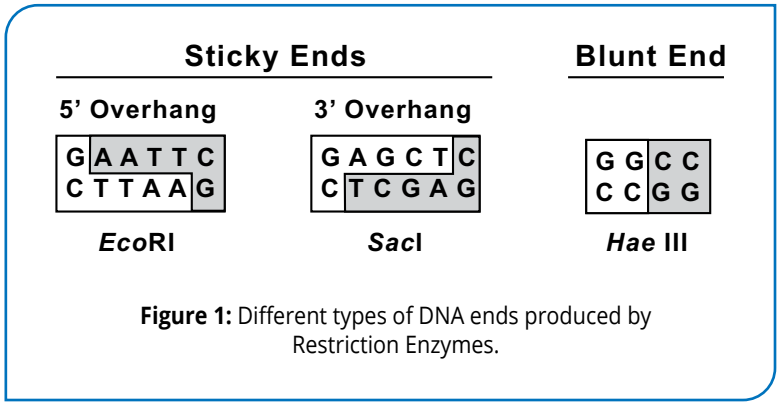
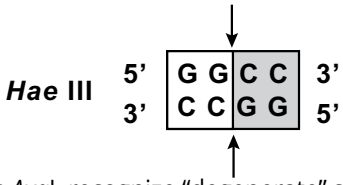
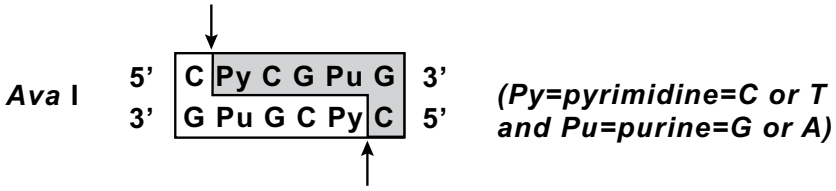


Figure 1: Different types of DNA ends produced by Restriction Enzymes.

In contrast to *Eco*RI, *Hae*III 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.

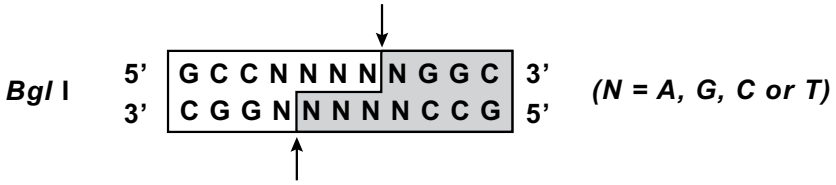


Some restriction enzymes, such as *Ava*I, recognize "degenerate" sites, which contain one or more variable positions.



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

There are even enzymes like *Bgl*I that recognize "hyphenated" sites, which are palindromic sequences separated by a number of completely variable bases.



The six G-C base pairs that *Bgl*I specifically recognizes must be separated by five base pairs of DNA; otherwise the enzyme cannot properly interact with the DNA to cleave its backbone. Because these five base pairs are not required to make up a specific sequence, *Bgl*I can recognize and cleave up to 1024 possible sequences!

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 (see figure). First, DNA molecules are added into depressions (or "wells") within a gel, 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 towards the positive electrode (Fig. 2)

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 "bands" within the gel. After the current is stopped, the bands can be visualized using a stain that sticks to DNA.

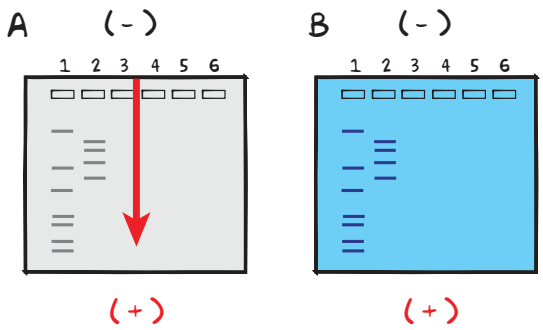


Figure 2:
Overview of Agarose Gel Electrophoresis.

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 through the gel more quickly than similarly-sized linear DNA, which prevents an accurate comparison of size.

SOUTHERN BLOT ANALYSIS

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-stranded form (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 adsorbed to the membrane, that can then be manipulated easier than gels.

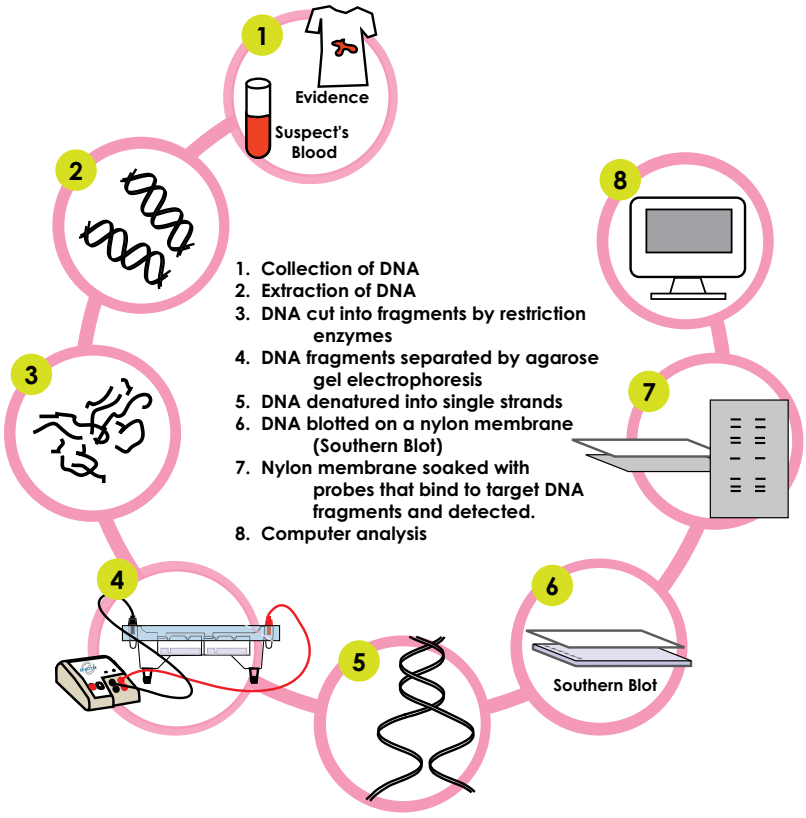


Figure 3: DNA Fingerprinting using RFLP and Southern blot analysis.

Analysis of the blotted 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 containing the single-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.

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 analyses performed 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 a thermostable DNA polymerase, such as *Taq* polymerase. Purified from 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 Mg2+. The primers are designed to correspond to the start and end of the DNA to be amplified, known as the "target".

The PCR reaction mixture (which contains the DNA polymerase, buffer, deoxynucleotides, primers, and template) 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°. In this step, known as "annealing", the primers, present in great excess to the template, bind to the separated DNA strands.

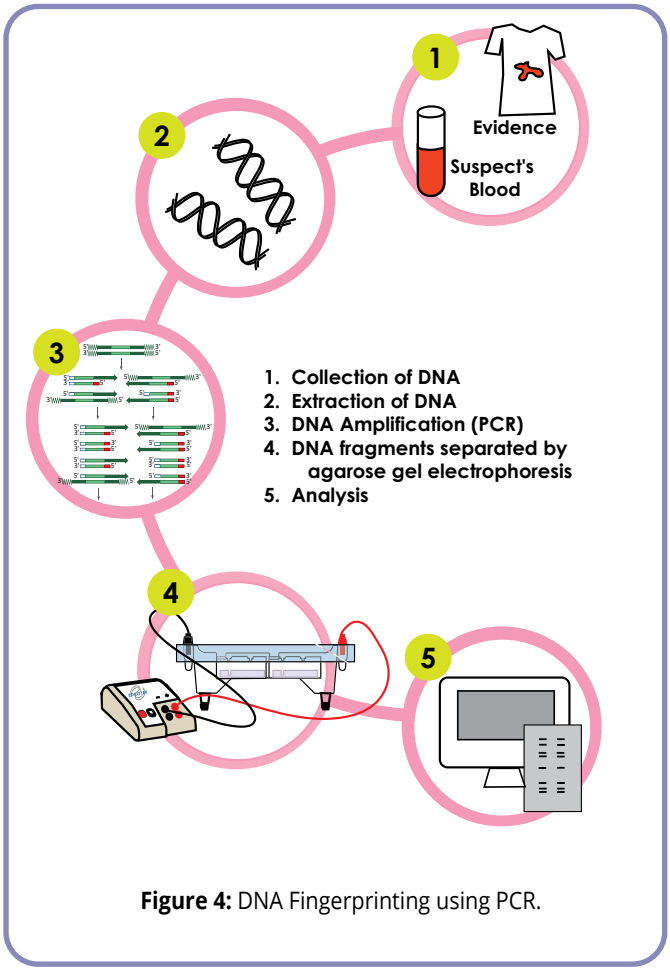


Figure 4: DNA Fingerprinting using PCR.

- In the third 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 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. Standard DNA Fragments 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.

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.

THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA.

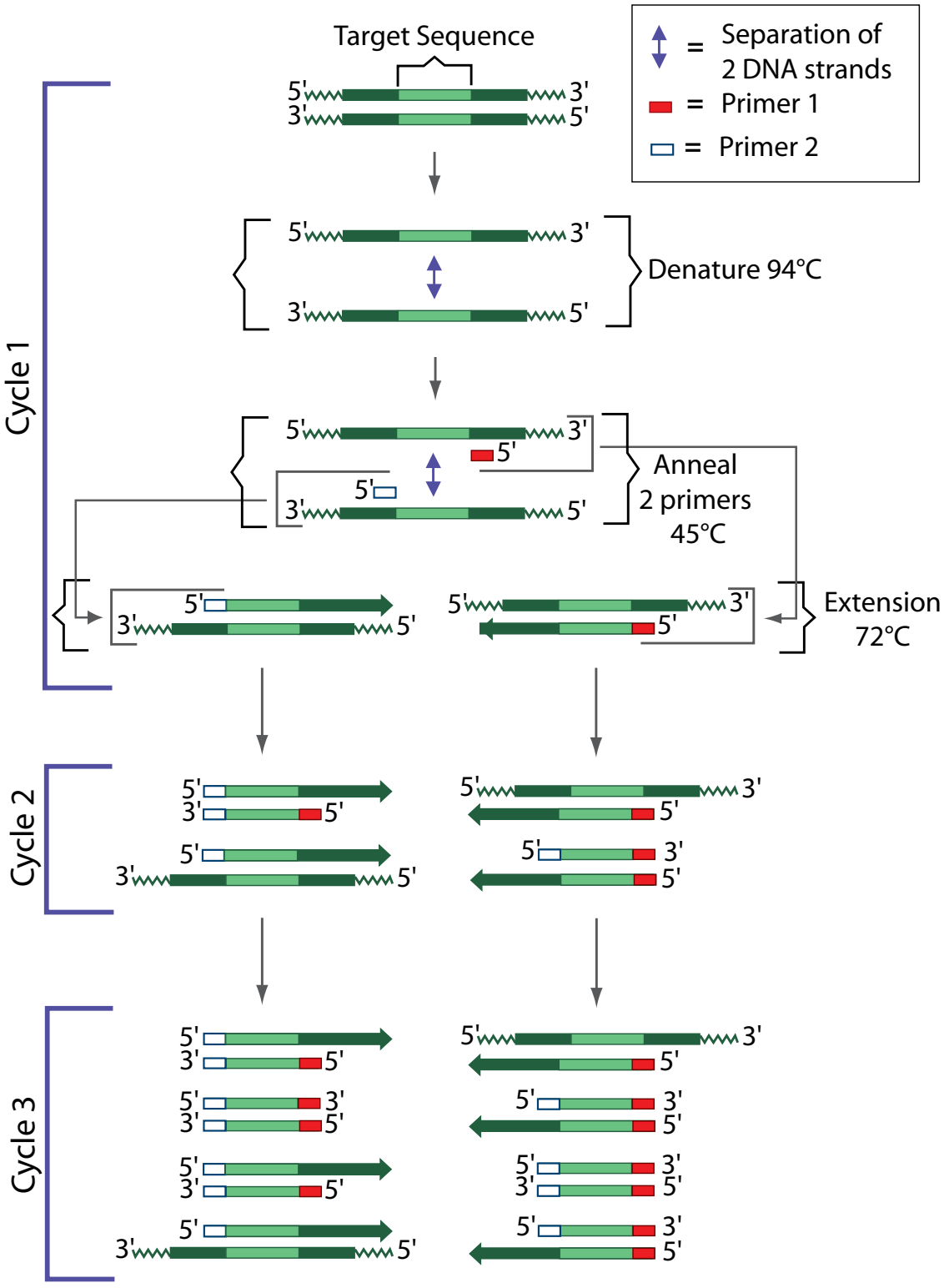


Figure 5: The Polymerase Chain Reaction

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this simulated forensic analysis is to develop an understanding of the use of restriction enzymes as applied to RFLP-based DNA fingerprinting.

LABORATORY SAFETY

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

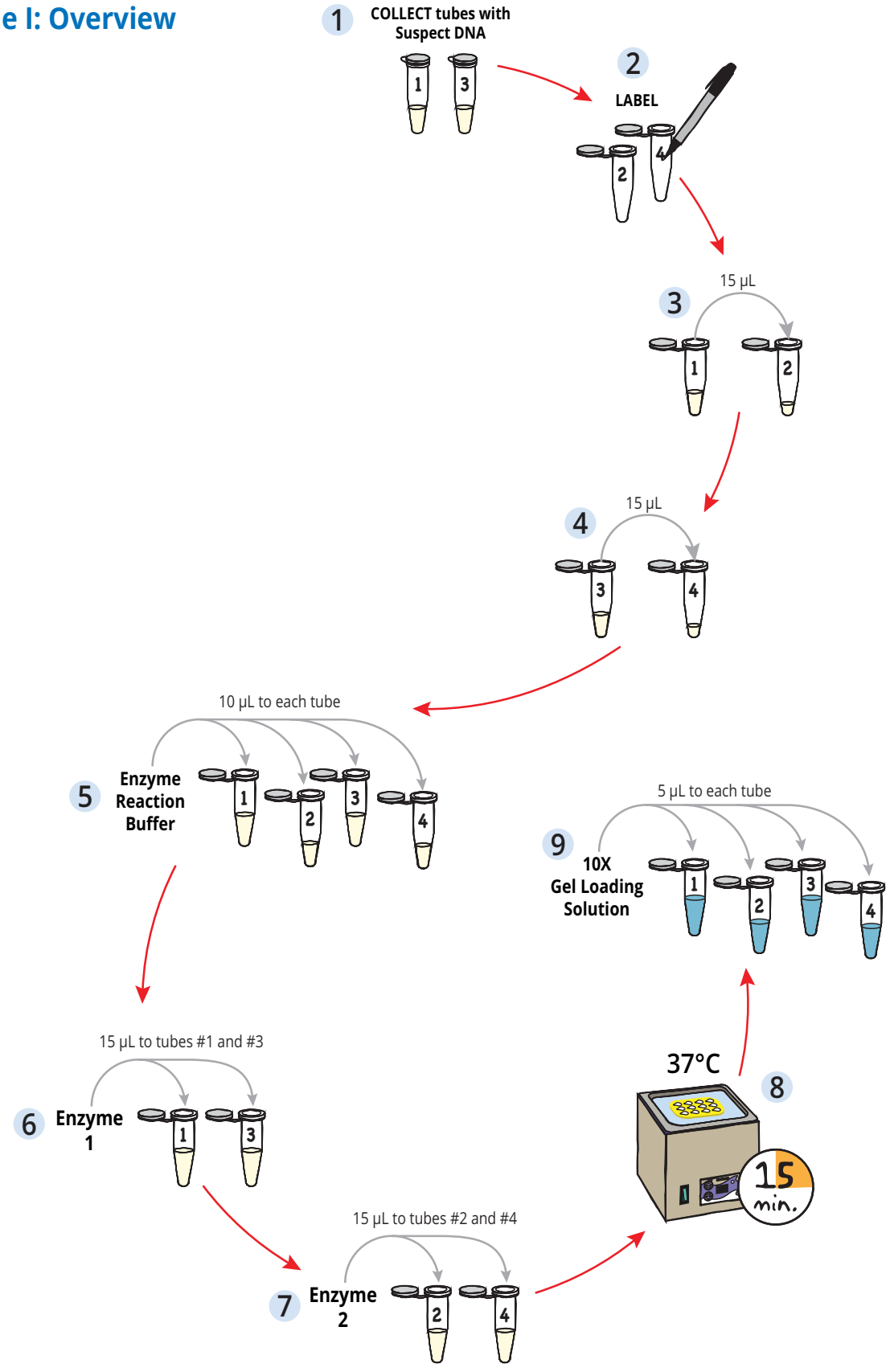
During the Experiment:

- Record your observations.

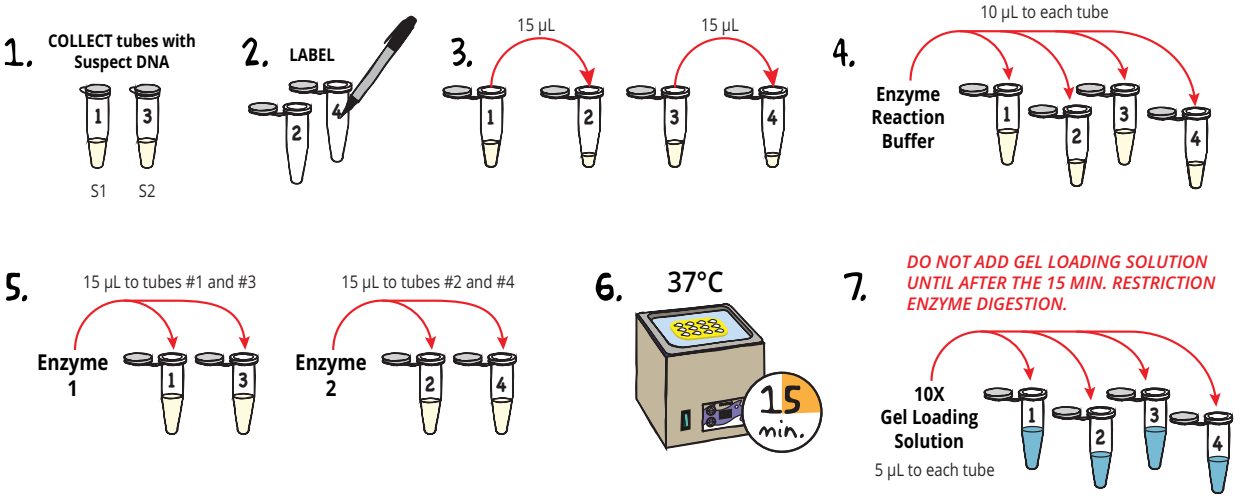
After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I: Overview



Module I: DNA Digestion with Restriction Enzymes



1. **COLLECT** two microtest tubes labeled #1 and #3 from your instructor. Tube #1 contains the DNA from Suspect 1 and Tube #3 contains the DNA from Suspect 2.
2. **LABEL** two empty microtest tubes as #2 and #4.
3. **TAP** tubes #1 and #3 on the lab bench to collect all the contents at the bottom of the tube. **TRANSFER** 15 µL from Tube #1 to Tube #2, then **TRANSFER** 15 µL from Tube #3 to Tube #4.
4. Use an adjustable volume micropipette to **DISPENSE** 10 µL of Enzyme Reaction Buffer (Rxn Buffer) to each of four reaction tubes labeled 1 - 4.
5. **ADD** the enzymes to the reaction tubes as summarized in Table 1, below. Use a **FRESH** micropipette tip for each enzyme transfer. ***NOTE: DO NOT ADD GEL LOADING SOLUTION AT THIS POINT!***
6. **PLACE** reaction tubes in a float and **INCUBATE** in a 37°C water bath for 15 minutes.


Wear gloves and safety goggles

After the incubation is completed:

7. **ADD** 5 µL of 10x gel loading solution to reaction tubes 1 - 4 to stop the reactions.
8. **COLLECT** the following tubes from your instructor, each tube will contain 35 µL:
   - DNA Standard Marker
   - DNA from crime scene digested with Enzyme 1
   - DNA from crime scene digested with Enzyme 2
9. **PROCEED** to gel electrophoresis and follow Table 2 on page 16 for the Gel Loading Scheme.

IMPORTANT NOTE: Do NOT add Gel loading solution until AFTER the 15 min. Restriction Enzyme Digestion.

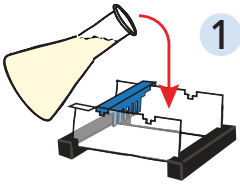
Table 1: Summary of Restriction Enzyme Digestion Reactions

Reaction Tube	Suspect 1 DNA	Suspect 2 DNA	Reaction Buffer	Enzyme 1	Enzyme 2	10X Gel Load
#1	15 µL	-	10 µL	15 µL	-	5 µL
#2	15 µL	-	10 µL	-	15 µL	5 µL
#3	-	15 µL	10 µL	15 µL	-	5 µL
#4	-	15 µL	10 µL	-	15 µL	5 µL

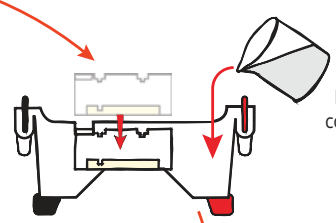
Module II: Overview

If your lab has a blue light or UV transilluminator, it is recommended that you cast gels with SYBR® Safe Stain. However, FlashBlue™ Stain is also included for those who prefer to visualize on a white light source.

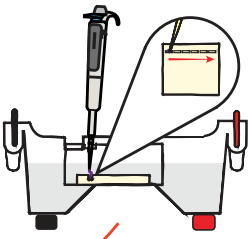
If using SYBR® Safe Stain to visualize gels, add DILUTED SYBR® to molten agarose before pouring gels.



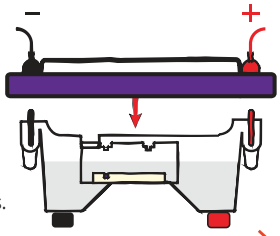
1 Prepare agarose gel in casting tray.



2 Remove end caps & comb, then submerge gel under buffer in electrophoresis chamber.



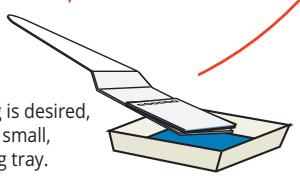
3 Load each sample in consecutive wells

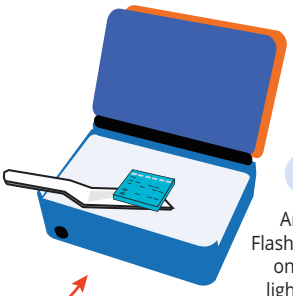


4 Attach safety cover & connect leads to power source to conduct electrophoresis.

5 If SYBR® Safe Stain was used, proceed to analysis on blue light or UV light source.

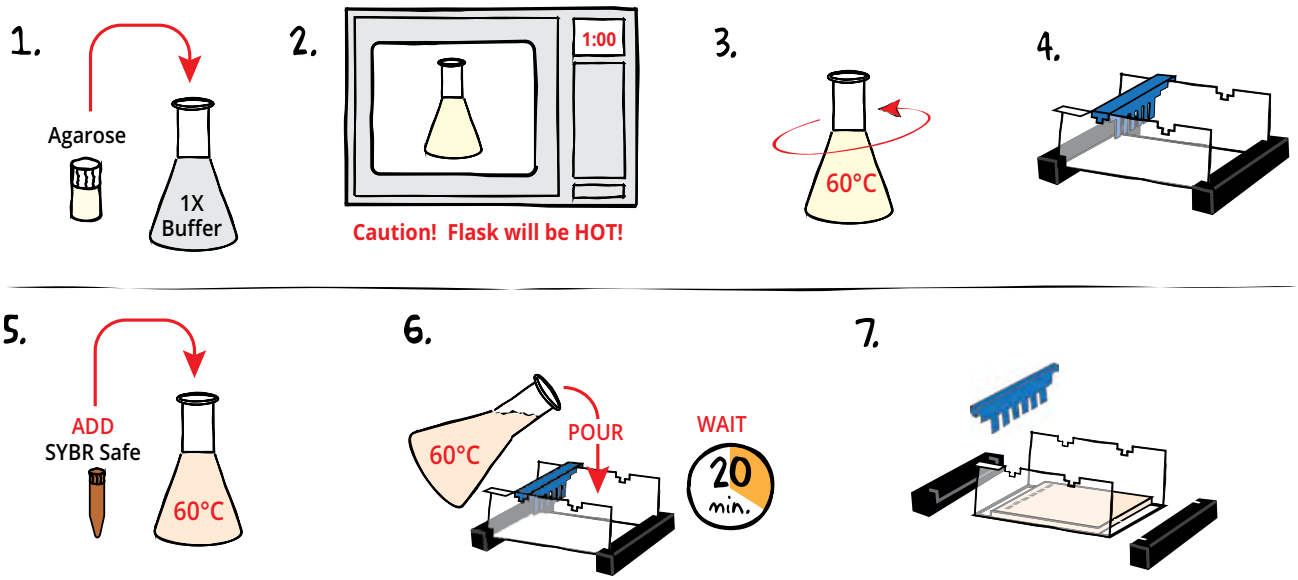
6 If FlashBlue™ Staining is desired, transfer the gel a small, clean gel-staining tray.





7 Analyze FlashBlue™ gels on a white light source.

Module II: Gel Electrophoresis of Restriction Fragments



NOTE: If you are casting your own gels, review the following instructions. If you are using pre-cast gels, proceed to Step 8.

Wear gloves and safety goggles

Reminder: This experiment requires 1.2% agarose gels cast with 8 wells.

1. **MIX** agarose powder with 1X buffer in a 250 mL flask (see Table A).
2. **DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
3. **COOL** agarose to 60°C with careful swirling to promote even dissipation of heat.
4. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.

IF STAINING WITH SYBR® SAFE STAIN, proceed to step 5. If NOT using SYBR®, proceed to step 6.

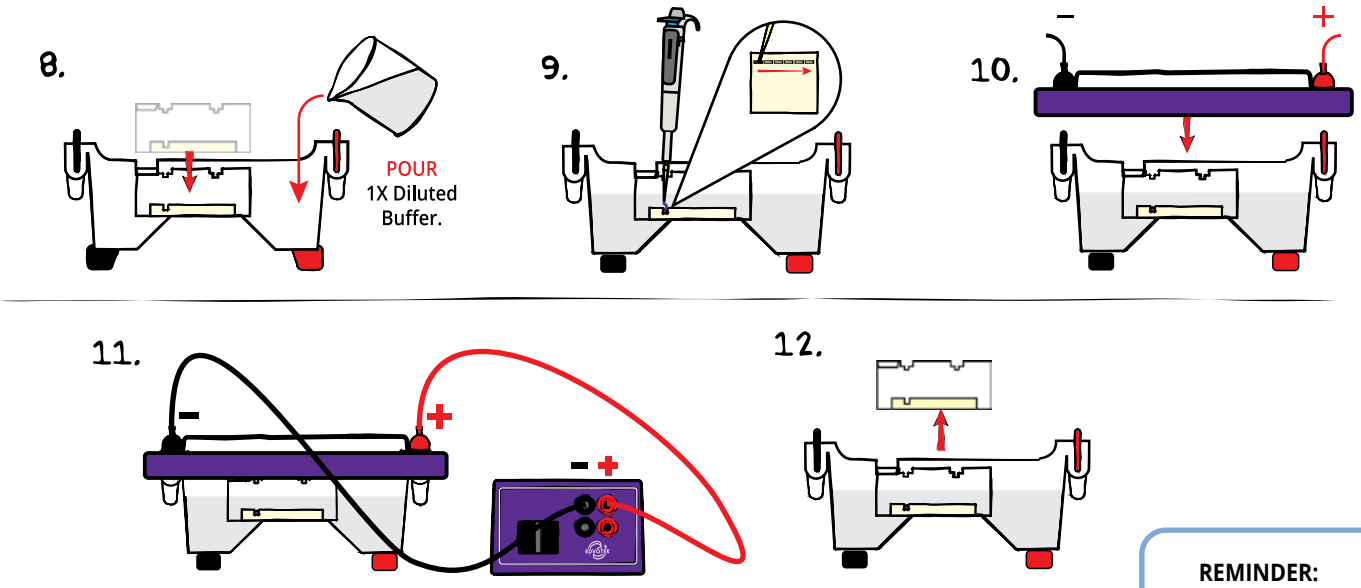
5. Before casting the gel, **ADD *DILUTED*** SYBR® Safe to the molten agarose and swirl to mix (see Table A).
6. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
7. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Table A — **Individual 1.2% UltraSpec-Agarose™ Gels with SYBR® Stain**

Size of Gel Casting Tray	Agarose	+ 1X Buffer	+ *DILUTED* SYBR® (if using)
7 x 7 cm	0.36 g	30 mL	30 µL
10 x 7 cm*	0.6 g	50 mL	50 µL
14 x 7 cm	0.72 g	60 mL	60 µL

** Recommended gel volume for the EDGE™.*

Module II: Gel Electrophoresis of Restriction Fragments, continued



REMINDER: Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

8. **PLACE** gel (on the tray) into electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
9. **LOAD** the entire sample volume (35 µL) into the well in the order indicated by Table 2.
10. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
11. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber. If SYBR® Safe Stain was used, proceed to **VISUALIZING THE SYBR® GEL** on page 17. If FlashBlue™ Staining is desired, proceed to page 18.

Table 2: Gel Loading Scheme

Lane	Sample	Vol. to Load
1	DNA Standard Marker	35 µL
2	Crime scene DNA digested with Enzyme 1	35 µL
3	Crime scene DNA digested with Enzyme 2	35 µL
4	Tube 1 - Suspect 1/Enzyme 1	35 µL
5	Tube 2 - Suspect 1/Enzyme 2	35 µL
6	Tube 3 - Suspect 2/Enzyme 1	35 µL
7	Tube 4 - Suspect 2/Enzyme 2	35 µL

Table B

1x Electrophoresis Buffer (Chamber Buffer)

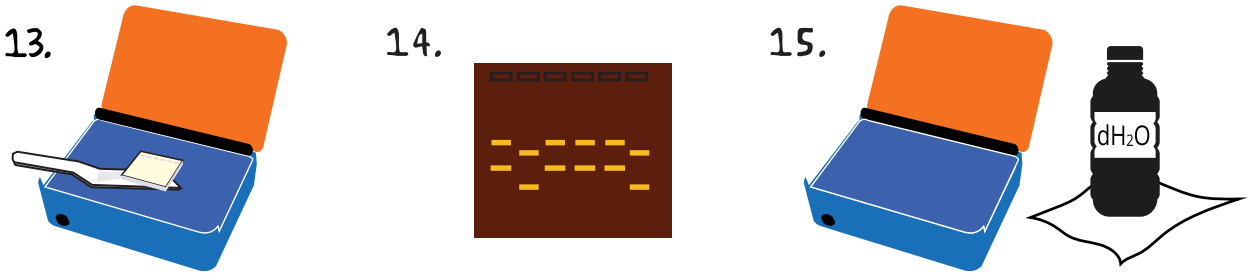
EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C

Time and Voltage Guidelines
(1.2% Agarose Gel)

	Electrophoresis Model: EDGE™	M12/M36
Volts	Min/Max (minutes)	Min/Max (minutes)
150	20/30	25/35
100	35/45	40/50
75	NA	55/65

Module II: Gel Electrophoresis of Restriction Fragments, continued



VISUALIZING THE SYBR® GEL

Be sure to wear UV goggles if using a UV transilluminator.

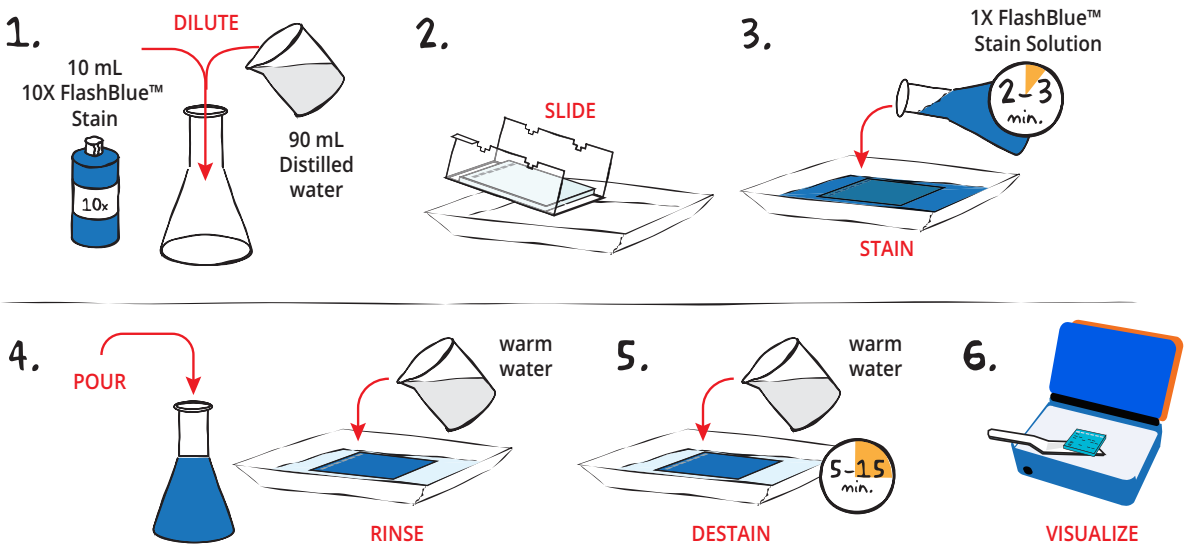
13. **SLIDE** gel off the casting tray onto the viewing surface of the transilluminator and turn the unit on. **ADJUST** the brightness to the desired level to maximize band visualization. DNA should appear as bright green bands on a dark background.

14. **PHOTOGRAPH** results.

15. **REMOVE** and **DISPOSE** of the gel and **CLEAN** the transilluminator surfaces with distilled water.

Module III: Staining Agarose Gels Using FlashBlue™ (OPTIONAL)

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV reactive DNA stains like SYBR® Safe. ***If staining with both SYBR® Safe and FlashBlue™, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.***



1. **DILUTE** 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. **MIX** well.
2. **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off the casting tray into a small, clean gel-staining tray.
3. **COVER** the gel with the 1X FlashBlue™ stain solution. **STAIN** the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
4. **POUR** the 1X FlashBlue™ back into the flask (the stain can be reused). **COVER** the gel with warm water (40-45°C). Gently **RINSE** the gel for 20-30 seconds. **POUR** off the water.
5. **COVER** the gel with clean, warm water (40-45°C). **DESTAIN** for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.
6. Carefully **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Wear gloves and safety goggles

ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

1. **DILUTE** 1 mL of 10X FlashBlue™ stain with 149 mL distilled water.
2. **COVER** the gel with diluted FlashBlue™ stain.
3. **SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
4. Carefully **REMOVE** the gel from the staining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Study Questions

1. Which suspect's DNA matches that found at the crime scene? Does this automatically mean that the suspect is guilty?
2. What possible experimental problems could occur to invalidate the results?
3. If only Restriction Enzyme 1 was used, would the interpretation be the same?