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THE **BIOTECHNOLOGY EDUCATION** COMPANY®

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- Better separation
- Easier band measurements
- No unused bands

NEW DNA Standard ladder sizes:
6751, 3652, 2827, 1568, 1118, 825, 630

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.

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Table of Contents

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

Experiment # 225 is designed for 6 groups.

Experiment Components

	Contents	Storage
A	Crime scene DNA sample, pre-cut with Restriction Enzyme 1	-20°C freezer
B	Crime scene DNA sample, pre-cut with Restriction Enzyme 2	-20°C freezer
(Samples A and B are ready for electrophoresis)		
C	Suspect #1 DNA sample	-20°C freezer
D	Suspect #2 DNA sample	-20°C freezer
E	DNA Standard Marker	-20°C freezer
F	Enzyme Reaction Buffer	4°C Refrigerator
G	Dryzymes™ Restriction Enzyme 1 (*Eco*RI)	4°C Refrigerator
H	Dryzymes™ Restriction Enzyme 2 (*Hin*dIII)	4°C Refrigerator
I	Reconstitution buffer	-20°C freezer
J	Enzyme Grade water	-20°C freezer

- 10x Gel Loading Solution
- UltraSpec-Agarose™ powder
- Concentrated electrophoresis buffer
- FlashBlue™ Liquid Stain
- InstaStain® Blue
- 1 ml pipet
- Microtipped Transfer Pipets

Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipets with tips
- Water bath (37°C or 45°C)
- Balance
- Hot plate, Bunsen burner or microwave oven
- DNA visualization system (white light)
- Small plastic trays or large weigh boats (for gel destaining)
- Safety goggles and disposable laboratory gloves
- Pipet pumps
- 20 ml and 250 ml beakers or flasks
- Hot gloves
- Marking pens
- Distilled or deionized water
- Ice

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

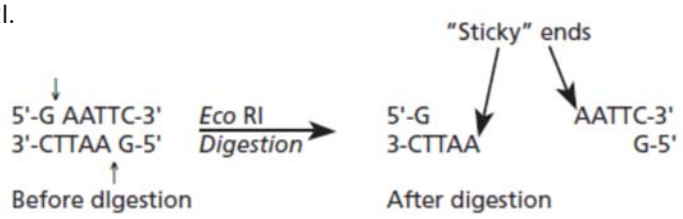
Table 1: Common Restriction Enzymes

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, *Eco*RI 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 44) base pairs, while an enzyme that recognizes a six base pairs long site (e.g., *Eco*RI) will cut once every 4096 (or 46) 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).

Background Information

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 (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′).

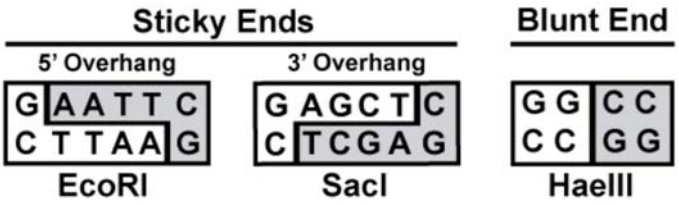
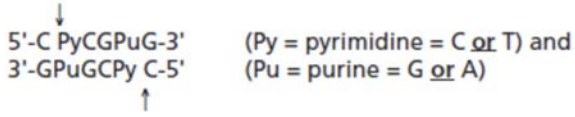


Figure 1: Different Types of DNA Ends Produced by Restriction Enzymes.

In contrast to *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.

Background Information

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

```
      ↓
5'-GCCNNNN NGGC-3'
3'-CGGN NNNNCCG-5'
        ↑
```

(N = A, G, C or T)

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 625 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).

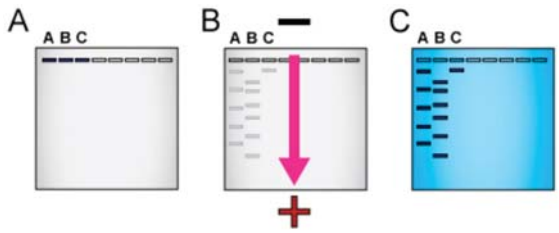


Figure 2: Overview of Agarose Gel Electrophoresis

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.

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.

Background Information

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.

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.

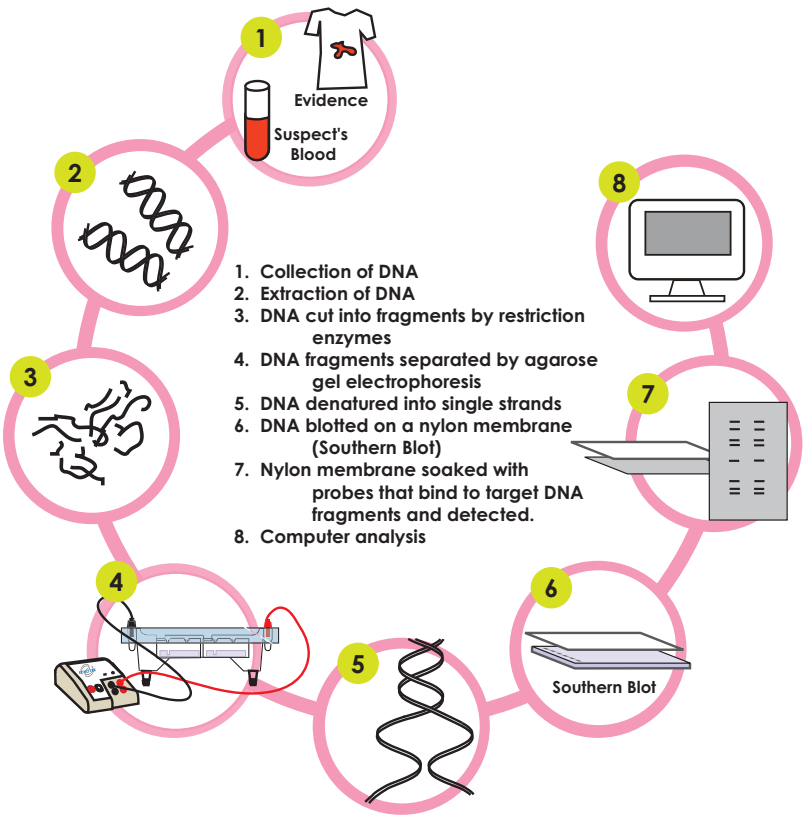


Figure 3: DNA Fingerprinting using RFLP and Southern blot analysis.

Background Information

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 Mg^{2+}. 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.

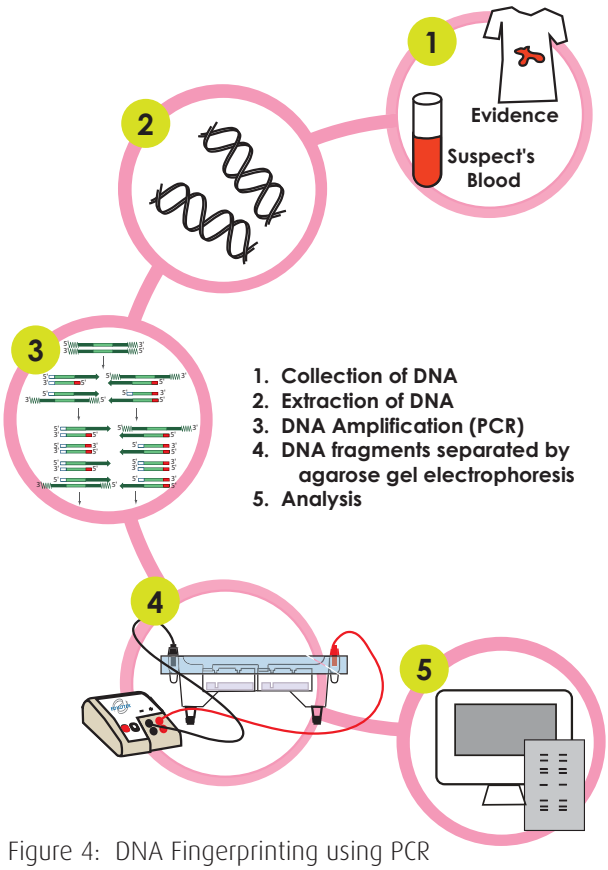


Figure 4: DNA Fingerprinting using PCR

Experiment Procedure

Background Information

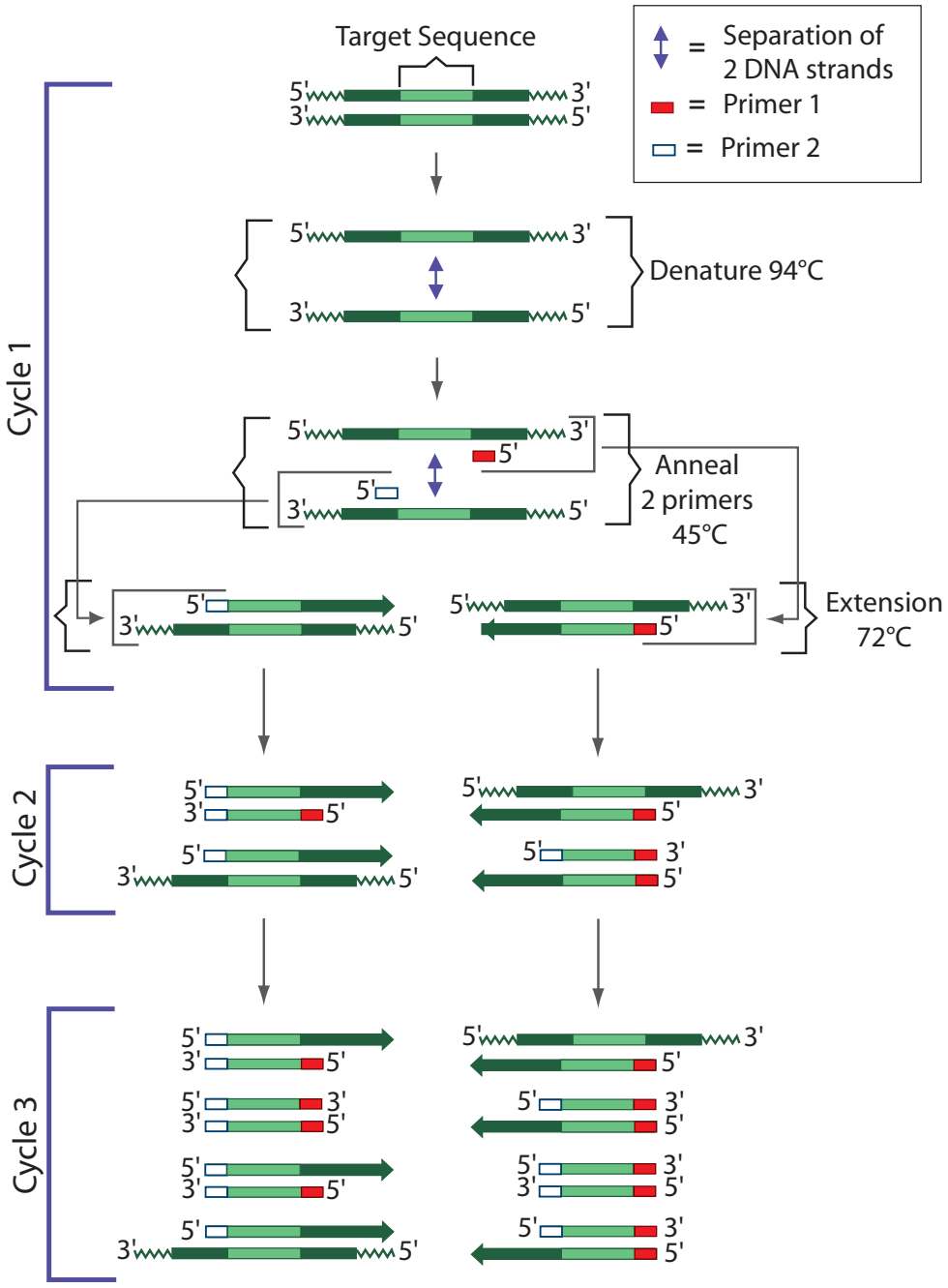


Figure 5: The Polymerase Chain Reaction

Background Information

- 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.

- 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. 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.

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.

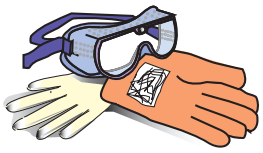
THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA.

Experiment Overview and General Instructions

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 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!

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.

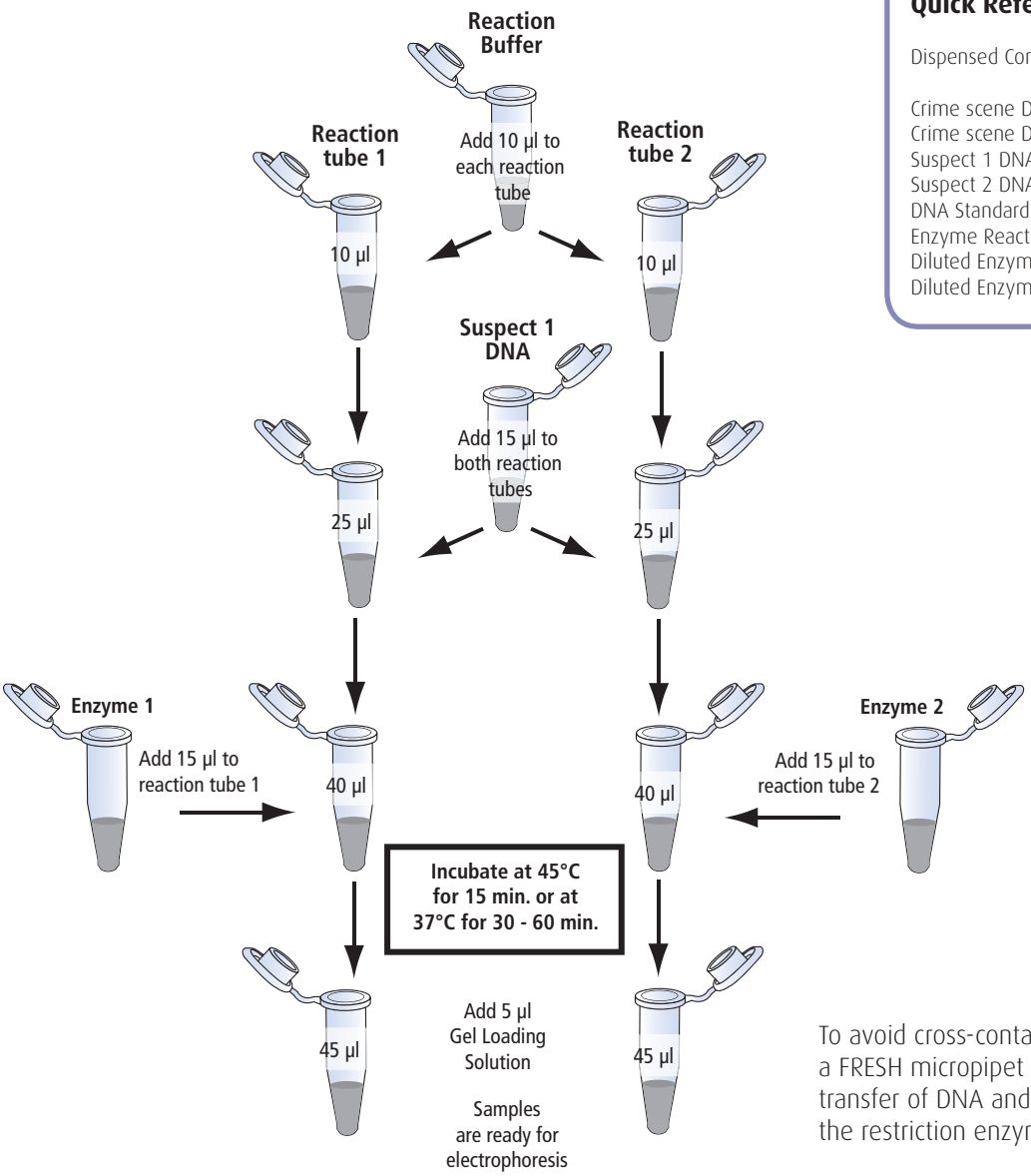
During the Experiment:
- Record your observations.

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.

Experiment Procedure

Crime Scene Investigation - Restriction Enzyme Digestion

In this experiment, 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).



Quick Reference:

Dispensed Components	Tube Label
Crime scene DNA 1	CS 1
Crime scene DNA 2	CS 2
Suspect 1 DNA	DNA 1
Suspect 2 DNA	DNA 2
DNA Standard Marker	Markers
Enzyme Reaction Buffer	Rxn Buffer
Diluted Enzyme 1	Enzyme 1
Diluted Enzyme 2	Enzyme 2

To avoid cross-contamination, use a FRESH micropipet tip for each transfer of DNA and enzyme to the restriction enzyme reaction.

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

Chart 1: 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.

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.

Experiment Procedure

Agarose Gel Electrophoresis

Prepare the Gel (see Appendices A, B, and C):

1. Prepare an agarose gel with specifications summarized below.

• Agarose gel concentration required:	0.8%
• Recommended gel size:	7 x 7 cm or 7 x 14 cm (two gels)
• Number of sample wells required:	7-8
• Well-former template (comb) placement (using EDVOTEK units):	For 7 x 7 cm gel - one 8-well comb in first set of notches For 7 x 14 cm gel - two standard 6-well combs in first and middle set of notches.

Load the Samples:

2. Load 40 µl of each of the DNA samples in the following manner (See Sample Loading Table, below).

   Optional Step: Heat the samples, including the DNA Standard Marker for two minutes at 65ºC. Allow the samples to cool for a few minutes.

Run the Gel:

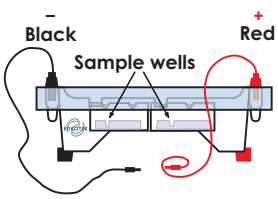
3. Close the lid of electrophoresis chamber and connect the electrodes. Set the power source to the required voltage and run the gel for the length of time specified by your instructor.

4. Check that the current is flowing correctly. Remember, 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.



SAMPLE LOADING FOR 7 x 14 cm GEL

First Row

Lane	Tube	
1	Markers	Standard DNA Marker
2	CS 1	DNA from crime scene cut with Enzyme 1
3	CS 2	DNA from crime scene cut with Enzyme 2
4	1	DNA from Suspect 1 cut with Enzyme 1
5	2	DNA from Suspect 1 cut with Enzyme 2

Second Row

Lane	Tube	
1	Markers	Standard DNA Marker
2	3	DNA from Suspect 2 cut with Enzyme 1
3	4	DNA from Suspect 2 cut with Enzyme 2

SAMPLE LOADING FOR 7 x 7 cm GEL

Lane	Tube	
1	Markers	Standard DNA Marker
2	CS 1	DNA from crime scene cut with Enzyme 1
3	CS 2	DNA from crime scene cut with Enzyme 2
4	1	DNA from Suspect 1 cut with Enzyme 1
5	2	DNA from Suspect 1 cut with Enzyme 2
6	3	DNA from Suspect 2 cut with Enzyme 1
7	4	DNA from Suspect 2 cut with Enzyme 2

Agarose Gel Electrophoresis

Stain the Gel:

6. Carefully remove the agarose gel and casting tray from the electrophoresis chamber.

7. Stain the gel according to the staining instruction **(See Appendices E-H).**

DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS!

View the Gel:

8. Examine your stained gel and document the results of your experiment.

9. This can be done by taking a picture of your gel or by tracing the gel onto a transparency. Be sure to include the outline of the gel and the sample wells.

Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

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?

NOTES:

Instructor's Guide

Notes to the Instructor:

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will learn to perform restriction enzyme analysis, load samples and run agarose gel electrophoresis. Analysis of the experiments will provide students the means to transform an abstract concept into a concrete explanation. Please visit our website for specific content and skill standards for various experiments.

EDUCATIONAL RESOURCES

Electrophoresis Hints, Help and Frequently Asked Questions

EDVOTEK Electrophoresis Experiments are easy to perform and are designed for maximum success in the classroom setting. However, even the most experienced students and teachers occasionally encounter experimental problems or difficulties. The EDVOTEK web site provides several suggestions and reminders for conducting electrophoresis, as well as answers to frequently asked electrophoresis questions.

Laboratory Extensions and Supplemental Activities

Laboratory extensions are easy to perform using EDVOTEK experiment kits. For laboratory extension suggestions, please check the EDVOTEK website, which is updated on a continuous basis with educational activities and resources.

Notes to the Instructor:

This experiment simulates a forensic case in which DNA samples from a hypothetical crime scene and suspects are digested by six-base cutting enzymes (*Eco* RI and *Hind* III). The objective is to analyze suspect DNA fingerprint patterns and compare them with "crime scene" samples. Each DNA sample will be cleaved with two restriction enzymes in separate reactions, and pairs of fragmentation patterns will serve as the fingerprints. The DNA fragmentation patterns will be analyzed in the stained agarose gel, without the need for Southern blot analysis.

This experiment module contains biologicals and reagents for six groups. The experimental procedures consist of two major parts: 1) restriction enzyme digestion of DNA, which is followed by 2) agarose gel electrophoresis.

Each laboratory group receives two predigested, ready-for-electrophoresis "crime scene" samples and the DNA Standard Marker. Four additional DNA samples are generated by performing restriction enzyme digestion reactions on the DNAs of two suspects.

If you have six (6) electrophoresis units, one for each of the six lab groups, electrophoresis can be performed simultaneously by all six groups. Alternatively, some lab groups can store their samples at 4°C and perform the electrophoresis at different times.

APPROXIMATE TIME REQUIREMENTS

1. Prelab prep and dispensing of biologicals and reagents take approximately 1-2 hours.
2. The approximate time required for students to perform the restriction enzyme digestion and prepare samples for electrophoresis is 50 minutes. The incubation time for restriction enzyme digestion may be extended to 60 minutes.
3. **Gel preparation:** Whether you choose to prepare the gel(s) in advance or have the students prepare their own, allow approximately 30-40 minutes for this procedure. Generally, 20 minutes of this time is required for gel solidification.
4. **Practice Gel Loading:** If your students are unfamiliar with using micropipets and sample loading techniques, a practice activity is suggested prior to conducting the experiment. EDVOTEK electrophoresis experiments contain a tube of practice gel loading solution for this purpose. Casting of a separate practice gel is highly recommended. This activity can require anywhere from 10 minutes to an entire laboratory session, depending upon the skill level of your students.
5. **Conducting Electrophoresis:** The approximate time for electrophoresis will vary from approximately 15 minutes to 2 hours. Different models of electrophoresis units will separate DNA at different rates depending upon its design configuration. Generally, the higher the voltage applied the faster the samples migrate. However, maximum voltage should not exceed the indicated recommendations. Refer to Table C.1 for specific Time and Voltage recommendations.

Table C.1 — Time and Voltage Guidelines (0.8% Gel)

	EDVOTEK Electrophoresis Model M6+	EDVOTEK Electrophoresis Model M12 & M36
Volts	Minimum / Maximum	Minimum / Maximum
150	15 / 20 min	25 / 35 min
125	20 / 30 min	35 / 45 min
70	35 / 45 min	60 / 90 min
50	50 / 80 min	95 / 130 min

Notes to the Instructor:

GEL STAINING AND DESTAINING AFTER ELECTROPHORESIS

This experiment features FlashBlue™ and InstaStain® Blue for gel staining after electrophoresis. It is a proprietary new staining method which saves time and reduces liquid waste. EDVOTEK also offers InstaStain® Ethidium Bromide and Protein InstaStain® for staining protein polyacrylamide gels.

Three options are provided for gel staining with FlashBlue™ liquid and InstaStain® Blue.

- **Method 1 - Staining and Visualization of DNA with FlashBlue™ liquid stain:**

  A simple and rapid staining procedure that will allow gels to be stained in less than 5 minutes.

- **Method 2 - One-step Staining and Destaining with InstaStain® Blue:**

  Agarose gels can be stained and destained in one easy step, which can be completed in approximately 3 hours, or can be left in liquid overnight.

- **Method 3 - Direct Staining with InstaStain® Blue:**

  Using InstaStain® Blue cards requires approximately 5-10 minutes for staining. DNA bands will become visible after destaining for approximately 20 minutes, and will become sharper with additional destaining. For the best photographic results, allow the gel to destain for several hours to overnight. This will allow the stained gel to "equilibrate" in the destaining solution, resulting in dark blue DNA bands contrasting against a uniformly light blue background.

Pre-Lab Preparations

PREPARATION OF BIOLOGICALS AND REAGENTS

1. Thaw all DNAs. Tap tubes on a table to get all the sample to the bottom of the tube.

2. Two tubes, components A and B, contain crime scene samples. These DNA samples have been cut with restriction enzymes and are ready for electrophoresis. Sample A represents "crime scene" DNA cut with Restriction Enzyme 1. Sample B represents "crime scene" DNA cut with Restriction Enzyme 2.

   - Label six tubes "CS 1" for the crime scene sample #1 (A).
   - Label six tubes "CS 2" for the crime scene sample #2 (B).
   - Dispense 45 µl of each crime scene sample in the appropriate tubes for each of the six lab groups.

3. Component E contains the DNA Standard Marker.

   - Label six tubes "Markers".
   - Dispense 85 µl of DNA Standard Marker to each tube for each of the six groups.

4. Component F is the Enzyme Reaction buffer.

   - Label six tubes "Rxn Buffer".
   - Dispense 45 µl of Enzyme Reaction buffer to each tube for each of the six groups.

Quick Reference: Components for Restriction Enzyme Digestion

A Crime scene DNA sample, pre-cut with Restriction Enzyme 1
B Crime scene DNA sample, pre-cut with Restriction Enzyme 2

(Samples A and B are ready for electrophoresis)

C Suspect #1 DNA sample
D Suspect #2 DNA sample
E DNA Standard Marker
F Enzyme Reaction Buffer
G Restriction Enzyme 1
H Restriction Enzyme 2

PREPARATION OF SUSPECT DNA

5. Using an automatic micropipet, dispense the two Suspect DNAs (C, D) for each of the six lab groups .

   - For each of 6 groups, label two tubes: "DNA 1", & "DNA 2".
   - Dispense 35 µl of each Suspect DNA to the appropriate tube.

Pre-Lab Preparations

PREPARATION OF DRYZYME™ RESTRICTION ENZYMES

Prepare restriction digests within 30 minutes of reconstituting Dryzymes™.

1. Make sure that the solid material is at the bottom of the tubes. If not, centrifuge the tubes in a microcentrifuge at full speed for 20 seconds or tap the tube on the lab bench.

2. Add 120 µl Reconstitution Buffer (I) to the solid at the bottom of each tube containing Dryzymes™.

3. Allow the samples to hydrate for 1 minute.

4. Mix the samples vigorously by flicking the tubes with your finger or by vortexing for 30 seconds until the solid appears to be completely dissolved.

5. Add 120 µl Enzyme Grade Water (J) to each of the tubes of rehydrated Dryzymes™.

6. Mix or vortex the samples and then centrifuge for 20 seconds or tap the tube on the lab bench.

   After the rehydration, check that no undissolved particulate matter remains. If not completely dissolved, repeat mixing or vortexing.

7. Label six tubes "Enzyme 1" and six tubes “Enzyme 2”.

8. Transfer 35 µl of diluted Restriction Enzyme 1 to each tube labeled "Enzyme 1". Cap the tubes and immediately put on ice.

9. Transfer 35 µl of diluted Restriction Enzyme 2 to each tube labeled "Enzyme 2". Cap the tubes and immediately put on ice.

Pre-Lab Preparations

Table 1: Summary of Biologicals and Reagents required for each of six groups

	Component	Label 6 tubes each	Dispense for each tube*	
A	Crime scene DNA 1	CS 1	45 µl	
B	Crime scene DNA 2	CS 2	45 µl	
C	Suspect 1 DNA	DNA 1	35 µl	
D	Suspect 2 DNA	DNA 2	35 µl	
E	DNA Standard Marker	Markers	85 µl	
F	Reaction Buffer	Rxn Buffer	45 µl	
I, J, G	Diluted Enzyme 1	Enzyme 1	35 µl	on ice
I, J, H	Diluted Enzyme 2	Enzyme 2	35 µl	on ice

* *Recommended dispensing volumes include a small amount of "excess", which is 5 µl more than the total volume required for the experiment.*

GENERAL PREPARATIONS

1. Allow ample time to equilibrate a water bath at 45°C or 37°C on the day of the experiment.

2. Each student group can perform 4 restriction enzyme reactions. Each student group should receive the following materials:

   - Reagents and biologicals summarized in Table 1
   - Automatic micropipet and tips
   - 4 microtest tubes with attached caps
   - Marking pen

PREPARING AGAROSE GELS

Preparing gels for electrophoresis

There are several options for preparing agarose gels for the electrophoresis experiments:

1. Individual Gel Casting: Each student lab group can be responsible for casting their own individual gel prior to conducting the experiment. See instructions for "Individual Gel Preparation" **(Appendix A)**.

2. Batch Gel Preparation: A batch of agarose gel can be prepared for sharing by the class. To save time, a larger quantity of UltraSpec-Agarose can be prepared for sharing by the class. See instructions for "Batch Gel Preparation" **(Appendix B)**.

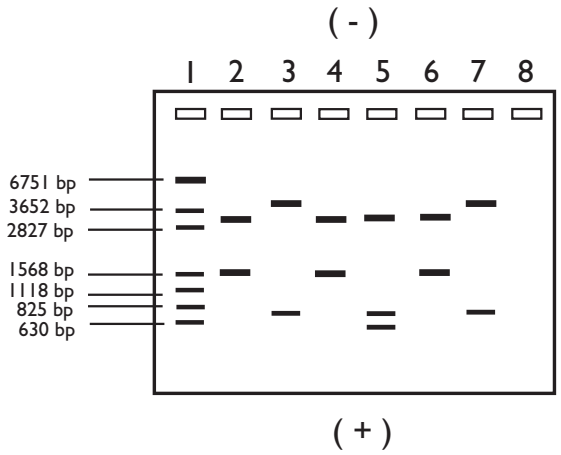
3. Preparing Gels in Advance:
   - Gels may be prepared ahead and stored for later use. Solidified gels can be stored **under** buffer in the refrigerator for up to 2 weeks.

     Do not store gels at -20°C. Freezing will destroy the gels.

   - Gels that have been removed from their trays for storage, should be "anchored" back to the tray with a few drops of hot, molten agarose before placing the gels into the apparatus for electrophoresis. This will prevent the gels from sliding around in the trays and the chambers.

Experiment Results and Analysis

GEL ELECTROPHORESIS RESULTS



The idealized schematic shows relative positions of DNA fragments. Actual results will yield broader bands of varying intensities. Smaller fragments will stain less efficiently and will appear as fainter bands. The idealized schematic shows the relative positions of the bands, but are not depicted to scale.

Lane	Tube	
1	Markers	DNA Standard Marker
2	CS 1	DNA from crime scene cut with Enzyme 1
3	CS 2	DNA from crime scene cut with Enzyme 2
4	1	DNA from Suspect 1 cut with Enzyme 1
5	2	DNA from Suspect 1 cut with Enzyme 2
6	3	DNA from Suspect 2 cut with Enzyme 1
7	4	DNA from Suspect 2 cut with Enzyme 2

Study Questions and Answers

1. **Which suspect DNA matches that found at the crime scene? Does this automatically mean that the suspect is guilty?**

   The DNA profile for Suspect 2 matches the DNA obtained at the crime scene. The results do not automatically mean that the suspect is guilty (see answers to questions 2 and 3).

2. **What possible experimental problems could occur to invalidate the results?**

   Experimental problems which could invalidate the results include contamination of DNA samples or incomplete cleavage by the restriction enzymes.

3. **If only Restriction Enzyme 1 was used, would the interpretation be the same?**

   The interpretation would not be the same if only one enzyme were used. For instance, both suspects have the same fragment pattern with Restriction Enzyme 1. The results would be inconclusive. As covered in the background information, in practice, several different probes containing different types of repetitious sequences are used in DNA profile analysis in order to satisfy certain statistical criteria for positive identification. The use of different restriction enzymes allow for accuracies in positive identifications of greater than one in 100 million.

Appendices

A Individual Preparation for 0.8 % Agarose Gel Electrophoresis

B Quantity Preparations for 0.8 % Agarose Gel Electrophoresis

C Agarose Gel Preparation Step by Step Guidelines

D Loading the Samples and Conducting Electrophoresis

E Staining & Visualization of DNA with FlashBlue™ Liquid Stain

F One-step Staining and Destaining of DNA with InstaStain® Blue

G Direct Staining of DNA with InstaStain® Blue

H General Notes on Staining & Destaining

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

Appendix
A

Individual Preparation for 0.8% Agarose Gel Electrophoresis
(DNA Staining with FlashBlue™ or InstaStain® Blue)

We provide concentrated (50x) Tris-Acetate-EDTA (TAE) buffer for agarose gel electrophoresis. Dilute the concentrate using 49 volumes of distilled or deionized water for every one volume of buffer concentrate. Prepare volume of buffer as required for your electrophoresis apparatus.

Individual Gel Casting and Buffer: Each laboratory group is responsible for preparing their own gel and buffer.

⇩ If preparing a 0.8% gel with concentrated (50x) buffer, use Table A.1

⇩ If preparing a 0.8% gel with diluted (1x) buffer, use Table A.2

Table A.1 Individual 0.8%* UltraSpec-Agarose™ Gel
DNA Staining with FlashBlue™ or InstaStain® Blue

Size of Gel (cm)	Amt of Agarose (g)	+ Concentrated Buffer (50x) (ml)	+ Distilled Water (ml)	= Total Volume (ml)
7 x 7	0.23	0.6	29.4	30
7 x 10	0.39	1.0	49.0	50
7 x 14	0.46	1.2	58.8	60

* 0.77 UltraSpec-Agarose™ gel percentage rounded up to 0.8%

Table A.2 Individual 0.8%* UltraSpec-Agarose™ Gel
DNA Staining with FlashBlue™ or InstaStain® Blue

Size of Gel (cm)	Amt of Agarose (g)	+ Diluted Buffer (1x) (ml)
7 x 7	0.23	30
7 x 10	0.39	50
7 x 14	0.46	60

Table B Electrophoresis (Chamber) Buffer

EDVOTEK Model #	Total Volume Required (ml)	Dilution: 50x Conc. Buffer (ml)	+ Distilled Water (ml)
M6+	300	6	294
M12	400	8	392
M36	1000	20	980

Prepare the agarose gel solution for a single gel in a 250 ml flask or beaker.

Note: The UltraSpec-Agarose™ bottle usually contains exactly three grams. If the amount of agarose is not specified on the label or if the bottle's plastic seal has been broken, weigh the agarose to ensure that the correct amount is used.

Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C.1 for 0.8% agarose gels. The time for electrophoresis will vary from approximately 15 minutes to 2 hours depending upon various factors. Conduct the electrophoresis for the length of time determined by your instructor.

Table C.1 Time and Voltage Guidelines (0.8% Gel)

	EDVOTEK Electrophoresis Model M6+	EDVOTEK Electrophoresis Model M12 & M36
Volts	Minimum / Maximum	Minimum / Maximum
150	15 / 20 min	25 / 35 min
125	20 / 30 min	35 / 45 min
70	35 / 45 min	60 / 90 min
50	50 / 80 min	95 / 130 min

Quantity Preparations for Agarose Gel Electrophoresis

Appendix B

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities and distributed among an entire class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

Bulk Electrophoresis Buffer

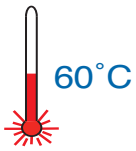
Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

Table D
Bulk Preparation of Electrophoresis Buffer

Concentrated Buffer (50x) (ml)	+	Distilled Water (ml)	=	Total Volume (ml)
60		2,940		3000 (3 L)

Batch Agarose Gels (0.8%)

1. Use a 500 ml flask to prepare the diluted gel buffer.
2. Pour 3.0 grams of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 60°C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.



6. Dispense the required volume of cooled agarose solution for casting each gel. The volume required is dependent upon the size of the gel bed and DNA staining method, which will be used. Refer to Appendix A for guidelines.
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

Table E.I
Batch Preparation of 0.8% UltraSpec-Agarose™

Amt of Agarose (g)	+	Concentrated Buffer (50X) (ml)	+	Distilled Water (ml)	=	Total Volume (ml)
3.0		7.5		382.5		390

Note: The UltraSpec-Agarose™ kit component is often labeled with the amount it contains. In many cases, the entire contents of the bottle is 3.0 grams. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

Preparing Gels in Advance

Solidified agarose gels can be removed from the casting trays and stored in the refrigerator for up to 2 weeks before performing the experiment. Keep gels covered in buffer to prevent them from drying out.

Gels that have been removed from their trays for storage should be "anchored" back to the tray. Place a few drops of molten agarose on the casting tray before replacing the gel. This will secure the gel to the tray and prevent it from sliding and/or floating in the electrophoresis chamber.

NEVER store an agarose gel at -20°C. FREEZING WILL DESTROY THE GEL!

Appendix

C

Agarose Gel Preparation - Step by Step Guidelines

1. Mix agarose powder with 1x electrophoresis buffer in a flask. Swirl the mixture to disperse clumps of agarose powder. Using a lab pen, mark the level of the solution volume on the outside of the flask.

   The amount of agarose and buffer required depends upon the size of the casting tray. Refer to the Appendix A for specifics.

2. Dissolve agarose by boiling the solution until it appears completely clear.

   Be sure to check the solution regularly while heating. If you see crystalline particles, the agarose is not completely dissolved. Larger total volumes of gel buffer solution will require longer heating time.

   A. Microwave method:
      - Cover the flask with plastic wrap to minimize evaporation.
      - Microwave the solution on High for 1 minute. BE CAREFUL—the flask may be hot! Carefully remove the flask from the microwave and swirl to mix.
      - Continue heating the solution in 15-second bursts until the agarose is completely dissolved.

   At high altitudes, use a microwave oven to reach boiling temperatures.

   B. Hot plate method:
      - Cover the flask with aluminum foil to minimize evaporation.
      - Swirling occasionally, bring the mixture to a boil over the burner, and remove from heat as soon as the agarose is completely dissolved.

3. Cool the agarose solution to 60°C with careful swirling to promote even dissipation of heat. The flask should now be warm to the touch, but not painfully hot to handle.

   If considerable evaporation has occurred during heating, add distilled water to bring the solution to the original volume and swirl to mix. Placing the bottle in a 60°C water bath, if available, will allow the agarose to cool, while preventing it from prematurely solidifying.

4. While agarose is cooling, close off the open ends of a clean and dry casting tray using rubber dams or tape.

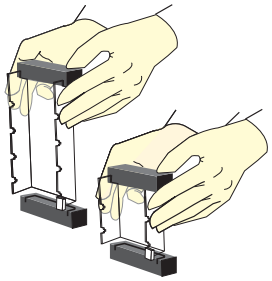
   A. Using rubber end caps:
   Position a rubber dam at each end of the bed. Make sure the dam makes firm contact with the sides and bottom of the bed.

   B. Using labeling or masking tape:
      - Extend one-inch wide tape over the sides and bottom edge of the tray.
      - Fold the extended tape edges back onto the sides and bottom. Apply pressure at contact points to establish a reliable seal.

If gel trays and rubber end caps are new, they may be initially somewhat difficult to assemble. Here is a helpful hint:

Place one of the black end caps with the wide "u" shaped slot facing up on the lab bench.

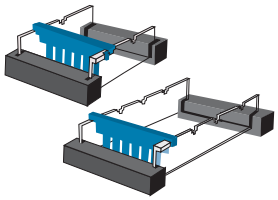
Push one of the corners of the gel tray into one of the ends of the black cap. Press down on the tray at an angle, working from one end to the other until the end of the tray completely fits into the black cap. Repeat the process with the other end of the gel tray and the other black end cap.



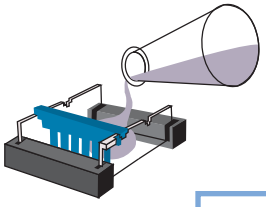
Agarose Gel Preparation Step by Step Guidelines, continued

Appendix C

5. 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.



6. Seal the sides of the gel tray to prevent agarose solution from leaking.
   - Using a transfer pipet, deposit a small amount of the agarose to the interior edge on both sides of the tray.
   - Wait approximately 1 minute for the agarose to solidify.

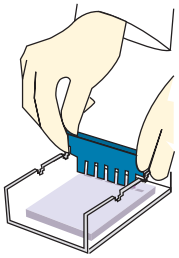


7. Once it has cooled to 60°C, slowly pour the agarose solution into the tray.

   DO NOT POUR BOILING HOT AGAROSE INTO THE GEL TRAY!
   Extreme heat can cause the tray to warp or crack!

8. Allow the gel to solidify completely.

   The gel will stiffen and become less transparent as it solidifies. The gel should thoroughly solidify within 20 minutes.



9. Slowly and carefully remove the comb and dams. Take particular care with the comb to prevent damage to the wells.

10. At this point, the gel is ready for electrophoresis (or storage under buffer in the refrigerator).

Appendix D

Loading the Samples and Conducting Electrophoresis

1. Load the samples as instructed by your instructor.
2. After the DNA samples are loaded, carefully snap the cover down onto the electrode terminals.

   Make sure that the negative and positive color-coded indicators on the cover and apparatus chamber are properly oriented.
3. Insert the plug of the black wire into the black input of the power source (negative input). Insert the plug of the red wire into the red input of the power source (positive input).
4. Set the power source at the required voltage and conduct electrophoresis for the length of time determined by your instructor. General guidelines are presented in Table C.1.
5. Check to see that current is flowing properly - you should see bubbles forming on the two platinum electrodes.
6. After the electrophoresis is completed, turn off the power, unplug the power source, disconnect the leads and remove the cover.
7. Remove the gel from the bed for staining.

About DNA Gel Staining

- After electrophoresis, the agarose gels require staining in order to visualize the separated DNA samples. This experiment features two proprietary stain called FlashBlue™ liquid stain and InstaStain® Blue cards.
- Check with your instructor regarding which staining method you should use.

Table C.1 Time and Voltage Guidelines (0.8% Gel)

	EDVOTEK Electrophoresis Model	
	M6+	M12 & M36
Volts	Minimum / Maximum	Minimum / Maximum
150	15 / 20 min	25 / 35 min
125	20 / 30 min	35 / 45 min
70	35 / 45 min	60 / 90 min
50	50 / 80 min	95 / 130 min

Staining and Visualization of DNA FlashBlue™ Liquid Stain

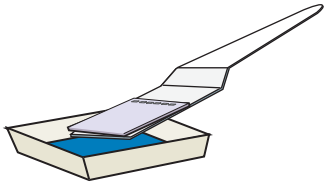
Appendix **E**

Preparation of FlashBlue™ Stain from Concentrated Solution

Wear Gloves and Goggles

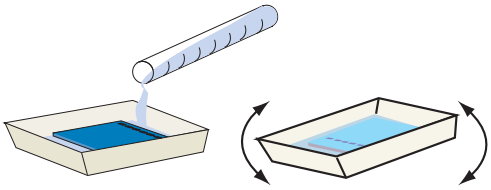
1. Dilute 10 ml of 10x FlashBlue™ with 90 ml of distilled or deionized water in a flask. Mix well.
2. Cover the flask and store it at room temperature until ready for gel staining.
3. Do not stain gel(s) in the electrophoresis apparatus.

Staining and Destaining



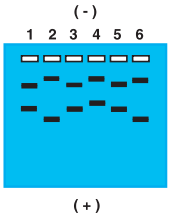
1. Remove the agarose gel from its bed and completely submerse the gel in a small, clean weigh boat or lid from pipet tip rack containing 75 ml of 1x FlashBlue™ stain. Add additional stain if needed to completely submerge the gel.
2. Stain the gel for 5 minutes.

   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.



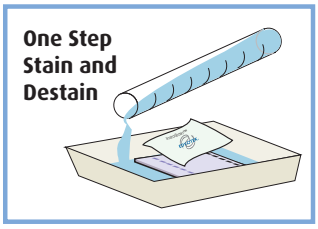
6. Carefully remove the gel from the destaining liquid and examine the gel on a Visible Light Gel Visualization System.

Storage and Disposal of Stain and Gel

See Appendix H.

Appendix

F



One-Step Staining and Destaining with InstaStain® Blue

Agarose gels can be stained and destained in approximately 3 hours using InstaStain® Blue.

1. Carefully slide the agarose gel from its tray into a small, clean tray containing at least 75 ml of distilled/deionized water or used electrophoresis buffer. The agarose gel should be completely submerged.

   DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS!

   Appropriate staining trays include large weigh boats and small, plastic food containers.

2. Gently float the InstaStain® Blue card on top of the liquid with the stain (blue side) facing toward the gel.

   Each InstaStain® Blue card will stain 49 cm^2 of gel (7 x 7 cm).

3. After 30 minutes, remove the InstaStain® Blue card.

4. Cover the tray with plastic wrap to prevent evaporation. Let the gel soak undisturbed in the liquid for at least three hours. The gel can destain overnight if necessary.

5. Carefully remove the gel from the staining tray and document results.

Storage and Disposal of Stain and Gel

See Appendix H.

InstaStain is a registered trademark of EDVOTEK, Inc.

Direct Staining of DNA with Instastain® Blue

Appendix G

STAINING OF DNA

Wear Gloves and Goggles

1. After electrophoresis, place the agarose gel on a flat surface covered with plastic wrap.
2. Wearing gloves, place the blue dye side of the InstaStain® Blue card(s) on the gel.
3. Firmly run your fingers several times over the entire surface of the InstaStain® card to establish good contact between the InstaStain® card and the gel.
4. To ensure continuous contact between the gel and the InstaStain® card, place a gel casting tray and weight, such as a small empty beaker, on top of the InstaStain® card.
5. Allow the InstaStain® Blue to sit on the gel for 5 to 10 minutes.
6. After staining, remove the InstaStain® card. If the color of the gel appears very light, wet the gel surface with buffer or distilled water and place the InstaStain® card on the gel for an additional 5 minutes.

DESTAINING AND VISUALIZATION OF DNA

1. Transfer the gel to a large weigh boat or small plastic container.
2. Destain with approximately 100 ml of distilled water to cover the gel.
3. Repeat destaining by changing the distilled water as needed.

   Larger DNA bands will initially be visible as dark blue bands against a lighter blue background. When the gel is completely destained, larger DNA bands will become sharper and smaller bands will be visible. With additional destaining, the entire background will become uniformly light blue. Destaining time may vary between 20 - 90 minutes.
4. Carefully remove the gel from the destain solution and examine the gel on a Visible Light Gel Visualization System. To optimize visibility, use the amber filter.
5. If the gel is too light and bands are difficult to see, repeat the staining and destaining procedures.

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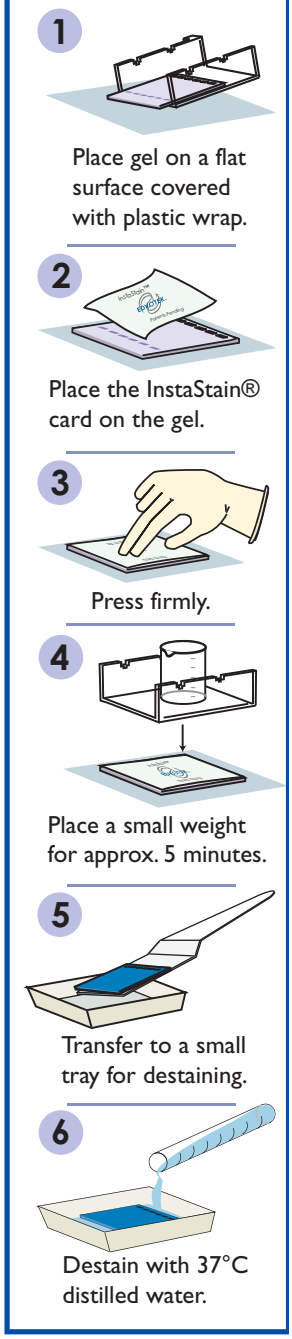
Storage and Disposal of Stain and Gel

See Appendix H.



InstaStain is a registered trademark of EDVOTEK, Inc.

Appendix
H

General Notes on Staining and Destaining with FlashBlue™ Liquid stain and InstaStain® Blue Cards

- 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.

Storage and Disposal of FlashBlue™ Liquid Stain, InstaStain® Blue Cards and Gels

- Stained gels may be stored in the refrigerator for several weeks. Place the gel in a sealable plastic bag with destaining liquid.
- DO NOT FREEZE AGAROSE GELS!
- Used InstaStain® cards and destained gels can be discarded in solid waste disposal.
- Destaining solutions can be disposed down the drain.