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THE BIOTECHNOLOGY EDUCATION COMPANY®

Includes EDVOTEK's All-NEW DNA Standard Marker

- Better separation
- Easier band measurements
- No unused bands

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

REVISED & UPDATED

Edvo-Kit #
206

Edvo-Kit #206

Restriction Enzyme Mapping

Experiment Objective:

In this experiment, students will develop an understanding of plasmid mapping using restriction enzymes. Results are analyzed using agarose gel electrophoresis and a standard curve.

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 Components

	Component	Storage	Check (✓)
A	Plasmid DNA for Restriction Digest	Freezer	❑
B	Restriction Enzyme Reaction Buffer	Freezer	❑
C	UltraPure Water	Freezer	❑
D	Restriction Enzyme Dilution Buffer	Freezer	❑
E	*Eco*RI Dryzyme	Freezer	❑
F	*Bam*HI Dryzyme	Freezer	❑
G	DNA Standard Marker	Freezer	❑

Store the following components at room temperature.

- UltraSpec-Agarose™ ❑
- Electrophoresis Buffer (50x) ❑
- 10x Gel Loading Solution ❑
- InstaStain® Ethidium Bromide ❑
- FlashBlue™ Stain ❑
- Microcentrifuge Tubes with attached caps ❑

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Experiment #206 is designed for 6 groups.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Requirements *(not included with this kit)*

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipets (5-50 µl) with tips
- Waterbath (37° C)
- Balance
- Microwave, hot plate or burner
- 250 ml flasks or beakers
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Distilled or deionized water
- Ice buckets and ice

For gel staining with InstaStain® Ethidium Bromide *(Preferred Method)*:

- UV transilluminator
- UV safety goggles

For gel staining with FlashBlue™ liquid stain:

- Small plastic trays or large weigh boats for destaining
- White light DNA visualization system

Background Information

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

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, and the next two letters are the first two letters of the species name of the organism. Other letters and numerals describe 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 shown in Table 1.)

Table 1: Restriction Enzyme Recognition Sites

Restriction Enzyme	Genus	Species	Strain	Recognition Site
Ava I	*Anabaena*	*variablis*	n/a	C^YCGUG
Bgl I	*Bacillus*	*globigii*	n/a	GCCNNNN^NGGC
*Eco*RI	*Escherichia*	*coli*	RY 13	G^AATTC
*Hae*III	*Haemophilus*	*aegyptius*	n/a	GG^CC
*Hin*dIII	*Haemophilus*	*influenzae*	Rd	A^AGCTT
Sac I	*Streptomyces*	*achromogenes*	n/a	GAGCT^C

Many restriction enzymes require Mg^{2+} for activity and recognize palindromic stretches of DNA, generally 4-8 base pairs long. The probability that a specific 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. In contrast, 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 has one or more restriction sites. For example, imagine *Eco*RI is used to digest human chromosomal DNA containing 3 billion base pairs and a small bacterial plasmid containing 5,000 base pairs. The enzyme 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).

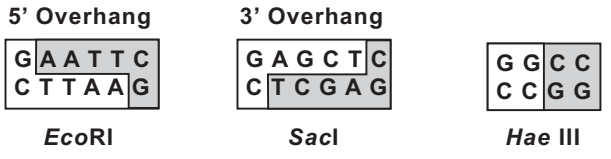
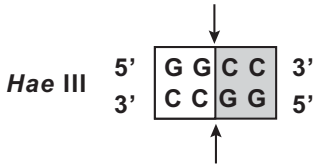


Figure 1: Different types of DNA ends produced by Restriction Enzymes.

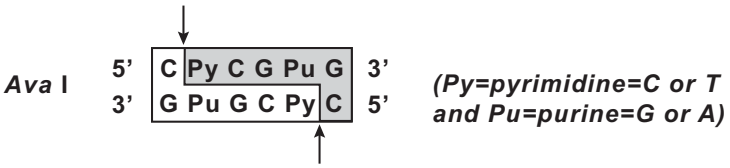
Digestion by a restriction enzyme generates DNA fragments with one of two types of DNA ends--"sticky" or "blunt" (Figure 1). To illustrate this, first consider the recognition site and cleavage pattern of *Eco*RI.

*Eco*RI cleaves between the G and neighboring A, as shown by the arrows in the left side of the figure. It is important to note 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′).

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.

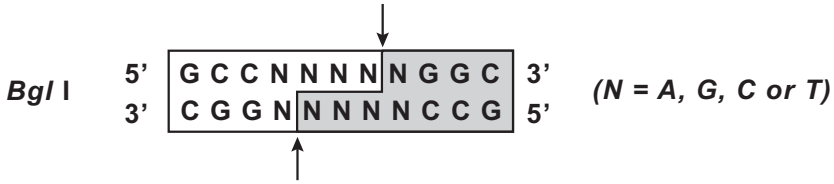


Some restriction enzymes, such as *Ava*I, recognize "degenerate" sites, which contain one or more variable positions.



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 several variable bases.



The six G-C base pairs recognized by *Bgl*I must be separated by five base pairs of DNA, otherwise the enzyme cannot cleave the phosphodiester backbone. Since the sequence of these base pairs is variable, *Bgl*I can recognize and cleave up to 625 possible sequences!

USING RESTRICTION ENZYMES TO MAP PLASMIDS

The locations of the restriction enzyme cleavage sites are important for molecular cloning experiments. This information is very important for downstream applications like creating recombinant DNA constructs. Digestion of plasmid DNA by a restriction enzyme will produce fragments of varying lengths depending on the locations of the recognition sites. This technique is performed in the biotechnology laboratory to create a "map" of the molecule. If we digest a plasmid with several restriction enzymes, we can determine the relative distance between the enzyme sites.

For example, consider a 5000 base pair, circular plasmid DNA containing single recognition sites for three separate enzymes - A, B, and C. When enzymes cut the plasmid, they generate linear DNA molecules. Different combinations of these enzymes will produce the following DNA fragments (in base pairs). Analysis of this data creates a map that explains the locations of the different restriction enzymes in the plasmid. When we analyze this data, we see that the combination of enzymes A and B generated the smallest fragment (500 bp) out of all the double digests. This suggests that the restriction site of B is the closest to that of A. Next, examining the other two double digests, we can determine that the restriction sites for enzymes A and C are 2000 base pairs apart (Figure 2), and that the restriction sites between enzymes B and C are 1500 base pairs apart.

A+B	A+C	B+C	A+B+C
4500	3000	3500	3000
500	2000	1500	1500
			500

Once we have determined the shortest relative distance between each of the restriction sites, we can assign their positions in the plasmid. As a starting point, the cleavage site for enzyme A is assigned to position zero, and the cleavage site for enzyme B is assigned to position 500. We also know the restriction site for enzyme C cannot between A and B, because the 500 base pair fragment remains after the triple digest (A+B+C).

Next, the data from the triple digest shows the 2000 base pair fragment found in the A+C digest is cleaved into 1500 and 500 base pair fragments. This suggests that the restriction site for enzyme B lies between the sites for enzyme A and C. This kind of logic enables the construction of a map from DNA fragment sizes (Figure 3).

If we move in a clockwise direction from A, the distance between A and B is 500 bp, B and C is 1500 bp, and C and A is 3000 bp (Figure 3). Note that the data from this experiment cannot tell us the absolute orientation of the cleavage sites since it can lead to an alternative map as shown in Figure 4. However, the relative positions between the cut sites stay the same (B is between A and C).

Under certain experimental conditions, the results of the restriction digest may complicate analysis of the data. The plasmid DNA may not be fully digested if there if an insufficient amount of enzyme is used or the reaction is stopped after a short time. In these situations, some restriction enzyme sites remain uncut (Figure 5). The partially digested pieces of DNA will create extra bands on the gel, which can complicate analysis in the restriction mapping experiment.

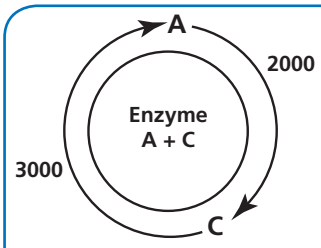


Figure 2: Plasmid Digest with Two Enzymes

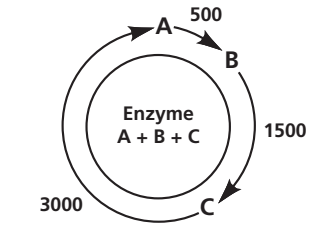


Figure 3: Creating a Restriction Map

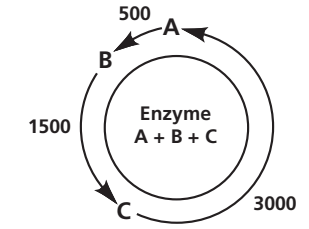


Figure 4: Alternative Restriction Map

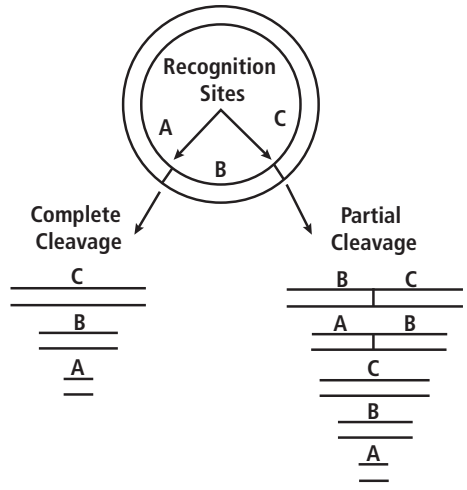


Figure 5: Partial Digest of Plasmid DNA.

ANALYSIS OF RESTRICTION DIGESTS

To analyze the mixture of DNA fragments, scientists use a technique called agarose gel electrophoresis. This technique separates DNA fragments according to size. First, a micropipet is used to dispense digested DNA molecules into depressions (or "wells") within a gel. Once loaded, 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 (Figure 6A).

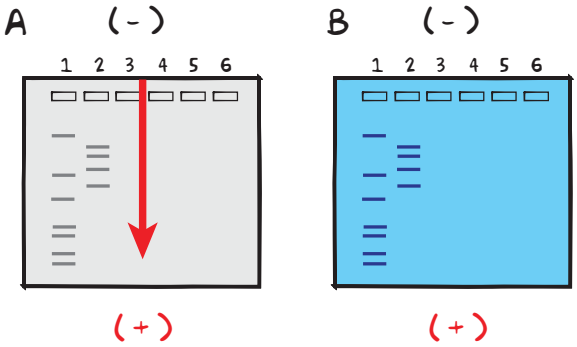


Figure 6: Overview of Agarose Gel Electrophoresis

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 are visualized using a stain that sticks to DNA (Figure 6B).

While electrophoresis is a powerful separation technique, it is not without its technical limitations. For example, unexpected bands may be present in the undigested plasmid DNA sample. This is because plasmids can exist in different conformations. In the cell, plasmid DNA is tightly wound in a compact secondary structure, which allows the DNA to be efficiently packaged. The "supercoiled" DNA will appear smaller than its molecular weight when analyzed by electrophoresis. In contrast, if the DNA backbone is broken during purification, the plasmid will lose its compact structure and run at the appropriate size. A plasmid sample containing both supercoiled and linear DNA will show two distinct bands when analyzed by electrophoresis.

After restriction digest, the plasmid mixture will produce the same series of DNA fragments when analyzed by electrophoresis. However, as mentioned previously, partial digests can complicate analysis in the restriction mapping experiment. Furthermore, if two different fragments are similar in size, they will migrate together through the gel and may appear as a single band. If digestion produces a broad distribution of DNA sizes, the fragments may appear as a smear. If two restriction enzyme sites are close to one another (i.e. within 30 base pairs), the fragment may not be visualized by standard agarose gel electrophoresis protocols.

In this experiment, students will determine the relative locations of restriction enzyme cleavage sites on a plasmid DNA. Each enzyme cuts the plasmid once or twice, but the relative positions of the cuts are unknown. First, students digest the plasmid DNA with different combinations of restriction enzymes. The restriction digests are analyzed using agarose gel electrophoresis. Each group builds a standard curve using a DNA standard marker, which allows them to calculate the lengths of the restriction fragments. Using the data, students create a restriction map that describes the relationship between the restriction enzymes.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students will develop an understanding of plasmid mapping using restriction enzymes. Results are analyzed using agarose gel electrophoresis and a standard curve.

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.

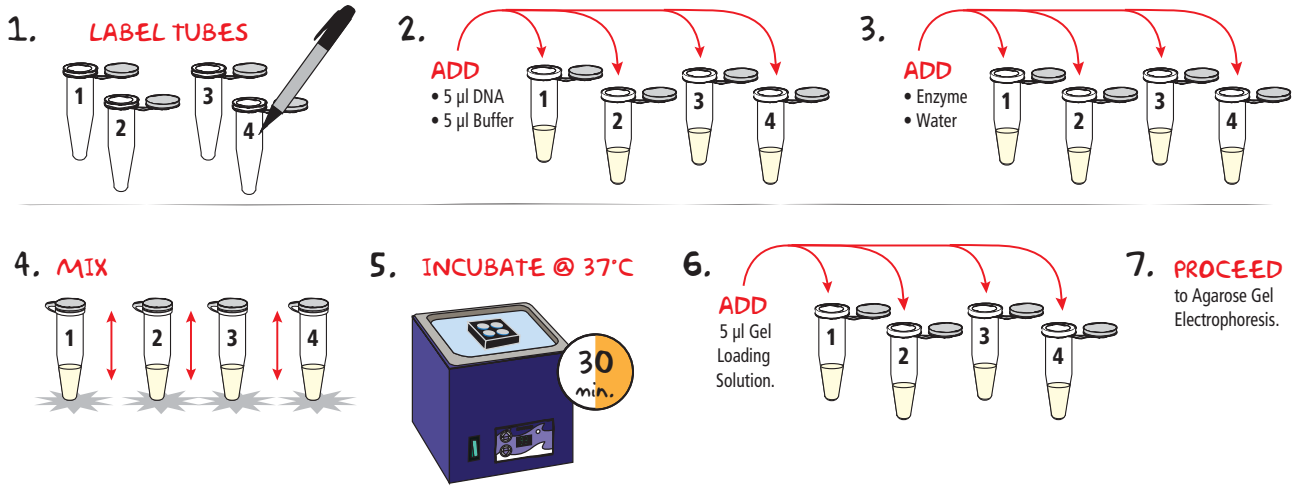
During the Experiment:

- 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: Restriction Enzyme Analysis



1. **LABEL** four 1.5 ml microcentrifuge tubes with your student group and the number 1, 2, 3, and 4.
2. **ADD** 5 µl of DNA and 5 µl of Restriction Enzyme Reaction Buffer to each of the four reaction tubes (1-4).
3. **ADD** water and enzyme to reaction tubes as summarized in the table below. Use a fresh micropipet tip for each transfer of enzyme or water.

Summary of Restriction Enzyme Digestion Reactions

Reaction Tube	Plasmid DNA	Restriction Enzyme Rxn Buff	EcoRI Enzyme	BamHI Enzyme	Ultra Pure Water	Final Reaction Volume
1	5 µl	5 µl	----	----	20 µl	30 µl
2	5 µl	5 µl	5 µl	----	15 µl	30 µl
3	5 µl	5 µl	----	5 µl	15 µl	30 µl
4	5 µl	5 µl	5 µl	5 µl	10 µl	30 µl

IMPORTANT:

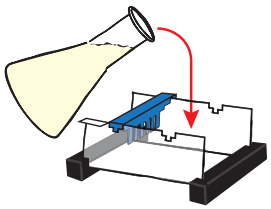
To prevent contamination, be sure to use a fresh pipet tip before going into the enzyme, DNA, and buffer stocks. Keep the enzymes on ice when not in use.

4. Thoroughly **MIX** the restriction digests by pipetting up and down or gently tapping the tubes.
5. **CAP** the tubes and **INCUBATE** the samples at 37°C for 30 minutes.
6. After the incubation, **ADD** 5 µl of 10x gel loading solution to each reaction tube. **CAP** the tubes and **MIX** by tapping the tube or vortexing vigorously.
7. **PROCEED** to Module II – Agarose Gel Electrophoresis.

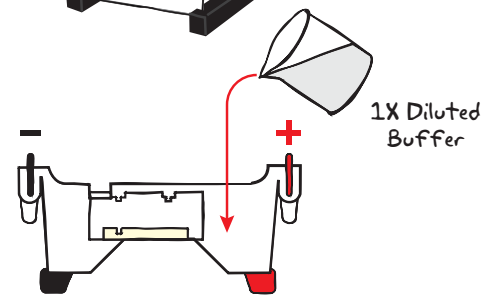
OPTIONAL STOPPING POINT:
The restriction digest samples can be stored at -20°C for electrophoresis at a later time.

Overview of Agarose Gel Electrophoresis & Staining

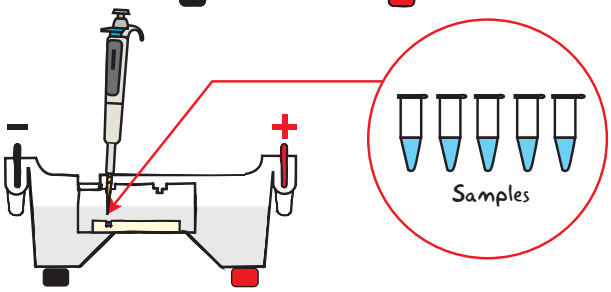
1 Prepare agarose gel in casting tray.



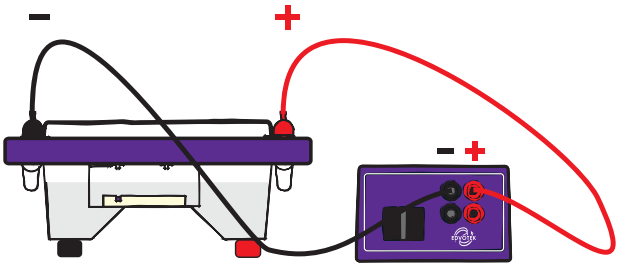
2 Remove end caps & comb. Place tray into the electrophoresis chamber. Add 1x buffer into the chamber to submerge the gel.



3 Using a variable micropipet, load each sample into consecutive wells.



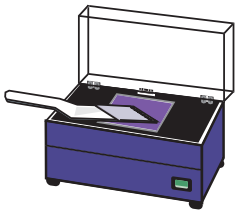
4 Attach safety cover, connect leads to power source and conduct electrophoresis.



5 After electrophoresis, transfer gel for staining with either InstaStain® Ethidium Bromide (Preferred) or FlashBlue™ Stain.

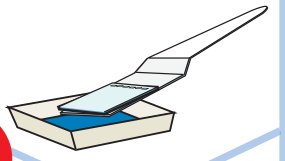
6 Visualize results on a U.V. Trans-illuminator (InstaStain® Ethidium Bromide) or a white light system (FlashBlue™ Stain).

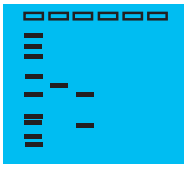
Preferred Method
InstaStain® Ethidium Bromide



or

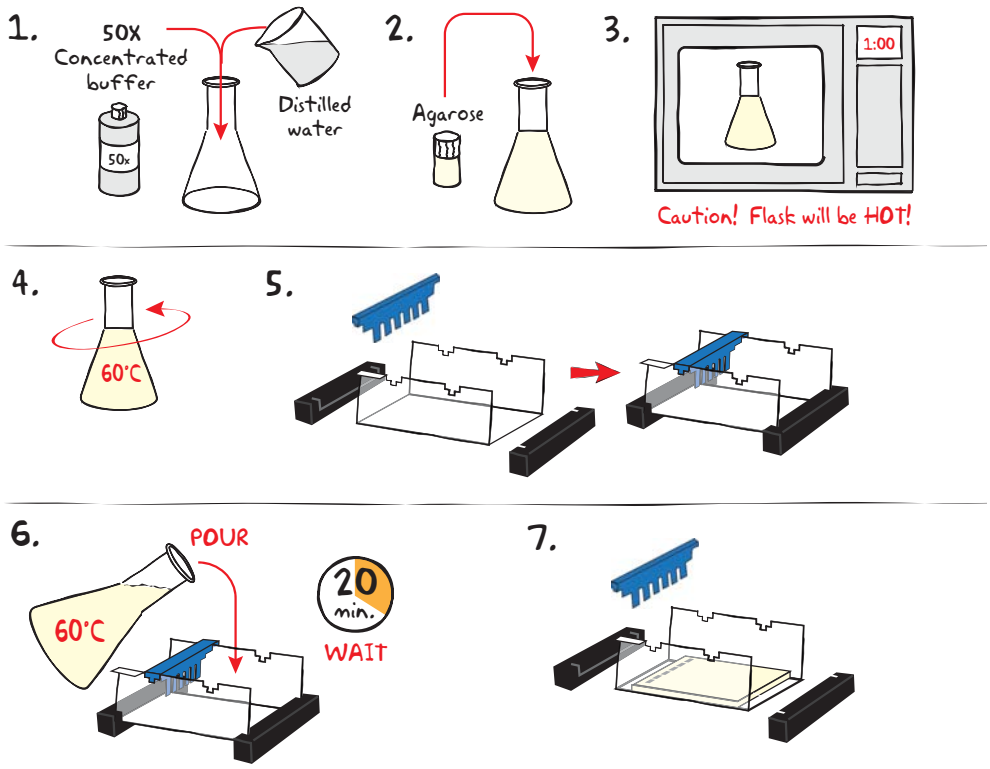
FlashBlue™ Stain





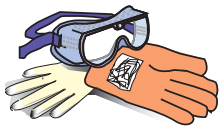
Gel pattern will vary depending upon experiment.

Module II: Agarose Gel Electrophoresis



IMPORTANT:
7 x 7 cm gels are recommended. Each gel can be shared by 1 student group. Place well-former template (comb) in the first set of notches.

If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at **www.edvotek.com**



Wear gloves and safety goggles

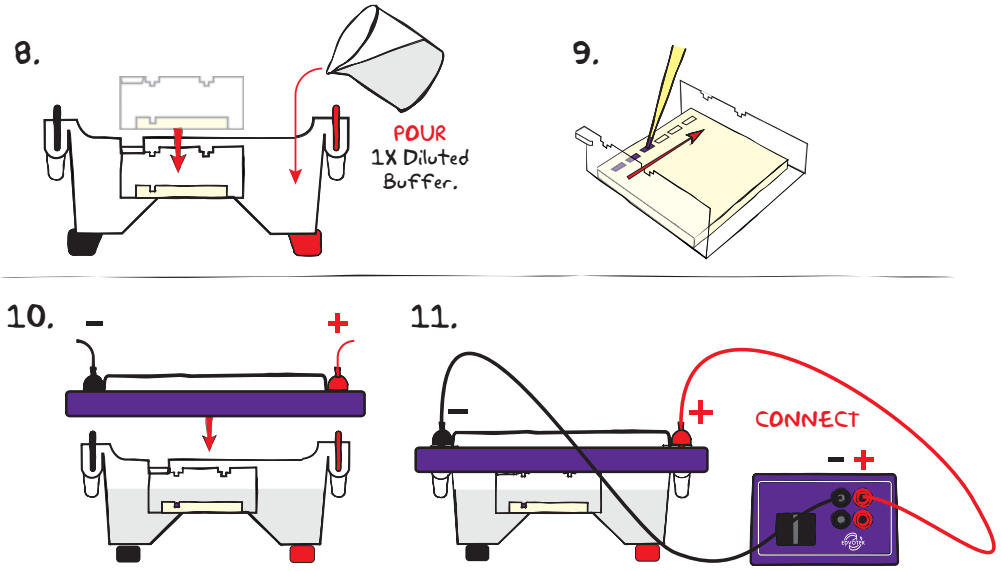
CASTING THE AGAROSE GEL

1. **DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
2. **MIX** agarose powder with 1X buffer in a 250 ml flask (see Table A).
3. **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).
4. **COOL** agarose to 60° C with careful swirling to promote even dissipation of heat.
5. 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.
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. Carefully **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Table A — Individual 0.8% UltraSpec-Agarose™ Gel

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

Module II: Agarose Gel Electrophoresis, continued



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

Wear gloves and safety goggles

RUNNING THE GEL

8. **PLACE** gel (on the casting tray) into electrophoresis chamber. **POUR** 1X Diluted Chamber Buffer into the electrophoresis chamber (See Table B for recommended volumes). Completely **SUBMERGE** the gel.
9. **LOAD** the entire sample volumes into the wells in consecutive order as indicated by Table 3.
10. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the 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). For best results, the blue tracking dye should migrate at least 3.5 cm from the wells.
12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and proceed to **STAINING** the agarose gel.

Table 3: Gel Loading

Lane 1	DNA Standard Marker
2	Reaction Tube 1
3	Reaction Tube 2
4	Reaction Tube 3
5	Reaction Tube 4

Table B 1x Electrophoresis Buffer (Chamber Buffer)

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

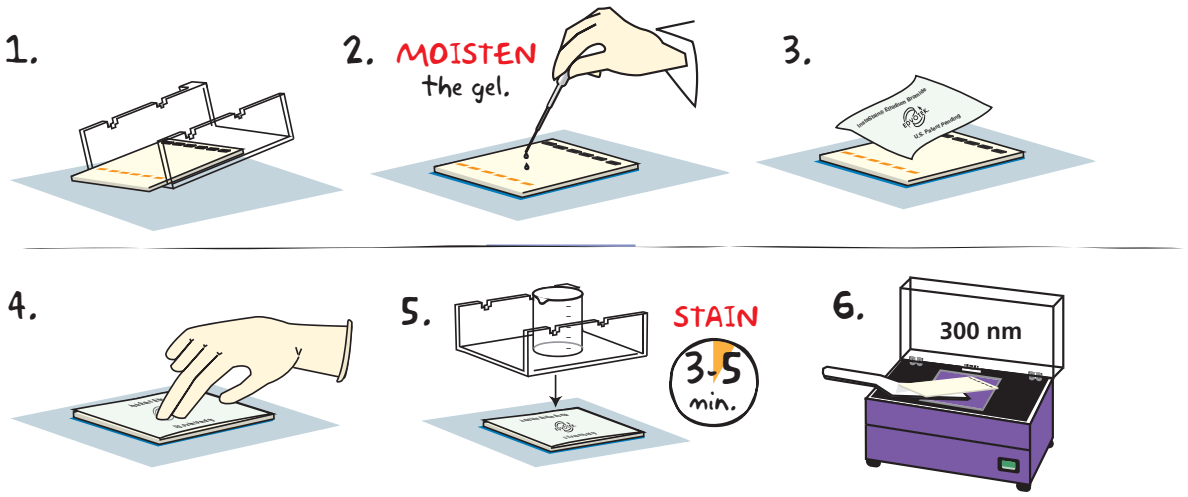
Table C Time & Voltage Guidelines (0.8% Agarose Gel)

	Electrophoresis Model		
Volts	M6+ Min. / Max.	M12 (new) Min. / Max.	M12 (classic) & M36 Min. / Max.
150	15/20 min.	20/30 min.	25 / 35 min.
125	20/30 min.	30/35 min.	35 / 45 min.
75	35 / 45 min.	55/70 min.	60 / 90 min.

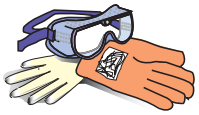
Module III-A: Staining Agarose Gels with InstaStain® Ethidium Bromide

InstaStain® ETBR

PREFERRED METHOD



1. Carefully **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray on to a piece of plastic wrap on a flat surface. **DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.**
2. **MOISTEN** the gel with a few drops of electrophoresis buffer.
3. Wearing gloves, **REMOVE** and **DISCARD** the clear plastic protective sheet from the unprinted side of the InstaStain® card(s). **PLACE** the unprinted side of the InstaStain® Ethidium Bromide card(s) on the gel. You will need one card to stain a 7 x 7 cm gel.
4. With a gloved hand, **REMOVE** air bubbles between the card and the gel by firmly running your fingers over the entire surface. Otherwise, those regions will not stain.
5. **PLACE** the casting tray on top of the gel/card stack. **PLACE** a small weight (i.e. an empty glass beaker) on top of the casting tray. This ensures that the InstaStain® Ethidium Bromide card is in direct contact with the gel surface. **STAIN** the gel for 3-5 minutes.
6. **REMOVE** the InstaStain® Ethidium Bromide card(s). **VISUALIZE** the gel using a mid-range ultraviolet transilluminator (300 nm). DNA should appear as bright orange bands on a dark background.

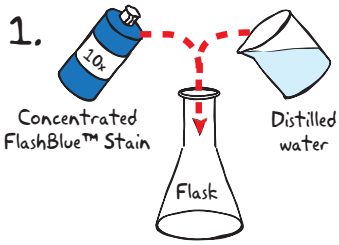


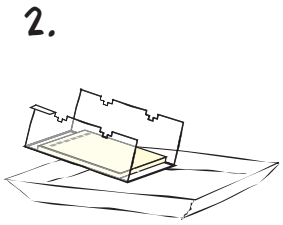
Wear gloves and UV safety goggles

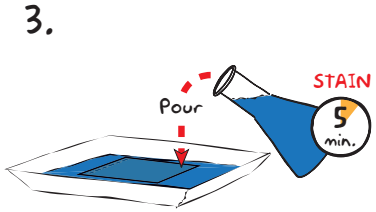
BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!

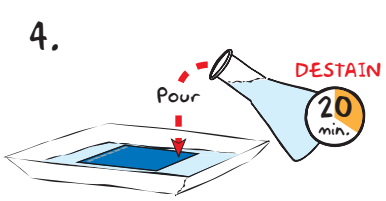
EDVOTEK®

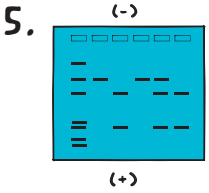
Module III-B: Staining Agarose Gels with FlashBlue™ Stain











1. **DILUTE** 10 ml of 10x concentrated FlashBlue™ with 90 ml of water in a flask and **MIX** well.
2. **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray into a small, clean gel-staining tray.
3. **COVER** the gel with the 1x FlashBlue™ stain solution. **STAIN** the gel for 5 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 5 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
4. **TRANSFER** the gel to a second small tray. **COVER** the gel with water. **DESTAIN** for at least 20 minutes with gentle shaking (longer periods will yield better results). Frequent changes of the water will accelerate destaining.
5. 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.

ALTERNATIVE PROTOCOL:

1. **DILUTE** one ml of concentrated FlashBlue™ stain with 149 ml dH_2O.
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.

Module IV: Size Determination of DNA Restriction Fragments

Agarose gel electrophoresis separates cut DNA into discrete bands, each comprising molecules of the same size. How can these results be used to determine the lengths of fragments in the restriction digests? Remember, as the length of a DNA molecule increases, the distance to which the molecule can migrate decreases because large DNA fragments cannot pass through the channels in the gel with ease. Therefore, the migration rate is inversely proportional to the length of the DNA fragment—more specifically, to the $\log_{10}$ of fragment length. To illustrate this, we ran a sample that contains DNA strands of known lengths called a "standard". We will measure the distance that each of these bands traveled to create a graph, known as a "standard curve", which can then be used to extrapolate the size of unknown DNA fragments.

Figure 7: Measure distance migrated from the lower edge of the well to the lower edge of each band.

1. MEASURE AND RECORD DISTANCES Using Standard DNA Fragments

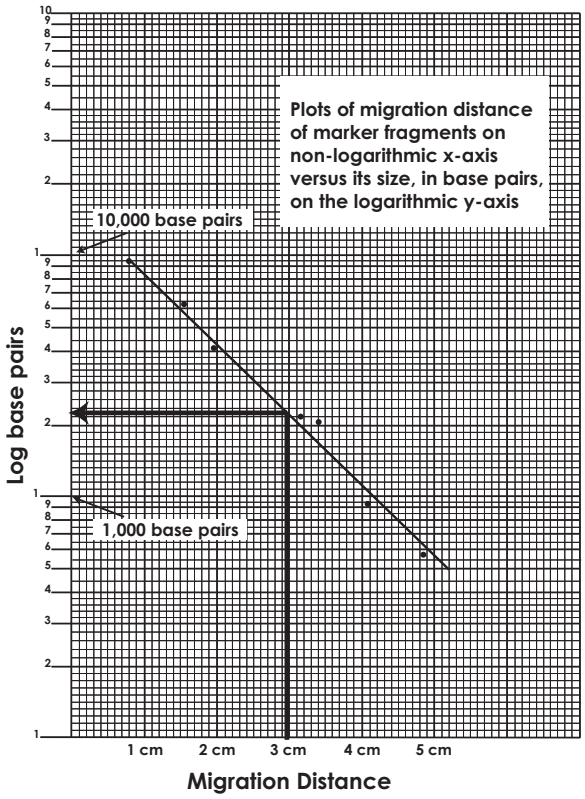
Measure the distance traveled by each Standard DNA Fragment from the lower edge of the sample well to the lower end of each band. Record the distance in centimeters (to the nearest millimeter) in your notebook. Repeat this for each DNA fragment in the standard.

2. GENERATE A STANDARD CURVE

Because migration rate is inversely proportional to the $\log_{10}$ of DNA length, plotting the data as a semi-log plot will produce a straight line and allow us to analyze an exponential range of fragment sizes. You will notice that the vertical axis of the semi-log plot appears atypical at first; the distance between numbers shrinks as the axis progresses from 1 to 9. This is because the axis represents a logarithmic scale. The first cycle on the y-axis corresponds to lengths from 100-1,000 base pairs, the second cycle measures 1,000-10,000 base pairs, and so on. To create a standard curve on the semi-log paper, plot the distance each Standard DNA fragment migrated on the x-axis (in mm) versus its size on the y-axis (in base pairs). Be sure to label the axes!

After all the points have been plotted, use a ruler or a straight edge to draw the best straight line possible through the points. The line should have approximately equal numbers of points scattered on each side of the line. It is okay if the line runs through some points (see Figure 8 for an example).

Figure 8: Semilog graph example

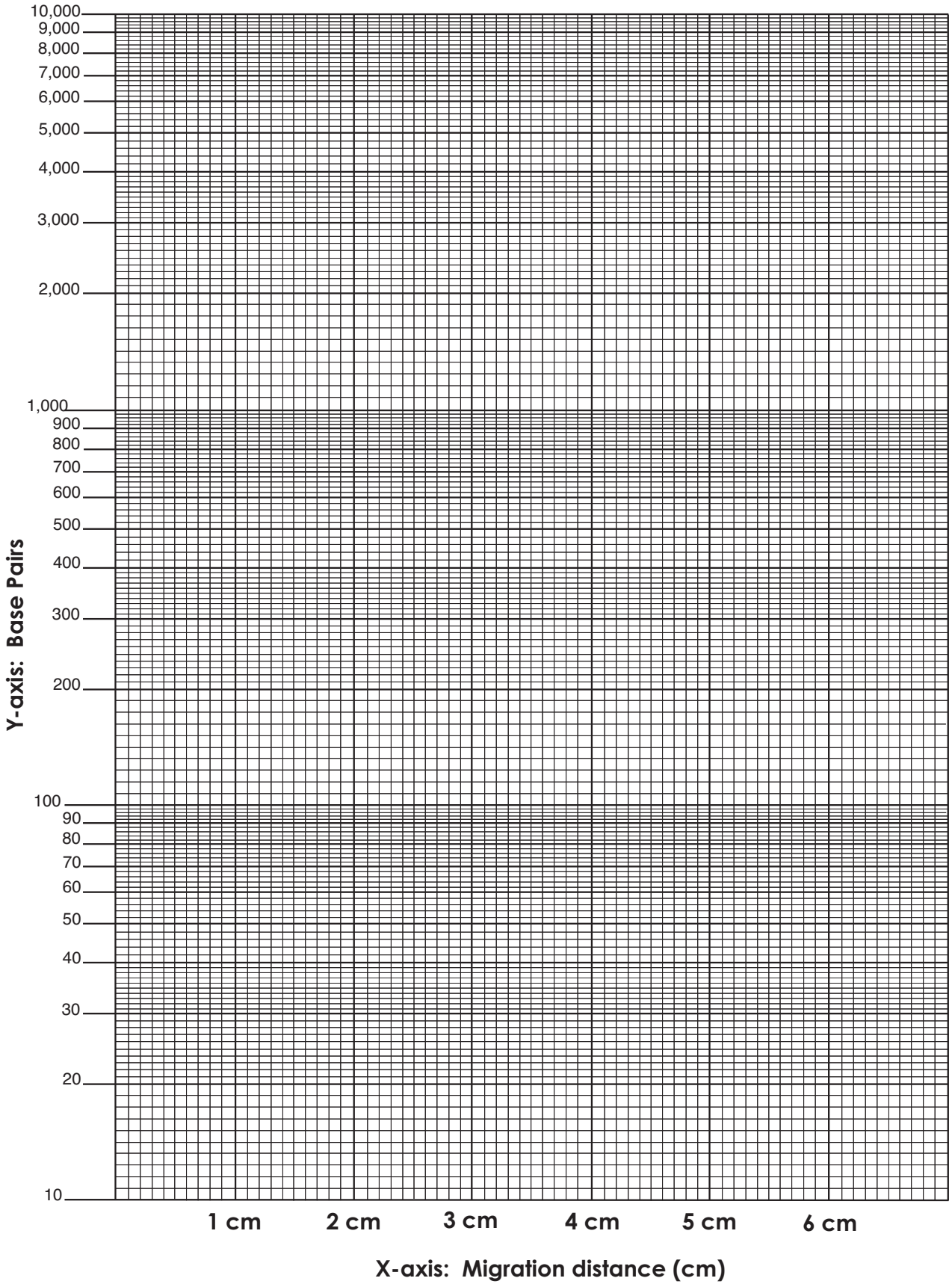


3. DETERMINE THE LENGTH OF EACH UNKNOWN FRAGMENT

a. Locate the migration distance of the unknown fragment on the x-axis of your semi-log graph. Draw a vertical line extending from that point until it intersects the line of your standard curve.
b. From the point of intersection, draw a second line, this time horizontally, toward the y-axis. The value at which this line intersects the y-axis represents the approximate size of the fragment in base pairs (refer to Figure 8 for an example). Make note of this in your lab notebook.
c. Repeat for each fragment in your unknown sample.

Quick Reference:
DNA Standard Marker sizes - length is expressed in base pairs.

6751, 3652, 2827, 1568, 1118, 825, 630



Study Questions

1. The restriction enzyme *Sau*3AI recognizes the following sequence: 5'-GATC-3'. On average, how often should this enzyme cleave DNA? In contrast, the restriction enzyme *Not*I recognizes the following sequence: 5'-GCGGCCGC-3'. On average, how often should this enzyme cleave DNA? Does *Not*I cleave DNA more frequently than *Sau*3AI?

2. An uncharacterized plasmid DNA was cleaved using several restriction enzymes individually and in various combinations. The DNA fragment sizes were determined by agarose gel electrophoresis and the restriction enzyme recognition sites were mapped. Subsequently, the DNA was sequenced and an extra recognition site was found for one of the enzymes. However, all the other mapping data was consistent with sequence data. What are the simplest explanations for this discrepancy? Assume the DNA sequence had no errors.

3. A plasmid was cleaved with several restriction enzymes, individually and in combinations. The following fragment sizes (base pairs) were determined by agarose gel electrophoresis.

Eco RI	4363	
Ava I	2182	
Pvu II	4363	
Pst I	4363	
Eco RI - *Ava* I	2938	1425
Eco RI - *Pst* I	3609	754
Ava I - *Pvu* II	3722	641
Ava I - *Pst* I	2182	
Pvu II - *Pst* I	2820	1543
Eco RI - *Pvu* II	2297	2066

   Make a restriction map based on this data.

   Note: There may be some slight discrepancy in summing up the total base pairs. Indicate the distances between sites. Why is only one band detected in the *Ava* I - *Pst* I co-digest?

4. Create a table detailing the results from the restriction digest experiments. Using this data, draw a restriction map of the plasmid.

Instructor's Guide

ADVANCE PREPARATION:

Preparation For:	What to do:	When:	Time Required:
Module I: Restriction Enzyme Analysis	Prepare and aliquot reagents	One day to 30 minutes before performing the experiment.	20 min.
	Equilibrate water bath	One to two hours before the experiment.	10 min.
	Prepare and aliquot restriction enzymes	No more than 30 minutes before use.	30 min.
Module II: Agarose Gel Electrophoresis	Prepare diluted electrophoresis buffer	Any time before the class period.	10 min.
	Prepare molten agarose and pour gels	One day to 30 minutes before performing the experiment.	45 min.
Module III: Staining Agarose Gels	Prepare staining components	The class period or overnight before the class period.	10 min.

Module I: Pre-Lab Preparations

Prepared reagents can be stored on ice if prepared on the day of the lab. If prepared in advance, store the reagents in the freezer (-20°C). Thaw completely before using.

1. Thaw tubes of Plasmid DNA for Restriction Digest (A), Restriction Enzyme Reaction Buffer (B), and UltraPure Water (C).
2. Dispense 25 µl of the Plasmid DNA for Restriction Digest (A) into 6 appropriately labeled microcentrifuge tubes. Cap the tubes.
3. Dispense 25 µl of the Restriction Enzyme Reaction buffer (B) into 6 appropriately labeled microcentrifuge tubes. Cap the tubes.
4. Dispense 70 µl of UltraPure Water (C) into 6 appropriately labeled microcentrifuge tubes. Cap the tubes.
5. Dispense 25 µl of 10x Gel Loading Solution into 6 appropriately labeled microcentrifuge tubes. Cap the tubes.

DAY OF THE LAB

Equilibrate a 37° C water bath.

Preparation of Dryzyme™ Restriction Enzymes

Perform the restriction digest within 30 minutes of reconstituting Dryzyme Restriction Enzymes (Components E and F).

1. Thaw UltraPure Water (C) and Restriction Enzyme Dilution Buffer (D) and place on ice.
2. Make sure that the solid material is at the bottom of the Dryzyme™ tubes (E and F). If not, gently tap the tube on the tabletop or centrifuge to collect the material at the bottom of the tube.
3. Within 30 minutes of starting the Module I experiment, add 75 µl Restriction Enzyme Dilution Buffer to the solid at the bottom of the Dryzyme™ tube and allow the sample to hydrate for one minute. Mix the sample vigorously by flicking the tube with your finger or vortexing for at least 30 seconds. Continue to mix until the solid appears to be completely dissolved. ***At this point, the enzyme can no longer be stored.*** It must be used as soon as possible. Keep the tube on ice until use.
4. Slowly add 75 µL UltraPure Water (C) to each tube of rehydrated Dryzyme™.
5. After adding the water, thoroughly mix the sample by vortexing or by pipetting up and down for twenty seconds.
6. Dispense 15 µl of the reconstituted *Eco*RI enzyme to 6 appropriately labeled microcentrifuge tubes. Repeat with *Bam*HI. Place tubes on ice. Use within 30 minutes.

Summary of Reagent Preparation

Component	Number of tubes	Volume per tube
Plasmid DNA	6	25 µl
Restriction Enzyme Reaction Buffer	6	25 µl
UltraPure Water	6	70 µl
Eco RI	6	15 µl
Bam HI	6	15 µl
10x Gel Loading Buffer	6	25 µl

FOR MODULE I
Each Group Requires:
1 tube of Plasmid DNA
1 tube of Reaction Enzyme Rxn Buffer
1 tube of UltraPure Water
1 tube of *Eco*RI (on ice)
1 tube of *Bam*HI (on ice)
1 tube of 10X Gel Loading Dye
4 microcentrifuge tubes
Transfer pipets OR micropipet with tips

Module II: Pre-Lab Preparations

AGAROSE GEL ELECTROPHORESIS

Each group will use one 0.8% gel with 6 sample wells for electrophoresis. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Individual Gel Preparation:

Before electrophoresis, each group should receive 40 µl DNA Standard Marker. Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Module I in the Student's Experimental Procedure. Students will need 50x Electrophoresis Buffer, distilled water and agarose powder.

Batch Gel Preparation:

To save time, a larger quantity of agarose solution can be prepared for sharing by the class. Electrophoresis buffer can also be prepared in bulk. See Appendix B.

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 freeze gels at -20° C as 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 molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

Additional Materials:

Aliquot 40 µl of DNA standard marker per student group.

NOTE:
Accurate pipetting is critical for maximizing successful experiment results. EDVOTEK 200 Series experiments are designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students are unfamiliar with using micropipets, we recommended performing Cat. #S-44, Micropipetting Basics or Cat. #S-43, DNA DuraGel™ prior to conducting this advanced level experiment.

FOR MODULE II
Each Student Group should receive:

- 50x Electrophoresis Buffer
- Distilled Water
- UltraSpec-Agarose™
- 35 µl DNA Standard Marker

NOTE:
QuickGuide instructions and guidelines for casting various agarose gels can be found our website.
www.edvotek.com/quick-guides

MODULE III: Pre-Lab Preparations

STAINING AGAROSE GELS

InstaStain® Ethidium Bromide *(PREFERRED METHOD)*

InstaStain® Ethidium Bromide provides the sensitivity of ethidium bromide while minimizing the volume of liquid waste generated by staining and destaining a gel. An agarose gel stained with InstaStain® Ethidium Bromide is ready for visualization in as little as 3 minutes! Each InstaStain® card will stain 49 cm^2 of gel (7 x 7 cm).

FOR MODULE III-A
Each Student Group should receive:
- 1 InstaStain® card per 7 x 7 cm gel

Use a mid-range ultraviolet transilluminator (Cat. #558) to visualize gels stained with InstaStain® Ethidium Bromide. **BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!**

Wear gloves and safety goggles

- Standard DNA markers should be visible after staining even if other DNA samples are faint or absent. If bands appear faint, repeat staining with a fresh InstaStain card for an additional 3-5 min. If markers are not visible, troubleshoot for problems with electrophoretic separation.
- Ethidium bromide is a listed mutagen. Wear gloves and protective eyewear when using this product. UV protective eyewear is required for visualization with a UV transilluminator.
- InstaStain® Ethidium Bromide cards and stained gels should be discarded using institutional guidelines for solid chemical waste.

FlashBlue™

FlashBlue™ can be used as an alternative to Ethidium Bromide in this experiment. However, FlashBlue™ is less sensitive than InstaStain® Ethidium Bromide and will take a longer time to obtain results.

FOR MODULE III-B
Each Student Group should receive:
- 10 ml 10X concentrated FlashBlue OR 100 mL 1x diluted FlashBlue
- Small plastic tray or weight boat
- Distilled or deionized water

FlashBlue™ stain, however, is optimized to shorten the time required for both staining and destaining steps. Agarose gels can be stained with diluted FlashBlue™ for 5 minutes and destained for only 20 minutes. For the best results, leave the gel in liquid 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. A white light box (Cat. #552) is recommended for visualizing gels stained with FlashBlue™.

- Stained gels may be stored in destaining liquid for several weeks with refrigeration, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels can be discarded in solid waste disposal. Destaining solutions can be disposed of down the drain.

Photodocumentation of DNA (Optional)

Once gels are stained, you may wish to photograph your results. There are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.

Please refer to the kit insert for the Answers to Study Questions

Appendices

A EDVOTEK® Troubleshooting Guide

B Bulk Preparation of Agarose Gels

Safety Data Sheets:

Now available for your convenient download on **www.edvotek.com/safety-data-sheets**

Appendix A

EDVOTEK® Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
The DNA did not digest	The restriction enzymes were not active.	Be sure that the restriction enzymes were diluted in the correct buffer.
		For optimal activity, prepare the enzymes within 30 minutes of use.
There are bands on my gels that can't be explained by the restriction digests.	Some bands may represent partially digested DNA.	The sample was not digested at the right temperature.
		The sample was not digested for the appropriate amount of time.
The ladder and student samples are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		Gels of higher concentration (>0.8%) require special attention when melting the agarose. Make sure that the solution is completely clear of "clumps" and glassy granules before pouring gels.
	The gel was not stained properly.	Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel, the DNA bands are faint.	The gel was not stained for a sufficient period of time.	Repeat staining protocol.
	DNA stained with FlashBlue or InstaStain Blue may fade over time.	Re-stain the gel with FlashBlue or InstaStain Blue.
	The background of the gel is too dark.	Destain gel for 5-10 minutes in distilled water.
After staining the gel, the ladder and control samples are visible on gel, but some student samples are not present.	Wrong volumes of DNA and enzyme added to restriction digest.	Practice using pipettes.
There is no separation between DNA bands, even though the tracking dye ran the appropriate distance.	The wrong percent gel was used for electrophoretic separation.	Be sure to prepare the correct percent agarose gel. For reference, the DNA samples should be analyzed using a 0.8% agarose gel.
DNA bands were not well resolved.	Tracking dye should migrate at least 3.5 cm from the wells to ensure adequate separation.	Be sure to run the gel at least 3.5 cm before staining and visualizing the DNA .

Appendix B
Bulk Preparation of Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the 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

50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 ml		2,940 ml	3000 ml (3 L)

Batch Agarose Gels (0.8%)

For quantity (batch) preparation of 0.8% agarose gels, see Table E.

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. Measure 30 ml for a 7 x 7 cm tray, 50 ml for a 7 x 10 cm tray, and 60 ml for a 7 x 14 cm tray. ***For this experiment, 7 x 7 cm gels are recommended.***
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.

Note:
The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. 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.

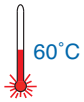


Table E — Batch Prep of 0.8% UltraSpec-Agarose™

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