EDVOTEK®

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 and Updated

Edvo-Kit #

212

Edvo-Kit #212

Cleavage of Lambda DNA with *Eco*RI Restriction Enzyme

Experiment Objective:

The objective of this experiment is to develop an understanding of restriction enzymes and agarose gel electrophoresis.

See page 3 for storage instructions.

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

Experiment Components

	Component	Storage	Check (✓)
A	Lambda phage DNA	-20° C Freezer	❑
B	Concentrated Restriction Enzyme Reaction Buffer	-20° C Freezer	❑
C	EDVOTEK® enzyme grade water	-20° C Freezer	❑
D	*Eco* RI Dryzymes™ endonuclease	-20° C Freezer with desiccant	❑
E	DNA Standard Marker	-20° C Freezer	❑
F	Reconstitution Buffer F	-20° C Freezer	❑
G	Reconstitution Buffer G	-20° C Freezer	❑

Store the following components at room temperature.

- 10x Gel Loading Solution ❑
- Practice Gel Loading Solution ❑
- UltraSpec-Agarose™ powder ❑
- 50x concentrated electrophoresis buffer ❑
- FlashBlue™ DNA Stain ❑
- InstaStain™ Blue cards ❑
- 1 ml pipets ❑
- Microtipped Transfer pipets ❑
- Microtest tubes with attached caps ❑
- Semi-log graph paper template ❑

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Experiment #212 contains biologicals for 10 groups to perform restriction enzyme digestion of Lambda DNA with *Eco* RI, and electrophoresis reagents sufficient for five gels.

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 and tips
- Waterbath (37°C & 65° C)
- Small plastic trays or large weigh boats for gel staining
- DNA Visualization System (visible light)
- Microwave, hot plate or burner
- Hot Gloves
- Pipet Pumps or bulbs
- 5 or 10 ml pipets
- 250 ml flasks or beakers
- 500 ml graduated cylinder
- Disposable gloves and safety goggles
- Distilled or Deionized Water
- Ice
- Metric rulers

Background Information

AN INTRODUCTION TO RESTRICTION ENZYMES

The discovery of restriction enzymes ushered in a new era of molecular genetics. These enzymes gave us the ability to cut DNA in a highly specific and reproducible way. This, in turn, ushered in the area of molecular cloning, mapping and sequencing the fine genetic structure. These procedures, in turn, made the Human Genome Project possible.

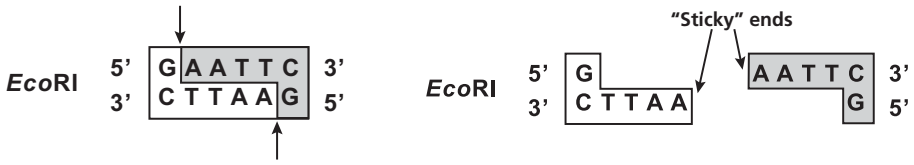
Restriction enzymes are endonucleases which catalyze the cleavage of the phosphate bonds within both strands of DNA. They require Mg^{+2} for activity and generate a 5 prime (5') phosphate and a 3 prime (3') hydroxyl group at the point of cleavage. The distinguishing feature of restriction enzymes is that they only cut at very specific palindromic sequences of bases. Restriction enzymes are produced by many different species of bacteria (including blue-green algae). Over 1500 restriction enzymes have been discovered and catalogued. More recently, intron-coded yeast mitochondrial endonucleases have been discovered that also cut DNA. The recognition sequences for these enzymes are such that they yield very few cuts in DNA and promise to be important new biological reagents.

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
*Hind*III	*Haemophilus*	*influenzae*	R_d	A^AGCTT
Sac I	*Streptomyces*	*achromogenes*	n/a	GAGCT^C

Table 1

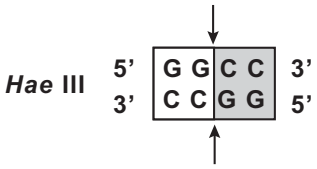
Restriction enzymes are named according to the organism from which they are isolated. This is done by using the first letter of the genus followed by the first two letters of the species. Only certain strains or sub-strains of a particular species may produce restriction enzymes. The type of strain or substrain sometimes follows the species designation in the name. Finally, a Roman numeral is always used to designate one out of possibly several different restriction enzymes produced by the same organism or by different substrains of the same strain.

A restriction enzyme requires a specific double-stranded recognition sequence of nucleotide bases to cut DNA. Recognition sites are usually 4 to 8 base pairs in length. Cleavage occurs within or near the site. The cleavage positions are indicated by arrows. Recognition sites are frequently symmetrical, i.e., both DNA strands in the site have the same base sequence when read 5' to 3'. Such sequences are called palindromes. Consider the recognition site and cleavage pattern of *Eco* RI as an example.

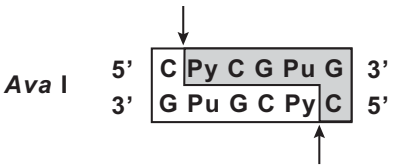


As you can see, *Eco* RI causes staggered cleavage of its site. The resulting ends of the DNA fragments are called "sticky" or "cohesive" because the single-stranded regions of the ends are complementary.

Some restriction enzymes, such as *Hae* III, introduce cuts that are opposite each other. This type of cleavage generates "blunt" ends.



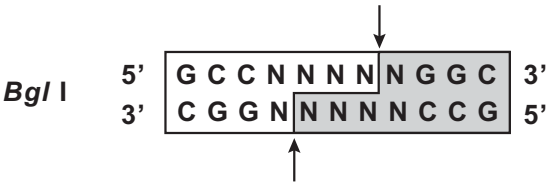
The recognition sites of some restriction enzymes contain variable base positions. For example, *Ava* I recognizes:



(Py=pyrimidine=C or T and Pu=purine=G or A)

Keep in mind that A pairs with T and G pairs with C. Consequently, there are four possible sequences *Ava* I recognizes. Recognition sites of this type are called degenerate.

There are some recognition sites that are divided by a certain number of totally variable bases. For example, *Bgl* I recognizes:



(N = A, G, C or T)

There are 625 possible sequences *Bgl* I can cleave. The only bases the enzyme truly "recognizes" are the six G-C base pairs at the ends, which form a palindrome. In the case of *Bgl* I, these true recognition bases must always be separated by 5 base pairs of DNA; otherwise, the enzyme cannot properly interact with the DNA and cleave it. Recognition sites like that of *Bgl* I are called hyphenated sites.

In general, the longer the DNA molecule, the greater the probability that a given recognition site will occur. Statistically, an enzyme will average one cut for every 4^n base pairs, where n is the length of the recognition site in base pairs. Thus, an enzyme that recognizes four nucleotides will cut DNA on average once every 256 (or 4^4) base pairs, while an enzyme that recognizes five base pairs will cut DNA once every 1024 base pairs (or 4^5). Chromosomal DNA, which can contain billions of base pairs, has many more recognition sites than a plasmid DNA containing only a few thousand base pairs. However, very large DNA is difficult to isolate intact. During handling, it is randomly sheared to fragments in the range of 50,000 to 100,000 base pairs.

Plasmids and many viral DNAs are circular molecules. If circular DNA contains one recognition site for a restriction enzyme, then it will open up to form a linear molecule when cleaved. By contrast, if a linear DNA molecule contains a single recognition site, it will be cleaved once to form 2 fragments. The size of the fragments produced depends on how far the sites are from each other. If a DNA molecule contains several recognition sites for a restriction enzyme, then under certain experimental conditions, it is possible that certain sites are cleaved and not others. These incompletely cleaved fragments of DNA are called partials. Partials can arise if low amounts of enzyme are used or the reaction is stopped after a short time. In reality, reactions containing partials also contain some molecules that have been completely cleaved.

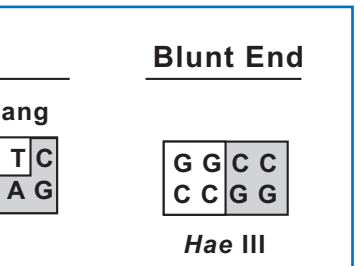


Figure 1: Different types of DNA ends produced by Restriction Enzymes.

Agarose gel electrophoresis is a powerful separation method frequently used to analyze DNA fragments generated by restriction enzymes. The gel consists of microscopic pores that act as a molecular sieve. Samples of DNA are loaded into wells made in the gel during casting. Direct current is then applied. Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode during electrophoresis. Linear DNA molecules are separated according to their size. The smaller the linear fragment, the faster it migrates. If the size of two fragments are similar or identical, they will migrate together in the gel. This is called a doublet. If DNA is cleaved many times the wide range of fragments produced will appear as a smear after electrophoresis. Other forms of DNA, such as circular and superhelical, are separated in the gel according to their size and shape.

The Lambda DNA used in this experiment is isolated as a linear molecule from the *E. coli* bacteriophage lambda. It contains approximately 49,000 base pairs and has 5 recognition sites for *Eco* RI, and 7 for *Hin*d III. The smaller fragments generated by a restriction enzyme, such as those generated by *Hin*d III, may not be visible after agarose gel electrophoresis. Since there is less mass in the bands containing smaller fragments, they stain with less intensity and may be undetectable.

Lambda phage DNA contains 10-16 base single-stranded regions at the 5' and 3' terminus which are self-complementary, called cos ends. To properly resolve lambda phage DNA fragments, they must be heated to 65° C before loading onto the gel. For example, the 4361 and 23130 base pair fragments will hybridize at the "cos" sites, and the amount of the 4361 base pair fragment will be decreased and hard to visualize on the stained gel.

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is to develop an understanding of restriction enzymes and agarose gel electrophoresis.

LABORATORY SAFETY

Wear gloves and safety goggles

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

LABORATORY NOTEBOOKS:

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

Before starting the Experiment:

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

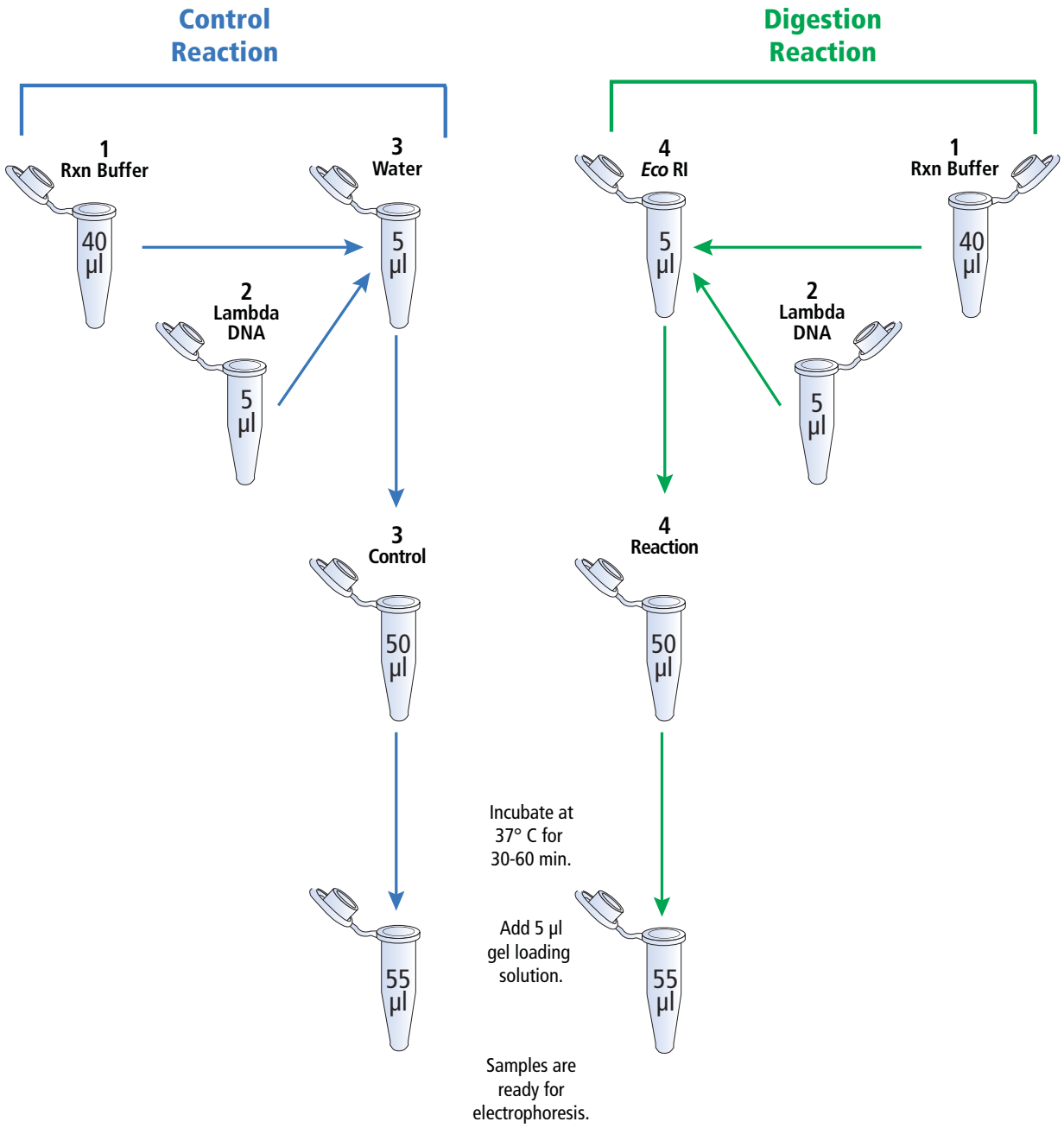
During the Experiment:

- Record your observations.

After the Experiment:

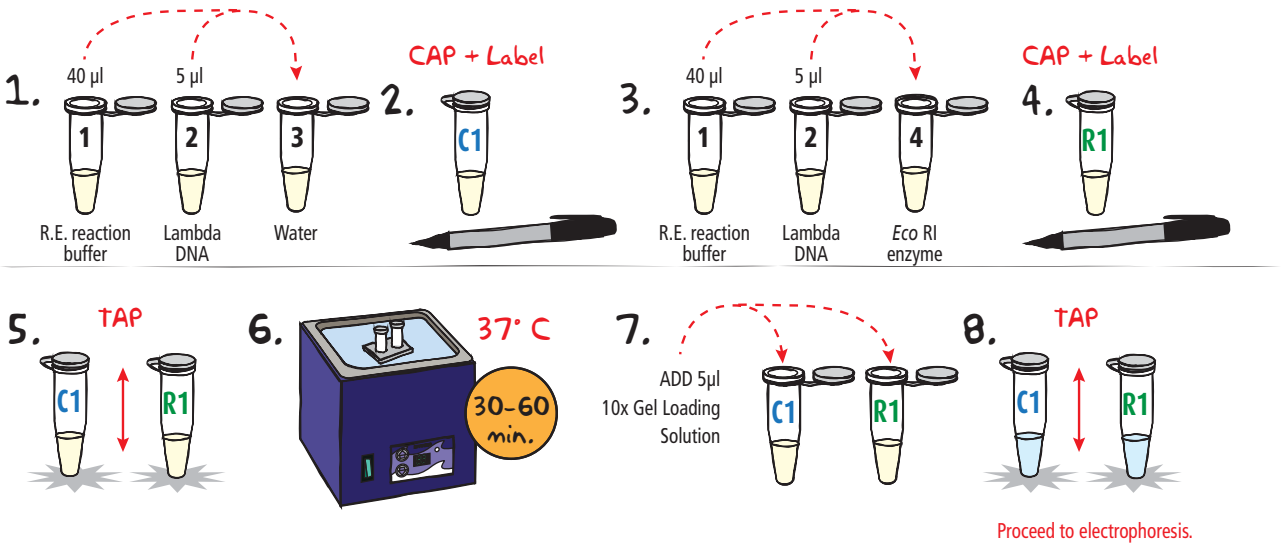
- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

MODULE I OVERVIEW



Tube numbers correspond to number assignments in Table 1 (page 9)

Module I: Restriction Digest of Lambda DNA



1. **TRANSFER** 40 µl Restriction Enzyme Reaction Buffer (tube 1) and 5 µL Lambda DNA (tube 2) to the tube labeled "3" (Water).
2. **CAP** the tube. **LABEL** this tube with "Control" and your group name/number.
3. **TRANSFER** 40 µl Restriction Enzyme Reaction Buffer (tube 1) and 5 µL Lambda DNA (tube 2) to the tube labeled "4" (*Eco* RI enzyme).
4. **CAP** the tube. **LABEL** this tube "Reaction" and your group name/number.
5. **MIX** the restriction digestion reactions by gently tapping the tubes.
6. **INCUBATE** the samples at 37° C for 30-60 minutes.
7. After the incubation, **ADD** 5 µl of 10x gel loading solution to each reaction tube.
8. **CAP** the tubes and **MIX** by tapping the tube or vortexing vigorously. **PROCEED** to electrophoresis with the two samples.

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

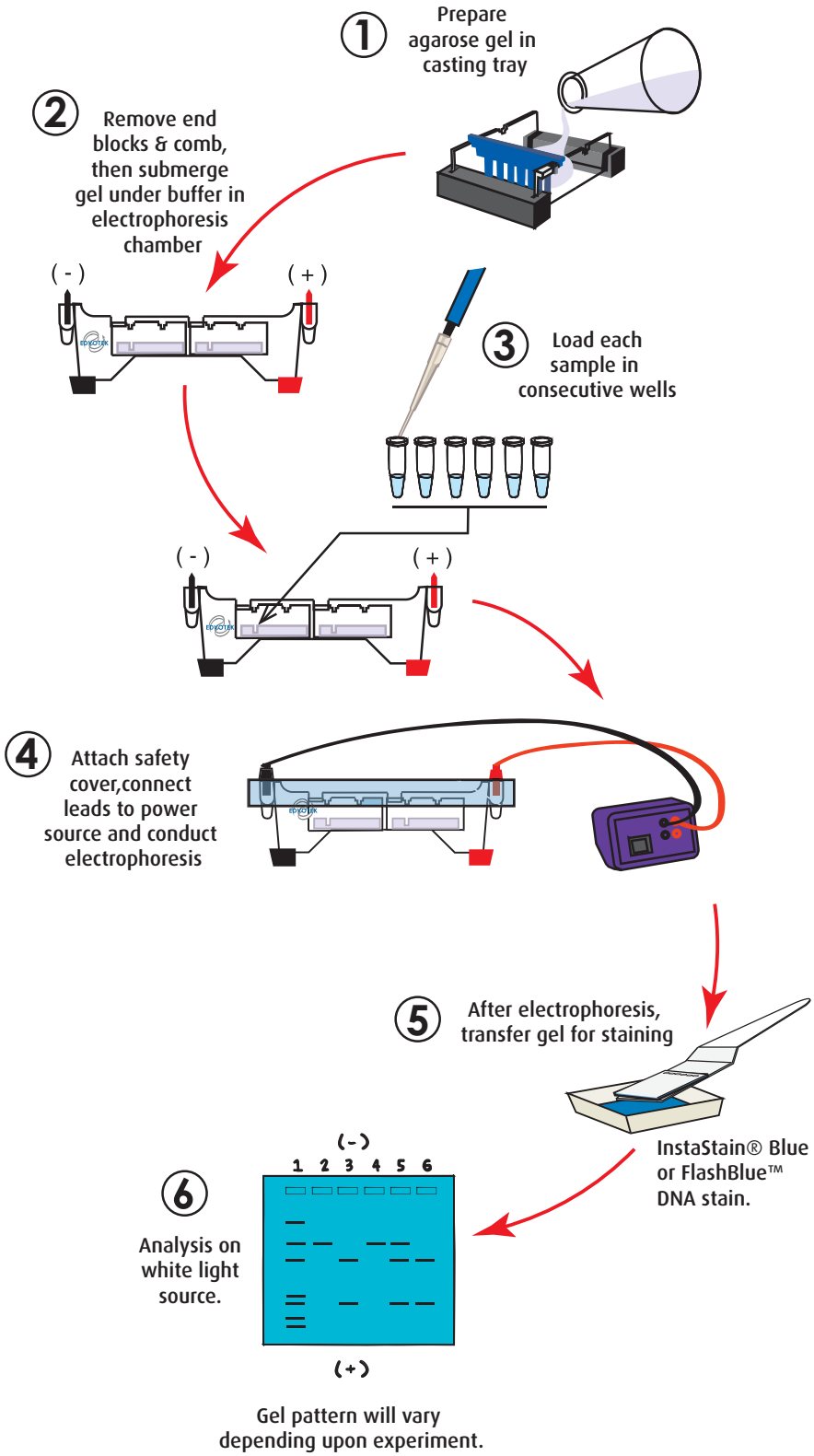
Summary of Restriction Enzyme Digestion Reactions

Tube	Tube 1 Reaction Buffer	Tube 2 Lambda DNA	Tube 3 Water	Tube 4 EcoRI	Reaction Volume
REACTION	40 µl	5 µl	----	5 µl	50 µl
CONTROL	40 µl	5 µl	5 µl	----	50 µl

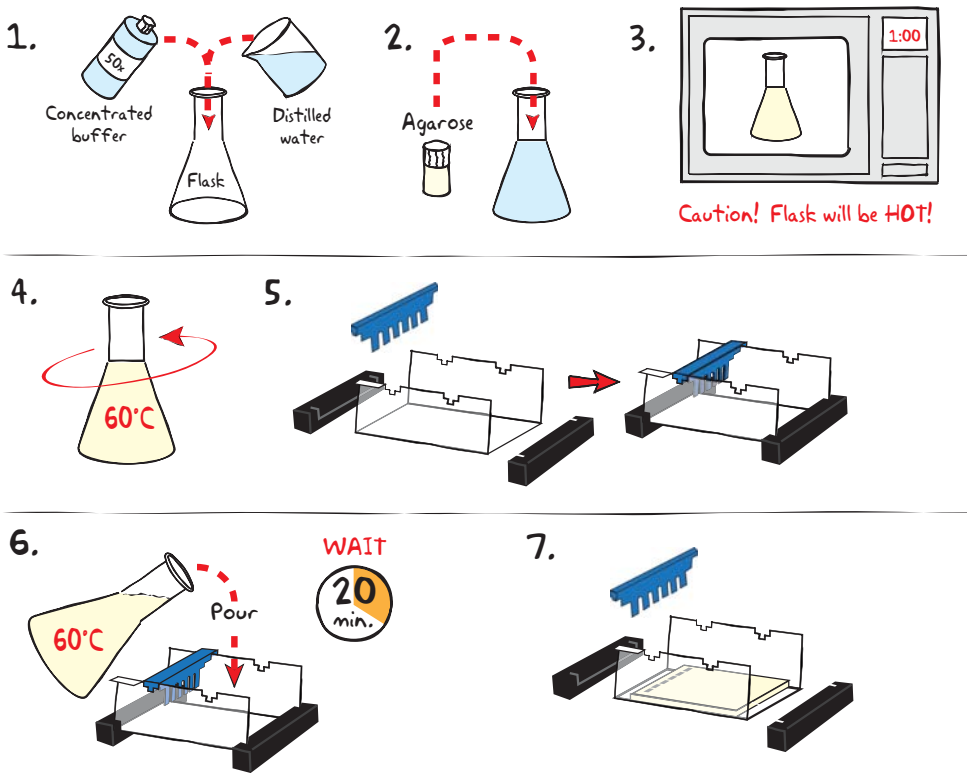
IMPORTANT:

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.

MODULE II OVERVIEW



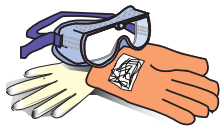
Module II: Agarose Gel Electrophoresis



IMPORTANT:

Each gel is shared by two groups.

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

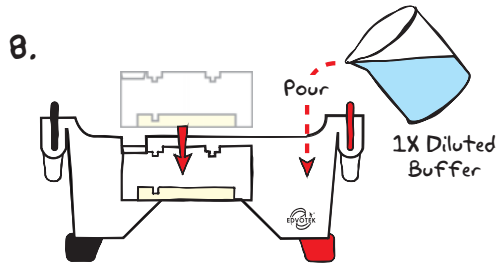
CASTING THE AGAROSE GEL

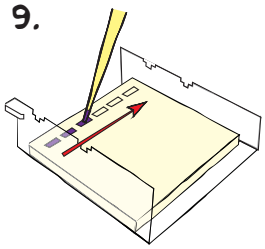
1. **DILUTE** concentrated 50X Electrophoresis buffer with distilled water (refer to Table A for correct volumes depending on the size of your gel casting tray).
2. **MIX** agarose powder with buffer solution in a 250 ml flask (refer to Table A).
3. **DISSOLVE** agarose powder by boiling the solution. **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. **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

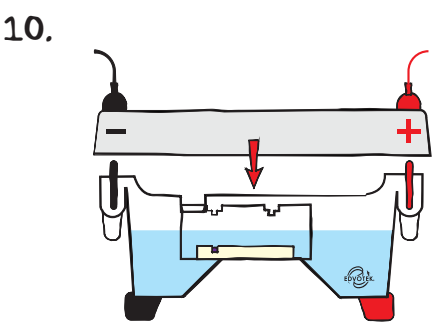
Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume
7 x 7 cm	0.6 ml	29.4 ml	0.23 g	30 ml
7 x 10 cm	1.0 ml	49.0 ml	0.39 g	50 ml
7 x 14 cm	1.2 ml	58.8 ml	0.46 g	60 ml

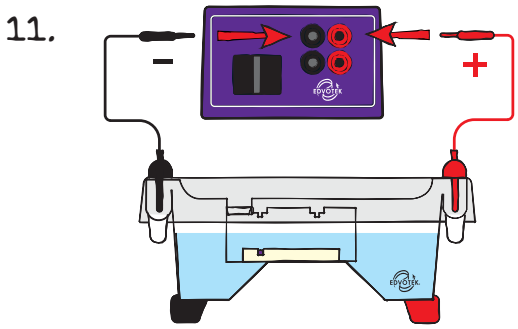
Module II: Agarose Gel Electrophoresis, continued





Wear gloves and safety goggles





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

RUNNING THE GEL

8. **PLACE** the gel (still on the tray) into the electrophoresis chamber. **COVER** the gel with 1X Electrophoresis Buffer (See Table B for recommended volumes). The gel should be completely submerged.
9. **LOAD** 40 µl of the sample into the well in the order indicated by Table 2, at right.
10. **PLACE** safety cover on the unit. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
11. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines). Allow the tracking dye to 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 instructions for **STAINING** the agarose gel.

Table 2: Gel Loading

Lane	
1	Group 1 Standard Marker
2	Group 1 Reaction
3	Group 1 Control
4	Group 2 Standard Marker
5	Group 2 Reaction
6	Group 2 Control

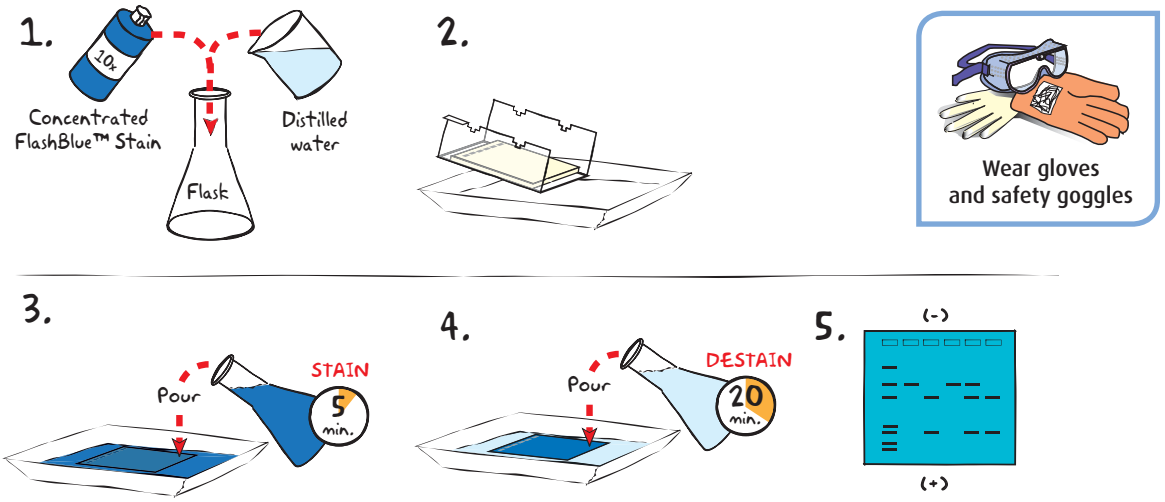
Table B — 1x Electrophoresis Buffer (Chamber Buffer)

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: M6+	M12 (new)	M12 (classic) & M36
Volts	Min. / Max.	Min. / Max.	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 Using FlashBlue™

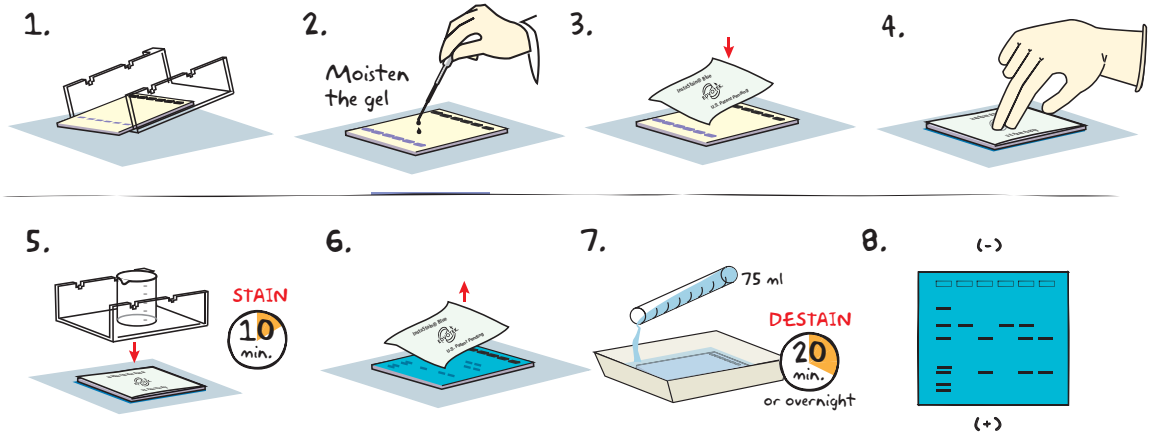


1. **DILUTE** 10 ml of 10x concentrated FlashBlue™ with 90 ml of water in a flask and **MIX** well.
2. **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray into a small, clean gel-staining tray.
3. **COVER** the gel with the 1x FlashBlue™ 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 III-B: Staining Agarose Gels Using InstaStain® Blue



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.
2. **MOISTEN** the gel with a few drops of electrophoresis buffer.
3. Wearing gloves, **PLACE** the blue side of the InstaStain® Blue card on the 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® Blue card is in direct contact with the gel surface. **STAIN** the gel for 10 minutes.
6. **REMOVE** the InstaStain® Blue card. If the color of the gel appears very light, reapply the InstaStain® Blue card to the gel for an additional five minutes.
7. **TRANSFER** the gel to a small, clean gel-staining tray. **COVER** the gel with about 75 mL of distilled water and **DESTAIN** for at least 20 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. To accelerate destaining, warm the distilled water to 37°C and change it frequently.
8. Carefully **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Wear gloves and safety goggles

NOTE:
DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.

ALTERNATIVE PROTOCOL:

1. Carefully **SLIDE** the agarose gel from its casting tray into a small, clean tray containing about 75 ml of distilled/deionized water or used electrophoresis buffer. The gel should be completely submerged.
2. Gently **FLOAT** the InstaStain® Blue card(s) 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. **COVER** the tray with plastic wrap to prevent evaporation. **SOAK** the gel in the staining liquid for at least 3 hours. The gel can remain in the liquid overnight if necessary.
4. Carefully **REMOVE** the gel from the staining tray. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

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 2: 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

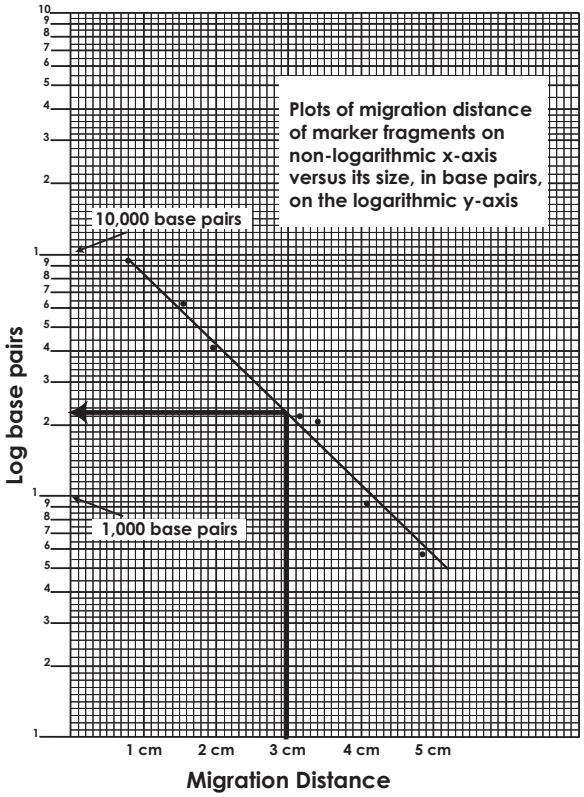
Measure the distance traveled by each Standard DNA Fragment from the lower edge of the sample well to the lower end of each band. Record the distance in centimeters (to the nearest millimeter) in your notebook. Repeat this for each DNA fragment in the standard.

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 3 for an example).

Figure 3: Semilog graph example

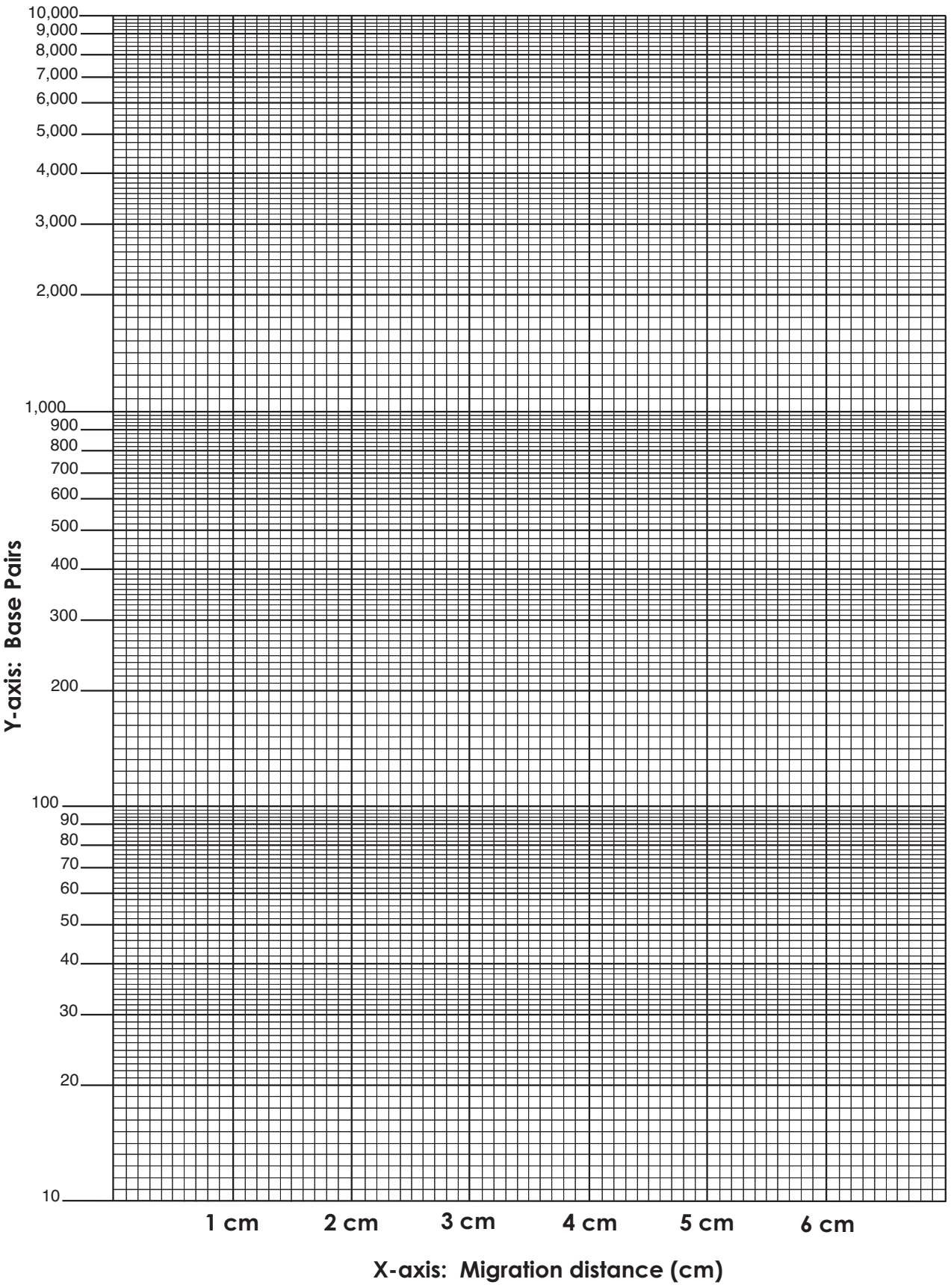


3. DETERMINE THE LENGTH OF EACH UNKNOWN FRAGMENT

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 3 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

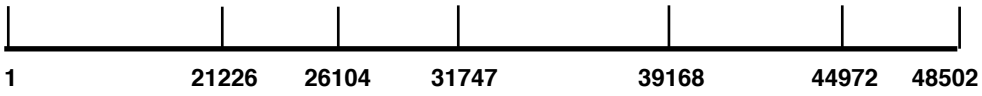
1. The restriction enzyme *Not*I recognizes the following sequence: 5'-GCGGCCGC-3'. On average, how often should this enzyme cleave DNA? The genome of the human malaria parasite *Plasmodium falciparum* is extremely A-T rich – As and Ts comprise about 80% of its genome. Would *Not*I cleave this DNA more or less frequently?

2. Why do you heat the Lambda DNA fragments prior to electrophoresis?

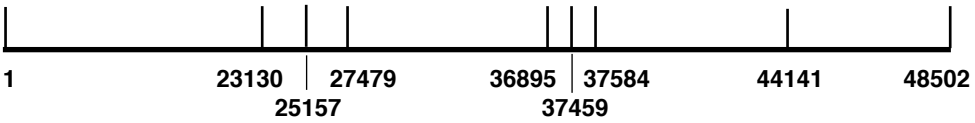
3. Predict the number of DNA fragments and their sizes if Lambda phage DNA were incubated and cleaved simultaneously with both *Hind* III and *Eco* RI (refer to the map below).

LAMBDA PHAGE DNA RESTRICTION ENZYME MAP
48,502 Base Pairs

A. *Eco*RI (5 Sites)



B. *Hind*III (7 Sites)



* The map is not drawn to scale. It serves to locate the relative sites of cleavage in base pairs.

Instructor's Guide

ADVANCE PREPARATION:

Preparation For:	What to do:	When:	Time Required:
Module I	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	30 minutes before use.	30 min.
Module II	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	Prepare staining components	The class period or overnight before the class period.	10 min.

Module I: Pre-Lab Preparations

1. Thaw the tubes of Lambda Phage DNA (A), Concentrated Restriction Enzyme Reaction Buffer (B), Enzyme Grade Water (C).
2. Prepare the Restriction Enzyme Reaction Buffer:
   a. Using a fresh micropipet tip, transfer 1.0 mL of Enzyme Grade Water (C) to a microcentrifuge tube.
   b. Add 126 µl (~0.13 mL) of Concentrated Restriction Enzyme Reaction Buffer (B) to the tube.
   c. Cap the tube and mix vigorously by flicking the tube with your finger or vortexing for at least 30 seconds.
   d. Dispense 80 µl of diluted reaction buffer into 10 microcentrifuge tubes "1". Cap the tubes.
3. Dispense 10 µl of the Lambda Phage DNA (A) into 10 microcentrifuge tubes "2". Cap the tubes.
4. Dispense 5 µl of the Enzyme Grade Water (C) into 10 microcentrifuge tubes "3". Cap the tubes.
5. Dispense 10 µl of 10x Gel Loading Solution into 10 microcentrifuge tubes.
6. Prepared reagents can be stored at room temperature if prepared on the day of the lab. If prepared in advance, store the reagents in the refrigerator (4° C).

DAY OF THE LAB

Equilibrate a 37° C water bath.

Preparation of Dryzyme™ *Eco*RI Restriction Enzyme

Prepare restriction digests within 30 minutes of reconstituting Dryzyme™ *Eco*RI

1. Thaw Reconstitution Buffer F and Reconstitution Buffer G and place on ice.
2. Make sure that the solid material is at the bottom of the Dryzyme™ tube (D). 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 30 µl Reconstitution Buffer (F) 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 30 µL Reconstitution Buffer G to the tube of rehydrated Dryzyme™. Be aware that Reconstitution Buffer G is somewhat viscous and may be difficult to pipet. Take care to ensure that the total volume of liquid is drawn into the pipet tip before adding the buffer to the reconstituted Dryzyme™.
5. After adding the buffer, mix the sample by vortexing or by pipetting up and down for twenty seconds.
6. Dispense 5 µL of the reconstituted EcoRI enzyme to 10 microcentrifuge tubes labeled "4". Place tubes on ice. Use within 30 minutes.

NOTE:
After the rehydration with Reconstitution Buffer F, check that no undissolved particulate matter remains. If not completely dissolved, repeat the mixing or vortexing step until no solid material is observed.

Summary of Reagent Preparation

Component	Number of tubes	Label	Volume per tube
Diluted Reaction Buffer	20	1	80
Lambda DNA	20	2	10
Enzyme Grade water	10	3	5
Eco RI	10	4	5

FOR MODULE I
Each Group Requires:

1 tube of Diluted Restriction Enzyme Reaction Buffer "1"
1 tube of Lambda DNA "2"
1 tube of Enzyme Grade Water "3"
1 tube of *Eco*RI (on ice) "4"
1 tube 10x Gel Loading Buffer
Transfer pipets OR micropipet with tips

Module II: Pre-Lab Preparations

AGAROSE GEL ELECTROPHORESIS

Two groups will share 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. Before electrophoresis, dispense 40 µl of DNA Standard Marker into 10 labeled microcentrifuge tubes. Distribute one tube per gel.

Individual Gel Preparation:

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.

NOTE:
Accurate pipetting is critical for maximizing successful experiment results. EDVOTEK Series 100 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 Group Requires:
- 50x Electrophoresis Buffer
- 1 tube DNA Standard Marker
- Distilled Water
- UltraSpec-Agarose™
- Samples from Module I

Module III: Pre-Lab Preparations

Wear gloves and safety goggles

MODULE III-A: STAINING AGAROSE GELS WITH FLASHBLUE™

FlashBlue™ stain is optimized to shorten the time required for both staining and de-staining 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.

FOR MODULE III-A
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

MODULE III-B: STAINING AGAROSE GELS WITH INSTASTAIN® BLUE

The easiest and most convenient DNA stain available is InstaStain® Blue. InstaStain® Blue does not require the formulation, storage and disposal of large volumes of liquid stain. Each InstaStain® Blue card contains a small amount of blue DNA stain. When the card is placed in water, the DNA stain is released. This solution simultaneously stains and destains the gel, providing uniform gel staining with minimal liquid waste and mess.

You can use a White Light Visualization System (Cat. #552) to visualize gels stained with InstaStain® Blue.

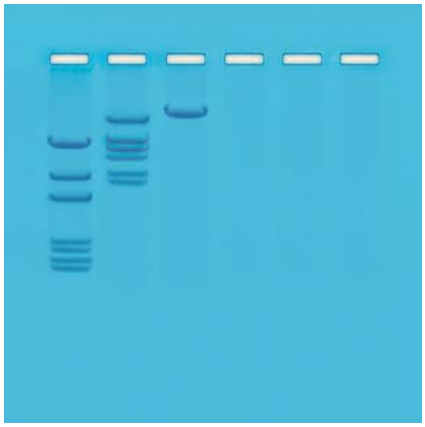
FOR MODULE III-B
Each Student Group should receive:

- 1 InstaStain® card per 7 x 7 cm gel

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.

Experiment Results and Analysis



Lane	Sample	Molecular Weight (bp)
1	DNA Standard Marker	6751, 3652, 2827,1568, 1118, 825, 630
2	Lambda DNA cut with *Eco*RI	21226, 7421, 5804, 5643, 4878, 3530
3	Lambda DNA (uncut)	48500

Note: This technique has a ± 10 - 15% margin of error.

NOTE:
Smaller bands appear fainter in color than larger bands because they stain less efficiently. A white light visualization system (Cat. #552) will aid with visualization.

* *This band may not appear on the gel and likely will not be visualized.*
* *Two bands appear as a single band.*

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.

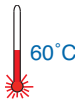
Table D — Bulk Preparation of Electrophoresis Buffer

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