EDVO-Kit: AP09

Biotechnology:
Restriction Enzyme
Analysis of DNA

See Page 3 for storage instructions.

EXPERIMENT OBJECTIVE:

The objective of this experiment is to develop a basic understanding of the role of restriction enzymes and agarose gel electrophoresis to cut and size DNA. Variations in cleavage patterns obtained from Lambda DNA digested with different restriction enzymes will serve as a “genetic fingerprint simulation”, which will then be analyzed by the students.
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Material Safety Data Sheets can be found on our website:
www.edvotek.com

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All 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.

THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA. None of the experiment components are derived from human sources.

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Experiment Components

The DNA samples for electrophoresis in experiment AP09 are packaged in one of the following ways:

1. Pre- aliquoted Quickstrip™ connected tubes, which contains:
   - A and D  Lambda DNA cut with Hind III
   - B and E  Lambda DNA cut with Eco RI
   - C and F  Lambda DNA (uncut)
   - G and H  blank

OR

2. Individual 1.5 ml (or 0.5 ml) microcentrifuge tubes, which contains:
   - A  Lambda DNA cut with Hind III
   - B  Lambda DNA cut with Eco RI
   - C  Lambda DNA (uncut)

REAGENTS & SUPPLIES

- UltraSpec-Agarose™ powder
- Concentrated electrophoresis buffer
- FlashBlue™ DNA Stain
- InstaStain® Blue cards
- Practice Gel Loading Solution
- 1 ml pipet
- Microtipped Transfer Pipets

Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipets with tips
- Balance
- Microwave, hot plate or burner
- Pipet pump
- 250 ml flasks or beakers
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Small plastic trays or large weigh boats (for gel destaining)
- DNA visualization system (white light)
- Distilled or deionized water

DNA samples are stable at room temperature. However, if the experiment will not be conducted within one month of receipt, it is recommended that the DNA samples be stored in the refrigerator.

DNA samples do not require heating prior to gel loading.
Background Information

The discovery of restriction enzymes began a new era of molecular genetics. These enzymes cut DNA in a highly specific and reproducible way. This, in turn, made molecular cloning, DNA mapping, sequencing and various genome projects possible.

Restriction enzymes are endonucleases that catalyze cleavage of phosphodiester 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 DNA at very specific base sequences. Restriction enzymes are produced by many different species of bacteria (including blue-green algae). Over 2,000 restriction enzymes have been discovered and characterized. More recently, intron-coded yeast mitochondrial endonucleases have been discovered that also cut DNA. The recognition sequences for these enzymes yield very few cuts in DNA and promise to be important new biological reagents for DNA analysis.

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.

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 specific enzyme recognition sites. 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 shown above, Eco RI causes staggered cleavage of its site. The resulting ends of the DNA fragments are also called “sticky” or “cohesive” ends.

This is because the single-stranded regions of the ends are complementary.

Some restriction enzymes, such as HaeIII, introduce cuts that are opposite each other. This type of cleavage generates “blunt” ends.
Background Information

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

\[ 5'\text{-C PyCGPuG-3'} \quad (\text{Py = pyrimidine = C or T) and} \]
\[ 3'\text{-GPuGCPy C-5'} \quad (\text{Pu = purine = G or A}) \]

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

\[ 5'\text{-GCCNNNN NGGC-3'} \]
\[ 3'\text{-CGGN NNNNCCG-5'} \quad (N = A, G, C or T) \]

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 forms 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 restriction enzyme recognition site will occur. The probability of DNA digestion is directly proportional to the size of the enzyme recognition palindrome. Thus, an enzyme that recognizes four nucleotides will cut DNA on average once every 256 base pairs, while an enzyme that recognizes five base pairs will cut DNA once every 1024 base pairs. Human chromosomal DNA contains 3 billion base pairs and has a large number of restriction enzyme recognition sites. Plasmid DNAs usually contain only a few thousand base pairs and contains fewer restriction enzyme sites.

Plasmids and many viral DNAs are circular and double-stranded. If circular DNA contains one recognition site for a restriction enzyme, when cleaved, it will form a linear molecule. By contrast, if a linear DNA molecule that contains a single recognition site is cleaved once, it will generate two fragments. The size of the fragments produced depends on how far the restriction enzyme sites are from each other. If a DNA molecule contains several recognition sites for a restriction enzyme, it is possible that under certain experimental conditions not all sites are cleaved. Incompletely cleaved fragments of DNA are called partial digestion products. These partials can arise if low amounts of enzyme are used or the reaction is stopped after a short time. Reactions containing “partials” usually contain molecules that have not been completely cleaved.
Background Information

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 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.

![Figure 3: Overview of Agarose Gel Electrophoresis](image)

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.

In this experiment, we will be analyzing the products of a restriction digest of linear DNA isolated from a virus that infects *E.coli*, known as bacteriophage Lambda. Lambda DNA, which contains approximately 49 kilobases (49,000 base pairs), is cut by the restriction enzymes *Eco* RI and *Hind* III five and seven times, respectively. Using agarose gel electrophoresis, we will estimate the lengths of these fragments, and then determine which enzyme was used to digest the DNA.
Background Information

Lambda DNA is used in this experiment that 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 *Hind* III. The smaller fragments generated by a restriction enzyme, such as those generated by *Hind* III digestion of Lambda DNA, may not be visible after separation on agarose gel electrophoresis. Smaller fragments will be first to run off the gel during electrophoresis. Since there is less mass in the bands containing smaller fragments, they stain with less intensity and may be less detectable. Stoichiometric cleavage of a pure sample of DNA results in equal amounts of fragments.

The discovery of restriction enzymes in the late 1960s had an enormous impact on molecular biology. Due to the exact specificity of restriction enzymes, and the specific cleavage patterns generated when various cut DNA fragments run on gels, restriction enzymes also enables for mapping of DNA. A common application of this is restriction fragment length polymorphism (RFLP), used for e.g. paternity testing. In RFLP, specific human genomic DNA-areas are cut by several restriction enzymes, and the fragments are subjected to electrophoresis. The generated fragment pattern is unique for a given individual, but shares certain similarities with patterns generated by related individuals.

Lambda DNA is 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 *Hind* III. The smaller fragments generated by a restriction enzyme, such as those generated by *Hind* III digestion of Lambda DNA, may not be visible after separation on agarose gel electrophoresis. Smaller fragments will be first to run off the gel during electrophoresis. Since there is less mass in the bands containing smaller fragments, they stain with less intensity and may be less detectable. Stoichiometric cleavage of a pure sample of DNA results in equal amounts of fragments.

The Lambda DNA cut with *Hind* III will be served as a marker, providing a set of RFLPs of known sizes (standard). Variations in cleavage patterns obtained from Lambda DNA digested with different restriction enzymes will be analyzed, along with the “uncut” lambda DNA.
Biotechnology: Restriction Enzyme Analysis of DNA

Experiment Overview and General Instructions

EXPERIMENT OBJECTIVE:

The objective of this experiment is to develop a basic understanding of the role of restriction enzymes and agarose gel electrophoresis to cut and size DNA. Variations in cleavage patterns obtained from Lambda DNA digested with different restriction enzymes will serve as a “genetic fingerprint simulation”, which will then be analyzed by the students.

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 Overview: Flow Chart

1. Prepare agarose gel in casting tray
2. Remove end blocks & comb, then submerge gel under buffer in electrophoresis chamber
3. Starting with Sample A, load each sample in consecutive wells
4. Attach safety cover, connect leads to power source and conduct electrophoresis
5. FlashBlue™ DNA stain
6. Analysis on white light source

Experiment Procedure

After electrophoresis, transfer gel for staining

Analysis on white light source
Biotechnology: Restriction Enzyme Analysis of DNA

**Experiment AP09**

**Experiment Procedure**

**Prepare the Gel**

1. Prepare an agarose gel with specifications summarized below. Your instructor will specify which DNA stain you will be using.

   - 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: 3
   - Placement of well-former template: first set of notches (7 x 7 cm) first & third set of notches (7 x 14 cm)

**Agarose Gel Electrophoresis**

<table>
<thead>
<tr>
<th>Lane</th>
<th>Tube</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A or D</td>
<td>Lambda DNA cut with <em>Hind</em> III</td>
</tr>
<tr>
<td>2</td>
<td>B or E</td>
<td>Lambda DNA cut with <em>Eco</em> RI</td>
</tr>
<tr>
<td>3</td>
<td>C or F</td>
<td>Lambda DNA (uncut)</td>
</tr>
</tbody>
</table>

2. Load the DNA samples in tubes A - C (or D - F) into the wells in consecutive order.

   - For gels to be stained with FlashBlue™ or InstaStain® Blue, fill wells with 35 - 38 µl.
   - For gels to be stained with InstaStain® Ethidium Bromide, fill wells with 18 - 20 µl.

3. After DNA samples are loaded, connect the apparatus to the D.C. power source and set the power source at the required voltage.

4. Check that current is flowing properly - you should see bubbles forming on the two platinum electrodes. Conduct electrophoresis for the length of time specified by your instructor.

5. After electrophoresis is completed, proceed to DNA staining and visualization. Refer to Appendix E, F, G, or H for the appropriate staining instructions.

6. Document the results of the gel by photodocumentation.

   Alternatively, place transparency film on the gel and trace it with a permanent marking pen. Remember to include the outline of the gel and the sample wells in addition to the migration pattern of the DNA bands.

**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.

- Black
- Red

- Sample wells
- Lane
- Tube

For gels to be stained with FlashBlue™ or InstaStain® Ethidium Bromide, prepare gels according to Appendix A.

Step-by-step guidelines for agarose gel preparation are summarized in Appendix C.
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 log10 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.

1. Measure and Record Migration Distances

Measure the distance traveled by each Lambda/Hind III digest 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. Do not measure the migration of the largest fragment, as this point will not be used to create the standard curve.

Measure and record the migration distances of each of fragments in unknown Lambda/Hind III digest sample in the same way you measured the standard bands.

2. Generate a Standard Curve.

Because migration rate is inversely proportional to the log10 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!
Size Determination of DNA Restriction Fragments

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 5 for an 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 5 for an example). Make note of this in your lab notebook.
   c. Repeat for each fragment in your unknown sample.

Quick Reference:
Lambda DNA cut with Hind III using a standard curve will be plotted on semi-log graph paper. The following are the sizes - length is expressed in base pairs.

23130  9416  6557  4361
2322  2027  564
Study Questions

1. How often would a restriction enzyme such as Not I, which has 8 nucleotides in its recognition sites 5’-GCGGCCGC-3’, cleave DNA on average? Would it cleave a species DNA more or less often if the DNA from that species were 70% A-T rich?

2. 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**

**A. Eco RI (5 Sites)**

| 1 | 21226 | 26104 | 31747 | 39168 | 44972 | 48502 |

**B. Hind III (7 Sites)**

| 1 | 2313 | 27479 | 36895 | 37584 | 44141 | 48502 |

The 125 bp fragment (resulted from digestion of the 38459 bp and 37584 bp sites) is too small to be visualized on the gel.
Biotechnology: Restriction Enzyme Analysis of DNA

Instructor’s Guide

Notes to the Instructor & Pre-Lab Preparations

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in planning and implementing 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. 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).

EDUCATIONAL RESOURCES, NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will learn to load samples and run agarose gel electrophoresis. Experiment analysis will provide students the means to transform an abstract concept into a concrete explanation.

EDVOTEK Ready-to-Load 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. EDVOTEK web site resources provide suggestions and valuable hints 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 example, a DNA sizing determination activity can be performed on any electrophoresis gel result if DNA markers are run in parallel with other DNA samples. For DNA Sizing instructions, and other laboratory extension suggestions, please refer to the EDVOTEK website.

Visit the EDVOTEK web site often for updated information.
Notes to the Instructor & Pre-Lab Preparations

APPROXIMATE TIME REQUIREMENTS

1. Gel preparation:
   Whether you choose to prepare the gel(s) in advance or have the students prepare their own, allow approximately 30 minutes for this procedure. Generally, 20 minutes of this time is required for gel solidification.

2. Micropipetting and Gel Loading:
   If your students are unfamiliar with using micropipets and sample loading techniques, a micropipetting or practice gel loading activity is suggested prior to conducting the experiment. Two suggested activities are:
   - EDVOTEK Expt. # S-44, Micropipetting Basics, focuses exclusively on using micropipets. Students learn pipetting techniques by preparing and delivering various dye mixtures to a special Pipet Card™.
   - Practice Gel Loading: EDVOTEK Series 100 electrophoresis experiments contain a tube of practice gel loading solution for this purpose. It is highly recommended that a separate agarose gel be cast for practice sample delivery. This activity can require anywhere from 10 minutes to an entire laboratory session, depending upon the skill level of your students.

3. 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. The Table C example at left shows Time and Voltage recommendations. Refer to Table C in Appendices A or B for specific experiment guidelines.

<table>
<thead>
<tr>
<th>Volts</th>
<th>EDVOTEK Electrophoresis Model</th>
<th>M6+ Minimum / Maximum</th>
<th>M12 &amp; M36 Minimum / Maximum</th>
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<tr>
<td>150</td>
<td>15 / 20 min</td>
<td>25 / 35 min</td>
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<td>125</td>
<td>20 / 30 min</td>
<td>35 / 45 min</td>
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<td>70</td>
<td>35 / 45 min</td>
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</tr>
<tr>
<td>50</td>
<td>50 / 80 min</td>
<td>95 / 130 min</td>
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APPROMOXIMATE TIME REQUIREMENTS

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<th>Activity</th>
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<tr>
<td>Day Before Lab</td>
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</tr>
<tr>
<td>Sample Preparation</td>
<td>20 min</td>
</tr>
<tr>
<td>Day of Lab</td>
<td></td>
</tr>
<tr>
<td>Gel Preparation*</td>
<td>30 min</td>
</tr>
<tr>
<td>Gel Loading</td>
<td>20 min</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>15 min – 2 hr</td>
</tr>
<tr>
<td>Staining</td>
<td>3 hr – overnight</td>
</tr>
<tr>
<td>Day After Lab</td>
<td></td>
</tr>
<tr>
<td>Document Results</td>
<td>20 min</td>
</tr>
<tr>
<td>Discussion</td>
<td>20 min</td>
</tr>
</tbody>
</table>

* This step can be performed in advance if there are time constraints.
Notes to the Instructor & Pre-Lab Preparations

PREPARING AGAROSE 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.

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”.

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.**

USING AGAROSE GELS THAT HAVE BEEN PREPARED IN ADVANCE

If gels have been removed from their trays for storage, they 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 gel from sliding around in the tray and/or floating around in the electrophoresis chamber.

AGAROSE GEL CONCENTRATION AND VOLUME

Gel concentration is one of many factors which affect the mobility of molecules during electrophoresis. Higher percentage gels are sturdier and easier to handle. However, the mobility of molecules and staining will take longer because of the tighter matrix of the gel. Gel volume varies depending on the size of the casting tray, as well as the type of stain to be used for DNA staining after electrophoresis. Gels which will be stained with InstaStain® Ethidium Bromide require less sample amount (volume) than gels that will be stained with FlashBlue™ or InstaStain® Blue.

This experiment requires a 0.8% gel. It is a common agarose gel concentration for separating dyes or DNA fragments in EDVOTEK experiments. Specifications for preparing a 0.8% gel to be stained with InstaStain® Ethidium Bromide, FlashBlue™, or InstaStain® Blue can be found in Appendix A.
Notes to the Instructor & Pre-Lab Preparations

GEL STAINING AND DESTAINING AFTER ELECTROPHORESIS

Option 1: FlashBlue™ liquid - Appendix E
This simple and rapid liquid staining and destaining procedure yields excellent visibility of DNA bands in less than 25 minutes (5 minutes staining, 20 minutes destaining).

Option 2: InstaStain® Blue cards, One-step Staining and Destaining- Appendix F
Agarose gels can be stained and destained in one easy step.

Option 3: InstaStain® Blue cards (Direct Staining) - Appendix G
Using InstaStain® Blue cards, staining is completed in approximately 5-10 minutes. DNA bands will become visible after destaining for approximately 20 minutes. Results 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.

Option 4: InstaStain® Ethidium Bromide - Appendix H
Staining with ethidium bromide is very sensitive and can detect as little as 5 to 10 nanograms of DNA with the use of a U.V. transilluminator. Ethidium Bromide is a dye that is commonly used by scientific researchers. It is a listed mutagen and forms a tight complex with DNA by intercalating between the bases within the double helix. The complex strongly fluoresces when exposed to ultraviolet light.

CAUTION: Ethidium Bromide is a listed mutagen. Disposal of the InstaStain® EtBr cards, which contain microgram amounts of ethidium bromide, is minimal compared to the large volume of liquid waste generated by traditional ethidium bromide staining procedures. Disposal of InstaStain® cards and gels should follow institutional guidelines for chemical waste.
Notes to the Instructor & Pre-Lab Preparations

READY-TO-LOAD DNA SAMPLES FOR ELECTROPHORESIS

No heating required before gel loading.

EDVOTEK offers the widest selection of electrophoresis experiments which minimize expensive equipment requirements and save valuable time for integrating important biotechnology concepts in the teaching laboratory. Ready-to-Load™ DNA experiments feature samples which are predigested with restriction enzymes and are stable at room temperature (however, refrigeration is recommended). DNA samples are ready for immediate delivery onto agarose gels for electrophoretic separation and do not require pre-heating in a waterbath.

Electrophoresis samples and reagents in EDVOTEK experiments are packaged in various formats. The samples in Series 100 and S-series electrophoresis experiments will be packaged in one of the following ways:

1) Pre-aliquoted Quickstrip™ connected tubes
   OR
2) Individual 1.5 ml (or 0.5 ml) microtest tubes

SAMPLES FORMAT: PRE-ALIQUOTED QUICKSTRIP™ CONNECTED TUBES

Convenient QuickStrip™ connected tubes contain pre-aliquoted ready-to-load samples. The samples are packaged in a microtiter block of tubes covered with a protective overlay. Follow the instructions below for preparation of QuickStrip™.

1. Use sharp scissors to separate the block of samples into individual strips as shown in the diagram at right.
   The number of samples per set will vary depending on the experiment. Some tubes may be empty.

2. Cut carefully between the rows of samples. Do not cut or puncture the protective overlay directly covering the sample tubes.

3. Next, cut each individual strip between samples C & D.
   - A & D contain Lambda cut with Hind III
   - B & E contain Lambda cut with Eco RI
   - C & F contain Lambda (uncut)
   - G & H are intentionally left blank

4. Each gel will require one strip of samples, either A - C or D - F.

5. Remind students to tap the tubes before gel loading to ensure that all of the sample is at the bottom of the tube.
Notes to the Instructor & Pre-Lab Preparations

SAMPLES FORMAT: INDIVIDUAL 1.5 ML MICROTEST TUBES

It is recommended that samples packaged in 1.5 ml individual microtest tubes be aliquoted for each gel. DNA Samples packaged in this format are available in three standard quantities:

- Standard experiment kit: 240 µl
- Bulk B-Series: 480 µl
- Bulk C Series: 960 µl

1. Check all sample volumes for possible evaporation. Samples will become more concentrated if evaporation has occurred.

2. If needed, tap or centrifuge the sample tubes. Then add distilled water to slightly above the following level:
   - 1.3 cm level for Standard experiment kit
   - 1.9 cm level for the B-Series
   - 2.8 cm level for the C-Series

3. Mix well by inverting and tapping the tubes several times.

4. After determining that the samples are at their proper total volumes, aliquot each sample into appropriately labeled 0.5 ml or 1.5 ml microtest tubes.
   - For gels to be stained with FlashBlue™ or InstaStain® Blue: 35-38 µl of each sample
   - For gels to be stained with InstaStain® Ethidium bromide: 18-20 µl of each sample

5. If students have difficulty retrieving the entire aliquoted volume of sample because some of it clings to the side walls of the tubes, remind students to make sure all of the sample is at the bottom of the tube before gel loading. They should centrifuge the samples tubes, or tap the tubes on the tabletop.
Experiment Results and Analysis

In the idealized schematic, the relative positions of Lambda DNA fragments are shown but are not depicted to scale.

Lane  Tube  Lambda DNA cut with *Hind* III (expressed in approximate base pairs)
1    A or D  23130  9416  6557  4361  2322
     2027  564*

2    B or E  Lambda DNA cut with *Eco* RI
   Expected *Eco* RI fragment sizes in base pairs:
   21226  7421  5804**  5643**  4878  3530

3    C or F  Lambda DNA (uncut)
          Note: This technique has a ± 10 - 15% margin of error.

* This band does 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  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  Staining and Visualization of DNA with InstaStain® Ethidium Bromide Cards
I  General Notes on Staining & Destaining
**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

<table>
<thead>
<tr>
<th>Size of Gel (cm)</th>
<th>Amt of Agarose (g)</th>
<th>Concentrated Buffer (50x) (ml)</th>
<th>Distilled Water (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 x 7</td>
<td>0.23</td>
<td>0.6</td>
<td>29.4</td>
<td>30</td>
</tr>
<tr>
<td>7 x 10</td>
<td>0.39</td>
<td>1.0</td>
<td>49.0</td>
<td>50</td>
</tr>
<tr>
<td>7 x 14</td>
<td>0.46</td>
<td>1.2</td>
<td>58.8</td>
<td>60</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Size of Gel (cm)</th>
<th>Amt of Agarose (g)</th>
<th>Diluted Buffer (1x) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 x 7</td>
<td>0.23</td>
<td>30</td>
</tr>
<tr>
<td>7 x 10</td>
<td>0.39</td>
<td>50</td>
</tr>
<tr>
<td>7 x 14</td>
<td>0.46</td>
<td>60</td>
</tr>
</tbody>
</table>

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.
Quantity Preparations for Agarose Gel Electrophoresis

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.

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.

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!
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.

   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.
Agarose Gel Preparation Step by Step Guidelines, continued

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).
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.

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**Table C.1**

<table>
<thead>
<tr>
<th>Voltage</th>
<th>EDVOTEK Electrophoresis Model M6+</th>
<th>M12 &amp; M36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum / Maximum</td>
<td>Minimum / Maximum</td>
</tr>
<tr>
<td>150</td>
<td>15 / 20 min</td>
<td>25 / 35 min</td>
</tr>
<tr>
<td>125</td>
<td>20 / 30 min</td>
<td>35 / 45 min</td>
</tr>
<tr>
<td>70</td>
<td>35 / 45 min</td>
<td>60 / 90 min</td>
</tr>
<tr>
<td>50</td>
<td>50 / 80 min</td>
<td>95 / 130 min</td>
</tr>
</tbody>
</table>
Staining and Visualization of DNA
FlashBlue™ Liquid Stain

Preparation of FlashBlue™ Stain from Concentrated Solution

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 submerge 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 de-staining 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 I.
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 cm² 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 I.

InstaStain is a registered trademark of EDVOTEK, Inc.
Direct Staining of DNA with Instastain® Blue

STAINING OF DNA

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.

Storage and Disposal of Stain and Gel

See Appendix I.

InstaStain is a registered trademark of EDVOTEK, Inc.
Staining and Visualization of DNA with InstaStain® Ethidium Bromide

Do not stain gel(s) in the electrophoresis chamber.

1. After electrophoresis, place the gel on a piece of plastic wrap on a flat surface. Moisten the gel with a few drops of electrophoresis buffer.

2. Wearing gloves, remove and discard the clear plastic protective sheet, and place the unprinted side of the InstaStain® Ethidium Bromide card(s) on the gel.

3. With a gloved hand, firmly run your fingers over the entire surface of the InstaStain® Ethidium Bromide card. Do this several times.

4. Place the gel casting tray and a small empty beaker on top to ensure that the InstaStain® Ethidium Bromide card maintains direct contact with the gel surface.

   Allow the InstaStain® Ethidium Bromide card to stain the gel for 3-5 minutes.

   Note: Staining time is optimized for 0.8-1.0% gels. Gels of higher concentrations will take longer to stain.

5. After 3-5 minutes, remove the InstaStain® Ethidium Bromide card. Transfer the gel to a ultraviolet (300 nm) transilluminator for viewing. Be sure to wear UV protective goggles.

   Caution: Ethidium Bromide is a listed mutagen.

Disposal of InstaStain® Ethidium Bromide

Disposal of InstaStain® Ethidium Bromide cards and gels should follow institutional guidelines for solid chemical waste.

Additional Notes About Staining:

- If bands appear faint, or if you are not using EDVOTEK UltraSpec-Agarose™, gels may take longer to stain with InstaStain® Ethidium Bromide. Repeat staining and increase the staining time for an additional 3-5 minutes.

- DNA markers should be visible after staining even if other DNA samples are faint or absent. If markers are not visible, troubleshoot for problems with the electrophoretic separation.
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.