EDVOTEK®

The Biotechnology Education Company ®

EDVO-Kit #

213

Cleavage of DNA with Restriction Enzymes

See page 3 for specific storage instructions.
Some items require freezer storage.

EXPERIMENT OBJECTIVE:

This experiment is an inquiry-based experiment to develop an understanding of DNA digestion by restriction enzymes and determining the size of DNA fragments by agarose gel electrophoresis.

Table of Contents

Experiment Components

This experiment is designed for 6 groups.

	Contents	Storage
A	*Eco* RI Dryzyme™ endonuclease	Refrigerated, desiccated
B	*Bam* HI Dryzyme™ endonuclease	Refrigerated, desiccated
C	Restriction enzyme dilution buffer	-20°C.
D	Restriction enzyme reaction buffer	Refrigerated
E	Water, qualified enzyme grade	-20°C.
F	Supercoiled plasmid DNA 1	-20°C.
G	Supercoiled plasmid DNA 2	-20°C.
H	Lambda DNA	-20°C.
I	Standard DNA fragments	-20°C.

Store all of the following at Room temperature:

- 10x Gel Loading Solution
- UltraSpec-Agarose™ powder
- 50x concentrated electrophoresis buffer
- InstaStain® Blue
- FlashBlue™ liquid stain
- 1 ml pipet
- 100 ml plastic graduated cylinder
- Microtest tubes with attached caps
- Semi-log graph paper template

Requirements

- Horizontal Electrophoresis Apparatus
- D.C. Power Supply
- Automatic Micropipet (5-50 µl) & Disposable tips
- Balance
- Microwave Oven, Hot Plate, or Burner
- White light visualization system*
- Photodocumentation system (optional)
- Waterbath
- Pipet pumps or bulbs
- 1 ml and 5 or 10 ml pipets
- Disposable gloves and safety goggles
- Hot gloves
- Small plastic trays or large weigh boats
- Lab Marking pens
- Lab glassware - 20 & 250 ml beakers, 100 & 500 ml graduated cylinder
- Metric rulers
- Microtipped transfer pipets
- Distilled or deionized water
- Ice

* If performing alternate staining with InstaStain® Ethidium Bromide (must be purchased separately, Cat. # 2001), a UV Transilluminator is required for DNA visualization.

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

Background Information

Sequence-specific, or Type II endonucleases, are commonly known as restriction enzymes. In contrast with nonspecific endonucleases, these enzymes generate reproducible fragments from DNA. They cleave double-stranded DNA by hydrolyzing two phosphodiester bonds (one per strand) within defined nucleotide sequences. Over 3,000 restriction enzymes, which are extracted from a variety of bacterial strains, have been discovered since the first report by H.O. Smith and collaborators.

Restriction Enzyme	Organism
Bgl I	*Bacillus globigii*
Bam HI	*Bacillus amyloliquefaciens* H
Eco RI	*Escherichia coli* RY13
Eco RII	*Escherichia coli* R 245
Hae III	*Haemophilus aegyptius*
Hind III	*Haemophilus influenzae* R4

The discovery of restriction enzymes ushered in a new era of molecular genetics. These enzymes cut the DNA molecule in a highly specific and reproducible way. This, in turn, has lead to the development of molecular cloning and the mapping of genetic structures.

Restriction enzymes are endonucleases which catalyze the cleavage of the 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 at very specific sequences of bases. Restriction enzymes are obtained from many different species of bacteria (including blue-green algae). To date, over 2,100 restriction enzymes have been discovered and catalogued.

The name of a restriction enzyme is derived from the genus and species of bacterium from which it is isolated. The first letter of the genus name and first two letters of the species are combined to form the enzyme name. This is followed by a strain designation if applicable. In many instances, a bacterial strain contains more than one restriction endonuclease. When this occurs, each enzyme is assigned a Roman numeral. For example, *Bam* HI was the first enzyme activity reported from *Bacillus amyloliquefaciens* strain H (see figure 1).

A restriction enzyme requires a specific double stranded recognition sequence of nucleotides 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.

Eco RI

```
          ↓
5'....GAATTC....3'        5'....G    AATTC....3'
3'....CTTAAG....5'        3'....CTTAA    G....5'
               ↑
```

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

Background Information

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

Hae III

```
          ↓
5'....GGCC....3'        5'....GG    CC....3'
3'....CCGG....5'        3'....CC    GG....5'
          ↑
```

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

Ava I

```
      ↓
5'....CPyCGPuG....3'
3'....GPuGCPyC....5'
      ↑
```

(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:

Bgl I

```
          ↓
5'....GCCNNNNNGGC....3'
3'....CGGNNNNNCCG....5'
          ↑
```

(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 recognition site will occur. Therefore, human chromosomal DNA, which contains three billion base pairs, has many more recognition sites than a plasmid DNA containing only several 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, when cleaved once it will generate two 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.

Background Information

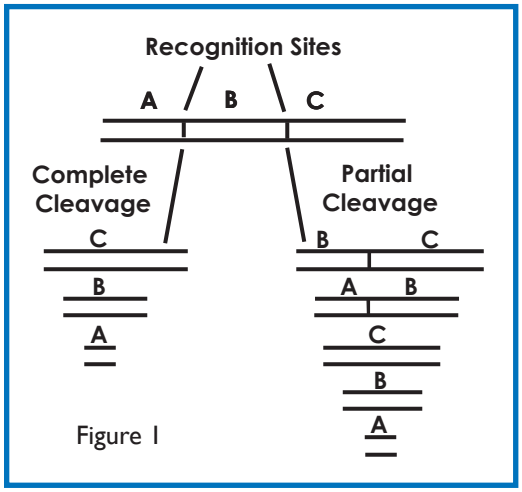


Figure I

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 2).

Supercoiled plasmid DNA has a more compact and entangled shape than its corresponding non-supercoiled forms (linear, nicked and relaxed circles). Under the electrophoresis conditions used in the experiments you will be conducting, supercoiled DNA migrates faster than its linear form and linear DNA migrates faster than its nicked circular form. Catenanes migrate more slowly than single circles that are nicked during electrophoresis. Dimers migrate faster than trimers, which migrate faster than tetramers, etc. Catenanes give rise to the same final restriction enzyme cleavage patterns as their uncatenated single forms.

Other forms of DNA, such as linear, circular or superhelical, are separated in the gel according to their charge, size and shape. One linear DNA example is Lambda DNA, a 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, may not be visible after separation on agarose gel electrophoresis. Smaller fragments will be first to run off the gel during electrophoresis. 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 equimolar amounts of fragments.

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.

This is an inquiry-based experiment where students will select to digest the three DNAs (two circular plasmid DNAs and a linear DNA) with *Eco* RI, *Bam* HI, or a mixture of the two enzymes and determine the size of the resulting fragments. Standard DNA fragments are provided to construct the standard curve.

Experiment Overview and General Instructions

EXPERIMENT OBJECTIVE:

This experiment is an inquiry-based experiment to develop an understanding of DNA digestion by restriction enzymes and determining the size of DNA fragments by agarose gel electrophoresis.

Wear Gloves and Goggles

LABORATORY SAFETY

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.
   - Although electrical current from the power source is automatically disrupted when the cover is removed from the electrophoresis apparatus, first turn off the power, then unplug the power source before disconnecting the leads and removing the cover.
   - Turn off power and unplug the equipment when not in use.
5. EDVOTEK injection-molded electrophoresis units do not have glued junctions that can develop potential leaks. However, in the unlikely event that a leak develops in any electrophoresis apparatus you are using, IMMEDIATELY SHUT OFF POWER. Do not use the apparatus.
6. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

Cleavage of DNA with Restriction Enzymes

1. Plan an experiment using the table on the next page or construct a table similar to it.

2. Label 5 reaction tubes starting with "2".

   The number "1" has been pre-assigned to Standard DNA markers which are ready for electrophoresis.

Each group should have the following materials

- Automatic micropipet & tips
- Microtest tubes
- Lab marker

Reagent or Biological	Tube label
• Restriction Enzyme Reaction Buffer	Rxn Buffer
• Plasmid DNA 1	DNA 1
• Plasmid DNA 2	DNA 2
• Lambda DNA	DNA 3
• Enzyme Grade Water	Water
• Standard DNA Fragments	Markers
• Diluted *Eco* RI	*Eco* RI
• Diluted *Bam* HI	*Bam* HI
• 10x Gel Loading solution	10x Gel Load

3. Add reaction buffer, DNA, water, and enzyme (always add the enzyme last) to reaction tubes you labeled. Use a FRESH micropipet tip for each transfer of DNA and enzyme.

4. Cap the reaction tubes and tap gently to mix. There should be no dense layer of enzyme solution at the bottom of the reaction tube.

5. Tap each tube on the lab bench or quick spin the tubes in a microcentrifuge to collect the contents at the bottom.

6. Incubate the tubes in a 37°C water bath for 30-60 minutes.

   Note: Extending the restriction enzyme digestion to 60 minutes will ensure complete cleavage of DNA.

7. After the 30-60 minute incubation is completed, add 5 µl of 10x gel loading solution to reaction tubes to stop the reactions. Cap and mix by tapping. The reactions are ready for electrophoresis.

Cleavage of DNA with Restriction Enzymes

Setting up DNA Digests (Select one or more digestions)

Reaction Tube	Reaction Buffer (5 µl)	DNA 1 (5 µl)	DNA 2 (5 µl)	DNA 3 (Lambda) (5 µl)	*Eco* RI (5 µl)	*Bam* HI (5 µl)	Qualified Water (to 50 µl)	Reaction Volume (50 µl)	10x Gel Load (5 µl)	Final Volume (55 µl)
1	-------- Standard DNA Markers are ready to load for electrophoresis --------									
2										
3										
4										
5										
6										

Reminder:
Do not cross-contaminate enzyme and DNA stocks by using the same pipet tip.

OPTIONAL STOPPING POINT

After addition of 10x gel loading solution, which stops the reaction, samples are ready for electrophoresis. The samples may be stored in the refrigerator for electrophoresis at a later time.

Agarose Gel Electrophoresis

Wear Gloves and Goggles

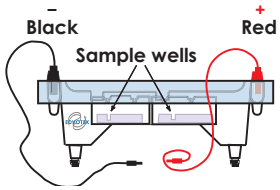
Prepare the Gel

1. Prepare a 0.8% agarose gel for electrophoresis and DNA staining with Flash-Blue ™ or InstaStain® Blue. Refer to Appendix A. Alternatively, If DNA will be stained with InstaStain® Ethidium bromide, refer to Appendix B.

- Recommended gel size: 7 x 7 cm or 7 x 14 cm
- Number of sample wells required: 6
- Placement of well-former template: first set of notches
- Agarose gel concentration required: 0.8%

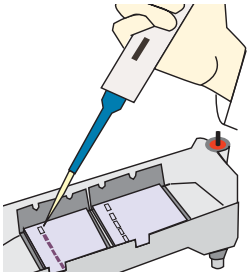
Reminders:

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.



After connecting the apparatus to the D.C. power source, check that current is flowing properly - you should see bubbles forming on the two electrodes.

Load the Samples



2. Before loading samples, equilibrate a 65°C waterbath or beaker of water for heating the tubes containing DNA fragments.

   At 65°C, non-specific aggregation due to sticky ends generated by restriction enzyme digestions will melt. This will result in sharp individual DNA bands upon separation by agarose gel electrophoresis.

3. Heat the samples, including the Standard DNA fragments for two minutes at 65°C. Allow the samples to cool for a few minutes.

4. Load 35 µl of each DNA sample in the following manner:

Lane	Tube label	
1	Markers	Standard DNA Fragments
2	2	DNA Digest 1
3	3	DNA Digest 2
4	4	DNA Digest 3
5	5	DNA Digest 4
6	6	DNA Digest 5

Run the Gel

5. After the DNA samples are loaded, set the power source at the required voltage and conduct electrophoresis for the length of time specified by your instructor.

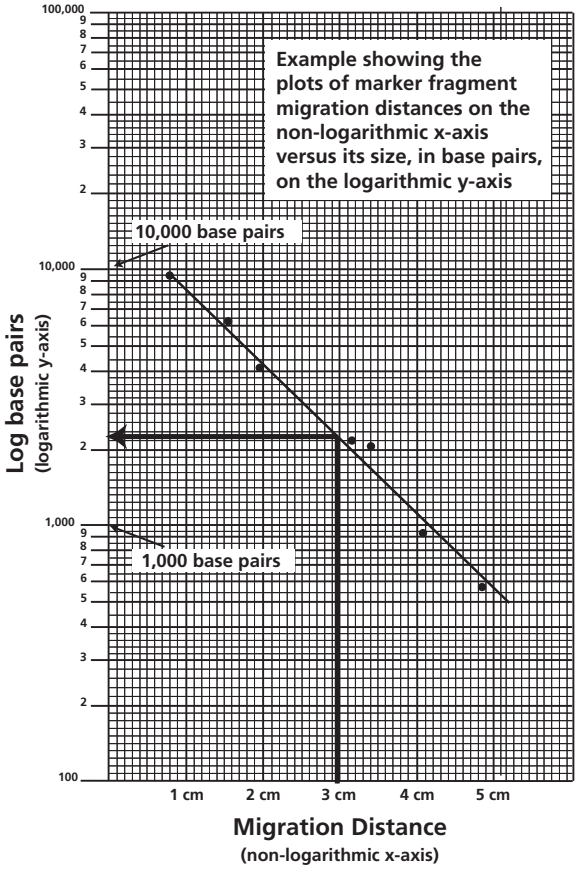
6. After electrophoresis is completed, proceed to DNA staining and visualization (See Appendices for staining options and instructions).

7. Document and analyze the gel results.

Experiment Procedure

Size Determination of DNA Restriction Fragments

The sizes of DNA restriction fragments can be determined by migration distances after electrophoresis. The sizes of the "unknowns" (DNA digests in lanes 2-6) will be extrapolated by graphing their migration distances relative to the Standard DNA Fragments, for which the size of each fragment is known. The assignment of sizes for DNA fragments separated by agarose gel electrophoresis can have a ±10% margin of error.



DNA FRAGMENT SIZE DETERMINATION

1. Measure and record the distance traveled in the agarose gel by each Standard DNA fragment (except the largest 23,130 bp fragment, which will not fit in a straight line in step 4).

   In each case, measure from the lower edge of the sample well to the lower edge of each band. Record the distance traveled in centimeters (to the nearest millimeter).

2. Label the semi-log graph paper:
   - Label the non-logarithmic horizontal x-axis "Migration Distance" in centimeters at equal intervals.
   - Label the logarithmic vertical y-axis "Log base pairs". Choose your scales so that the data points are well spread out. Assume the first cycle on the y-axis represents 100-1,000 base pairs and the second cycle represents 1,000-10,000 base pairs.

3. For each Standard DNA fragment, plot the measured migration distance on the x-axis versus its size in base pairs, on the y-axis.

4. Draw the best average straight line through all the points. The line should have roughly equal numbers of points scattered on each side of the line. Some points may be right on the line (see semi-log example at left).

Quick Reference:

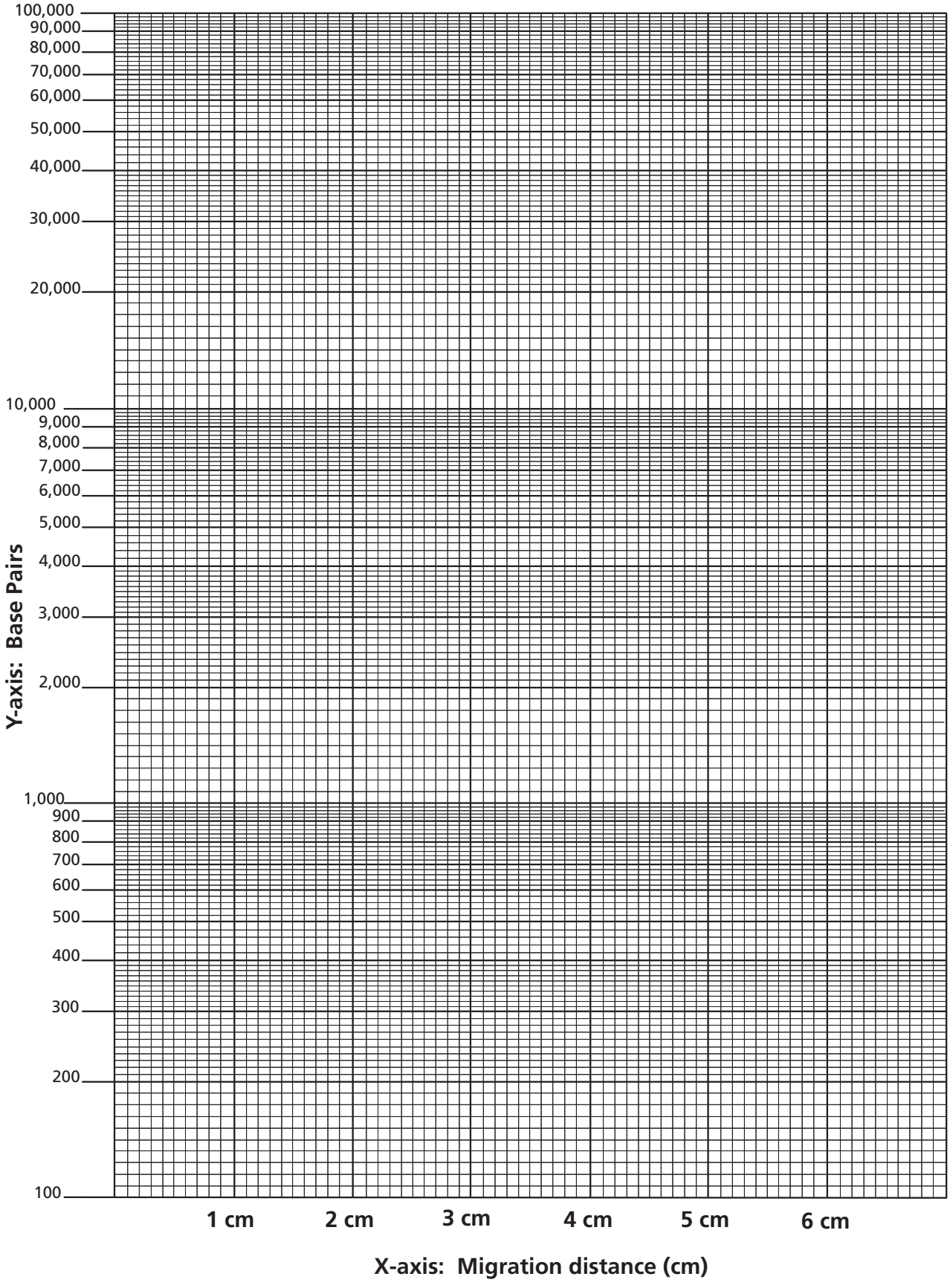
Standard DNA fragment sizes - length is expressed in base pairs.

23130	9416	6557
4361	3000	2322
2027	725	570

5. Measure the migration distance of each of the DNA fragments from the three restriction enzyme digestions (reactions 2, 3 and 4).

6. Using the graph of the Standard DNA fragments, determine the sizes in base pairs of each fragment from the restriction enzyme digestion reactions. Find the migration distance of a fragment on the x-axis - draw vertical line from that point until standard graph line is intersected. From the point of intersection, draw a second line horizontally to the y-axis and determine the approximate size of the fragment in base pairs (see semi-log example at left).

Cleavage of DNA with Restriction Enzymes



Experiment Analysis & Study Questions

Observe and record the results of your experiment in your laboratory notebook or on a separate worksheet. Continue and answer the following study questions:

1. Can the size of a supercoiled plasmid DNA be determined by using standard DNA fragments electrophoresed in parallel? Why?

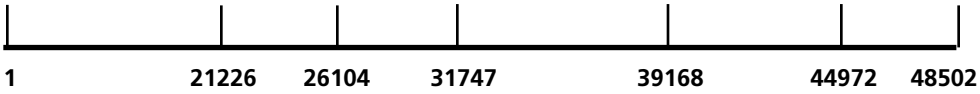
2. An unknown DNA molecule was cleaved using several restriction enzymes individually and in various combinations. The DNA fragment sizes were determined by agarose gel electrophoresis and the restriction enzyme recognition sites were mapped. Subsequently, the DNA was sequenced and an extra recognition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

3. Why are the Lambda DNA fragments heated prior to electrophoresis?

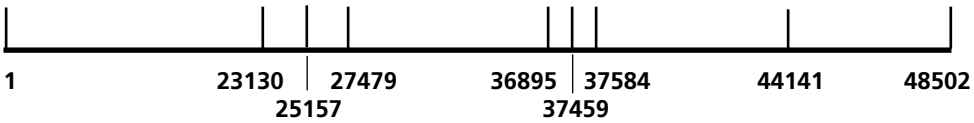
4. Predict the number of DNA fragments and their sizes if Lambda phage DNA were incubated and cleaved simultaneously with both *Hind* III and *Eco* RI (refer to the map below).

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

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 containing DNA markers run in parallel with other DNA samples. For DNA Sizing instructions, and other laboratory extension suggestions, please refer to the EDVOTEK website.

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

Visit the EDVOTEK web site often for continuously updated information.

Pre-Lab Preparations

The experimental procedures consist of two major parts:

1) Restriction enzyme digestion of DNA
2) Agarose gel electrophoresis

Each group receives biologicals and reagents to plan and perform restriction enzyme digestion reactions, which will be submitted to agarose gel electrophoresis. After electrophoresis, students analyze their gel and determine the locations of restriction enzyme cleavage sites on two circular plasmid DNAs.

APPROXIMATE TIME REQUIREMENTS

- Prelab preparation and dispensing of biologicals and reagents take approximately 1 hour.
- Allow 50-75 minutes for the restriction enzyme digestion and preparation of samples for electrophoresis. Extending the restriction enzyme digest incubation time to 60 minutes will ensure complete cleavage of DNA.
- Approximate time for electrophoresis will vary from 40 minutes to 2 hours. See Agarose Gel Electrophoresis Hints and Help, Appendix I.

PREPARATION OF DNA AND MARKER SAMPLES

1. Thaw the following components on ice:
   - Restriction enzyme dilution buffer (C)
   - Restriction enzyme reaction buffer (D)
   - Enzyme grade water (E)
   - Plasmid DNAs (F & G)
   - Lambda DNA (H)

Quick Reference

Summary of Biologicals and Reagents for each group

D	Restriction Enzyme Reaction Buffer	Rxn Buffer	30 µl
F	Plasmid DNA 1	DNA 1	30 µl
G	Plasmid DNA 2	DNA 2	30 µl
H	Lambda DNA	DNA 3	30 µl
E	Enzyme Grade Water	Water	40 µl
I	Standard DNA Fragments	Markers	35 µl
A	Diluted *Eco* RI	*Eco* RI	30 µl on ice
B	Diluted *Bam* HI	*Bam* HI	30 µl on ice

2. Tap the tubes with your fingers or on a table to get all contents to the bottom of the tube. Put them back on ice.
3. Label tubes and dispense the DNAs.
   - 30 µl of plasmid DNA 1 to 6 microtest tubes labeled "DNA 1"
   - 30 µl of plasmid DNA 2 to 6 microtest tubes labeled "DNA 2".
   - 30 µl of Lambda DNA to 6 microtest tubes labeled "DNA 3"
4. Label 6 microtest tubes "Marker" and dispense 35 µl of the Standard DNA Fragments (I) to each tube.

Pre-Lab Preparations

RECONSTITUTION OF DRYZYME™ RESTRICTION ENZYMES

Students should perform the restriction enzyme digests within 30 minutes of reconstituting the Dryzymes™.

Prepare two Dryzymes™ *Eco* RI (A) and *Bam* HI (B) as specified in steps 5 - 10.

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

6. Add 100 µl of Restriction Enzyme Dilution Buffer (C) to the solid at the bottom of each tube containing Dryzymes™ and allow the samples to hydrate for 1 minute.

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

Each group should have the following materials

- Automatic micropipet & tips
- Microtest tubes
- Lab marker

Reagent or Biological	Tube label
• Restriction Enzyme Reaction Buffer	Rxn Buffer
• Plasmid DNA 1	DNA 1
• Plasmid DNA 2	DNA 2
• Lambda DNA	DNA 3
• Enzyme Grade Water	Water
• Standard DNA Fragments	Markers
• Diluted *Eco* RI	*Eco* RI
• Diluted *Bam* HI	*Bam* HI
• 10x Gel Loading solution	10x Gel Load

8. Add 100 µl of Enzyme Grade Water (E) to each of the tubes of rehydrated Dryzymes™.

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

10. Keep the reconstituted restriction enzyme on ice. Students should perform the restriction enzyme digests within 30 minutes.

ASSEMBLY OF STUDENT MATERIALS

11. The table at the left summarizes the materials each student group will require for the restriction enzyme digestion part of this experiment.

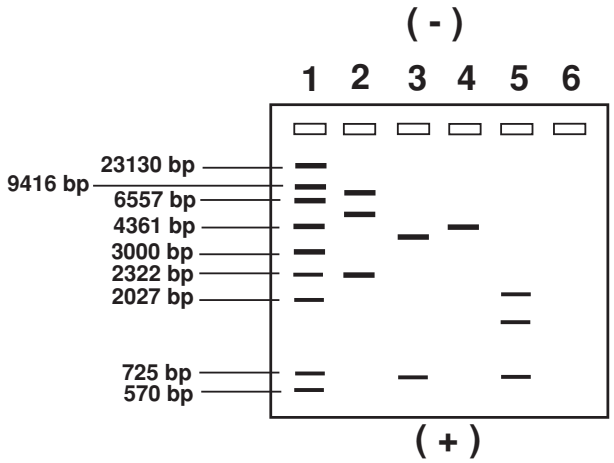
    Once reconstituted, the restriction enzymes are labile. Keep them cold and minimize handling. Prepare the enzymes no more than 30 minutes before use. Once diluted, enzymes must be used.

    Do not cross-contaminate enzyme and DNA stocks by using the same pipet tip.

Expected Results

Results will vary based on the DNA and enzymes used. What follows is an example of a specific set of results from a digestion. The representative results show the relative positions of the DNA restriction fragments. Actual results will yield bands of varying sizes and intensities.

The migration rate of DNA fragments is inversely proportional to the log of their size in base pairs. However, the 23,130 base pair fragment is usually not included in the standard curve. Larger DNA fragments tend to migrate faster than one would predict. This is potentiated by increasing gel porosity and electric field strength. The percentage of agarose used for this experiment minimizes this effect.



Lane 1	Standard DNA Fragments (expressed in approximate base pairs)			
	23130 9416 2322 2027	6557 725	4361 570	3000
Lane 2	Plasmid - superhelical DNAs cannot be precisely measured using linear standard fragments.			
Lane 3	Restriction Enzyme 1	3710 bp 630 bp	± ±	556 95
Lane 4	Restriction Enzyme 2	4340 bp	±	650
Lane 5	Restriction Enzyme 1 & 2	2,080 bp 1630 bp 630 bp	± ± ±	312 245 95

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

Appendices

A 0.8 % Agarose Gel Electrophoresis Reference Tables For DNA Staining with FlashBlue™ or InstaStain® Blue

B 0.8% Agarose Gel Electrophoresis Reference Tables For DNA Staining with InstaStain® Ethidium Bromide

C Buffer and Agarose Quantity Preparations

D Agarose Gel Preparation

E Staining and Visualization of DNA - FlashBlue™

F Staining and Visualization of DNA - InstaStain® Blue

G Staining and Visualization of DNA - InstaStain® Ethidium Bromide

H Electrophoresis Hints and Help

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

0.8% Agarose Gel Electrophoresis Reference Tables
(DNA Staining with FlashBlue™ or InstaStain® Blue)

Appendix
A

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

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

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

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

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

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

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

For DNA analysis, the recommended electrophoresis buffer is Tris-acetate-EDTA, pH 7.8. The formula for diluting EDVOTEK (50x) concentrated buffer is one volume of buffer concentrate to every 49 volumes of distilled or deionized water. Prepare buffer as required for your electrophoresis unit.

Table B Electrophoresis (Chamber) Buffer

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

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

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

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 20 minutes to 2 hours depending upon various factors. Conduct the electrophoresis for the length of time determined by your instructor.

EDVOTEK®

Appendix **B**

0.8% Agarose Gel Electrophoresis Reference Tables
(DNA Staining with InstaStain® Ethidium Bromide)

If preparing a 0.8% gel with concentrated (50x) buffer, use Table A.3.

If preparing a 0.8% gel with diluted (1x) buffer, use Table A.4.

Table A.3 Individual 0.8%* UltraSpec-Agarose™ Gel
DNA Staining with InstaStain® Ethidium Bromide

Size of Gel (cm)	Amt of Agarose (g)	+	Concentrated Buffer (50x) (ml)	+	Distilled Water (ml)	=	Total Volume (ml)
7 x 7	0.15		0.4		19.6		20
7 x 14	0.3		0.8		39.2		40

Table A.4 Individual 0.8%* UltraSpec-Agarose™ Gel
DNA Staining with InstaStain® Ethidium Bromide

Size of Gel (cm)	Amt of Agarose (g)	+	Diluted Buffer (1x) (ml)
7 x 7	0.15		20
7 x 14	0.3		40

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

For DNA analysis, the recommended electrophoresis buffer is Tris-acetate-EDTA, pH 7.8. The formula for diluting EDVOTEK (50x) concentrated buffer is one volume of buffer concentrate to every 49 volumes of distilled or deionized water. Prepare buffer as required for your electrophoresis unit.

Table B Electrophoresis (Chamber) Buffer

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

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

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

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.

EDVOTEK®

Buffer and Agarose Quantity Preparations

Appendix
C

To save time, electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be stored for use at a later time and solidified agarose can be remelted.

Table D
Bulk Preparation of Electrophoresis Buffer

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

BULK ELECTROPHORESIS BUFFER

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

PREPARING AGAROSE GELS BY BATCH

For quantity (batch) preparation of 0.8% agarose gel solution, refer to Table E.1.

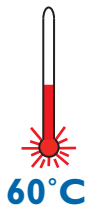
1. Use a 500 ml Pyrex flask or beaker to prepare the diluted gel buffer
2. Pour the appropriate amount of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.

Table E.1
Batch Preparation of 0.8%* UltraSpec-Agarose™

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

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

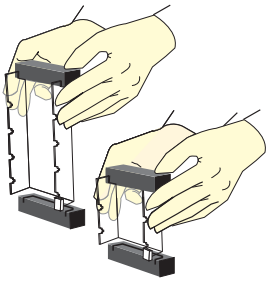
4. Heat the agarose solution in the same manner as described for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 60°C with swirling to promote even dissipation of heat. If evaporation has occurred, 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.
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.



Appendix
D

Agarose Gel Preparation Step by Step Guidelines

EDVOTEK electrophoresis units include 7 x 7 cm or 7 x 14 cm gel casting trays.



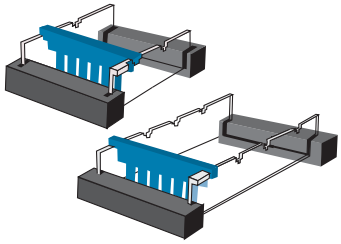
Preparing the Gel bed

1. Close off the open ends of a clean and dry gel bed (casting tray) by using rubber dams or tape.

   A. Using Rubber dams:
   - Place a rubber dam on each end of the bed. Make sure the rubber dam fits firmly in contact with the sides and bottom of the bed.

   B. Taping with labeling or masking tape:
   - Extend 3/4 inch wide tape over the sides and bottom edge of the bed.
   - Fold the extended tape edges back onto the sides and bottom. Press contact points firmly to form a good seal.

2. Place a well-former template (comb) in the first set of notches at the end of the bed. Make sure the comb sits firmly and evenly across the bed.



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

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

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

Casting Agarose Gels

3. Use a 250 ml flask or beaker to prepare the gel solution.

4. Refer to the appropriate Reference Table (i.e. 0.8%, 1.0% or 2.0%) for agarose gel preparation. Add the specified amount of agarose powder and buffer. Swirl the mixture to disperse clumps of agarose powder.

5. With a lab marking pen, indicate the level of the solution volume on the outside of the flask.

6. Heat the mixture to dissolve the agarose powder.

   A. Microwave method:
   - Cover the flask with plastic wrap to minimize evaporation.
   - Heat the mixture on High for 1 minute.
   - Swirl the mixture and heat on High in bursts of 25 seconds until all the agarose is completely dissolved.

   B. Hot plate method:
   - Cover the flask with aluminum foil to minimize evaporation.
   - Heat the mixture to boiling over a burner with occasional swirling. Boil until all the agarose is completely dissolved.

   Continue heating until the final solution appears clear (like water) without any undissolved particles. Check the solution carefully. If you see "crystal" particles, the agarose is not completely dissolved.

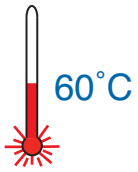
At high altitudes, it is recommended to use a microwave oven to reach boiling temperatures.

Agarose Gel Preparation
Step by Step Guidelines, continued

Appendix
D

7. Cool the agarose solution to 60°C with careful swirling to promote even dissipation of heat. If detectable evaporation has occurred, add distilled water to bring the solution up to the original volume marked in step 5.

DO NOT POUR BOILING HOT AGAROSE INTO THE GEL BED.

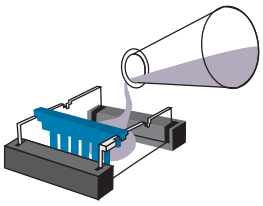


Hot agarose solution may irreversibly warp the bed.

After the gel is cooled to 60°C:

- **If you are using rubber dams, go to step 9.**
- **If you are using tape, continue with step 8.**

8. Seal the interface of the gel bed and tape to prevent agarose solution from leaking.
   - Use a transfer pipet to deposit a small amount of the cooled agarose to both inside ends of the bed.
   - Wait approximately 1 minute for the agarose to solidify.

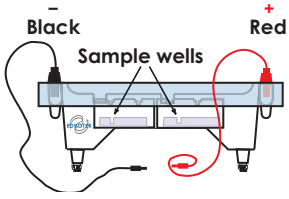


9. Place the bed on a level surface and pour the cooled agarose solution into the bed.

10. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes.

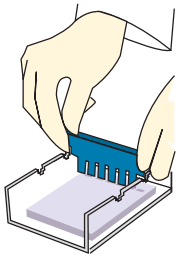
Preparing the gel for electrophoresis

11. After the gel is completely solidified, carefully and slowly remove the rubber dams or tape from the gel bed. Be especially careful not to damage or tear the gel wells when removing the rubber dams. A thin plastic knife, spatula or pipet tip can be inserted between the gel and the dams to break possible surface tension.



During electrophoresis, the DNA samples migrate through the agarose gel towards the positive electrode.

12. Remove the comb by slowly pulling straight up. Do this carefully and evenly to prevent tearing the sample wells.



13. Place the gel (on its bed) into the electrophoresis chamber, properly oriented, centered and level on the platform.

14. Fill the electrophoresis apparatus chamber with the appropriate amount of diluted (1x) electrophoresis buffer (refer to Table B on the Appendix page provided by your instructor).

15. Make sure that the gel is completely submerged under buffer before proceeding to loading the samples and conducting electrophoresis.

Appendix

E

Staining and Visualization of DNA
FlashBlue™ Liquid Stain

DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.

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.

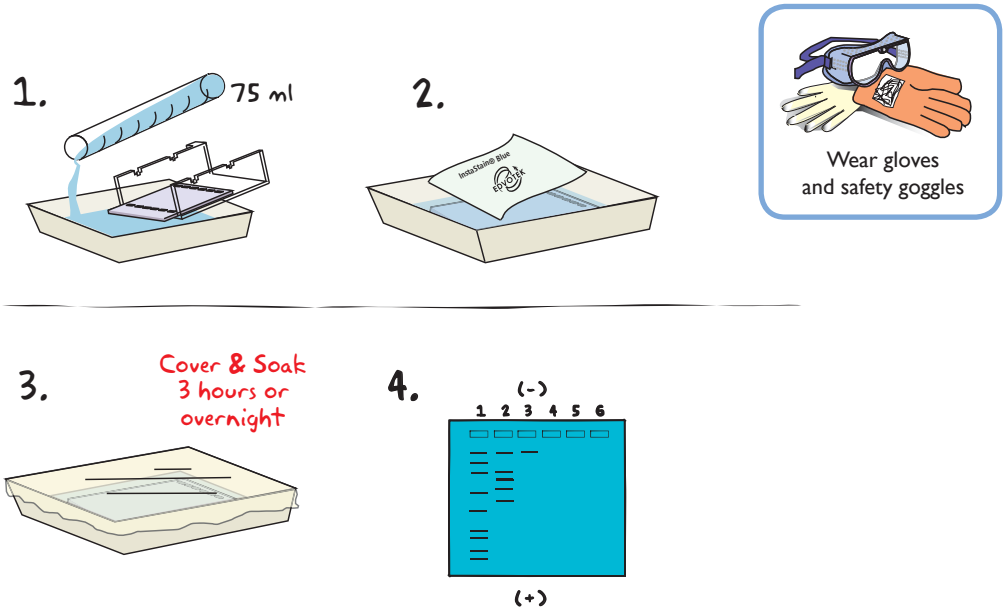
5. **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Alternate 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.

Staining and Visualization of DNA
InstaStain® Blue Stain

Appendix
F



DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.

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

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

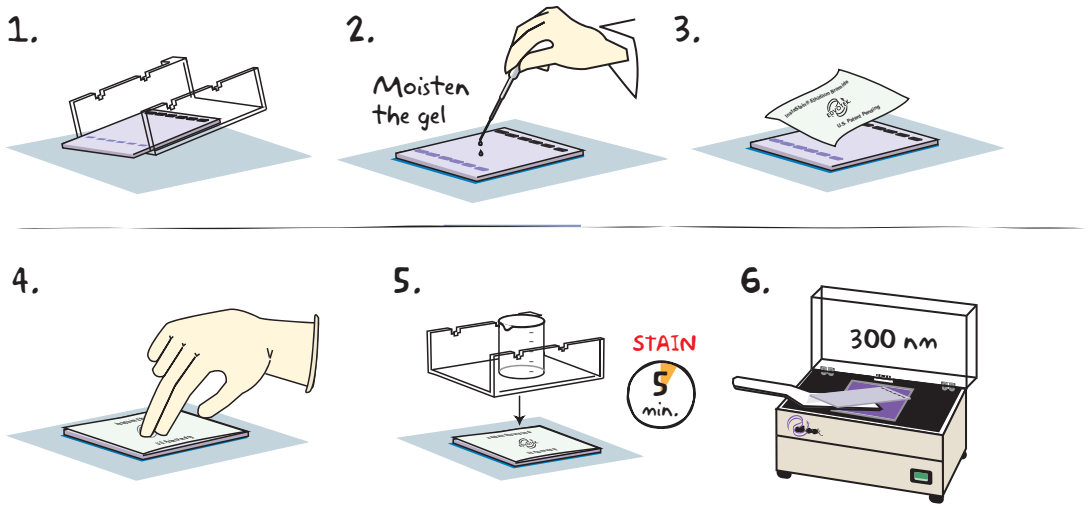
2. Gently **FLOAT** the InstaStain® Blue card(s) on top of the liquid with the stain (blue side) facing the gel. Each InstaStain® Blue card will stain 49 cm^2 of gel (7 x 7 cm). **REMOVE** the InstaStain® card(s) after 30 seconds.

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 and **DOCUMENT** results.

Appendix
G

Staining and Visualization of DNA
InstaStain® Ethidium Bromide Cards



WEAR GLOVES AND GOGGLES WHEN USING THIS PRODUCT.

1. Carefully **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray on to a piece of plastic wrap on a flat surface.

   DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.

2. **MOISTEN** the gel with a few drops of electrophoresis buffer.

3. Wearing gloves, **REMOVE** and **DISCARD** the clear plastic protective sheet from the unprinted side of the InstaStain® card(s). **PLACE** the unprinted side of the InstaStain® Ethidium Bromide card(s) on the gel. You will need 2 cards to stain a 7 x 14 cm gel.

4. With a gloved hand, **REMOVE** air bubbles between the card and the gel by firmly running your fingers over the entire surface. Otherwise, those regions will not stain.

5. **PLACE** the casting tray on top of the gel/card stack. **PLACE** a small weight (i.e. an empty glass beaker) on top of the casting tray. This ensures that the InstaStain® Ethidium Bromide card is in direct contact with the gel surface. **STAIN** the gel for 3-5 min. for an 0.8% gel or 8-10 min. for a gel 1.0% or greater.

6. **REMOVE** the InstaStain® Ethidium Bromide card(s). **VISUALIZE** the gel using a long wavelength ultra-violet transilluminator (300 nm). DNA should appear as bright orange bands on a dark background.

   BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!

Agarose Gel Electrophoresis Hints and Help

Appendix
H

TO MAXIMIZE SUCCESS:

1. The approximate time for electrophoresis will vary from experiment to experiment. A variety of factors, including gel concentration, will influence electrophoresis time. Generally, the higher the voltage applied, the faster the samples migrate. However, depending upon the apparatus configuration and the distance between the two electrodes, individual electrophoresis units will separate DNA at different rates.

2. Do not move the apparatus after the samples have been loaded.
   - Moving the apparatus will dislodge the samples from the wells into the buffer and will compromise results.
   - If it is absolutely necessary to move the apparatus during electrophoresis, you may safely do so after the tracking dye has migrated at least 1 cm from the wells into the gel.

3. For optimal DNA fragment separation, do not use voltages higher than 125 volts for agarose gel electrophoresis. Higher voltages can overheat and melt the gel.

4. The DNA samples contain tracking dye, which moves through the gel ahead of most DNA (except extremely small fragments). Migration of the tracking dye will become clearly visible in the gel after approximately 10-15 minutes.

5. If DNA fragments are similar in size, fragments will migrate close to one another.
   - In general, longer electrophoretic runs will increase the separation between fragments of similar size.
   - Experiments which involve measurement of fragment molecular size or weight should be run at the recommended optimal time to ensure adequate separation.

6. Electrophoresis should be terminated when the tracking dye has moved a minimum of 3.5 to 4 centimeters from the wells for 7 x 7 cm gels, or 5-8 centimeters for 7 x 14 cm gels. Terminate the electrophoresis before the tracking dye moves off the end of the gel.
   - For optimal results, stain the gel immediately after electrophoresis.
   - For convenience, the power source can be connected to a household automatic light timer to terminate the electrophoretic separation and avoid running samples off the end of the gel.

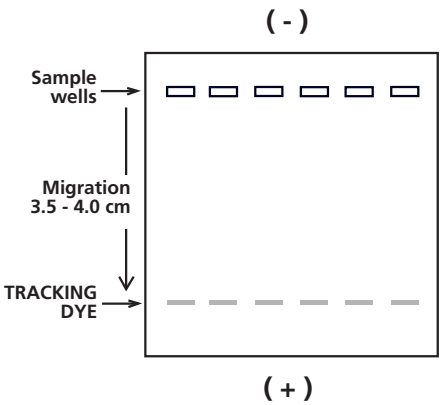


Figure is not drawn to scale.

Appendix
H

Agarose Gel Electrophoresis Hints and Help

TO AVOID COMMON PROBLEMS:

To avoid potential problems, some suggestions and reminders are listed below.

7. Use only distilled or deionized water to prepare buffers and gels. Do not use tap water.

8. To ensure that DNA bands are well resolved, make sure the gel formulation is correct and that electrophoresis is conducted for the recommended optimal amount of time.

9. Correctly dilute the concentrated buffer for preparation of both the gel and electrophoresis (chamber) buffer. Remember that without buffer in the gel, there will be no DNA mobility. Check that the gel is completely submerged under buffer during electrophoresis.

10. For optimal results, use fresh electrophoresis buffer prepared according to instructions.

11. Before performing the actual experiment, practice sample delivery techniques to avoid diluting the sample with buffer during gel loading.

12. To avoid loss of DNA fragments into the buffer, make sure the gel is properly oriented so the samples are electrophoresed from the negative electrode (cathode) towards the positive electrode (anode).

13. To avoid obtaining gel results that are missing small DNA fragments (small fragments move faster), remember that the tracking dye in the sample moves through the gel ahead of the smallest DNA fragments. Terminate the electrophoresis before the tracking dye moves off the end of the gel.

14. If DNA bands appear faint after staining and destaining, repeat the procedure. Staining for a longer period of time will not harm the gel. Restained gels may require longer destaining.

CARE AND MAINTENANCE OF THE ELECTROPHORESIS APPARATUS

15. The temperature of the melted agarose which is poured into the bed during gel casting should not exceed 60°C. Hot agarose solution may irreversibly warp the casting tray.

16. Avoid touching the fragile platinum electrodes.

17. Power should always be turned off and leads disconnected from the power source when the cover is removed from the apparatus.

18. To clean the apparatus chamber, gel casting tray and combs, rinse thoroughly with tap water. Give the items a final rinse with distilled water. Let them air dry. Do not use detergents of any kind, or expose the apparatus to alcohols.

19. EDVOTEK injection-molded electrophoresis units do not have glued junctions that can develop potential leaks. In the unlikely event that a leak develops in any electrophoresis apparatus you are using, IMMEDIATELY SHUT OFF POWER. Do not use the apparatus.