EDVOTEK®

THE **BIOTECHNOLOGY EDUCATION** COMPANY®

REVISED & UPDATED

Edvo-Kit # 302

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Purification of the Restriction Enzyme *Eco* RI

Experiment Objective:

In this experiment, students will purify a restriction endonuclease, test its enzyme activity, and visualize the test results by agarose gel electrophoresis.

See page 3 for storage instructions.

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

Experiment Components

Components		Storage	Check (✓)
A	*E. coli* RY (*Eco* RI) Extract (lyophilized)	Freezer	❑
B	DEAE-Cellulose	Room temperature	❑
C	10x Equilibration Buffer	Freezer	❑
D	50% Glycerol	Freezer	❑
E	KCl	Room temperature	❑
F	*Eco* RI Reaction Buffer	Freezer	❑
G	Qualified Water	Freezer	❑
H	Lambda DNA	Freezer	❑
I	Lambda/*Eco* RI Marker	Freezer	❑
J	*Eco* RI Dilution Buffer	Freezer	❑

This experiment is designed for 5 lab groups.

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

REAGENTS & SUPPLIES
Store all components below at room temperature.

- UltraSpec-Agarose™ powder ❑
- Concentrated electrophoresis buffer ❑
- 10x Gel Loading Solution ❑
- Chromatography columns ❑
- SYBR® Safe Stain ❑
- 5 mL Snap-top microcentrifuge tubes ❑
- 1.5 mL Snap-top microcentrifuge tubes ❑
- 50 mL Conical tube ❑

STORAGE OF PERISHABLES

This experiment includes perishable components which were sent on wet ice. Store these components at -20°C (-4°F). Please note what type of freezer you have and store components accordingly.

Frost-free Freezer

Most refrigerator/freezers in homes are frost free. This means the freezer goes through warming cycles to eliminate frost (defrost cycle). If using this type of freezer, keep the enzymes in the foam chest (with the ice brick) in which they were sent. This will help maintain the enzymes at -20°C when the freezer goes through the defrost cycle.

Non Frost-free Freezer

These older model freezers, which are still sold but are harder to find, do not go through warming cycles. Therefore, ice will build up on freezer walls over time. If using this type of freezer, check to make sure that it maintains temperature at -20°C.

Requirements *(items not included with the experiment)*

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipettes with tips
- Balance
- Water bath
- Ring stand and clamps
- 13 x 100 mm glass test tubes
- Assorted laboratory glassware
- Permanent markers and tape
- 1.5 mL microtest tubes
- Microwave, hot plate or burner
- Pipette pump
- 250 mL flasks or beakers
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Distilled or deionized water
- LED Transilluminator (Cat, 557 TruBlu™ LED Transilluminator, recommended)

Background Information

PURIFICATION OF THE RESTRICTION ENZYME *Eco* RI

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

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.

Restriction Enzyme	Recognition Site
Bam HI *Bacillus amyloliquefaciens* H	5'-GGATCC-3' 3'-CCTAGG-5'
Bgl I *Escherichia coli* RY13	5'-GCCNNNNNGGC-3' 3'-CGGNNNNNCCG-5'
Eco RI *Bacillus globigii*	5'-GAATTC-3' 3'-CTTAAG-5'
Hae III *Haemophilus aegyptius*	5'-GGCC-3' 3'-CCGG-5'
Hind III *Haemophilus influenzae* R4	5'-AAGCTT-3' 3'-TTCGAA-5'

Figure 1: Examples of Restriction Enzymes and their recognition sites.

Most restriction enzymes are composed of two polypoeptides of equal subunits with molecular weights of 20,000-25,000 or single polypeptides with molecular weights of 30,000-35,000. Enzyme activities can be differentiated from each other by their characteristic digestion patterns of small viral DNAs. The DNA from bacteriophage lambda is the most widely used substrate for screening restriction enzymes. Because it is often difficult to determine a characteristic pattern from a lambda digest, smaller DNAs, such as the replicative form of bacteriophage ØX174 and SV40 DNA are also used as substrates. The resulting DNA restriction enzyme digests are displayed on agarose gels and visualized by staining with ethidium bromide.

A given recognition sequence in DNA can often be cleaved by more than one restriction enzyme. The term "isoschizomers" describes a group of restriction enzymes that recognize the same sequence in DNA. The sequences recognized by these enzymes are for the most part centrosymmetric "palindromic" sequences that are usually hexamers, pentamers, or tetramers. Several Type II restriction enzymes recognize DNA at a specific site and hydrolyze phosphodiester bonds at a defined distance from that site. An example of this group of enzymes is *Bgl* I, which recognizes a sequence containing two groups of specified residues separated by completely unspecified residues - GCCNNNNNGGC; it therefore generates DNA fragments with variable end groups.

There is considerable diversity in the fragment termini produced in cleavage by Type II endonucleases that recognize and cleave within the same sequence. In some cases, the 5' extension may be as short as two nucleotides or as long as five. Points of cleavage on each strand may be opposite each other; this results in blunt (square ends). Several restriction endonucleases produce 3' extensions of two to four nucleotides. However, all Type II endonucleases produce fragments with a 5'-terminal phosphate and a 3'-terminal hydroxyl residue (Figure 1).

Enzymes in the Type II restriction enzyme family are amenable to purification by chromatographic procedures. Ion exchangers at nearly neutral pH are used as separation matrices after extracts have been freed of cellular nucleic acids. At this stage of purification, short-term assays often make it possible to visualize enzyme fractions that contain restriction enzymes. A variety of enzymes have been fractionated with affinity chromatography. This method takes advantage of biospecific interactions not offered by conventional fractionation methods. The advantages of affinity chromatography are speed of purification and often protection against denaturation during fractionation.

Effects of Reaction Conditions on Restriction Enzymes

Several reports have described apparent changes in specificity of restriction endonucleases in association with altered reaction environments. Conditions that alter specificity have included changes in ionic concentration, pH of the reaction buffer, and the amounts of glycerol in the storage and the reaction mixture. For example, when lambda DNA is incubated with *Eco* RI or *Bam* HI in the presence of glycerol at various concentrations, a progressive change in the DNA digestion pattern is observed.

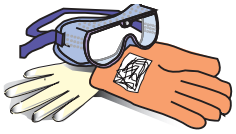
A change in recognition specificity of enzymes include *Bam* HI and *Eco* RI activity. The second activity is designated as ".1" (as *Bam* HI.1). A similar activity is displayed by *Eco* RI. Increasing the pH of the reaction from 7.0 to 9.0 in the absence of monovalent cations stimulates alternate activities. Decreases in the ionic strength have a similar effect.

Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will purify a restriction endonuclease, test its enzyme activity, and visualize the test results by agarose gel electrophoresis.

LABORATORY SAFETY



- Wear gloves and goggles while working in the laboratory.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.

LABORATORY NOTEBOOKS

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

Before starting the Experiment:

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

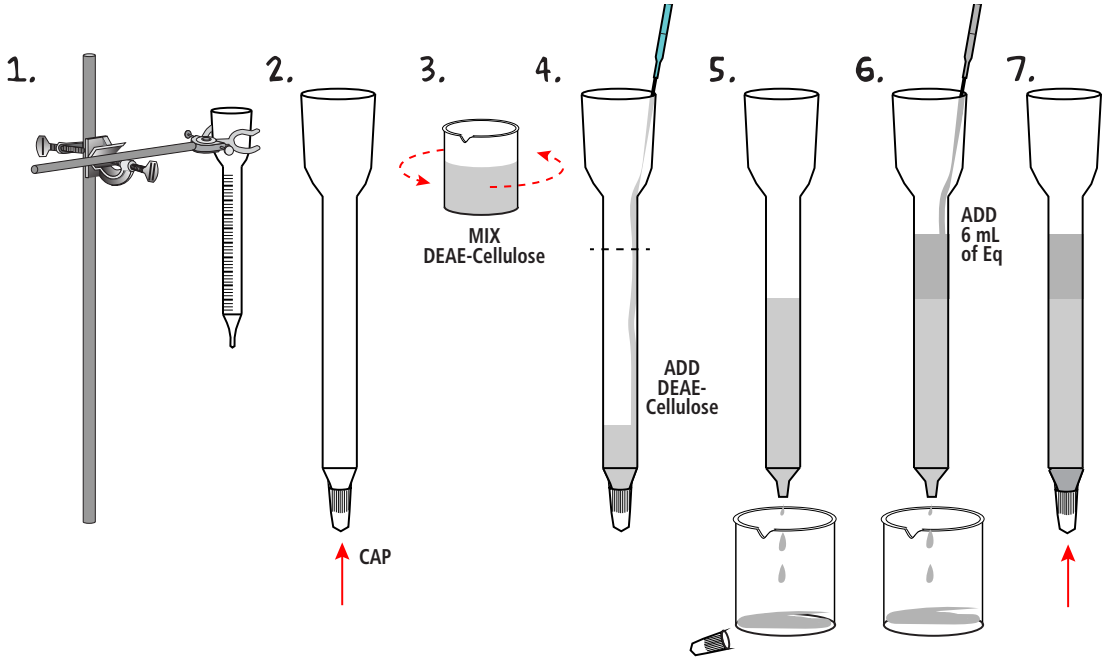
During the Experiment:

- Record your observations in your lab notebook.

After the Experiment:

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

Module I: Partial Purification of *Eco* RI



PACKING AND EQUILIBRATING THE COLUMN

1. Vertically **MOUNT** the column on a ring stand. Make sure it is straight.
2. **SLIDE** the cap onto the spout at the bottom of the column.
3. **MIX** 1.5 mL DEAE-Cellulose (ion-exchanger matrix) thoroughly by swirling or gently stirring. If your DEAE-Cellulose is in a tube, pipette up and down to **MIX** thoroughly.
4. Carefully **PIPETTE** the mixed DEAE-Cellulose into the column by letting it stream down the inside walls of the column.

   If the flow is stopped by an air pocket, **STOP** adding the DEAE-Cellulose and firmly **TAP** the column until the air is removed and the exchanger flows down. Continue adding the exchanger.

5. **PLACE** an empty beaker under the column to **COLLECT** wash material and **REMOVE** the cap from the bottom of the column. Allow the matrix to pack into the column.
6. **WASH** the packed column with 6 mL of Eq (1x equilibration buffer).

   Do not allow the column to go dry!

7. **SLIDE** the cap onto the spout and make sure it does not drip.

NOTE:

The loading of the column and subsequent elution will be done at room temperature. The elution buffers and the fractions collected will be stored on ice as they elute from the column.

Do not allow the column to go dry!

Module I: Partial Purification of *Eco* RI, continued

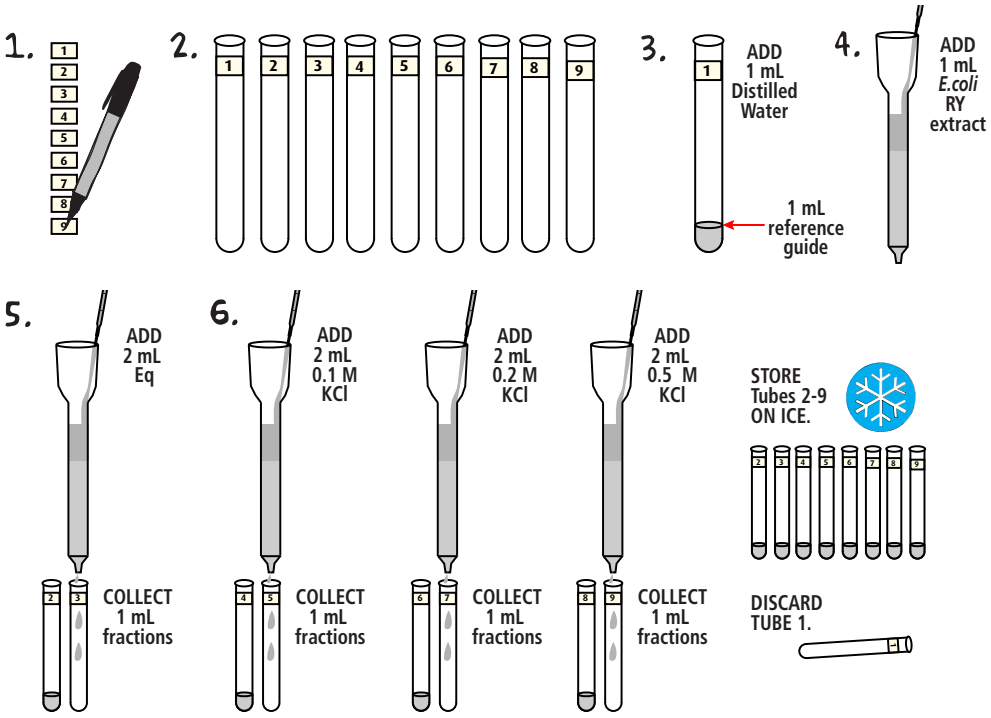


TABLE 1: Key for Identifying Fractions

Tube	Fraction
1	1 mL reference guide
2	(no salt)
3	(no salt)
4	0.1 M KCl
5	0.1 M KCl
6	0.2 M KCl
7	0.2 M KCl
8	0.5 M KCl
9	0.5 M KCl

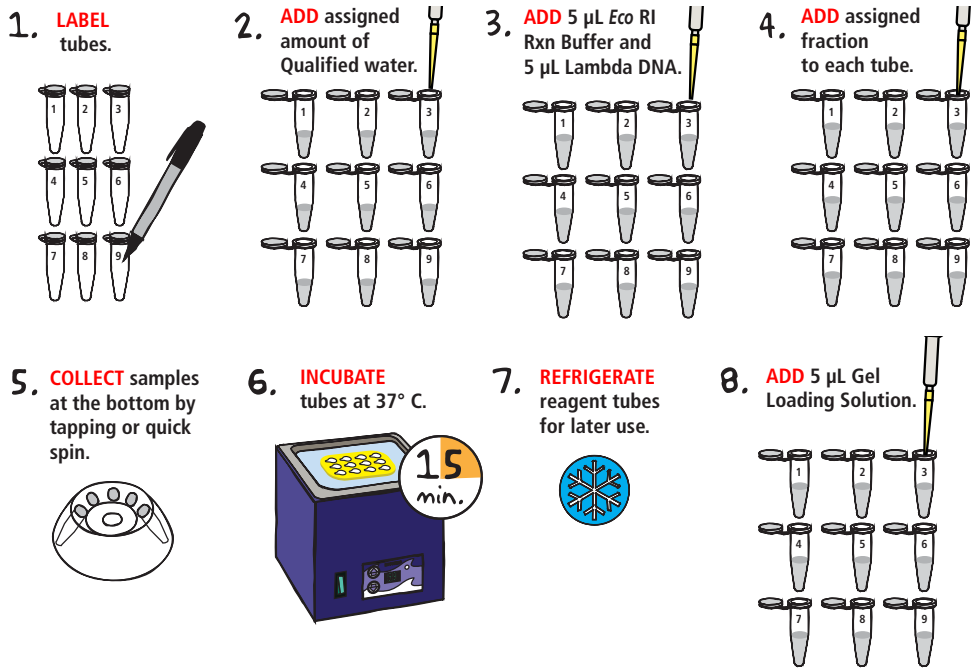
COLLECTING COLUMN FRACTIONS

1. **LABEL** nine pieces of tape with numbers 1 through 9 with a permanent lab marker.
2. **ADHERE** labels to 13x100 mm test tubes. Table 1 indicates which tubes to use for the different fractions.
3. **ADD** 1 mL of distilled water to tube #1 and use this as a reference guide for collecting the eluted fractions.
4. Slowly **LOAD** the column with 1 mL of *E. coli* RY extract. **UNCAP** the column and allow the extra to completely enter the matrix. **RE-CAP** the column when it has completely entered.
5. Slowly **ADD** 2 mL of Eq to the column to remove protein that is in the flow through. **UNCAP** the column and **COLLECT** two fractions (1 mL each) into the tubes labeled 2 and 3 and **STORE** immediately on ice.
6. Sequentially **ELUTE** the column with the following buffers. In each case, **COLLECT** 1 mL fractions into the appropriate tubes and **STORE** fractions on ice immediately upon collection. **Do not let the column to go dry!**
   - Slowly **ADD** 2 mL of 0.1 M KCl. **COLLECT** two 1 mL fractions into tubes 4 and 5. **STORE** immediately on ice.
   - Slowly **ADD** 2 mL of 0.2 M KCl. **COLLECT** two 1 mL fractions into tubes 6 and 7. **STORE** immediately on ice.
   - Slowly **ADD** 2 mL of 0.5 M KCl. **COLLECT** two 1 mL fractions into tubes 8 and 9. **STORE** immediately on ice. **DISCARD** tube #1.

OPTIONAL STOPPING POINT

If time does not permit you to continue with *Eco* RI Activity analysis, you may freeze the fractions at -20°C and perform the assays at a later time. Thaw the fractions at room temperature and immediately place on ice. Continue with the analysis of *Eco* RI Activity.

Module II: Analysis of *Eco* RI Activity (First Assay)



Lambda DNA will be incubated with the fractions collected from Module I and the samples will be electrophoresed in an agarose gel to determine the peak activity of *Eco* RI endonuclease. Lambda DNA cut with *Eco* RI yields a characteristic and recognizable fragmentation pattern.

1. **OBTAIN** from your instructor the reagent tubes listed in Table 2 plus the fraction tubes you collected (Tubes 2-9) in Module I.
2. With a permanent marker, **LABEL** 9 microcentrifuge tubes 1-9. Put your initials and group number on each tube. Each group will assay *Eco* RI using either 2 µL, 4 µL, 6 µL, 8 µL, or 10 µL of your eluted cell fraction as assigned by your instructor. In Table 3 on the next page, the "x" equals your assigned volume for analysis.
3. Use an automatic micropipette to **ADD** (40-x µL) of Qualified water to each of the 9 tubes. For example, if your group was assigned 2 µL you would add 38 µL (40-2=38) of water to the tubes.
4. Use an automatic micropipette to **ADD** 5 µL of the *Eco* RI Rxn Buffer **AND** 5 µL of Lambda DNA to each of the 9 tubes.
5. Use a clean pipette tip for each fraction and **ADD** either 2 µL, 4 µL, 6 µL, 8 µL, or 10 µL as assigned, from each cell fraction from Module I to the appropriate tube. **PLACE** the remainder of your fractions on ice or in the refrigerator to save as they will be used in Module IV.
6. **CAP** the tubes tightly and tap on the lab bench to **COLLECT** samples at the bottom of the tubes or quick spin balanced tubes in a microcentrifuge.
7. **INCUBATE** in a 37°C water bath for 15 minutes.
8. Make sure your set of reagent tubes are labeled with your initials or group number and **STORE** in the refrigerator for later use in the second assay.
9. After the 15 minute incubation is complete, **ADD** 5 µL of 10x gel loading solution to each tube to stop the reactions. This prepares the *Eco* RI digestion products for separation by agarose gel electrophoresis.

Module II: Analysis of *Eco* RI Activity (First Assay), continued

TABLE 2:
Reagents for the Incubation of *Eco* RI with Lambda DNA

Qualified Water (G)	1 mL	on ice
Eco RI Rxn Buffer (F)	100 µL	on ice
Lambda DNA (H)	100 µL	on ice
10x Gel Loading Sol.	100 µL	
Lambda/Eco RI Marker	45 µL	

IMPORTANT NOTE:
Reagents listed in Table 2 will be used for two assays. Label your set of reagent tubes with your initials or group number and store reagents in the refrigerator between assays. When reagents are removed from refrigerator, store them on ice.

TABLE 3: Sequence for Restriction Enzyme Reactions

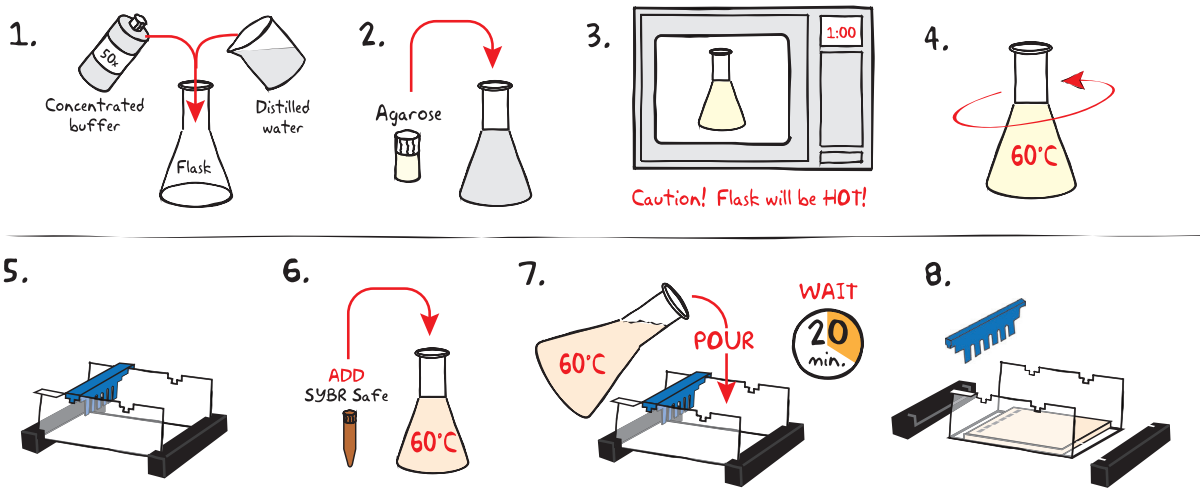
Rxn Tube	Qualified Water (µL)	Eco RI Reaction Buffer (µL)	Lambda DNA (µL)	Fraction* (amt. assigned)	Reaction Volume (µL)	37°C Incubation (minutes)	10x Gel Load (µL)**
1	40	5	5	None	50	15	5
2	(40 - x)	5	5	x µL tube 2 (no salt)	50	15	5
3	(40 - x)	5	5	x µL tube 3 (no salt)	50	15	5
4	(40 - x)	5	5	x µL tube 4 (0.1 M KCl)	50	15	5
5	(40 - x)	5	5	x µL tube 5 (0.1 M KCl)	50	15	5
6	(40 - x)	5	5	x µL tube 6 (0.2 M KCl)	50	15	5
7	(40 - x)	5	5	x µL tube 7 (0.2 M KCl)	50	15	5
8	(40 - x)	5	5	x µL tube 8 (0.5 M KCl)	50	15	5
9	(40 - x)	5	5	x µL tube 9 (0.5 M KCl)	50	15	5

** Volumes of Eco RI in fractions should be varied among different groups within the range of 2 to 10 µL, with 2 µL increments. Water in the assay should be adjusted accordingly.*

*** To be added after incubation at 37°C.*

OPTIONAL STOPPING POINT
If time does not permit you to continue with agarose gel electrophoresis at this time, you may freeze the fractions at -20°C and perform the electrophoresis at a later date. Thaw the fractions at room temperature and heat the samples at 65°C before loading the gel.

Module III: Analyzing the First Assay by Electrophoresis



PREPARING THE AGAROSE GEL WITH SYBR® SAFE STAIN

1. **DILUTE** the concentrated (50X) electrophoresis buffer with distilled water to create 1X buffer (see Table A).
2. **MIX** the agarose powder with 1X buffer in a 250 mL flask (see Table A).
3. **DISSOLVE** the agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. **COOL** the agarose to 60°C by carefully swirling the flask to promote even dissipation of heat.
5. While the agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the comb in the appropriate notch.
6. Before casting the gel, **ADD** the diluted SYBR® Safe stain to the cooled molten agarose and swirl to mix (see Table A).
7. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
8. **REMOVE** the end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Table A — Individual 1.0% UltraSpec-Agarose™ Gel with Diluted SYBR® Safe Stain

Size of Gel Casting tray	Concentrated Buffer (50x)	+	Distilled Water	+	Amt of Agarose	=	TOTAL Volume	Add DILUTED SYBR® (Step 6)
7 x 7 cm	0.5 mL		24.5 mL		0.25g		25 mL	25 µL
7 x 14 cm	1.0 mL		49.0 mL		0.50 g		50 mL	50 µL

Wear gloves and safety goggles

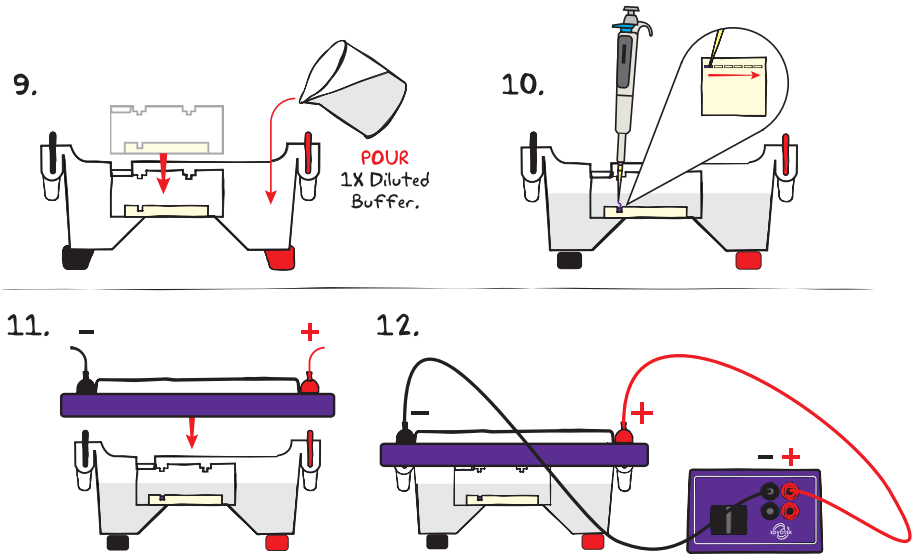
IMPORTANT:

7 x 14 cm gels are recommended. Place the comb in the first set of notches.

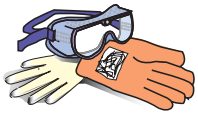
If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at **www.edvotek.com**

NOTE:
The listed volumes of SYBR® Safe are for stain that has already been diluted by the instructor.

Module III: Analyzing the First Assay by Electrophoresis, continued



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



Wear gloves and safety goggles

RUNNING THE GEL

9. **PLACE** the gel (on the tray) into the electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Gel Loading Table - First Assay for recommended volumes). The gel should be completely submerged.
10. **LOAD** 20 µL of each sample into the wells in the order indicated by the Gel Loading Table, right.
11. **CHECK** that the gel is properly oriented, then **PLACE** the safety cover onto the chamber. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** the leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and visualize the gel.

Gel Loading Table - First Assay

LANE	TUBE	SAMPLE
1	Marker	Lambda *Eco* RI Marker
2	1	Uncut Lambda DNA
3	2	Lambda + Fraction 2(no salt)
4	3	Lambda + Fraction 3(no salt)
5	4	Lambda + Fraction 4(0.1 M KCl)
6	5	Lambda + Fraction 5(0.1 M KCl)
7	6	Lambda + Fraction 6(0.2 M KCl)
8	7	Lambda + Fraction 7(0.2 M KCl)
9	8	Lambda + Fraction 8(0.5 M KCl)
10	9	Lambda + Fraction 9 (0.5 M KCl)

OPTIONAL STOPPING POINT

Gels can be stored for several days. Place gel in a watertight plastic bag with 2 mL of electrophoresis buffer and store in the refrigerator.

Table B — 1x Electrophoresis Buffer (Chamber Buffer)

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

Table C — Time and Voltage Guidelines (1.0% - 7 x 14 cm Agarose Gel)

Volts	Recommended Time: Minimum	Maximum
150	40 min.	55 min.
125	55 min.	1 hour 15 min.
70	2 hours 15 min.	3 hours

Module IV: Quantification of *Eco* RI Activity (Second Assay)

Units of enzyme activity are defined by convention. A restriction enzyme unit is defined as the amount of enzyme activity that will digest 1 µg of Lambda DNA at 37°C within one hour under the defined assay conditions. To determine the total units of *Eco* RI purified in this experiment, re-assay pooled enzyme fractions at various enzyme dilutions to determine the minimum amount of enzyme that yields complete digestion of 1 µg of Lambda DNA.

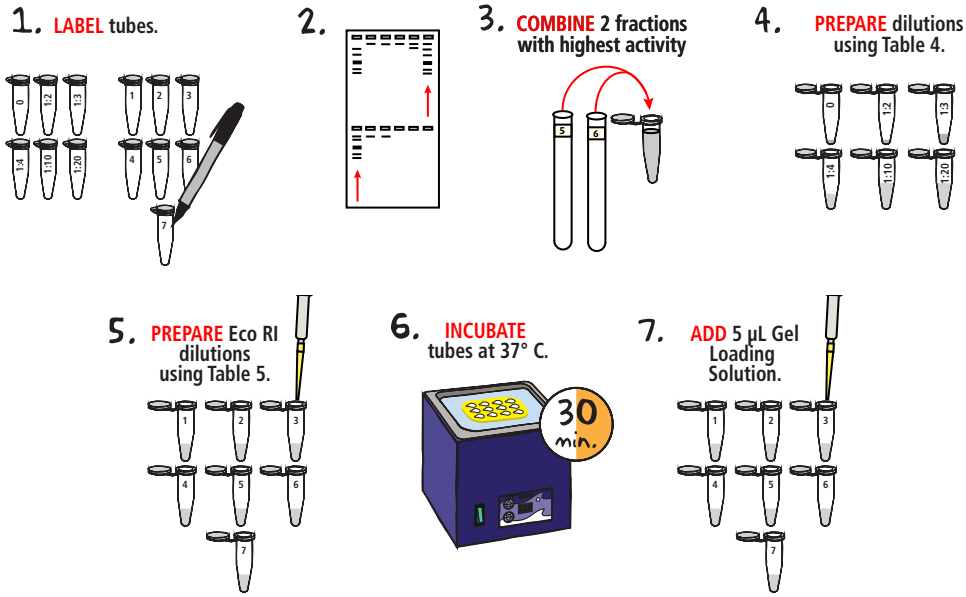


TABLE 4: Dilution of Pooled *Eco* RI

Dilution Factor	Pooled Enzyme Fraction	+ *Eco* RI Dilution Buffer	= Total Volume
0	10 µL	0 µL	10 µL
1:2	5 µL	5 µL	10 µL
1:3	10 µL	20 µL	30 µL
1:4	10 µL	30 µL	40 µL
1:10	10 µL	90 µL	100 µL
1:20	5 µL	95 µL	100 µL

For the Second Assay, each group should **OBTAIN** the following: 13 new 1.5 mL microcentrifuge tubes, a 5 mL snap-top tube, their fractions from Module I, their leftover Reagents from Module II, and 250 µL *Eco* RI Dilution buffer (Component J). ***NOTE: Store all fractions and reagents on ice.***

1. With a permanent marker, **LABEL** 6 microtest tubes: 0, 1:2, 1:3, 1:4, 1:10, and 1:20. **LABEL** 7 microtest tubes numbers 1 through 7.
2. By looking at your agarose gel results from Module III, **DETERMINE** which two cell fractions contain the highest *Eco* RI activity (more activity = more digestion of the plasmid). **CHOOSE** the 2 fractions with the most activity.
3. **COMBINE** the 2 fractions into a 5 mL snap-top tube. **CAP** and gently **INVERT** to **MIX**. This is your pooled enzyme fraction.
4. Using the tubes labeled 0, 1:2, 1:3, 1:4, 1:10, and 1:20, **PREPARE** the *Eco* RI initial dilutions according to Table 4.
5. Using the *Eco* RI initial dilutions from the previous step, **PREPARE** the assay dilutions in tubes labeled 1 through 7 (as outlined in Table 5).
6. **INCUBATE** tubes 1 through 7 in a 37°C water bath for 30 minutes
7. After the 30 minute incubation is complete, **ADD** 5 µL 10x Gel Loading Solution to tubes 1 through 7 to stop the reactions. This prepares the *Eco* RI digestion products for separation by agarose gel electrophoresis.

TABLE 5: Assay to Determine Total Units of *Eco* RI

Rxn Tube #	*Eco* RI Dilution (from Table 4)	+ Qualified Water	+ *Eco* RI Reaction Buffer	+ Lambda DNA	= Reaction Volume
1	None	40 µL	5 µL	5 µL	50 µL
2	10 µL of 0	30 µL	5 µL	5 µL	50 µL
3	10 µL of 1:2	30 µL	5 µL	5 µL	50 µL
4	10 µL of 1:3	30 µL	5 µL	5 µL	50 µL
5	10 µL of 1:4	30 µL	5 µL	5 µL	50 µL
6	10 µL of 1:10	30 µL	5 µL	5 µL	50 µL
7	10 µL of 1:20	30 µL	5 µL	5 µL	50 µL

Module V: Analyzing the Second Assay by Electrophoresis

PREPARING AN AGAROSE GEL FOR THE SECOND ASSAY

1. **PREPARE** a 1.0% agarose gel for the second assay according to instructions previously described in Module III.
2. **OBTAIN** tubes 1 through 7 from Module IV and a tube of Lambda *Eco* RI Marker.
3. **LOAD** 20 µL of each sample in the following manner:

Gel Loading Table - Second Assay		
LANE	TUBE	SAMPLE
1	Marker	Lambda *Eco* RI Marker
2	1	Uncut Lambda DNA
3	2	Lambda + Undiluted *Eco* RI
4	3	Lambda + 1:2 Dilution
5	4	Lambda + 1:3 Dilution
6	5	Lambda + 1:4 Dilution
7	6	Lambda + 1:10 Dilution
8	7	Lambda + 1:20 Dilution

4. After the samples are loaded, **CONDUCT** electrophoresis and **VISUALIZE** the gel.
5. **EXAMINE** the gel or take a **PHOTOGRAPH** to determine which lane gives complete digestion determined as follows:
   - No undigested or partially digested lambda DNA is visible.
   - All the DNA digestion products (5 bands) are visible.

Module VI: Activity Determination in Units

Restriction enzyme unit = amount of enzyme activity that will digest 1 µg of lambda DNA at 37°C within one hour·

DETERMINATION OF TOTAL ACTIVITY

Total units (units) is the amount of enzyme activity recovered from the preparation. It does not indicate the level of enzyme purity.

$$\text{Total Activity (units)} = \frac{\text{Pooled volume (µL)}}{\text{Volume used for assay (µL)}} \times \text{Dilution factor}$$

Example for Determining Total units.

Pooled volume is 9 mL = 9000 µL
Eco RI volume for assay = 10 µL
Dilution factor = 4

$$\frac{9000\ \mu L}{10\ \mu L} \times 4 = 3600 \text{ units}$$

for a 30 minute digestion

Conversion for a 1 hour digestion assay:

Total Activity units = 3600 units X 2 = 7200

SPECIFIC ACTIVITY DETERMINATION (OPTIONAL)

Specific activity is defined as the number of enzyme units per mg of total protein in the enzyme fraction. The less total protein the *Eco* RI fraction contains, the higher is its specific activity.

- For this experiment we have equated 1.0 absorbance unit at A_{280}. In 9 ml, the amount of protein is 0.2 mg/ml X 9 mL = 1.8 ml.

$$\text{Specific Activity} = \frac{\text{Total units}}{\text{mg of protein}}$$

Example for Determining Specific Activity

Total units: 7200 units for the total volume of 9 ml
Total mg. of protein - 1.8 mg

$$\text{Specific Activity} = \frac{7200 \text{ units}}{1.8 \text{ mg}} = 4{,}000 \text{ units/mg}$$

Study Questions

1. What is the recognition site for *Eco* RI?
2. How is *E. coli* host DNA protected against action of the *Eco* RI endonuclease?
3. How many *Eco* RI sites are there in lambda DNA?
4. What is the difference between total activity versus specific activity?

Instructor's Guide

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site. In addition, Technical Service is available from 8:00 am to 5:30 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

EDUCATIONAL RESOURCES

Electrophoresis Hints, Help and Frequently Asked Questions

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

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

Notes to the Instructor

MICROPIPETTING BASICS AND PRACTICE GEL LOADING

Accurate pipeting is critical for maximizing successful experiment results. EDVOTEK Series 300 experiments are designed for students who have had previous experience with agarose gel electrophoresis and micropipeting techniques. If your students are unfamiliar with using micropipettes, EDVOTEK highly recommends that students perform Experiment # S-44, Micropipetting Basics, or other Series 100 or 200 electrophoresis experiment prior to conducting this advanced level experiment.

APPROXIMATE TIME REQUIREMENTS

- **Pre-lab preparations**
  Pre-lab preparations and dispensing of biologicals and reagents take approximately 1-2 hours.

- **Restriction Enzyme Digestion**
  The approximate time required for students to perform the restriction enzyme digestion and prepare samples for electrophoresis is 50-75 minutes. Extending the restriction enzyme digest incubation time to 60 minutes will help ensure complete cleavage of DNA.

- **Agarose Gel preparation**
  Whether you choose to prepare the gel(s) in advance or have the students prepare their own, allow approximately 30-40 minutes for this procedure. Generally, 20 minutes of this time is required for gel solidification. See section "Options for Preparing Agarose Gels" below.

- **Conducting Electrophoresis**
  The approximate time for electrophoresis will vary. 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. Follow manufacturer's recommendations. Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C.

Table C
Time and Voltage Guidelines
(1.0% – 7 x 14 cm Agarose Gel)

Volts	Recommended Time Minimum	Maximum
150	40 min.	55 min.
125	55 min.	1 hour 15 min.
70	2 hours 15 min.	3 hours

PreLab Preparations: Module I

PARTIAL PURIFICATION OF *Eco* RI
(Packing the Column and Collecting Fractions)

DEAE-Cellulose Matrix

1. **MIX** the DEAE-Cellulose well so that it is evenly suspended.

2. **ALIQUOT** 1.5 mL for each of the five groups. **STORE** on ice.

The 10x equilibration buffer used to hydrate the DEAE-Cellulose contains potassium phosphate, pH 7.4, EDTA, and β-mercaptoethanol.

Buffers

3. **PREPARE** 500 mL of 1x equilibration buffer (Eq) in a 600 mL flask or beaker. To prepare, **ADD** the following and **STIR** thoroughly:

350 mL	Distilled water
50 mL	10x Equilibration buffer (C)
100 mL	50% glycerol (D)

4. **ALIQUOT** 8.5 Eq buffer for each group. **STORE** on ice. **USE** the remaining Eq buffer to make buffers in step 5.

5. To make Eq + KCl Buffers, **MIX** the following:

	Eq Buffer (1X)	KCl
0.1 M KCl	100 mL	0.75 g
0.2 M KCl	100 mL	1.5 g
0.5 M KCl	100 mL	3.75 g

6. **ALIQUOT** 2.5 mL of each KCl solution per group. **STORE** on ice.

E. coli Cell Extract Containing *Eco* RI Restriction Enzyme

7. **RE-HYDRATE** the sample by adding 0.5 mL of distilled or deionized water to tube component A and let sit for 5 minutes.

8. **MIX** vigorously by vortexing and transfer the entire contents to a 50 mL conical tube. **RINSE** tube A six times - each time with 1 mL of 1x Equilibration buffer (diluted Component C) and **ADD** the rinse material to the 50 mL conical tube. **MIX** the tube well.

9. **LABEL** 5 tubes "*E. coli* RY extract". **ALIQUOT** 1 mL of the re-hydrated extract for each of the student groups. **STORE** on ice.

FOR MODULE I
Each group will need:

- 1.5 mL DEAE-Cellulose
- 8.5 mL Eq Buffer
- 2.5 mL 0.1 M KCl
- 2.5 mL 0.2 M KCl
- 2.5 mL 0.5 M KCl
- 1 mL *E. coli* RY extract
- 1 Chromatography column
- 1 Ring stand with clamp
- 9 Test tubes (13 x 100 mm)
- Automatic micropipette & tips

PreLab Preparations: Modules II & IV

ANALYSIS AND QUANTIFICATION OF *Eco* RI ACTIVITY (First and Second Assays)

Incubation of Fractions with Lambda DNA

Important: Students should be reminded that the reagents they receive are for two assays.

1. **LABEL** 5 tubes "water" and **DISPENSE** 1 mL Qualified Water (G) into the tubes. **STORE** on ice.
2. **LABEL** 5 tubes "*Eco* RI Rxn Buffer" and **DISPENSE** 100 µL of *Eco* RI Reaction Buffer (F) into the tubes. **STORE** on ice.
3. **LABEL** 5 tubes "Lambda DNA" and **DISPENSE** 100 µL of Lambda DNA (H) into the tubes. **STORE** on ice.
4. **LABEL** 5 tubes "10x Gel Load" and **DISPENSE** 100 µL 10x Gel Loading Solution into the tubes.
5. **LABEL** 5 tubes "*Eco* RI Diln Buffer" and **DISPENSE** 250 µL of *Eco* RI Dilution Buffer (J) into the tubes. **STORE** on ice.
6. **PREPARE** a 37°C water bath for *Eco* RI activity analysis.

QUICK REFERENCE

Summary of Reagent Preparations for First & Second Assays

Reagent:	Use for:	Amount:	Store:
Qualified Water (G)	Modules II and IV	1 mL	on ice
Eco RI Rxn Buffer (F)	Modules II and IV	100 µL	on ice
Lambda DNA (H)	Modules II and IV	100 µL	on ice
10x Gel Loading Solution	Modules II and IV	100 µL	---
Eco RI Dilution Buffer (J)	Module IV	250 µL	on ice

NOTE: These reagents are for two assays. If Modules II and IV are performed on different days, store remaining reagents in the refrigerator

FOR MODULE II
Each group will need:

- Qualified Water
- Eco RI Rxn Buffer
- Lambda DNA
- 10X Gel loading solution
- 9 snap-top microcentrifuge tubes

FOR MODULE IV
Each group will need:

- Qualified Water
- Eco RI Rxn Buffer
- Lambda DNA
- 10X Gel loading solution
- Eco RI Dilution Buffer
- 13 snap-top microcentrifuge tubes
- One 5 mL microcentrifuge tube

PreLab Preparations: Modules III & V

AGAROSE GEL ELECTROPHORESIS

Preparation of Agarose Gels:

This experiment requires one 1.0% agarose gel per student group for Module III and another agarose gel per student group for Module V. For best results, we recommend using 7 x 14 cm gels. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Individual Gel Preparation
Each student group can be responsible for casting their own individual gels prior to conducting the experiment (see Module III). Students will need 50X concentrated buffer, distilled water, agarose powder, and SYBR® Safe stain.

Batch Gel Preparation
To save time, a larger quantity of agarose solution can be prepared for sharing by the class (see Appendix B).

SYBR® Safe Stain Preparation
Prepare diluted SYBR® Safe by adding 500 µL of 1X electrophoresis buffer to the tube of concentrated SYBR® Safe and tapping the tube several times to mix. For individual gel preparation, each group will need 50 µL of the diluted SYBR® Safe for a 7 x 14 cm gel. ***Each group will require 50 µL for Module III and 50 µL for Module V.*** For Batch Gel Preparation, you will use the entire tube of SYBR® Safe (see Appendix B).

Preparing Gels in Advance
Gels may be prepared ahead and stored for later use. Solidified gels can be stored for up to a week in the refrigerator in plastic bags containing a small amount of buffer to prevent drying. We recommend adding only 2 mL of buffer to the bag to prevent SYBR® Safe Stain from diffusing out of the gel.

Do not store gels at -20° C because freezing will destroy them.

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

Additional Materials
Each agarose gel should be loaded with the Lambda *Eco* RI marker.

- Pipette 45 µL of the Lambda *Eco* RI marker (I) into labeled microcentrifuge tubes and distribute one tube per group. This will be used for both Module III and Module V.

NOTE:
Accurate pipetting is critical for good experiment results. This experiment is designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students do not know how to use micropipettes, we recommended performing Cat. #S-44, Micropipetting Basics or Cat. #S-43, DNA DuraGel™ prior to conducting this advanced level experiment.

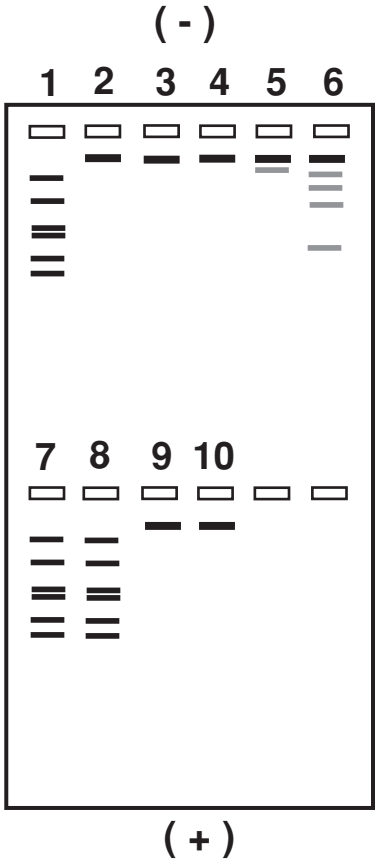
FOR MODULES III & V
Each group will need:

- 50X concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- Diluted SYBR® Safe Stain
- Lambda Eco RI marker

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

Experiment Results and Analysis

In the idealized schematic, the relative positions of DNA fragments are shown but are not depicted to scale. The schematic depicts an idealized gel result for identifying column fractions with *Eco* RI activity.



Lane	Tube	Sample
1	Marker	Lambda *Eco* RI Markers
2	1	Uncut Lambda DNA
3	2	Lambda + 2 (no salt)
4	3	Lambda + 3 (no salt)
5	4	Lambda + 4 (0.1 M KCl)
6	5	Lambda + 5 (0.1 M KCl)
7	6	Lambda + 6 (0.2 M KCl)
8	7	Lambda + 7 (0.2 M KCl)
9	8	Lambda + 8 (0.5 M KCl)
10	9	Lambda + 9 (0.5 M KCl)

* Results may vary between different groups and from the schematic depicted to the left. Some bands may be faint and thus difficult to see. You may also see extra bands due to partial digestion of the DNA. The amount of activity in the flow through may also vary.

Results of the second assay will show varying results depending upon the amount of purified enzyme activity.

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

Appendices

A 1.0% Agarose Gel Preparation Reference Tables

B 1.0% Quantity Preparations for Agarose Gel Electrophoresis

Safety Data Sheets:
Now available for your convenient download on **www.edvotek.com/Safety-Data-Sheets**

Appendix A

1.0% Agarose Gel Preparation Reference Tables

Table A Individual 1.0% UltraSpec-Agarose™ Gel with Diluted SYBR® Safe Stain

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	Add DILUTED SYBR® (Step 6)
7 x 7 cm	0.5 mL	24.5 mL	0.25g	25 mL	25 µL
7 x 14 cm	1.0 mL	49.0 mL	0.50 g	50 mL	50 µL

NOTE: For this experiment, 7 x 14 cm gels are highly recommended.

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 1x Electrophoresis Buffer (Chamber Buffer)

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

Table C Time and Voltage Guidelines (1.0% - 7 x 14 cm Agarose Gel)

Volts	Recommended Time: Minimum	Maximum
150	40 min.	55 min.
125	55 min.	1 hour 15 min.
70	2 hours 15 min.	3 hours

Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C. The approximate time for electrophoresis will vary depending upon various factors. Conduct electrophoresis for the length of time determined by your instructor.

Appendix B

1.0% Agarose Gel Electrophoresis Quantity Preparations

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities that the whole class can share. Leftover diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

BULK ELECTROPHORESIS BUFFER

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

Table D — Bulk Preparation of 1X Electrophoresis Buffer

50x Conc. Buffer	+	Distilled Water	Total Volume 1X Buffer
60 mL		2,940 mL	3000 mL (3 L)

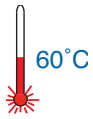
BATCH AGAROSE GELS (1.0%)

For quantity (batch) preparation of 1.0% agarose gels, reference **Table E**.

Table E — Batch Preparation of 1.0% UltraSpec-Agarose™

50x Conc. Buffer	+	Distilled Water	+	Amt of Agarose	+	Amt of SYBR®	=	Total Volume
6.0 ml		294 ml		3.0 g		300 µL		300 ml
8.0 ml		392 ml		4.0 g		400 µL		400 ml
10 ml		490 ml		5.0 g		500 µL		500 ml

1. Use a 500 mL flask to prepare the 1X electrophoresis buffer.
2. Pour the measured UltraSpec-Agarose™ into the prepared buffer. Refer to **Table E** for the mass. 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. If staining with SYBR® Safe, add the volume of diluted SYBR Safe (prepared on page 22) indicated in Table E, to the cooled agarose.
7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 25 mL for each 7 x 7 cm gel, 50 mL for each 7 x 14 cm gel. ***For this experiment, 7 x 14 cm gels are recommended.***
8. 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. Alternatively, gels can be stored in water-tight plastic bags with 2 mL of 1X electrophoresis buffer for up to 1 week in the refrigerator.

NOTE:
The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

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