EXPERIMENT 10: Restriction Digestion and Analysis of Lambda DNA

OBJECTIVE:

DNA splicing, the cutting and linking of DNA molecules, is one of the basic tools of modern biotechnology. The basic concept behind DNA splicing is to remove a functional DNA fragment from one organism and to combine it with the DNA of another organism in order to study how the gene works. The desired result of gene splicing is for the recipient organism to carry out the genetic instructions provided by its newly acquired gene.

The first part of this type of experiment involves the cutting (or digestion) of a DNA strand using enzymes called restriction endonucleases. These enzymes each cut at a specific palindromic sequence present in the DNA strand. Here, we will be digesting Lambda DNA with three different restriction enzymes and examining their cutting pattern using agarose electrophoresis.

MATERIALS:

- Electrophoresis buffer (TAE or TBE)
- Electrophoresis-grade Agarose
- 10X loading dye
- DNA molecular weight markers
- Sub-Cell GT Agarose Gel Electrophoresis System
- Casting platform
- Well combs
- DC Power supply
- Ethidium Bromide Solution (10 mg/mL)
- Enzymes (PstI / EcoRI / HindIII)
- Lambda DNA
- Microtubes
- Pipettor and Tips
- Ice
- 65°C Water Bath
- 37°C Water Bath
- Polaroid Camera
- B&W Film

SAFETY CONCERNS:

1. **Ethidium Bromide:**

Ethidium Bromide (EB) is a potent mutagen and moderately toxic after an acute exposure. EB can be absorbed through skin, so it is important to avoid any direct contact with the chemical. You MUST WEAR GLOVES AND SAFETY GOGGLES while handling this material.

EB is also an irritant to the skin, eyes, mouth, and upper respiratory tract. It should be stored away from strong oxidizing agents in a cool, dry place, and the container must be kept undamaged and tightly closed. You will find it in the hood for this experiment.

Unwanted solid EB, gels, and all working solutions must be disposed of as specified by the Health and Safety Department. For this experiment, you should allow your gel to dry by evaporation in the hood prior to disposal. Once dry, place the gel in a Ziploc bag, then in the Bio Hazard waste can. Disposal of EB into the sanitary sewer (sink drains) or the general trash is PROHIBITED.
2. **UV Illuminator:**

UV rays can cause burns on the skin as well as damage to your eyes. You should wear gloves, goggles and a lab coat when working with the UV Illuminator.

The safety shield will block most of the illumination, but you should take care to limit your exposure to the UV rays.

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**PROTOCOL**

This protocol has been adapted from the BioRAD protocol that accompanies the “Restriction Digestion and Analysis of Lambda DNA” kit. The full manual for that kit can be found on the web:


**Part I: Digestion of the Samples**

1. Label 4 microtubes and place in a foam holder at your bench.

   \[ \begin{align*}
   L & = \text{Uncut lambda DNA} \\
   P & = \text{PstI restriction digest of lambda DNA} \\
   E & = \text{EcoRI restriction digest of lambda DNA} \\
   H & = \text{HindIII restriction digest of lambda DNA}
   \end{align*} \]

2. Using a fresh tip for each sample, transfer 4.0 µl of uncut Lambda DNA to each of the four tubes

3. Add 5.0 µl of the restriction buffer to each of the four tubes.

4. Add 1.0 µl of the appropriate restriction enzyme to the appropriate tube.
   - **DO NOT** add any enzyme to the L sample (the uncut Lambda DNA sample)
   - Change tips whenever you switch reagents, or, if the tip touches any of the liquid in one of the tubes accidentally. When in doubt, change the tip! DNA goes in the tube before the enzyme. Always add the enzyme last.

5. Tightly cap each tube. Mix the components by gently flicking the tubes with your finger. If there is a centrifuge available, pulse the tubes for two seconds to force the liquid into the bottom of the tube to mix and combine reactants. (Be sure the tubes are in a **BALANCED** arrangement in the rotor).

6. Place the tubes in the foam micro tube holder and incubate them at 37°C for 30 minutes.
Table 1: Reaction Components

<table>
<thead>
<tr>
<th>Tube</th>
<th>Lambda DNA</th>
<th>Restriction Buffer</th>
<th>PstI</th>
<th>EcoRI</th>
<th>HindIII</th>
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**This is an excellent time to prep your Agarose gel!**

**Part II: Preparing a 1% agarose gel:**

1. Measure 40 mL of TAE buffer in a graduated cylinder and pour it into a 250 mL flask.
2. Weigh 0.4 grams of electrophoresis grade agarose and add it to the buffer solution in the flask and swirl it gently.
3. Melt the agarose by heating it in a microwave or hot water bath. If you use a microwave, make sure you monitor the melting so that the solution does not boil over, making a large mess.
4. Cool the agarose to about 50-55°C in a water bath for about 5-10 minutes (until it doesn’t burn the surface of your inner forearm). Swirl the agarose occasionally while it cools to avoid lump and bubble formation.
5. Add one drop of the ethidium bromide solution to your cooled agarose solution.
   - **BE VERY CAREFUL** with the ethidium bromide solution. You **MUST WEAR GLOVES** and add the solution in the hood.
   - If you spill the solution let you instructor know **IMMEDIATELY!!**
6. Place the gel tray into the gel box. Insert the black wedges so that the open ends of the tray are blocked off.
   - You can also use tape to close off the ends of the tray then remove the tape once the gel is set!
7. Place the comb in the slots on one end of the tray.
8. Using a pipet, put a small amount of the cooled agarose along the line where the wedge meets the tray to seal it off. Watch for leaks. If there are leaks, remove the wedge, clean the tray, and then try again.
9. Once you are sure there are no leaks, pour the remaining agarose into the tray and let it cool until it is solid (it should appear milky white).

10. Carefully pull straight upward on the comb to remove it once the agarose has solidified.

11. Remove the tray from the box and replace it so that the ends of the gel are open to the chambers on either side of the gel box.

12. Add one drop of Ethidium Bromide solution to your running buffer. (BE CAREFUL!) Pour buffer solution (TBE or TAE) into the gel box so that there is an equal amount of buffer solution in either chamber and there is about 2-3 mm of solution above the gel.

**Part III: Prepare your samples for Electrophoresis:**

1. Following incubation, obtain your four micro test tubes L, P, E, and H and place them in the foam micro test tube holder at your laboratory desk.

2. Using a pipettor, transfer 2.0 µl of sample loading dye to each of the tubes marked L, P, E, and H in the foam tube holder. Use a fresh tip with each sample to avoid contamination.

3. The DNA samples and the sample loading dye must be thoroughly mixed in each tube before placing the samples in the gel wells for electrophoresis.
   - Hold the top of a microtube between the index finger and thumb of one hand and flicking the bottom of the tube gently with the index finger of the other hand.

4. Collect the liquid in the bottom of the tube by tapping it gently on your laboratory bench.

5. Place the four tubes into the microcentrifuge. Be sure that the tubes are in a balanced arrangement in the rotor. If you do not understand how to do this, ask your instructor.

6. Pulse-spin the tubes (hold the button for a few seconds). This forces all of the components to the bottom of the tube.

7. Obtain the DNA marker (M) from your instructor.

8. Heat all samples at 65°C for 5 minutes and then place the samples on ice — this results in better separation of the DNA bands.

**Part V: Loading the gel**

1. Set your pipettor to 10.0 µl and carefully pipette the DNA\loadings dye mixture into a well of your gel.

2. Pipette 10.0 µl of DNA ladder into another well of the gel.
3. Prepare a table in your laboratory notebook indicating which lanes contain which samples as well as the DNA ladder.

**Part VI: Running the gel**

1. Connect the lid to the gel box (make sure that the negative pole is on the same side as the wells of your gel!) and then connect the positive and negative leads into the appropriate connection on the power supply.

2. Turn the power supply on and adjust the voltage to about 120 volts.
   - You can confirm that current is flowing by observing bubbles coming off the electrodes. **DNA will migrate towards the anode, which is usually colored red.**

3. Check that the loading dye is running toward the correct end of your gel after a few minutes.

4. Run the gel until the first dye is near the end of your gel.

5. Switch the power supply off, remove the lid, and carefully lift your gel tray out.

**Part VII: Band Visualization**

Ethidium Bromide was added to the agarose prior to pouring the gel and to the running buffer. This compound will fluoresce reddish-brown when exposed to UV light. For visualization of your bands, we will be taking a photograph of the gel while being irradiated with UV light. As Ethidium Bromide is a potential mutagen, you need to use gloves and safety goggles when handling your gels.

1. Place your gel tray into a Tupperware box for transport to the UV illuminator. This will be located in the Biochem Prep room for this lab.

2. Carefully slide the gel off the tray onto the illuminator.

3. Close the clear lid over your gel. The lid will block the UV rays, but you still want to be careful of prolonged exposure of any skin or your eyes to the UV rays.

4. Turn on the illuminator and determine whether your bands are visible. You may need to turn off the light in the prep room for the best visualization.

5. Once you have determined that you bands are present, turn off the illuminator and prep the camera for use.

6. Make sure that the Ethidium Bromide filter is attached to the camera lens. This filter should read “40.5 mm 15 Deep Yellow” on its edge.
7. Determine whether there is film in the camera. You should see white tabs sticking out of the film compartment if there are exposures left. Ask your instructor for assistance if the camera is empty.

8. On the camera, set the Aperture (top dial) between 16 and 11. Set the Exposure time (bottom dial) to “1”.

9. Lift the clear lid of the illuminator and place the camera directly over your gel.

10. Turn off the prep room light, then turn on the illuminator. With the illuminator on, pull the trigger on the camera and hold until you hear the mechanism finish.

11. Turn off the illuminator, then turn on the room light and remove the camera.

12. Pull the top white tab to remove the picture. Leave it in its sleeve for at least one minute for development.

13. After a minute, remove your picture and determine whether you can see the bands on the gel.

14. If there is a problem, consult the instructor.

15. If you are finished, move your gel back to the box and wipe off the illuminator with a little ethanol and a paper towel. Dispose of the paper towel in the hazardous water.

16. Place your gel (in the box) in the hood and allow to dry until the next lab period. Once dry, place in a Ziploc bag and place in the bio-hazard trash.

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**Part VIII: Analysis of DNA Fragments**

Similar to the procedure used for the SDS-PAGE gels, we will be measuring the distance that the bands traveled then using a standard curve to determine the Base Pairs.

1. Measure the distance traveled by all bands and put in the data table below.

2. Using EXCEL, prepare a standard curve for the DNA marker (see Table below for base pair of the bands), then determine the base pairs of your various DNA bands in the various lanes.

   - Remember to check your R² value for your standard curve. You may need to draw two standard curves, one for the larger data points (Bands 1-3) and one for the smaller data points (Bands 4-6). Estimation of the base pair size should then be made from the appropriate curve.

3. Using this information, draw a map of the Lambda DNA, showing the location of the
restriction sites you observed of the restriction enzymes used in this experiment (PstI; EcoRI; and HindIII). In addition, indicate the size the Lambda DNA and the size of the DNA fragments that resulted from the restriction digest.

Write Up for Notebook:

- Discuss the different enzymes used and their role in the digestion of foreign DNA.
- What evidence do you have that each enzyme cuts at a different site?
- Using the Table below (from the Bio-Rad site) discuss the following:
  - How well did your standard curve (or curves) work at giving the base pair size of the various bands?
  - Why do you think you got less cuts that those listed in the table below? For example PstI should have cut 29 times. Did you see this many bands? Why or why not?
  - Which of the fragments below do you think you visualized?
  - What do you think would happen if you accidentally put two restriction enzymes into a single digest reactions? This technique is called a “Double Digest”.
- Comment on any problems encountered in this experiment and suggest remedies for these problems.
- FYI: The full lambda bacteriophage genomic DNA sequence is available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov under accession number J02459))

<table>
<thead>
<tr>
<th>Uncut lambda DNA</th>
<th>PstI lambda digest</th>
<th>EcoRI lambda digest</th>
<th>HindIII lambda digest</th>
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<tbody>
<tr>
<td>48,502 bp</td>
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