

Lab 1: Restriction Analysis of Phage Lambda DNA

DNA molecules have two types of properties: those that are dependent on the repeating part of the structure, and those that are dependent on the nucleotide sequence. In this laboratory exercise, we will use sequence specific restriction enzymes to digest the phage lambda genome into a number of discrete fragments and then separate the fragments by size in an agarose gel matrix. The electrophoretic and staining properties of the DNA fragments do not depend on the sequence of base pairs.

Objectives

- 1) Review dilution and concentration calculations.
- 2) Review micropipettor technique.
- 3) Understand the properties of the components of a restriction digest.
- 4) Generate, collect, and analyze fragment-length data.
- 5) Answer the lab question and write a lab report.

Lab Question

Three restriction enzymes are provided for your use: *Pst* I, *Hind* III, and *Eco*R I. They have been randomized and labeled X, Y, and Z. Use restriction fragment analysis of the phage lambda genome to determine which restriction enzyme is in which tube.

Introduction

Restriction enzymes are bacterial defense proteins that digest DNA by making staggered double-stranded cuts at specific sequences. This allows bacteria to prevent viral replication by digesting the viral genome before it can be replicated or transcribed. The restriction enzymes we will use in this laboratory are homodimers that recognize DNA sequences that show two-fold symmetry (Table 1).

Table 1. Restriction Sites

<i>Eco</i> R I	5' ...G [^] AATTC ... 3' 3' ...CTTAA [^] G... 5'
<i>Hind</i> III	5' ...A [^] AGCTT... 3' 3' ...TTCA [^] A... 5'
<i>Pst</i> I	5' ...CTGCA [^] G... 3' 3' ...G [^] ACGTC... 5'

These six-base sequences occur about once per 4000 base pairs of random-sequence DNA, which makes them useful for digesting medium to large plasmid and phage DNA molecules into a relatively small number of fragments. Since each of the three enzymes has a different recognition sequence, they will cut any substrate at different locations and result in a different collection of fragments.

You will use linear phage lambda DNA as your substrate. The intact genome is 48,502 base pairs in length. During the lab period, you will digest this genome with restriction enzymes X, Y, and Z.

The products of your restriction digest will be separated on a 0.9% agarose gel. In this gel, DNA fragments are loaded in a well, and an electric field is applied. The negatively-charged DNA molecules move toward the positive electrode, and are separated on the

basis of size as larger fragments are retarded by the gel matrix. DNA is visualized by ethidium bromide staining in the presence of ultraviolet light, and data is captured using a digital camera. Then, you can construct a standard curve using the electrophoretic mobility of fragments of known size from the same agarose gel and use this curve to predict the sized of your fragments from their mobility.

Finally, you can go the Genbank sequence database, search for, find, and download an electronic copy of the sequence of the lambda genome. Using this sequence data and the known recognition sequences of the restriction enzymes you are using, you can predict the fragments that will result from each digestion. You can compare the patterns of fragments you saw with the patterns predicted for each restriction enzyme and deduce the identity of the enzymes in tubes X, Y, and Z.

You should complete this exercise with a lab partner. The entire exercise takes about four to six hours over two lab periods, although it may take much longer if you are not skilled at calculating concentrations or at using the computers to find and manipulate sequence data. There are several long waits, for restriction, gel casting, electrophoresis, etc. At your option, you may start early or come to open lab on Wednesday afternoons to complete your project. Whichever you choose, your lab report is due Monday 2 February in lab. Each lab group should work independently

You will also need a bound lab notebook to record your procedures and results. Theses notebooks are available at the bookstore, and should be purchased before beginning the first lab.

Procedure

Section A - Restriction Digest

The following reagents are provided. All must be kept in ice water at all times. The restriction enzymes are extremely vulnerable to even small increases in temperature, and must be used by the entire class. Please do not jeopardize the grades of others by careless handling or contamination of restriction enzymes.

~500 µg / ml Lambda DNA
Restriction Enzymes X, Y, and Z
10 x Buffer X, Y, and Z
Distilled water

In your lab notebook, plan how you will construct a 1/2 ml reaction tube that contains 20 µl total volume with 1 µg of lambda DNA, 1x buffer, and 1 µl of enzyme. Then, reacquaint yourself with the use of the micropipettors. Once you are certain you know what you are doing, get your instructor's approval on the line below and set up your reaction. Mistakes at this point will cost many hours latter on, so be careful! Add the enzyme last (Why?). Mix by pipetting up and down gently, centrifuge briefly to collect, place the tube in a 37° C water bath for one hour. After the incubation, pulse again in the centrifuge, and place the tube at room temperature.

Section A Approval: _____

Section B - Agarose Gel Preparation

The following reagents are provided.

Agarose Powder
50 x TAE
Distilled Water
Ethidium Bromide Stock - Caution!

In your lab notebook, plan how you will construct a 0.8% agarose gel in 1 x TAE, with 100 mL total volume. Get your instructor's approval, combine the reagents, and heat to dissolve the agarose. Following the instructions on the next page, cast the gel, and allow to solidify for 40 minutes at room temperature.

Section B Approval: _____

Section C - Electrophoresis

Remove the gel from the casting chamber and place it in the electrophoresis chamber with the wells near the black electrode. Fill the chamber with 1x TAE to cove the gel with 2 mm of buffer. Carefully remove the comb.

Removable tray (UVTP) gel casting using a Gel Caster or Mini-Gel Caster

1. Level the Gel Caster or Mini-Gel Caster using the leveling feet in the gel caster and the leveling bubble provided.
2. Disengage and slide the movable wall to the open end of the Gel Caster or Mini-Gel Caster by turning and lifting the cam peg upward.

Note: If casting more than one gel with the Gel Caster, add the removable gel casting wall to the gel caster. The removable wall will allow casting of two 15 x 10 cm trays, four 7 x 10 cm trays or one 15 x 10 cm and one 15 x 15 cm trays.

3. Place the open edge of the UVTP tray against the fixed wall of the Gel Caster or Mini-Gel Caster.
4. Slide the movable wall against the edge of the UVTP tray (Figure 2.1).
5. To seal the open tray ends, engage the cam peg by turning and pressing downward simultaneously.
6. When the cam peg has dropped into the appropriate slot, turn the peg in either direction until resistance is felt. This action seals the edges of the tray for casting.
7. Place the comb(s) into the appropriate slot(s) of the tray.

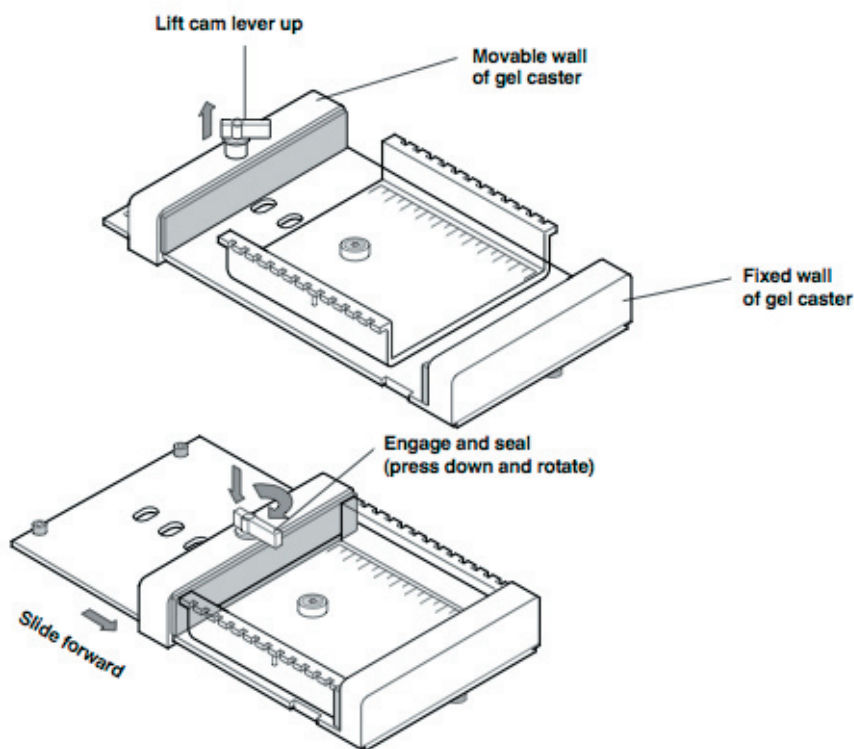


Fig. 2.1. Sealing the UVTP tray for gel casting.

8. Prepare the desired concentration and amount of agarose in 1x electrophoresis buffer (see Section 2.1). When the agarose solution has cooled to 50–60 °C pour the molten agarose between the gates.

Warning: Hot agarose (>60 °C) may cause the tray to warp or craze and will decrease the lifetime of the tray. Warping may also result in sample wells of uneven depth.

Before you begin, answer the following questions: Is phage lambda linear or circular? What are *cos* sites? How could their behavior affect your results? Is there anything you can do to control the behavior of these sequences?

Label four 500 μ l microtubes as “M” (marker), X, Y, and Z for each of your digests. Do not mix the products of the digests. Construct a marker tube containing 2 μ l marker, 4 μ l loading buffer, and 14 μ l distilled water. Construct three experimental tubes by combining 8 μ l of your digestion, 4 μ l of loading buffer, and 8 μ l distilled water. Carefully load the gel by pipetting 20 μ l of each of the tubes into separate wells. Record the lane order in your notebook. Close the electrophoresis chamber and connect the leads. Run the gel at 100 volts. When the dye has migrated to the appropriate position, remove the gel from the electrophoresis chamber and stain in the ethidium bromide solution for 15 min., wash the gel in distilled water for 10 minutes, and photograph on the transilluminator / digital camera. Save your file in the class folder.

Section D - Standard Curve

Print your gel photograph and construct a standard curve by plotting in Excel the log molecular weight versus the distance migrated for the band. Then use your standard curve to predict the molecular weights of all of the fragments in each of your digests.

Section E - Finding Your Unknowns

This part of the exercise requires computer analysis of sequence data. You may complete this portion in the lab or elsewhere, but it is recommended that you work in the lab. You should get a start before the lab period, if possible. Most students will not be able to complete this section in the time allotted. If you need extra time, the lab will be open on Wednesday 12 January and Wednesday 19 January for your use. Set up a user account for your lab group on one of the lab computers. Start early, and see your instructor for help.

You will need the following web resources:

NCBI Entrez
www.ncbi.nlm.nih.gov

Webcutter
<http://www.firstmarket.com/cutter/cut2.html>

RestrictionMapper
<http://www.restrictionmapper.org/>

Go to the NCBI web site to retrieve the DNA sequence of the entire phage lambda genome. Paste or save this sequence into a local file so that you can use it when needed. Trim the file so that it contains only the base sequence of the DNA you are using as a substrate. On the lab computer, save the sequence as a file, open in MS Word, option-select the sequence only, copy and paste it into a new file, and save the new file as text only. You may want to turn off the spelling and grammar checks for this document. Then, use webcutter or RestrictionMapper to predict fragment lengths for the three restriction enzymes you are using. Remember to think about the potential issues posed by *cos* sites. Upload or paste your file, set the analysis parameters (command-select your three restriction enzymes), and run the analysis. You can use the positions of the cut sites on the linear DNA to predict the fragment sizes in each of your digests and solve your unknown.

Assignment - Lab Report

Write a complete lab report with introduction, methods, results, and discussion. Your report should use the format you learned in Biology 107 and Biology 320. External sources will be required for your introduction. The results section must include all of your data, presented in proper graph/table/photograph format, and must include an answer to the lab question that is supported by your data. Do not work with other lab groups. You may work with your lab partner, but each student must write her or his own report, including graphs, tables, and photograph labels. Any students who turn in copied work will be penalized. Remember to submit your entire lab report to turnitin.com before the due date.