Biology 324 Laboratory 1 Leaf/Moyer
Winter 2000

Restriction Digestion of λ DNA

The first way that a DNA molecule is characterized is by mapping restriction sites. This requires a detailed understanding of the distance between restriction sites. To determine the size of a restriction fragment, DNA is resolved by electrophoresis and it's migration is compared to known samples. In this lab, you will cut bacteriophage DNA with Hind III and determine the size of the Hind III fragments with a Hi lo Molecular Markers by agarose gel electrophoresis. Please note that you should be familiar with how to set up restriction digest tables before starting this lab.

Experimental Protocol For Two Lab Periods

1) Set up restriction digestion
2) pouring agarose gel
3) preparing DNA samples
4) electrophoresis
5) photographing gel under UV light
6) analyzing gel with NIH Image
7) graphing mobility versus size of restriction fragments

If you are able to start the restriction digestion by 2:30 pm, you should be able to continue to step 5. If you start digestions after 2:30 pm, you will continue step 3 next week.

Each person will set up one restriction digest of DNA cut with either Hind III. Each group of 2 share a gel and a gel box.

Reagents:

Sterile water- in bottle at bench
10X H buffer- in eppendorf tube on ice
100 mM DTT- in eppendorf tube on ice
Restriction enzymes, Hind III and Eco R1- in -20 degree freezer
10X sample buffer
5X TBE Gel buffer

Equipment

Sterile eppendorf tubes
Sterile pipette tips
Pipettemen
Power Supplies
mini- gel boxes

Setting up restriction digest table:

1) Calculate the amount of the following reagents for the restriction digestion of 1 µg of phage DNA in a total volume of 20 µl. **Before you start your reaction, you must have your restriction digest table approved by DSL.**

Water
DNA (250 ng/µl) - need 1 µg
10X H buffer - final concentration is 1X
100 mM DTT - final concentration is 1mM
Hind III (10 u/µl) - want 5X what is theoretically needed

Initial your tubes!

Restriction Digestion

1). Add the appropriate amount of these in reagents in the above order into a marked eppendorf tube. We will help you in getting the restriction enzymes. ALWAYS KEEP RESTRICTION ENZYMES ON ICE.

2) After adding the reagents to the eppendorf tube, spin the tube a few seconds in the microfuge to get all the solutions to the bottom of the tube.
Then flick the tube gently to mix and place it in a floater in a water bath on the side bench.

3). Let the restriction digest go for 1 hour at 37 degrees in a water bath.

(Note that if you start after 2:30 pm, you won't pour a gel until next week).

Pouring agarose gels

1) Place the comb into the plastic gel tray. The comb has teeth which will form the wells where the DNA samples are loaded. **The bottom of the teeth should be very close but not touching the bottom of the plastic gel tray.**

2) Make an 1% agarose solution in 50 ml of 0.5 X TBE, Melt the agarose solution in a glass beaker by microwaving it 1 to 2 minutes at 30% power. Let the agarose solution cool to 65 degrees.

3) The instructor will show you how to add 5 µl of 10 mg/ml EtBR. ETBR IS A MUTAGEN AND MAY CAUSE CANCER. DISPOSE OF ALL ETBR CONTAMINATED MATERIALS IN THE DESIGNATED CONTAINER IN HOOD.

4) Pour in the molten agarose. **The agarose should fill a little less than 1/2 of the gel tray.**

5) Let agarose cool to room temperature and harden. As it does so, it will turn cloudy. After 10-15 minutes at room temperature, the gel will be ready for electrophoresis.

Preparing the λ Hind III-cut DNA and loading samples onto gel

1) Determine what the concentration of restriction fragments are in the restriction digestion.

Use the table below to determine the appropriate amount of DNA, water and 10XSB. (The **10X sample buffer** is dense solution with a dye. The dye helps track how far the DNA has migrated over time).

Reagent	Concentration	Final conc. or amt.	Volume
DNA		400 ng	
10XSB	10X	1X	
water			
TOTAL VOLUME			20 µl

2) Spin the eppendorf tubes few seconds in the microfuge to centrifuge the solution to the bottom of the tube. Your instructor will help you with microfuge.

3) Before loading the DNA samples into the 1.0 % gel, each person should practice loading 1X sample buffer into the "practice" gel.

After the practice gel has set, place the gel in a horizontal gel box and pour the gel buffer (0.5X TBE) to barely cover the gel.

Each person should practice loading 20 µl of a 1X sample buffer into the gel 2 times or so.

4) After this practice gel loading, add your DNA samples into the wells of the 1.0% agarose gel.

Keep track of where the samples were loaded on the gel.

Electrophoresis

1) After loading, hook up the red electrode to the "bottom" of the gel box, and the black electrode to "top" of the gel box .

2) Turn on the power supply. **Set the voltage for 75 V.** You should see that the blue dye (bromphenol blue) migrating out of the well towards the "bottom" of the gel. (towards the positive electrode).

3) Let the gel electrophorese until bromphenol blue is about 2/3 down the length of the gel, towards the anode, about 40-50 minutes.

Turn off the power supply, disconnect electrodes from the power supply, and view the gel under UV light box.

REMEMBER THAT THE GEL AND GEL BUFFER ARE CONTAMINATED WITH ETBR.

Visualizing the DNA under UV light

In order to see the DNA, the gel will be placed on the Ultra Lum trans-illuminator The gel will be archived using the Ultra Lum system. You will be shown how to use the hardware and software in lab by instructor or TA.
box. The trans-illuminator has a bank of UV lights underneath a quartz glass plate. When it is on, UV light goes through translucent gel, DNA fluoresces orangish-pink. Because the amount of EtBr bound determines the degree of staining, a larger fragment will stain more intensely than a smaller fragment.

SEE ACCOMPANYING HANDOUTS FOR ULTRALUM AND NIH IMAGE DIRECTIONS.

Always wear a face shield or safety glasses when using the UV light.

ALWAYS PUT YOUR GEL ON SARAN WRAP ON UV LIGHT BOX. NEVER PUT A NAKED GEL ONTO THE UV LIGHT BOX.

CLEANING UP

POUR ETBR-CONTAMINATED GEL BUFFER INTO THE LARGE BOTTLE MARKED "ETBR CONTAMINATED 0.5 X TBE"

RINSE OUT ELECTROPHORESIS CHAMBERS

PUT AGAROSE GELS IN BAG MARKED "ETBR CONTAMINATED".

WIPE DOWN YOUR LAB BENCH
QUESTIONS:
1) What size is the bacteriophage Hind III fragments?
2) Is the standard curve linear for all sizes of DNA fragments?
3) What sizes of DNA are resolved poorly on this gel system?
4) How could the resolution of poorly resolved DNA fragments be improved?

POTENTIAL TEST QUESTIONS:
1) Dilution and solution calculations
2) anything over laboratory
3) pp. 39 to 50 from Handout "Basic Tools and Techniques of DNA Science"