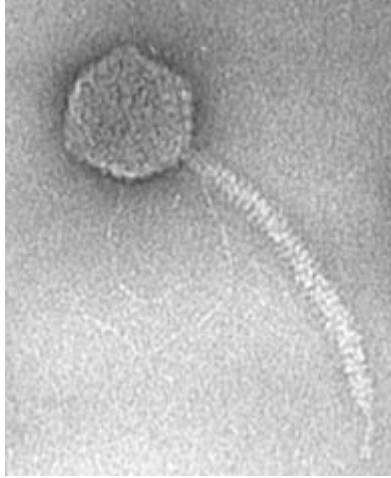


BIOL/CHEM 475 Spring 2007
RESTRICTION ENDONUCLEASES AND BACTERIOPHAGE λ



Lambda (λ) is a temperate *Escherichia coli* bacteriophage. Optional read about phage λ :
<http://www.asm.org/division/M/fax/LamFax.html>
http://en.wikipedia.org/wiki/Lambda_phage
Bacteriophage were important model organisms in the development of the field of molecular genetics.

Major discoveries made with bacteriophage:

<http://www.asm.org/division/M/blurbs/Secrets.html>

If the above link doesn't impress you, try this one:

<http://fire.biol.wvu.edu/trent/trent/fecesbacteriophage.pdf>

☞ The goals of this laboratory exercise are to introduce you to:

1. Restriction enzymes and agarose gel electrophoresis
2. Photographing gels using the Ultralum and the Zoombrowser gel documentation systems
3. Working up protocols and keeping accurate, legible and complete records in your lab notebook

In Lab Today (Week 1):

- Work up protocol for restriction digests of bacteriophage λ genomic DNA → **Step 1 below, Leaf's rules (at end of handout), and handout on reagent volume calculations (digest tables).**
- Set up and incubate restriction digests
- Work up recipe for agarose mini-gel (and get gel mix prepped if time) → **Step 2**
- Work out volumes of DNA to load on gel and recipes for loading each sample → **Step 3**

Before Week 2's Lab:

- Read about restriction enzymes in your biochemistry or genetics textbook or online
- Record in your notebook for each restriction enzyme (1) its recognition site and (2) the composition of the buffers used for the digest. The restriction enzymes that you will be using were purchased from either New England

Biolabs or Promega. You can obtain technical information about each enzyme and the composition of the buffers used at the web sites from these companies (see links on 475 web site).

- Start working on analysis of data from *High resolution agarose gel electrophoresis of restriction digests of λ DNA* (see 475 web site)

In Lab Week 2:

- Analyze restriction digests using agarose gel electrophoresis → **Steps 4 & 6**
- Photograph & print out gel using Ultralum (CCD camera) or Zoombrowser EX (digital camera) → **Steps 5**
- Record all gel info in notebook
- Start next exercise

STEP 1 REAGENTS FOR RESTRICTION ENDONUCLEASE DIGESTS:

- ❖ λ DNA (check label on tube for concentration). *Virion DNA is linear and double-stranded (48502 nt) with 12 bp single-stranded complementary 5'-ends.*
- ❖ Various restriction endonucleases. Check the label on the tube for the number of units per μ l. One unit of restriction enzyme is defined as the amount of enzyme required to produce a complete digest of one μ g of substrate DNA in 60 minutes at the appropriate temperature. The general rule of thumb is to use 5X the minimal amount of enzyme needed.
- ❖ 10X buffers for the various enzymes. Note which company (*New England Biolabs* or *Promega*) supplied the enzymes. You will need this information to look up the composition of the 10X buffers online. Alternatively, check the product sheets which should be posted in the lab. Be sure to note the name of the buffer – for example, buffer B or Multi-core buffer, etc.
- ❖ *Read the Leaf notes at the end of this handout before you get started.*

Restriction digests with Hind III, Pst I and Eco RI

In your lab notebook, work up a protocol for three restriction digests of lambda genomic DNA. Each digest will have one restriction enzyme (listed above).

RESTRICTION ENZYMES MUST BE KEPT ON ICE.

The total volume of each digest should be 20 μ l.

- You should have ~300 ng of λ DNA in each digest. Check the board for the concentration of the stock of λ DNA and doublecheck that it matches the label on the tube that you take your aliquot from.
- Be sure to match the 10X buffers with their cognate enzymes. The final buffer concentration should be 1X. Check the chalkboard to find out if you should add BSA (bovine serum albumin) or DTT (dithiothreitol) to your digests.

- Calculate how much enzyme you need for 5 fold excess. Use a minimum of 1 μ l of enzyme per digest. You will probably be using more than 5X excess but this is the molecular biologist way. **The volume of enzyme added should never exceed 10% of the total volume of the reaction.** *In your lab notebook record the #units/ μ l for each enzyme.*
- You must use nanopure water (in your private stash of reagents).
- Before you get started, have an instructor doublecheck your digest recipe. Mix together the nanopure water, 10X buffer and DNA and then add the enzyme. Mix your digest carefully with a pipetperson. (The storage buffer for the enzyme contains 50% glycerol, so your enzyme will sink right to the bottom of your tube.) If you've splattered the contents of the tube up on the sides, briefly (5 seconds) centrifuge the contents in a microcentrifuge.
- Place digest at 37 $^{\circ}$ C and incubate for 1-2 hours. Store in freezer until next week.

STEP 2 Preparation of agarose minigel

(see also glassware notes on pg 5)

WEEK 1: In your lab notebook, work up a recipe for an agarose minigel. You can use tap distilled water for preparing the gel and the running buffer.

- Figure out your gel volume by measuring the area of the gel bed and assuming that you want your gel to be about 0.5 cm thick
- The gel should be 0.8% agarose cast in 1 X TBE (tris-borate electrophoresis buffer). The TBE stock will be 5X or 10X TBE. This buffer will be made for you the first week. After that you will have to make up your own.

WEEK 2: Make up agarose solution in a 200 ml flask.

- Stuff the top of the flask with kimwipes and microwave -- gently --- until the solution boils. *Be sure that all of the agarose flakes are in solution.*
- After the solution has cooled and right before you pour the gel, add ethidium bromide* to a final concentration of 0.5 μ g/ml. The stock of ethidium bromide is 10mg/ml. Discard etbr contaminated pipet tip in the appropriate waste container.
- Swirl the agarose gently before pouring.
- Use a comb with wide teeth.

* **Working with ethidium bromide:** You must wear gloves whenever you are working with ethidium bromide. It is a mutagen. Treat it with respect. All ethidium bromide contaminated waste should be placed in the proper waste containers (in fume hood).

STEP 3 Preparing samples for loading on minigel:

- ❖ Remove ~150 ng of DNA from each restriction digest. Mix with 6X gel loading dye. The final concentration of the loading dye should be 1X, so you may (or may not) need to add water to your sample. [Alternatively, you could add the appropriate amount of loading dye to the entire reaction and then load part of it.]
- ❖ Also prepare a control sample of 100 ng of uncut λ DNA
- ❖ You will also run one lane of the HI-Lo size standards which are already mixed with gel-loading dye

STEP 4 MiniGel electrophoresis:

- ❖ The running buffer will be 1 X TBE.
- ❖ Carefully load your samples. Be sure to record the location of each sample on the gel. Run the minigel at 80-100 volts. Double check that there are bubbles at the negative pole and that there is current flowing through the gel.
- ❖ Stop the gel when the bromophenol blue dye is about 3/4 of the way down the gel. Check the BPB migration table to determine what size fragments will be running ahead of or behind the dye front. Record voltage, current, length of gel run position of dye when gel run ended.

STEP 5 PHOTOGRAPHING GELS:


- ❖ The gel should be visualized on a ultraviolet transilluminator and photographed as soon as it is removed from the gel box. Be sure to place the gel in a plastic box or on a tray to transport it to one of the transilluminators. Don't forget that the gel buffer is now contaminated with ethidium from the gel.
- ❖ You will receive other handouts describing the use of the Ultralum and Zoombrowser equipment.

Ultraviolet light at 254 nm is absorbed by DNA and transmitted to the bound ethidium dye. The energy is re-emitted at 590 nm in the red-orange region of the visible spectrum. The fluorescence of ethidium-DNA complexes is much greater than that of unbound dye.

Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded. Wear protective goggles or a safety face mask that efficiently blocks the UV light if the transilluminator is not fully enclosed in a light-tight box.

STEP 6 CLEAN-UP

- Take care of glass/plasticwear as described on next page
- Agarose gels should be discarded in the EtBr solid waste containers.
- Gel tank buffer should be discarded in the Et Br liquid waste container. Alternatively, you can decant it into a large bottle and use it again. The gel tanks should be rinsed with tap distilled water and allowed to drain on paper towels on the lab bench. Ditto with gel beds and combs. Remember to wear gloves at all times.
- Wipe down your lab bench.

 ***A note about glassware:*** With the exceptions noted below, all dirty glass/plasticware should be placed on the cart at the front of the lab.

EXCEPTIONS:

- If you have used glassware for measuring tap distilled water, do not put it on the dirty glassware cart. Drain water and return to general circulation.
- When preparing 1X TBE use large graduated cylinders and flasks that are labelled TBE only. After you have prepared your diluted TBE solution, decant into gel box and/or storage bottle. Rinse out cylinders&flasks with tap distilled water and return to general circulation – with TBE label intact. [Likewise for smaller cylinders and flasks – although each pair can probably lay claim to these and keep them as private stash in their cabinet.]
- **Ethidium-bromide contaminated glassware.** Each pair of students should keep a 200 ml flask for preparing agarose gels. This flask should be labelled EtBr contaminated. After you have poured your gel*, rinse the flask with a small volume of hot tap water and discard this rinse into the EtBr liquid waste container. Rinse the flask again with tap distilled and discard down the drain. Retain flask with label in your private glassware stash – do not put on the dirty glassware cart. *If left-over agarose sets up in the flask, as a little water and nuke it -- then discard in the liquid waste.

Appendix A

Stock Reagents for agarose gel electrophoresis:

6x sample loading dye:

15% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol, and 50 mM EDTA

5X TBE: Tris borate electrophoresis buffer:

54 g	Tris Base
27.5 g	Boric Acid
20 ml	0.5 M EDTA, pH 8

Add distilled (from the tap)H₂O to obtain a final volume of **1 Liter**

Check final pH – it should be ~ 8.3

Ethidium Bromide at 0.5 µg/ml final (10mg/ml stock)