

DNA, RESTRICTION ENZYMES, AND GEL ELECTROPHORESIS

INTRODUCTION

In this two-day lab you will explore the many properties of DNA. On the first day you will use salmon sperm DNA to demonstrate the properties of denaturation and renaturation. Next you will perform extractions of DNA from both salmon sperm and E. coli to demonstrate how DNA can be precipitated and isolated from a pure solution or from living cells. Then you will use restriction enzymes to cleave DNA from the virus bacteriophage λ . On day two you will analyze your restriction digests by gel electrophoresis.

DAY 1

SALMON SPERM DNA

EQUIPMENT

Small test tube & test tube gripper	2 stirring rods
1 ml squeeze pipette	~100 mg salmon sperm DNA
2M NaCl	100°C water bath
TE buffer	

DNA DENATURATION

1. Examine your piece of purified DNA. Probe and poke it with the stirring pipette.

Note its appearance and consistency.

2. Place 1 ml of Tris EDTA (TE) buffer into the test tube. The Tris serves to buffer the solution at about pH 8.0 while the EDTA chelates Mg^{++} which is a necessary cofactor for many DNAases and thereby protects the DNA from enzymatic degradation.
3. Stir and shake the mixture with the pipette tip to dissolve the DNA and get a feeling for the viscosity of the dissolved DNA at room temperature. This usually takes about ten minutes

Record your observations.

4. Using the test tube holder, carefully hold the tube in the 70°C water bath, stir to note any changes in viscosity.

Record your observations.

5. Move the tube to the 95°C water bath. After 2 minutes, stir and note any changes in viscosity.

Record your observations.

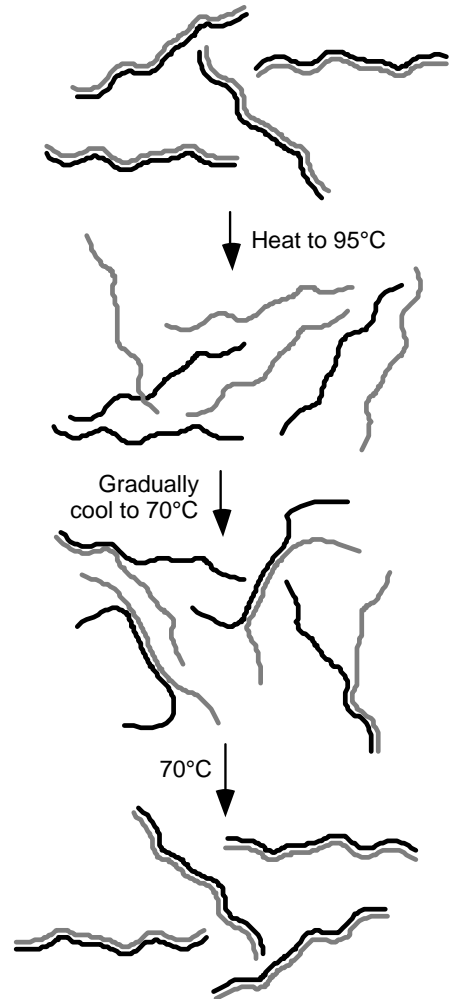
6. Remove the tube from 95°C and hold in the air for 15 seconds and then move to the 70°C water bath and note the changes. Record your observations. Allow the tube to cool to room temperature and save the DNA sample for the next part.

DISCUSSION

You should observe that the DNA solution becomes much less viscous at 95°C than at lower temperatures. This is often referred to as “melting” or “denaturation”. At about 95°C, the Hydrogen bonds holding the two strands of DNA together are broken and the strands separate creating single stranded DNA which is a much less viscous solution. Upon cooling, the hydrogen bonds will reform according to the rules of base pairing, and the viscosity returns. This process is called renaturation, reassociation, or annealing. If the cooling is gradual, the two strands align properly and reform all the original base pairs. If the temperature is lowered abruptly, regions of partial complementarity form base pairs leaving stretches that are not base paired.

SPOOLING THE DNA

1. Make sure your stirring pipette is very clean and rinsed with DI H₂O.
2. Add 2 ml of TE buffer to your DNA sample from above and mix well.
3. Pipette 0.2 ml of the 2M NaCl solution into the tube and mix well.
4. Very carefully add 3 ml of cold 100% Ethyl Alcohol so that the alcohol forms a layer on top of the DNA solution. This is best accomplished by allowing the alcohol to trickle down the side of the tube as it is added. Don't let the two layers mix!
5. While holding the tube so that you can easily see the two layers, gently twirl your stirring pipette at the alcohol-DNA solution interface. You should see the DNA precipitating onto the glass at the interface. Continue twirling and collecting the DNA on the stirring rod (kind of like winding spaghetti on a fork). This is called “spooling out” the DNA.



Describe the appearance of the DNA during this process.

EXTRACTION OF DNA FROM *E. COLI*

EQUIPMENT

Overnight <i>E. coli</i> culture in test tube	2 Pasteur pipettes
SDS solution	100% Ethyl alcohol
2M NaCl solution	

PROCEDURE

1. Obtain your tube of overnight culture of *E. coli* from the refrigerator.
2. Pipette 0.2 ml of the 2M NaCl solution into the tube and mix well.
3. Add 10% SDS dropwise, shaking the tube after each drop, until the tube clears (do not add more than 20 drops). SDS is a detergent that disrupts the plasma membranes of the bacterial cells. The solution clears as the cells break down and the cell contents, including DNA go into solution.
4. Very carefully add an equal volume of cold 100% Ethyl Alcohol so that the alcohol forms a layer on top of the DNA solution. This is best accomplished by allowing the alcohol to trickle down the side of the tube as it is added. Don't let the two layers mix!
5. As you did with the salmon sperm DNA, gently twirl your stirring pipette at the alcohol-DNA solution interface and spool out the DNA.

Note the appearance of the DNA during this process to the salmon sperm DNA.

6. After you have had enough spooling, replace the cap onto the test tube and gently mix by inverting the tube slowly back and forth.

Note what you see happening.

MAKING A RESTRICTION DIGEST

EQUIPMENT

0.075 $\mu\text{g}/\mu\text{l}$ λ DNA (purified DNA from Lambda phage)
Restriction enzymes: <i>EcoRI</i> <i>Bam</i> H1 and <i>Hind</i> III, 1 μl each in separate microfuge tubes
2X restriction buffer
1 microfuge tube
2-20 μl micropipet with yellow tips

PROCEDURE

1. Obtain a set of microfuge tubes and label them "*Eco*; *Bam*; *Hin* ; and *control*" (or just E, B, H, C)
2. Use Table 1 as a guide to prepare the tubes.

Table 1. Guide to loading the tubes				
REAGENTS	TUBES			
	<i>“Eco”</i>	<i>“Bam”</i>	<i>“Hin”</i>	<i>“control”</i>
λ DNA	4 μ l	4 μ l	4 μ l	4 μ l
Buffer	4 μ l	4 μ l	4 μ l	4 μ l
<i>EcoRI</i>	2 μ l	-	-	-
<i>BamHI</i>	-	2 μ l	-	-
<i>HindIII</i>	-	-	2 μ l	-
H ₂ O	-	-	-	2 μ l

- Add 4 μ l λ DNA to each tube.
- Next add 4 μ l buffer to each tube. Use a fresh tip for each tube.
- Add 2 μ l of the appropriate enzyme to the correct tube. Use a fresh tip for each tube.
- Use a fresh tip to add water to the control tube.
- Close tube tops and mix the tubes by placing in microfuge and spinning for few seconds.
- Place the tubes in a 37°C water bath and incubate for at least 20 minutes.
- When the restriction enzyme incubation is complete, remove the tubes from the water bath.
- Add 2 μ l of loading dye to each reaction tube.
- Close tops and mix with pulse on the microfuge.
- Place tubes in refrigerator until next lab.

DAY 2

PREPARING FOR AGAROSE GEL ELECTROPHORESIS

- Observe the instructor's demonstration of casting the gel during the restriction enzyme incubation.
- Seal the ends of the gel tray and place the well-forming comb in its proper position.
- Obtain the flask of liquid agarose. CHECK TO MAKE SURE THE SOLUTION HAS NOT COOLED AND FORMED FLAKES.
- Slowly pour the agarose into the tray to a depth of about 5 mm. The gel should only cover about 1/3 the height of the comb teeth. Use a micropipet tip to remove any bubbles before gel solidifies.
- When the agarose has set, remove the end-seals of the casting tray. Leave the comb in place.
- Place the gel tray in the electrophoresis box. If two gel trays go in the box, wait until both are in place.
- Fill the box with TBE buffer to a level that just covers the surface of the gel. TBE buffer can carry an electrical current.
- Gently remove the comb.
- Make sure that the sample wells are completely submerged. Add more TBE buffer if needed.

LOADING AND RUNNING THE GEL

- Observe the instructor's demonstration of loading the electrophoresis gel.

- Set the micropipet to 12 μ l.
- Carefully add 12 μ l of each tube to the appropriate well in your gel. Some work groups may have to share a gel, so the first group should add their tubes beginning on the second lane from the left and working right. The second group should begin with the second lane from the right and work from right to left.
- Cover the electrophoresis box making sure that the wells are on the cathodal side (-) and the DNA will run to the anodal (+) side. Plug the box into the power supply.
- Turn on the power supply and set it to 100-150 volts. The lower the voltage, the better the resolution, but the longer it takes.
- Run the gel until the dye mark has progressed about 2/3 of the gel (about 1 hour). **Meanwhile work on your restriction maps for *Eco* and *Bam* below!**
- Turn off power supply and disconnect.
- Remove the gel tray and proceed to the staining procedure below.

STAINING THE GEL

- The stain you will be using is ethidium bromide, a suspected **carcinogen**. It works by intercalating between the strands of DNA and will glow under UV light. **You must wear nitrile gloves (the blue ones) and protective eyewear when handling EtBr solutions.** You will also be assisted by the lab technician during this procedure.
- Submerge your gel in EtBr solution in a plastic container. Gently agitate for 5 min. Pour back used EtBr solution into the stock bottle.
- Destain gel by submerging gel in diH₂O. Gently agitate for 5 min.
- Pour off liquid and view gel under the UV lightbox. Be sure the protective cover is closed before turning on the UV light.
- Your lab technician will assist you in photographing your gel. Be sure to write your team's name on the list provided. Your photograph will be posted online (or provided to you as an image file) for your lab report.
- An "ideal" gel is shown in Figure 2.

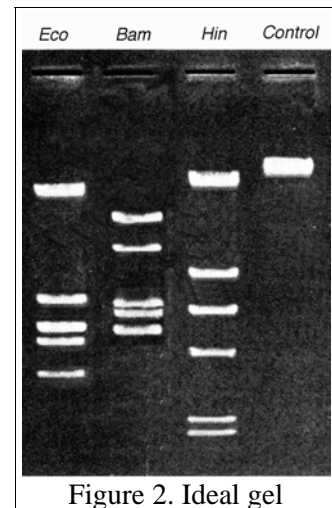


Figure 2. Ideal gel

ANALYSIS

The λ chromosome is 48,502 base pairs in length. Different restriction enzymes cleave at various positions along the chromosome. There are seven restriction sites for *Hind*III enzyme and they are shown in Figure 1. Also shown are the sizes of the eight resulting fragments from a *Hind*III restriction digest.

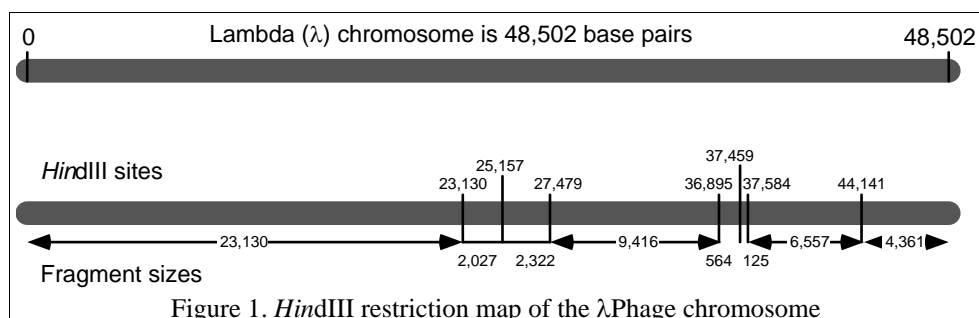


Figure 1. *Hind*III restriction map of the λ Phage chromosome

During electrophoresis, linear DNA fragments migrate at rates inversely proportional to the log of their molecular weights. For simplicity's sake, base pair length is usually substituted for molecular weight. If a mixture of known fragment sizes is run in one lane of a gel, then a standard curve relating distance migrated and base pair size can be drawn up and used to estimate fragment sizes of digests in other lanes on the same gel. *HindIII* digests of λ DNA is often used as such a standard because it gives a good range of fragment sizes and is predictable.

You will notice that the staining intensity decreases as the fragment size decreases. This simply reflects the fact that there is less DNA in these bands for the stain to interact with.

Very small DNA fragments will often run off the end of the gel, and will therefore not appear in the picture.

DNA fragments that are similar in size may show up as one thick band in the gel. These are called doublets.

MAKE RESTRICTION MAPS FOR THE *ECORI* AND *BAMHI* RESTRICTION ENZYMES

1. A linear restriction map for *HindIII* is shown in Figure 1 above.
2. The map begins at one end of the chromosome (the 0 end) and proceeds to the other end that is 48,502 base pairs away.
3. Restriction sites are given as the number of base pairs they are from the zero end.
4. The size of the restriction fragments may be determined by subtracting the site closest to the zero end (the smaller number) from the one further down the chromosome (the larger number). Fragment sizes are also shown in Figure 1.
5. Restriction sites for *EcoRI* are: 21,226; 26,104; 31,747; 39,168; and 44,972
6. Restriction sites for *Bam HI* are: 5,505; 22,346; 27,972; 34,499; and 41,732
7. Using the restriction site data given above, make linear restriction maps for λ DNA for *EcoRI*, and *Bam HI* enzymes.

MAKE A STANDARD CURVE TO ESTIMATE RESTRICTION FRAGMENT SIZE

1. With a metric ruler, measure the distance migrated (in mm) of the bands in the “*Hin*” lane on your gel.
2. Record these measurements in column 1 of Table 2 next to the fragment size represented by the band. Note that the 564 and 125 sized fragments may have migrated off the gel and are not visible.

HindIII		EcoRI			Bam H1		
Dist. (mm)	Actual Base Pairs	Dist. (mm)	Est.Base Pairs	Actual Base Pairs	Dist. (mm)	Est.Base Pairs	Actual Base Pairs
	23,130						
	9,416						
	6,557						
	4,361						
	2,322						
	2,027						
	564						
	125						

3. Start a spreadsheet that has the "Actual Base Pairs" from Table 2 in the first column and the distance migrated in the second column.
4. You are going to make a standard curve graph with the fragment size (in number of base pairs) on the X axis and migration distance on the Y axis.
5. If you plotted these two variables in a standard curve you would find the relationship is not linear (it's logarithmic) and it would not make a suitable standard curve.
6. You can make the graph roughly a straight line by having *Excel* plot the X axis in log scale.
7. When the graph is drawn, click on the X axis to select it. Then select "Selected Axis..." from the "Format" pull down menu.
8. Click on the "Scale" tab as in Figure 3.
9. Select "Logarithmic scale" and make the other settings as in Figure 3 or as you see fit for your data.
10. Adjust the scale on Y axis scale so that only the plotted area is shown on the graph (don't make it logarithmic).
11. Add an appropriate trendline to your graph.
12. You should end up with a graph that will allow you to calculate fragment sizes based on distance migrated for your experimental lanes on your gel.
13. Save this graph and make a printout to use in the next section.

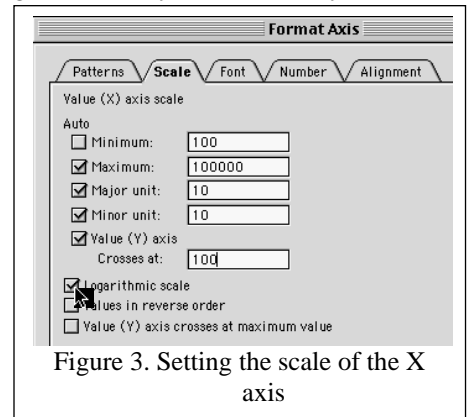


Figure 3. Setting the scale of the X axis

ESTIMATE THE FRAGMENT SIZES IN THE *ECO* AND *BAM* LANES

1. Measure the distance migrated for each band in the *Eco* and *Bam* lanes, and record in the appropriate columns in Table 2.
2. Calculate the corresponding fragment size using your standard curve equation in base pairs. Record this number in Table 2 under "Est. base pairs."
3. From the restriction maps you made for *Eco* and *Bam* above, record the fragments sizes you would expect for each, in descending order, in the appropriate "Actual Base Pairs" column in Table 2.
4. You should find that there are more fragments in the "Actual Base Pairs" column than bands that you observed in the lane. This is because fragments of similar size show up as a "doublet" band or smaller bands migrated off the gel completely.
5. Determine which bands in the *Eco* and *Bam* lanes are doublets.

QUESTIONS FOR YOUR REPORT

(20 pts)

1. Present an abstract of your work. Your abstract should include a brief description (1/2 page) of 1) what you set out to learn, 2) what procedures you performed, and 3) a summary of your data or discoveries.
2. Explain how the viscosity of your salmon sperm DNA solution changed as it was heated to 95°C and then cooled. Give an explanation for your observations.
3. Present your linear restriction maps for λ DNA for *EcoRI*, and *BamH1* enzymes. Be sure to include all restriction fragment cut sites and all fragment sizes.
4. Present a picture of your gel from this experiment. Clearly label all lanes and bands found on the gel (you may just number the bands in each lane). Compare your gel to the picture of the “Ideal Gel” in Figure 2. Note any differences (i.e. missing or extra bands).
5. Present your standard curve that shows the relationship between distance migrated and fragment size for the *HindIII* restriction fragments. Explain how you will calculate your unknown values for *EcoRI*, and *BamH1* lanes.
6. Present a spreadsheet containing all the data from Table 2. How did your estimates compare to the actual sizes you calculated from the restriction map? Explain any differences.
7. Explain what restriction enzymes are and how they work. Discuss one important application of restriction enzymes in molecular biology.
8. What are two functions of the blue gel loading dye?
9. What would happen if the electrodes were reversed during gel electrophoresis? What would happen if deionized water was used instead of TBE buffer in the gel boxes?
10. Why do the bands of DNA near the bottom of the gel appear fainter than bands near the top? Explain in terms of how we visualize DNA in a gel.