

Texas A&M University-Corpus Christi
CHEM4402 Biochemistry II Laboratory
Laboratory 8: DNA Restriction Digest (II) and DNA Sequencing (I)

We have made considerable progress in our analysis of the gene for green fluorescent protein. From our original sequence we designed primers and amplified the gene using the polymerase chain reaction, a powerful technique for making many copies of a given DNA sequence. We cloned the amplified DNA by covalently ligating it to a bacterial plasmid vector using DNA ligase. Plasmid DNA vectors, as you recall, are small, circular DNA molecules that have the capacity to be replicated independently of the genomic (chromosomal) DNA. Some plasmids have been genetically engineered to also allow expression of RNA from cloned DNA fragments, which is the template for protein synthesis in all cells.

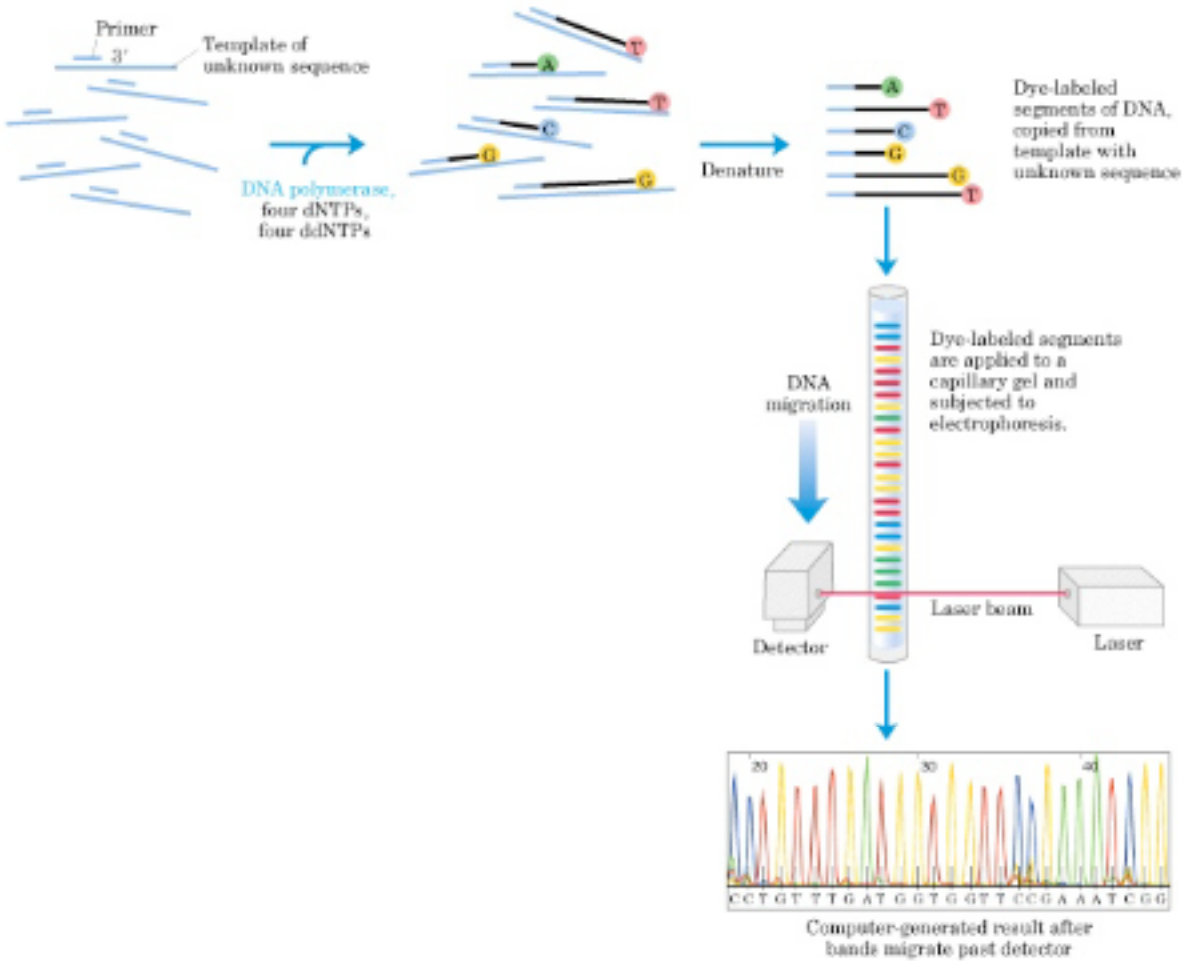
Selectable markers (genes for antibiotic resistance and galactosidase) contained within the plasmid DNA allowed us to identify bacterial clones that (1) were resistant to a particular antibiotic, and (2) contained a DNA fragment of sufficient size to disrupt the β -galactosidase gene, the site of insertion during the ligation reaction. Today's procedure will take this verification one step further by verifying whether the the ligated fragment from one of the "positive" clones truly is our GFP PCR fragment of interest. We will perform this verification procedure by running the products from our various restriction digests (EcoRI, EcoRI + Csp45I, Csp45I) out on an agarose gel to examine the size of the fragments generated. If the sizes of the fragments generated by the digest match the sizes expected from our "virtual" digest, we can be pretty sure that the clone truly does contain the DNA product of interest. Sites for EcoRI have been genetically engineered into our plasmid to occur on both sides of the PCR fragment insertion site. By digesting our plasmid with EcoRI, we should have released the ligated PCR fragment from the plasmid, making it easier to identify by size analysis on an agarose gel. Csp45I is our diagnostic enzyme. We know from our "virtual" digest that it should cut our GFP fragment only once, and produce fragments of specific length. Since it would be highly unlikely that another, arbitrary DNA fragment of similar size would also have a Csp45I restriction site *in the same location*, restriction fragment analysis is a fairly reliable method for verification of fragment identity.

We will also be performing the first part of our DNA sequencing lab, by estimating the amount of plasmid DNA that has been isolated and then performing the cycle sequencing reaction using a PCR procedure. DNA sequencing is the ultimate verification procedure, bridging the identification gap from "probably or probably not" the correct DNA fragment (restriction enzyme digest verification) to "is or is not". DNA sequencing is a biochemical procedure for identifying the sequence of nucleotides in a particular segment of DNA (figure 1). The process is essentially a variation on a combination of PCR and electrophoresis. A series of dye-labeled DNA fragments, which differ in length by a single base pair, are generated from a target DNA sequence using PCR. In a sequencing PCR ("cycle sequencing") reaction, however, a portion of the nucleotides used to synthesize copies of the DNA target have two chemical modifications. The first involves reduction of the ribose 3'-OH group to -3'-H, which prevents the attachment of any additional nucleotide to the nascent polymer. Thus, whenever one of these "terminator" nucleotides is incorporated, extension of the copied DNA fragment is ended. By adjusting the concentration of these "terminator" nucleotides a series of DNA fragments are produced that

range in size from the length of the oligonucleotide primer + 1 nucleotide to the length of the full-size PCR product (though 1500 nucleotides is presently the upper limit for a typical sequencing reaction). The second modification involves the attachment of a fluorescent dye to a proportion of the nucleotides. When passed through a laser, excitation of the dye causes it to emit light of a characteristic wavelength (fluoresce). As four different dye labels are used (one to identify each of the A, T, G and C bases), the emission spectra are interpreted by computer as corresponding to either the A, T, G or C bases when DNA fragments containing these labelled, terminator dyes cross the detector.

The DNA fragments are separated using capillary electrophoresis which, like gel electrophoresis, separates molecules on the basis of their size to charge ratio. The fragments will come off of the capillary (analogous to the gel in agarose gel electrophoresis) in order of size, from smallest to largest. The power of the capillary separation is that fragments which differ in size by only one nucleotide are cleanly separated, allowing the computer to “read” the PCR products as if it were starting at base 1 of the target sequence and continuing through to the end of the PCR fragment.

Figure 1. Cycle Sequencing and Capillary electrophoresis analysis of amplified fragments.



Part I - Electrophoretic Analysis of Restriction Digest Results and estimation of plasmid DNA concentration

Materials

Agarose	Electrophoresis power supply
1X TAE Buffer	125 ml erlenmeyer flask
Gel box, tray and comb	ethidium bromide (10 mg/ml solution)
Hot plate	DNA molecular weight markers
DNA loading buffer	lambda DNA marker
sterile H ₂ O	

Procedure

1. Make **40 ml** of a **2%** agarose solution in a 125 ml erlenmeyer flask (0.8 g agarose, 40 ml 1X TAE (Tris – Acetic acid – EDTA buffer))
2. Add stir bar to agarose solution. Heat on hot plate until it melts. While waiting, set up gel tray as you did in your electrophoresis & ligation lab (see your instructor if you need review)
3. Place melted agarose in a **water bath** to cool for **2 minutes**.
4. Add **4 ul** of **ethidium bromide** to the gel solution (**caution – ethidium bromide is a mutagen. Handle very carefully and wear gloves**).
5. Pour melted agarose solution into a gel tray as indicated by your instructor. Be sure the tray contains a **10-well comb** which will produce the wells that you will use to load your DNA samples. Rinse erlenmeyer flask with bleach solution, then wash with warm soapy water.
6. Once your gel has set (it will have an opaque, grey-white appearance), place it in the electrophoresis tank (wells closest to the **black (-) electrode**). Remember to lower the dams on either end of the gel tray. Cover the gel with 250 ml of 1X TAE buffer.
7. Add 5 ul of DNA loading buffer to each of your digest samples (EcoRI, EcoRI + Csp45I, Csp45I) . Mix **gently** by tapping the microcentrifuge tube with your finger.
8. Load the following samples in your gel, **left to right**: (1) 10 ul of DNA molecular weight markers (2) 25 ul of “E” tube (Eco RI digest) (3) 25 ul of “EC” (EcoRI/Csp45I double digest) (4) 25 ul of “C” (Csp45I digest) (5) 10 ul of lambda DNA marker.
9. Insert lid of electrophoresis tank. Connect electrodes to power supply.
10. Set power supply to 100 V. Start. Allow gel to run for **75 minutes**.
11. After your gel has finished running, stop the power supply (press running man figure again). Turn off the power supply and remove the electrodes connected to the gel box.

Remove the top of the gel box and remove the gel (wear gloves). Proceed with your instructor to take a photo of your gel.

12. Using your gel picture, estimate the **concentration of plasmid DNA by comparing the intensity of the Csp45I digest sample to the 4.4 Kb band on the lambda DNA sample** (figure 3). This will be a very rough estimation (“as intense”, “1/2 as intense”, “2 times as intense”, etc.). Because it is a commercial standard, we know the **4.4 Kb** band contains **32 femtomoles** (fmol; 10^{-15} mole) of DNA. Therefore, if you decide that your DNA is 2 times as intense, then you would estimate that you had $2 \times 32 = 64$ fmole of DNA in your Csp45I digest.
13. Once you have estimated the approximate amount of DNA in your Csp45I digest, back-calculate to estimate the concentration of DNA in your plasmid prep. Recall that we used 10 ul of plasmid DNA in each digest. Therefore, if you estimate that your Csp45I digest contained 64 fmol of DNA that would mean that the concentration of DNA in your plasmid prep is 6.4 fmol/ul (64 fmole/10 ul). The cycle sequencing reaction calls for 50 fmol of DNA.
14. **Carefully** rinse out your gel box, tray and comb with distilled water. Place on a paper towel to dry. **DO NOT HAND DRY** (this can break the electrodes). Proceed to part II, **DNA Sequencing (I)**.

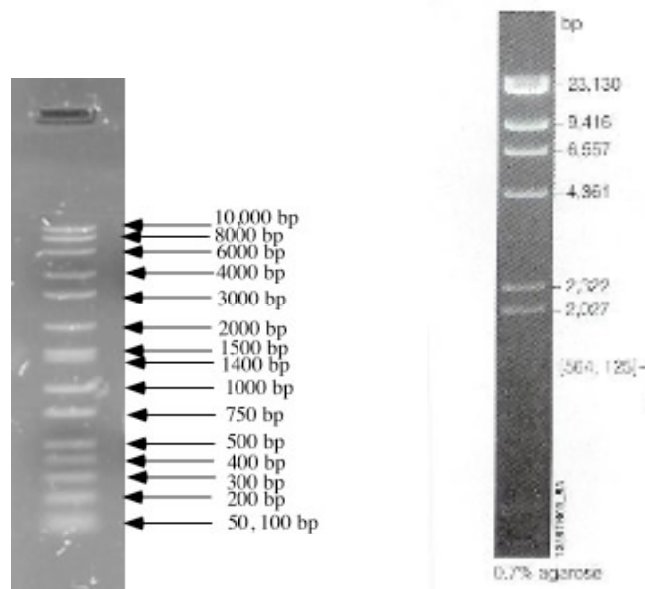


Figure 3. Molecular Weight Markers (left:PCR, plasmid DNA digestion, right: lambda DNA (bp))

Part II - DNA sequencing (I)

Materials

Plasmid DNA prep	sterile PCR tube
Sterile dH ₂ O	Sequencing Primer (SP)
Sequencing Master Mix (MM)	Styrofoam cup
ice	Thermal cycler

Procedure *NOTE: Keep all reagents, other than plasmid DNA template, on ice*

1. Calculate the volume of plasmid prep that will provide 50 fmol of plasmid DNA. If your DNA concentration is less than 5 fmol/ul use the entire volume of your remaining plasmid prep (up to 10 ul).
2. Transfer the appropriate volume of plasmid DNA (50 fmol) to a PCR tube. Label with your initials. Your instructor will then collect the samples and place them in a **65°C heating block** (thermocycler) **for 5 min.**
3. Allow your plasmid DNA to cool to room temperature on your benchtop (3 min.)
4. Add the following reagents to your PCR tube, in the order below

Sterile H ₂ O	0-9.5 ul (enough to bring total volume (including plasmid DNA) to 20 ul)
Sequencing Primer (1.6 pmol/uL) (SP)	2.0 uL
Sequencing Master Mix (MM)	8.0 uL
Total volume	20.0 uL

6. Mix reagents by gently stirring with your pipet tip. Your instructor will collect the tubes for centrifugation prior to the PCR cycle sequencing reaction.

7. Place samples in the PCR machine. The thermal cycling conditions are as follows:

Step 1 96°C for 20 seconds

Step 2 50°C for 20 seconds

Step 3 60°C for 4 minutes

Step 4 return to Step 1, 30 times

Step 6 4°C hold until samples are removed to freezer (instructor)

