

## Experiment 2

### *Plasmid DNA Isolation, Restriction Digestion and Gel Electrophoresis*

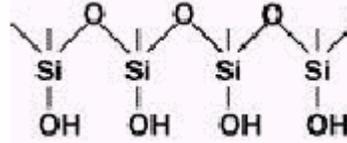
#### **Plasmid DNA isolation introduction:**

The application of molecular biology techniques to the analysis of complex genomes depends on the ability to prepare pure plasmid DNA. Most plasmid DNA isolation techniques come in two flavors, simple - low quality DNA preparations and more complex, time-consuming high quality DNA preparations. For many DNA manipulations such as restriction enzyme analysis, subcloning and agarose gel electrophoresis, the simple methods are sufficient. The high quality preparations are required for most DNA sequencing, PCR manipulations, transformation and other techniques.

The alkaline lysis preparation is the most commonly used method for isolating small amounts of plasmid DNA, often called minipreps. This method uses SDS as a weak detergent to denature the cells in the presence of NaOH, which acts to hydrolyze the cell wall and other cellular molecules. The high pH is neutralized by the addition of potassium acetate. The potassium has an additional effect on the sample. Potassium ions interact with the SDS making the detergent insoluble. The SDS will easily precipitate and can be separated by centrifugation. In doing so the insoluble SDS traps the larger genomic DNA and removes it from the supernatant. This leaves the plasmid DNA and RNA in solution. The RNA is often removed by digestion with the addition of RNaseA. This leaves only proteins, carbohydrates and RNA nucleoside monomers in solution. A primary alcohol, such as ethanol or propanol is used to precipitate the DNA. This is accomplished by the reordering of water, making the DNA aggregate and become insoluble. The result is a somewhat pure pellet of DNA that can be resuspended in a mildly buffered solution or water.

Newer and more popular methods now exist that take advantage of the differences in physical properties of plasmid DNA and the contaminants found in the alkaline lysis method. Recently, a diatomaceous earth-based method was used to isolate the plasmid from cell lysate using the alkaline lysis method. This is also called a silica gel method. For this treatment the DNA pellet is resuspended in RNaseA to remove the RNA by digestion. This is necessary because the RNA will compete with DNA for binding to the

diatomaceous earth. After RNaseA treatment, the DNA containing supernatant is bound to the diatomaceous earth in a chaotropic buffer, often guanadine chloride or urea. The chaotropic buffer will force the silica (diatomaceous earth) to interact



hydrophobically with the DNA. Purification using silica-technology is based on a simple bind-wash-elute procedure. Nucleic acids are adsorbed to the silica-gel membrane in the presence of high concentrations of chaotropic salts, which remove water from hydrated molecules in solution. Polysaccharides and proteins do not adsorb and are removed. After a wash step, pure nucleic acids are eluted under low-salt conditions in small volumes, ready for immediate use without further concentration.

The diatomaceous earth is centrifuged and contaminants are left behind in the supernatant. DNA is eluted during incubation at 65°C and then removed from the particles.

#### **Restriction Digestion Analysis:**

Restriction Digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction Endonucleases (sometimes just called Restriction Enzymes or RE's). These special enzymes recognize specific sequences in the DNA molecule (for example GATATC) wherever that sequence occurs in the DNA.

Restriction Digests begin by mixing the DNA and the RE, but it's unfortunately not quite as simple as that. Restriction Enzymes are delicate and need to be treated carefully. Because enzymes are proteins and proteins denature as the temperature is increased, RE's are always stored in a freezer until they are used. In fact, all of the ingredients in a Restriction Digest are kept on ice until it's time for the reaction to begin. The actual reaction conditions vary from one enzyme to the next, and include temperature, NaCl and/or MgCl<sub>2</sub> concentration, pH, etc. All of these variables except temperature are optimized by

mixing the enzyme and DNA with a buffer specific for the enzyme of choice. Once all the ingredients are mixed in the reaction tube, the tube is incubated at the Restriction Enzyme's optimal temperature for 1 hour or longer. Once the Restriction Digest is completed, agarose gel electrophoresis is performed to separate the digest fragments by size and visualize the fragments and perhaps purify them for further experiments.

### Agarose Gel Electrophoresis

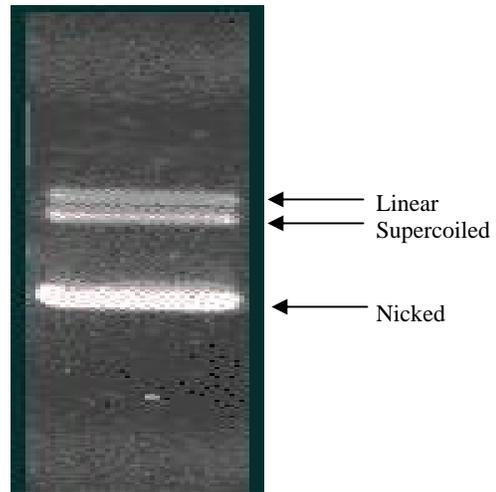
Mobility of a molecule under the influence of an electric field "is determined by its charge, its formula weight, the pore size of the matrix material and the strength of the electric field". This holds true for the electrophoresis of DNA in agarose gels. Because DNA and RNA have constant anionic charge/mass ratios (one phosphate for every nucleotide linkage) they travel through the gel at a constant speed in response to an electric current. The DNA polymers of various sizes (sometimes called DNA fragments) are separated by the molecular sieving action of the pores in the agarose gel.

Agarose is used as the matrix in DNA electrophoresis because it can be used to form much larger pores than polyacrylamide. The lower the percentage of agarose or polyacrylamide, the larger the pores.

DNA migrates differently depending on whether it is circular or linear. Linear DNA migrates through the agarose pores in an end-on-end fashion and snakes its way through the pores. Circular supercoiled DNA is more compact and migrates through the pores more easily. **Linear DNA almost always migrates more slowly than circular supercoiled DNA.**

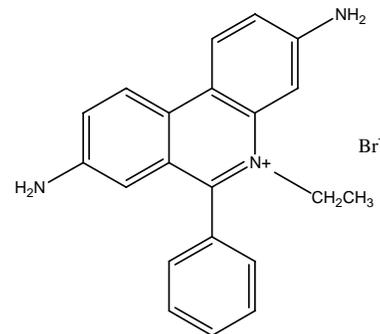
There are primarily two types of circular DNA, closed and nicked. Closed circular DNA has all of its nucleic acids linked with phosphodiester bonds and is supercoiled. Nicked circular DNA has at least one broken phosphodiester linkage. Nicked DNA is sometimes also called relaxed DNA because some of the tension present in covalently closed and superhelically twisted DNA has been released. Closed circular DNA can be supercoiled. A single nick in one strand is sufficient to release the tension caused by all degrees of supercoiling. Even though all of these DNA forms have the same number of nucleotides and the same formula weight, they migrate differently during

gel electrophoresis. You should also be aware that they do not necessarily migrate in the order presented in the figure above. Depending on conditions the linear form may be faster than supercoiled DNA or slower than nicked DNA.



### Detection of Nucleic Acids in Gels:

The most popular method for the visualization of double stranded DNA (or RNA) in agarose gels is the fluorescent dye ethidium bromide (see below).

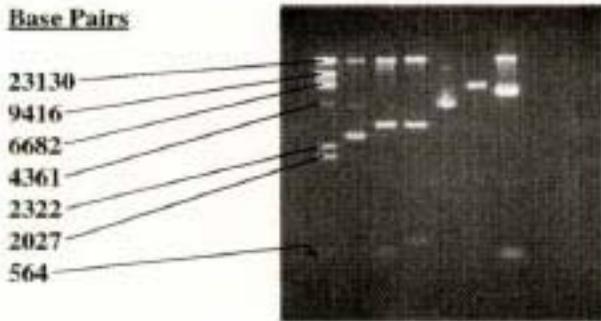


This dye is a planar molecule and intercalates between the stacked base pairs of DNA. When the dye becomes fixed in its position and enters the hydrophobic interior of the DNA, it becomes more fluorescent than free dye in solution. The dye absorbs UV light at 300 to 360 nm and emits light at 590 nm in the red-orange region of the visible spectrum.

Single-stranded DNA (or RNA) also will bind dye and can be detected by this method, but not nearly as well as double-stranded DNA. The affinity of the dye for single-stranded DNA is relatively low and the fluorescent yield is poor.

### Electrophoresis standard curve:

The number of base pairs in a DNA (or RNA) molecule is determined by comparison to the mobilities of standards. This method is only



accurate for linear DNA molecules because the molecular sieving properties of the gel are determined by the end-to-end length of the DNA molecule and for the reasons discussed above. The distance migrated by several linear DNA molecules of a known number of base pairs is measured and a standard curve plotted as log (base pairs) versus distance migrated. The number of base pairs in the unknown DNA fragment is then determined by comparing the distance it migrated to the standard curve.

### Experimental Procedures (DNA Isolation):

#### 1) Select a well-isolated colony for inoculation.

This will ensure maintenance of the plasmid and that the plasmid is from a single cell. Inoculate into 5 mL of LB media containing 100 µg/mL ampicillin and grow overnight at 37°C. This is best started in the morning the day before starting the purification.

2) **Transfer 1.5 mL of your cells to a 1.5 mL microfuge tubes.** Harvest the cells by centrifugation (max rpm in the microfuge) for 2 minutes at room temperature. Remove all traces of supernatant by inverting the centrifuge tube until all media has been drained. The pellet can then be frozen at -20°C for later use.

#### 3) **Resuspend the bacterial pellet in 200 µL of buffer Cell Resuspension Buffer.**

Use a 200 µL pipettor and rinse the pellet in and out of the pipet with the tip placed near or at the bottom of the container. Go slowly; do not aspirate the material into the pipet. Make sure that the bacterial pellet is completely resuspended and no clumps remain.

4) **Add 200 µL of Cell Lysis Buffer and mix the sample by inversion 10 times and incubate at room temp for 5 min. DO NOT VORTEX!** Vortexing will result in shearing of the genomic DNA, leaving free chromosomal fragments to contaminate the plasmid DNA. This will result in a co-purification of both DNA types and will ruin any further experiments. The unlysed cells will be very thick, almost like snot. The solution should become slightly clearer if the cells have lysed. Incubate for exactly 5 minutes, longer will lead to inappropriate degradation of genomic DNA. SDS solubilizes the phospholipid and protein components. NaOH denatures the DNA as well as proteins. Optimal lysis time allows maximal release of plasmid DNA from the cells without release of genomic DNA. Long exposure to alkaline conditions may cause the plasmid DNA to become irreversibly denatured. This DNA will run faster on a gel and is resistant to restriction enzyme digestion.

#### 5) **Add 200 µL of neutralization buffer and mix immediately but gently by inversion (5 - 6 times).**

After addition of this buffer the solution becomes cloudy and very viscous. The lysate is neutralized by the addition of acidic potassium acetate in the neutralization buffer. The high salt (K<sup>+</sup>) concentration causes the SDS to become insoluble and precipitate. Plasmid DNA is too small to be trapped in the precipitate while genomic DNA and protein is. Any remaining SDS in solution will inhibit DNA binding to the column later so make certain that the solution is well mixed.

6) **Mix the sample by inversion 5-6 more times and centrifuge (max rpm in the microfuge) for 10 min. at room temperature. Remove the supernatant immediately and place in a new sterile 1.5 ml tube.** It is important that the supernatant is clear and free of cellular debris. The supernatant (cleared lysate) at this step contains the plasmid DNA.

7) **Prepare spin column.** Attach a spin column to a 10 mL sterile syringe barrel after removing the plunger. Mix the matrix by vortexing or repeated shaking and inversion of the bottle to insure that it is completely suspended. The silica has already been equilibrated in binding buffer.

8) **Add 1 mL of thoroughly suspended matrix to the cleared supernatant from the previous step**

and pipet up and down to mix. Pipet this mixture into your column/syringe from the previous step.

9) **Push the mixture through the mini column with the plunger.** This will trap the plasmid DNA in the mini column. Remove the column, remove the plunger, and place the column back on the syringe. Immediately wash the column with 2 mL of wash solution by pushing it through the syringe barrel with the plunger.

10) **Complete the wash.** Remove the spin column from the syringe barrel and place it in a sterile 1.5 mL centrifuge tube. Place the tube/column into the microfuge and spin for 2 minutes to remove any residual traces of ethanol.

11) **Elute the DNA from the matrix.** Remove the spin column and discard the microcentrifuge tube. Place the column in a new 1.5 mL microfuge tube. Add 50  $\mu\text{L}$  of 65°C deionized water. Promptly elute the DNA for 1 minute at top speed. The decreased concentration of salts and increased temperature allow the DNA to be solubilized and decreases the affinity to the hydrophobic silica matrix. Save the eluted DNA.

13) **Determine the concentration of DNA in the sample.** Blank the spec with 995  $\mu\text{L}$  of water at 260 nm and 280nm. Add 5  $\mu\text{L}$  (exactly) of DNA to the 995  $\mu\text{L}$  of water in the quartz cuvette. Determine the absorbance at 260 and 280 nm. Given that double stranded DNA has an absorbance at 260 nm of 1 for 50 $\mu\text{g}/\text{mL}$ , calculate the concentration of the DNA. The actual purity of the sample can be determined by taking the ratio of absorbance at 260 to 280 nm. If the ratio is greater than 1.8 the absorption is due to nucleic acids. A high quality prep should have a ratio of 2.0 to 1.8. If the ratio is below 1.6 there may be proteins or other organic contaminants and the DNA can be extracted by phenol/chloroform extraction for a final clean up.

14) **Analyze the sample by agarose minigel.** The class will run one minigel. Add 5  $\mu\text{L}$  of DNA Sample buffer to 10  $\mu\text{L}$  of your pure DNA and load the whole sample. Include a DNA ladder by adding 5  $\mu\text{L}$  of sample buffer to 3  $\mu\text{L}$  of DNA marker.

#### Buffers

*Cell Resuspension Buffer:*

- 50 mM Tris-Cl, pH 8.0

- 10 mM EDTA 100  $\mu\text{g}/\text{ml}$
- DNase free RNase

*Cell Lysis Buffer*

- 200 mM NaOH
- 1% SDS store at room temp

*Neutralization Solution*

- 3.0 mM potassium acetate, pH 5.

*Equilibration Buffer (Matrix storage buffer)*

- 6 M guanidine hydrochloride.

*Wash buffer*

- 80% isopropanol diluted with water.
- Ethanol can be used but is more expensive.

#### Experimental Procedures (Restriction Digests and Electrophoresis):

##### Setting up restriction digest.

You will need to get this part of the experiment going before you pour the gel as it takes approximately 45 minutes of incubation time. Setup the following in small eppendorf tubes:

Tube	DNA	10x Buffer	EcoRI	HindIII	Water
L	5 $\mu\text{L}$	--	--	--	10 $\mu\text{L}$
E	5 $\mu\text{L}$	1.5 $\mu\text{L}$	1.5 $\mu\text{L}$	--	7 $\mu\text{L}$
H	5 $\mu\text{L}$	1.5 $\mu\text{L}$	--	1.5 $\mu\text{L}$	7 $\mu\text{L}$
C	5 $\mu\text{L}$	1.5 $\mu\text{L}$	1.5 $\mu\text{L}$	1.5 $\mu\text{L}$	5.5 $\mu\text{L}$
Plasmid	5 $\mu\text{L}$	1.5 $\mu\text{L}$	1.5 $\mu\text{L}$	--	7 $\mu\text{L}$

After you have set these tubes up, they will need to incubate at 37°C for 45 minutes.  $\lambda$  /Hind III/EcoRI DNA fragments will be used as the standards for this experiment. Lambda DNA cut with HindIII/EcoRI consists of double stranded DNA fragments with the following number of basepairs: 21,226; 5148; 4973; 4268; 3530; 2027; 1904; 1584; 1375; 947; 831; 564; and 125.

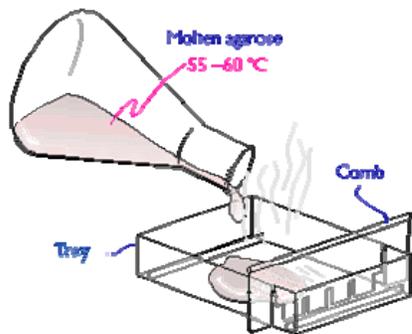
##### Gel Preparation

You will be provided with the following stock solutions:

- 10x TBE buffer/L: 108g Tris, 55g Boric acid, 40 mL of 0.5M EDTA pH 8
- Agarose powder

This apparatus was designed so that you could use the buffer chamber as a gel-casting tray. Place the gel tray in the chamber so that the black gaskets press against the walls of the unit. You will be using a 1% agarose solution in 1X TBE

buffer. Make sure the cap is loose on the agarose container and then heat the solution until in the microwave until it starts to boil. Remove the solution from the microwave and swirl it to make sure the agarose has all dissolved. Repeat the heating procedure until the solution is completely clear. Let the solution cool to about 50°C and then pour 40 mL of the warm agarose into the tray. Insert the comb into its position at one end of the gel. Use the comb with the fewest teeth. This particular apparatus is designed so that there is 1.0 mm agarose between the bottom of the teeth and the base of the gel. This insures that the sample wells are completely sealed when the gel has solidified. It will take about 15 min for the gel to solidify and cool somewhat; it should appear uniformly opalescent.



After the gel has solidified, lift the casting tray and place it in the buffer chamber. Pour in enough buffer to completely cover the gel to a 5 mm depth. It will take about 300 ml of 1x TBE solution. Carefully remove the comb by lifting it straight up.

### Preparing the samples:

You will make up your samples by adding 5  $\mu$ L tracking dye to each of your restriction digests. The entire sample will be loaded onto the gel. To fix a sample of your plasmid, take 5  $\mu$ L of the plasmid and add 5  $\mu$ L of the tracking dye. Load the entire sample.

Load onto the gel the following:

- Markers ( $\lambda$  DNA/HindIII/EcoRI)
- Uncut  $\lambda$  DNA
- EcoRI cut DNA
- Hind III cut  $\lambda$  DNA
- EcoRI/HindIII cut  $\lambda$  DNA
- Your uncut plasmid DNA

Using a micropipette, load all of each of your samples into separate wells on the gel.

Carefully slide the safety lid onto the unit. This will connect the power cords to the electrodes. Turn on the power supply and run the gel at 115 volts until tracking dye is almost to the bottom of the gel (~1hr).

When the run is complete, turn off the power supply, and slide off the safety lid. The casting tray can be used as a convenient carrier for your gel. Place the gel in a container and stain with a couple drops of 5 mg/mL ethidium bromide for ~5 minutes. Place the gel and its carrier on the UV transilluminator and look at the gel through the safety cover. Be sure to wear goggles to protect your eyes from the UV light. Also, don't expose your skin to the UV light too long or you'll get sunburn.

### Results and discussion section of your lab report:

Plot the logarithm of the number of base pairs of the standard ( $\lambda$ /Hind III/EcoRI DNA) on the y-axis vs. distance migrated (x-axis). Do a linear regression analysis and write the equation of the line on the plot.

Include in report:

1. What was the size of your DNA fragments (using your standard curve obtained from the  $\lambda$ /Hind III/EcoRI DNA)? A nice table would be a good idea here.
2. What was the size of your plasmid DNA?
3. Be sure to attach the photograph of the gel and indicate your partner's name.