Exercise 20
GEL ELECTROPHORESIS OF DNA SAMPLES
(Plasmids, PCR products & Restriction Fragments)

Introduction

Gel electrophoresis is a technique or procedure allowing DNA fragments to be separated on the basis of size, stained and observed with the naked eye. It has numerous applications in biotechnology. DNA samples may be subjected to gel electrophoresis following digestion with restriction enzymes to verify that digestion has proceeded to completion and to characterize the size and number of the digestion products formed. DNA samples amplified using the polymerase chain reaction (PCR) and those formed through ligation procedures are often subjected to gel electrophoresis to determine their size and/or purity. Automated nucleotide sequencing methods and DNA fingerprinting techniques also involve gel electrophoresis.

Gel electrophoresis involves loading DNA samples into wells (depressions) located at one end of a gel composed of **agarose** (purified agar) or polyacrylamide, and subjecting them to an electric current. Since DNA molecules, like many other organic compounds, carry a slight negative charge, they will migrate in an electric field toward the positive pole or **anode**. DNA fragments of different lengths travel at different rates within the agarose or polyacrylamide because the gel molecules tend to interfere with their migration to some extent. For this reason, DNA fragments subjected to electrophoresis tend to separate themselves into distinct **bands** representing different size categories. Smaller DNA fragments travel more rapidly through gels than do larger fragments, so will travel greater distances in the same amount of time.

Samples of DNA that have been prepared for electrophoresis are not visible in solution, so in order to keep track of their progress through a gel, it is necessary to combine them with some type of dye, or to run along with them dyes that migrate at a similar rate (**tracking or loading dyes**). In this laboratory we will mix our DNA samples with a loading/tracking dye containing two components, **xylene cyanol** and **bromophenol-blue**. The xylene cyanol migrates at a rate similar to 3000-4000bp DNA fragments, while bromophenol-blue migrates at a rate similar to 100-300bp fragments. The plasmids isolated in the previous exercise are approximately 3,000 to 5,000bp and the PCR **amplicons** (products) obtained are approximately 1,500bp, so when subjected to electrophoresis in combination with these dyes can be expected to form bands that will lie in the vicinity of (between) the two blue-colored bands. **Note** – It is possible to use only the bromophenol-blue dye and still keep track of our DNA, since most DNA samples should be located between the bromophenol-blue band and the wells.

Once the DNA fragments have been separated into discrete size categories, they must be stained in some manner so they can be observed. In this laboratory, we will stain DNA samples with **ethidium bromide**, or **GelRed**, chemicals that produce an orange or red fluorescence respectively when subjected to ultra violet light. Stained gels will then be placed over a UV light source (**transilluminator**), and photographed so observations, measurements, etc., can be completed.

Note – Ethidium bromide is recognized as a strongly mutagenic agent because of its ability to intercalate into DNA molecules. For this reason, no students will be allowed to handle this chemical, and all gels containing DNA stained with ethidium bromide will be handled with extreme care and disposed of as hazardous waste. GelRed has been engineered to prevent or substantially reduce mutation by making it incapable of crossing cell membranes; however, GelRed gels will be disposed of as hazardous waste.

Since gel electrophoresis allows DNA samples to be observed, it is a technique frequently applied in research facilities Students in this class will use gel electrophoresis during two laboratory sessions, and to complete independent investigations involving DNA analysis. During this introductory exercise (Exercise A), students will make agarose gels, and will subject samples of plasmid DNA (from Exercise 19) and PCR product DNA (16S ribosomal DNA) to gel electrophoresis. In order to verify the sizes of plasmid and PCR product DNA, DNA from bacteriophage lambda, precut into **known** size fragments with the restriction endonuclease *Hin*dIII will be used as a **size standard** for calibration (a **DNA ladder**).

During a later class session (Exercise B), gel electrophoresis will be used to determine the success of restriction endonuclease digestion of PCR product DNA. During that exercise, multiple samples of PCR product (16S ribosomal DNA) cut with the restriction enzyme AluI will be electrophoresed, and their RFLP patterns compared as a means of determining bacterial identity. DNA from bacteriophage lambda, precut into known size fragments with the restriction endonuclease *Pst*I or a 1Kb DNA ladder will be used as a standard for calibration.

Materials:

TBE electrophoresis buffer: 90mM TRIS-HCl pH 8.2, 2.5mM EDTA (disodium salt), 89 mM boric acid.
Reaction Stop Mix and Loading dye: 5 M urea, 10% glycerol, 0.5% SDS, 0.025% xylene cyanol, 0.025% bromphenol-blue.
Agarose: Molten 1% solution in TBE buffer or pre-made agarose gels.
Ethidium bromide (or equivalent): 4 micrograms/ml. Ethidium bromide should be treated as a mutagen (compound capable of causing genetic mutation) and should not be allowed to contact bare skin at any time.
Lambda standard DNA: Precut with *Hin*d III, *Pst*I or other known restriction enzymes.
Student-extracted plasmid DNA.
PCR product DNA digested with *Alu*I and undigested amplicons.
Digital pipettes and sterile tips (.5-10µl volume)
Electrophoresis gel boxes and power supplies
Ultra violet transilluminator
Ice buckets and ice

Procedure (Exercise A):

1. Prepare 20mL of agarose per group, and then make a gel by pouring 12mL of molten agarose onto a clean, glass slide around a plastic comb as instructed. This is most readily accomplished if the agarose is transferred using a 10ml pipette and if the glass is elevated from the table surface by a pair of coins. After the gel has cooled, gently remove the comb by lifting it out. Be careful not to damage the solidified agar.

2. Place the gel in an electrophoresis chamber (gel box) containing TBE buffer, without removing it from the glass. Be careful to position the gel such that the wells are nearest the negative pole or cathode.

3. Obtain your tubes of plasmid DNA prepared during a previous exercise and add 20µL of 10mM Tris buffer to the bottom of each one. Allow the DNA to hydrate for at least 10 minutes and then **vortex mix the samples for 30 seconds.** Place these tubes in the ice bath.

4. Obtain the tube containing your PCR product DNA (amplicon) prepared during a previous exercise (numbers on tube caps correspond with numbers recorded for PUNK2). Thaw these samples and place them in the ice bath.

5. Obtain a small piece of parafilm and place it on the counter top near your gel box. Small samples of DNA will be mixed with loading dye on this surface.

6. Using a digital pipette and a clean, sterile tip, place six, 5μL samples of loading dye in a line on the parafilm. Be careful to space these six samples far enough apart that they will not run together.

7. Using a new, clean, sterile pipette tip, obtain a 5μL sample of Lambda standard DNA and place this next to one of the dye samples on the parafilm. Note – The droplets of dye and DNA should be the same size.

8. Using the same tip, gently roll the DNA sample into the dye droplet next to it. You may mix the DNA with the dye by moving the sample into and out of the tip two or three times, but avoid making bubbles.

9. Pick up 5μL of the DNA/dye mix and load it into well #4 of the gel as directed. **Remember** – You must release the DNA sample below the surface of the buffer solution in order to fill the well. If the pipette tip is allowed to contact the surface, the DNA sample can spread over the buffer surface and be lost. Using the same pipette tip, pick up the remaining 5μL of the DNA/dye mix and load it into the same well (it is important to load the entire 10μL volume).

10. Using another clean, sterile pipette tip, take 5μL of DNA from the **bottom** of the blue tube containing pUC19 plasmid DNA, and place this next to a drop of loading dye on the parafilm. Compare droplet sizes to insure that you have transferred the correct volume of DNA. Using the same tip, load the plasmid sample into lane #1 of the gel. Record this placement.

11. Using another clean, sterile pipette tip, take 5μL of DNA from the **bottom** of the orange tube containing pGEM plasmid DNA, and place this next to a drop of loading dye on the parafilm. Compare droplet sizes to insure that you have transferred the correct volume of DNA. Using the same tip, load this plasmid sample into lane #2 of the gel. Record this placement.

12. Using another clean, sterile pipette tip, take 5μL of DNA from the **bottom** of the green tube containing pGLO plasmid DNA, and place this next to a drop of loading dye on the parafilm. Compare droplet sizes to insure that you have transferred the correct volume of DNA. Using the same tip, load this plasmid sample into lane #3 of the gel. Record this placement.

13. Using separate, clean, sterile pipette tips for each PCR product sample, mix these with dye and load them into wells #5, and #6 of the gel. Record PUNK2 numbers for PCR product samples. If there are three students in your group, one PCR amplicon will be run in an alternate gel. Locate this gel, load your PCR amplicon and record the lane number as instructed.

    When all DNA samples have been transferred to the gel, each group will have three samples of plasmid DNA, one sample of bacteriophage lambda DNA and two samples of PCR product DNA arranged in this order (from lane one to lane six). **If your DNA samples are arranged differently, or placed in an alternate gel, be sure to record the location and identity of each one**.

14. Once the DNA samples have been loaded, apply power to the electrophoresis chamber (place the lid on the box, and turn the power supply on). Adjust the power supply so that the voltage applied to the electrodes is about 45 volts (flat bottom box) or 110 volts (deep well chamber).

    Important note – Electrophoresis requires the use of fairly substantial amounts of electric current. Although the gel boxes used in this laboratory are fairly accident proof by design, students are advised that making contact with the buffer solution and the electric current at the same time should be avoided. **Do not turn the power on until the lid is fully on the box.**

15. During the "run" (electrophoresis), the bromophenol-blue and xylene cyanol dye samples will resolve into separate colored bands. The electric current will be maintained until the blue-violet band (bromophenol-blue) moves about three quarters of the way to the end of the gel.

16. Your instructor will transfer the gel to a staining tray containing ethidium bromide or GelRed in TBE buffer solution. The stain will be allowed to penetrate the gel for 5-10 minutes, and then the gel will be removed from the tray and rinsed. **Note – Ethidium bromide is a mutagen**, so individuals working with stained gels must wear gloves at all times.

17. Stained gels can be visualized with ultra violet transillumination. If time permits, you may observe your gel; otherwise, photographs will be taken and will be used for future observation, measurement, and analysis.

18. Compare the results obtained for the various DNA samples used (compare the lanes of your gel). Compare also your results with those obtained by other members of the class.

    Note – The bacteriophage lambda (θλ) DNA when digested (cut) with the restriction enzyme *Hin*dIII will yield fragments of the following sizes:

23,130 base pairs	2,322 base pairs
9,416 base pairs	2,027 base pairs
6,557 base pairs	564 base pairs
4,361 base pairs	125 base pairs

    The band containing fragments that are 125 base pairs in length will not be visible in your gel, however all of the other bands should be clearly visible.

The plasmid pUC19 is about 2.7 kb (kilobases) in length (2,686 bp), the plasmid pBR322 is about 4.4 kb in length (4,363 bp) and the plasmid pGLO is about 5400 kb in length (5371 bp). A plasmid sample completely digested with restriction enzymes (linear DNA) will typically form a single band within its respective lane, but **undigested samples often form multiple bands**. This is because ccc-DNA tends to **supercoil**, i.e., twist into a tight spiral, and will move through agarose at a pace faster than that expected for cut DNA. Uncut plasmids may be "relaxed" when "nicked" by gyrase (topoisomerase) enzymes (one strand is cut, forming a loose, floppy circle of DNA). These migrate more slowly than cut plasmid DNA. Sometimes plasmids also attach to each other (the loops interconnect) and so will move through the gel at a pace much slower than the cut DNA. The bands traveling the greatest distance in the gel (fastest moving bands) provide the most accurate representation of relative plasmid sizes. By comparing the position of each plasmid's **fastest band** (leading edge of color) with the bands formed by the **lambda standard** you can determine each plasmid's apparent size when supercoiled. Bands visible behind the smallest sample represent "nicked" circular DNA or interconnected plasmids, because our plasmid samples are not cut.

Procedure (Exercise B):

1. Obtain a pre-made agarose gel and place it correctly in a gel box (electrophoresis chamber) with the appropriate quantity of 1X TBE buffer solution.

2. Obtain your tubes of digested PCR product DNA (prepared during a previous exercise), one tube of bacteriophage lambda or other calibration standard provided, and one tube of loading dye. Thaw these samples and place them on ice.

3. Obtain a small piece of parafilm and place six, 5μL samples of loading dye down the middle in an evenly spaced line.

4. Using a different clean, sterile pipette tip for each sample, load five, 5μL samples of digested PCR product DNA and one 5μL sample of calibration standard DNA into the gel as indicated below. Place the standard sample at one end (lane #1 or lane #6).

   Note – The pre-made agarose gels have thin wells that are somewhat more difficult to load than other gels. Each will accommodate 10μL of liquid, but not if the pipette-tip is in the well. **Remember** to release each DNA sample below the surface of the buffer solution, and to move the tip to the side before lifting it out to avoid having surface tension pull the DNA samples from the wells.

   a. Using a new, clean, sterile pipette tip, obtain a 5μL sample of DNA and place this next to one of the dye samples on the parafilm. Note – The droplets of dye and DNA should be the same size.
   b. Using the same tip, gently roll the DNA sample into the dye droplet next to it. You may mix the DNA with the dye by moving the sample into and out of the tip two or three times, but avoid making bubbles.
   c. Pick up 5μL of the DNA/dye mix and load it into a well of the gel; then using the same tip, pick up the remaining DNA from the parafilm and load that into the same well. It is important to load the entire 10μL volume of DNA/dye mix into the well.

5. Record the locations of each DNA sample (which sample is in which well) on a slip of paper and tape this to the bench top near the gel box.

6. Once the DNA samples have been loaded and labeled, close the box, turn on the power and check the voltage. Your gels will run for approximately 2.5 hours, then each gel will be stained with ethidium bromide and photographed while exposed to ultra violet light.

7. Compare the results obtained for the various DNA samples run. Remember that samples of 16S ribosomal DNA (PCR product) from different bacteria will yield different RFLP patterns when cut with the same enzyme (in this case AluI) because their nucleotide sequences are different. Determine the size of each RFLP band in your lane by comparing these to the standard, and then check to see if the gel data matches data obtained from nucleotide sequence analysis and computer assisted simulated digestion. Group cooperation will be required.

Questions:

1. What is gel electrophoresis? What is this procedure used for?

2. Why do DNA molecules migrate when subjected to an electric current? In which direction do they travel (toward the anode or cathode)?

3. Which of the following DNA fragments would you expect to travel a greater distance from the loading well within a given period of time; a 500 base pair fragment, or a 300 base pair fragment? Why?

4. What are the approximate fragment sizes formed when bacteriophage lambda DNA is cut with *Hin*dIII? Which of the various plasmids used is larger in size?

5. Is the sample of PCR product DNA larger or smaller than the plasmid samples used?

6. Is it possible to distinguish between cut and uncut samples of plasmid DNA by observing these in an electrophoresis gel? If so, which will appear larger, and why?

7. What is the relationship between RFLP and DNA fingerprints?

8. Did the number and sizes of RFLP bands observed in your gel match the expected number and size as determined through nucleotide sequence analysis and computer assisted simulated digestion of DNA? If not, suggest multiple reasons why they did not?

Name_____ **Lab Section**_____

WORKSHEET
Exercise 19 & 20
The Miniscreen & Gel Electrophoresis

Goals: _____
_____
_____

Materials & Methods:

A. Miniscreen
Exercise 19
Organism used: _____

B. Gel Electrophoresis
Exercise 20, Procedure A

Gels were made of _____% agarose and used buffer _____.

Plasmid sample (from Ex. 19) was loaded onto gel _____, lane _____.

PCR sample (from Ex. 16) was loaded onto gel _____, lane _____.

Data & Results:

Attach a picture of the gel in the space below. If your samples were on two separate gels, be sure to include a picture of each. Label all lanes and samples.

Conclusions:

Was the miniscreen successful?_____ Explain. _____
_____
_____
_____

Was the PCR successful?_____ Explain. _____
_____
_____
_____

Additional comments and conclusions regarding the success of the gel electrophoresis: ____
_____
_____
_____
_____