**Section 3, DNA Module:**

**Agarose Gel Electrophoresis**

Objective:

Separate DNA fragments by size, to determine if restriction digestion of the pKan and pGFPuv plasmids was successful.

Background:

 Separating molecules for analysis is important in many experiments. Agarose gel electrophoresis is a technique for separating molecules based on differences in their sizes. Agarose, a carbohydrate extracted from red algae, is heated in a solution of buffer and then cooled to make a gel. This gel contains tiny pores, through which molecules can pass. Small molecules will move through the gel more quickly than large molecules. After running the gel for a period of time, researchers can visualize the molecules and determine which molecules moved farther than others. Running a “ladder” of molecules of known sizes on the same gel provides a reference for size estimations of molecules in an experimental sample. When DNA molecules are being analyzed, the ladder consists of linear DNA fragments of different lengths.

 In electrophoresis, an electric current drives the movement of charged molecules. Thus, in order for sample molecules to move during agarose gel electrophoresis, either the sample molecules must be charged already, due to their chemical structure, or a charged reagent can be bound to the sample molecules to give them a charge. The phosphate groups in the DNA backbone give DNA a net negative charge, so DNA placed within an electric field will move away from the negative electrode (black) toward the positive electrode (red). Again, when the current is driving DNA molecules through the matrix of an agarose gel, smaller molecules will move faster.

Protocol:

1. Obtain a flask containing 0.5 g of agarose. Share the flask with other lab groups as explained by your instructor.

*Note that solid agarose has the appearance of a fine white powder. Unless stated otherwise, all chemicals (such as agarose) were purchased from Fisher Scientific (Fair Lawn, New Jersey, U.S.A.).*

1. Add 50 mL of TAE buffer to the flask containing the agarose.

*These amounts will result in a 1% agarose gel. (In general, percentages are calculated as mass per volume, so that 1 g of agarose in 100 mL,* i.e. *1 g / 100 mL, is equivalent to 1%.)*

*The TAE buffer consists of 40 mM Tris base, 20 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA).*

1. Take the flask of TAE plus agarose to the microwave, and watch as an instructor heats the flask to prepare molten (60°C) agarose.

*Look for the agarose to change from an opaque, white powder to clear “lenses” in the TAE buffer, and finally to completely clear solution as the agarose melts.*

1. Following the directions of the teaching staff, prepare the casting tray in the electrophoresis apparatus to receive the molten agarose. Check that the ends of the casting tray are completely sealed.
2. Wait until the molten agarose is just warm to the touch, and then add 5 µL of SYBR Safe DNA-staining dye (bright red) to the 50 mL of molten agarose.

*SYBR Safe, purchased from Invitrogen (Carlsbad, CA), is a substitute for ethidium bromide, which is commonly used to visualize DNA in gels.*

*Ethidium bromide is a mutagen that acts by intercalating between base pairs of DNA, where ethidium bromide’s fluorescence allows the DNA to be visible under UV light. Unfortunately, ethidium bromide can also penetrate skin and intercalate into your DNA, where the chemical can introduce mutations during replication. Anyone handling ethidium bromide or equipment or solutions contaminated with ethidium bromide MUST WEAR GLOVES!*

*Although SYBR Safe is (as the name implies) much safer than ethidium bromide, you should still take the precaution of always wearing gloves when there is a chance of coming into contact with SYBR Safe.*

1. Quickly but carefully pour the molten agarose, now containing SYBR Safe, into the casting tray.
2. Place a comb with narrow teeth into the slot near the end of the tray.
3. Pop any bubbles with a pipet tip or move bubbles to the sides of the gel before it cools.
4. Allow the gel to completely solidify with the electrophoresis chamber open. (The agarose solution will change from clear to opaque white.)
5. While you are waiting, prepare your DNA samples. First, thaw your tubes of intact pKan and pGFPuv plasmids.
6. Next, obtain the tubes containing restriction-digested pKan and pGFPuv.

*The intact plasmids, as well as the restriction-digested plasmids (after the overnight digestion), were stored at -20°C.*

1. Balancing the tubes against each other, spin them briefly in a microcentrifuge to collect the liquid at the bottom of the tubes.
2. Obtain four clean 1.5 mL tubes, and label them “uncut pKan”, “digested pKan”, “uncut pGFPuv”, and “digested pGFPuv”. It is very important to keep track of which tube is which!
3. Add 8 µL of RODI H2O to the “uncut pKan” and to the “uncut pGFPuv” tubes.
4. Remove 2 µL of intact, uncut pKan DNA from your original pKan miniprep, and place this volume into the “uncut pKan” tube. The volume of liquid in the tube will be a total of 10 µL.

1. Repeat step 14, but with pGFPuv: Place 2 µL of intact, uncut pGFPuv into the “uncut pGFPuv” tube, bringing the total volume to 10 µL.
2. Now pipet 10 µL of restriction-digested pKan into the “digested pKan” tube.
3. Pipet 10 µL of restriction-digested pGFPuv into the “digested pGFPuv” tube.
4. To each of the four tubes labeled in step 13, add 2 µL of 6X DNA loading dye (deep blue). DO NOT add loading dye to any of the other tubes.

*The 6X DNA loading dye consists of 50% glycerol, 5% xylene cyanol, 5% bromophenol blue, and 0.05 M EDTA, pH 8.0.*

*The glycerol increases the density of the DNA sample, making it sink to the bottom of the well in the agarose gel. Xylene cyanol and bromophenol blue are dyes which together give the DNA sample a deep blue color, so that you can easily see the sample while loading. Xylene cyanol and bromophenol blue also migrate within the gel, giving an indication of the progress of electrophoresis.*

1. Mix all tubes by vortexing briefly, and spin the tubes for a few seconds in a microcentrifuge to collect the liquid at the bottom of the tubes.
2. When the gel is completely solidified, carefully remove the comb by gently lifting it straight up.
3. Re-orient the gel in the electrophoresis apparatus, so that the open ends of the tray face the electrodes.
4. Pour enough TAE buffer (perhaps 250-300 mL) into the gel tray to just submerge the gel.
5. Obtain a tube of 1 kb ladder (also called a size marker) from the stock cart. The ladder will already have blue loading dye added to it.

*We will use a one-kilobase (1 kb) ladder to measure fragment sizes (Figure 3). This particular ladder is supplied by New England Biolabs (Ipswich, MA). The brightest band in this ladder corresponds to linear DNA molecules that are 3.0 kb long.*

1. Prepare to load your samples on the gel in the order listed in Table III below.

*Note: As a general guideline, samples containing molecules whose sizes you need to measure accurately (i.e. the restriction-digested plasmids) should be placed close to the ladder. Sometimes samples in lanes near the sides of the gel will run slightly differently than samples in the middle of the gel, so we will skip the first lane.*

**Table III.** Samples to be loaded on the agarose gel.

**Figure 3.** One-kilobase (kb) DNA ladder separated by agarose gel electrophoresis. Sizes of marker fragments are indicated in kilobases to the right of the gel.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Lane Number** | **1** | **2** | **3** | **4** | **5** | **6** |
| LaneContents | no sample (skip) | uncut pKan | digested pKan | 1 kb ladder | digested pGFPuv | uncut pGFPuv |

1. The teaching staff will demonstrate how to stabilize one’s hands when loading the gel. It is important to place the pipet tip vertically just inside the top of the well, but not too far down, because the tip can puncture the bottom of the gel, causing the sample to leak out (Figure 4).

A

B

C

D

1. Practice loading liquid into wells using the extra gels and practice solution. Holding the pipettor vertically, or nearly so, place the end of a tip containing blue practice solution under the surface of the TAE buffer and within a well on the gel (Figure 4A). Then slowly press the plunger to the first stop. Wait a few seconds for the sample to settle into the well; then, lift the pipet tip out of the liquid, while still holding the plunger at the first stop. Do not push the plunger to the second stop; doing so will introduce bubbles and may push your sample back out of the well.
2. Taking turns with another group, load 12 µL of each of the four experimental samples (uncut and restriction-digested pKan and pGFPuv) and of the 1 kb ladder (Table III). One group should use the top half of the gel, and the other group should use the bottom half.
3. Once the gel is loaded with samples, connect the electrical leads (black to negative and red to positive), turn on the power switch, and set the apparatus to 110 volts. Bubbles should rise from the electrodes once you switch on the current.

**Figure 4.** Tip positioning for loading samples into wells on an agarose gel. Panel **A** illustrates correct positioning of the tip, while panels **B**-**D** illustrate common errors. (**A**) The end of the tip containing sample is below the surface of the buffer (wavy line) and approximately in the middle of the rectangular well. Puncturing the side (**B**) or bottom (**C**) of the well or pipetting sample before the end of the tip is fully within the well (**D**) can all result in the loss of sample.

1. A few minutes after starting the current, check that the samples, as indicated by the blue dye, are moving in the correct direction, into the gel and not in the direction of the buffer reservoirs.
2. Allow the gel to run until the blue dye has moved two-thirds of the way down the gel (30-60 minutes).
3. Turn off the power, and disconnect the leads.
4. With gloved hands, lift your gel out of the electrophoresis chamber. Take the gel (still in its tray) to the gel imaging system. It is helpful to have a teammate (without gloves) open doors for you. Note that the gels are slippery and can easily slide onto the floor if you are not careful.
5. Slide your gel off of its tray and onto the ultraviolet transilluminator, which serves as the gel imaging platform.

***Caution:*** *Ultraviolet (UV) light can damage your eyes. Never look directly at an unshielded UV light source without adequate eye protection. View only with a safety shield that absorbs the harmful wavelengths.*

1. The teaching staff will help you to capture an image of the gel using a digital camera.
2. With your group, analyze the results of the agarose gel electrophoresis. Determine whether the mini-preps and restriction digestions of pKan and pGFPuv were successful, or whether you will need one or both back-up plasmids to proceed with the next experiments.
3. Follow the instructions given for disposing of gels, running buffer, and other supplies. Wash your hands after completing the experiment.