Agarose gels are used to analyze DNA molecules. These gels are simple to construct, because they rely only on the gelling properties of agarose. Molecules are separated by size and visualized with fluorescent intercalating dyes. In this lab, you will analyze the products of the PCR reactions from the previous lab.

**Objectives**

At the end of this lab, students should be able to:

- prepare an agarose gels for separating DNA molecules.
- separate DNA molecules by electrophoresis.
- visualize DNA molecules on agarose gels using intercalating dyes.
- estimate the approximate sizes of DNA molecules using size standards.
Agarose gels provide a simple method for analyzing preparations of DNA. DNA molecules share the same charge/mass ratio, which imparts similar electrophoretic properties to linear DNA molecules of widely varying lengths. We will use “molecule” to refer to a linear piece of DNA, but keep in mind that a single DNA “molecule” may contain the sequences of multiple genes or parts of genes.

**Agarose gels are porous matrices**

Agarose is a polysaccharide purified from red algae, or seaweed. Agarose is more highly purified (and significantly more expensive!) than agar, which is obtained from the same seaweed. Agarose molecules are long, linear polymers of a repeating disaccharide D-galactose and 3,6-anhydro-α-L-galactopyranose (right). A typical agarose molecule contains over one hundred subunits. The agarose used for electrophoresis has been highly purified. The purification process removes contaminants that would interfere with the enzymes used in molecular cloning, such as restriction endonucleases. The process also generates an agarose preparation with desirable electrophoretic properties and minimal background fluorescence, which is important for visualizing DNA molecules.

Agarose molecules are able to form gels with relatively defined pore sizes because of the chemical properties of agarose molecules. Agarose demonstrates hysteresis - its melting temperature is higher than its gelling temperature. Agarose molecules dissolve at about 90°C, forming random coils in solution. Gels form when the temperature falls to approximately 40°C. As the gel forms, the agarose molecules first assemble into helical fibers, which then further aggregate to form networks of supercoiled helices stabilized by hydrogen bonds. The sizes of the pores, which typically range from 50 to 200 nm, depend on the concentration of agarose. As the agarose concentration increases, the average diameter of the pore decreases.

**Several factors affect the migration of DNA through agarose gels**

Because of the negative charge of the phosphate residues in the DNA backbone, DNA molecules move toward the positive pole (anode) of the electrophoresis apparatus. The actual migration rate of DNA molecules in a particular experiment is affected by multiple factors. Some of these factors are intrinsic to the DNA molecules, while other factors relate to the electrophoretic conditions. Intrinsic factors include both the length and conformation of the DNA molecules that are being analyzed. Within a certain size range dictated by the gel conditions, the migration rate of linear DNA molecules is inversely proportional to the log_{10} (number of base pairs). The migration of more structured DNA molecules, such as supercoiled plasmids, is much less predictable. The migration rates of these more highly structured DNAs are influenced by the density of coils, the presence of nicks, and other structural features.
The migration rates of DNA molecules in agarose gels are also affected by the composition of the gel. The migration rate of a DNA molecule decreases as the concentration of agarose in the gel increases. Researchers commonly adjust the agarose concentration to optimize the resolution of DNA molecules within a particular size range. The two buffers commonly used in labs, TAE (Tris: acetate: EDTA) and TBE (Tris: borate: EDTA), also affect electrophoresis rates.

Because of this inherent variability, researchers ALWAYS include a lane of DNA standards with known sizes on the same gel as the samples being analyzed. Importantly, these standards need to have a similar structure (e.g. linear or supercoiled) and to be subjected to the same chemical modifications as the DNA samples being analyzed.

**Fluorescent intercalating agents are used to visualize DNA molecules in gels**

Nucleic acids are visualized by fluorescent dyes that bind strongly to DNA. The dyes are intercalating agents that insert into the DNA helix and into structured regions of single-stranded nucleic acids. The fluorescence of these dyes increases by an order of magnitude when they bind nucleic acids, so the background fluorescence on agarose gels is usually low. In this class, we will use ethidium bromide (EtBr) to visualize DNA fragments. EtBr absorbs light in the ultraviolet (UV) range and emits orange light. Gels are viewed with special transilluminators containing UV lights. EtBr is a light-sensitive compound, so stocks are stored in the dark.

**Standards are used to estimate the sizes of DNA molecules**

The figure on the right shows a lane from an EtBr-stained 1% agarose gel containing DNA size standards. The 1 kb ladder shown in this student gel is a proprietary mixture of linear DNA fragments ranging in size from 0.5 to 10 kilobases (kb). The staining intensity of bands on agarose gels reflects the quantity of DNA in the band, because EtBr intercalates fairly evenly along the length of linear DNA molecules. As you can see, this particular mixture contains similar quantities of each DNA fragment, with the exception of the 3 kb fragment, which has ~2.5 more DNA than the other fragments. The greater intensity of the 3 kb fragment serves as a useful orientation marker in situations where smaller fragments might have run off the end of the gel or when some markers are not well resolved from one another. (These are common occurrences!)

Note that the shortest molecules on the gel are more well separated from one another than the longer molecules. The PCR products that you will be analyzing in this lab are mostly in the 400-1000 bp range. To increase the resolution of these molecules, we will use 1.25% agarose gels. (This will reduce the resolution of larger DNA molecules). Note also that smaller bands appear fuzzier on the stained gel, because they have been more affected by random diffusion as they have migrated through the network of agarose polymers.
Chapter 8

Prepare the agarose gel

In this lab, you will use agarose gels to separate DNA molecules produced in PCR reactions. These PCR products should be well-resolved on 1.25% agarose gels prepared in TAE buffer, which provide good separation of molecules that are smaller than 2 kb. Place the casting tray into the gel apparatus. If you are using the BioRad apparatuses, position the black wedges at each end of the casting tray.

1. Determine the amount of agarose that you will need for a 1.25% (1.25 g/100 mL) gel that fits your casting platform. Most of the gel apparatuses in the lab are the BioRad Mini-Sub GT systems with a 7 cm x 7 cm casting tray that accommodates a 30 mL gel. You may need to adjust the size of your gel if you are using a different apparatus. Check your calculations with your teammates before you proceed.

2. Fill a graduated cylinder with the appropriate volume of TAE buffer. Pour the solution into a small flask.

3. Weigh out the appropriate amount of agarose. Sprinkle the agarose onto the surface of the TAE in the flask. Note: the agarose will not dissolve until it is heated.

4. Dissolve the agarose by heating the solution for intervals of 15-20 seconds in a microwave oven. After each interval, remove the flask and gently swirl it around a bit to disperse the contents. Note if the agarose particles are still apparent or if the agarose has dissolved. The best gels are made from agarose that has NOT been overcooked.

SAFETY NOTE: The agarose solution will be very HOT when you remove it from the microwave! Use caution when handling the flask. Be particularly careful not to contact the steam that will be coming through the opening of the flask. Fold several paper towels and wrap them around the neck of the flask when you handle it. If you do happen to spill some hot agarose on your skin, wash it immediately with cold water and alert your TA.

5. Allow the agarose solution to cool until you can comfortably touch the flask with your hands. Agarose solutions over 60°C will warp the casting tray! Pour the gel. Place the sample comb in place. Do not move the casting platform until the gel sets. You will know that the gel is set when it becomes opaque. Allow the gel to cure for about 20 minutes after it sets.
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Agarose gel electrophoresis

**Sample preparation**

Prepare your samples for electrophoresis while the gel is curing by adding concentrated loading buffer. The loading buffer contains two dyes, bromophenol blue and xylene cyanol. During electrophoresis, the dyes will migrate with “apparent” molecular weights of ~5 kb and ~0.5 kb, respectively. The loading buffer also contains glycerol, which makes the sample dense enough to sink to the bottom of the sample well.

Add 4 µL of 6X loading buffer directly to each of the 20 µL PCR reactions from the last lab. Briefly, centrifuge each tube to mix the dye and samples, if necessary. You will use half of each sample in your gels. *Store the remaining sample in the refrigerator.*

**Load and run the agarose gel**

1. When the gel has set, *carefully* remove the comb and the black wedges.
2. Orient the gel in the electrophoresis tank such that the wells (holes made by the comb) are oriented toward the black (negative) electrode. DNA molecules will move from the well toward the red (positive) electrode. Fill the tank with enough TAE buffer to submerge the gel (approx. 275-300 mL).
3. Load one sample to each well, which can accommodate ~20 µL. Load 10-12 µL of a PCR sample to each lane. Try to avoid air bubbles as you load the samples.
4. Load 5 µL molecular weight standard to one lane of the gel. Make sure that you have accurately recorded the location of each sample in the gel.
5. Place the lid on the electrophoresis tank and connect the electrodes to the power supply (black-to-black and red-to-red). *Make sure that the polarity is correct before continuing!* 
6. Turn on the power and apply a constant voltage of 125 V.
7. Pay careful attention to the gel as it runs. Turn off the power when the bromophenol blue is ~ 1 cm from the end of the gel. *Do not allow the dye to run off the gel, since small DNA molecules will be lost.* (Think about the size of your PCR products.)

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**Proper set-up of an agarose gel**

The gel is submerged in running buffer. Samples will electrophorese toward the postivie (red) pole. Red electrical leads are attached to the positive pole of the power pack. Samples are loaded into wells.
Stain and analyze the agarose gel

SAFETY NOTE: Wear disposable gloves when staining gels. Gloves are important when working with intercalating dyes, which are potential mutagens.

1. Remove the gel from the apparatus and transfer the gel to a small tray. Cover the gel with deionized water. Add 5 µL of EtBr solution (10 mg/mL) to the tray. What is the approximate concentration of EtBr in the staining solution?

2. Place the tray on a rocking platform and rock gently for 20-30 minutes.

3. Drain the EtBr solution into the appropriate waste container in the fume hood.

4. Cover the gel with deionized water and rock gently for 1 minute.

5. With a spatula, carefully place the gel on the transilluminator and close the cover to the Gel-Logic apparatus. (Drain the wash solution into the waste container.)

6. Turn on the transilluminator light and photograph the gel according to the posted instructions. Turn off the transilluminator immediately after you photograph the gel. Save the picture and email a copy to yourself. (If no bands are apparent, the staining can be repeated for a longer period of time.)

7. Open the door of the GelLogic apparatus. Use the spatula to transfer the gel to a waste container set up for EtBr-stained gels.

8. Determine the approximate length of the DNA molecules in your samples by comparing their migration to that of the standards. Are the sizes consistent with your expectations?

Test yourself

A research group has just received four strains from a one of their collaborators. Following their usual practice, a student is using colony PCR to confirm the identities of the four strains A-D before starting work. The lab has diagnostic primers for all four strains. The expected sizes of the PCR products for strains A-D are 680 bp, 1100 bp, 572 bp and 462 bp, respectively. Unfortunately, the student who runs the gel forgets to write down which sample has been run in each lane. Analyze the gel on the right. No PCR product can be detected in lane 5. The position of the 3 kb marker is indicated. Place size labels for a few of the other markers. Which strain’s PCR product has been loaded into:

Lane 2  Lane 3  Lane 4  Lane 5

Explain your reasoning. What might the lack of a product in Lane 5 indicate?