

Agarose Gel Electrophoresis

In the laboratory today, we will examine the products of the PCRs we ran last week, using agarose gel electrophoresis and the nucleic acid specific stain ethidium bromide. In general, electrophoresis is a technique that separates molecules on the basis of their different rates of movement in an applied electric field through a porous semisolid matrix. The semisolid matrix we will use today is a gel made from agarose and buffered water. Agarose is a polysaccharide consisting of a linear polymer (repeating units) of D-galactose and 3,6-anhydro L-galactose (Fig. 1). Commercially, agarose is extracted from seaweed and purified for use in electrophoresis. The movement of molecules through an agarose gel is dependent on the size and charge of molecules and the pore sizes present in the agarose gel. At neutral pH, DNA, RNA, and proteins migrate toward the anode (positive electrode) when an electric field is applied across the gel. Small, highly negatively charged molecules migrate faster through agarose gels than large, less negatively charged molecules. Rates that molecules move through the gel can also be effected by the sizes of pores in the agarose gel. Decreasing pore sizes increases the separation of small and large molecules during electrophoresis (Table 1). Pore size can be decreased by increasing the percentage of agarose in the gel.. For example, the pore sizes are smaller in a 3% agarose gel than in a 1% agarose gel.

Figure 1
Basic structure of Agarose

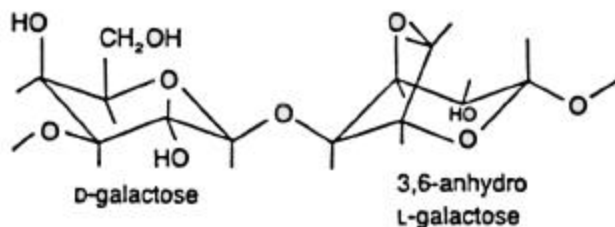


Table 1. Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kilobase [Kb; 1 Kb = 1000 base pairs])
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7.0
1.2	0.4-6.0
1.5	0.2-3.0
2	0.1-2.0

The movement of molecules through the gel is also affected by the electrophoresis buffer. Two important parameters of the buffer are its composition and its ionic strength. The electrical conductance of the gel is dependent on the presence of ions. Therefore, without the presence of the buffer, the current running through the gel would be very small and molecules would migrate either very, very slowly or not at all. Conversely, a buffer with too high an ionic strength produces a very high electrical conductance and sig-

nificant amounts of heat. The heat that is produced by passing the electrical current through the gel can be hot enough to denature the DNA so that it runs through the gel as single strands instead of double strands or the heat may even melt the gel. The buffer we will use today is a **0.5X TBE** solution containing 0.045M Tris-borate and 0.001M EDTA. To make our 0.5X buffer we will dilute a 10X TBE Buffer stock solution. Do you remember the functions of Tris and EDTA?

We will use the GIBCO/BRL Horizon 58 Horizontal Gel Electrophoresis Apparatus for our agarose electrophoresis today.

I. Assembly for Gel Casting

A. Assemble the gel unit (Fig. 2) on a level surface (check with bubble level) following the procedure outlined below.

B. Open the safety interlocking lid and insert the buffer tray in the tray support stand.

C. Place the gel casting deck in the center of the buffer tray with the outermost, well visualization strip (red stripe) towards the left (negative) electrode.

D. Slide the gel casting dams (wedges) down in the "V"-shape grooves of the buffer tray. Keep the flat edges of the wedges against the gel casting deck. Apply gentle pressure simultaneously to both wedges to seat the sealing surfaces against the sides of the gel deck. **Do not force the gel casting dams down, as this may displace the gel deck out of level.**

E. Insert the comb into the desired comb position slot, with the teeth in line with a red stripe. The combs for this unit have 8-teeth that are 1 mm wide and 0.8 mm thick, 8-teeth that are 1 mm wide and 1.5 mm thick, or 14-teeth that are 0.5 mm wide and 0.8 mm thick.

Figure 2

Gel Apparatus

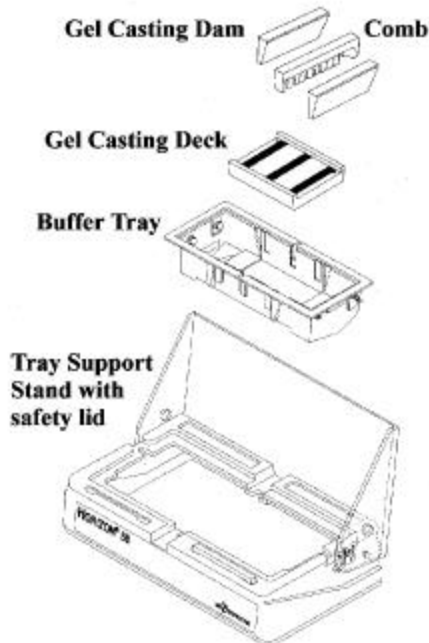


Figure 1. Horizon 58 Horizontal Gel Electrophoresis Apparatus.

II. Preparing 1% Agarose for Gels

A. Weigh out 0.2 g of agarose into a 125 ml flask.

B. Add 25 ml of autoclaved ddH₂O to the flask.

C. Microwave the flask for 1-2 min. Make sure that the mixture does not boil over. Stop the microwave every 30 s and swirl the flask. Continue to heat until the agarose is completely dissolved.

D. After the agarose has completely dissolved, measure the volume of the contents of flask using a graduated cylinder.

E. Bring the volume up to **19** mL with autoclaved ddH₂O and return the contents to the 125 ml flask.

F. Add 1 mL of 10X TBE and swirl the flask.

F. Microwave if necessary for 15-20 s.

III. Pouring the Gel

A. Pour the contents of the flask into the gel casting deck. Pour carefully so that not many air bubbles are created. Also make sure that the entire bottom of the casting deck is covered.

B. Remove any air bubbles with a pipet.

C. Allow the gel apparatus to stand undisturbed until the agarose gel solidifies

IV. Preparing the Samples

We will mix our DNA with a loading buffer to prepare the sample for loading onto the agarose gel. The loading buffer contains glycerol and two dyes, bromophenol blue and xylene cyanol (the recipe for a 5X concentration of this loading buffer is shown below in Table 2). We use a loading buffer for two reasons. First, the glycerol in the loading buffer helps to keep our DNA in the loading well. The teeth of the comb that are placed in the gel will form a series of wells or depressions in the gel when the comb is removed. These wells represent the spaces in the gel that will hold our sample when we load the gel. When we remove the comb, 0.5X TBE buffer will fill the wells. As we add our sample to the wells the volume of our sample displaces the buffer in the wells. Glycerol is denser than water, and consequently, the loading buffer very readily displaces the 0.5X TBE buffer in the wells. Since our samples of DNA are mixed with the loading buffer they tend to stay in the wells instead of diffusing away. The second reason we use a loading buffer is that the two dyes that are in the loading buffer will help us track how the gel is running. When 0.5% to 1.4% agarose gels are used with 0.5X TBE, the bromophenol blue front runs at about the same position in the gel as 300 bp dsDNA and the xylene cyanol front runs at about the same position in the gel as 4,000bp dsDNA.

Table 2. Recipe for 5X Loading Buffer for DNA Samples

Reagent	Amount	Final Conc.
Glycerol	3.0 mL	%
Bromophenol Blue	0.025 g	%
Xylene Cyanol FF	0.025 g	%
Autoclaved ddH ₂ O	up to 10 mL	

Use the table below to determine how much 5X loading buffer and autoclaved ddH₂O you need to add to your sample.

Table 3. Sample Preparation

Component	Volume (μL)	Final Conc.
Autoclaved ddH ₂ O		---
5X Loading Buffer		1X
DNA Sample	4	----
Total Volume	5	----

V. 1 Kb DNA Ladder

In addition to our DNA samples, we will run a lane on the gel that contains a series of DNA fragments of known size (Fig. 3). A subset of these fragments are small, being between 75 and 517 bp long. The remaining DNA fragments differ in size by about 1000 bp or 1 kilobase pairs (1 Kb). The dsDNA fragment generated by our RT-PCR should be how long? Where would we expect our fragment to be in the gel relative to the DNA fragments in the 1 Kb ladder?

Knowing that the 1 Kb ladder DNA is at a stock concentration of 1 μg/ml, how much of each component would you use below to prepare 10 μL of 1X loading buffer containing 1 Kb DNA ladder at a concentration of 0.05 μg/μL.

Table 4. DNA Standard Preparation

Component	Volume (μL)	Final Conc.
Autoclaved ddH ₂ O		---
5X Loading Buffer		1X
1 Kb DNA Ladder	1	0.05 μg/μL
Total Volume	5	----

VI. Running the Gel

A. Carefully remove the casting dams (wedges) from the casting deck. Make sure that you do not disturb the comb.

B. Pour about 140 mL of 0.5X TBE into the buffer tray. The surface of the gel should be covered with 1 to 2 mm of electrophoresis buffer (0.5X TBE).

C. Gently remove the comb. To avoid tearing the bottom of the wells, gently wiggle the comb to free the teeth from the gel. Slightly lift up one side of the comb, then the other. Where do you think your sample goes if you tear out the bottom of the wells?

D. Remove any trapped air bubbles in the wells to ensure that the wells fill with buffer.

E. Use a micropipet to load the samples onto the floor of the wells. Try not to bump the gel apparatus while you load your samples. If you do bump the apparatus you may dislodge your samples from the wells.

F. Close the safety interlock lid.

G. Connect the gel apparatus to the power supply. Red is positive and black is negative. Toward which of these electrodes are our samples going to run?? That's right they are going to run toward the positive electrode. So connect the red wire to the right side of the apparatus and the black negative wire to the left side of the apparatus.

H. Switch on the power supply and set the voltage using the knob to about 100 Volts (101-104) which should be about 16-20 mamps (check with toggle switch). You should see bubbles being generated at the negative electrode.

I. Run the gel until the bromophenol blue front begins to migrate out of the gel.

J. To stop the gel turn off the power supply.

VII. Staining the gel with Ethidium Bromide and Visualizing the DNA Bands

We will stain the DNA bands in our gel using ethidium bromide. Ethidium bromide intercalates (inserts itself) between A-T base pairs and fluoresces when illuminated with UV light. **Ethidium bromide is known to be a frame shift mutagen.** Consequently, you do not want to let your skin come into contact with ethidium bromide. Always wear gloves when handling gels stained with ethidium bromide. In addition, UV light is also dangerous. UV light can damage your eyes. **Therefore, never look at a UV light source without eye protection!!!!!!!**

A. To stain the gel with ethidium bromide, slide the gel off the casting deck into solution of ethidium bromide at a concentration of 0.5 µg/ml. Leave the gel soaking the stain for about 5 min.



B. Destain the gel by transferring the gel to a container partially filled with ddH₂O. You do not have to use autoclaved water unless you are going to destain for a very prolonged period of time. Allow the gel to destain for about 3 min.

C. Place the gel on the UV trans-illuminator and lower the protective plastic lid.

D. Turn on the illuminator. You should see DNA bands in the lanes that you loaded.

E. To take a picture, lift up the protective plastic lid and place the camera unit on top. The camera should be seated so that the gun handle is pointing toward you. Turn on the UV trans-illuminator and slowly squeeze the trigger on the camera. The camera settings should be F5.6 at 1 sec. After tripping the shutter, turn off the UV illuminator, and pull the white tab on the side of the camera. Pull out your picture and wait 30 sec. Separate your picture from the backing. The picture does not have to be coated. Just throw away the unwanted portions of the film.

F. If you are done with gel, carefully wrap it up in paper towels throw it away in the biohazards trash.

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1 Kb DNA Ladder
 Cat. No. 15615-016
 Lot No. JF0710 250 µg; 1.0 µg/µl
 Store at -20°C.

Description:
 The 1 Kb DNA Ladder (U.S. Patent No. 4,403,036) is suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. The bands of the ladder each contain from 1 to 12 repeats of a 1018-bp DNA fragment. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1636 bp. The 1636-bp band contains 10% of the mass applied to the gel. The ladder may be radioactively labeled by one of the following methods: (i) Partial exonucleolytic degradation and resynthesis with T4 DNA polymerase. This method is preferred because higher specific activity is achieved with less ³²P input; (ii) Labeling the 5' ends with T4 polynucleotide kinase; (iii) Filling in the 3' recessed ends with *E. coli* DNA polymerase I or the large fragment of DNA polymerase I.

Storage Buffer:
 10 mM Tris-HCl (pH 7.5)
 50 mM NaCl
 0.1 mM EDTA

Recommended Procedure:
 A final concentration of 20 mM NaCl is recommended. Apply approximately 0.1 µg of ladder per mm lane width. Do not heat before loading.

Quality Control Data:
 Agarose gel analysis shows that all bands larger than 500 bp are distinguishable.

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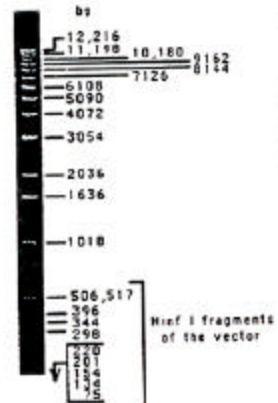
Structure of Fragment (1):



Notes:
 During 1.2% agarose gel electrophoresis with Tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates together with the 506/517 bp doublet band.

The 1636 bp band and all bands less than 1000 bp are generated from pBR322.

If the ionic strength of the sample is too low, blurring of the bands can occur.



1 Kb DNA Ladder
 4.5 µg/lane
 0.5% agarose gel
 stained with ethidium bromide

Cat. No. 15615-016

Figure 3. 1 Kb DNA Ladder