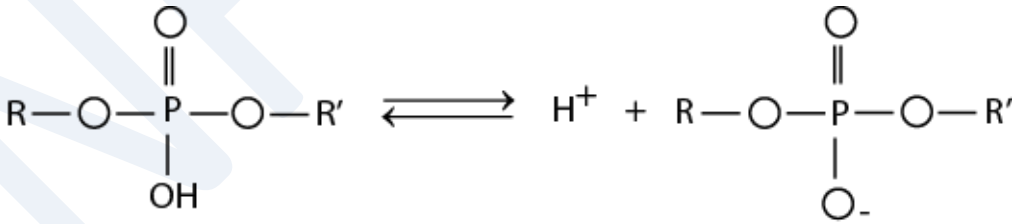
Lecture 27: Agarose Gel Electrophoresis for DNA analysis

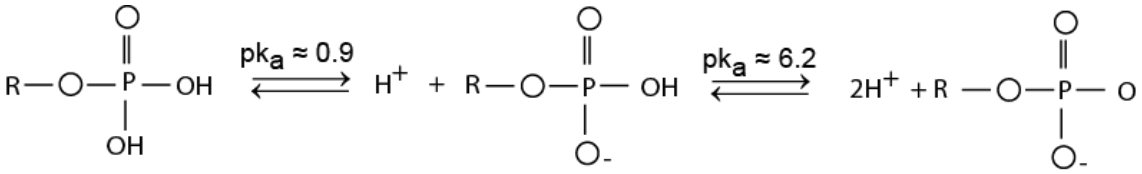
During Lecture 9 and 10 we have studied basics of protein electrophoresis. Recalling our discussion during lecture, protein needs to be boiled with SDS to give uniform negative charge. This enables protein to move toward positive electrode when electric field is applied. Polyacrylamide was used as solid support for separation. We also discussed why a solid support is needed for electrophoratic separation (please refer our earlier lecture to refresh the concepts).

Let us see how DNA electrophoresis differs from protein electrophoresis.

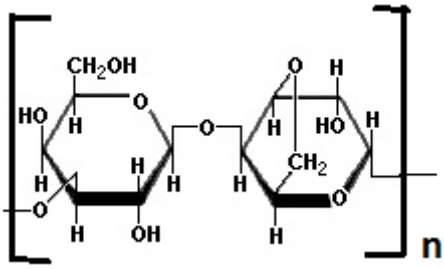
- Basic theory of the electrophoresis is valid in case of DNA electrophoresis as well.
- Generally agarose is used for DNA separation. As size of the DNA is lot bigger than proteins, a stable solid support with bigger pore size is required. Size of polyacrylamide pores is smaller for this purpose. Note: Agarose gel can also be used for separation of very large protein/protein complex. Agarose gel electrophoresis is most suitable for separation of DNAs/RNAs in the range of 100bp to about 15kb. Polyacrylamide gels matrix may be used for separation of small DNAs or RNAs.
- DNA already has uniform negative charge. If you recall DNA structure from our last class, two nucleotides are connected together by a phosophodiester bond which gives one negative charges.



Here, we are ignoring one negative charge due to phosphate at 5’ end as this is very small compare to net charge provided by phosophodiester bonds in a polynucleotide.



There are several physical methods available for separating nucleic acid (DNA and RNA) based on its size. Gel electrophoresis is a separation technique which is purely based on charge and size. Agarose gel electrophoresis is eventually one of the traditional methods of separating and analyzing nucleic acid. In this method, gel made from agarose acts as a separating medium. Agarose which is linear polymer agarose is a polysaccharide, whose monomeric unit is a disaccharide of D-galactose and 3,6-anhydro-L-galactopyranose.



Agarose is in powdered form, and is insoluble in water at room temperature. It gets dissolved in boiling water and when it starts to cool, it undergoes cross-linking (H-bonding) and results in polymerization (agarose gel matrix). Extent of cross-linking depends on percentage of agarose (higher percentage results in higher cross-linking thus more sieving effect due to small pore size).

As discussed early in the lecture, the charge of the DNA is negative and therefore it migrates to the anode (positively charged electrode), if a voltage is applied. Nucleic acid molecules are separated by applying an electric field to move the DNA through an agarose gel matrix. The migration rate of the DNA is mainly affected by the factors such as size of the DNA, agarose concentration used and conformation of the DNA.

The migration of the molecules in gel electrophoresis is directly proportional to the size of the molecule. The gel sieves the movement of the molecules based on their size. Small molecules migrate faster and then bigger ones as small molecules can move more easily through gel pores. Due to difference in the migration rate of various size DNA molecules in gel DNA fragments are separated based on sizes. The size of the fragments can be determined by running standard DNA ladder run in parallel. The relationship of migration rate to size is linear throughout most of the gel, except for very largest fragments. The fragments such as genomic DNA which are very big in size shows difficulty in penetration through the gel pores. Hence, does not show linearity. The precise relationship between migration rate and size can be calculated as rate is inversely proportional to the $\log_{10}$ of the number of base pairs in a DNA fragment.

DNA fragments of length ranging from 50 base pair to several million base pair can be separated using agarose gel electrophoresis. Migration rate of the fragments also depends on the concentration of agarose used to prepare gel. The concentration of agarose is inversely proportional to the rate of migration of the DNA fragments, i.e. lower the concentration, the faster is the DNA migration rate and *vice versa*. Generally used agarose concentration is 0.7% to separate DNA fragments of range 2- 10 kb and 2% agarose for separation of small fragments such as 0.1- 1 kb. Low percent gels are weak and high percent gels are often brittle. Standard 1 % agarose gels are common for many applications, which can resolve DNA fragments from 0.5-30 kb in length.

The above explained relationship between migration rate and size of DNA is applicable only to linear DNA fragments. Generally DNA can exist in three forms: linear form, opencircular form and supercoiled form. The linear DNA may be the product of PCR amplification (Fig. 3) or the restriction digestion product. But plasmid DNA is the one which are mostly studied. In *in-vivo* plasmids exist as highly supercoiled form to enable it to fit inside the cell. When the plasmid preparation is done plasmid DNA can exist in all the three conformations i.e. linear, opencircular and supercoiled forms. In a good careful plasmid preparation, most of the DNA will remain supercoiled along with the open circular and linear form. The open circular form of plasmid exist due to the break in only one of the strands, the DNA will exist as circular. The break will allow rotation around the phosphodiester back bone of DNA due to which supercoils will be released. The plasmid exists in the linear form when there is a cut in the circular DNA. The supercoiled

form of plasmid DNA sustains less friction when run in agarose gel matrix. Therefore, for the same size plasmid DNA, supercoiled DNA form will runs faster than open circular form and linear form. When a pool of plasmid sample containing supercoiled, opencircular and linear form is run on agarose gel, supercoiled DNA will run faster which is the bottom most band, next is the nicked form i.e. opencircular form which is the middle band and the top most band in the gel is the linear or relaxed form (Fig. 4). In unicellular, plasmids tend to recombined with themselves to produce smaller multimers. Therefore, an uncut plasmid produces a complex banding pattern on an agarose gel. When the plasmid is cut with a single restriction enzyme, the super coiled, open circular and multimer forms are reduced to a linear conformation. The amount of different conformation of the plasmid depends on number of manipulations that are incurred in plasmid preparation. If the plasmid preparation is not good linearization of supercoiled DNA may occur because of the nick produced in the plasmid in preparation process. Due to which in a plasmid preparation, the amount of supercoiled DNA may be small amount to nil, which can be clearly seen on agarose gels. Some times improper handling or storage of the isolated DNA may degrade, which can be detected as a smear when run on the agarose gel.

Agarose gels are horizontally laid and are completely covered by running buffer; hence they are referred to as submarine gels. The agarose gels are prepared in a gel casting trays which are of different sizes. The small gel slab are used for quick check in which the resolution is not great and runs for 30 to 40 minutes. Big gels are used for good resolution or separation of different sized DNA fragments, which generally runs for longer time, 60 minutes or more.

Electrophoresis buffer

For the electrophoresis of DNA many buffers have been recommended. But the most commonly used are TAE (Tris-acetate-EDTA) and TEB (Tris-botate-EDTA). Due to the difference in ionic strength of these two buffers, DNA fragments will migrate at different rates. The gels must be prepared in the same buffer in which the gels are run i.e. either TAE or TBE buffer. A buffer not only provides ions to support the conductivity, but also establish a pH. If you use concentrated buffer, enough heat may generate to melt the gel. The working concentration of the buffer is 1X and stock is prepared for 50X.

Preparing a agarose gel

Agarose gels are prepared as percentage weight/volume solutions. Thus to prepare standard 1% agarose gel 1 gram of agarose is dissolve in 100 ml of buffer. For bigger gels just scale up the volume accordingly. Agarose do not dissolve in the buffer, rather it has to be melted by boiling which is typically done in a microwave oven. While melting agarose, care should be taken to prevent boil over. To visualize the DNA on agarose gel UV- fluorescent dye is required. Ethidium bromide is a florescent dye that intercalates between bases of nucleic acids and allows convenient detection of DNA fragments in agarose under UV light. After agarose has cooled to about 60°C, a final concentration of 0.5 µg/ml ethidium bromide is added to agarose solution before pouring the agarose into the gel tray which is sealed and come is positioned. The agarose solution is allowed to cool at room temperature for an hour to solidify gel. Comb is carefully removed from the solidified gel and seal of the gel tray is removed. The gel is placed in the buffer tank carefully with buffer completely submerge the gel (Fig 1)

Figure 1: A. Agarose gel casting unit. **B.** Solidified agarose gel.

Running a Gel

Load your DNA samples in corresponding wells in the gel. Remember that DNA is negatively charged and runs towards the positive electrode. The gel wells should be nearer towards black electrode and farther from red electrode (DNA should run to the red end). Turn on the power to run the gel with the voltage set at 60-80 volts for 40 minutes for small gels and 90-100 volts for 1-2 hours for larger gels. Agarose gels run at high voltage may result in melting the gel and distortion of bands. One can confirm that the gel is running by checking for bubbles from the electrodes. Switch off the power at the end of the run. The next step is visualization of gel for bands (Fig. 2)

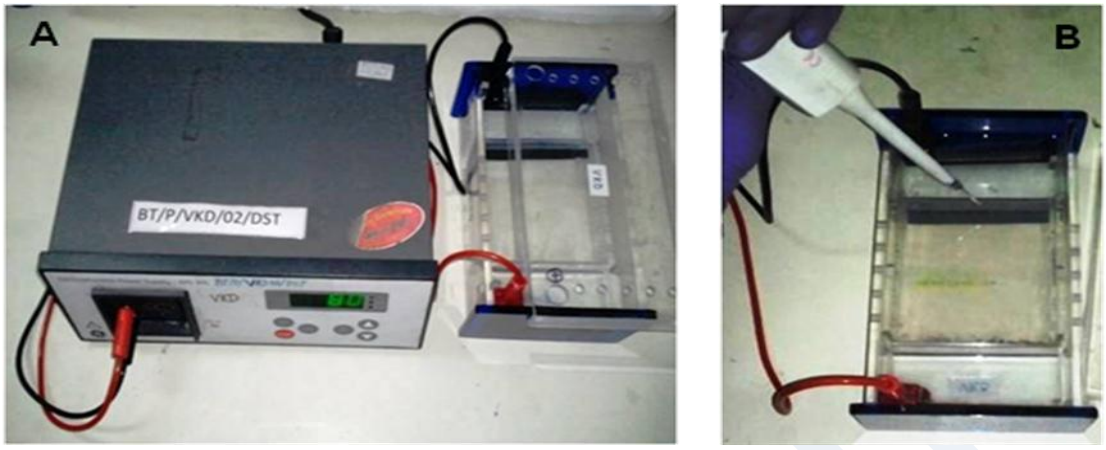


Figure 2: A. Agarose gel running unit. **B.** Loading of agarose gel.

Visualization

Ethidium bromide (EtBr) is traditionally used as a dye that binds to DNA and fluoresces under ultraviolet light. EtBr causes mutation and must be handled as hazardous waste. Due to the hazardous nature of the EtBr, recently non-toxic dyes have been introduced in which the gels have to be stained first and then destained to visualize the bands. But, if you are using EtBr as a dye to visualize DNA bands you need to be more careful in handling as to prevent the hazardous effect of EtBr. The EtBr gels have to be disposed separately in hazard wastes disposal bags.

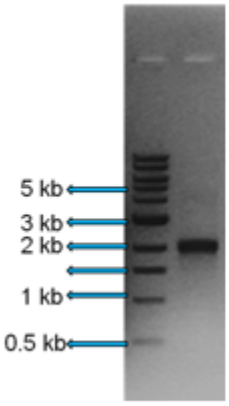


Fig. 3: PCR amplified linear fragment of DNA run parallel with standard marker.

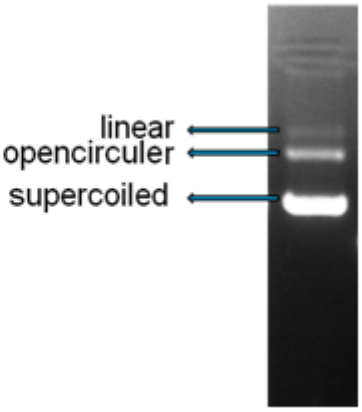


Fig. 4: Plasmid DNA checked on agarose gel showing all the three conformations.

Analyzing a Gel

Generally agarose gels are visualized using a standard control to check for whether bands have generated or not. Bands that are closer to the wells are more compressed than bands away from the wells. Standard marker helps in finding the approximate size of the linear fragments of DNA. Linear duplex DNA fragments travel through agarose gels at a rate which is inversely proportional to the log of their molecular weight.

$$M_r \propto 1/\log(Mw)$$

Example 1: Compare molecular mass vs. expected migration rate:

Molecular Mass (kDa)	log (Molec. Mass)	1/log (Molec. Mass) i.e. relative M_r (Migration Rate)
20000	4.3	0.23
15000	4.1	0.24
10000	4.0	0.25
5000	3.7	0.27
1000	3.0	0.33

There is an inverse linear relationship between the logarithm of the electrophoretic mobility and gel concentration.

$$\text{Log } (M_r) \propto 1/\text{Gel \%}$$

Example 2: Compare gel percentage vs. expected migration rate (Mr)

Gel %	1/Gel %	inv log(1/Gel %) (i.e. relative M_r)
2.0	0.5	3.2
1.5	0.66	4.6
1.0	1.0	10.0
0.5	2.0	100.0

Assignment

1. What is the function of the agarose gel in electrophoresis?

   A. Stabilizes DNA.
   B. Helps in DNA migration.
   C. Provides matrix for separation.
   D. Provides charge to DNA.

2. What are the functions of the buffer in electrophoresis?

   A. Stabilizes DNA.
   B. Provides matrix for separation.
   C. Helps in DNA migration
   D. Provides ionic charge and pH.

3. What are the functions of the EtBr in agarose gel electrophoresis?

   A. Increases mobility of DNA.
   B. Helps in visualizing of DNA.
   C. Increases conductivity.
   D. Helps in separation of DNA by size.

4. Toward which pole does DNA migrate when electric current is run through the gel and why?

   A. From +ve pole to -ve pole as DNA is +ve charged.
   B. From -ve pole to +ve pole as DNA is +ve charged.
   C. From +ve pole to -ve pole as DNA is -ve charged.
   D. From -ve pole to +ve pole as DNA is -ve charged.

5. What are the three main factors effecting the migration rate of DNA on grarose gel?

   A. DNA concentration, voltage, and buffer concentration.
   B. Gel percentage, buffer concentration and voltage.
   C. Size of DNA, gel concentration and conformations of DNA.
   D. Length of the gel, buffer concentration and DNA concentration.

6. Give the relation between migration rate (M_r) and gel concentration (gel %).

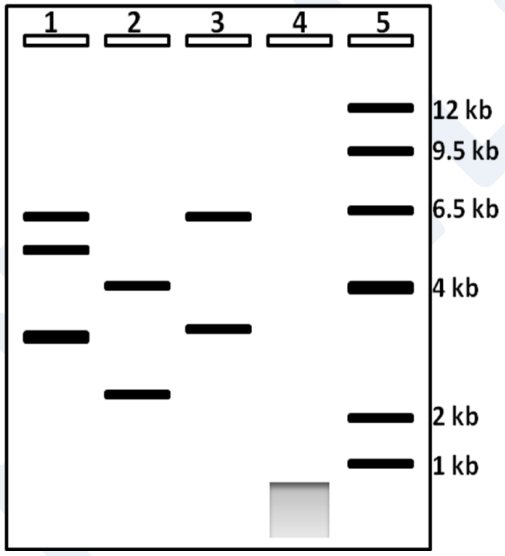
   A. $\log(\text{gel\%}) \propto 1/\log[M_r]$
   B. $\log(M_r) \propto 1/[\text{gel\%}]$
   C. $1/\log(M_r) \propto 1/[\text{gel\%}]$

D. log (M_r) ∝ [gel%]

7. Give the relation between migration rate (M_r) and molecular weight (Mw).

A. $1/M_r \propto 1/\log (Mw)$
B. $\log M_r \propto 1/\log (Mw)$
C. $1/\log M_r \propto \log (Mw)$
D. $M_r \propto 1/\log (Mw)$

8. Plasmid was isolated from bacterial cells and was subjected to restriction digestion using three different restriction enzymes as shown in the gel below. Standards are in Lane 5 and the uncut control is in Lane 1, and roughly the same amount of total DNA in sample lanes 1-4. Considering the given data answer the questions below.



i. Give the order of the DNA conformation in lane 1 from top to bottom?

A. Opencircular, supercoiled and linear.
B. Supercoiled, opencircular and linear.
C. Linear, supercoiled and opencircular.
D. Linear, opencircular and supercoiled.

ii. Approximately how big is your original plasmid?

A. 10 kb
B. 3.5 kb
C. 6.5 kb

D. 9.5 kb

iii. How many times did the enzyme cut in Lane 2 digest the plasmid?

A. 1
B. 2
C. 3
D. 4

iv. How many times did the enzyme used in Lane 3 digest the plasmid? Does the data seem reasonable?

A. 2 times and the data are reasonable.
B. 1time and the data are reasonable.
C. 2 times and the data are not reasonable.
D. 1 time and the data are not reasonable.

v. What are some likely explanations for the smearing detected in Lane 4?

A. Due to absence of DNA
B. Due to DNA degradation
C. Due to restriction digestion
D. Reason B or A

Answers to question 1-C; 2-D; 3-B; 4-D; 5-C; 6-B; 7-D

Answers to question 8: i-D; II-C; iii-B; iv-C; v-D