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Principles of Nucleic Acid Separation by Agarose Gel Electrophoresis

Muhittin Yılmaz*, Cem Ozic and İlhami Gok
University of Kafkas, Department of Biology, Faculty of Sciences, Kars, Turkey

1. Introduction

1.1 Principles of nucleic acid separation by agarose gel electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. (Kryndushkin et al., 2003). Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired "band" from a stained gel viewed with a UV transilluminator (Sharp et al., 1973).

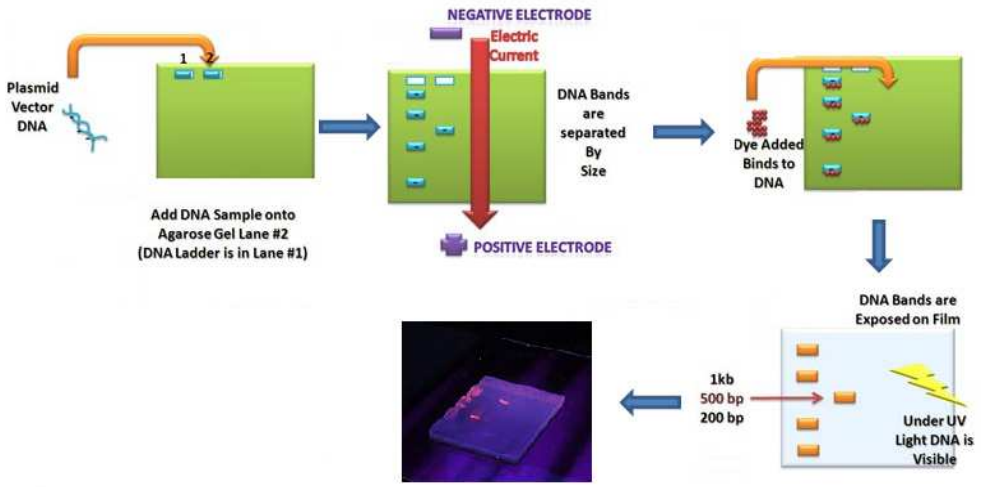

Fig. 1. Agarose gel electrophoresis method (modified from http://www.molecularstation.com/agarose-gel-electrophoresis).

In order to visualize nucleic acid molecules in agarose gels, ethidium bromide or SYBR Green are commonly used dyes. Illumination of the agarose gels with 300-nm UV light is subsequently used for visualizing the stained nucleic acids. Throughout this chapter, the common methods for staining and visualization of DNA are described in details.

Agarose gel electrophoresis provides multiple advantages that make it widely popular. For example, nucleic acids are not chemically altered during the size separation process and agarose gels can easily be viewed and handled. Furthermore, samples can be recovered and extracted from the gels easily for further studies. Still another advantage is that the resulting gel could be stored in a plastic bag and refrigerated after the experiment, there may be limits. Depending on buffer during electrophoresis in order to generate a suitable electric current and to reduce the heat generated by electric current can be considered as limitations of electrophoretic techniques (Sharp et al., 1973; Boffey, 1984; Lodge et al. 2007).

1.2 Application

The agarose gel electrophoresis is widely employed to estimate the size of DNA fragments after digesting with restriction enzymes, e.g. in restriction mapping of cloned DNA. It has also been a routine tool in molecular genetics diagnosis or genetic fingerprinting via analyses of PCR products. Separation of restricted genomic DNA prior to Southern blot and separation of RNA prior to Northern blot are also dependent on agarose gel electrophoresis.

Agarose gel electrophoresis is commonly used to resolve circular DNA with different supercoiling topology, and to resolve fragments that differ due to DNA synthesis. DNA damage due to increased cross-linking proportionally reduces electrophoretic DNA migration (Blasiak et al., 2000; Lu & Morimoto, 2009).

In addition to providing an excellent medium for fragment size analyses, agarose gels allow purification of DNA fragments. Since purification of DNA fragments size separated in an agarose gel is necessary for a number molecular techniques such as cloning, it is vital to be able to purify fragments of interest from the gel (Sharp et al. 1973).

Increasing the agarose concentration of a gel decreases the migration speed and thus separates the smaller DNA molecules makes more easily. Increasing the voltage, however, accelerates the movement of DNA molecules. Nonetheless, elevating the currency voltage is associated with the lower resolution of the bands and the elevated possibility of melting the gel (above about 5 to 8 V/cm).

1.3 Visualization

Ethidium bromide (EtBr -Figure2.) is the common dye for nucleic acid visualization. The early protocol that describes the usage of Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridiniumbromide-) for staining DNA and RNA in agarose gels dates as far back as 1970s (Sharp et al., 1973). Although the with a lower efficiency compare to the double- stranded DNA, EtBr is also used to stain single- stranded DNA or RNA. Under UV illumination, the maximum excitation and fluorescence emission of EtBr can be obtained from 500- 590 nm. Exposing DNA to UV fluorescence should be performed rapidly because nucleic acids degrade by long exposures and thus, the sharpness of the bands would be negatively affected.

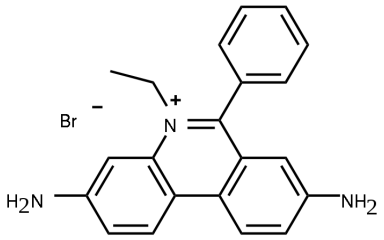

Fig. 2. Chemical formula of ethidium bromide.

An alternative dsDNA stain is SYBR Green I, produced by Invitrogen. Despite the fact that SYBR Green is more expensive, it is 25 times more sensitive than ethidium bromide (Jin et al., 1994). SYBR Safe, a variant of SYBR Green, has been shown to have low levels of mutagenicity and toxicity compared with ethidium bromide (Madruga et al., 1997) while providing similar sensitivity levels EtBr (Madruga et al., 1997). Nevertheless, similar to the SYBR Green, SYBR Safe is also more expensive when compared to EtBr.

Since EtBr stained DNA is not visible in natural light, negatively charged loading buffers are commonly added to DNA prior to loading to the gel. Loading buffers are particularly useful because they are visible in natural light and they co-sediment with DNA. Xylene cyanol and Bromophenol blue are the two common dyes used as loading buffers and they run about the same speed as DNA fragments that are 5000 bp and 300 bp respectively. The other less frequently used progress markers are Cresol Red and Orange G which run at about 125 bp and 50 bp, respectively.

If some of the bands after size separation in an agarose gel are intended to be purified for further analyses, it is advisable to avoid the exposure of gel with UV light. As an alternative, a blue light excitation source could be used. A blue excitable stain is therefore required for such cases. SYBR Green or Gel Green stains could serve for the purpose. Blue light is also convenient for visualization, because it is safe and also it passes through transparent plastic and glass.

1.4 Preparing and running standard agarose DNA gels

Several electrophoresis buffers can be used for fractionating nucleic acid such as, Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE) (Sharp et al., 1973; Boffey, 1984; Lodge et al., 2007). TAE gel buffer systems are more convenient than TBE systems, if post-separation methods are the ultimate goal of running a gel (Rapley, 2000). For gel preparation, agarose powder electrophoresis grade is mixed with electrophoresis buffer to the desired concentrations (usually with a range of 0,5-2%) then heated in a microwave oven until completely dissolved. Ethidium bromide is usually added to the gel at concentration of 0.5 ug/ml for nucleic acid visualization. The mixture is cooled to 60^0C and poured into the casting tray for solidification. Immediately after the gel solidification, the comb is removed. The gel is kept in its plastic during electrophoresis and PCR product mixed with loading dye is placed in the wells. As nucleic acids are negatively charged, wells should be placed towards the negative electrode. At the same time, ethidium bromide migrates in the reverse direction, meets and couples with DNA fragments. DNA fragments are visualized by staining with ethidium bromide when adequate migration has occurred. Then, this fluorescent dye intercalates between bases of DNA and RNA (Corley, 2005).

Linear DNA fragments migrate through agarose gels with a velocity that is inversely proportional to the log10 of their molecular weight (Sambrook & Russel, 2001). Circular forms of plasmids migrate in agarose gels differently compared to linear DNA of the same size. Typically, uncut plasmids will migrate faster than the same plasmid when linearized (Sambrook & Russel, 2001).

The several factors listed below are effecting the mobility of DNA fragments in agarose gels.

1.4.1 Agarose concentration

Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several mega bases (Mb) using specialized apparatus. In the gel, the distance between DNA bands of a given length is determined by the percent agarose. Higher concentrations have the disadvantage of long run times. PFGE is used to separate higher Mw by applying different voltage.

Most agarose gels are prepared with the agarose concentrations ranging 0.7% (good separation or resolution of large 5–10kb DNA fragments) to 2% (good resolution for small 0.2–1kb fragments) (Table 1- Lewis, 2011).

Agarose Concentration in Gel (% [w/v])	Range of Separation of Linear DNA Molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Table 1. The suggested agarose concentrations for separation of different ranges of Linear DNA molecules (Lewis, 2011).

1.4.2 Voltage

Migration of fragments in an agarose gel depends on the difference in electric current. Different optimal voltages are required for different fragment sizes. For instance, the best resolution for fragments larger than 2 kb could be obtained by applying no more than 5 volts per cm to the gel (Sharp et al., 1973; Boffey, 1984; Lodge et al., 2007;Harrington 1993; Lane et al., 1992).

1.4.3 Electrophoresis buffer

Various buffers are used for agarose electrophoresis. The two most common buffers for nucleic acids are Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE). DNA fragments migrate with different rates in these two buffers due to differences in ionic strength. Buffers not only establish an ideal pH, but provide ions to support conductivity. In general, the ideal buffer should produce less heat, have a long life and a good conductivity. For example, deviations from the optimal concentration of the buffer (over concentrated) could produce enough heat to melt the gel (Sharp et al., 1973; Boffey, 1984; Lodge et al., 2007; Harrington 1993; Lane et al. 1992).

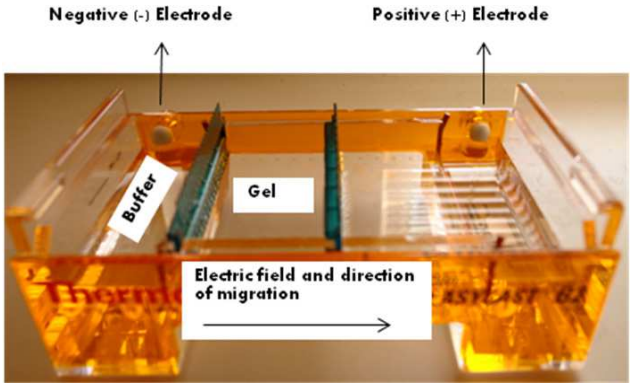

Fig. 3. Schematic illustration of a typical horizontal gel electrophoresis setup for the separation of nucleic acids.

The two buffers vary according to the advantages and disadvantages. For instance, Borate has disadvantages as it polymerizes and/or interacts with cis diols found in RNA. TAE on the other hand has the lowest buffering capacity but provides the best resolution for larger DNA which implies the need for lower voltage and more time with a better product. Lithium Borate (LB) - relatively new buffer and is ineffective in resolving fragments larger than 5 kbp. However, with its low conductivity, a much higher voltage could be used (up to 35 V/cm) and this high voltage leads a shorter analysis time for routine electrophoresis.

1.4.4 Effect of ethidium bromide

Ethidium bromide is a fluorescent dye and it intercalates between nucleic acids bases and provides opportunity to easily detect nucleic acid fragments in gels (Sharp et al. 1973; Boffey, 1984; Lodge et al. 2007; Harrington, 1993; Lane et al., 1992).The gel subsequently is being illuminated with an ultraviolet lamp usually by placing it on a light box. An apparatus integrated with the illumination system is used to take images of the gel with the presence of UV illumination. The gel can be subsequently photographed usually with a digital camera and images are usually shown in black and white, despite the fact that the stained nucleic acid fluoresces reddish-orange.

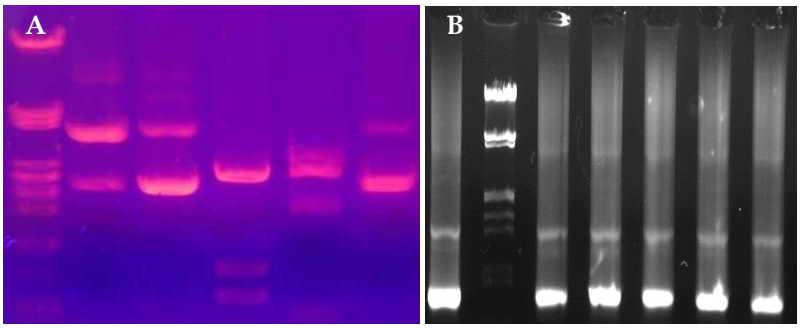

Fig. 4. Gel electrophoresis based image analysis**.** Agarose gels, stained by Ethidium bromide (A) and UV light (B).

For more Imaged agarose gels can be analyzed using image analysis tools after high resolution scan. An example for an open access image analysis tool is Image J provided by NIH (http://rsbweb.nih.gov/ij/docs/user-guide.pdf). Ethidium bromide fluoresces orange when intercalating DNA and when exposed to UV light (Figure 4).

Protocol 1: Agarose Gel Electrophoresis (Modified from Sambrook &Russel 2001)

2. Materials

Nucleic Acids and Oligonucleotides; DNA samples**,** DNA size standards and PCR product
Buffers and Solutions; Agarose solutions, Electrophoresis buffer**,** DNA staining solution and 6x Gel-loading buffer
DNA Staining Solution; Ethidium bromide (10 mg/ml) or SYBR Green.

Ethidium Bromide: Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the %1 (10 mg/ml) solution to a dark bottle and store at room temperature. Ethidium bromide is a powerful mutagen and toxic.

SYBR Green: SYBR Green (Molecular Probes) is supplied as a stock solution of unknown concentration in dimethylsulfoxide. Agarose gels are stained in a working solution of SYBR Green, which is a 1:10,000 dilution of SYBR Green nucleic acid stain in electrophoresis buffer. Prepare working stocks of SYBR Green daily and store in the dark at regulated room temperature.

Electrophoresis Buffer; TAE, TPE and TBE

TAE; Prepare a 10x stock solution in 1 liter of H_2O:

48.4 g Tris base [tris(hydroxymethyl)aminomethane]
11.4 ml glacial acetic acid (17.4 M)
20 ml of 0.5 M EDTA or 3.7 g EDTA, disodium salt.

Dissolve all in 800 ml deionized water and mass up to 1 liter, store in room temperature and the solution should be diluted to 1X prior to use [100 ml (10 x stock) up to 1 liter deionized water].

TBE; Prepare a 10x stock solution in 1 liter of H_2O:

48.4 g Tris base [tris(hydroxymethyl)aminomethane]
55 g of boric acid
40 ml of 0.5 M EDTA (pH 8.0)

TPE; Prepare a 10x stock solution in 1 liter of H_2O:

108 g Tris base
15.5 ml of 85% (1.679 g/ml) phosphoric acid
40 ml of 0.5 M EDTA (pH 8.0)
The 1x working solution is 90 mM Tris-phosphate/2 mM EDTA.

6x Gel-loading Buffer I

0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
40% (w/v) sucrose in H_2O

2.1 Method

1. Prepare a solution of agarose in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA sample(s).
2. If using a glass bottle, loose the cap. Heat the mixture in a boiling-water bath or a microwave oven until the agarose dissolves.
3. Use insulated gloves to transfer the flask into a water bath at 55°C. When the melted gel has cooled, add ethidium bromide to a final concentration of 0.5 μg/ml. Mix the gel solution thoroughly by gentle swirling.
4. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.
5. Pour the warm agarose solution into the mold.
6. Allow the gel to polymerize completely (20-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.
7. Place the gel into the electrophoresis device and enough electrophoresis buffers to cover the gel to a depth of approx. 1 mm.
8. Mix the sample by loading dye with a ration 1:5 or 1:10.
9. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or a drawn-out Pasteur pipette or glass capillary tube. Load size standards into slots on both the right and left sides of the gel.
10. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1-5 V/cm. If the leads have been attached correctly, bubbles should be generated at the anode and cathode, and within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated for distance through the often to the last third og the gel.
11. When the DNA samples or dyes have migrated for a sufficient distance through the gel, turn off the electric current and remove the leads and lid from the gel tank. Otherwise, stain the gel by immersing it in electrophoresis buffer or H_2O containing ethidium bromide (0.5 μg/ml) for 20-45 minutes at room temperature or by soaking in a 1:10,000-fold dilution of SYBR Green stock solution in electrophoresis buffer.

3. Detection of DNA in agarose gels

Nucleic acids running on an electrophoresis can be detected by staining with a dye and visualized under 300-nm UV light. Staining and visualization of DNA are conducted by using either ethidium bromide or SYBR Green. The most convenient and commonly used method to visualize DNA in agarose a gel is ethidium bromide. Ethidium bromide can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA). However, the resolution of single-stranded nucleic acid is relatively low and the fluorescent yield is poor compared to the SYBR Green. In fact, most fluorescence associated with staining single-stranded DNA or RNA is attributable to binding of the dye to short intrastrand duplexes in the molecules (Sambrook &Russel 2001).

The banding pattern of DNA resolved through the gel by recorded images. Images of ethidium bromide stained gels may be captured by using transmitted or incident UV light.

However, the amount of nicking of the DNA is much lower at 302 nm compared to 254 nm. If SYBR Green used instead of ethidium bromide another 10-20-fold increase in the sensitivity using conventional image taking techniques is in the range of possibility. Detection of DNAs stained with this dye requires the use of a yellow or green gelatin or cellophane filter with the camera along with the illumination with 300-nm UV light.

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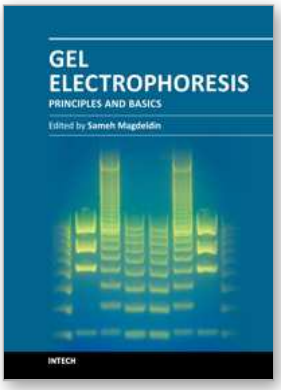

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Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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