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Review Article

Introduction of Electrophoresis Process

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ABSTRACT
Electrophoresis is the process of moving charged molecules in solution by applying an electric field across the mixture. Electrophoresis is often classified according to the presence or absence of a solid supporting medium or matrix through which the charged molecules move in the electrophoretic system. The fundamental driving force of electrophoresis is the voltage applied to the system. Gel electrophoresis is a laboratory method used for a variety of protocols including sequencing, RFLP analysis, marker analysis, DNA fingerprinting and DNA purification. It is based on two key principles: 1) DNA has an overall negative charge (due to the phosphate backbone) and thus will migrate towards a positive charge. 2) A gel acts as a sort of microscopic sieve; smaller DNA fragments will travel through the gel more rapidly than larger fragments. Thus DNA can be separated based on the length (size) of the DNA segment. In this study we worked in Desert laboratory in semnan university for analysis effect of temperature on gle electrophoresis. We conducted that method of electrophoresis used to separate proteins or nucleic acids (DNA and RNA) across a temperature at 4°C for 10-15 minutes. poured gel at 4°C for 10-15 minutes OR let sit at room temperature for 20-30 minutes, until it has completely solidified.
Key words: Electrophoresis, Gel Electrophoresis, DNA Molecules

INTRODUCTION

Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field.[1][2][3][4][5][6] This electrokinetic phenomenon was observed for the first time in 1807 by Ferdinand Frederic Reuss (Moscow State University),[7] who noticed that the application of a constant electric field caused clay particles dispersed in water to migrate. It is ultimately caused by the presence of a charged interface between the particle surface and the surrounding fluid. It is the basis for a number of analytical techniques used in biochemistry for separating molecules by size, charge, or binding affinity.

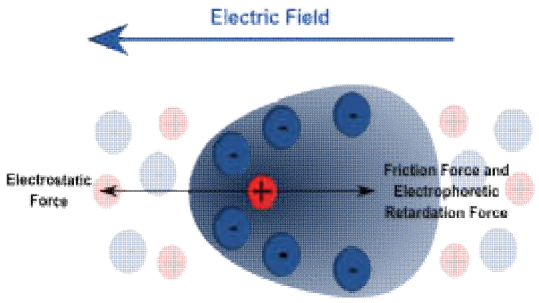

Fig 1 : Illustration of electrophoresis

Electrophoresis of positively charged particles (cations) is called **cataphoresis**, while electrophoresis of negatively charged particles (anions) is called **anaphoresis**. Electrophoresis is a technique used in laboratories in order to separate macromolecules based on size. The technique applies a negative charge so proteins move towards a positive charge. This is used for both DNA and RNA analysis. Polyacrylamide gel electrophoresis (PAGE) has a clearer resolution than agarose and is more suitable for quantitative analysis. In this technique DNA foot-printing can identify how proteins bind to DNA. It can be used to separate proteins by size, density and purity. It can also be used for plasmid analysis, which develops our understanding of bacteria becoming resistant to antibiotics

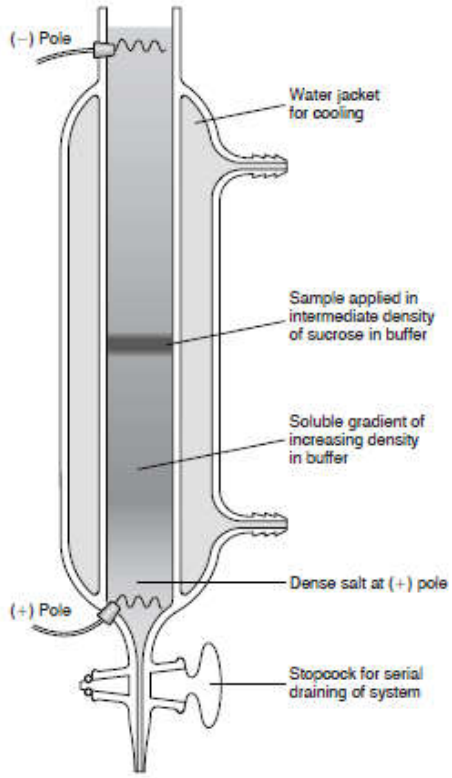

Fig 2 : Solution of electrophoresis system

History of electrophoresis

The **history of electrophoresis** begins in earnest with the work of Arne Tiselius in the 1930s, and new separation processes and chemical analysis techniques based on electrophoresis continue to be developed into the 21st century. Tiselius, with support from the Rockefeller Foundation, developed the "Tiselius apparatus" for moving boundary electrophoresis, which was described in 1937 in the well-known paper "A New Apparatus for Electrophoretic Analysis of Colloidal Mixtures".[1] The method spread slowly until the advent of effective zone electrophoresis methods in the 1940s and 1950s, which used filter paper or gels as supporting media. By the 1960s, increasingly sophisticated gel electrophoresis methods made it possible to separate biological molecules based on minute physical and chemical differences, helping to drive the rise of molecular biology. Gel electrophoresis and related techniques became the basis for a wide range of biochemical methods, such as protein fingerprinting, Southern blot and similar blotting procedures, DNA sequencing, and many more. Early work with the basic principle of electrophoresis dates to the early 19th century, based on Faraday's laws of electrolysis proposed in the late 18th century and other early electrochemistry. Experiments by Johann Wilhelm Hittorf, Walther Nernst, and Friedrich Kohlrausch to measure the properties and behavior of small ions moving through aqueous solutions under the influence of an electric field led to general mathematical descriptions of the electrochemistry of aqueous solutions. Kohlrausch created equations for varying concentrations of charged particles moving through solution, including sharp moving boundaries of migrating particles. By the beginning of the 20th century, electrochemists had found that such moving boundaries of charged particles could be created with U-shaped glass tubes.[2]

Methods of optical detection of moving boundaries in liquids had been developed by August Toepler in the 1860s; Toepler measured the schlieren (*shadows*) or slight variations in optical properties that in inhomogeneous solutions. This method combined with the theoretical and experimental methods for creating and analysing charged moving boundaries would form the basis of Tiselius's moving boundary electrophoresis method.[3]

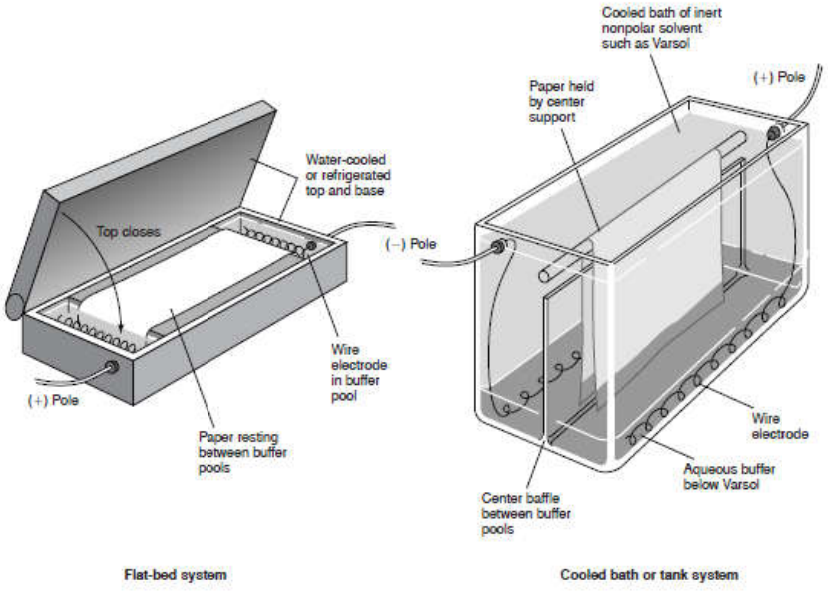

Fig 3: electrophoresis systems.

The apparatus designed by Arne Tiselius enabled a range of new applications of electrophoresis in analyzing chemical mixtures. Its development, significantly funded by the Rockefeller Foundation, was an extension of Tiselius's earlier PhD studies. With more assistance from the Rockefeller Foundation, the expensive Tiselius apparatus was built at a number of major centers of chemical research. By the late 1940s, new electrophoresis methods were beginning to address some of the shortcomings of the moving boundary electrophoresis of the Tiselius apparatus, which was not capable of completely separating electrophoretically similar compounds. Rather than charged molecules moving freely through solutions, the new methods used solid or gel matrices to separate compounds into discrete and stable bands (zones); in 1950 Tiselius dubbed these methods "zone electrophoresis".

Zone electrophoresis found widespread application in biochemistry after Oliver Smithies introduced starch gel as an electrophoretic substrate in 1955. Starch gel (and later polyacrylamide and other gels) enabled the efficient separation of proteins, making it possible with relatively simple technology to analyze complex protein mixtures and identify minute differences in related proteins. Suspended particles have an electric surface charge, strongly affected by surface adsorbed species,[8] on which an external electric field exerts an electrostatic Coulomb force. According to the double layer theory, all surface charges in fluids are screened by a diffuse layer of ions, which has the same absolute charge but opposite sign with respect to that of the surface charge. The electric field also exerts a force on the ions in the diffuse layer which has direction opposite to that acting on the surface charge. This latter force is not actually applied to the particle, but to the ions in the diffuse layer located at some distance from the particle surface, and part of it is transferred all the way to the particle surface through viscous stress. This part of the force is also called electrophoretic retardation force. When the electric field is applied and the charged particle to be analyzed is at steady movement through the diffuse layer, the total resulting force is zero :

$$F'_{tot} = 0 = F'_{el} + F'_{f} + F'_{ret}$$

Considering the drag on the moving particles due to the viscosity of the dispersant, in the case of low Reynolds number and moderate electric field strength E, the drift velocity of a dispersed particle v is simply proportional to the applied field, which leaves the electrophoretic mobility μ_e defined as:

$$\mu_e = \frac{v}{E}.$$

The most well known and widely used theory of electrophoresis was developed in 1903 by Smoluchowski[9]

$$\mu_e = \frac{\varepsilon_r \varepsilon_0 \zeta}{\eta},$$

where ε_r is the dielectric constant of the dispersion medium, ε_0 is the permittivity of free space ($C^2\ N^{-1}\ m^{-2}$), η is dynamic viscosity of the dispersion medium (Pa s), and ζ is zeta potential (i.e., the electrokinetic potential of the slipping plane in the double layer). The Smoluchowski theory is very

powerful because it works for dispersed particles of any shape at any concentration. Unfortunately, it has limitations on its validity. It follows, for instance, from the fact that it does not include Debye length κ^{-1}. However, Debye length must be important for electrophoresis, as follows immediately from the Figure on the right. Increasing thickness of the double layer (DL) leads to removing point of retardation force further from the particle surface. The thicker DL, the smaller retardation force must be.

Detailed theoretical analysis proved that the Smoluchowski theory is valid only for sufficiently thin DL, when particle radius a is much greater than the Debye length :

$$a\kappa \gg 1.$$

This model of "thin Double Layer" offers tremendous simplifications not only for electrophoresis theory but for many other electrokinetic theories. This model is valid for most aqueous systems, where the Debye length is usually only a few nanometers. It only breaks for nano-colloids in solution with ionic strength close to water. The Smoluchowski theory also neglects the contributions from surface conductivity. This is expressed in modern theory as condition of small Dukhin number:

$$Du \ll 1$$

In the effort of expanding the range of validity of electrophoretic theories, the opposite asymptotic case was considered, when Debye length is larger than particle radius:

$$a\kappa < 1.$$

Under this condition of a "thick Double Layer", Hückel[10] predicted the following relation for electrophoretic mobility:

$$\mu_e = \frac{2\varepsilon_r\varepsilon_0\zeta}{3\eta}.$$

This model can be useful for some nanoparticles and non-polar fluids, where Debye length is much larger than in the usual cases.

There are several analytical theories that incorporate surface conductivity and eliminate the restriction of a small Dukhin number, pioneered by Overbeek[11] and Booth.[12] Modern, rigorous theories valid for any Zeta potential and often any $a\kappa$ stem mostly from Dukhin-Semenikhin theory.[13] In the **thin Double Layer** limit, these theories confirm the numerical solution to the problem provided by O'Brien and White.[14]

GEL ELECTROPHORESIS OF NUCLEIC ACIDS

Nucleic acid electrophoresis is an analytical technique used to separate DNA or RNA fragments by size and reactivity. Nucleic acid molecules which are to be analyzed are set upon a viscous medium, the gel, where an electric field induces the nucleic acids to migrate toward the anode, due to the net negative charge of the sugar-phosphate backbone of the nucleic acid chain. The separation of these fragments is accomplished by exploiting the mobilities with which different sized molecules are able to pass through the gel. Longer molecules migrate more slowly because they experience more resistance within the gel. Because the size of the molecule affects its mobility, smaller fragments end up nearer to the anode than longer ones in a given period. After some time, the voltage is removed and the fragmentation gradient is analyzed. For larger separations between similar sized fragments, either the voltage or run time can be increased. Extended runs across a low voltage gel yield the most accurate resolution. Voltage is, however, not the sole factor in determining electrophoresis of nucleic acids. The nucleic acid to be separated can be prepared in several ways before separation by electrophoresis. In the case of large DNA molecules, the DNA is frequently cut into smaller fragments using a DNA restriction endonuclease (or restriction enzyme). In other instances, such as PCR amplified samples, enzymes present in the sample that might affect the separation of the molecules are removed through various means before analysis. Once the nucleic acid is properly prepared, the samples of the nucleic acid solution are placed in the wells of the gel and a voltage is applied across the gel for a specified amount of time.

The DNA fragments of different lengths are visualized using a fluorescent dye specific for DNA, such as ethidium bromide. The gel shows bands corresponding to different nucleic acid molecules populations with different molecular weight. Fragment size is usually reported in "nucleotides", "base pairs" or "kb" (for thousands of base pairs) depending upon whether single- or double-stranded nucleic acid has been separated. Fragment size determination is typically done by comparison to commercially available DNA markers containing linear DNA fragments of known length. The types of gel most commonly used for nucleic acid electrophoresis are agarose (for relatively long DNA molecules) and polyacrylamide (for high resolution of short DNA molecules, for example in DNA sequencing). Gels have conventionally been run in a "slab" format such as that shown in the figure, but capillary electrophoresis has become important for

applications such as high-throughput DNA sequencing. Electrophoresis techniques used in the assessment of DNA damage include alkaline gel electrophoresis and pulsed field gel electrophoresis.
For short DNA segments such as 20 to 60 bp double stranded DNA, running them in Polyacrylamide gel (PAGE) will give better resolution(native condition).[1] Similarly, RNA and single stranded DNA can be run and visualised by PAGE gels containing denaturing agents such as Urea. PAGE gels are widely used in techniques such as DNA foot printing, EMSA and other DNA-protein interaction techniques.. The measurement and analysis are mostly done with a specialized gel analysis software. Capillary electrophoresis results are typically displayed in a trace view called an electropherogram.

FACTORS AFFECTING MIGRATION OF NUCLEIC ACIDS
A number of factors can affect the migration of nucleic acids: the dimension of the gel pores, the voltage used, the ionic strength of the buffer, and the concentration intercalating dye such as ethidium bromide if used during electrophoresis.[2]
Size of DNA
The gel sieves the DNA by the size of the DNA molecule whereby smaller molecules travel faster. Double-stranded DNA moves at a rate that is approximately inversely proportional to the logarithm of the number of base pairs. This relationship however breaks down with very large DNA fragments and it is not possible to separate them using standard agarose gel electrophoresis. The limit of resolution depends on gel composition and field strength.[3] and the mobility of larger circular DNA may be more strongly affected than linear DNA by the pore size of the gel.[4] Separation of very large DNA fragments requires pulse field gel electrophoresis (PFGE). In field inversion gel electrophoresis (FIGE, a kind of PFGE), it is possible to have "band inversion" - where large molecules may move faster than small molecules.

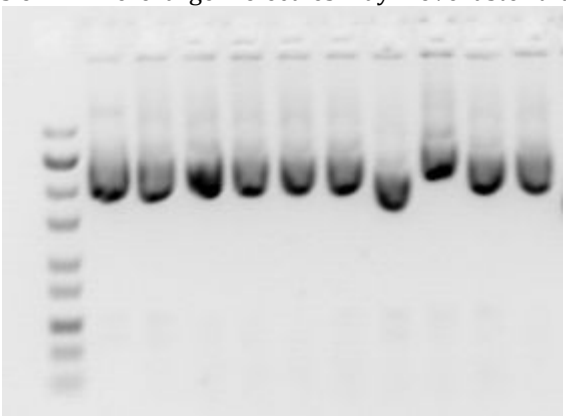

Fig 4: Gels of plasmid molecules

Gels of plasmid preparations usually show a major band of supercoiled DNA with other fainter bands in the same lane. Note that by convention DNA gel is displayed with smaller DNA fragments near the bottom of the gel. This is because historically DNA were run vertically and the smaller DNA fragments move downwards faster.
Conformation of DNA[edit]
The conformation of the DNA molecule can significantly affect the movement of the DNA, for example, supercoiled DNA usually moves faster than relaxed DNA because it is tightly coiled and hence more compact. In a normal plasmid DNA preparation, multiple forms of DNA may be present,[5] and gel from the electrophoresis of the plasmids would normally show a main band which would be the negatively supercoiled form, while other forms of DNA may appear as minor fainter bands. These minor bands may be nicked DNA (open circular form) and the relaxed closed circular form which normally run slower than supercoiled DNA, and the single-stranded form (which can sometimes appear depending on the preparation methods) may move ahead of the supercoiled DNA. The rate at which the various forms move however can change using different electrophoresis conditions, for example linear DNA may run faster or slower than supercoiled DNA depending on conditions,[6] and the mobility of larger circular DNA may be more strongly affected than linear DNA by the pore size of the gel.[4] Unless supercoiled DNA markers are used, the size of a circular DNA like plasmid therefore may be more accurately gauged after it has been linearized by restriction digest.
DNA damage due to increased cross-linking will also reduce electrophoretic DNA migration in a dose-dependent way.[7][8]
Concentration of ethidium bromide

Circular DNA are more strongly affected by ethidium bromide concentration than linear DNA if ethidium bromide is present in the gel during electrophoresis. All naturally occurring DNA circles are underwound, but ethidium bromide which intercalates into circular DNA can change the charge, length, as well as the superhelicity of the DNA molecule, therefore its presence during electrophoresis can affect its movement in gel. Increasing ethidium bromide intercalated into the DNA can change it from a negatively supercoiled molecule into a fully relaxed form, then to positively coiled superhelix at maximum intercalation.[9] Agarose gel electrophoresis can be used to resolve circular DNA with different supercoiling topology.

Gel concentration

The concentration of the gel determines the pore size of the gel which affect the migration of DNA. The resolution of the DNA changes with the percentage concentration of the gel. Increasing the agarose concentration of a gel reduces the migration speed and improves separation of smaller DNA molecules, while lowering gel concentration permits large DNA molecules to be separated. For a standard agarose gel electrophoresis, a 0.7% gives good separation or resolution of large 5–10kb DNA fragments, while 2% gel gives good resolution for small 0.2–1kb fragments. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel would be more appropriate for resolving small fragments. High concentrations gel however requires longer run times (sometimes days) and high percentage gels are often brittle and may not set evenly. High percentage agarose gels should be run with PFGE or FIGE. Low percentage gels (0.1–0.2%) are fragile and may break. 1% gels are common for many applications.[10]

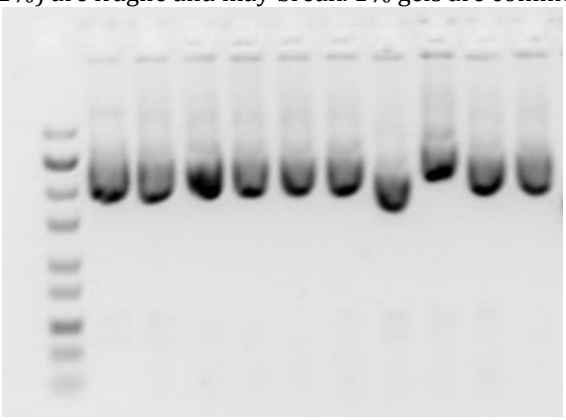

Fig 5: Gels of plasmid preparations usually show a major band of supercoiled DNA with other fainter bands in the same lane. Note that by convention DNA gel is displayed with smaller DNA fragments near the bottom of the gel. This is because historically DNA were run vertically and the smaller DNA fragments move downwards faster

Applied field

At low voltages, the rate of migration of the DNA is proportional to the voltage applied, i.e. the higher the voltage, the faster the DNA moves. However, in increasing electric field strength, the mobility of high-molecular-weight DNA fragments increases differentially, and the effective range of separation decreases and resolution therefore is lower at high voltage. For optimal resolution of DNA > 2kb in size in standard gel electrophoresis, 5 to 8 V/cm is recommended.[6] Voltage is also limited by the fact that it heats the gel and may cause the gel to melt if a gel is run at high voltage for prolonged period, particularly for low-melting point agarose gel.

The mobility of DNA however may change in an unsteady field. In a field that is periodically reversed, the mobility of DNA of a particular size may drop significantly at a particular cycling frequency.[11] This phenomenon can result in band inversion whereby larger DNA fragments move faster than smaller ones in PFGE.

MECHANISM OF MIGRATION AND SEPARATION

The negative charge of its phosphate backbone moves the DNA towards the positively charged anode during electrophoresis. However, the migration of DNA molecules in solution, in the absence of a gel matrix, is independent of molecular weight during electrophoresis, i.e. there is no separation by size without a gel matrix.[12] Hydrodynamic interaction between different parts of the DNA are cut off by streaming counterions moving in the opposite direction, so no mechanism exists to generate a dependence of velocity on length on a scale larger than screening length of about 10 nm.[11] This makes it different from other processes such as sedimentation or diffusion where long-ranged hydrodynamic interaction are important. The gel matrix is therefore responsible for the separation of DNA by size during electrophoresis, however the precise mechanism responsible the separation is not entirely clear. A number of models exists for the mechanism of separation of biomolecules in gel matrix, a widely accepted

one is the Ogston model which treats the polymer matrix as a sieve consisting of randomly distributed network of inter-connected pores.[13] A globular protein or a random coil DNA moves through the connected pores large enough to accommodate its passage, and the movement of larger molecules is more likely to be impeded and slowed down by collisions with the gel matrix, and the molecules of different sizes can therefore be separated in this process of sieving.[11]

The Ogston model however breaks down for large molecules whereby the pores are significantly smaller than size of the molecule. For DNA molecules of size greater than 1 kb, a reptation model (or its variants) is most commonly used. This model assumes that the DNA can crawl in a "snake-like" fashion (hence "reptation") through the pores as an elongated molecule. At higher electric field strength, this turned into a biased reptation model, whereby the leading end of the molecule become strongly biased in the forward direction, and this leading edge pulls the rest of the molecule along. In the fully biased mode, the mobility reached a saturation point and DNA beyond a certain size cannot be separated.[13] Perfect parallel alignment of the chain with the field however is not observed in practice as that would mean the same mobility for long and short molecules.[11] Further refinement of the biased reptation model takes into account of the internal fluctuations of the chain.[14]

The biased reptation model has also been used to explain the mobility of DNA in PFGE. The orientation of the DNA is progressively built up by reptation after the onset of a field, and the time it reached the steady state velocity is dependent on the size of the molecule. When the field is changed, larger molecules take longer to reorientate, it is therefore possible to discriminate between the long chains that cannot reach its steady state velocity from the short ones that travel most of the time in steady velocity.[14] Other models, however, also exist.

Real-time fluorescence microscopy of stained molecules showed more subtle dynamics during electrophoresis, with the DNA showing considerable elasticity as it alternately stretching in the direction of the applied field and then contracting into a ball, or becoming hooked into a U-shape when it gets caught on the polymer fibres.[15][16] This observation may be termed the "caterpillar" model.[17] Other model proposes that the DNA gets entangled with the polymer matrix, and the larger the molecule, the more likely it is to become entangled and its movement impeded.[18]

Visualization

The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into the major groove of DNA (or RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20 ng DNA becomes distinctly visible. EtBr is a known mutagen, and safer alternatives are available, such as GelRed, produced by Biotium, which binds to the minor groove.[18]

SYBR Green I is another dsDNA stain, produced by Invitrogen. It is more expensive, but 25 times more sensitive, and possibly safer than EtBr, though there is no data addressing its mutagenicity or toxicity in humans.

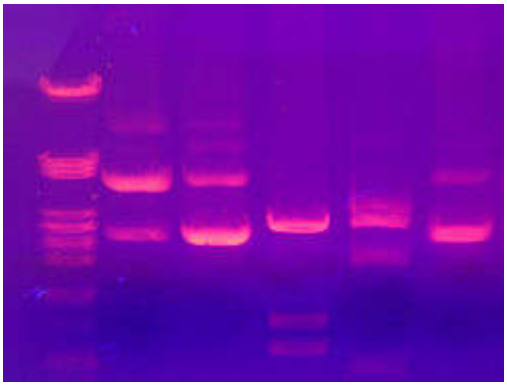

Fig 6 : DNA gel electrophoresis

SYBR Safe is a variant of SYBR Green that has been shown to have low enough levels of mutagenicity and toxicity to be deemed nonhazardous waste under U.S. Federal regulations. It has similar sensitivity levels to EtBr, but, like SYBR Green, is significantly more expensive. In countries where safe disposal of hazardous waste is mandatory, the costs of EtBr disposal can easily outstrip the initial price difference, however.

Since EtBr stained DNA is not visible in natural light, scientists mix DNA with negatively charged **loading buffers** before adding the mixture to the gel. Loading buffers are useful because they are visible in natural light (as opposed to UV light for EtBr stained DNA), and they co-sediment with DNA (meaning they move

at the same speed as DNA of a certain length). Xylene cyanol and Bromophenol blue are common dyes found in loading buffers; they run about the same speed as DNA fragments that are 5000 bp and 300 bp in length respectively, but the precise position varies with percentage of the gel. Other less frequently used progress markers are Cresol Red and Orange G which run at about 125 bp and 50 bp, respectively. Visualization can also be achieved by transferring DNA after SDS-PAGE to a nitrocellulose membrane followed by exposure to a hybridization probe. This process is termed Southern blotting. For fluorescent dyes, after electrophoresis the gel is illuminated with an ultraviolet lamp (usually by placing it on a light box, while using protective gear to limit exposure to ultraviolet radiation). The illuminator apparatus mostly also contains imaging apparatus that takes an image of the gel, after illumination with UV radiation. The ethidium bromide fluoresces reddish-orange in the presence of DNA, since it has intercalated with the DNA. The DNA band can also be cut out of the gel, and can then be dissolved to retrieve the purified DNA. The gel can then be photographed usually with a digital or polaroid camera. Although the stained nucleic acid fluoresces reddish-orange, images are usually shown in black and white (see figures). UV damage to the sample can reduce the efficiency of subsequent manipulation of the sample, such as ligation and cloning. This can be avoided by using a blue light excitation source with a blue-excitable stain such as SYBR Green or GelGreen.. Gel electrophoresis research often takes advantage of software-based image analysis tools.

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