

Gel Electrophoresis

Contents

A Brief Historical Introduction	1
Agarose Gel Electrophoresis of Nucleic Acids	3
Polyacrylamide Gel Electrophoresis (PAGE)	5
Recipes for Electrophoresis Tracking Dyes *	6
A Final Note About Agarose.....	6
References	7

A Brief Historical Introduction

The term “electrophoresis” was originally meant to refer to the migration of charged particles in an electrical field. The alternative term “ionophoresis” had been reserved for the migration of lower molecular weight substances in stabilized media such as gels and powders [1]. Today, the general term electrophoresis covers all applications regardless of the material being studied and the medium being used.

Studies by W.B. Hardy around the turn of the twentieth century established that many biologically important molecules, such as enzymes and other proteins, displayed characteristic electrophoretic mobilities [2, 3]. The demonstration that biomolecules migrated in predictable ways in an electrical field attracted a great deal of interest among biochemists. For example, Michaelis [4] was able to use electrophoresis to determine the isoelectric points of various enzymes. The isoelectric point, pI , is loosely defined as the pH at which a protein will no longer migrate in an electrical field. It is important to note here that electrophoresis made such determinations possible before researchers were able to actually purify the proteins themselves! In fact, as electrophoresis methods improved, it was found that some substances that many had believed were well defined entities via other means such as crystallization were yielding multiple components in an electrical field. This meant that some “pure” substances were actually composed of two or more previously unsuspected subunits.

While the majority of the early electrophoretic studies of biomolecules were carried out in liquid phase, von Klobusitzky and Konig [5] successfully applied an electrophoretic field to paper strips saturated with an electrolytic solution to separate components of snake venom. This “paper electrophoresis” pre-dates by several years the technique of “paper chromatography” with which it is usually associated [6]. During the late 1940s and early 1950s, this solid phase electrophoresis was used to characterize a vast array of biologically important substances including the amino acids [7]. The advantage of the solid support electrophoresis over free solution electrophoresis lies in the ability of the

solid support to stabilize migration. Such stabilization led to the development of a precise mathematical theory of molecular migration. It was clear from the outset that time and the intensity of the electrical field were major determinants of the mobility of a substance on paper strips. If, however, those two factors were to be held constant, the mobility of a molecule, u , can be written as,

$$U = Qd / 4\pi r^2 \eta$$

where Q is the charge on the molecule, d is distance, r is the radius of the molecule, and η is the viscosity of the solution used to wet the strips. The radius of the molecule could be experimentally determined from the observed diffusion constant. Note here that the radius of the molecule is in the denominator of the mobility expression and is squared. This means that the relationship between molecular size and mobility is **non-linear**. That is, all other factors being equal, mobility increases as the inverse square of the size of the molecule. This will become important later in discussions of electrophoretic separations of nucleic acids.

The types of apparatus designed for paper electrophoresis are nearly identical to those in use today for gel electrophoresis. Two of these paper electrophoresis rigs are shown below in Figure 1.

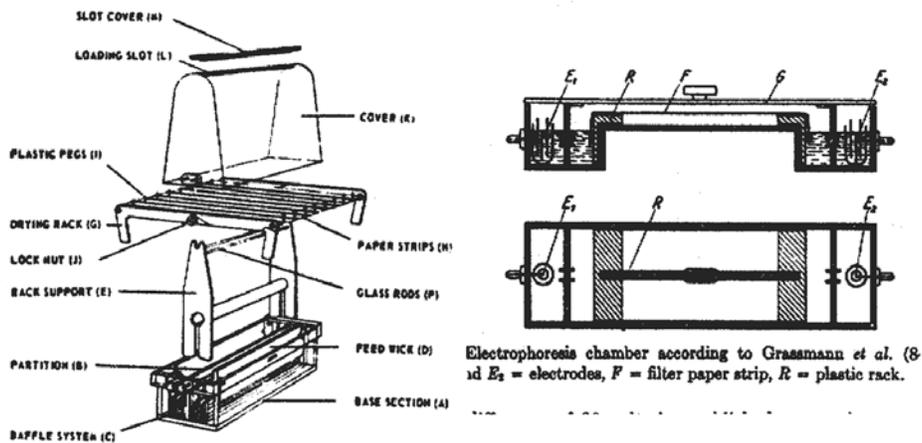


Figure 1. Diagrams of two paper electrophoresis rigs. The vertical apparatus on the left is from Williams et al. [8] and the horizontal apparatus on the right is from Grassmann et al. [9]. Compare the horizontal rig design to any modern horizontal gel electrophoresis set up.

While paper and other solid support materials proved to be an advantage over free solutions for the electrophoretic analysis of biomolecules, gels were adopted later because gels not only minimized diffusion better than paper supports they actually participated in the separation process by interacting with the migrating particles. Gels

can be thought of as semi-solid matrices whose pore sizes aid in separation. The semi-solid nature of the gel participates through a process known as **molecular sieving**. The three common media for gel electrophoresis are starch, polyacrylamide, and agarose. Of these, the starch gel medium is the least versatile whereas a wide range of separation effects can be achieved using the other two media.

There are limitations to the use of both polyacrylamide and agarose but these are effectively minimized when the material to be analyzed is a nucleic acid. Even very small nucleic acids (i.e., oligonucleotides) are easily separated in an electrical field by one or the other medium. The primary criteria for choosing polyacrylamide or agarose gel electrophoresis are length and whether or not the nucleic acid is single stranded or double stranded. Short, single-stranded DNAs like oligonucleotides require polyacrylamide whereas long (>100bp), double stranded DNAs are best resolved on agarose.

Agarose Gel Electrophoresis of Nucleic Acids

Nucleic acids are polymers composed of individual nucleotide units. The units are connected via phosphate diester linkages of the backbone sugars. The net effect of these linkages is to give the polymers a net negative charge. From the earliest days of electrophoresis it has been axiomatic that molecules carrying an electrical charge will migrate in an electrical field in a predictable manner. When subjected to an electrical field, a molecule carrying a net negative charge will migrate toward the positive pole and a molecule with a net positive charge will migrate toward the negative pole. In a semi-solid matrix like agarose, the equation describing mobility can be re-interpreted, at least heuristically, by defining η as gel density or concentration and r as the length of the molecule. Thus, when they are placed in the semi-solid matrix of a gel, nucleic acids will migrate toward the positive pole in a predictable and reproducible manner that can be described as a negative exponential function of length. That is to say, shorter molecules will migrate faster and longer molecules will migrate slower. Indeed, in the case of a nucleic acid in a gel in an electrical field, every other element of the migration expression is a constant and mobility is completely determined by molecular length.

As a matter of practice, it is difficult to accurately resolve double-stranded nucleic acids smaller than about 100 bases in an agarose gel because the sieving properties of agarose are not fine enough. On the other end of the scale, molecules longer than about 25,000 bp but shorter than around 2,000,000 bp will all run at the same rate. This is called **limiting mobility**. Nucleic acid molecules longer than 2,000,000bp will not even enter an agarose gel. Thus, the effective size range for agarose gel electrophoresis of double stranded nucleic acids is between 100bp and 25,000bp. In this range the behavior of the molecule is precise and predictable. This behavior is shown in figure 2. As can be seen there is minimal separation of the larger fragments but resolution improves as the fragments get smaller. While this phenomenon has been known for many years, what was not known was how the nucleic acid molecules actually moved in

the gel matrix. In the 1980s a theory was put forward that nucleic acids migrated through the gel much the same way that a snake moves. That is, the leading edge moves forward and pulls the rest of the molecule with it. In this model, as the molecule gets longer resistance to being pulled along increases. This resistance is further increased by the interaction of the molecule with the gel matrix. As has been discussed above, the increase in resistance is non-linear. This model, called “biased reptation,” is sufficient to explain all of the behavior of a nucleic acid in a semi-solid matrix [10, 11]. In 1989 a group at the University of Washington put this theory to the test. They filmed DNA molecules moving through an agarose gel. Their films showed both reptation and the nucleic acid/gel matrix interactions [12].

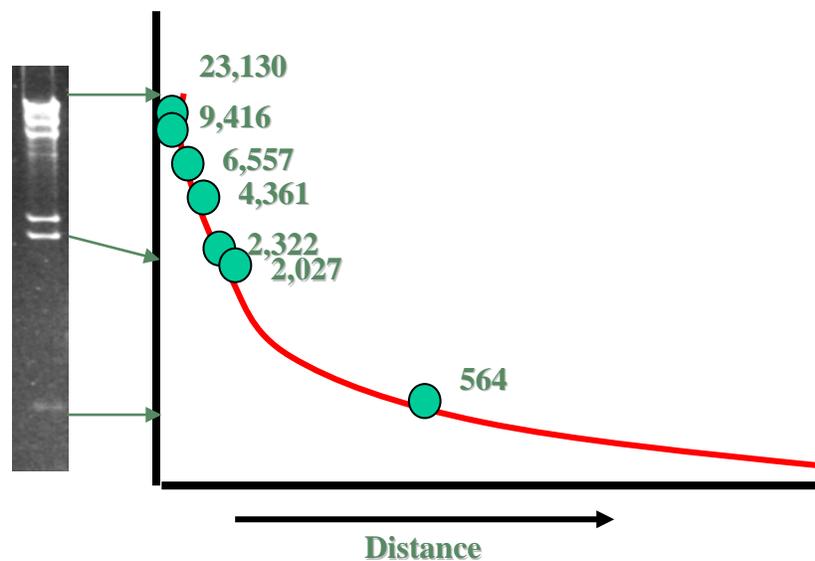


Figure 2. Electrophoretic mobility of DNA restriction fragments in an agarose gel. The DNA is from bacteriophage lambda digested with the restriction enzyme Hind III. The graph shows migration distance in centimeters by size of the restriction fragment in base pairs.

In the mid-1980s a number of methods were developed to electrophoretically analyze nucleic acid molecules in the limiting mobility size range. The solution involved artificially introducing a size dependent mobility on nucleic acid molecules by altering the electrophoretic field. The first such alteration involved simply switching the polarity of the field in a regular pattern. Carle et al. [13] showed that periodic reversals of polarity would induce the molecules to make U-turns in the gel. Even at very large sizes, this turning would permit separation of molecules. If the length of time the molecules were reversed was about one-third the time they were oriented forward, for example three seconds forward and one second back, molecules as large as 2,000,000bp could be resolved in a standard agarose gel in a few hours. The first practical demonstration of

this method, called Field Inversion Gel Electrophoresis (FIGE), was to completely resolve intact yeast chromosomes [14]. Since then, a variety of methods collectively termed pulsed-field gel electrophoresis, have been developed [15].

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel is the result of polymerizing acrylamide monomers into long chains and then cross-linking the chains with a bifunctional compound. A number of these bifunctional cross-linking compounds are known including ethylene diacrylate, N,N'-bisacrylcystamine (BAC), and N,N'-diallyltartardiamide (DATD). However, the most generally useful compound is N,N'-methylene bisacrylamide (bis meaning two).

Polymerization of acrylamide and bisacrylamide is catalyzed in the presence of either ammonium persulphate or riboflavin. In addition, the compound N,N,N,N'-tetramethylethyldiamine (TEMED) or, less commonly, 3-dimethylamino propionitrile (DMAPN), are introduced to accelerate the polymerization process. In the ammonium persulphate-TEMED system that is conventionally employed, TEMED catalyzes the formation of free radicals from persulphate and these free radicals initiate polymerization. Since the free base of TEMED is required, polymerization can be slowed at lower pH and can be prevented entirely at very low pH. Polymerization rates can be increased by increasing the TEMED or persulphate concentrations. Also, temperature has a direct relationship with speed of polymerization. For this reason, the persulphate and the acrylamides are usually stored at -20°C .

In contrast to persulphate polymerization, the use of riboflavin-TEMED requires light to initiate polymer formation. Light causes photo-decomposition of riboflavin which generates the necessary free radicals.

As with agarose, the sieving properties of polyacrylamide gels depends upon the effective pore size of the gel. Effective pore size decreases as the concentration of the acrylamide increases. As pore size decreases the ability of the gel to resolve smaller and smaller molecules increases. Polyacrylamide gels have effective pore sizes that are much smaller than agarose gels at any concentration and, thus, they are ideal for resolving nucleic acids in the size range of oligonucleotides. The relationship between gel concentration and resolving power can be simply demonstrated by observing the behavior of tracking dyes in gels. The two common dyes are **bromophenol blue** and **xylene cyanole**. These chemicals have a net negative charge and they will migrate in gels the same way that nucleic acids do. The usefulness of these dyes is that they will migrate as if they were nucleic acids of different lengths depending upon gel type and concentration. This phenomenon is shown in Table 1.

Table 1
Migration of tracking dyes in gel electrophoresis.

The numbers given below each dye reflect the approximate length of a DNA molecule with which the dye will migrate in a gel of the stated type and concentration.

<u>Gel Type and Concentration</u>		<u>Bromophenol Blue</u>	<u>Xylene cyanole</u>
Agarose	0.8%	500	3500
Acrylamide	3.5%	100	460
	5.0%	65	260
	8.0%	45	160
	12.0%	20	70
	15.0%	15	60
	20.0%	12	45

Recipes for Electrophoresis Tracking Dyes *

Dye I (6X concentrate): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 40% (v/v) sucrose in sterile water. Store at 4°C.

Dye II (6X concentrate): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% (v/v) Ficoll Type 400 in sterile water. Store at RT.

Dye III (6X concentrate): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in sterile water. Store at 4°C.

*recipes adapted from Maniatis et al. [16].

A Final Note About Agarose

During the 1980s and 1990s a considerable effort was made on the part of several companies to develop “designer” agarose. These are different formulations that have very specific sieving properties. Among these are products like SeaKem, SeaPlaque, NuSeive, MetaPhor, and so on. We have tried many of these with varying degrees of success. One type that does work very well for us is the so-called Low Melting Point (LMP) agaroses. Compared to a 1.4% standard agarose, 1.2% LMP gels give superior resolution in the all-important 100bp to 500bp size range.

Anyone interested in these various types of agarose should visit the BioWhittaker/Cambrex web site (www.cambrex.com) and search on “agarose.”

References

1. Tiselius A. (1959) Introduction. In: M. Bier (ed.) *Electrophoresis: Theory, Methods, and Applications*. New York: Academic Press. xv–xx.
2. Hardy WB. (1899) On the coagulation of proteid by electricity. *J Physiol*, 24: 288–304.
3. Hardy WB. (1905) Colloidal solution. The globulins. *J Physiol*, 33: 251–337.
4. Michaelis L. (1909) Elektrische überführung von fermenten. *Biochemische Zeitschrift*, 16: 81–86.
5. von Klobusitzky D and König P. (1939) Biochemische studien über die gifte der schlangengattung Bothrops. VI. *Arch Exp Pathol Pharmacol*, 192: 271–275.
6. Consden R, Gordon AH, and Matrin AJP. (1944) Qualitative analysis of proteins: a partition chromatography method using paper. *Biochem J*, 38: 224–232.
7. Wunderly C. (1959) Gel structure and the diffusion of proteins. *Clin Chim Acta*, 4: 754–759.
8. Williams FG, Pickles EG, and Durrum EL. (1955) Improved hanging-strip paper electrophoresis technique. *Science*, 121: 829–832.
9. Grassmann W, Hannig K, and Knedel M. (1955) Über ein verfahren zur elektrophoretischen bestimmung der serum proteine auf filtrierpapeir. *Deutsche Medizinische Wochenschrift*, 76: 333–339.
10. Lerman LS and Frisch HL. (1982) Why does the electrophoretic mobility of DNA in gels vary with the length of the molecule? *Biopolymers*, 21: 995–997.
11. Lumpkin OJ, Dejardin P, and Zimm BH. (1985) Theory of gel electrophoresis of DNA. *Biopolymers*, 24: 1573–1593.
12. Smith SB, Aldridge PK, and Callis JB. (1989) Observation of individual DNA molecules undergoing gel electrophoresis. *Science*, 243: 203–206.
13. Carle GF, Frank M, and Olson MV. (1986) Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science*, 232: 65–68.
14. Carle GF and Olson MV. (1984) Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. *Nucleic Acids Res*, 25: 564–567.
15. Lai E, Birren BW, et al. (1989) Pulsed field gel electrophoresis. *Biotechniques*, 7: 34–42.
16. Maniatis T, Fritsch EF, and Sambrook J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.