

Discriminatory Power of Agarose Gel Electrophoresis in DNA Fragments Analysis

Seow Ven Lee and Abdul Rani Bahaman

*Putra University
Malaysia*

1. Introduction

Since the first invention back in 1930s (Tiselius, 1937), electrophoresis methods have diversified significantly and new techniques along with applications are still being developed till date. This method, through agarose or polyacrylamide gels, is the most commonly used for sorting of biological macromolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein, from both bacteria and viruses. Though agarose gels have a lower resolving power than polyacrylamide gels, they can separate DNAs from 50 bp to several mega bases in length on gels of various concentrations and configurations. Polyacrylamide gels are most effective for separation of small DNA fragments (5 to 500 bp).

Overall, the term electrophoresis refers to the movement of a charged particle through a matrix in an electrical field while the electrophoretic mobility depends both on net charge and on molecular radius (Rickwood & Hames, 1982). The target fragments are separated under identical buffer, temperature and time duration but using different gel concentrations. For agarose, the gel concentration is defined in g/100 ml (Hjertén, 1962) while the pore size of a polyacrylamide gel can be controlled by the total amount of acrylamide present (%T, where T = total concentration of acrylamide and bisacrylamide monomer) and the degree of cross-linking (%C, where C = bisacrylamide concentration) (Rüchel, 1978). This procedure, in fact, resembles a sieving process where the gels act as a sieving medium, defining nucleic acids based on fragment size in response to an electric current. The gels can be casted in a variety of shapes, sizes, and porosities. The choice within these parameters depends primarily on the fragments to be separated.

For many years, agar gel electrophoretic media have been used to facilitate characterization of biological mixtures and diagnosis of disease. Despite an enormous repertoire of methodologies has been developed for advance determination of nucleic acids, conventional procedures such as agarose gel electrophoresis remained an exclusive method to separate, identify and purify DNA fragments in the life sciences research. The technique is easy to perform, require relatively inexpensive equipment and yet offer excellent analytical performance characteristics. This perhaps reasons agarose gel electrophoresis as one of the most readily performed, irreplaceable and widely available molecular methods particularly in technology-restricted settings.

A further increase in the use of agarose gels was observed concurrent with the rapidly expanding suite of restriction enzymes. In restriction fragment length polymorphism (RFLP) analysis, agarose gel electrophoresis has been an important tool in characterizing digested DNA fragments based on fragment size, excluding the need for expensive gene sequencing. However, improvement over the conventional practice is needed to achieve better discriminatory power of agarose gel electrophoresis in DNA fragment analysis. In cases of frequent target variation, agarose gel electrophoresis may offer the only practical means of positive, reproducible detection as well as characterization.

2. Gel electrophoresis

Electrophoresis is the motion of colloidal particles relative to a fluid medium under the influence of an electric field that is uniformly spaced. This electrokinetic phenomenon was first noted by Reuss in 1809 (Reuss, 1809) and remains widely used in an array of practical devices and processes to produce macro-scale effects. Examples of applications and operations include measurements, electrophoretic deposition, electrophoretic fingerprinting, as well as gel electrophoresis.

In principle, when a direct current power supply is introduced, molecules and particles, usually in aqueous solution, will migrate towards the direction of the electrode bearing the opposite charge. Since they are varying in terms of masses and charges, different molecules and particles of a mixture will migrate at different velocities and therefore, will be separated into single fractions. Basically, the mobility of nucleic acids in gels is influenced by factors such as gel concentrations, buffer conditions, sizes and conformations, with little influence from base composition or sequence.

2.1 Effects of voltage, current and power on gel electrophoresis

Two equations which are applicable to the use of power supplies for electrophoresis of macromolecules include Ohm's Law and the Second Law of electrophoresis (Millikan & Bishop, 1917). These two laws and the interactions of these parameters (volts, current, and watts) are critical to understanding electrophoresis.

- Ohm's Law

$$\text{Current (I)} = \text{Voltage (V)} / \text{Resistance (R)}$$

Ohm's Law states that current is directly proportional to the voltage and is inversely proportional to the resistance. Resistance of the system is determined by the buffers used, the type and configurations of the gels being run, and the total volume of all the gels being run.

- Second Law

$$\text{Watts (W)} = \text{Current (I)} \times \text{Voltage (V)}$$

The Second Law states that power or watts is equal to the product of the current and voltage. Since $V = I \times R$, this can also be written as $\text{Watts} = I^2 \times R$.

There are advantages and disadvantages for setting each of the critical parameters as the limiting factor in electrophoresis. During electrophoresis one of the parameters is held

Discriminatory Power of Agarose Gel Electrophoresis in DNA Fragments Analysis

constant and the other two are allowed to vary as the resistance of the electrophoretic system changes (Table 1). Under normal conditions in horizontal systems, there is little change in resistance. Nonetheless, when high voltage is applied or extended runs in horizontal systems, this resistance can decrease.

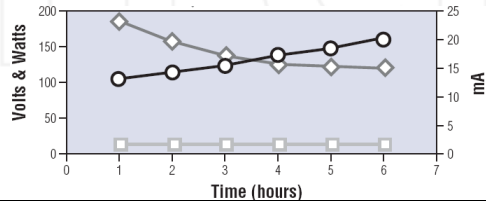
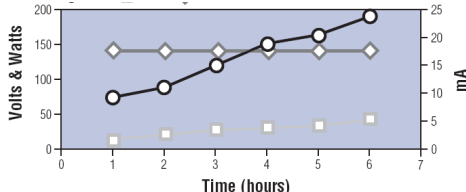
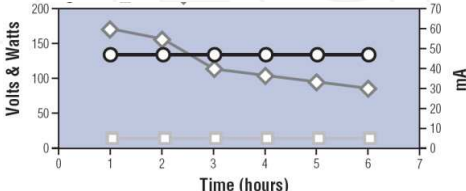
Parameters		Description																												
Constant	Variable																													
Watts	Volts and Current	<p>When the current decreases disproportionately (due to buffer or hardware problem), the power supply will increase the voltage to compensate. Therefore resulting in a decrease in the velocity of the samples. Mobility of samples from the calculation of watt-hours will be unpredictable but the generation of heat will remain uniform in this condition.</p>  <table border="1"> <caption>Data for Constant Watts Graph</caption> <thead> <tr> <th>Time (hours)</th> <th>Volts</th> <th>Watts</th> <th>Current (mA)</th> </tr> </thead> <tbody> <tr><td>1</td><td>100</td><td>10</td><td>22</td></tr> <tr><td>2</td><td>110</td><td>10</td><td>18</td></tr> <tr><td>3</td><td>120</td><td>10</td><td>15</td></tr> <tr><td>4</td><td>130</td><td>10</td><td>12</td></tr> <tr><td>5</td><td>140</td><td>10</td><td>12</td></tr> <tr><td>6</td><td>160</td><td>10</td><td>12</td></tr> </tbody> </table>	Time (hours)	Volts	Watts	Current (mA)	1	100	10	22	2	110	10	18	3	120	10	15	4	130	10	12	5	140	10	12	6	160	10	12
Time (hours)	Volts	Watts	Current (mA)																											
1	100	10	22																											
2	110	10	18																											
3	120	10	15																											
4	130	10	12																											
5	140	10	12																											
6	160	10	12																											
Current	Volts and Watts	<p>The samples will migrate at a constant rate. However, voltage and wattage will increase as the resistance increases (due to buffer leaking or damaged lead), resulting in an increase in heat generation during the run.</p>  <table border="1"> <caption>Data for Constant Current Graph</caption> <thead> <tr> <th>Time (hours)</th> <th>Volts</th> <th>Watts</th> <th>Current (mA)</th> </tr> </thead> <tbody> <tr><td>1</td><td>70</td><td>10</td><td>15</td></tr> <tr><td>2</td><td>85</td><td>13</td><td>15</td></tr> <tr><td>3</td><td>115</td><td>17</td><td>15</td></tr> <tr><td>4</td><td>145</td><td>22</td><td>15</td></tr> <tr><td>5</td><td>160</td><td>24</td><td>15</td></tr> <tr><td>6</td><td>180</td><td>27</td><td>15</td></tr> </tbody> </table>	Time (hours)	Volts	Watts	Current (mA)	1	70	10	15	2	85	13	15	3	115	17	15	4	145	22	15	5	160	24	15	6	180	27	15
Time (hours)	Volts	Watts	Current (mA)																											
1	70	10	15																											
2	85	13	15																											
3	115	17	15																											
4	145	22	15																											
5	160	24	15																											
6	180	27	15																											
Voltage	Current and Watts	<p>The current and wattage will decrease as the resistance increases, resulting in a decrease of heat and samples migration. Since the voltage is constant, if the resistance increases dramatically (due to apparatus malfunction), the current and wattage will fall, and when the resistance increase so much that the power supply will not be able to compensate, it will shut off.</p>  <table border="1"> <caption>Data for Constant Voltage Graph</caption> <thead> <tr> <th>Time (hours)</th> <th>Volts</th> <th>Watts</th> <th>Current (mA)</th> </tr> </thead> <tbody> <tr><td>1</td><td>130</td><td>78</td><td>60</td></tr> <tr><td>2</td><td>130</td><td>52</td><td>40</td></tr> <tr><td>3</td><td>130</td><td>39</td><td>30</td></tr> <tr><td>4</td><td>130</td><td>39</td><td>30</td></tr> <tr><td>5</td><td>130</td><td>39</td><td>30</td></tr> <tr><td>6</td><td>130</td><td>39</td><td>30</td></tr> </tbody> </table>	Time (hours)	Volts	Watts	Current (mA)	1	130	78	60	2	130	52	40	3	130	39	30	4	130	39	30	5	130	39	30	6	130	39	30
Time (hours)	Volts	Watts	Current (mA)																											
1	130	78	60																											
2	130	52	40																											
3	130	39	30																											
4	130	39	30																											
5	130	39	30																											
6	130	39	30																											

Table 1. Interactive effects of voltage, current and power on gel electrophoresis (Rickwood & Hames, 1982). □ Watts; ○ Volts; ◇ Current (mA).

2.2 Electrophoresis buffers

During electrophoresis, water will be electrolyzed; generating protons at the anode while hydroxyl ions at the cathode. This effect leads to the cathodal end of the electrophoresis chamber becomes basic and the anodal end acidic. The use of a buffering system, therefore, is required when charged molecules are electrophoresed through a separation medium.

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. Because the pH of these buffers is neutral, the phosphate backbone of DNA which possesses a net negative charge will migrate towards the anode. In the absence of ions (e.g., if water is substituted for electrophoresis buffer in gel or in the electrophoresis tank), electrical conductivity is minimal and DNA migrates slowly, if at all. In buffer of high ionic strength (e.g., high concentration of electrophoresis buffer, such as 10X, is mistakenly used), electrical conductance becomes very efficient and significant amounts of heat will be generated, even when moderate voltages are applied (Sambrook & Russell, 2001). In the worst case, the gel melts and the DNA denatures.

2.2.1 Type of running buffers

Several different buffers are available for electrophoreses of nature, double-stranded DNA (dsDNA). There are: Tris-acetate with EDTA (TAE; 40 mM Tris-acetate, 1 mM EDTA), Tris-borate with EDTA (TBE; 89 mM Tris-borate, 2 mM EDTA) and Tris-phosphate with EDTA (TPE) at concentration of ~50 mM and pH 7.5-7.8. The two more commonly used buffers are TAE and TBE. Despite the apparent similarity of TAE and TBE buffers, each has different properties which make it best suited for different applications (see table 2).

Buffer	Properties	Descriptions
TAE	Low buffering capacity - periodic replacement of the buffer may be necessary for extended electrophoretic times (>6 hours).	Use when DNA is to be recovered. Better resolution for high molecular-weight and supercoiled DNAs than TBE or TPE. Migration rate of linear dsDNA fragments is ~10% faster than TBE or TPE. Slightly cheaper than TBE or TPE.
TBE	High buffering capacity - no recirculation required for extended run times.	Suitable for electrophoresis of small (<1 kb) DNA when DNA recovery is not required. Increased resolution of small (<1 kb) DNA. Decreased DNA mobility.
TPE	High buffering capacity - no recirculation required for extended run times.	Does not interfere with DNA recovery procedures (but cannot be used when recovered DNA will be used in a phosphate-sensitive reaction).

Table 2. Properties of TAE, TBE and TPE buffer systems.

Generally, all of these buffers work well and the choice among them is largely a matter of personal preference. Both TAE (1X) or TBE (1X or 0.5X) buffer is suitable for use when the DNA fragments to be separated is less than 12-15 kb or when the recovery of DNA is not needed for subsequent experiment (Miura et al., 1999). For larger DNA, the best buffer to

use for electrophoresis is TAE in combination with low field strength (1-2 V/cm). During these extended electrophoretic runs, larger apparent gel porosity, lower EEO and low field strength decrease the tendency of large DNA to smear. Since TAE has the lowest buffering capacity of the three, periodic replacement of the buffer may be necessary for extended electrophoretic times. Exhaustion of TAE can cause the anodic portion of the gel to become acidic and the bromophenol blue migrating through the gel towards the anode will change in colour from bluish-purple to yellow (Sambrook & Russell, 2001). This change begins at pH 4.6 and is complete at pH 3.0.

Meanwhile, whichever buffer is used, the depth over the gel in a horizontal electrophoretic system should be 3-5 mm. Excessive buffer depths will decrease DNA mobility, promote band distortion and can cause excessive heating within the system. Conversely, the gel may dry out if less amount of buffer is applied during electrophoresis.

The rate of buffer depletion is influenced by the buffer used and its buffering capacity. Evidence of buffer depletion is gel melting, smearing of DNA and/or overheating. TBE (0.5X) buffer has greater buffering capacity than 1X TAE buffer at the pH used because the acid dissociation constant (pK_a) of borate is closer to the initial buffer pH than that of acetate. Effects of buffer depletion and development of a pH gradient can be reduced via buffer recirculation. This is usually necessary only when electrophoresis is done for extended times or the electrophoresis buffer has a low buffering capacity.

3. Electrophoresis through agarose gel

Agarose gel electrophoresis is the benchmark technique for separation and purification of nucleic acids as well as RFLP-analysis (Sambrook & Russell, 2001). In contrast to polyacrylamide gels, the resolution of agarose gels is inferior but possesses greater range of separation. Small DNA fragments (50-20,000 bp) are best resolved in agarose gels run in a horizontal configuration with an electric field of constant strength and direction. Most often, this straightforward method is thought to be the basic routine tool that is integral in a molecular biology laboratory and can be mastered in a short period of time. Besides, agarose gels are easily cast and handled as compared to other matrices, resulting to it being one of the standard physical methods for work with proteins and nucleic acids. In procedures involving nucleic acids, agarose mostly becomes the preferred matrix due to the neutral charge, chemically inert and not exhibiting electroendosmosis (EEO), making it less likely to interact with biomolecules.

During electrophoresis, DNA is forced to migrate through a highly cross-linked agarose matrix in response to an electric current. The migration of DNA molecules towards the anode (the positive pole) occurs mainly due to the naturally occurring negative charge carried by their sugar phosphate backbone (Lodish et al., 2000) and is primarily size-dependent. The porosity of agarose (determined by agarose concentration in the gel) is responsible for much of its DNA separation properties. Under these conditions, the migration velocity of the DNA fragments decreases as their length increases and is proportional to the strength of the electric field (Calladine et al., 1991; Fangman, 1978; McDonnell et al., 1977). This association, however, cannot be applied once the size of DNA fragments surpasses a maximum value, which is defined basically by the composition of the gel and the electric field strength (Hervet & Bean, 1987).

Most often, agarose gels are referred to as submarine gels because the slab is submerged completely in electrophoresis running buffer in a horizontal electrophoresis apparatus (Westermeier, 2005). This is to prevent the event of gel from drying out.



Fig. 1. The “submarine” technique for the separation of nucleic acids.

3.1 Structure of agarose

Agarose is a linear polymer composed of alternating residues of 1,3-linked β -D-galactose and 1,4-linked 3,6-anhydro- α -L-galactose (Sambrook & Russell, 2001), infrequently substituted with pyruvate, sulfate and methyl esters (Figure 2).

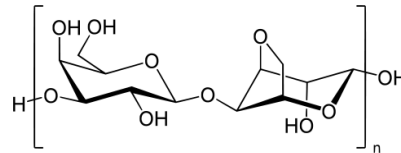


Fig. 2. Chemical structure of an agarose polymer.

These molecules, in solution at high temperatures, have a random coil structure (Modrich, 1979; Roberts, 1976; Zeiger et al., 1972). Upon cooling, the agarose chains form helical fibers that aggregate into supercoiled structure with a radius of 20-30 nm. Subsequent solidification of agarose will result in three-dimensional mesh of channels whose diameters range from 50 nm to >200 nm (Kirkpatrick, 1990).

3.2 Properties of agarose

The charged groups present on the polysaccharide, pyruvates and sulfates are responsible for many of the agarose properties. Commercially prepared agarose polymers are believed to contain approximately 800 galactose residues per chain. However, agarose is not homogeneous (varies in each batch and manufacturers). Lower grades of agarose may be contaminated with other polysaccharides, as well as salts and proteins. This variability can affect the gelling and/or melting temperature during agarose preparation, the sieving of nucleic acids, and the recovery of DNA from gel for subsequent enzymatic reactions. By careful selection of raw materials, these properties can be controlled to meet specific needs. Following are the few properties which often influence the electrophoresis through agarose gel.

3.2.1 Electroendosmosis (EEO)

Electroendosmosis (EEO) is a functional measure of the number of sulfate and pyruvate residues present in a given agarose gel (Peter & J.A. Upcroft, 1993; Sambrook & Russell,

2001). This phenomenon occurs during electrophoresis when the anticonvective medium (the agarose in this case) has a fixed negative charge. In an electric field, the hydrated positive ions associated with the fixed anionic groups in the agarose gel migrate toward the cathode. Water is thus pulled along with the positive ions, and migration of negative molecules such as DNA is retarded. The effects of EEO are seen mostly in resolution of fragments >10 kb.

3.2.2 Melting and gelling temperature

The gelling temperature as well as energy needed to melt an agarose gel increases in proportion with the gel concentration. For this reason, gelling or re-melting temperatures are expressed at a given agarose concentration. This property is of practical value since it is possible to vary gelling and melting parameters by using lower or higher concentrations of agarose. The dependence of gelling and melting temperatures on concentration is most pronounced at concentrations less than 1%.

3.2.3 Gelation

The mechanism for gelation of agarose involves a shift from a random coil in solution to a double helix in the initial stages of gelation and then to bundles of double helices in the final stage (Arnott et al., 1974; Rees, 1972). The average pore size varies with concentration and type of agarose, but is typically 100 to 300 nm.

3.2.4 Gel strength

One of the most important factors contributing to the success of agarose as an anticonvection medium is its ability to exhibit high gel strength at low concentrations. Gel strength is defined as the force (expressed in g/cm²), that must be applied to fracture an agarose gel of a standard concentration. As there are several test methods used to measure gel strength, a direct comparison of gel strength values between different manufacturers is sometimes difficult. The gel strength of a specific lot of agarose will decrease over time because of the spontaneous hydrolysis of the agarose polysaccharide chains.

3.3 Parameters affecting the migration rate of DNA through agarose gels

Although agarose gel electrophoresis is a simple and easy to perform technique, gel performance and the ability to resolve DNA fragments can be influenced by both characteristics of the gel itself (agarose concentration, class, and grade), conditions under which the electrophoresis is run (voltage applied to the gel, loading and running buffers used, and duration of the electrophoretic run), and the characteristics of the nucleic acid fragments being separated (quantity, size, and conformation) (Sambrook & Russell, 2001).

3.3.1 Characteristics of agarose gels

Since agarose is not homogeneous, the concentration and the choice of agarose best used in a gel can be assessed mainly based on the size of the DNA to be analyzed and the need for subsequent manipulations post-electrophoresis (Peter & J.A. Upcroft, 1993; Sambrook & Russell, 2001). Agarose is commercially available in many grades with the more expensive

grades containing lower levels of contaminating polysaccharides, salts, and proteins, all potentially affecting gel performance. Depending on application, different preparations, such as standard (high melting point) and low melting point (LMP) preparative grade can be obtained. Molecular Biology grade agarose powders are generally suitable for analytical separation of DNA.

Agarose (%)	Effective range of resolution of linear DNA fragments (kb)
0.5	30 to 1
0.7	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

Table 3. Appropriate agarose concentrations for separating DNA fragments of various sizes (Sambrook & Russell, 2001).

Agarose concentration plays an important role in electrophoretic separations. The gel's porosity is directly related to the concentration of agarose in the medium and it determines the size range of DNA molecules that can be adequately resolved. In a given size, a linear DNA fragment will migrate at different rates through gels containing different concentrations of agarose (Figure 3).

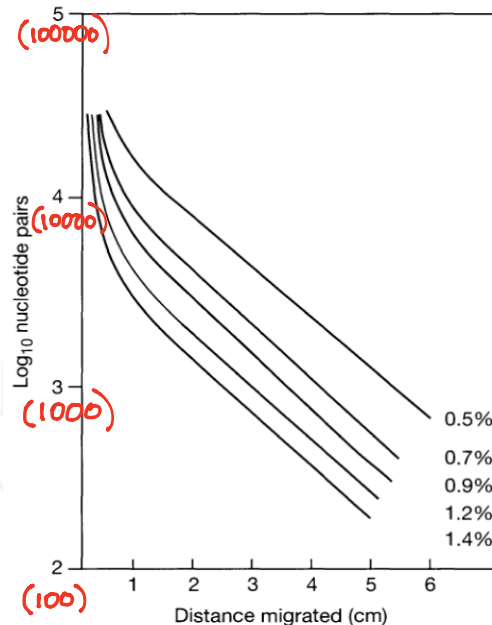


Fig. 3. The relationship between the size of DNA and its electrophoretic mobility (Buffer: 0.5X TBE, 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide, electrophoresis 1 V/cm for 16 hours (Sambrook & Russell, 2001).

3.3.2 Voltage, buffers and electrophoretic time

The voltage applied, which is defined in V/cm, is determined basing on the distance between the electrodes, not the gel length. DNA molecules exposed to this electric field will migrate toward the anode due to the negatively charged phosphates along the DNA backbone. The migration velocity is limited by the frictional force imposed by the gel matrix. Since the distribution of phosphate is very regular across the length of nucleic acids, these molecules have a constant charge to mass ratio and therefore, travel through agarose at a rate that is proportional to the applied voltage (Sambrook & Russell, 2001). In general, it is the size of the DNA that determines the rate at which it passes through the gel, thereby allowing an effective separation of DNA fragment-length mixtures by electrophoresis. Within a range, the higher the applied voltage, the faster the samples migrate. However, if the voltage is too high, band streaking, especially for DNA ≥ 12 -15 kb, may result. Conversely, when the voltage is too low, the mobility of small (≤ 1 kb) DNA is reduced and band broadening will occur due to dispersion and diffusion. The following table provides a quick reference for optimal voltage for DNA electrophoresis. The types of running buffers have been described in section 2.2.1.

Size	Voltage	Buffer	
		Recovery	Analytical
≤ 1 kb	5 V/cm	TAE	TBE
1 kb to 12 kb	4 - 10 V/cm	TAE	TAE/TBE
> 12 kb	1 - 2 V/cm	TAE	TBE

← primarily what we use

Table 4. Recommended voltages and buffers related to DNA size and application.

The most common means of monitoring the progress of an electrophoretic separation is by following the migration of tracking dyes that are incorporated into the loading buffer. Besides to increase the density of the samples, gel loading buffer is used to ensure that the DNA fall evenly into the well. Two widely used dyes displaying different electrophoretic mobilities are bromophenol blue and xylene cyanol. Xylene cyanol typically migrates with DNA fragments around 5 kb while bromophenol blue usually co-migrates with DNA molecules around 0.5 kb. Both dyes can interfere with the visualization of the fragments that co-migrate with them.

Basically, electrophoretic time is accessed based on the migration of target DNA fragments. The gel should be run until the band of interest has migrated 40% - 60% down the length of the gel. A decrease in resolution at the lower third of the gel occurs mainly due to band broadening resulting from dispersion and diffusion. Resolution may also be decreased in smaller gels, since longer electrophoretic runs result in greater separation between two fragments.

3.3.3 Characteristics of DNAs

The migration rate of dsDNA is inversely proportional to the \log_{10} of the length of the DNA strand, such that smaller molecules of nucleic acid move more quickly than large ones (Helling et al., 1974). Larger molecules easily become entangled in the matrix, resulting in greater frictional drag, and hence, they migrate through the pores of the gel less efficiently than smaller molecules.

Close (superhelical) circular (form I), nicked circular (form II), and linear duplex (form III) DNAs of the same molecular weight migrate at different rates through agarose gels (Thorne, 1966). The relative mobility of the three forms not only depends primarily on the concentration and type of agarose used to make gel, they are also influenced by the applied current and the ionic strength of the buffer as well as the density of superhelical twists in the form I DNA (Johnson & Grossman, 1977). Under certain circumstances, form I DNA may migrate faster than form III DNA and vice versa. Therefore, in most cases, the best way to distinguish between the two conformational forms of DNA is simply to include in the gel a sample of untreated DNA plasmid and a sample of the same plasmid that has been linearized by digestion with a restriction enzyme that cleaves the DNA in only one site (Sambrook & Russell, 2001).

3.3.4 Presence of ethidium bromide in the gel and electrophoresis buffer

Ethidium bromide, an intercalating dye, is the most convenient and commonly used method to visualize DNA in agarose gel. It allows very small quantities of DNA to be detected (<10 ng) (Sambrook & Russell, 2001). The dye, however, is believed to be a potent mutagen since it contains a tricyclic planar group that intercalates between the stack bases of DNA, thus deforming the DNA. At saturations in solutions of high ionic strength, approximately one ethidium molecule is intercalated per 2.5 bp (Waring, 1965).

Ethidium bromide binds to DNA with little or no sequence preference (Sambrook & Russell, 2001). Upon insertion into the helix, this dye lies perpendicular to the helical axis and makes van der Waals contacts with the base pairs above and below. The fixed position of the planar group and its close proximity to the bases cause dye bound to DNA, resulting to an increase in fluorescent yield. Ethidium bromide-stained DNA is detected by ultraviolet radiation. At 254 nm, UV light is absorbed by the DNA and transmitted to the dye; at 302 nm, and 366 nm, UV light is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum (Le Pecq & Paoletti, 1967).

This fluorescent dye can be incorporated into the gel and running buffer (0.5µg/ml) for direct visualization of DNA with only a minor loss of resolution. However, for optimal resolution, lowest background, and sharpest bands, it is suggested to stain the gel with ethidium bromide post electrophoresis. Ethidium bromide detects both single- and double stranded nucleic acids but the affinity for single-stranded DNA is relatively low compared to dsDNA. Meanwhile, the dye can cause a decrease in the negative charge of the dsDNA while increase in both its stiffness and length. Although the electrophoretic mobility of linear dsDNA will consequently be reduced by a factor of approximately 15% in the presence of this dye (Sharp et al., 1973), the ability to examine the gel directly via UV illuminator during or at the end of the run is a great advantage.

3.4 Determination of DNA fragments in agarose gel electrophoresis

Besides all mentioned earlier, factors such as gel casting, quantity of DNA loaded, presence of molecular weight marker as well as the choice of gel stains may also engage in determining DNA fragments in an agarose gel electrophoresis. For optimal resolution, horizontal gels should not be casted more than 3-4 mm thick. Gel thickness has a profound

We will use SYBR stain instead; it is another intercalating dye →

effect on the resolution of smaller fragments. The smaller DNA fragments in the 10 mm thick gel are fuzzy, whereas in the 3 mm thick gel the resolution is sharp throughout the gel. There is also a higher background staining in gels thicker than 5 mm. In addition, the width (thickness) of the comb used to form wells in agarose gels can affect the resolution of DNA fragments. A thin comb (≤ 1 mm) will result in sharper DNA bands while with a thicker comb, more volume can be added to the well but the separated DNA bands may be broader.

The quantity of DNA that can be loaded on a gel depends on the well capacity (the width or the depth of the well) and the distribution of DNA fragments (the number and size of target fragments). Overloaded DNA may result in trailing and smearing effect, a problem that will become more pronounced as the size of the DNA increases (>10 kb). Most important is the quantity of DNA in the bands of interest. Among the samples loaded onto a gel, at least one lane should contain one molecular weight marker, a series of DNA fragments of known sizes, so that a standard curve can be constructed to allow the calculation of the sizes of unknown DNA fragments.

Detection of DNA in an agarose gel is typically accomplished through the use of stains, applied either post-electrophoresis or incorporated into the gel before current is applied. The more commonly used stain for direct visualization of DNA in gels is ethidium bromide. This stain is frequently added to the gel and running buffer prior to electrophoresis. While this has a slight effect on the mobility of the DNA, it eliminates the need to stain the gel upon completion of the separation. An added advantage to running gels with ethidium bromide is that the mobility of the DNA can be monitored throughout the run until the desired separation is achieved. The least amount of dsDNA in a single band that can be reliably detected with ethidium bromide is approximately 10 ng. Unlike ethidium bromide, other stains such as GelStar® and SYBR® Green I are highly sensitive fluorescent stains and they fluoresce only upon binding to nucleic acids. The GelStar® nucleic acid stain is able to detect approximately 20 pg of dsDNA while with SYBR® Green I stain detects approximately 60 pg (Williams, 2001).

4. DNA fragments analysis through agarose gel electrophoresis

Agarose gel electrophoresis is a fundamental method in all scientific investigations involving nucleic acids. In RFLP analysis of chromosomal DNA, this method has been a valuable tool in localization of genes for genetic disorders, genome mapping, determination of diseases, and paternity test. Although sequencing has been used progressively for such purposes, the simplicity of the practice together with straightforward results interpretation of DNA fragments has indirectly made it a persistent choice for genetic analyses.

The analysis of RFLP is known to exploit variations in homologous DNA sequences. In fact, this technique has been a basic tool in molecular epidemiology and phylogenetic studies that grow concurrent with the rapidly expanding suite of restriction enzymes. The large number of enzymes available and the extent to which this method has been practiced worldwide revealed that RFLP is well defined for many species of organisms.

Generally, the procedure of RFLP analysis involves digestion of DNA fragments, followed by separation through agarose gel electrophoresis and detection of fragments containing specific genes using one of several methods, including DNA staining of the gel. While this

method is easy to adopt, the results obtained can be interpreted in a straightforward manner with little ambiguity, if any.

Restriction enzyme analysis of chromosomal DNA as a means of characterization and diagnosis of bacterial infections has been a valuable tool for decades. This reproducible technique with no highly sophisticated equipment and critical optimization required is suitable to be used ubiquitously. Nonetheless, improvements are essential for more rapid diagnosis. Although isolation procedures for bacterial chromosomal DNA are becoming more advanced, the process of gel electrophoresis remains unchanged. Present ideology, therefore, is generated and discussed with an attempt to strengthen the discriminatory power of agarose gel electrophoresis through modification of agarose medium preparation.

4.1 Technical background

With an avalanche of restriction endonucleases available, the choice of enzyme used in any particular RFLP analysis depends mainly on the target nucleotide acid sequence of interest as well as cost applied. The analysis of RFLP requires no specialized equipment beyond that normally used for agarose gel electrophoresis (unless southern blot hybridization is involved). Only a water bath or incubator is needed for the enzyme digestion process. Time requirements for restriction enzyme digestion depend on the cutting efficiency of the enzyme and the amount of DNA used in the digestion.

Generally, agarose gels are cast at a single concentration by melting the agarose in the presence of a desired buffer (Tris-acetate-EDTA or Tris-borate-EDTA) that establishes a pH and provides ions to support conductivity. The concentration and type of agarose best used in a gel can be assessed based on DNA fragment sizes to be resolved. Meanwhile, the conventional method of gel preparation in RFLP analysis requires large gels to obtain a good resolution for both large and small DNA fragments. Large gels are generally hard to handle and require lower voltage to avoid over-heating. Hence, in the traditional way of RFLP analysis, achieving a fine distribution of banding patterns is truly time consuming.

The voltage applied to complete an electrophoretic separation is one of the parameters that has the greatest effect in influencing the time duration in agarose gel electrophoresis. Most large agarose gels are subjected to run overnight (>16 hr) at very low voltages (1-1.5 V/cm). While gels can be run much faster, particularly if the gel apparatus is cooled, resolution of larger DNA fragments is lost when higher voltages are used. Because the resolution required depends on the relative molecular weights of the fragments of interest, the time required for adequate separation is best determined empirically.

4.2 Modified agarose gel preparation

The groundwork of agarose gel electrophoresis in RFLP analysis is easy and straightforward. But via traditional practice, to accomplish a fine dissemination of banding patterns is somehow onerous and cumbersome. Based on the principles and parameters as discussed earlier, this method of gel casting is developed to provide better separation of DNA fragments with yet a shorter electrophoretic time. It is demonstrated that the art of gel casting may influence the migration rate of nucleic acids through a matrix despite the charge to mass ratio (Z) of all species is uniform (Lee & Bahaman, 2010). This method, however, is

only well-suited for DNA analysis that demand the interrogation of medium range fragments (100-20,000 bp).

4.2.1 Gel casting

This technique of gel preparation involves two different concentrations of agarose gel. The gel concentrations were determined mainly based on the characteristics of agarose gel (section 3.3.1), earlier studies performed (O Hara et al., 1985; Venkatesha & Ramadass, 2001), as well as experiment-based optimization (Lee & Bahaman, 2010). Combination of high and low agarose concentrations would facilitate distinct migration of both smaller and larger fragments of DNA at a consistent voltage (4.5 V/cm). In addition, this method enables the observation of distinct banding patterns, exclude the need of preparing large gels which are difficult to handle. The technique of gel casting for modified gel preparation is demonstrated in Figure 4.

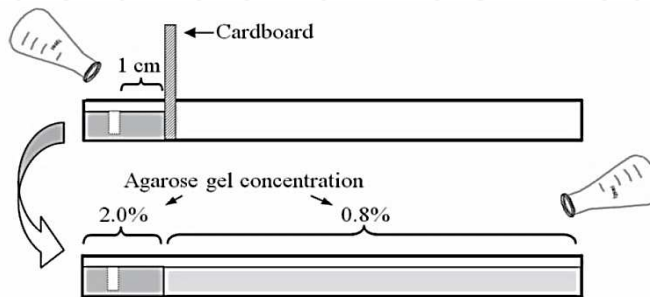


Fig. 4. Technique of modified gel preparation in agarose gel electrophoresis.

By having 2% agarose concentration as standard, smaller fragments were able to migrate faster and farther than the larger fragments to get into the lower agarose concentration. As described earlier, the concentration of agarose involved here (2%) was determined through experiment-based optimization. This part of gel basically serves as a sieve to facilitate the migration of small fragments into the lower agarose concentration while slowing down the larger fragments. Unlike the traditional method, distinct migration of DNA fragments (both large and small sizes) can be observed without the need for long-run of gel electrophoresis. This method promotes distinctive separation of DNA fragments (100-20,000 bp).

4.2.2 Interpretation of DNA fragments

Modified agarose gel preparation is proven to be able to provide better separation for both large and small DNA fragments at high voltage (4.5 V/cm). Figure 5 shows the results of agarose gel electrophoresis on two different gel preparations performed under analogous conditions (gel size, voltage and time). In this case, Gel A was prepared using a single agarose concentration (0.8%) to represent the traditional method of gel preparation for RFLP analysis while Gel B represents the modified method which consists of two different gel concentrations (2% + 0.8%). The electrophoresis process for both gel preparations were conducted under identical conditions: 4.5 V/cm for 1.7 hours (the duration of the electrophoretic run may vary, depending on the desired separation of DNA fragments).

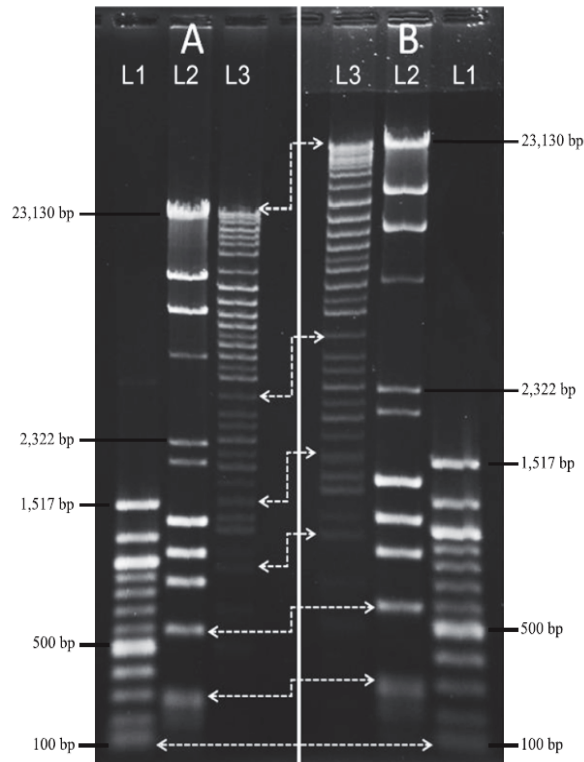


Fig. 5. Migration of DNA fragments in agarose gel electrophoresis via traditional method (A: 0.8% gel) and the modified method (B: 2% + 0.8% gel). Lane 1-3: 1kb DNA marker, λ DNA *Hind*III plus Φ X174 DNA-*Hae*III marker and DNA analytical marker, respectively.

As noted, though the migration rate of the smallest fragment (100 bp) was similar in both gels, the separation of subsequent DNA fragments in Gel B improved regardless of the fragment size. This indicated that the dual concentration gel was able to facilitate the migration of smaller DNA fragments efficiently as well as to provide distinctive separation of large DNA fragments without bias. Besides, higher voltage could be introduced via this method to shorten the electrophoresis time required. Conversely, the traditional method of gel preparation in RFLP analysis requires large gels to obtain a good resolution for both large and small DNA fragments. With this method, a good resolution could be achieved for both the short and long fragments within a comparatively short period of running time without resorting to large agarose gels.

5. Conclusion

It will become increasingly important to be able to choose and carry out the appropriate electrophoresis technique for specific separation problems. Various attempts have been made to improve the current electrophoresis method. By applying two different agarose concentrations, electrophoresis can be conducted rapidly with a higher voltage while

providing a better resolution of DNA fragments. The only drawback of this suggested technique is perhaps the method of preparing a gel with two agarose concentrations which might be slightly time-consuming and tedious as compared to the traditional method of gel preparation for RFLP analysis. However, the technique described in this section not only able to provide similar resolution of small fragments as can be achieved by the traditional RFLP analysis, yet a remarkably better resolution for larger DNA fragments. Hence, this modified technique is useful in the rapid analysis of an array of medium range DNA fragments (100-20,000 bp). With agarose gel electrophoresis, high separation efficiency can be achieved using a relatively limited amount of equipment.

6. Acknowledgement

The authors would like to thank the Faculty of Veterinary Medicine, Universiti Putra Malaysia for providing necessary facilities to carry out the research work and Yeong Fatt Shang for vetting the manuscript.

7. References

- Arnott, S., Fulmer, A., Scott, W. E., Dea, I. C., Moorhouse, R., & Rees, D. A. (1974). The agarose double helix and its function in agarose gel structure. *Journal of Molecular Biology*, 90: 269-284.
- Calladine, C. R., Collis, C. M., Drew, H. R., & Mott, M. R. (1991). A study of electrophoretic mobility of DNA in agarose and polyacrylamide gels. *Journal of Molecular Biology*, 221: 981-1005.
- Fangman, W. L. (1978). Separation of very large DNA molecules by gel electrophoresis. *Nucleic Acids Research*, 5: 653-665.
- Helling, R. B., Goodman, H. M., & Boyer, H. W. (1974). Analysis of R. EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose gel electrophoresis. *Journal of Virology*, 14:1235-1244.
- Hervet, H., & Bean, C. P. (1987). Electrophoretic mobility of lambda phage HindIII and HaeIII DNA fragments in agarose gels: A detailed study. *Biopolymers*, 26: 727-742.
- Hjertén, S. (1962). Chromatographic separation according to size of macromolecules and cell particles on columns of agarose suspensions. *Archives of Biochemistry and Biophysics*, 99: 466-475.
- Johnson, P. H., & Grossman, L. I. (1977). Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-stranded DNAs. *Biochemistry*, 16: 4217-4225.
- Kirkpatrick, F. H. (1990). Overview of agarose gel properties. *Current Communications in Cell and Molecular Biology*, 1: 9-22.
- Le Pecq, J. B., & Paoletti, C. (1967). A fluorescent complex between ethidium bromide and nucleic acids. *Journal of Molecular Biology*, 27 :87-106.
- Lee, S. V., & Bahaman, A. R. (2010). Modified gel preparation for distinct DNA fragment analysis in agarose gel electrophoresis. *Tropical Biomedicine*, 27: 351-354.
- Lodish, H., Berk, A., Zipursky, L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). *Molecular Cell Biology* (4th ed.). New York: W. H. Freeman.
- Marshall, R. B., Whitton, B. E., & Robinson, A. J. (1981). Identification of *Leptospira* serovars by restriction endonuclease analysis. *Journal of Medical Microbiology*, 14: 163-166.

- McDonell, M. W., Simon, M. N., & Studier, F. W. (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *Journal of Molecular Biology*, 110: 119-146.
- Millikan, R. A., & Bishop, E. S. (1917). *Elements of electricity, a practical discussion of the fundamental laws and phenomena of electricity and their practical applications in the business and industrial world*. Chicago: American Technical Society.
- Miura, Y., Wake, H., & Kato, T. (1999). TBE, or not TBE; that is the question: Beneficial usage of tris-borate for obtaining a higher resolution of small DNA fragments by agarose gel electrophoresis. *Nagoya Medical Journal*, 43: 1-6.
- Modrich, P. (1979). Structures and mechanisms of DNA restriction and modification enzymes. *Quarterly Reviews of Biophysics*, 12: 315-369.
- O Hara, M. J., Collins, D. M., & De Lise, G. W. (1985). Restriction endonuclease analysis of *Brucella ovis* and other *Brucella* species. *Veterinary Microbiology*, 10: 425-429.
- Rees, D. A. (1972). Shapely Polysaccharides. *Biochemical Journal*, 126: 257-273.
- Reuss, F. F. (1809). Sur un nouvel effet de l'électricité galvanique. *Mémoires de la Société Impériale des Naturalistes de Moscou*, 2: 327-337.
- Rickwood, D., & Hames, B. D. (1982). *Gel electrophoresis of nucleic acids : a practical approach*. Washington: Oxford University Press.
- Roberts, R. J. (1976). Restriction endonucleases. *Critical Reviews in Biochemistry*, 4: 123-164.
- Rüchel, R. (1978). Transmission-electron microscopic observations of freeze-etched polyacrylamide gels. *Journal of Chromatography*, 166: 563-575.
- Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.
- Sharp, P. A., Sugden, B., & Sambrook, J. (1973). Detection of two restriction endonuclease activities in *Haemophilus parainfluenza* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry*, 12: 3055-3063.
- Thorne, H. V. (1966). Electrophoretic Separation of Polyoma Virus DNA from Host Cell DNA. *Virology*, 29: 234-239.
- Tiselius, A. (1937). A new apparatus for electrophoretic analysis of colloidal mixtures. *Transactions of the Faraday Society*, 33: 524-531.
- Upcroft, P., & Upcroft, J. A. (1993). Comparison of properties of agarose for electrophoresis. *Journal of Chromatography*, 618: 79-93.
- Venkatesha, M. D., & Ramadass, P. (2001). Identification of leptospiral isolates by bacterial restriction endonuclease DNA analysis (BRENDAs). *Indian Journal of Medical Microbiology*, 19: 26-29.
- Waring, M. J. (1965). Complex formation between ethidium bromide and nucleic acids. *Journal of Molecular Biology*, 13: 269-82.
- Westermeyer, R. (2005). *Electrophoresis in Practice: A Guide to Methods and Applications of DNA and Protein Separations* (4th ed.). Weinheim: WILEY-VCH Verlag GmbH & Co.
- Williams, L. R. (2001). Staining nucleic acids and proteins in electrophoresis gels. *Biotechnic and Histochemistry*, 76: 127-132.
- Zeiger, R. S., Salomon, R., Dingman, C. W., & Peacock, A. C. (1972). Role of base composition in the electrophoresis of microbial and crab DNA in polyacrylamide gels. *Nature - New Biology*, 238: 65-69.