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Session 5

Agarose Gel Electrophoresis

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ВСЕМИРНАЯ ОРГАНИЗАЦИЯ ЗДРАВООХРАНЕНИЯ
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Introduction

Gel electrophoresis is a method that separates macromolecules on the basis of size, electric charge and other physical properties. The term electrophoresis describes the migration of charged particles under the influence of an electric field. “Electro” refers to electricity and “Phoresis”, from the Greek word *phoros*, meaning, "to carry across." Thus, gel electrophoresis refers to a technique in which molecules are forced across a span of gel, motivated by an electrical current. The driving force for electrophoresis is the voltage applied to electrodes at either end of the gel. The properties of a molecule determine how rapidly an electric field can move it through a gelatinous medium.

Many important biological macromolecules (e.g. amino acids, peptides, proteins, nucleotides and nucleic acids) possess ionisable groups and, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode. For example, when an electric field is applied across a gel at neutral pH, the negatively charged phosphate groups of the DNA cause it to migrate toward the anode (Westermeier, 1997).

Electrophoresis through agarose is a standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures. Furthermore, the location of DNA within the gel can be determined by staining with a low concentration of ethidium bromide, a fluorescent intercalating dye.

The following sections will outline the physical principles, components (gel matrix, buffer, loading buffer and marker) and procedures for the preparation of agarose gel electrophoresis (Sambrook *et al.*, 1989).

Physical principles of agarose gel electrophoresis

Gel electrophoresis is a technique used for the separation of nucleic acids and proteins. Separation of macromolecules depends upon two variables: charge and mass. When a biological sample, such as DNA, is mixed in a buffer solution and applied to a gel, these two variables act together. The electrical current from one electrode repels the molecules while the other electrode simultaneously attracts the molecules. The frictional force of the gel material acts as a "molecular sieve", separating the molecules by size. During electrophoresis, macromolecules are

forced to move through the pores and their rate of migration through the electric field depends on the following:

- the strength of the field
- the size and shape of the molecules
- the relative hydrophobicity of the samples
- the ionic strength and temperature of the buffer in which the molecules are moving.

To completely understand the separation of charged particles in gel electrophoresis, it is important to look at the simple equations relating to electrophoresis. When a voltage is applied across the electrodes, a potential gradient, *E*, is generated and can be expressed by the equation:

$$E = V/d \quad (1)$$

where *V*, measured in volts, is the applied voltage and *d* the distance in cm between the electrodes.

When the potential gradient, *E,* is applied, a force, *F*, on a charged molecule is generated and is expressed by the equation:

$$F = Eq \quad (2)$$

where *q* is the charge in coulombs bearing on the molecule. It is this force, measured in Newtons that drives a charged molecule towards an electrode.

There is also a frictional resistance that slows down the movement of charged molecules. This frictional force is a function of:

- the hydrodynamic size of the molecule
- the shape of the molecule
- the pore size of the medium in which electrophoresis is taking place
- the viscosity of the buffer

The velocity *v* of a charged molecule in an electric field is a function of the potential gradient, charge and frictional force of the molecule and can be expressed by the equation:

$$v = Eq / f \quad (3)$$

where *f* is the frictional coefficient.

The electrophoretic mobility, *M,* of an ion can then be defined by the ion's velocity divided by the potential gradient:

$$M = v / E \quad (4)$$

In addition, from equation (3) one can see that electrophoretic mobility *M* can be equivalently expressed as the charge of the molecule, *q*, divided by the frictional coefficient, *f*:

$$M = q / f \quad (5)$$

When a potential difference is applied, molecules with different overall charges will begin to separate due to their different electrophoretic mobilities. The electrophoretic mobility is a significant and characteristic parameter of a charged molecule or particle and depends on the pK value of the charged group and the size of the molecule or particle. Even molecules with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces. Linear double stranded DNA migrates through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs. Larger molecules migrate more slowly because of the greater frictional drag and because of the less efficient movement through the pores of the gel.

The current in the solution between the electrodes is conducted mainly by the buffer ions with a small proportion being conducted by the sample ions. The relationship between current *I*, voltage *V*, and resistance *R* is expressed as in Ohm's law:

$$R = V / I \quad (6)$$

This equation demonstrates that for a given resistance *R*, it is possible to accelerate an electrophoretic separation by increasing the applied voltage *V*, which would result in a corresponding increase in the current flow *I*. The distance migrated will be proportional to both current and time. However, the increase in voltage, *V*, and the corresponding increase in current, *I*, would cause one of the major problems for most forms of electrophoresis, namely the generation of heat. This can be illustrated by the following equation in which the power, *W*, (measured in Watts) generated during the electrophoresis is equal to the product of the resistance times the square of the current:

$$W = I^2R \quad (7)$$

Since most of the power produced in the electrophoretic process is dissipated as heat the following detrimental effects can result:

- an increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples
- the formation of convection currents, which leads to mixing of separated samples;
- thermal instability of samples that are rather sensitive to heat (e.g. denaturation of DNA)
- a decrease of buffer viscosity hence a reduction in the resistance of the medium

Components of agarose gel electrophoresis

Agarose

Agarose, a natural colloid extracted from seaweed, is a linear polysaccharide (average molecular mass ~12,000 Da) made up of the basic repeated unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. Agarose is very fragile and easily destroyed by handling. Agarose gels have large "pore" sizes and are used primarily to separate large molecules with a molecular mass greater than 200 kDa.

Agarose gels process quickly, but with limited resolution since the bands formed in the agarose gels tend to be fuzzy/diffuse and spread apart. This is a result of pore size and cannot be controlled. Agarose gels are obtained by suspending dry powdered agarose in an aqueous buffer, then boiling the mixture until the agarose melts into a clear solution. The solution is then poured onto a gel-tray and allowed to cool to room temperature to form a rigid gel. Upon hardening, the agarose forms a matrix whose density is determined by its concentration.

Electrophoresis buffer

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions, electrical conductance is minimal and DNA migrates slowly, if at all. In a buffer of high ionic strength electrical conductance is very efficient and a significant amount of heat is generated. In the worst circumstance, the gel melts and the DNA denatures.

Several buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50 mM (pH 7.5 - 7.8). Electrophoresis buffers are usually prepared as concentrated solutions and stored at room temperature. TBE was originally used at a working strength of 1x for agarose gel electrophoresis. However, a working solution of 0.5x provides more than enough buffering power and almost all agarose gel electrophoresis is now carried out using this buffer concentration.

Agarose concentration

A DNA fragment of a given size migrates at different rates through gels depending on the concentration of agarose. For a specific concentration of agarose and/or buffer, it is possible to separate DNA segments containing between 20 and 50,000 bp. In

horizontal gels, agarose is usually used at concentrations between 0.7% and 3% (see Table 1).

Table 1. Recommended agarose gel concentration for resolving linear DNA molecules

% agarose	DNA size range (bp)
0.75	10.000 - 15.000
1.0	500 - 10.000
1.25	300 - 5000
1.5	200 - 4000
2.0	100 - 2500
2.5	50 - 1000

Marker DNA

For a given voltage, agarose gel and buffer concentrations, the migration distance depends on the molecular weight of the starting material. Therefore, a marker DNA of known size should be loaded into slots on both the right and left sides of the gel. A marker generally contains a defined number of known DNA segments, which makes it easier to determine the size of the unknown DNAs if any systematic distortion of the gel should occur during the electrophoresis.

Loading buffer

The DNA samples to be loaded onto the agarose gel are first mixed with a loading buffer usually comprising water, sucrose, and a dye (e.g. xylene cyanole, bromophenol blue, bromocresol green, etc.). The maximum amount of DNA that can be loaded depends on the number of fragments. The minimum amount of DNA that can be detected by photography of ethidium bromide stained gels is about 2 ng in a 0.5-cm wide band. If there is more than 500 ng of DNA in a band of this width, the slot will be overloaded, resulting in smearing. The loading buffer serves three purposes:

- increases the density of the sample ensuring that the DNA drops evenly into the well
- adds colour to the sample, thereby simplifying the loading process
- imparts a dye to the sample that, in an electric field, moves toward the anode at a predictable rate

Experimental

Caution: Ethidium bromide is a powerful mutagen/carcinogen and is moderately toxic. Gloves should always be worn when handling solutions and gels containing ethidium bromide.

Equipment

- Horizontal electrophoresis unit with power supply
- Microwave oven or heating stirrer
- Micropipettes
- 1.5 ml reaction tubes
- Balance capable of 0.1 g measurements
- Spatulas
- Rack for reaction tubes
- Glassware
- Transilluminator (UV radiation, 312 nm)
- Instruments for documentation (e.g. Polaroid camera or a video recorder)

Reagents

- Agarose, suitable for DNA electrophoresis
- Tris[hydroxymethyl] aminomethane (Tris) CAS 77-68-1
- Boric acid
- Na_2EDTA CAS 6381-92-6
- Ethidium bromide CAS 1239-45-8
- Sucrose
- Xylene cyanole FF CAS 2650-17-1
- DNA markers:
    - Lambda DNA *Eco*RI/*Hind*III digested *(or other similar suitable marker)*
    - 100 bp DNA ladder

10x TBE buffer (1 litre)

Tris[hydroxymethyl] aminomethane (Tris)	54.0 g
Boric acid	27.5 g
Na_2EDTA	7.44 g

- Mix reagent to deionised water to obtain a 1 litre solution at pH 8.3
- Store at room temperature

6x loading buffer (10 ml)

Xylene cyanole FF	0.025 g
Sucrose	4 g

- Add sucrose and Xylene cyanole FF to deionised water to obtain 10 ml of solution.
- Mix the solution, autoclave and store at 4°C.

Procedure

- Seal the edges of a clean, dry plastic gel-tray either with tape or other means. Position the appropriate comb so that complete wells are formed when the agarose solution is added
- Dilute 10x TBE buffer to prepare the appropriate amount of 0.5x TBE buffer to fill the electrophoresis tank and to prepare the gel
- Weigh powdered agarose according to Table 2 and add it to an appropriate amount of 0.5x TBE buffer in an Erlenmeyer flask with a loose-fitting cap (usually 150 ml gel solution for a 15 x 15 cm gel-tray and 100 ml gel for a 15 x 10 cm gel-tray)

Table 2. Agarose gel concentrations used during the course

	GMO3 GMO4	ZEIN3 ZEIN4	p35S-cf3 p35S-cr4	HA-*nos*118-f HA-*nos*118-r	CRYIA3 CRYIA4	GM07 GM08	mg3 mg4	Genomic DNA
0.8 - 1%								X
1.5%	X	X						
2.0%			X	X	X	X	X	

- Heat the slurry in a microwave oven or in a boiling water bath until the agarose dissolves (check the volume of the solution after heating)
- Cool the mixture to 50 - 60°C and add ethidium bromide (from a stock solution of 10 mg/ml) to a final concentration of 0.2 µg/ml and mix thoroughly
- Pour the solution into the gel-tray and allow the gel to set. The amount of gel used should correspond to a depth of approximately 3 - 5 mm
- After the gel is completely set, carefully remove the comb and the tape and place the gel in the electrophoresis tank.
- Add enough 0.5x TBE buffer to the electrophoresis unit to cover the gel to a depth of about 2 - 5 mm

Prepare samples and marker for genomic DNA as follows:

sample		***marker***	
water	3 µl	water	6 µl
loading buffer	2 µl	loading buffer	2 µl
sample	5 µl	λ DNA *Eco*RI / *Hind*III	2 µl
	10 µl		10 µl

Prepare samples and marker for PCR products as follows:

sample		***marker***	
loading buffer	2 µl	100 bp DNA ladder	15 µl
sample	8 µl		
	10 µl		

- Load 10 µl of each sample into consecutive wells and the appropriate DNA marker into the first and last lane
- Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode and apply a voltage of 5 - 10 V/cm
- Run the gel until the xylene cyanole has migrated the appropriate distance through the gel (~ 40 - 60 minutes)
- Turn off the current; remove the leads and the lid from the gel tank. Place the gel on a UV lightbox and photograph the gel
- Discard the gel into the provided ethidium bromide solid waste bin

References

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Westermeier, R. (1997). Electrophoresis in Practice: a Guide to Methods and Applications of DNA and Protein Separation, VCH, Weinheim.