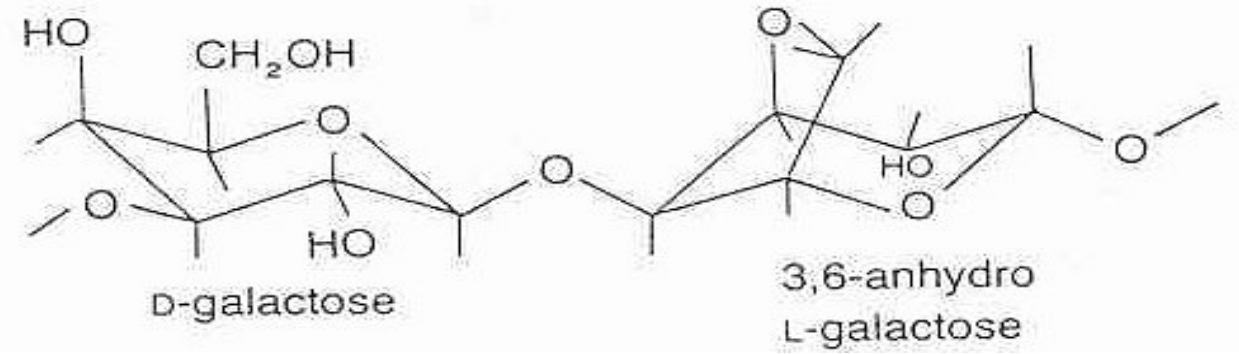
AGAROSEL GEL ELECTROPHORESIS

Agarose, which is extracted from seaweed, is a linear polymer whose basic structure is



Commercially available agrose is not completely pure; it is contaminated with other polysaccharides, salts, and proteins. The amount of contamination varies from batch to batch of agarose and from manufacturer to manufacturer.

The difference can affect → The migration of the DNA

→ The ability of the DNA recovered from gel to serve as substrate in enzymatic reactions.

Most manufactures now prepare special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide.

Also some manufacturers sell chemically modified forms of agarose that gel and melt at low temperature without significant deterioration in the strength of the hardened gel. Such chemically modified agarose are used for preparative electrophoresis of DNA and for digestion of DNA with restriction enzymes in situ. Gels made with agarose of this type have a greater resolving power than gels made with normal agarose.

Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allow to harden. When an electric field is applied across the gel, DNA, which is negatively charged at netural pH, migrate toward the anode.

Factors Affecting The Rate of DNA Migration In Agarose Gels:

1] Molecular size of the DNA:

Larger molecules migrate more slowly than smaller molecules.

Because of → greater frictional drag
→ they worm their way through the pores of the gel.

2] Agarose concentration:

A linear DNA fragment of a given size migrate at different rates through gels containing different concentrations of agarose.

By using gels of different concentrations, it is possible to resolve a wide size range of DNA molecules.

Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

3] Conformation of the DNA:

Superhelical circular DNAs
Nicked circular DNAs
Linear DNAs
→ of the same molecular weight →

Migrate through agarose gels at different rates.

The relative mobilites of the three forms depend on:

- The agarose concentration in the gel (primarily)
- Strength of the applied current
- Ionic strength of the buffer

An unambiguous method for identifying the different conformational forms of DNA is to carry out electrophoresis in the presence of increasing quantities of ethidium bromide. As the concentration of ethidium bromide increases, more of the dye become bound to the DNA.

4] Applied voltage:

At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA increase differentially.

5] Direction of the electric field:

DNA molecules larger than 50-100 kb in length migrate through agarose at the same rate if the direction of the electric field remains constant.

6] Base composition and temperature:

The electrophoretic behavior of DNA in agarose gels is not significantly affected by either the base composition of the DNA or the temperature at which the gel is run.

In general, agarose gels are run at room temperature.

7] Presence of intercalating dyes:

Ethidium bromide, a fluorescent dye that is used to detect DNA in agarose gels, reduces the electrophoretic mobility of linear DNA by about 15%.

The dye intercalates between stacked base pairs, extending the length of linear and nicked circular DNA molecules and making them more rigid.

N.B.: Ethidium bromide is a carcinogen and should be handled with care.

8] Composition of the 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 very slowly.

In buffers of high ionic strength, electrical conductance is very efficiend and significant amount of heat are generated. In the worst case, the gel melts and the DNA denatures.

Several different buffers are available for electrophoresis (TAE, TPE& TBE)

Commonly Used Electrophoresis Buffers

Buffer	Working Solution	Concentrated stock solution (per liter)
Tris-acetate (TAE)	1x:0.04$_M$ Tris-acetate 0.001 $_M$ EDTA	50x:242$_g$ Tris base 57.1ml glacial acetic acid 100ml 0.5$_M$ EDTA (pH 8.0)
Tris-phosphate (TPE)	1x:0.09$_M$ Tris-phosphate 0.002 $_M$ EDTA	10x:108$_g$ Tris base 15.5ml 85% phosphoric acid (1.679g/ml) 40 ml 0.5$_M$ EDTA (pH 8.0)
Tris-borate[a] (TBE)	0.5x:0.045$_M$ Tris-borate 0.001 $_M$ EDTA	5x:54$_g$ Tris base 27.5 $_g$ boric acid 20 ml 0.5$_M$ EDTA (pH 8.0)
Alkaline[b]	1x:50$_{MN}$ NaOH 1 m$_M$ EDTA	1x:5ml 10$_N$ NaOH 2 ml 0.5$_M$ EDTA (pH 8.0)

Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature.

Apparatuses Used For Agrose Gel Electrophoresis:

The most commonly used configuration is the horizontal slab gel, which is superior to the old-fashioned vertical gels in several respects:

a) Low agarose concentration can be used.

b) Gels can be cast in a wide variety of sizes.

c) The gels are very simple to lead, pour, and handle.

d) The apparatus is durable and inexpensive to construct or buy.

Horizontal slab gels are usually poured on a glass plate or plastic tray that can be installed on a platform in the electrophoresis tank.

Among the factors to be considered when buying an electrophoresis tank are:

1- It should be easy to examine the gel by ultraviolet light during the run.
2- The apparatus should be supplied with a variety of combs to generate different numbers of wells of different sizes.
3- The apparatus should be fitted with a lid containing shielded electrical connections.
4- The apparatus should be fitted with outlets to allow electrophoresis buffer to be removed easily and completely.

5- The outlets should be designed to allow circulation of buffer between the anodic and cathodic chambers.

Preparation And Examination of Agarose Gels:

A) Preparation of an Agarose Gel:

1- Seal the edges of a clean, dry, glas plate (plastic) with tape so as to form a mold. Set the mold on a horizontal section of the bench.

2- Prepare sufficient electrophoresis buffer to fill the electrophoresis tank and to prepare the gel. Add the correct amount of powdered agarose to a measured quantity of electrophoresis buffer in glass beaker.

3- Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves.

4- Cool the solution to 60°C, and, if desired, add ethidium bromide (from a stock solution of 10 mg/ml in water) to a final concentration of 0.5µg/ml and mix thoroughly.

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye.

5- Position the comb 0.5 – 1.0 mm above the plate so that a complete well is formed when the agarose is added.

6- Pour the warm agarose solution into the mold. The gel should be between 3mm and 5 mm thick. Check to see that there are no air bubbles under or between the teeth of the comb.

7- After the gel is completely set (30-45 minutes at room temperature), carefully remove the comb and tape and mount the gel in the electrophoresis tank.

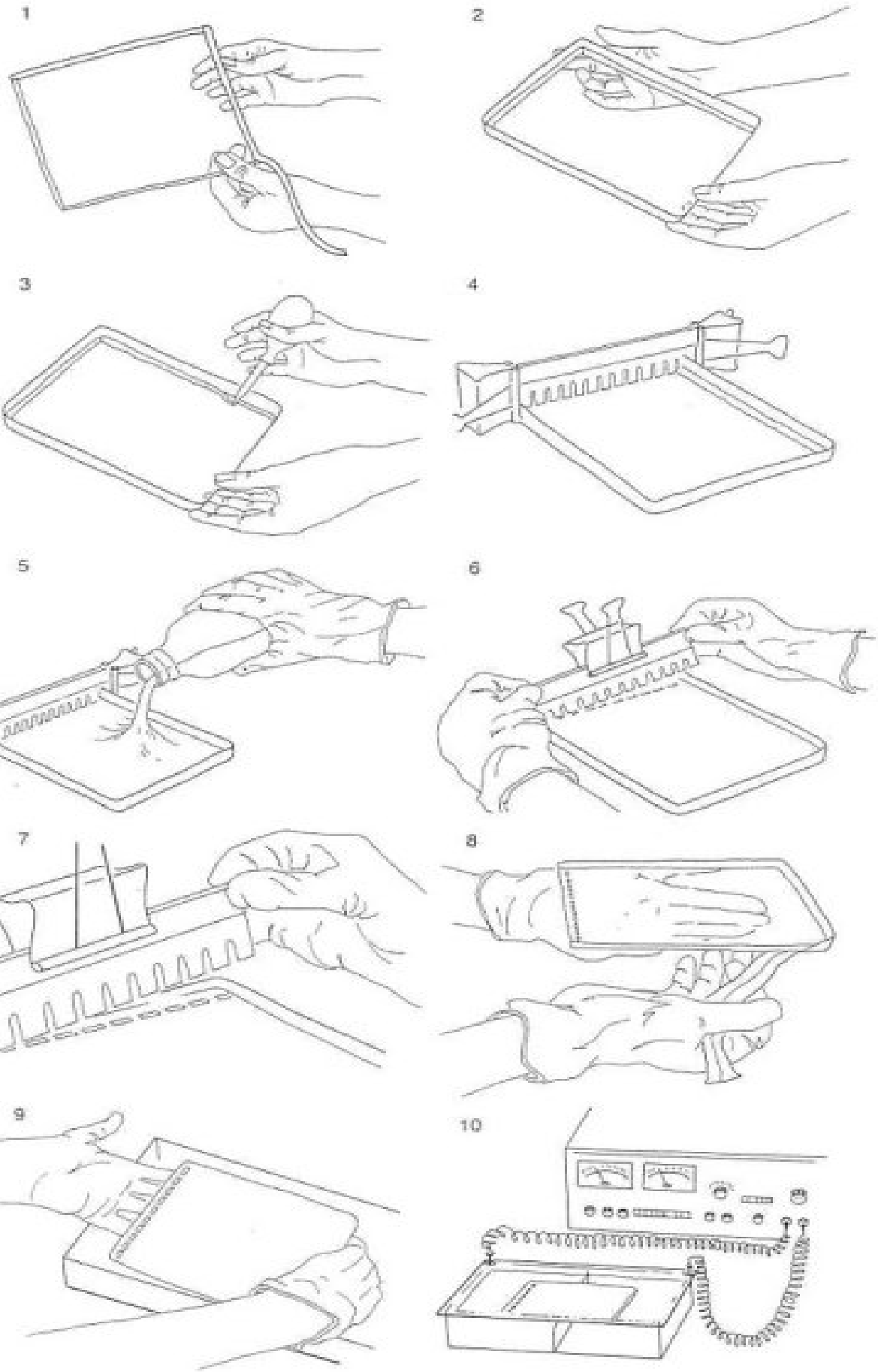
8- Add just enough electrophoresis buffer to cover the gel to a depth of about 1 mm.

9- Mix the samples of DNA with the desired gel-loading buffer. Slowly load the mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipetter.

Gel-loading Buffers

Buffer type	6x Buffer	Storage temperature
I	0.25% bromophenol blue 0.25% xylene cyanol FF 40% (w/v) sucrose in water	4°C
II	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 400; Pharmacia) in water	Room temp
III	0.25% bromophenol blue 0.25% xylene cyanol FF 30% glycerol in water	4°C
IV	0.25% bromophenol blue 40% (w/v) sucrose in water	4°C
V	*Alkaline loading buffer* 300_{mN} NaOH 6_{mM} EDTA 18% Ficoll (Type 400; Pharmacia) in water 0.15% bromocresol green 0.25% xylene cyanol FF	4°C

These gel-loding buffers serve three purposes: They increase the density of the sample, ensuring that the DNA drops evenly into the well; they add color to the sample.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10- Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a suitable voltage.

If the leads have been attached correctly, bubbles should be generated at the anode and cathodes and, within a few minutes, the bromophenole blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue have migrated the appropriate distance through the gel.

11- Turn off the electric current and remove the leads and lid from the gel tank. If ethidium bromide was present in the gel and electrophoresis buffer, examine the gel by ultraviolet light and photograph the gel.

B) Photography:

Photographs of gels may be made using ultraviolet light.

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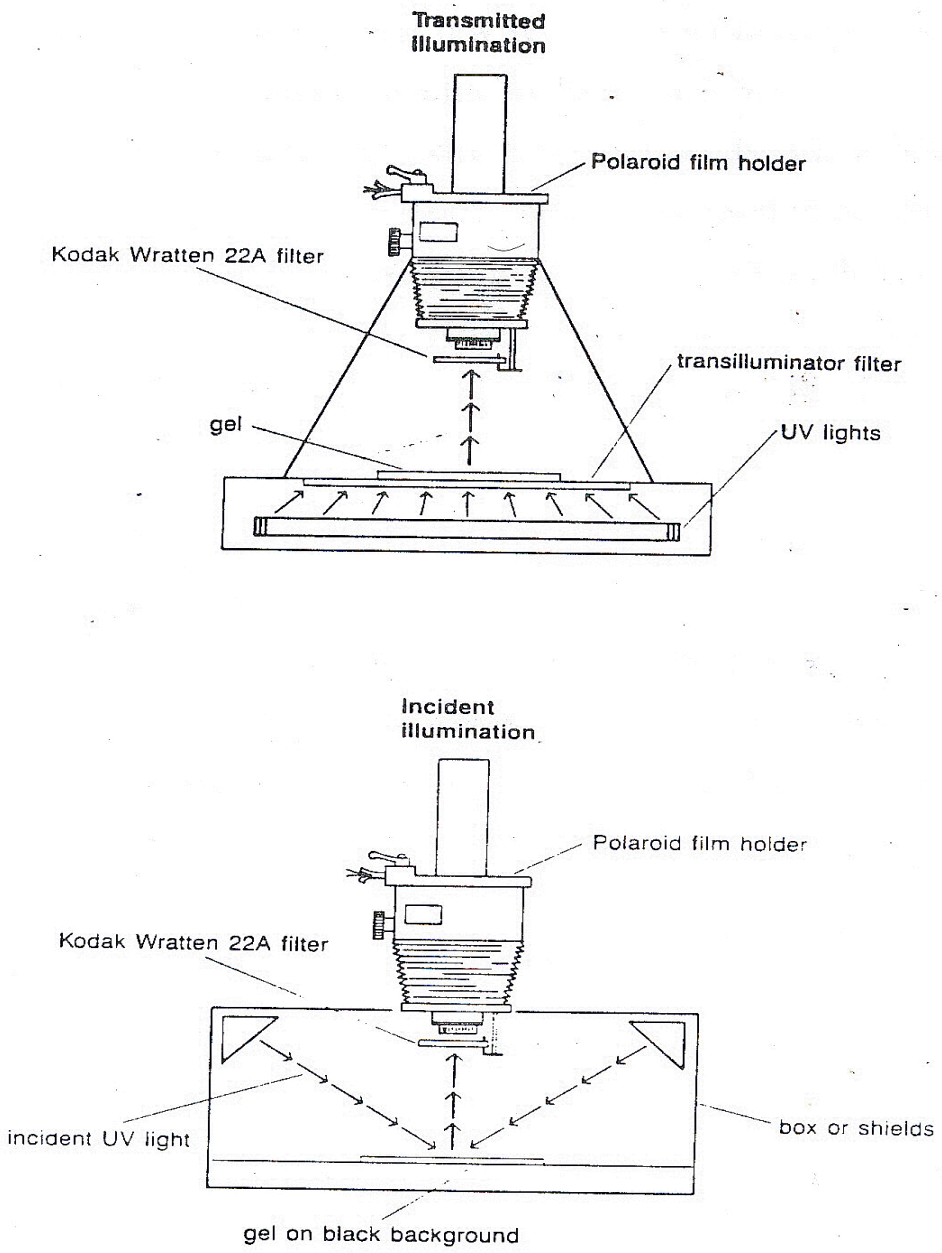

FIGURE

Photography of gels by ultraviolet illumination. The top diagram shows the arrangement of ultraviolet light source, the gel, and the camera that is used for photography by transmitted light. The bottom diagram shows the arrangement that is used for photography by incident light.

The most sensitive film is Polaroid type 57 or 667. With an efficient ultraviolet light source, filter, and a good lens (f = 135 mm), an exposure of a few seconds is sufficient to obtain images of bands containing as little as 10 ng of DNA.

For detection of extremely faint bands, a lens with a shorter focal length (F = 75 mm), should be used in combination with conventional wet-process film. This allows the lens to be moved closer to the gel, concentrates the image on a smaller area of film, and allows for flexibility in developing and printing the image.

Caution:

Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles that efficiently blocks ultraviolet light.

Staining DNA in Agarose Gels:

The most convenient method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide.

Because the fluorescent yield of ethidium bromide: DNA complexes is much greater than that of unbound dye, small amounts of DNA can be detected in the presence of free ethidium bromide in the gel.

Ethidium bromide can be used to detect both single-and-double stranded nucleic acids (both DNA and RNA).

Ethidium bromide is usually prepared as a stock solution of 10 mg/ml in water, which is stored at room temperature in dark bottles or bottles wrapped in aluminum foil. The dye is usually incorporated into the gel and the electrophoresis buffer at a concentration of 0.5 µg/ml.

Decontamination of Ethidium Bromide Solutions:

Caution:

Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, this solution should be decontaminated by one of the following methods:

A) Decontamination of concentrated solutions of ethidium bromide

(i.e., solution containing > 0.5 mg/ml)

Method (1)

This method reduces the mutagenic activity of ethidium bromide.

a. Add sufficient water to reduce the concentration of ethidium bromide to < 0.5 mg/ml.
b. To the resulting solution, add 0.2 volume of fresh 5% hypophosphorous acid and 0.12 volume of fresh 0.5 M sodium nitrite. Mix carefully. (The pH of solution is < 3.0).
c. After incubation for 24 hours at room temperature, add a large excess of 1 M sodium bicarbonate. The solution may now be discarded.

Method (2)

This method reduces the mutagenic activity of ethidium bromide.

a. Add sufficient water to reduce the concentration of ethidium bromide to ‹ 0.5 mg/ml.
b. Add 1 volume of 0.5 M $KMn0_4$. Mix carefully, and then add 1 volume of 2.5 N Hcl. Mix carefully, and allow the solution to stand at room temperature for several hours.
c. Add 1 volume of 2.5N Na0H. Mix carefully, and then discard the solution.

B) Decontamination of dilute solutions of ethidium bromide

(e.g., electrophoresis buffer containing 0.5 µg/ml ethidium bromide)

Method

a. Add 100 mg of powdered activated charcoal for each 100 ml of solution.
b. Store the solution for 1 hour at room temperature, shaking it intermittently.
c. Filter the solution through a Whatman No. 1 filter, and discard the filtrate.
d. Seal the filter and activated charcoal in a plastic bag, and dispose of the bag in the hazardous waste.