Scientific Practice-MCBG 2036
2018
Lecture 1-2

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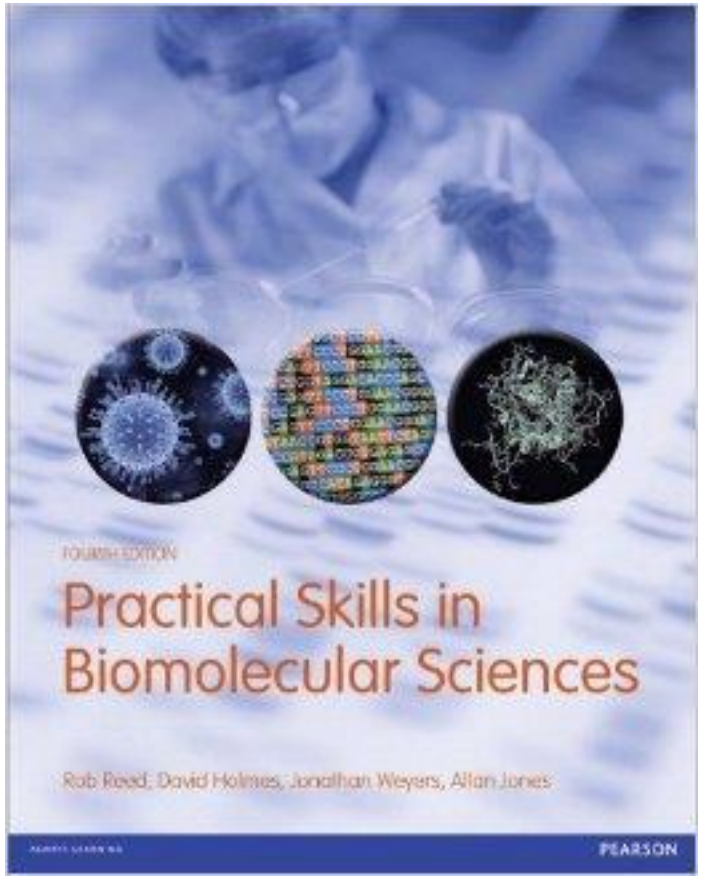
Consultation Hours:
Thursday-Friday: 13H30- 14H00

Prescribed Book



Other freely available book

Biochemistry Laboratory- by Rodney Boyer

http://www.e-library.esut.edu.ng/uploads/pdf/2745698718-biochemistry-laboratory-modern-theory-and-techniques.pdf

Electrophoresis- expected outcomes

BROADLY

- Understand general principles of electrophoresis
  - Electrophoresis of proteins
  - Electrophoresis of nucleic acids

SPECIFICALLY (Lecture 1 and 2)

- define ‘electrophoresis’
- describe the principle and important types of electrophoretic methods
- explain the principle and components of electrophoresis
- explain various uses of electrophoresis

Electrophoresis

- Electro refers to electron flow or current.
- Phoresis refers to movement.
- Thus Electrophoresis is movement under electric current.
- This technique therefore can separate molecules which can move in an electric field i.e. charged molecules.

Note: Positively charged molecules will move towards the negative pole while negatively charged molecules move towards the positive pole

Electrophoresis

Electrophoresis is a method whereby charged molecules in solution, chiefly proteins and nucleic acids, migrate in response to an electrical field.

Nucleic acid moves from a negative to a positive pole.

Their rate of migration through the electrical field, depends on:

- the strength of the field (voltage)
- on the net charge (anions [-vely charged] or cations [+vely charged])
- Size (large or small), and shape (globular, linear etc.) of the molecules,
- the ionic strength, viscosity, and temperature of the medium in which the molecules are moving.

Father of Electrophoresis

Arne Tiselius

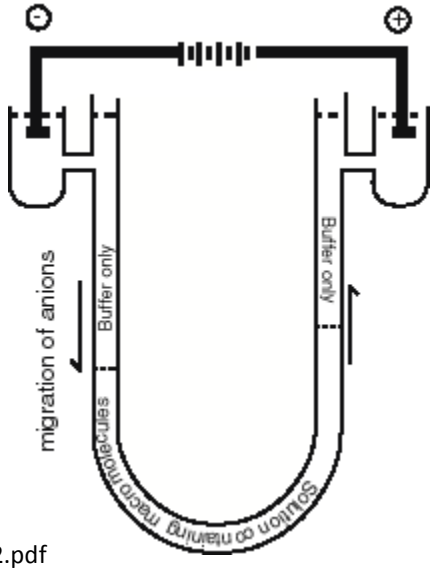
(Sweden, 1902-1971)

The Nobel Prize in Chemistry 1948

"for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins"

This type of cell is essentially a bent glass tube with electrolyte reservoirs containing the cathode and anode, and a buffer containing the macromolecules that need electrophoresed.

He tested horse serum in the apparatus and found 4 distinct bands consisting of albumin and 3 globulin components, which he named "α," "β," and "γ."



http://www.phys.sinica.edu.tw/TIGP-NANO/Course/2010_Spring/Classnotes/AAC_Chap%203%20Principles%20of%20Electrophoresis%20New_20100422.pdf

Principle of Electrophoresis

- **Cations (+ve)** migrate to **Cathode** (negative terminal/electrode)
- **Anions (-ve)** migrate to **Anode** (positive terminal/electrode)

Migration represented by equation:

$$\mu = \frac{qE}{r}$$

Where:

μ = Mobility

q= net charge

E= electric field in volt/cm

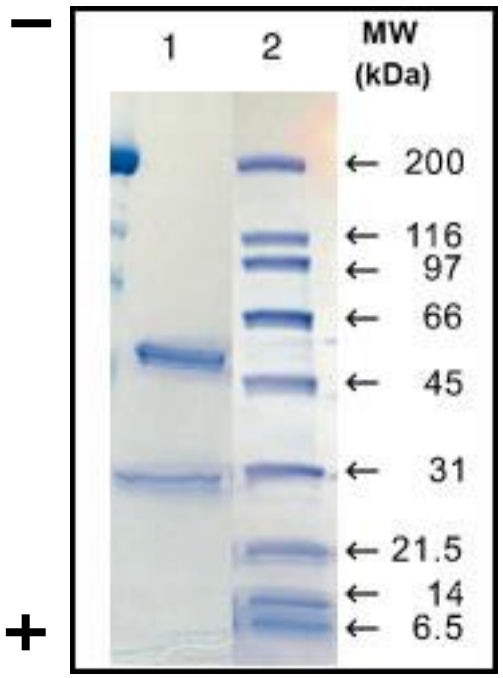
r = molecular radius

'r' is also called as frictional coefficient 'f' and is dependent on size and shape of molecule

Gel Electrophoresis

Most common method of separation in biological lab.

Separation is based upon the mobility of charged macromolecules under the influence of an electric field



Gel made of:

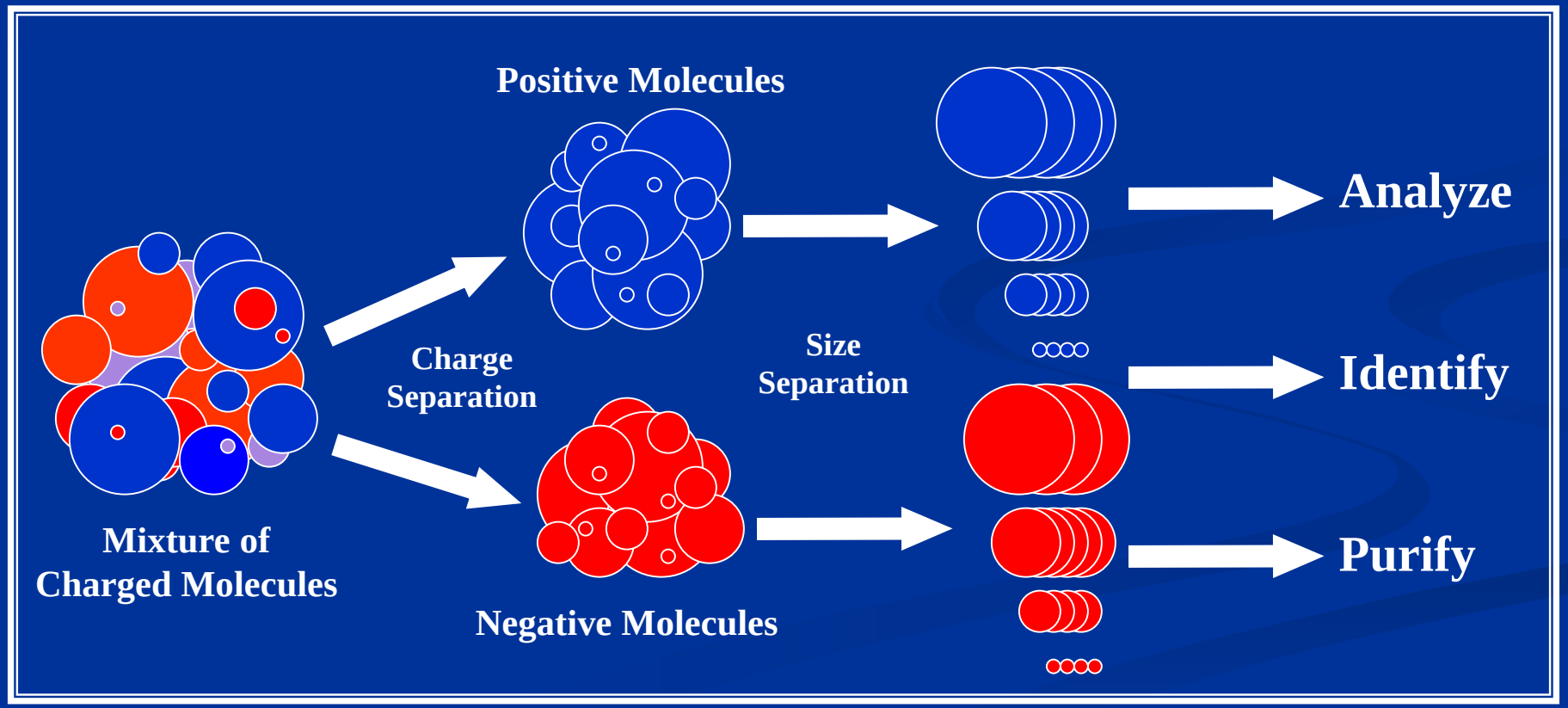
Agarose or polyacrylamide

The net charge of a sample molecule determines its direction of movement and significantly affects its mobility.

Modified from http://www.phys.sinica.edu.tw/TIGP-NANO/Course/2010_Spring/Classnotes/AAC_Chap%203%20Principles%20of%20Electrophoresis%20New_20100422.pdf

Separation of a Mixture of Charged Molecules

Charged molecules are separated based on their electrical charge and size within a matrix

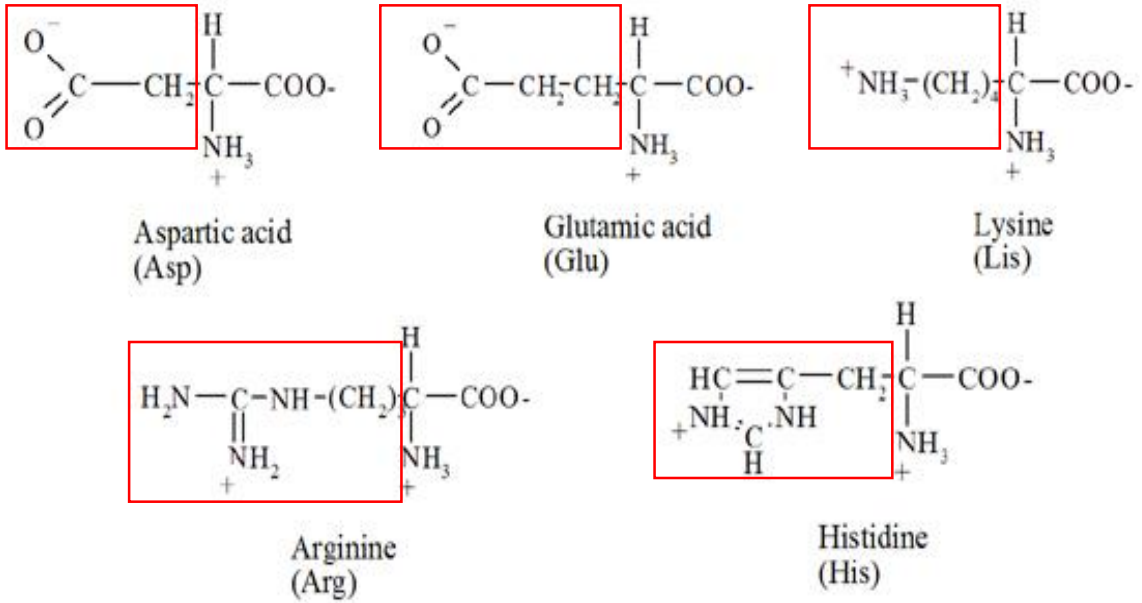


Where are the charges from? - Proteins

- These 5 amino acids: are very polar.
- are hydrophilic (or neutral, for histidine) and nearly always found on the outside of proteins.
- can be engaged in ionic bonds (through electrostatic attraction).

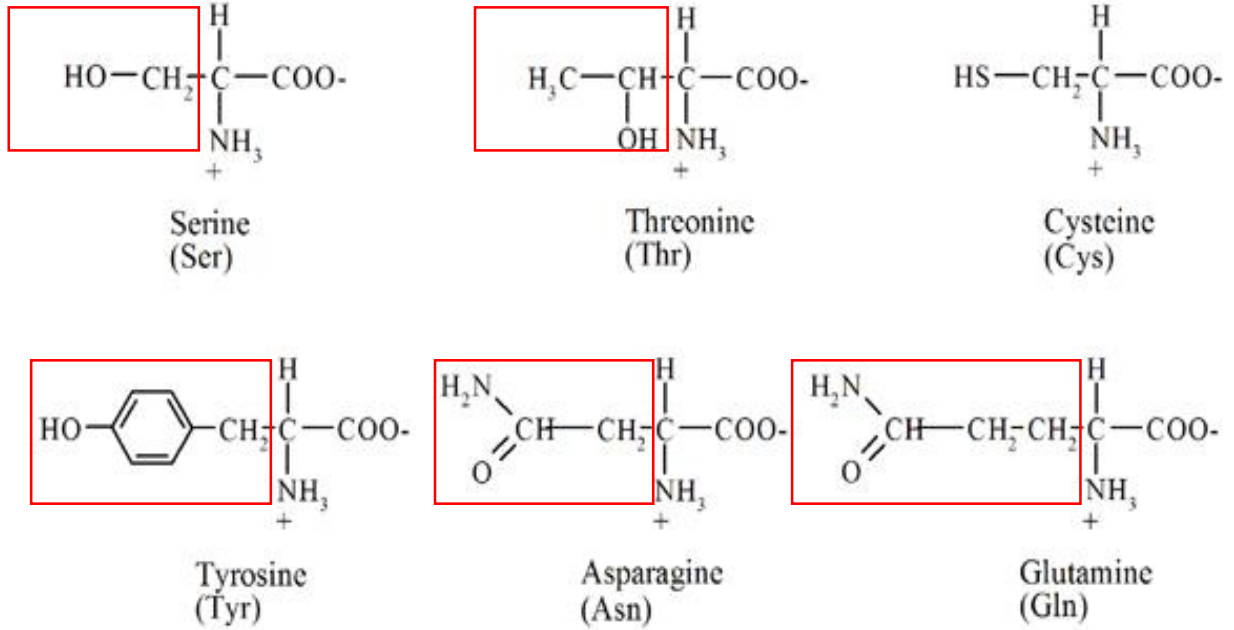
Charged side chains

Group 3 – Amino acids with charged R groups



- These 5 amino acids: are polar.
- are hydrophilic (Q, N) or neutral (S, T, Y) and are usually on the outside of proteins.
- are frequently engaged in hydrogen bonds.

Group 2 – Amino acids with uncharged polar R groups

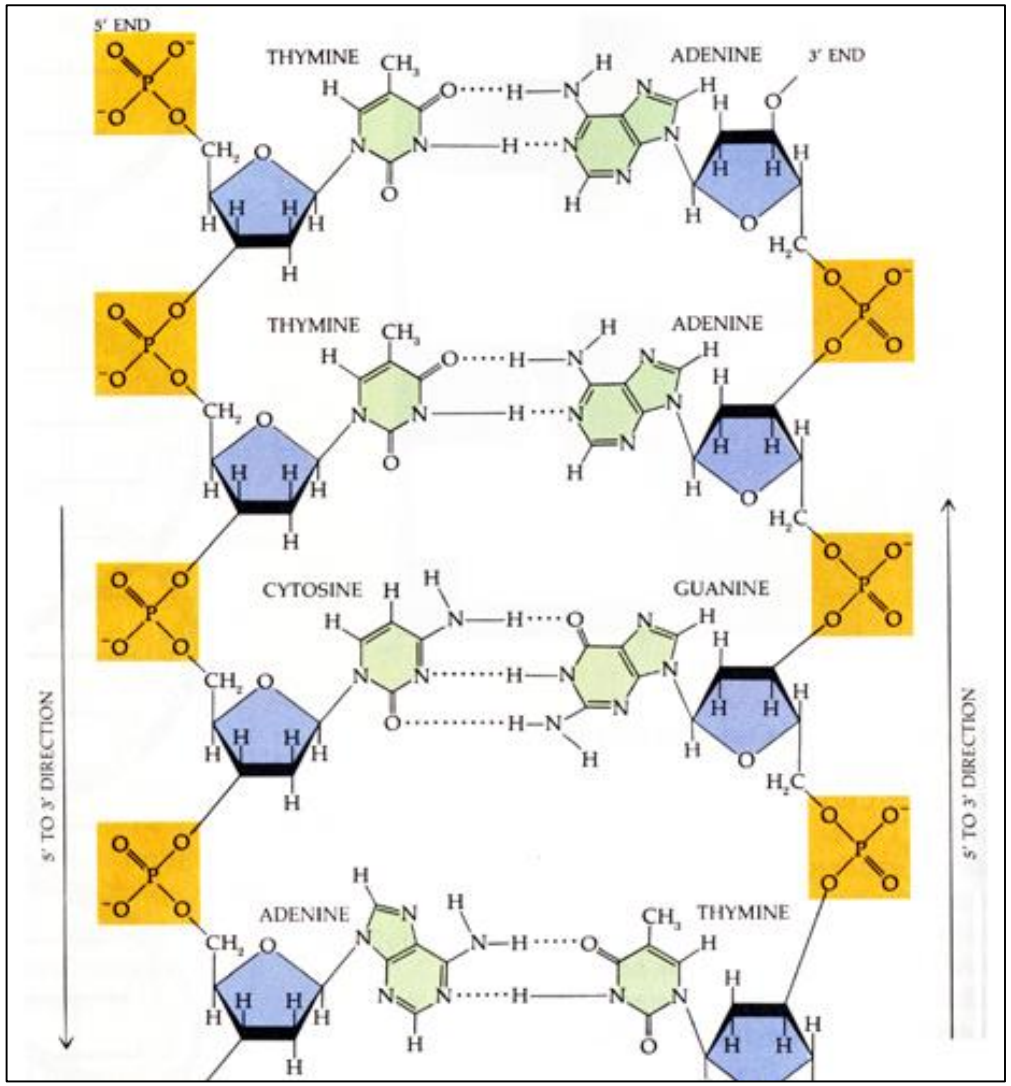


At physiological pH, some amino acid R-groups are charged, because of dissociation or association of a proton by, e.g., a carboxyl or amino group.

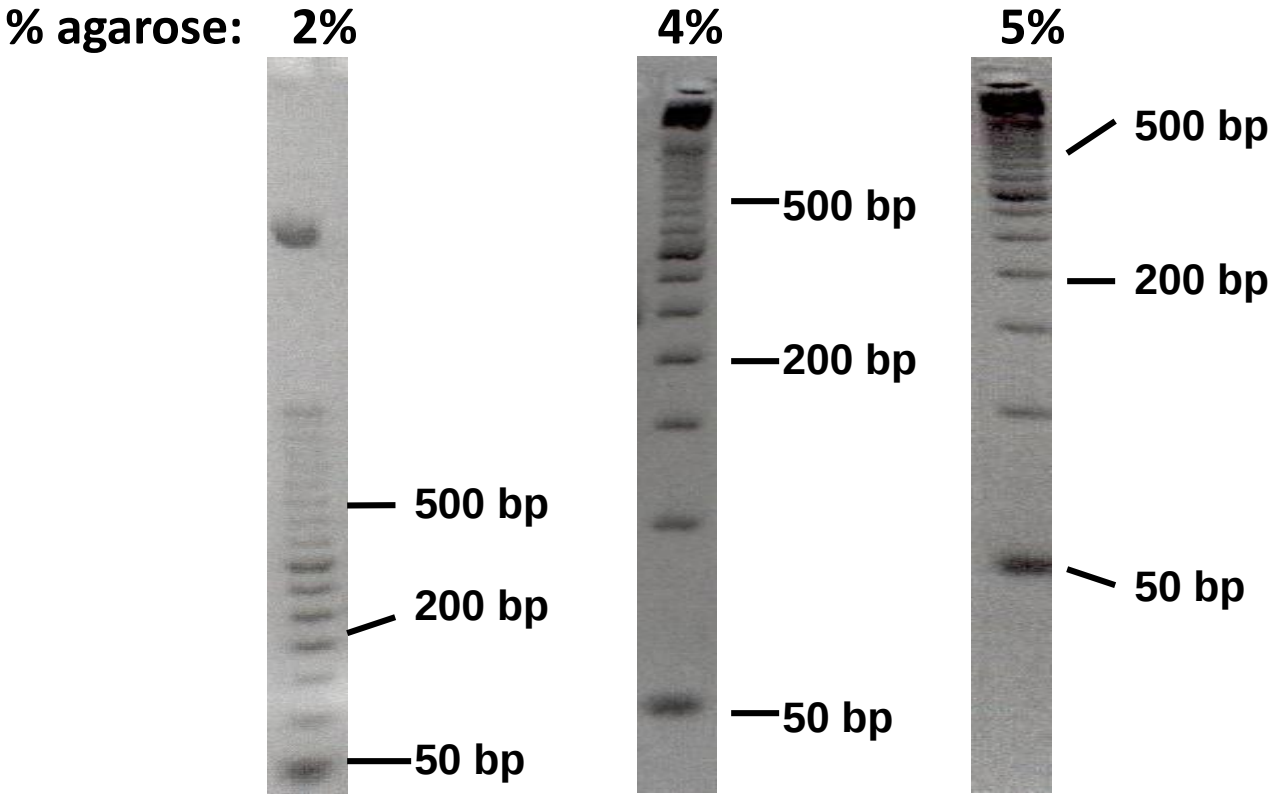
Modified from http://www.phys.sinica.edu.tw/TIGP-NANO/Course/2010_Spring/Classnotes/AAC_Chap%203%20Principles%20of%20Electrophoresis%20New_20100422.pdf

Where are the charges from? DNA

DNA Backbone



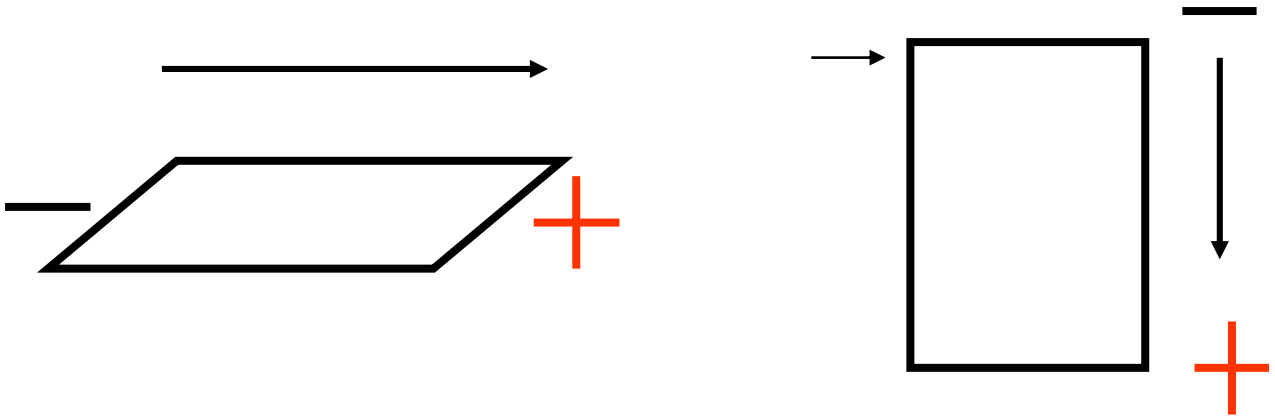
The movement of molecules is impeded in the gel so that molecules will collect or form a band according to their speed of migration.



The concentration of gel/buffer will affect the resolution of fragments of different size ranges

Gel Electrophoresis

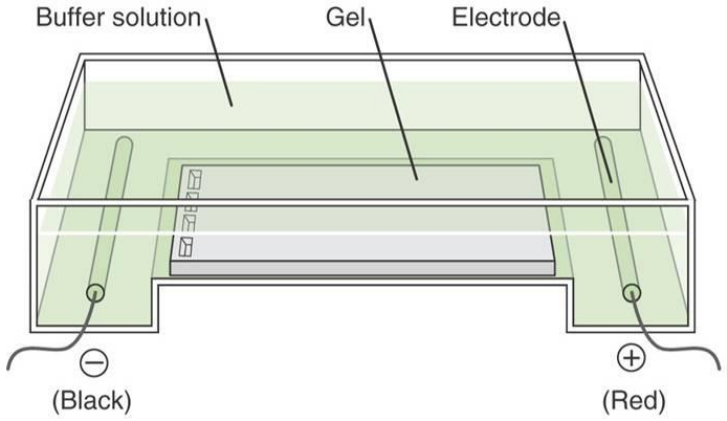
- Slab gel electrophoresis can have either a horizontal or vertical format.

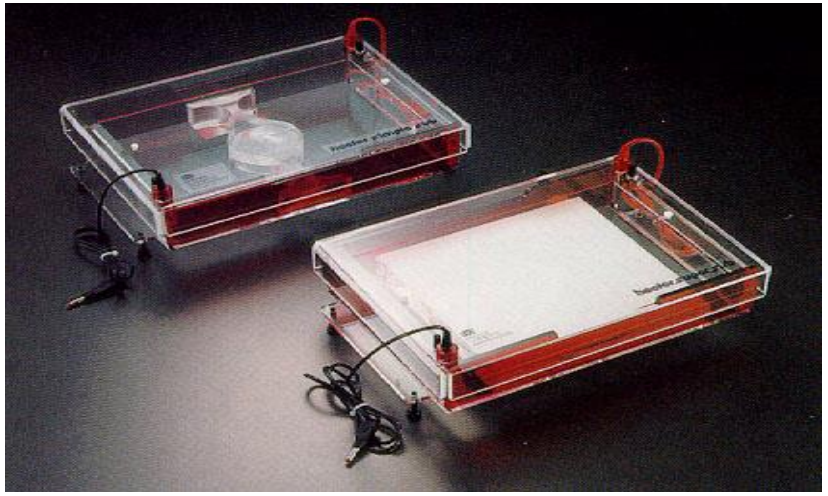


- Sample is introduced into wells at the top of the gel.

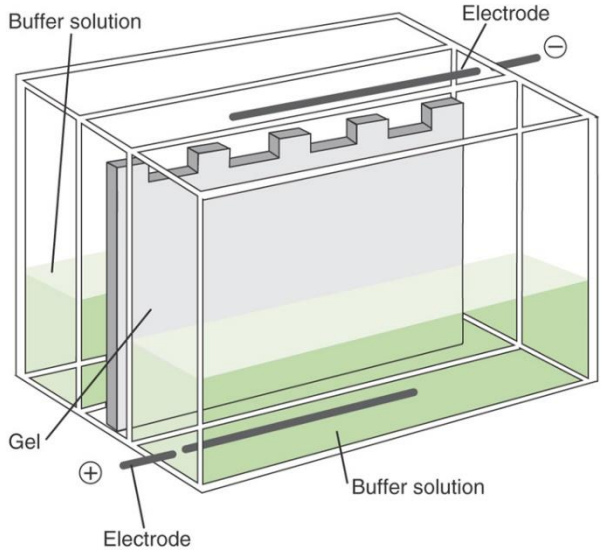
Basic Electrophoresis Apparatus

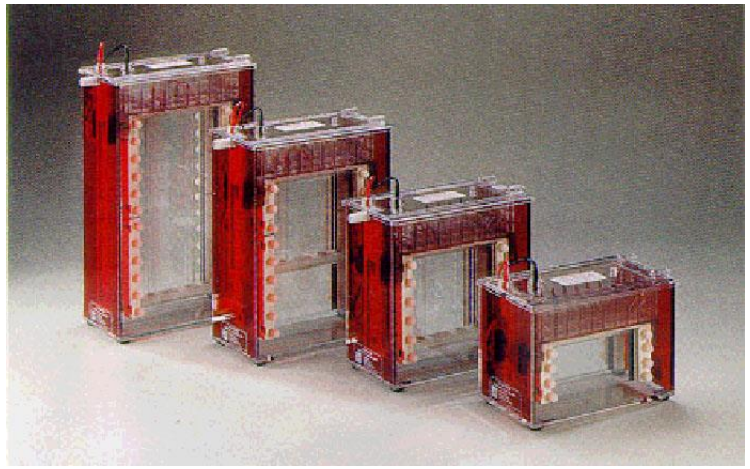
Horizontal or submarine gel





Vertical gel

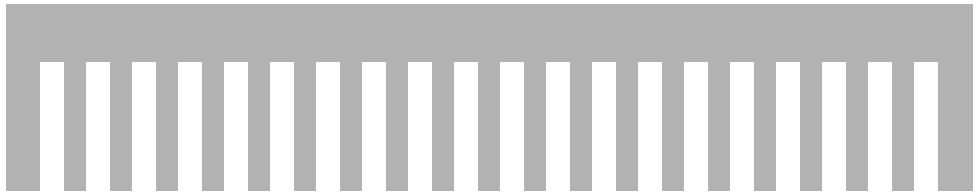




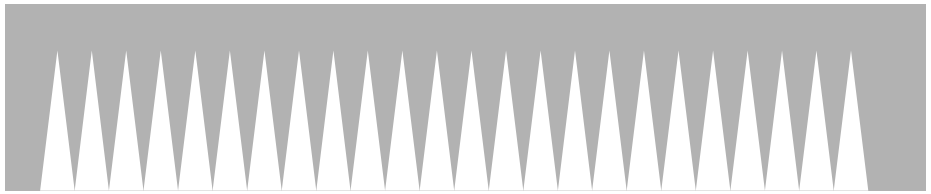
Adapted from www.fasdavis.com

Basic Electrophoresis Apparatus

Combs are used to put wells in the cast gel for sample loading.



- Regular comb: wells separated by an “ear” of gel



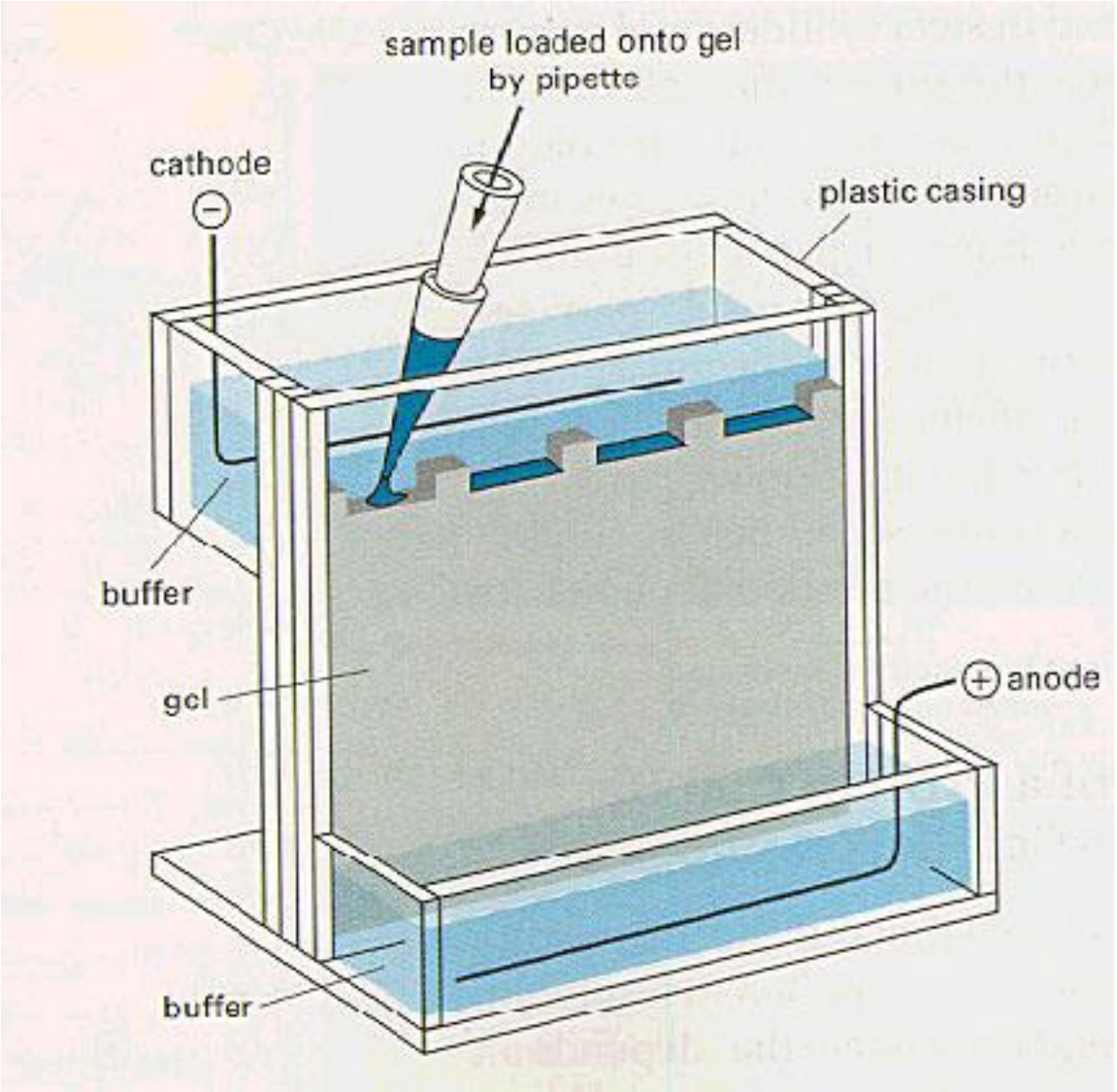
- Houndstooth comb: wells immediately adjacent

Running a Gel

- Use the proper gel concentration for sample size range.
  - 0.5–5% agarose
  - 3.5–20% polyacrylamide
- Use the proper comb (well) and gel size.

Running a Gel

In most electrophoresis units, the gel is mounted between two buffer chambers containing separate electrodes so that the **only electrical connection between the two chambers is through the gel**.

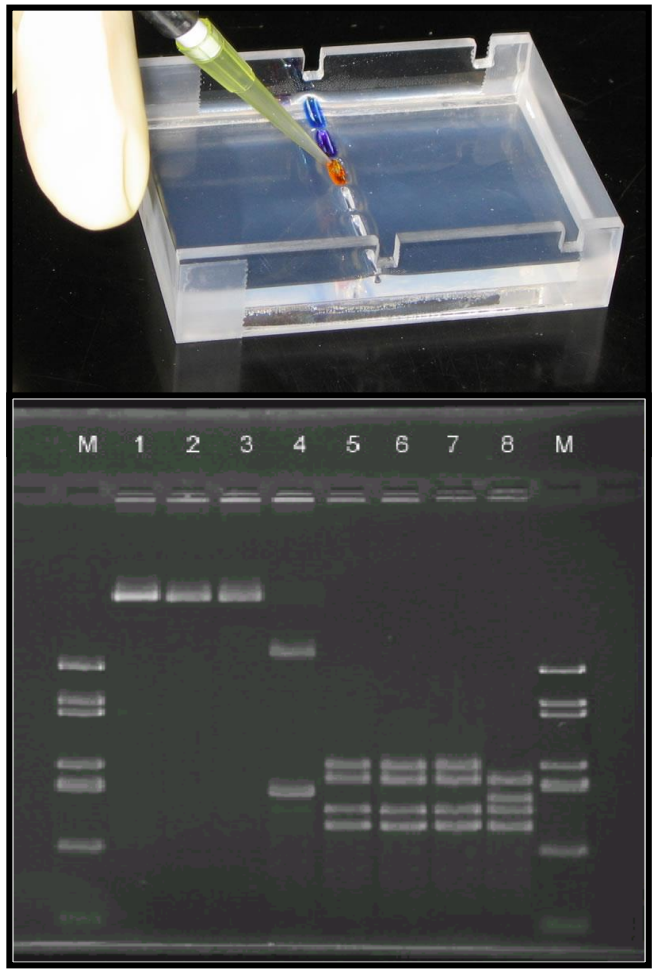


Adapted from www.fsdavis .com

Running a Gel

Load sample mixed with tracking dye (dye + density agent)

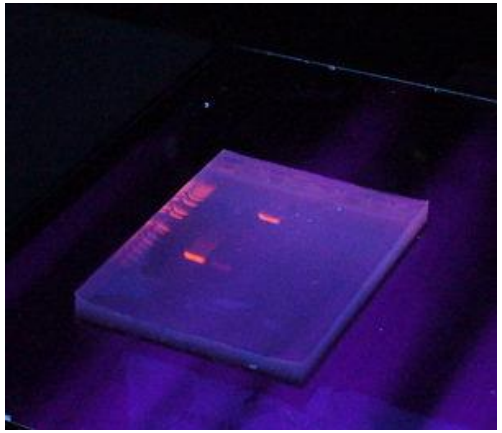
Adapted from www.fsdavis .com



http://www.antibiotic.ru/en/mbio/pub/images/p233r2.gif

Running a Gel

- Detect bands by staining during or after electrophoresis, several dyes available, examples are:
- Ethidium bromide: for double-stranded DNA
- SyBr green or SyBr gold: for single- or double-stranded DNA or for RNA
- Silver stain: more sensitive for single- or double-stranded DNA or for RNA and proteins
- Coomassie blue: for proteins



https://en.wikipedia.org/wiki/Ethidium_bromide

Interrelation of Resistance (R), Voltage (E), Current (I) and Power (P)

- Two basic electrical equations are important in electrophoresis
  - The first is Ohm's Law, $E = IR$
  - The second is $P = EI$

  This can also be expressed as $P = I^2R$

  Ohm's law demonstrates that an increase in voltage with a constant resistance will increase the current.

  Current produces heat, which must be dissipated or it will be absorbed by the system. Excessive heat can cause the DNA sample to migrate irregularly within the sample lane, resulting in the generation of sample bands that "smile." In very severe cases the gel can literally fall apart.

- In electrophoresis, one electrical parameter, either current, voltage, or power, is always held constant

Consequences

- Under constant current conditions (velocity is directly proportional to current), the velocity of the molecules is maintained, but heat is generated.

- Under constant voltage conditions, the velocity slows, but no additional heat is generated during the course of the run

- Under constant power conditions, the velocity slows but heating is kept constant

The Net Charge is Determined by the pH of the Medium

- Proteins are amphoteric compounds, that is, they contain both acidic and basic residues

- Each protein has its own characteristic charge properties depending on the number and kinds of amino acids carrying amino or carboxyl groups

- Nucleic acids, unlike proteins, are not amphoteric. They remain negative at any pH used for electrophoresis

Temperature and Electrophoresis

Important at every stage of electrophoresis

- During Polymerization
  - Exothermic Reaction
  - Gel irregularities
  - Pore size
- During Electrophoresis
  - Denaturation of proteins
  - Smile effect
  - Temperature Regulation of Buffers

Electrophoresis Buffers

- Carry current and protect samples during electrophoresis.
- Tris Borate EDTA (TBE), Tris Acetate EDTA (TAE), Tris Phosphate EDTA (TPE) used most often for DNA.
- 10 mM sodium phosphate buffer used for RNA.
- Buffer additives modify sample molecules.
  - Formamide, urea (denaturing agents)

More on buffers-

Extra resource: https://worldwide.promega.com/resources/product-guides-and-selectors/protocols-and-applications-guide/buffers-for-biochemical-reactions/

Summary

- Electrophoresis is used to separate molecules by charge.
- Nucleic acid fragments/proteins can be resolved on agarose or polyacrylamide gels.
- The net charge of a sample molecule determines its direction of movement and significantly affects its mobility.
- Many factors affect electrophoresis; such as pH, temperature, voltage/current/pore size of gels etc.

https://www.youtube.com/watch?v=eImaWphK2h4

How gel electrophoresis works?

- http://www.sumanasinc.com/webcontent/animations/content/gelelectrophoresis.html

  Includes a quiz as well

- Casting and running of gel

https://www.youtube.com/watch?v=59aIHlUoNQs

What is the Role of the Solid Support Matrix?

- It inhibits convection and diffusion, which would otherwise impede separation of molecules.
- It allows a permanent record of results through staining after run (also fixing using ‘trichloroacetic acid’ in case of proteins).
- It can provide additional separation through molecular sieving.

The gel (matrix)

- The gel (matrix) itself is composed of either agarose or polyacrylamide.

- Polyacrylamide is a cross-linked polymer of acrylamide.
  - Acrylamide is a potent neurotoxin and should be handled with care!

Types of electrophoresis

Two major types of electrophoresis (dependent on matrix type):

–agarose gel electrophoresis

–polyacrylamide gel electrophoresis

Both forms of electrophoresis can be modified for different applications including:

- nucleic acid sequencing
- analysis of protein purity
- separation of DNA and RNA
- determination of pI (isoelectric point) of proteins
- estimation of native size of proteins

Agarose - separation of large molecules
8 kD to 800,000 kD

Polyacrylamide - separation of smaller molecules
0.2 kD to 500 kD

Masses of proteins are often expressed in daltons. For example, a protein with a molecular weight of 64,000 g/mol has a mass of 64,000 daltons or 64 kDa in short.

Agarose and Polyacrylamide

- Although agarose and polyacrylamide differ greatly in their physical and chemical structures, they both make porous gels.

- A porous gel acts as a sieve by retarding or, in some cases, by completely obstructing the movement of macromolecules while allowing smaller molecules to migrate freely.

- By preparing a gel with a restrictive pore size, the operator can take advantage of molecular size differences among proteins

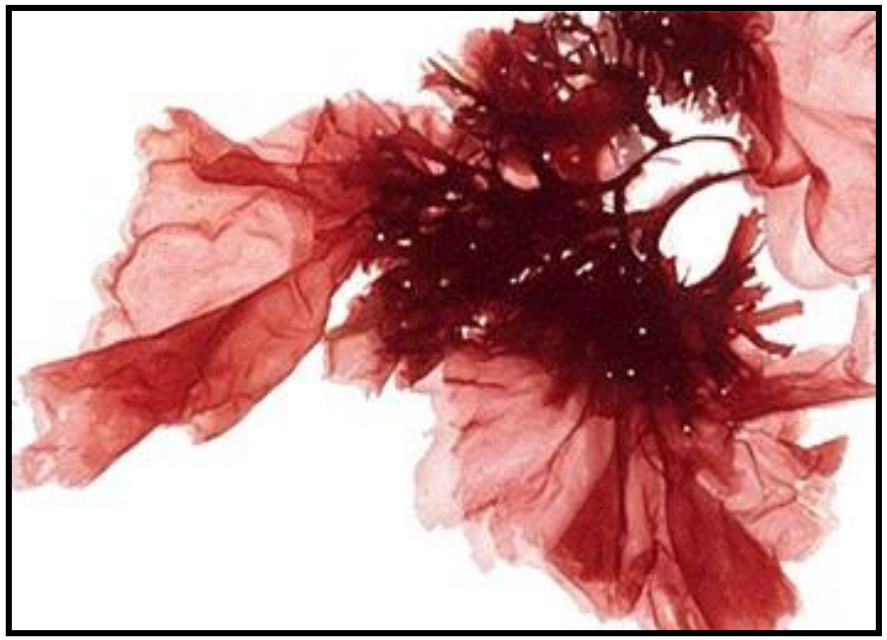
Agarose and Polyacrylamide

- Because the pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids, large proteins and protein complexes

- Polyacrylamide, which makes a small pore gel, is used to separate most proteins and small oligonucleotides

- Both are relatively electrically neutral

Agarose

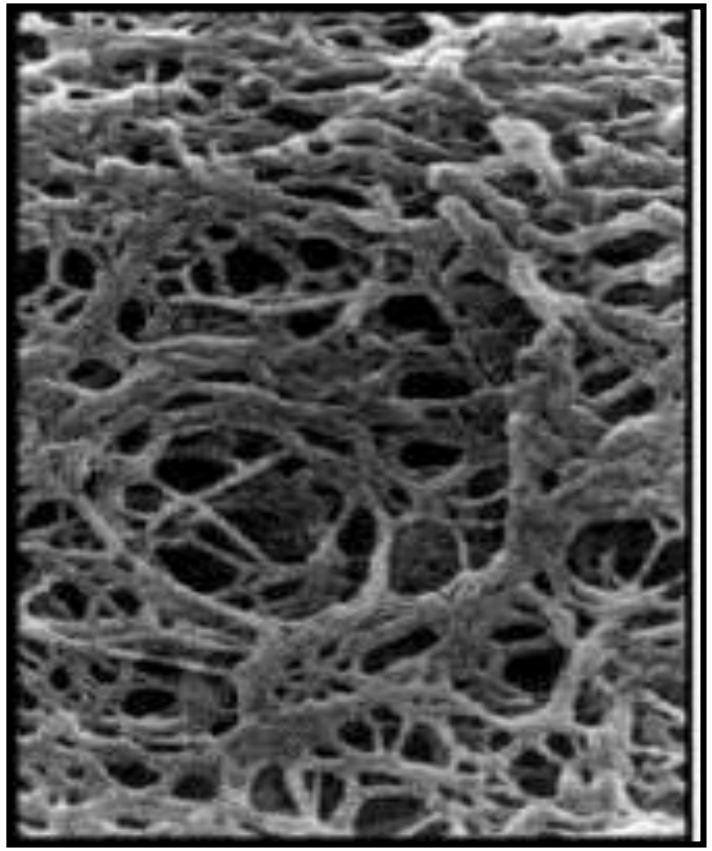


http://www.steve.gb.com/images/

- Neutral polysaccharide
- Harvested from Rhodophyceae algal cell walls

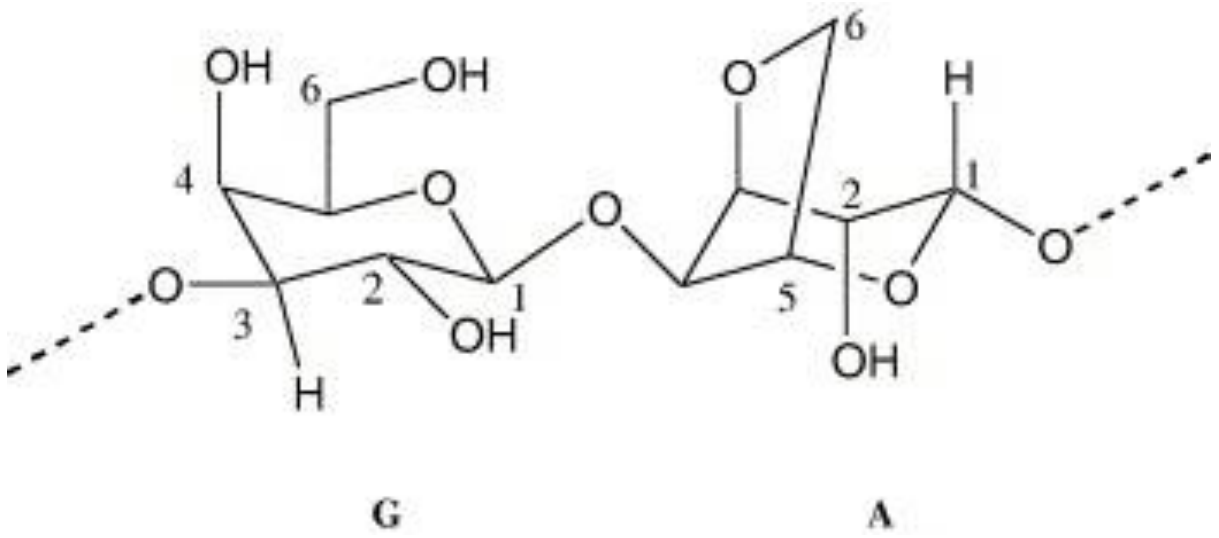
Agarose

- Alternating sugar units form linear chains
- Cross-linking chains via hydrogen bonds forms porous matrix
- Heat breaks matrix apart
- Cooling forms a matrix with an “average” pore size



http://www.bioscience-beads.com/images

Structure of the Repeating Unit of Agarose, 3,6-anhydro-L-galactose

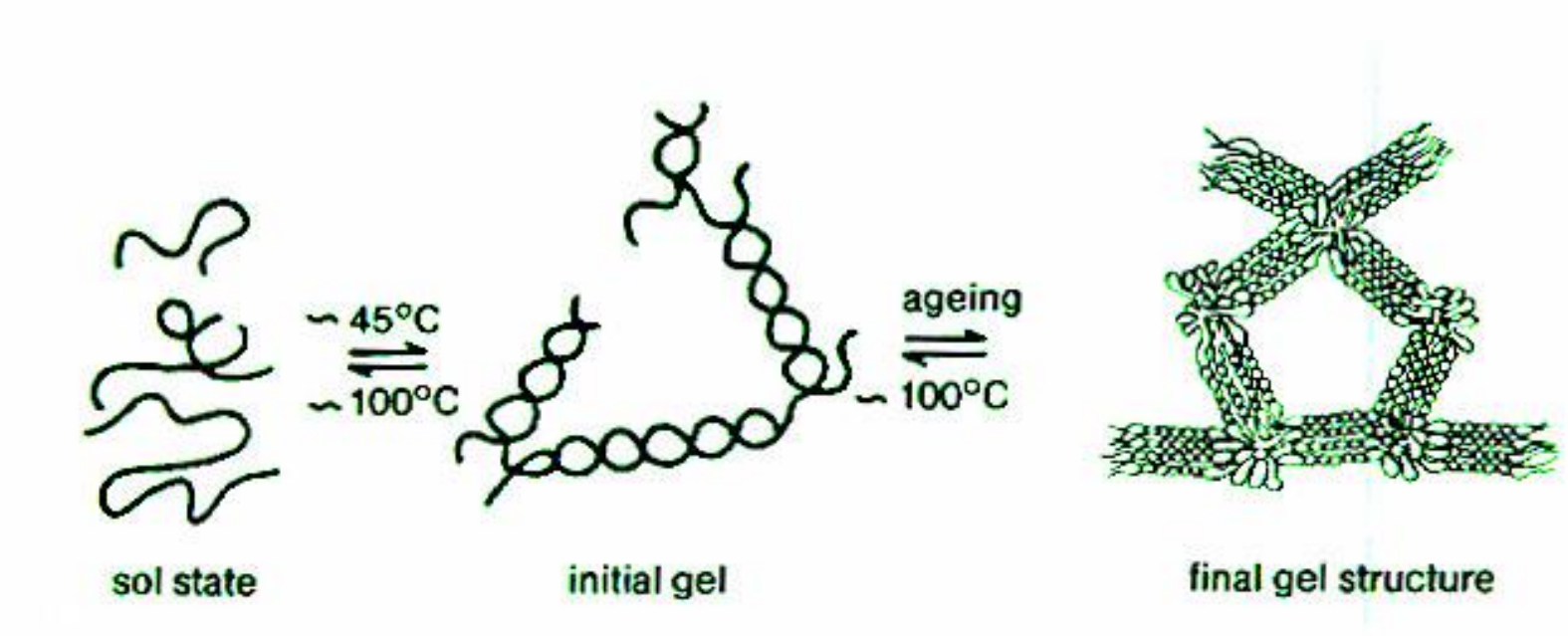


Basic disaccharide repeating units of agarose

G: 1,3-β-d-galactose

A: 1,4-α-l-3,6-anhydrogalactose

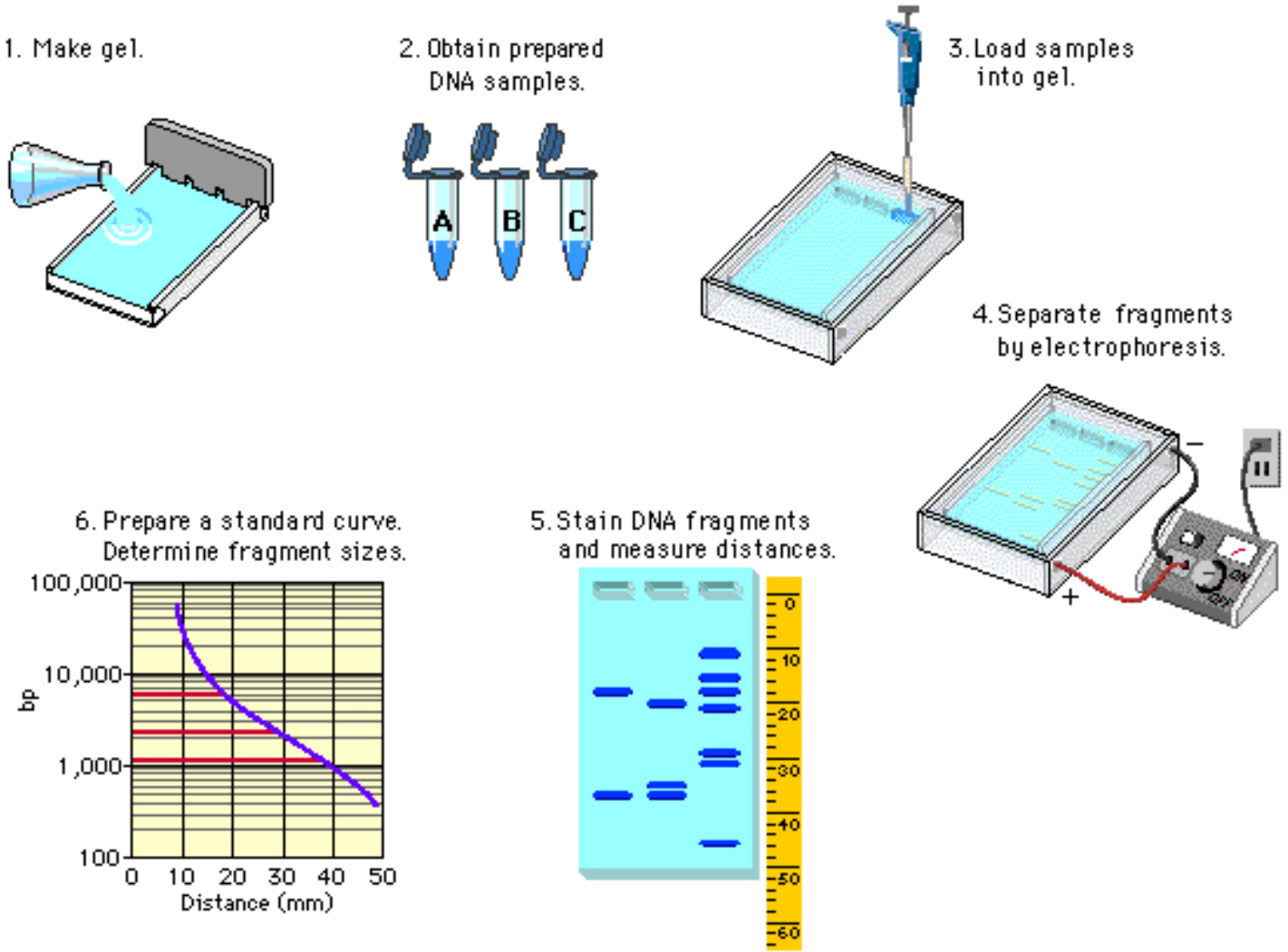
Gel Structure of Agarose



Agarose Gels

- Agarose dissolves when added to boiling liquid. It remains in a liquid state until the temperature is lowered to about 40° C at which point it gels
- The pore size may be predetermined by adjusting the concentration of agarose in the gel
- Agarose gels are fragile, however. They are actually hydrocolloids, and they are held together by the formation of weak hydrogen and hydrophobic bonds

Agarose gel electrophoresis of nucleic acids

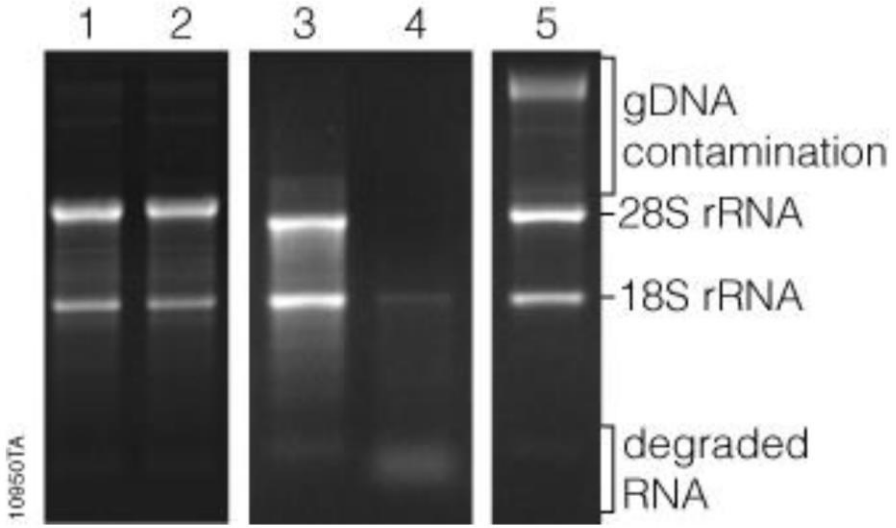


http://www.phschool.com/science/biology_place/labbench/lab6/images/overview.gif

Electrophoresis of RNA

- RNA is separated on basis of size like DNA
- But RNA has to be heated in dilute formamide or glyoxal to prevent formation of secondary structures.
- Buffers for RNA electrophoresis contain formaldehyde. i.e RNA are run in “denaturing gels”.
- **RNA quality can be determined by electrophoresis**
  - **2:1 ratio of 28s to 18s**

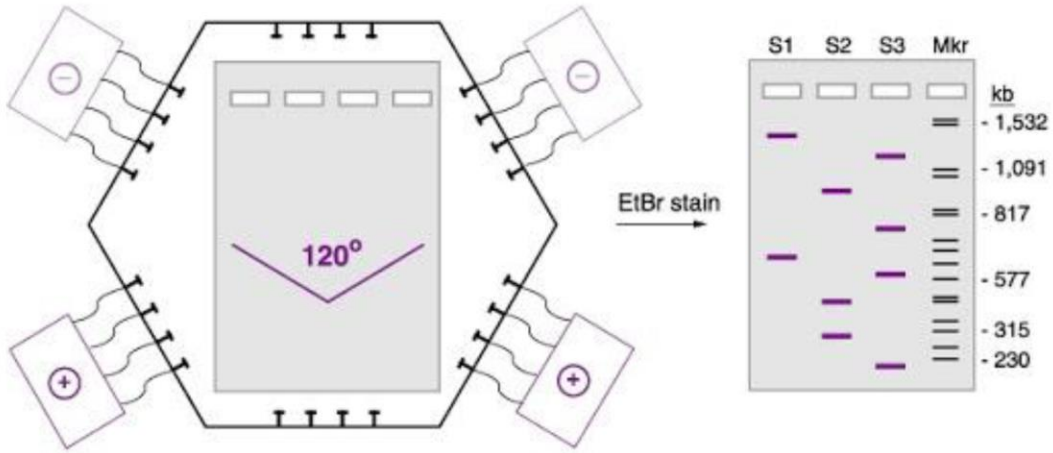
Since 28s is degraded earlier that 18s
28s = 5Kb
18s= 2Kb



https://worldwide.promega.com/resources/pubhub/methods-of-rna-quality-assessment/

Electrophoresis of larger DNA fragments

- Large DNA fragments of upto 12Mb are separated by **pulsed field gel electrophoresis (PFGE).**
- PFGE differs from conventional electrophoresis as it used two or more alternating electric fields.
- Larger molecules will take a longer time to reorientate than smaller molecules hence separation is on the basis of size.

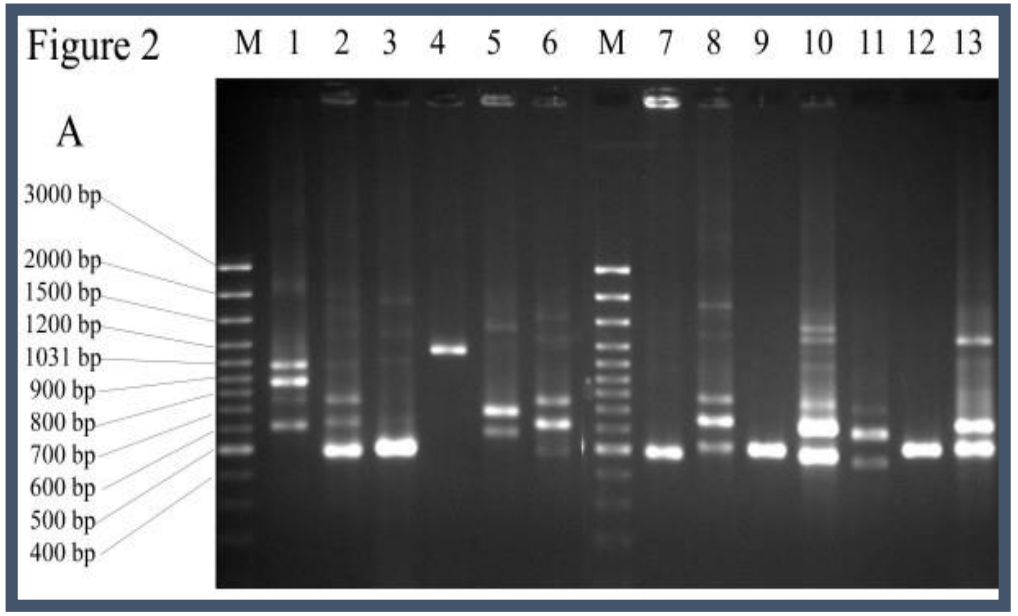


Electric field alternates 120° every 90 seconds for 18 to 24 hours at 14° C

http://uvmgg.wikia.com/wiki/Pulsed_Field_Gel_Electrophoresis

Interpreting results?

- Molecular weight markers are DNA fragments of known size
- Comparison of sample bands to markers allows…
  - Visible confirmation of desired product
  - Quantification of sample DNA

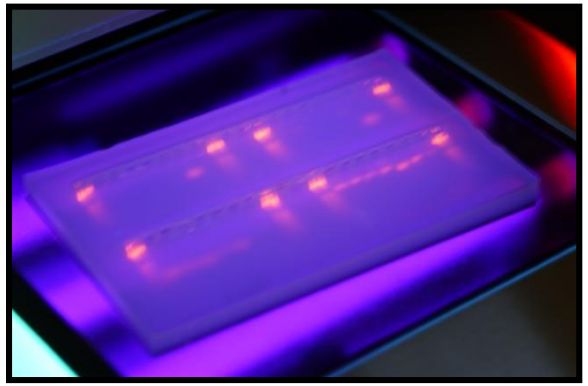


http://www.biomedcentral.com/content/figures/1471-2180-5-63-2.jpg

Visualizing DNA

Ethidium Bromide binds to DNA

Fluorescence under UV light makes bands visible



http://www.lanl.gov/news/albums/bioscience/AnthraxUVplate.sized.jpg

Summary of agarose gels

- DNA (-ve) migrates towards the anode (positive terminal/electrode) .

Rate of migration depends on several factors:

–molecular size of nucleic acid (smaller migrate faster)

–agarose concentration (high concentration for smaller molecules)

–conformation of nucleic acid (supercoiled migrate faster)

–applied voltage

–Temperature (high temp can result in faster run but smiley gels)

–presence of intercalating dyes

–Ionic strength of electrophoretic buffer (e.g 50xTAE vs water)