GENETIC ENGINEERING

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Lecture(2)

Working with nucleic acids

- Before examining some of the specific techniques used in gene manipulation, it is useful to consider the basic methods required for :
  - Handling.
  - Quantifying.
  - Analyzing nucleic acid molecules.
- It is often difficult to make the link between theoretical and practical aspects of a subject, and an appreciation of the methods used in routine work with nucleic acids this may be occurred with help of more detailed description for techniques of gene cloning and analysis.

Laboratory requirements

One of the striking aspects of gene manipulation technology is that many of the procedures can be carried out with a fairly basic laboratory setup. The requirements can be summarized under three headings:

- **1. General laboratory facilities.**
- **2. Cell culture and containment .**
- **3. Processing and analysis.**

Isolation of DNA and RNA

Every gene manipulation experiment requires a source of nucleic acid, in the form of either DNA or RNA. It is therefore important that reliable methods are available for isolating these components from cells. There are three basic requirements:

- (1) opening the cells in the sample to expose the nucleic acids for further processing.
- (2) separation of the nucleic acids from other cell components.
- (3) recovery of the nucleic acid in purified form.

- A variety of techniques may be used, ranging from simple procedures with few steps up to more complex purifications involving several different stages.

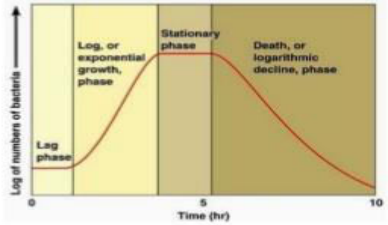
APPLICATIONS

DNA extraction is done for:

1. Tissue typing for organ transplant.
2. Detection of pathogens
3. Human identity testing
4. Genetic research
5. PCR
6. Restriction fragment length polymerization.
7. Hyberdization method.

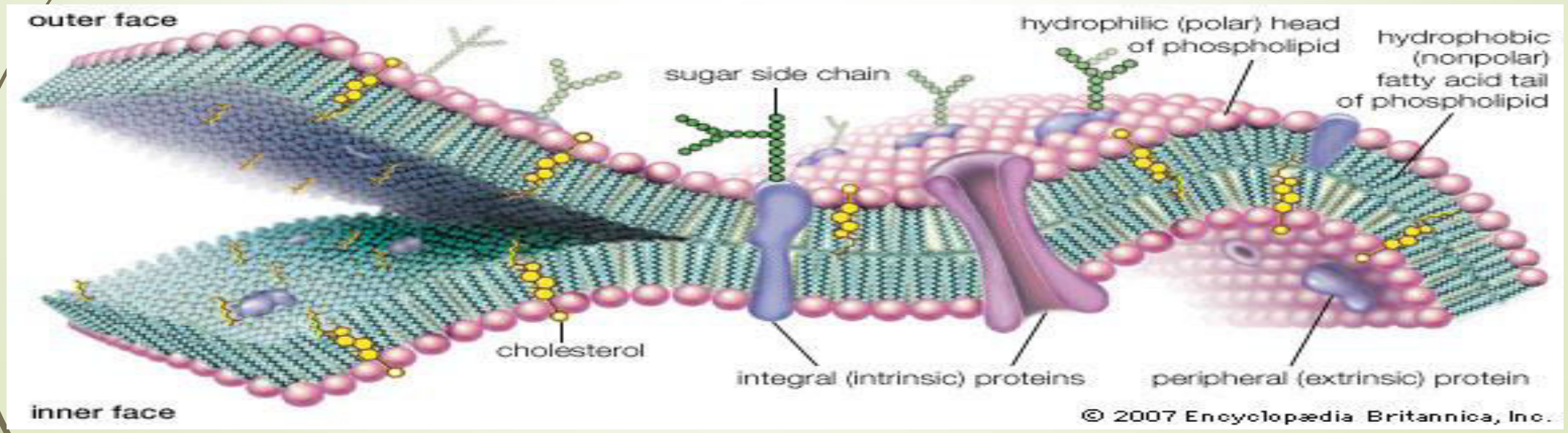
State of DNA

- Cell cycle has four phases
  1. Lag phase
  2. log phase
  3. Stationary phase
  4. Death phase



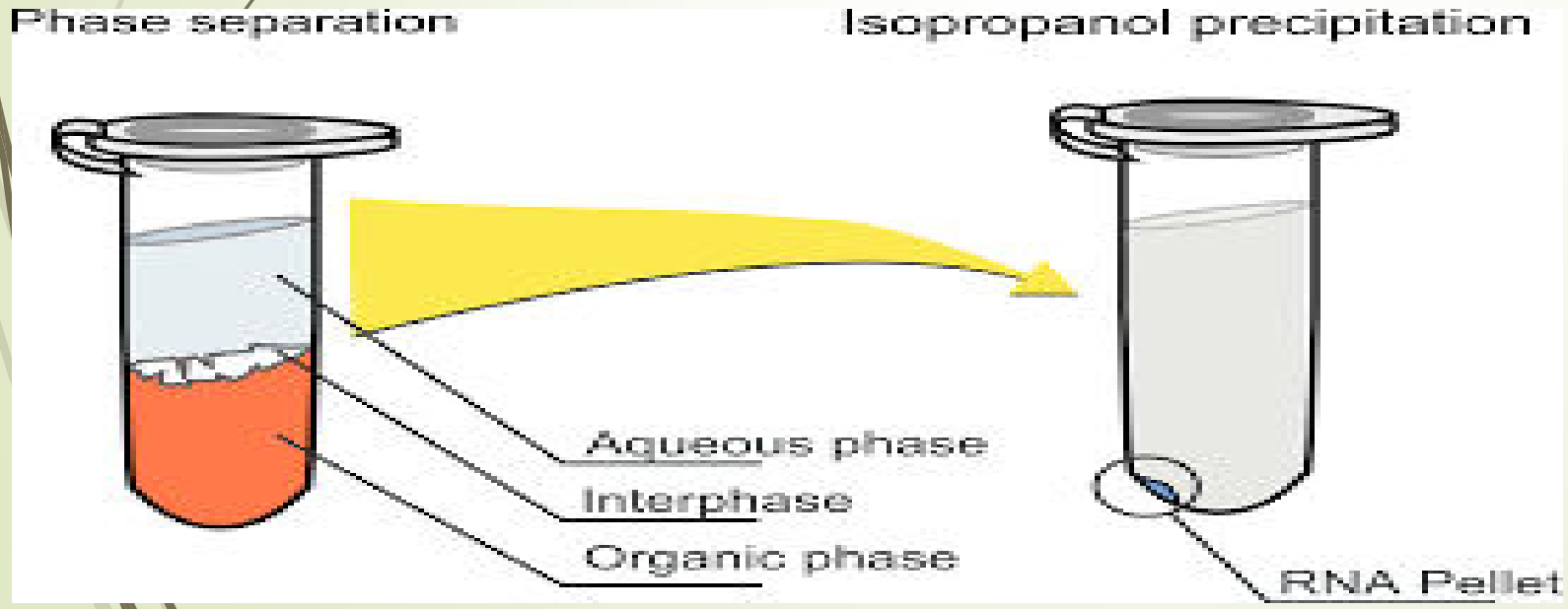
Isolation of DNA and RNA

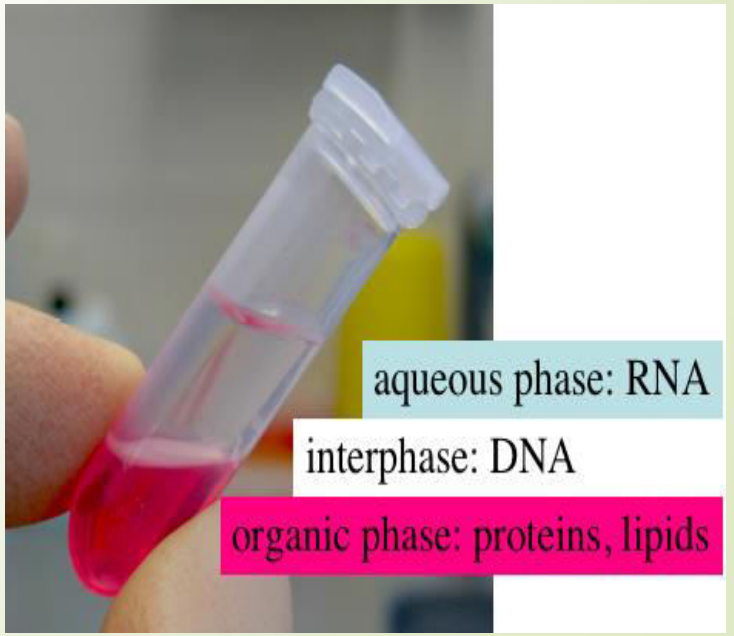
- **The first step** in any isolation protocol is *disruption* of the starting material, which may be **viral, bacterial, plant, or animal.**
  - The method used to open cells should be as gentle as possible, preferably utilising enzymatic degradation of cell wall material (if present) Cells have to be opened to enable nucleic acids to be isolated; opening cells should be done as gently as possible to avoid shearing large DNA molecules.
  - and detergent lysis of cell membranes. If more vigorous methods of cell disruption are required (as is the case with some types of plant cell material.



Isolation of DNA and RNA

- Following cell disruption, most methods involve a *deproteinisation stage*.
- This can be achieved by one or more extractions using phenol or phenol/chloroform mixtures. On the formation of an emulsion and subsequent centrifugation to separate the phases, protein molecules partition into the phenol phase and accumulate at the interface. The nucleic acids remain mostly in the upper aqueous phase and may be precipitated from solution using isopropanol or ethanol. Some techniques do not require the use of phenolic mixtures and are safer and more pleasant to use than phenol-based extraction media.





Isolation of DNA and RNA

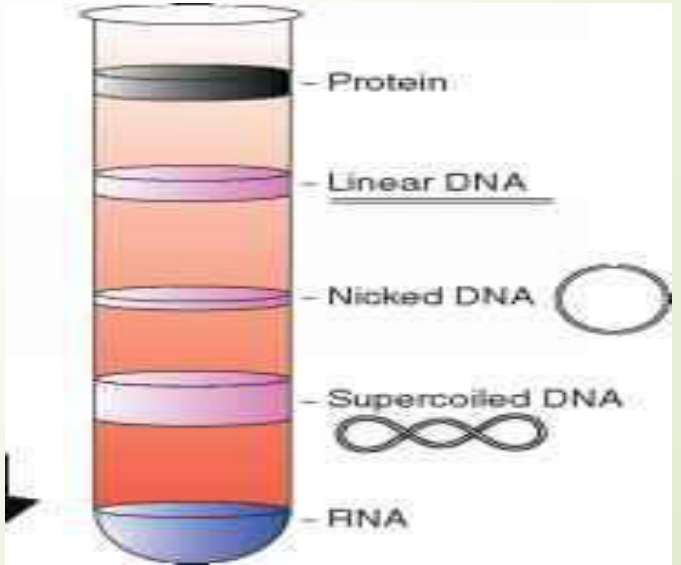
- If a DNA preparation is required, the enzyme ribonuclease (RNase) can be used to digest the RNA in the preparation.
- If mRNA is needed Once broken open, cell preparations can be deproteinised and the nucleic acids purified by a range of techniques.
- Some applications require highly purified nucleic acid preparations; some may be able to use partially purified DNA or RNA.
- for cDNA synthesis, a further purification can be performed by affinity chromatography using oligo(dT)-cellulose to bind the poly(A) tails of eukaryotic mRNAs . This gives substantial enrichment for mRNA and enables most of the contaminating DNA, rRNA, and tRNA to be removed.

Isolation of DNA and RNA

The technique of gradient centrifugation is often used to prepare DNA, particularly plasmid DNA (pDNA). In this technique a caesium chloride (CsCl) solution containing the DNA preparation is spun at high speed in an ultracentrifuge.

Over a long period (up to 48 h in some cases) a density gradient is formed and the pDNA forms a band. Solutions of nucleic acids are used to enable very small amounts to be handled easily, measured, and dispensed. At one position in the centrifuge tube. The band may be taken off and the CsCl removed by dialysis to give a pure preparation of pDNA.

As an alternative to gradient centrifugation, size exclusion chromatography (gel filtration) or similar techniques may be used.



Handling and quantification of nucleic acids

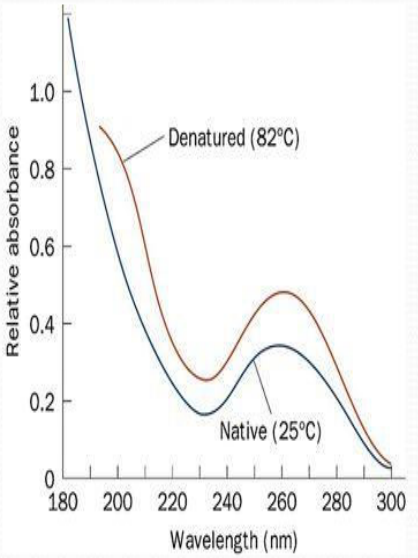
It is often necessary to use very small amounts of nucleic acid (typically micro-, nano-, or picograms) during a cloning experiment.

- It is obviously impossible to handle these amounts directly, so most of the measurements that are done involve the use of *aqueous solutions of DNA and RNA*.
- The concentration of a solution of nucleic acid can be determined by measuring the absorbance at 260 nm, using a spectrophotometer.
  - An A260 of 1.0 is equivalent to a concentration of 50 µg ml−1 for double-stranded DNA
  - or 40 µg ml−1 for single stranded DNA or RNA.
  - If the A280 is also determined
- the A260/A280 ratio indicates if there are contaminants present, such as residual phenol or protein. The A260/A280 ratio should be around 1.8 for pure DNA and 2.0 for pure RNA preparations.

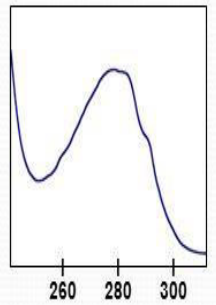
micro	µ	1000^{-2}	10^{-6}	0.000 001
nano	n	1000^{-3}	10^{-9}	0.000 000 001
pico	p	1000^{-4}	10^{-12}	0.000 000 000 001

Quantification of Nucleic Acids

- Spectrophotometric, based on absorbance at 260 nm.
- OD_{260} of 1.0 =
  - 50 µg/mL double-stranded DNA*.
  - 37 µg/mL single-stranded DNA.
  - 40 µg/mL RNA.
- OD_{260} can be problematic:
  - Protein absorbs strongly at 280 nm.
  - Protein is the most common contaminant in DNA preparations.
- Purity / contamination can be estimated via OD_{260}:OD_{280} ratio.
  - 1.7 – 2.0 is good.
  - 1.0 is bad (mostly protein).

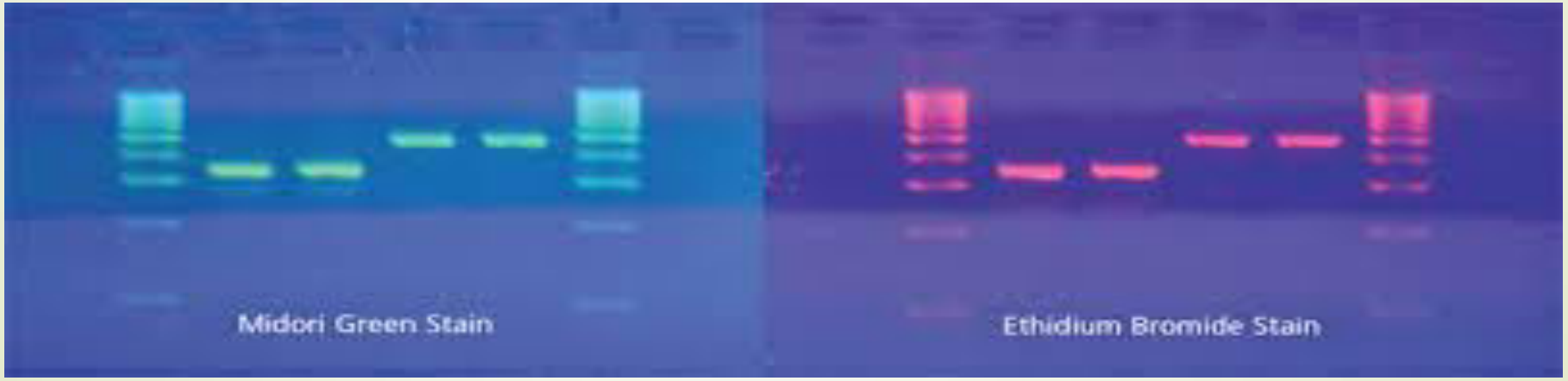


*µg/mL = ABS_{260} x 50



Handling and quantification of nucleic acids

- In addition to spectrophotometric methods, the concentration of DNA may be estimated by *monitoring the fluorescence* of bound ethidium bromide.
- This dye binds between the DNA bases (intercalates) and fluoresces orange when illuminated with ultraviolet (uv) light.
- By comparing the fluorescence of the sample with that of a series of standards, an estimate of the concentration may be obtained.
- This method can detect as little as 1--5 ng of DNA and may be used when uv-absorbing contaminants make spectrophotometric measurements impossible. Having determined the concentration of a solution of nucleic acid, any amount (in theory) may be dispensed by taking the appropriate volume of solution.



Handling and quantification of nucleic acids

- In this way nanogram or picogram amounts may be dispensed with reasonable accuracy. Precipitation of nucleic acids is an essential technique that is Nucleic acids can be concentrated by using alcohol to precipitate the DNA or RNA from solution; the precipitate is recovered by centrifugation and can then be processed as required. used in a variety of applications. The two most commonly used precipitants are isopropanol and ethanol, ethanol being the preferred choice for most applications. When added to a DNA solution in a ratio, by volume, of 2:1 in the presence of 0.2 M salt, ethanol causes the nucleic acids to come out of solution. Although it used to be thought that low temperatures (−20◦C or −70◦C) were necessary, this is not an absolute requirement, and 0◦C appears to be adequate. After precipitation the nucleic acid can be recovered by centrifugation, which causes a pellet of nucleic acid material to form at the bottom of the tube. The pellet can be dried and the nucleic acid resuspended in the buffer appropriate to the next stage of the experiment.

Labelling of nucleic acids

- A major problem encountered in many cloning procedures is that of keeping track of the small amounts of nucleic acid involved.
- This problem is magnified at each stage of the process, because losses mean that the amount of material usually diminishes after each step.
- One way of tracing the material is to label the nucleic acid with a marker of some sort, so that the material can be identified at each stage of the procedure.

Types of label – radioactive or not

- Radioactive tracers have been used extensively in biochemistry and molecular biology for a long time, and procedures are now well established.
- The most common isotopes used are:
  - tritium (3H)
  - carbon-14 (14C)
  - sulphur-35 (35S)
  - phosphorus-32 (32P).
- Tritium and 14C are low-energy emitters, with 35S being a ‘medium’-energy emitter and 32P being a high-energy emitter. Thus, 32P is more hazardous than Radioactive isotopes are often used to label nucleic acids, although they are more hazardous than non-radioactive labelling methods. the other isotopes and requires particular care in use.

Types of label – radioactive or not

1. There are also strict statutory requirements for the storage and disposal of radioactive waste materials. Partly because of the inherent dangers of working with high-energy isotopes, the use of alternative technologies such as:
   - fluorescent dyes
   - enzyme-linked labels
2. Although these methods do offer advantages for particular applications (such as DNA sequencing), for routine tracing experiments a radioactive label is still often the preferred choice. In this case the term radiolabelling is often used to describe the technique.
3. One way of tracing DNA and RNA samples is to label the nucleic acid with a radioactive molecule (usually a deoxynucleoside triphosphate (dNTP), labelled with 3H or 32P), so that portions of each reaction may be counted in a scintillation counter to determine the amount of nucleic acid present. This is usually done by calculation, taking into account the amount of radioactivity present in the sample.

Types of label – radioactive or not

- A second application of radiolabelling is in the production of highly radioactive nucleic acid molecules for use in hybridisation experiments. Such molecules are known as radioactive probes and Radioactive probes are very useful for identifying specific DNA or RNA sequences. have a variety of uses. The difference between labelling for tracing purposes and labelling for probes is largely one of specific activity, that is, the measure of how radioactive the molecule is. For tracing purposes, a low specific activity will suffice, but for probes a high specific activity is necessary. In probe preparation the radioactive label is usually the high-energy ß-emitter 32P.

End labelling

- In the end labelling technique, the enzyme polynucleotide kinase is used to transfer the terminal phosphate group of ATP onto 5 -hydroxyl termini of nucleic acid molecules. If the ATP donor is radioactively labelled, this produces a labelled nucleic acid of relatively low specific activity, as only the termini of each molecule become radioactive.

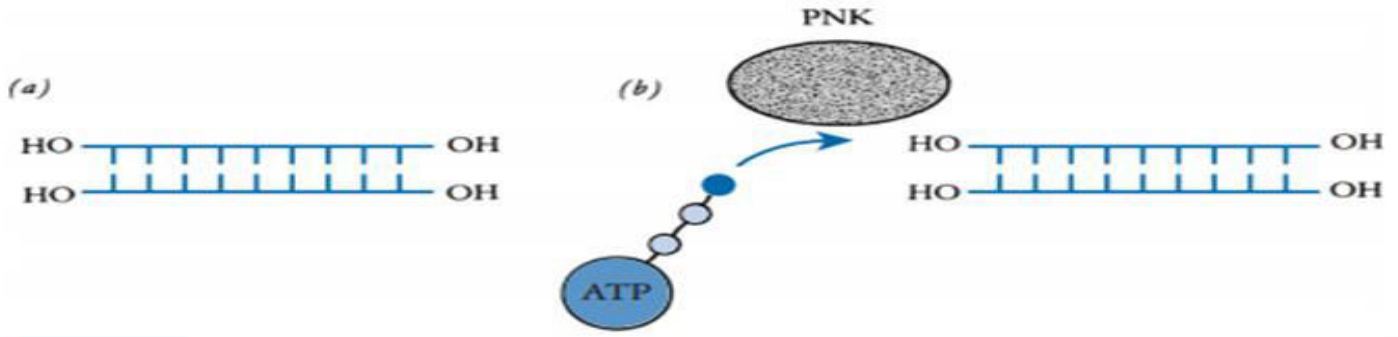


Fig. 3.2 End labelling DNA using polynucleotide kinase (PNK). (*a*) DNA is dephosphorylated using phosphatase to generate 5′-OH groups. (*b*) The terminal phosphate of [γ-^{32}P]ATP (solid circle) is then transferred to the 5′ terminus by PNK. The reaction can also occur as an exchange reaction with 5′-phosphate termini.

Nick translation

- Nick translation relies on the ability of the enzyme DNA polymerase I (to translate (move along the DNA) a nick created in the phosphodiester backbone of the DNA double helix. Nicks may occur naturally or may be caused by a low concentration of the nuclease DNase I in the reaction mixture. DNA polymerase I catalyses a strand-replacement reaction that incorporates new dNTPs into the DNA chain. If one of the dNTPs supplied is radioactive, the result is a highly labelled DNA molecule.

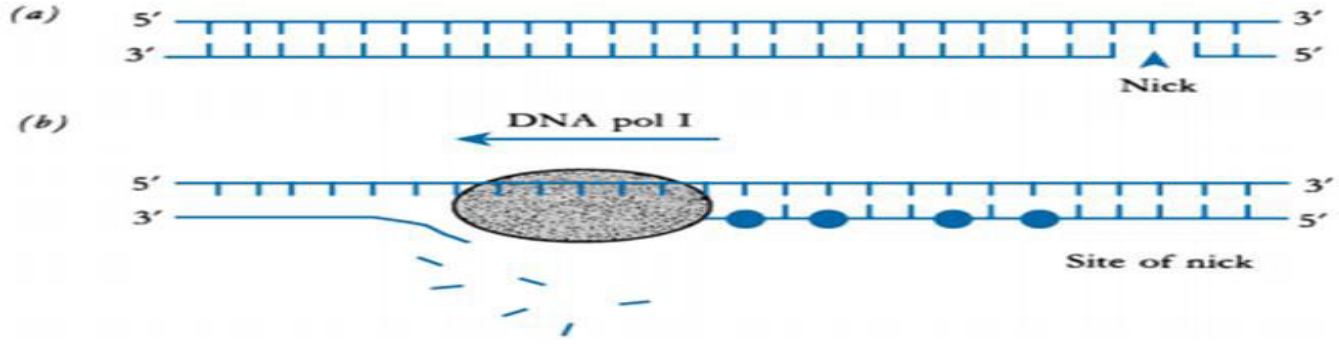


Fig. 3.3 Labelling DNA by nick translation. (*a*) A single-strand nick is introduced into the phosphodiester backbone of a DNA fragment using DNase I. (*b*) DNA polymerase I then synthesises a copy of the template strand, degrading the non-template strand with its 5′→3′ exonuclease activity. If [α-^{32}P]dNTP is supplied this will be incorporated into the newly synthesised strand (solid circles).

Labelling by primer extension

Labelling by primer extension refers to a technique that uses random oligonucleotides (usually hexadeoxyribonucleotide molecules -- sequences of six deoxynucleotides) to prime synthesis of a DNA strand In most labelling reactions not all the radioactive dNTP is incorporated into the target sequence, and non-incorporated isotope is usually removed before using the probe. by DNA polymerase. The DNA to be labelled is denatured by heating, and the oligonucleotide primers annealed to the single-stranded DNAs. The Klenow fragment of DNA polymerase .can then synthesise a copy of the template, primed from the 3 -hydroxyl group of the oligonucleotide. If a labelled dNTP is incorporated, DNA of very high specific activity is produced . In a radiolabelling reaction it is often desirable to separate the labelled DNA from the unincorporated nucleotides present in the reaction mixture. A simple way of doing this is to carry out a smallscale gel filtration step using a suitable medium. The whole process can be carried out in a Pasteur pipette, with the labelled DNA coming through the column first, followed by the free nucleotides. Fractions can be collected and monitored for radioactivity, and the data used to calculate total activity of the DNA, specific activity, and percentage incorporation of the isotope.

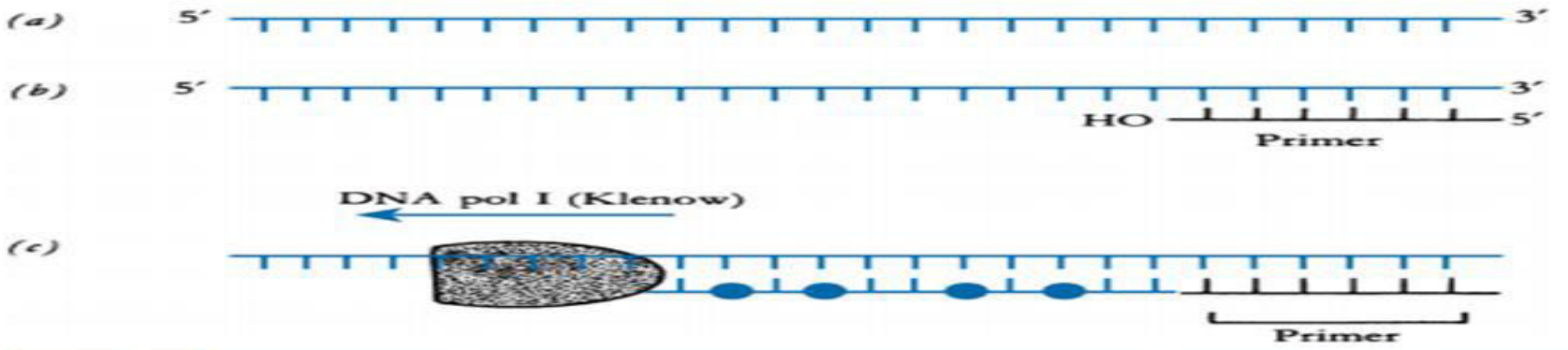
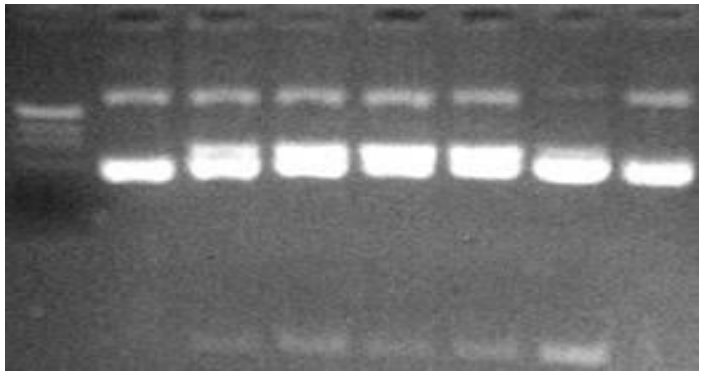
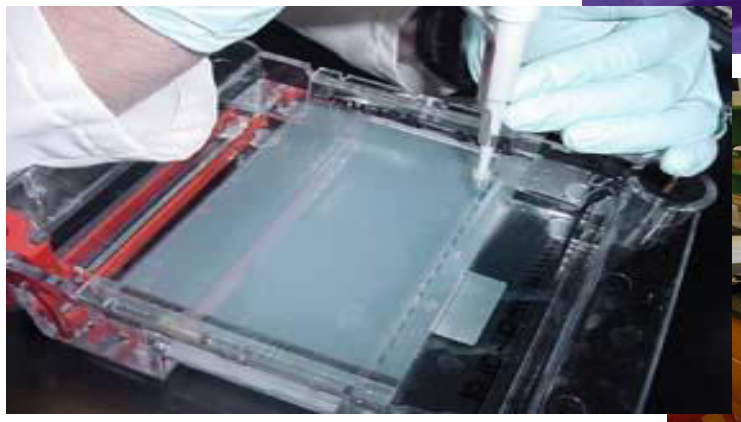
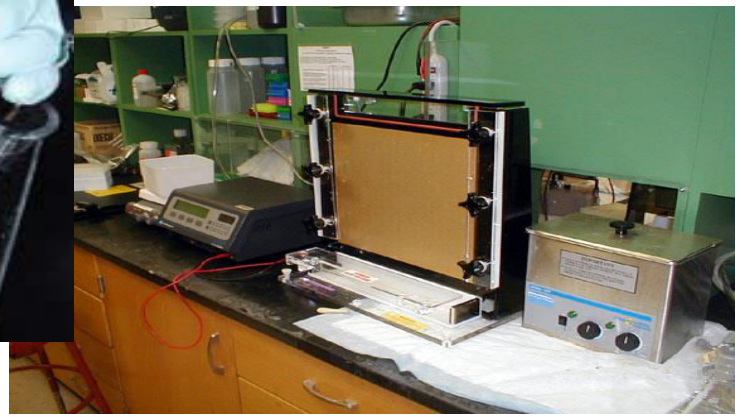


Fig. 3.4 Labelling DNA by primer extension (oligolabelling). (*a*) DNA is denatured to give single-stranded molecules. (*b*) An oligonucleotide primer is then added to give a short double-stranded region with a free 3′-OH group. (*c*) The Klenow fragment of DNA polymerase I can then synthesise a copy of the template strand from the primer, incorporating [α-^{32}P]dNTP (solid circles) to produce a labelled molecule with a very high specific activity.



Gel Electrophoresis





Developed by Science Behind Our Food

University of Georgia

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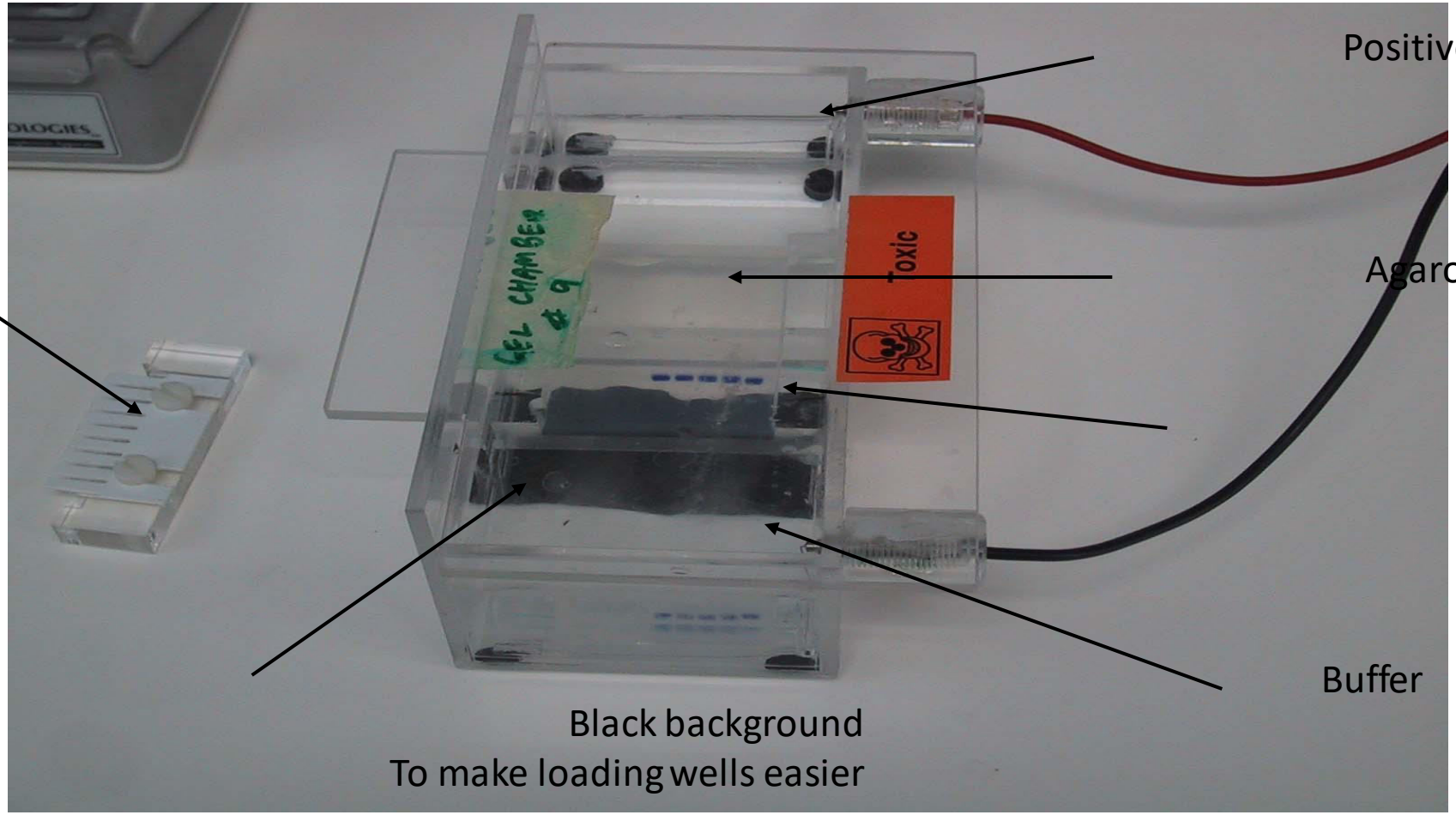
Electrophoresis

- **Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation.**

A gel being run



Positive electrode

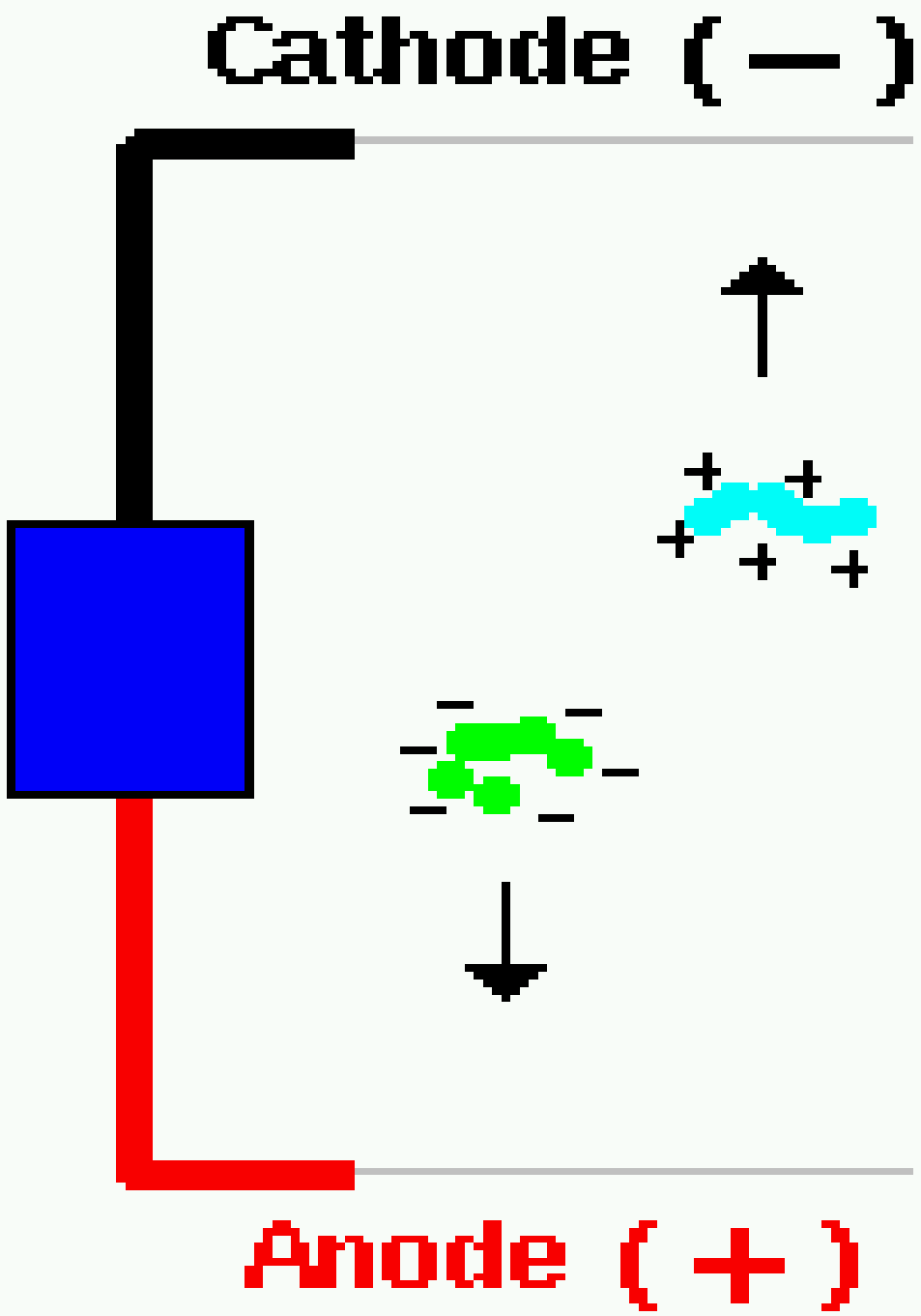
Comb

Agarose block

DNA loaded in wells in the agarose

Buffer

Black background
To make loading wells easier



- When charged molecules are placed in an electric field, they migrate toward either the positive (anode) or negative (cathode) pole according to their charge.

- Difference between DNA/RNA and proteins

Proteins and nucleic acids are electrophoresed within a matrix or "gel".

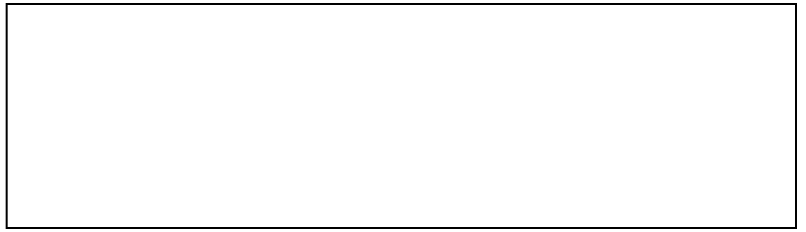
*agarose or polyacrylamide

Agarose

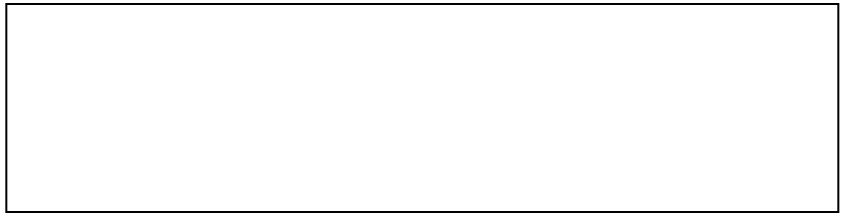
- polysaccharide extracted from seaweed. It is typically used at concentrations of 0.6 to 2%. The higher agarose concentration the "stiffer" the gel. Agarose gels are extremely easy to prepare: you simply mix agarose powder with buffer solution, melt it by heating, and pour the gel.
- non-toxic.

Agarose Gel

- A porous material derived from red seaweed
- Acts as a sieve for separating DNA fragments; smaller fragments travel faster than large fragments
- Concentration affects DNA migration
  - Low conc. = larger pores→ better resolution of larger DNA fragments
  - High conc. = smaller pores→ better resolution of smaller DNA fragments



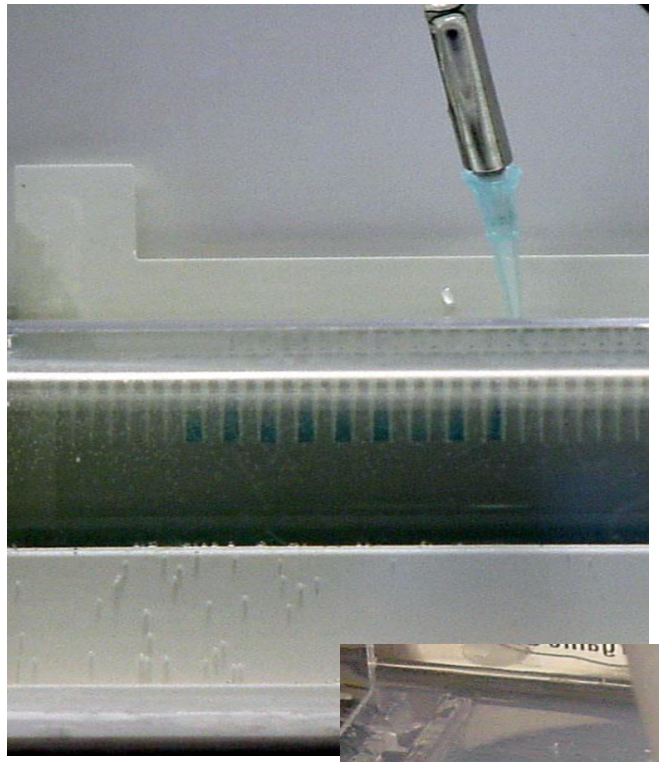
1% agarose

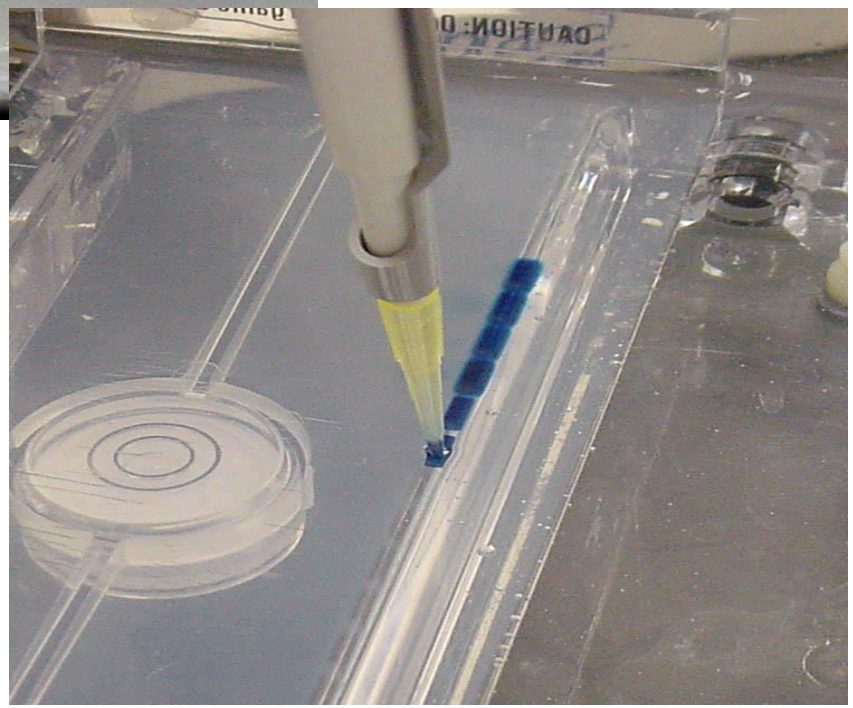


2% agarose

Loading Dye

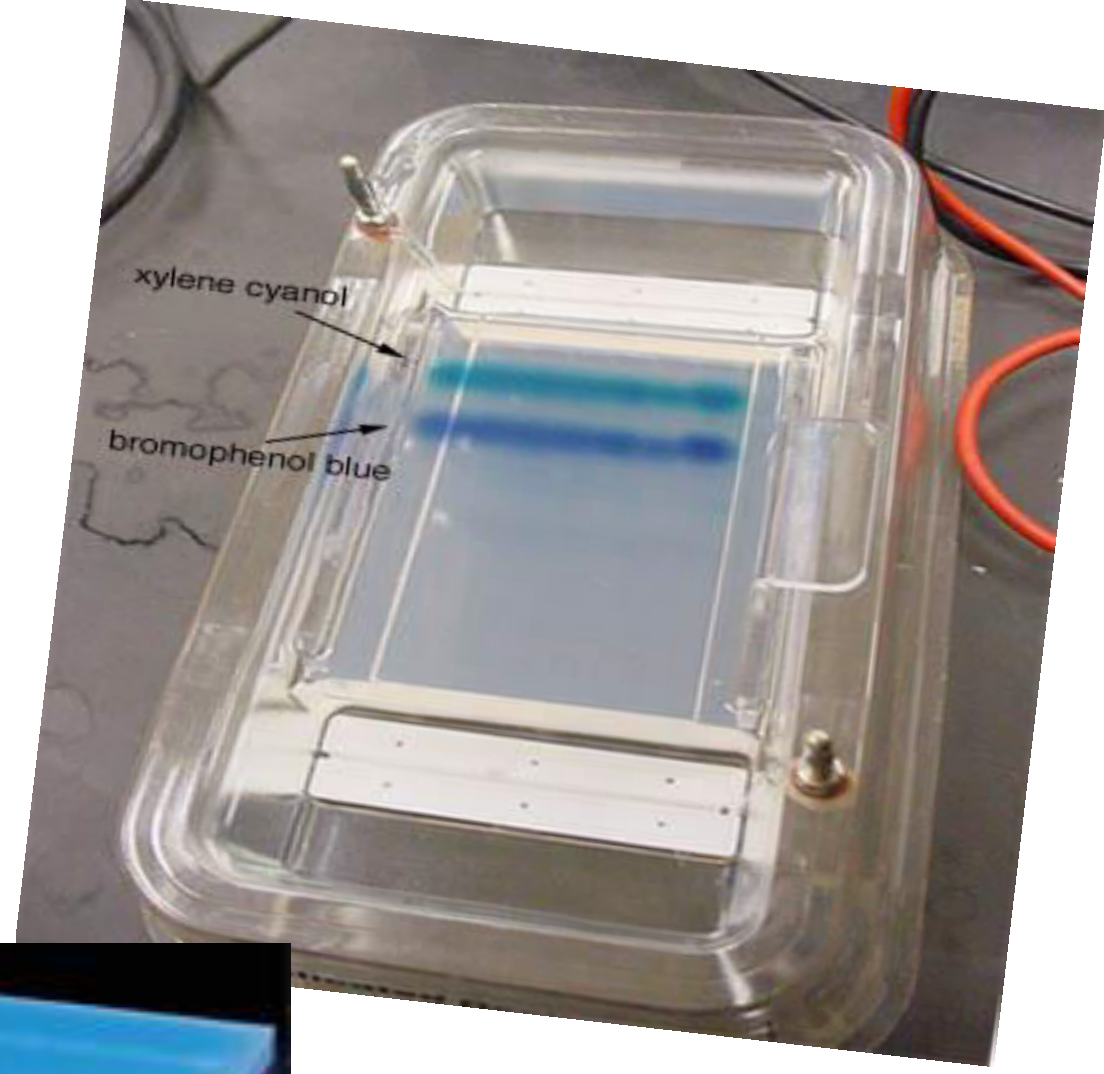
- DNA samples are loaded into a gel AFTER the tank has been filled with buffer, covering the gel
- Contains a dense substance, such as glycerol, to allow the sample to "fall" into the sample wells
- Contains one or two tracking dyes, which migrate in the gel and allow monitoring of how far the electrophoresis has proceeded.





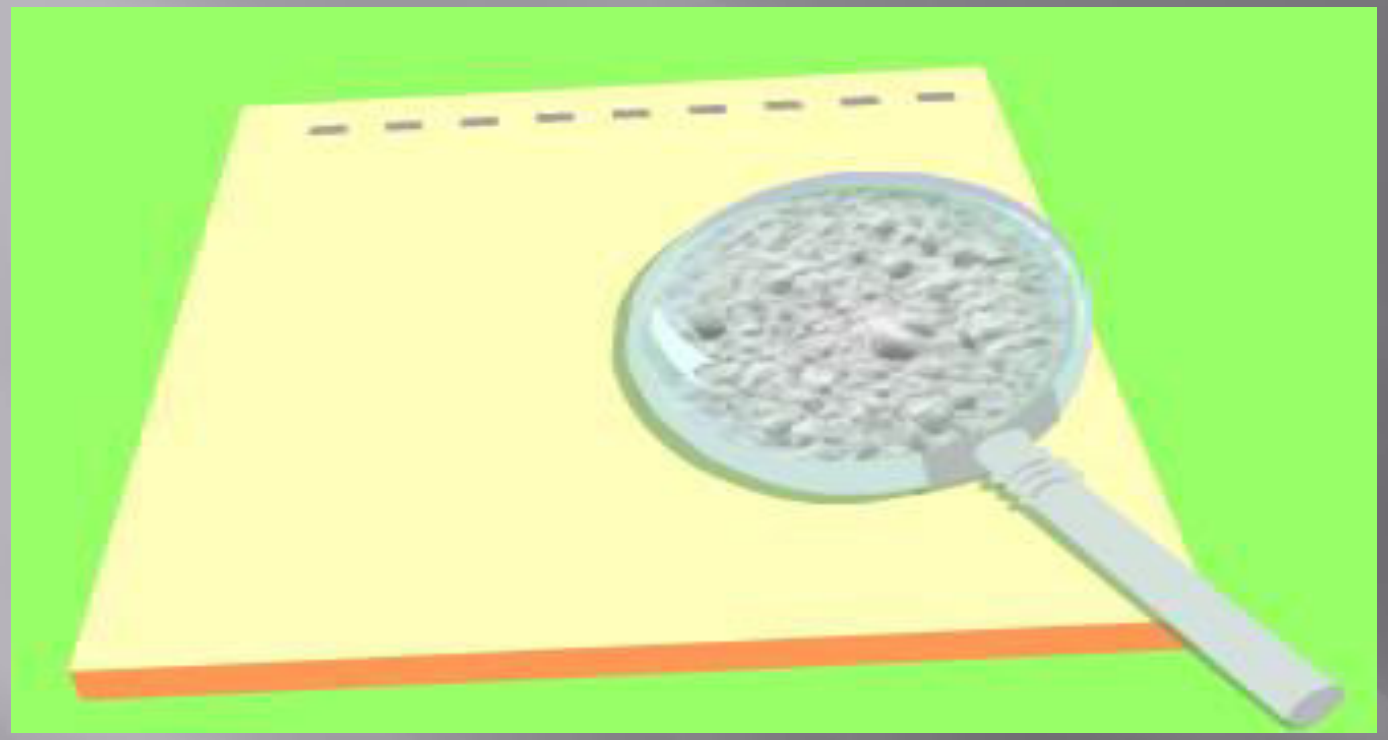
DNA Staining

- Allows DNA visualization after gel electrophoresis
- Ethidium Bromide
- Bio-Safe DNA stains
  - In gel staining



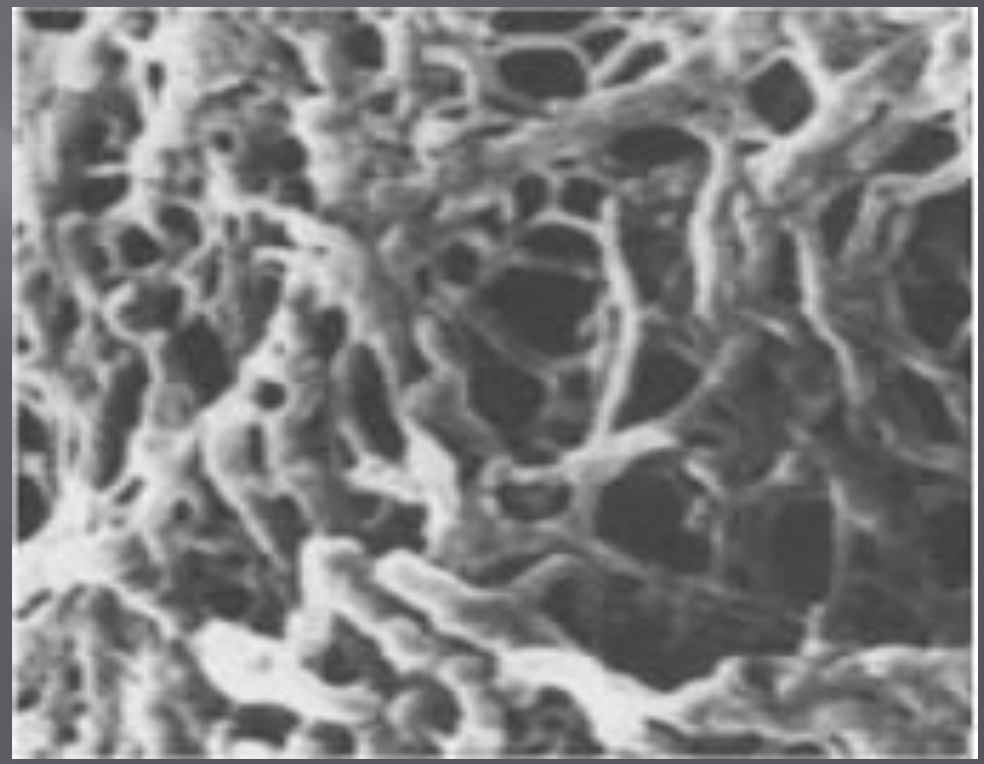
Polyacrylamide

- is a cross-linked polymer of acrylamide. 3.5 and 20%.
- Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates.
- **Acrylamide is a potent neurotoxin and should be handled with care** .



- Polymerized agarose is porous, allowing for the movement of DNA

Scanning Electron Micrograph of → Agarose Gel (1×1 μm)



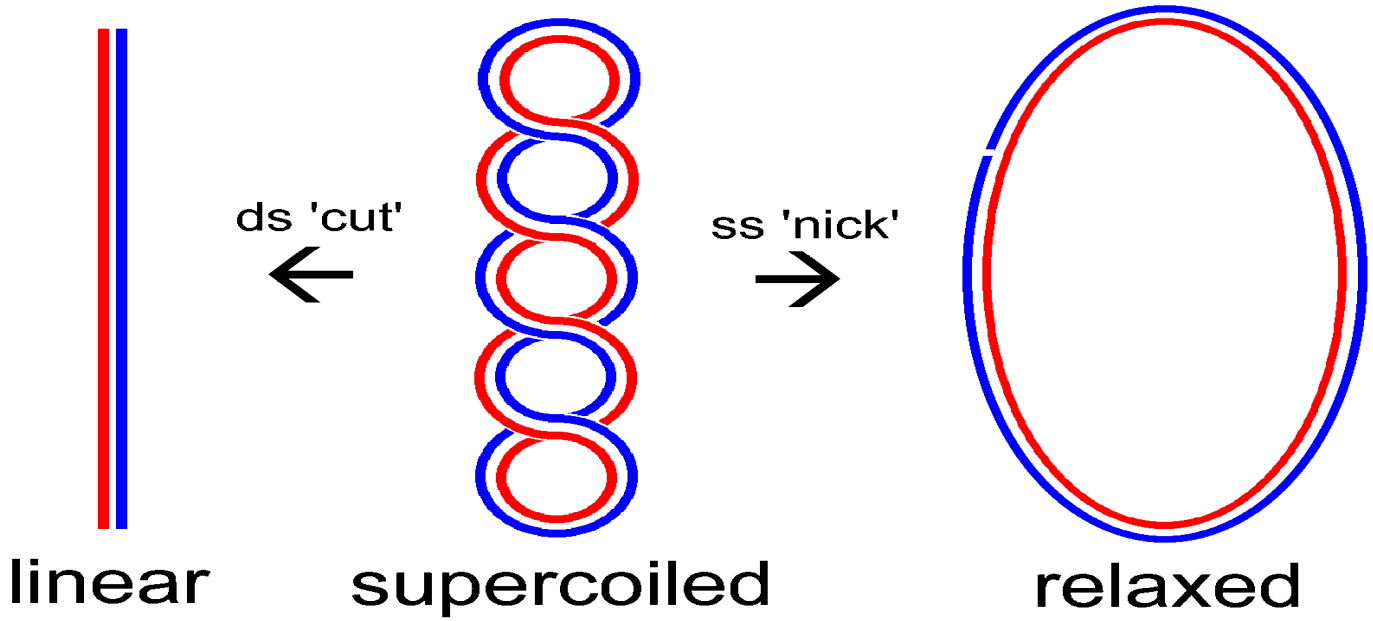
Uses

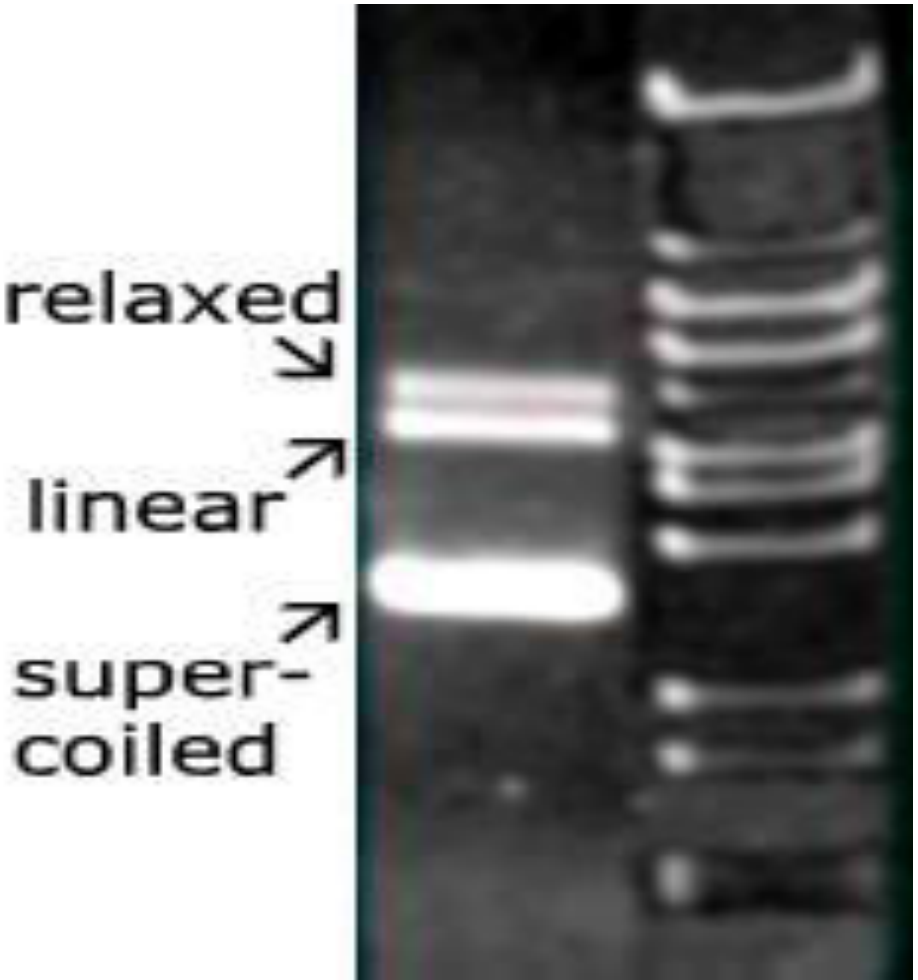
- **Polyacrylamide gels have a rather small range of separation, but very high resolving power.** polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.
- Agarose is used to separate DNA fragments from about 60 bp upward to 100,000 or so bp.

Visualization of DNA (Agarose)

- **Ethidium bromide**, a fluorescent dye used for staining nucleic acids.
- teratogen and suspected carcinogen and should be handled carefully.
- Transilluminator (an ultraviolet light box)

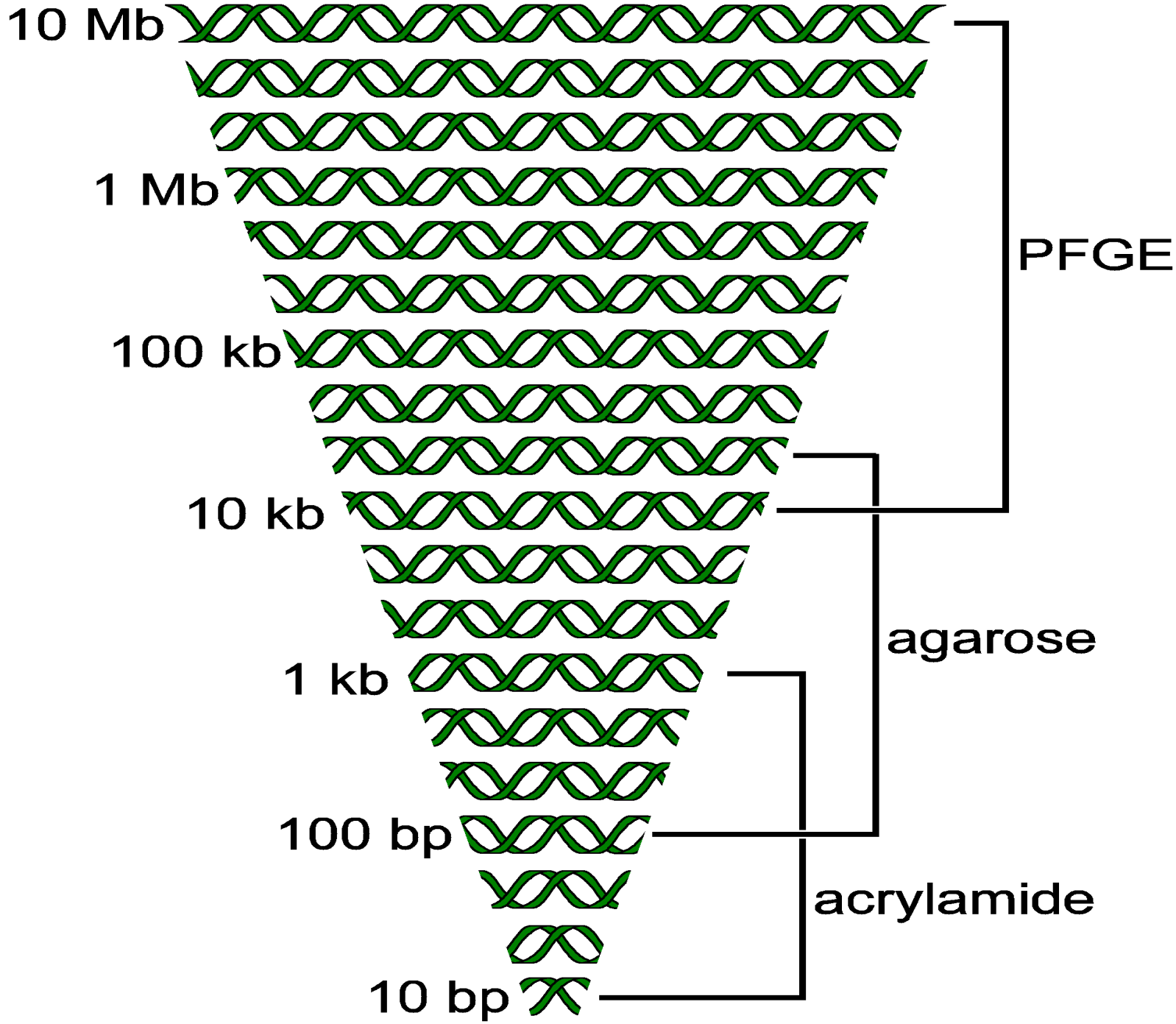
Circular vs. Linear DNA





PFGE - Pulsed Field Gel Electrophoresis

- Horizontal gel electrophoresis is the most common means of separating DNA molecules 0.1 to 30 kb in size. This technique employs a continuous and homogeneous electrical field and an agarose matrix to achieve separation.
- Since all DNA molecules have a similar charge:mass ratio and hence, a similar velocity when subjected to a voltage gradient in an aqueous solution, it is the sieving properties of agarose gel that determines the degree of separation between DNA molecules of different sizes.
- In general, linear DNA molecules of smaller size percolate through the pores/channels of the agarose matrix with less drag than the larger molecules


10 Mb
1 Mb
100 kb
10 kb
1 kb
100 bp
10 bp
PFGE
agarose
acrylamide