قسم التقنيات الاحيائية

المادة: مبادئ الهندسة الوراثية

المرحلة الرابعة

الكورس الاول

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Refreshment of Lecture one Gene Anatomy

1-What is the gene

2-How to identify that gene

3-The structure of the gene

4-Types of genes

5-Gene direction

-Definition Genetic Engineering

Genetic engineering: the process of using recombinant DNA technology to alter (changing or manipulating) their genetic makeup of an organisms it involves the direct manipulation of one or more genes most often a gene from another species is added to an organisms' genome to give it desired phenotype.

Recombinant DNA: fragments of DNA from two different species such as bacterium and a mammals spliced together in the laboratory into single molecule .

-What is a Gene?

Gene defined as the basic unit of heredity a sequence of DNA nucleotides on a chromosome that encodes A protein tRNA or rRNA molecule.

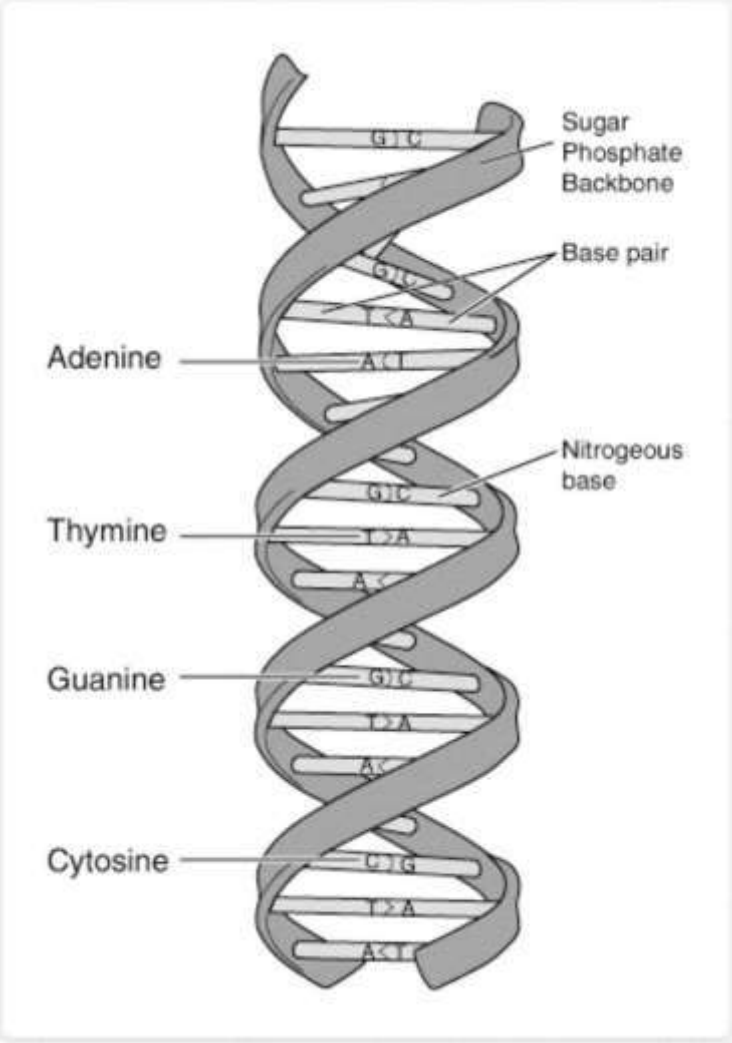
Base pair: bp two complementary nitrogenous molecules that are connected by hydrogen bonds.

A :Adenine

T: Thiamine

G: Guanine

C: Cytosine



DNA structure

Q/ When we say base pair and when we say nucleotide?

Answer:

A nucleotide is composed of a phosphate group, 5-carbon sugar, and nitrogenous base. A nitrogenous base is formed by either a single ring pyrimidine or a double ring purine.

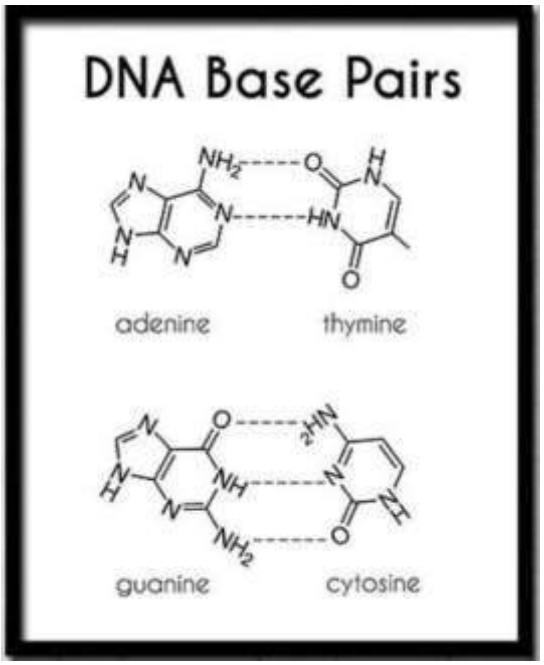
Types of bonds in DNA molecules:

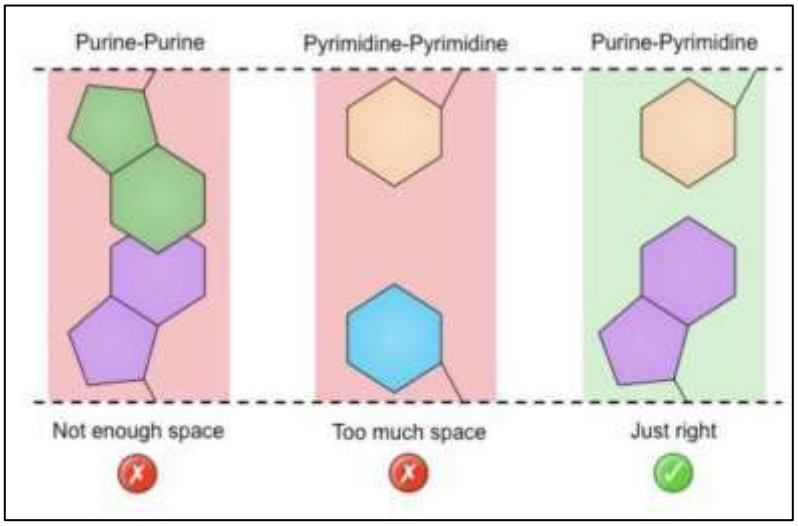
1. Hydrogen bond between strands of dsDNA

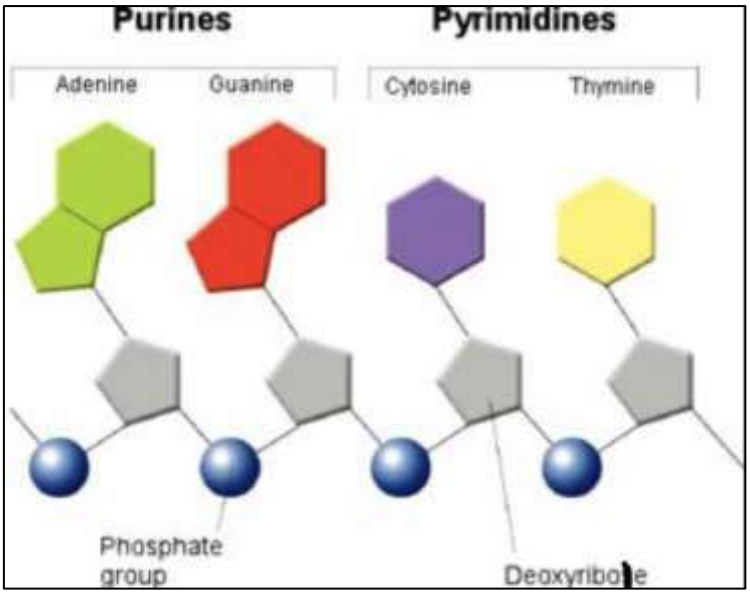
2. Glycosidic bond between sugar (deoxyribose in DNA or ribose in RNA) and nitrogen bases.

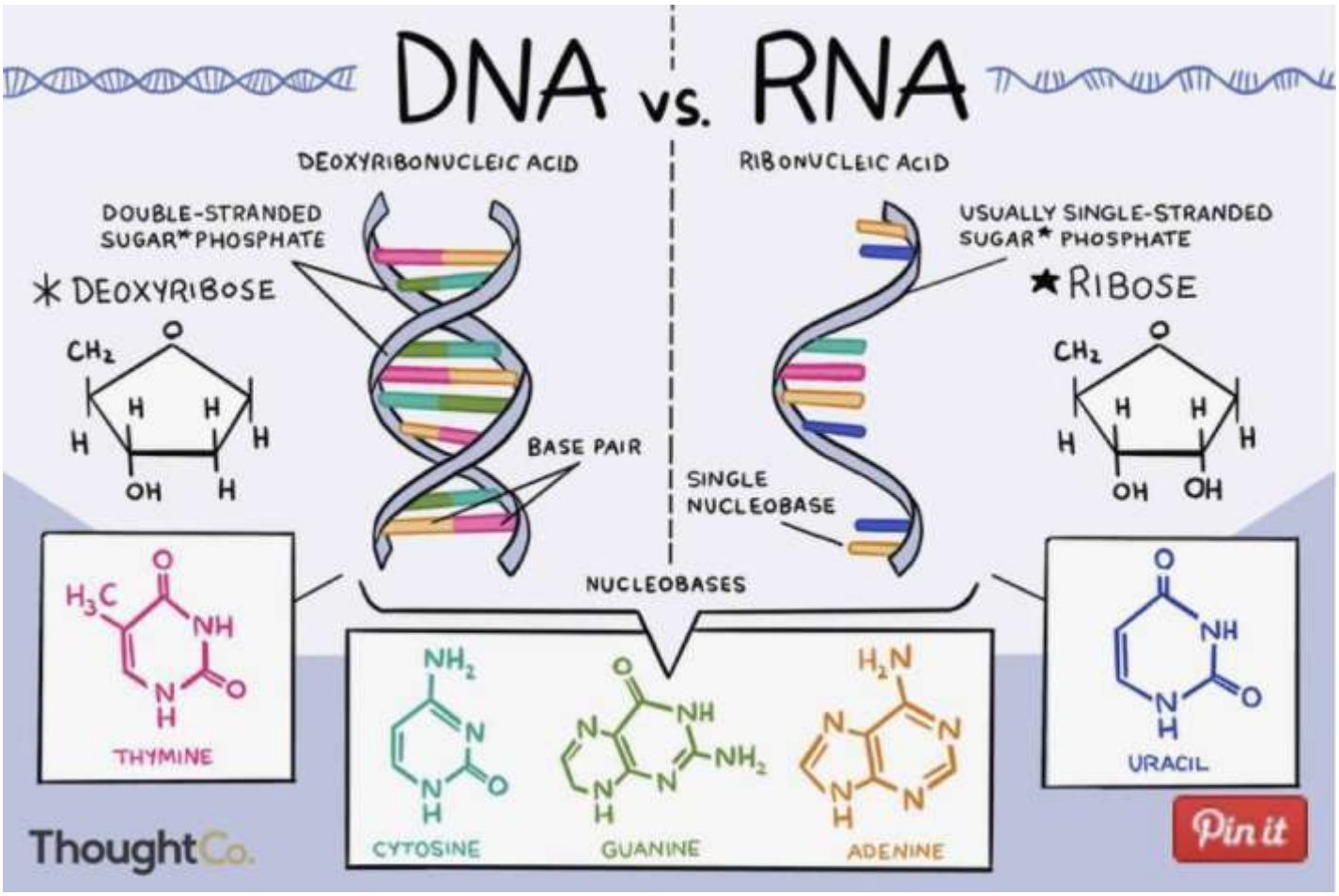
3. Phosphodiester bond between two nucleotides in same strand.

Not the hydrogen bonds in the figure below









Start codon: ATG

Stop codons:TAA, TGA, TAG

5’-AAACCCATGCGACCCATGCGTTTTCCCTCCTAAAAACCC-3’

3’-TTTGGGTACGCTGGGTACGCAAAAGGGAGGATTTTTGGG-5’

5’-TTACCC**ATG**CGACCCATGCGTTTTCCCTCC**TAA**AAACCCCAT-3’

3’-**AAT**GGGTACGCTGGGTACGCAAAAGGGAGGATTTTTGGG**GTA**-5’

Gene 1:

mRNA:5’ AUG CGA CCC AUG CGU UUU CCC UCC UAA3’

Condons number -1= amino acids number

=8 a.a

Peptides bond = amino acids number -1

Peptide bonds =7

Gene 2:

mRNA5’ AUG GGG UUU UUA GGA GGG AAA ACG CAU GGG UCG CAU GGG UAA3’

14-1 =13 aa

13-1= 12 peptide bond

Can we call the following gene?

5’-ATGCCTTCCTGGCCCGAGCTATAA-3’

3’...TACGGAAGGACCGGGCTCGATATT...5’

Maine types of the genes:

1. Structural genes
2. Regulatory genes
3. Housekeeping genes
4.Psedogenes.

Molecular size:

DNA Molecular size =how many base pairs in the DNA

RNA Molecular size = how may bases in the RNA

5’-ATGCCTTCCTGGCCCGAGCTATAA-3’

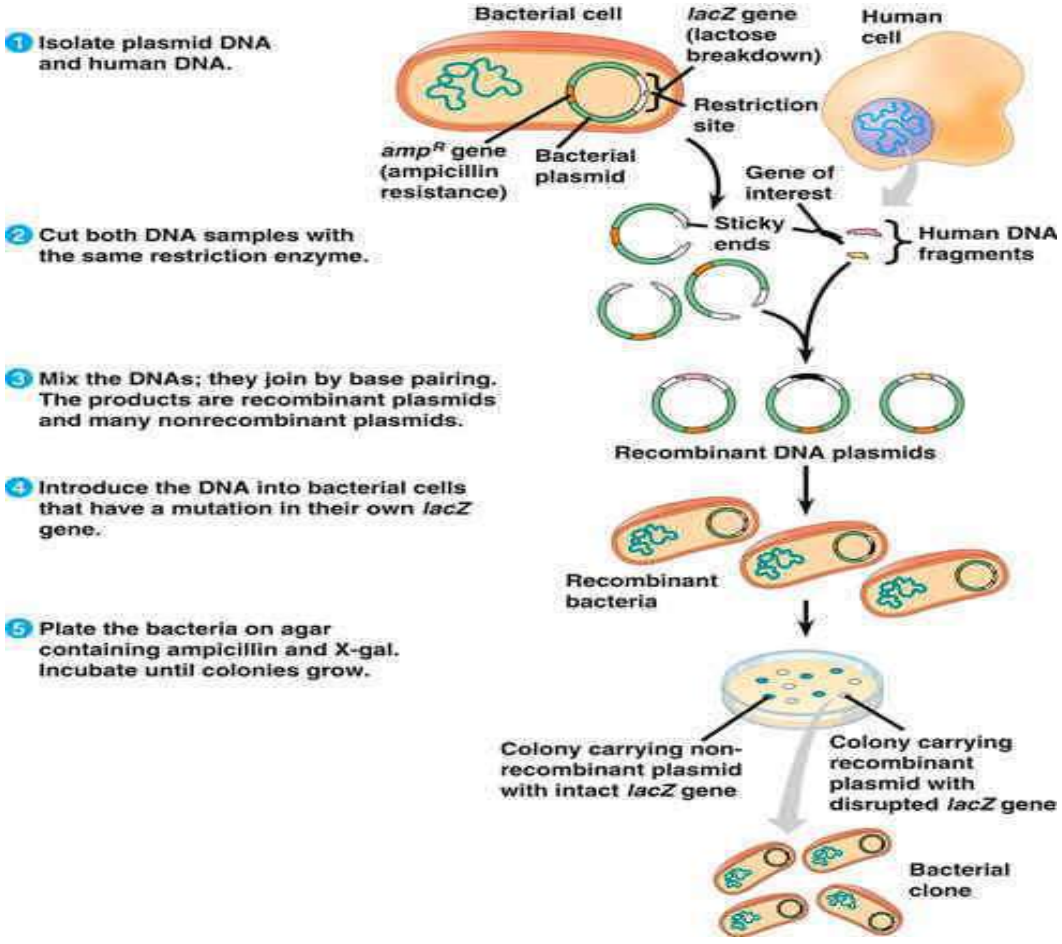
3’...TACGGAAGGACCGGGCTCGATATT...5’

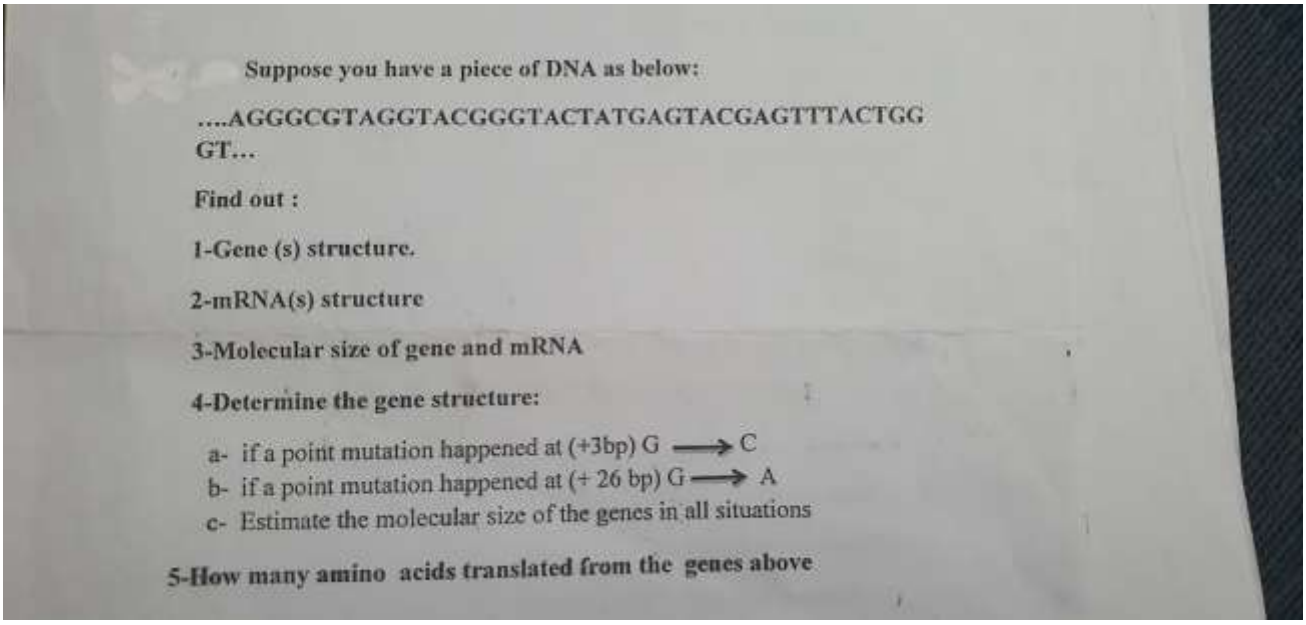
Lec.2 Principle of genetic engineering

Steps of cloning process

Cloning of any gene could be summarized by the following seven steps:

1- Isolation and purification of foreign DNA or target DNA (passenger DNA).
2- Suitable cloning vector isolation and purification, most of used vectors are plasmid or viruses.
3- Cutting DNA molecules by suitable restriction enzymes (R.E.).
4- Joining DNA molecules by DNA ligase.
5- Monitoring the cutting and joining of DNA molecules.
6- Transformation or transfection (introducing the recombinant molecules into the host).
7- Isolation and characterization of recombinants





Suppose you have a piece of DNA as below:

....AGGGCGTAGGTACGGGTACTATGAGTACGAGTTTACTGG GT...

Find out :

1-Gene (s) structure.

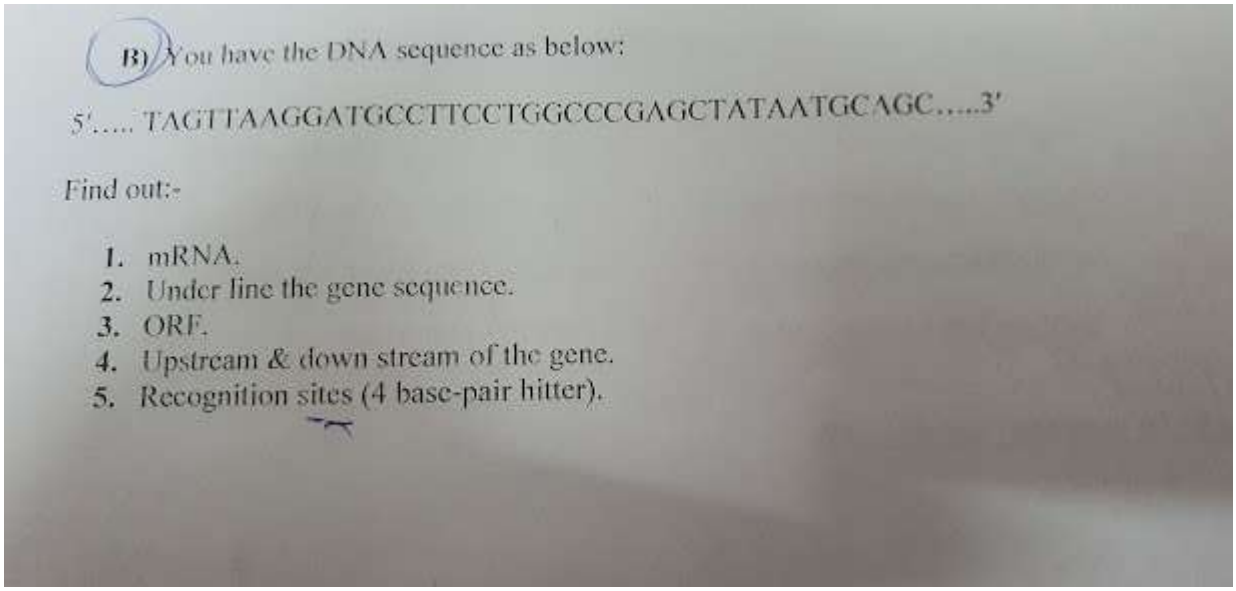
2-mRNA(s) structure

3-Molecular size of gene and mRNA

4-Determine the gene structure:

a- if a point mutation happened at (+3bp) G ⟶ C
b- if a point mutation happened at (+ 26 bp) G ⟶ A
c- Estimate the molecular size of the genes in all situations

5-How many amino acids translated from the genes above



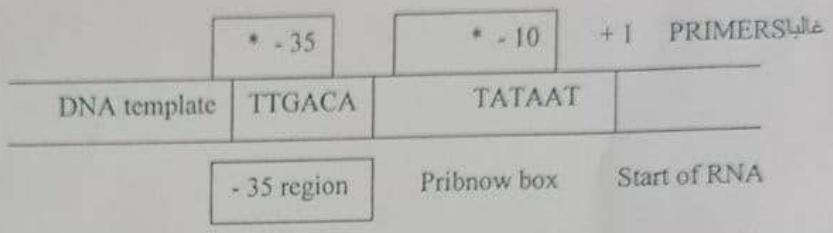
B) You have the DNA sequence as below:

5'..... TAGTTAAGGATGCCTTCCTGGCCCGAGCTATAATGCAGC.....3'

Find out:-

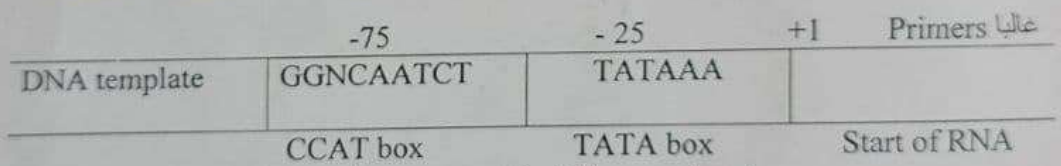
1. mRNA.
2. Under line the gene sequence.
3. ORF.
4. Upstream & down stream of the gene.
5. Recognition sites (4 base-pair hitter).

DNA templates contain regions called promoter site specifically bind RNA polymerase and determine where transcription begins



* Consenses Sequence

Procaryotic promoter site

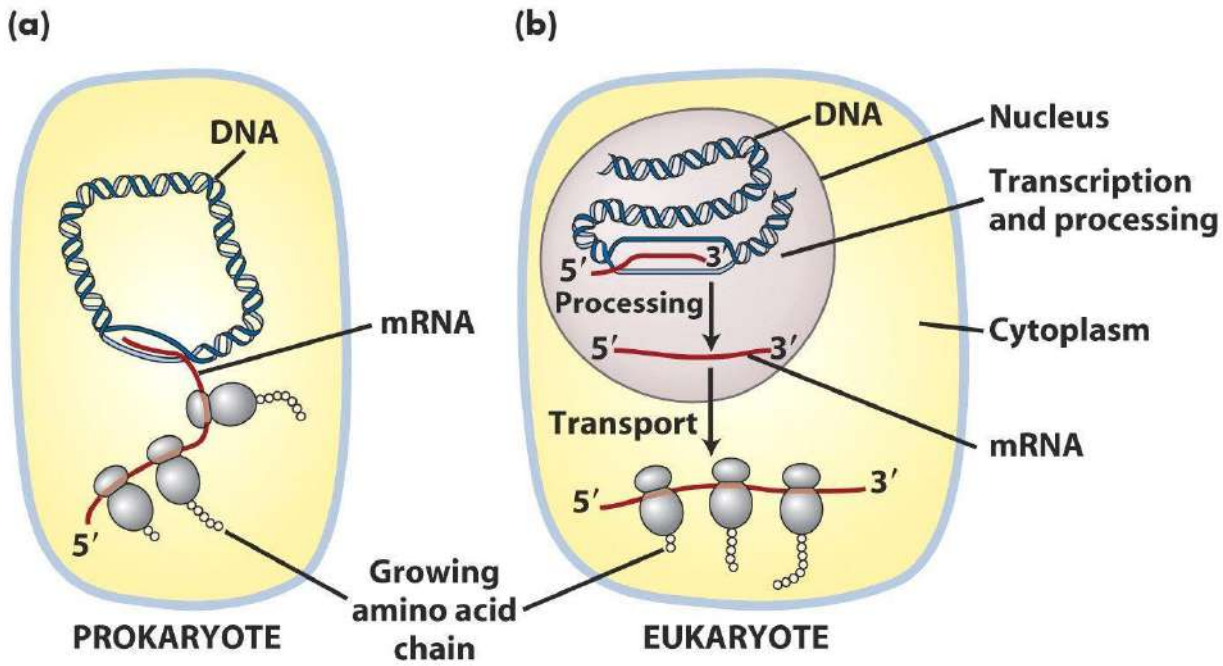


Eukaryotic Promoter site

Isolation and purification of nucleic acids (genomic DNA)

Introduction

Every gene manipulation procedure requires genetic material like DNA and RNA. Nucleic acids occur naturally in association with proteins and lipoprotein organelles. The dissociation of a nucleoprotein into nucleic acid and protein moieties and their subsequent separation, are the essential steps in the isolation of all species of nucleic acids. Isolation of nucleic acids is followed by quantitation of nucleic acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture.



Isolating the genetic material (DNA) from cells (bacterial, viral, plant or animal) involves three basic steps:

- Rupturing of cell membrane to release the cellular components and DNA
- Separation of the nucleic acids from other cellular components
- Purification of nucleic acids

Isolation and Purification of Genomic DNA

Genomic DNA is found in the nucleus of all living cells with the structure of double-stranded DNA. The isolation of genomic DNA differs in animals and plant cells. DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells. The amount and purity of extracted DNA depends on the nature of the cell.

The method of isolation of genomic DNA from a bacterium comprises following steps

1. Bacterial culture growth and harvest.
2. Cell wall rupture and cell extract preparation.
3. DNA Purification from the cell extract.
4. Concentration of DNA solution.

Growth and harvest of bacterial culture

Bacterial cell culture is more convenient than any other microbe, as it requires only liquid medium (broth) containing essential nutrients at optimal concentrations, for the growth and division of bacterial cells. The bacterial cells are usually grown on a complex medium like Luria-Bertani (LB), in which the medium composition is difficult to decipher. Later, the cells are separated by centrifugation and resuspended in 1% or less

of the initial culture volume.

Preparation of cell extract

Bacterial cell is surrounded by an additional layer called cell wall, apart from plasma membrane with some species of *E. coli* comprising multilayered cell wall. The lysis of cell wall to release the genetic material i.e. DNA can be achieved by following ways-

- Physical method by mechanical forces.
- Chemical method by metal chelating agents i.e. EDTA and surfactant i.e. SDS or enzyme (e.g. lysozyme).

Lysozyme

- Present in egg-white, salivary secretion and tears.
- Catalyzes the breakdown of cell wall i.e. the peptidoglycan layer.

EDTA (Ethylene diamine tetra-acetic acid)

- A chelating agent necessary for destabilizing the integrity of cell wall.
- Inhibits the cellular enzymes that degrade DNA.

SDS (Sodium dodecyl sulphate)

- Helps in removal of lipid molecules and denaturation of membrane proteins.

Sucrose 25%

Generally, a mixture of EDTA, lysozyme and sucrose 25% are used. Cell lysis is followed by centrifugation to pellet down the cell wall fractions leaving a clear supernatant containing cell extract.

Purification of DNA

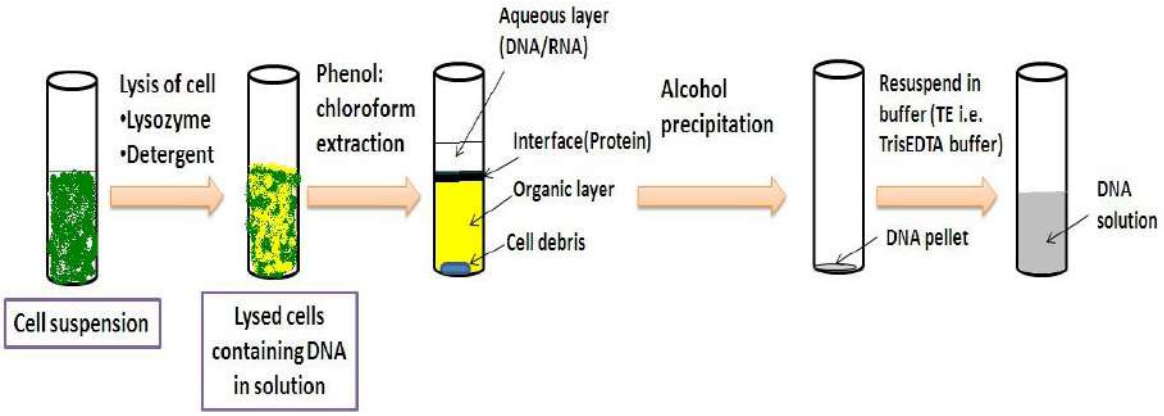
In addition to DNA, a cell extract contains significant quantities of protein and RNA which can be further purified by following methods-

1. Organic extraction and enzymatic digestion for the removal of contaminants

It involves the addition of a mixture of phenol and chloroform (1:1) to the cell lysate for protein separation. The proteins aggregate as a white mass in between the aqueous phase containing DNA and RNA, and the organic layer. Treatment of lysate with pronase or protease, in addition to phenol/chloroform, ensures complete removal of proteins from the extract. The RNA can be effectively removed by using Ribonuclease, an enzyme which rapidly degrades RNA into its ribonucleotide subunits. Repeated phenol extraction is not desirable, as it damages the DNA.

2. Using ion-exchange chromatography

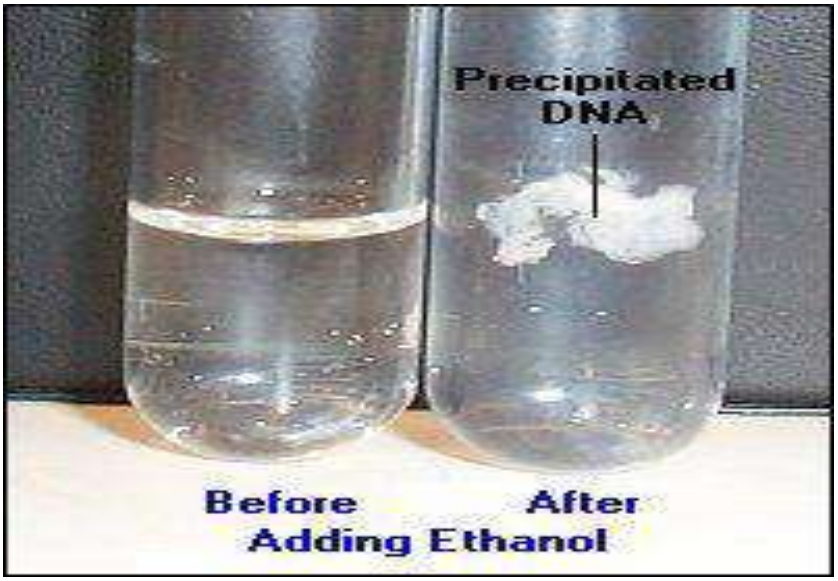
This involves the separation of ions and polar molecules (proteins, small nucleotides and amino acids) based on their charge. DNA carrying negative charge binds to the cationic resin or matrix which can be eluted from the column by salt gradient. Gradual increase in salt concentration detaches molecules from the resin one after another.



Preparation of genomic DNA

Concentration of DNA samples

Concentration of DNA can be done using ethanol along with salts such as sodium acetate, potassium acetate etc. These salts provide metal ions like sodium ions (Na+), potassium ions (K+) which help in aggregation and hence precipitation of DNA molecules.



Isolation and purification of nucleic acids (plasmid DNA)

Isolation and Purification of Plasmid DNA

Plasmids are circular, double stranded extra cellular DNA molecules of bacterium and most commonly used in recombinant DNA technology. The isolation of plasmid DNA involves three major steps-

1. Growth of the bacterial cell.
2. Harvesting and lysis of the bacteria.
3. Purification of the plasmid DNA.

1. Growth of the bacterial cell

It involves growth of the bacterial cells in a media containing essential nutrients.

2. Harvest and lysis of bacteria

Lysis of bacteria results in the precipitation of DNA and cellular proteins. Addition of acetate-containing neutralization buffer results in the precipitation of large and less supercoiled chromosomal DNA and proteins leaving the small bacterial DNA plasmids in solution.

3. Purification of Plasmid DNA

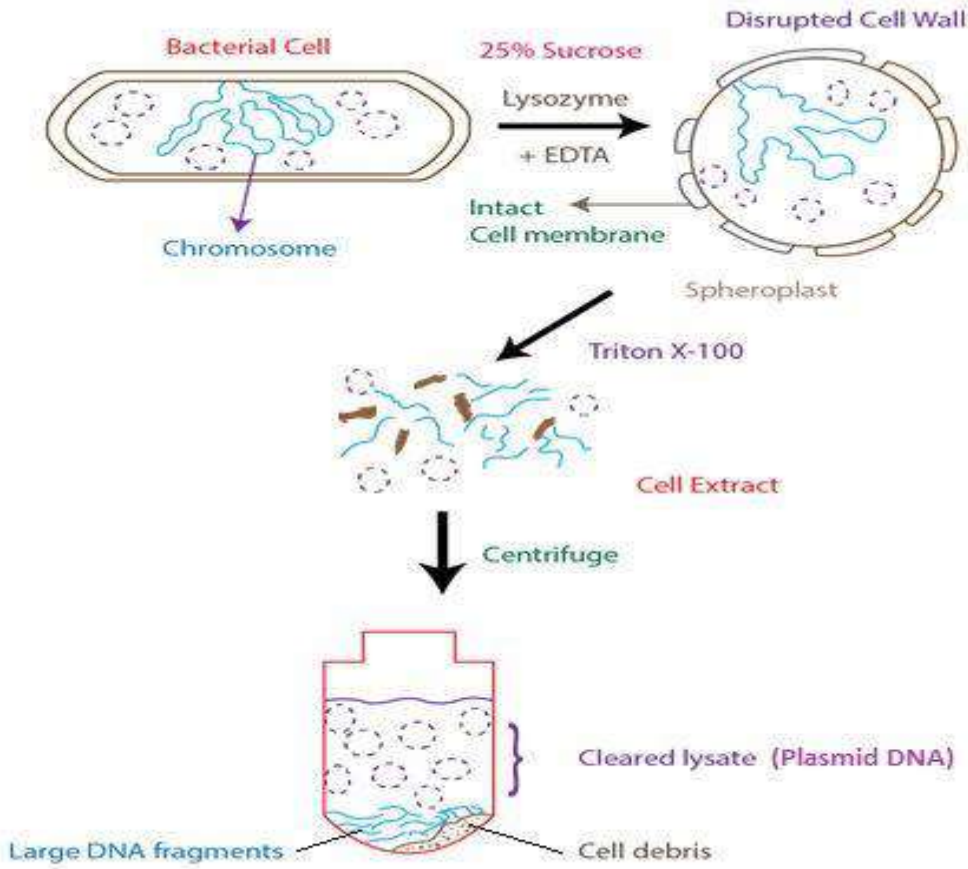
This step is same for both plasmid and genomic but former involves an additional step i.e. the separation of plasmid DNA from the large bacterial chromosomal DNA.

Methods for separation of plasmid DNA

Separation of plasmid DNA is based on the several features like size and conformation of plasmid DNA and bacterial DNA. Plasmids are much smaller than the bacterial main chromosomes, the largest plasmids being only 8% of the size of the *E. coli* chromosome. The separation of small molecules (i.e. plasmids) from larger ones (i.e. bacterial chromosome) is based on the fact that plasmids and the bacterial chromosomes are circular but bacterial chromosomes break into linear fragments during the preparation of the cell extract resulting in separation of pure plasmids. The methods of separation of plasmid DNA are described as below-

1. Separation based on size difference

- It involves lysis of cells with lysozyme and EDTA in the presence of 25% sucrose.
- Cells with partially degraded cell walls are formed that retain an intact cytoplasmic membrane called as spheroplasts.
- Cell lysis is then induced by the addition of a non-ionic detergent (e.g. Triton X- 100) or ionic detergents (e.g. SDS) causing chromosomal breakage.
- Bacterial chromosome attached to cell membrane, upon lysis gets removed with the cell debris.
- A cleared lysate consisting almost entirely of plasmid DNA is formed with very little breakage of the bacterial DNA.



Separation of plasmid DNA on the basis of size.

2. Separation based on conformation

Plasmids are supercoiled molecules formed by partial unwinding of double helix of the plasmid DNA during the plasmid replication process by enzymes called topoisomerases. The supercoiled conformation can be maintained when both polynucleotide strands are intact, hence called covalently closed-circular (ccc) DNA. If one of the polynucleotide strands is broken, the double helix reverts to its normal relaxed state taking an alternative conformation, called open-circular (oc). Super coiling is important in plasmid preparation due to the

easy separation of supercoiled molecules from non-supercoiled ones.

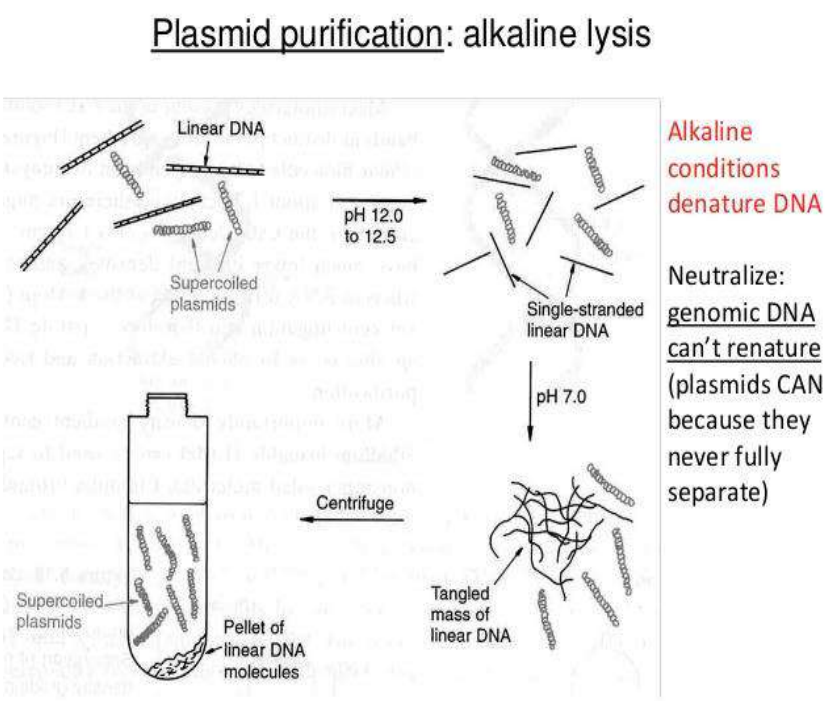
The commonly used methods of separation based on conformation are as

follows- **(a). Alkaline denaturation method**

- This method is based on maintaining a very narrow pH range for the denaturation of non-supercoiled DNA but not the supercoiled plasmid
- Addition of sodium hydroxide to cell extract or cleared lysate (pH12.0-12.5) results in disruption of the hydrogen bonds of non-supercoiled DNA molecules.
- As a result, the double helix unwinds and two polynucleotide chains separate.
- Further addition of acid causes the aggregation of these denatured bacterial DNA strands into a tangled mass which can be pelleted by centrifugation, leaving plasmid DNA in the supernatant.

Advantage

- Most of the RNA and protein under defined conditions (specifically cell lysis by SDS and neutralization with sodium acetate) can be removed by the centrifugation.
- No requirement of organic extraction.



Separation of plasmid DNA by Alkaline denaturation method

(b). Ethidium bromide-cesium chloride density gradient centrifugation

- Density gradient centrifugation can separate DNA, RNA and protein. It is a very efficient method for obtaining pure plasmid DNA.
- A density gradient is produced by centrifuging a solution of cesium chloride at a very high speed which pulls the CsCl ions towards the bottom. This process is referred as isopycnic centrifugation.
- The DNA migrates to the point at which it has density similar to that of CsCl i.e.1.7 g/cm^3 in the gradient.
- In contrast, protein molecules having lower buoyant densities float at the top of the tube whereas RNA gets pelleted at the bottom.

Density gradient centrifugation in the presence of ethidium bromide (EtBr) can be used to separate supercoiled DNA from non-super coiled molecules. Ethidium bromide is an intercalating dye that binds to DNA molecules causing partial unwinding of the double helix. Supercoiled DNA have very little freedom to unwind due to absence of free ends and bind to a limited amount of EtBr resulting in very less decrease in buoyant density (0.085 g/cm^3) than that of linear DNA (0.125 g/cm^3). As a result, they form a distinct band separated from the linear bacterial DNA. The EtBr bound to DNA is then extracted by n-butanol and the CsCl is removed by dialysis.

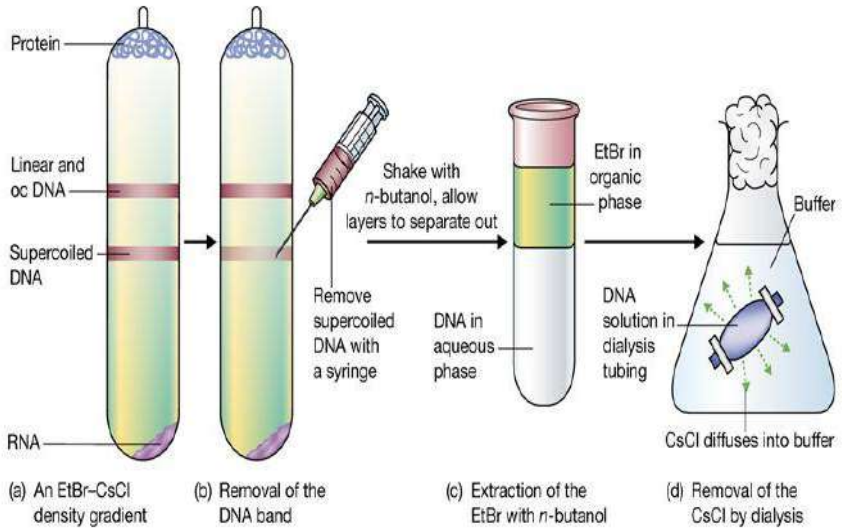


Figure 3.16
Purification of plasmid DNA by EtBr–CsCl density gradient centrifugation.

Determine the concentration and purity of a DNA sample

DNA yield can be assessed using various methods including absorbance (optical density), agarose gel electrophoresis, or use of fluorescent DNA-binding dyes. All three methods are convenient, but have varying requirements in terms of equipment needed, ease of use, and calculations to consider.

1- Absorbance Methods

The most common technique to determine DNA yield and purity is measurement of absorbance. Although it could be argued that fluorescence measurement is easier, absorbance measurement is simple, and requires commonly available laboratory equipment. All that is needed for the absorbance method is a spectrophotometer equipped with a UV lamp, UV-transparent cuvettes (depending on the instrument) and a solution of purified DNA. Absorbance readings are performed at 260nm (A_{260}) where DNA absorbs light most strongly, and the number generated allows one to estimate the concentration of the solution. DNA concentration is estimated by measuring the absorbance at 260nm, adjusting the A_{260} measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50µg/ml pure dsDNA.

Concentration (µg/ml) = (A_{260} reading (O.D260)) × dilution factor × 50µg/ml

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield (µg) = DNA concentration × total sample volume (ml)

However, DNA is not the only molecule that can absorb UV light at 260nm. Since RNA also has a great absorbance at 260nm, and the aromatic amino acids present

in protein absorb at 280nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260nm.

To evaluate DNA purity, measure absorbance from 230nm to 320nm to detect other possible contaminants. The most common purity calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present.

DNA purity (A_{260}/A_{280}) = (A_{260} reading) ÷ (A_{280} reading)

Strong absorbance around 230nm can indicate that organic compounds or salts are present in the purified DNA. A ratio of 260nm to 230nm can help evaluate the level of salt carryover in the purified DNA.

A reading at 320nm will indicate if there is turbidity in the solution, another indication of possible contamination. Therefore, taking a spectrum of readings from 230nm to 320nm is most informative.

2- Fluorescence Methods

The widespread availability of fluorometers and fluorescent DNA-binding dyes makes fluorescence measurement another popular option for determining of DNA yield and concentration. Fluorescence methods are more sensitive than absorbance, particularly for low-concentration samples, and the use of DNA-binding dyes allows more specific measurement of DNA than spectrophotometric methods allows. Hoechst bisbenzimidazole dyes, PicoGreen® and QuantiFluor™ dsDNA dyes selectively bind double-stranded DNA. The availability of single-tube and microplate fluorometers gives flexibility for reading samples in PCR tubes, cuvettes or multiwell plates and makes fluorescence measurement a convenient modern alternative to the more traditional absorbance methods.

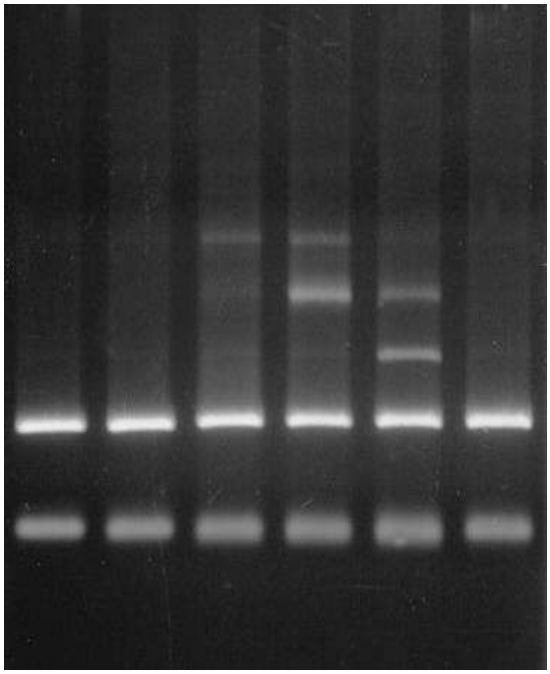
Materials required for fluorescence methods are: a fluorescent DNA binding dye, a fluorometer to detect the dyes, and appropriate DNA standards. Depending on the dye selected, size qualifications may apply, and the limit of detection may vary. The usual caveats for handling fluorescent compounds also apply—photobleaching and quenching will affect the signal.

3- Agarose Gel Electrophoresis

Agarose gel electrophoresis is another way to quickly estimate DNA concentration. To use this method, a horizontal gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer (e.g., 1X TAE) and an intercalating DNA dye along with appropriately sized DNA standards are required. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field. The negatively charged DNA backbone migrates toward the anode. Since small DNA fragments migrate faster, the DNA is separated by size. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity. Any RNA, nucleotides and protein in the sample migrate at different rates compared to the DNA so the band(s) containing the DNA will be distinct.

Concentration and yield can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard. For example, if a 2µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100ng standard, then the solution concentration is 50ng/µl (100ng divided by 2µl). Standards used for quantitation should be labeled

as such and be the same size as the sample DNA being analyzed. In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR® Green is required. Because ethidium bromide is a known mutagen, precautions need to be taken for its proper use and disposal.



Gel electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge.

Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by the charge in agarose because the pores of the gel are too small to sieve proteins.

Using an electric field, molecules (such as DNA) can be made to move through a gel made of agarose or polyacrylamide. The electric field consists of a negative charge at one end which pushes the molecules through the gel, and a positive charge at the other end that pulls the molecules through the gel. The molecules being sorted are dispensed into a well in the gel material. The gel is placed in an electrophoresis chamber, which is then connected to a power source. When the electric field is applied, the larger molecules move more slowly through the gel while the smaller molecules move faster. The different sized molecules form distinct bands on the gel.

The term "gel" in this instance refers to the matrix used to contain, then separate the target molecules. In most cases, the gel is a crosslinked polymer whose composition and porosity are chosen based on the specific weight and composition of the target to be analyzed. When separating proteins or small nucleic acids (DNA, RNA) the gel is usually composed of different concentrations of acrylamide and a cross-linker, producing different sized mesh networks of polyacrylamide. When separating larger nucleic acids (greater than a few hundred bases), the preferred matrix is purified agarose. In both cases, the

gel forms a solid, yet porous matrix. Acrylamide, in contrast to polyacrylamide, is a neurotoxin and must be handled using appropriate safety precautions to avoid poisoning. Agarose is composed of long unbranched chains of uncharged carbohydrate without cross-links resulting in a gel with large pores allowing for the separation of macromolecules.

The types of gel most typically used are agarose and polyacrylamide gels. Each type of gel is well-suited to different types and sizes of the analyte. Polyacrylamide gels are usually used for proteins and have very high resolving power for small fragments of DNA (5-500 bp). Agarose gels, on the other hand, have lower resolving power for DNA but have a greater range of separation, and are therefore used for DNA fragments of usually 50–20,000 bp in size. Polyacrylamide gels are run in a vertical configuration while agarose gels are typically run horizontally in a submarine mode. They also differ in their casting methodology, as agarose sets thermally, while polyacrylamide forms in a chemical polymerization reaction.

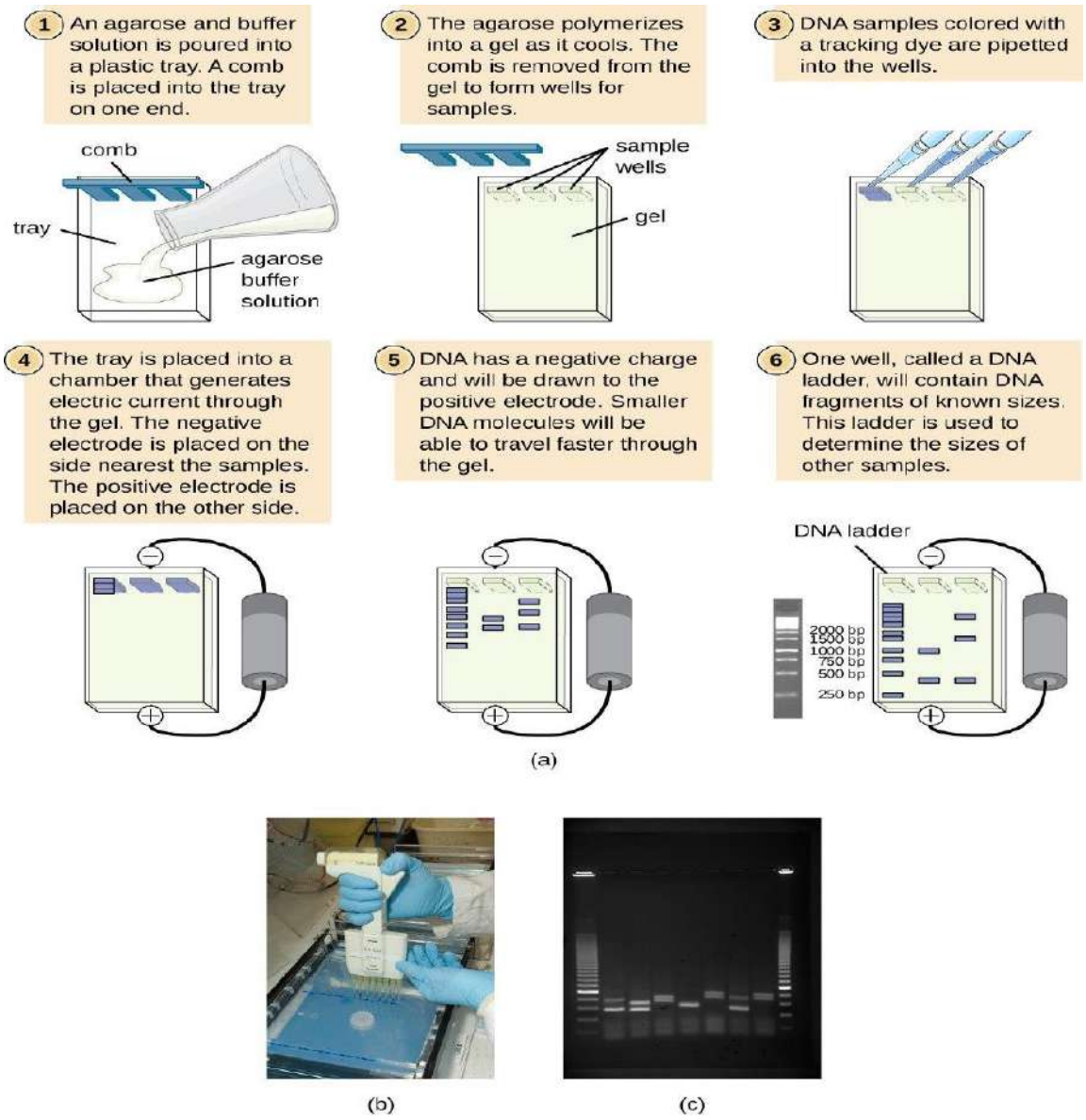
Agarose

Agarose gels are made from the natural polysaccharide polymers extracted from seaweed. Agarose gels are easily cast and handled compared to other matrices because the gel setting is a physical rather than chemical change. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

Agarose gels do not have a uniform pore size, but are optimal for electrophoresis of proteins that are larger than 200 kDa. Agarose gel electrophoresis can also be used for the separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases).

The distance between DNA bands of different lengths is influenced by the percent agarose in the gel, with higher percentages requiring longer run times.

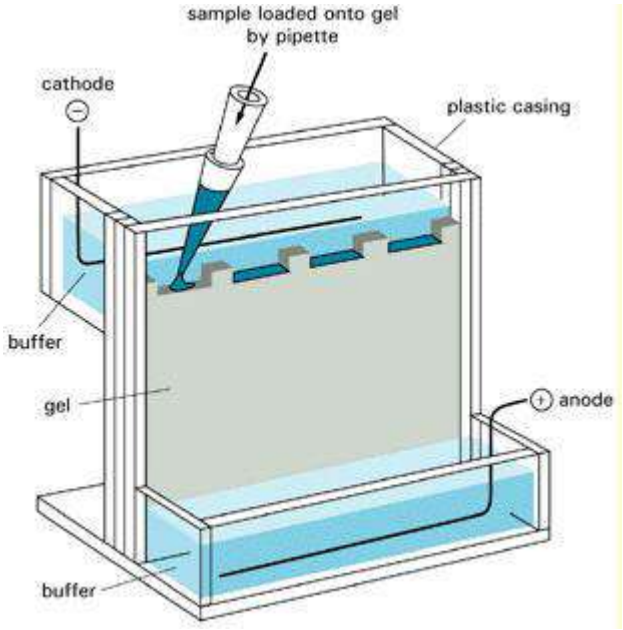
"Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly.

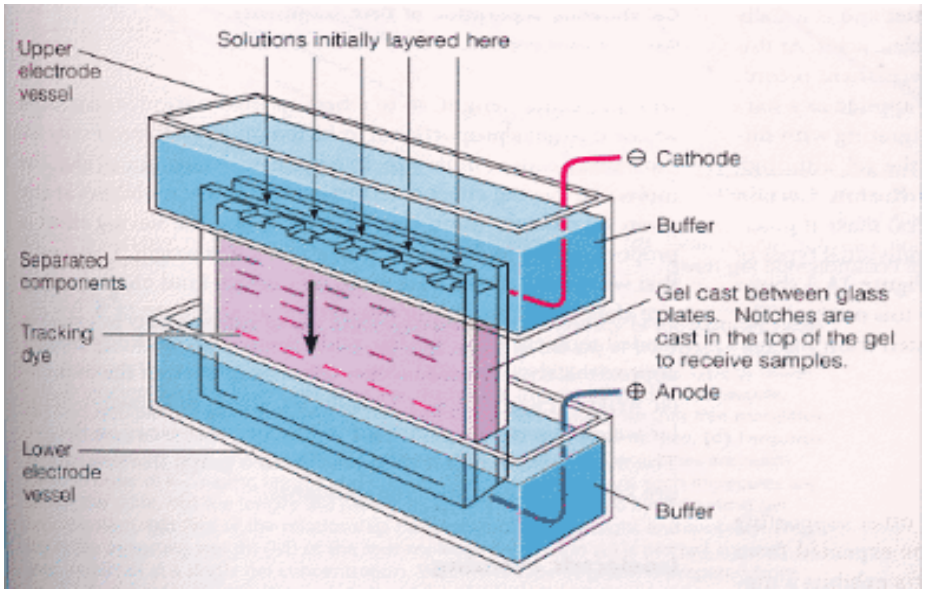


Polyacrylamide

Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Care must be used when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered forms.

The percentage chosen depends on the size of the protein that one wishes to identify in the sample. The smaller the known weight, the higher the percentage that should be used. Changes on the buffer system of the gel can help to further resolve proteins of very small sizes.





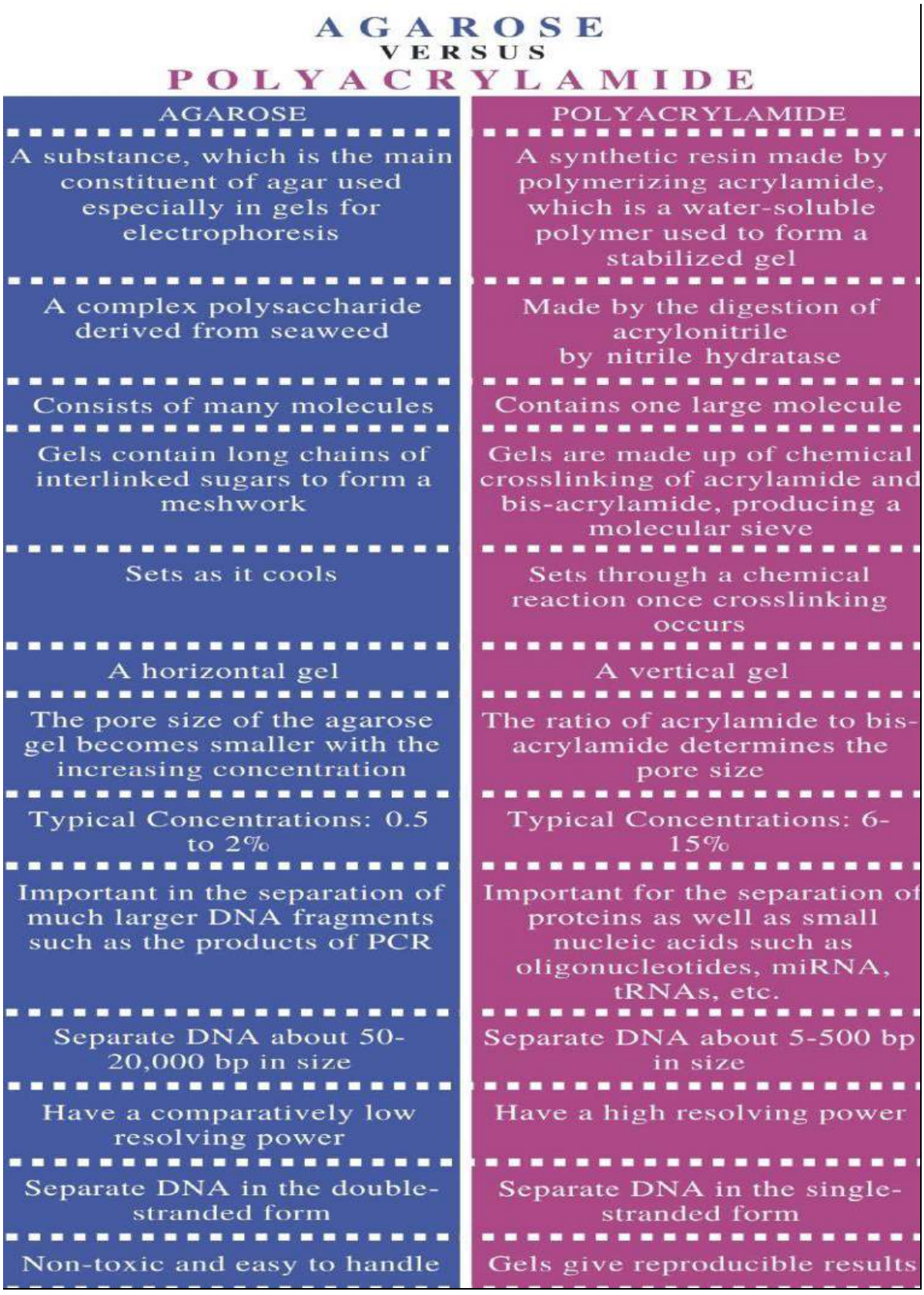
Buffers

Buffers in gel electrophoresis are used to provide ions that carry a current and to maintain the pH at a relatively constant value. These buffers have plenty of ions in them, which is necessary for the passage of electricity through them. Something like distilled water or benzene contains few ions, which is not ideal for the use in electrophoresis. There are a number of buffers used for electrophoresis. The most common being, for nucleic acids Tris/Acetate/EDTA (TAE), Tris/Borate/EDTA (TBE). TAE has the lowest buffering capacity but

provides the best resolution for larger DNA. This means a lower voltage and more time, but a better product.

Gel-electrophoresis applications

Gel-electrophoresis is used in forensics, molecular biology, genetics, microbiology and biochemistry. The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a computer operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel.



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Restriction enzyme

A restriction enzyme, restriction endonuclease, or *restrictase* is an enzyme that cleaves DNA into fragments at or near specific recognition sites within molecules known as restriction sites. Restriction enzymes are commonly classified into five types, which differ in their structure and whether they cut their DNA substrate at their recognition site, or if the recognition and cleavage sites are separate from one another. To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. These enzymes are found in bacteria and archaea and provide a defence mechanism against invading viruses. Inside a prokaryote, the restriction enzymes selectively cut up *foreign* DNA in a process called *restriction digestion*; meanwhile, host DNA is protected by a modification enzyme (a methyl transferase) that modifies the prokaryotic DNA and blocks cleavage. Together, these two processes form the restriction modification system.

Recognition site

Restriction enzymes recognize a specific sequence of nucleotide and produce a double-stranded cut in the DNA. The recognition sequences can also be classified by the number of bases in its recognition site, usually between 4 and 8 bases. Many of them are palindromic, meaning the base sequence reads the same backwards and forwards. In theory, there are two types of palindromic sequences that can be possible in DNA. The *mirror-like* palindrome is similar to those found in ordinary text, in which a sequence reads the same forward and backward on a single strand of DNA, as in GTAATG. The *inverted repeat* palindrome is also a sequence that reads the same forward and backward, but the forward and backward sequences are found in complementary DNA strands (i.e., of double-stranded DNA), as in GTATAC (GTATAC being complementary to

CATATG). Inverted repeat palindromes are more common and have greater biological importance than mirror-like palindromes.

Types of digestion produces:

EcoRI digestion produces "sticky" ends,

Examples [edit]

See also: List of restriction enzyme cutting sites

Examples of restriction enzymes include:[75]

Enzyme	Source	Recognition Sequence	Cut
EcoRI	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'
EcoRII	*Escherichia coli*	5'CCWGG 3'GGWCC	5'--- CCWGG---3' 3'---GGWCC ---5'
BamHI	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'
HindIII	*Haemophilus influenzae*	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'
TaqI	*Thermus aquaticus*	5'TCGA 3'AGCT	5'---T CGA---3' 3'---AGC T---5'

Whereas SmaI restriction enzyme cleavage produces "blunt" ends:

```
CCC|GGG
GGG|CCC
```

PvuII*	*Proteus vulgaris*	5'CAGCTG 3'GTCGAC	5'---CAG CTG---3' 3'---GTC GAC---5'
SmaI*	*Serratia marcescens*	5'CCCGGG 3'GGGCCC	5'---CCC GGG---3' 3'---GGG CCC---5'
HaeIII*	*Haemophilus aegyptius*	5'GGCC 3'CCGG	5'---GG CC---3' 3'---CC GG---5'

Recognition sequences in DNA differ for each restriction enzyme, producing differences in the length, sequence and strand orientation (5' end or 3' end) of a sticky-end "overhang" of an enzyme restriction.

Different restriction enzymes that recognize the same sequence are known as neoschizomers. These often cleave in different locales of the sequence.

For example, SmaI (CCC/GGG) and XmaI (C/CCGGG) are neoschizomers of each other.

Different enzymes that recognize and cleave in the same location are known as isoschizomers.

For example, SphI (CGTAC/G) and BbuI (CGTAC/G) are isoschizomers of each other.

Nomenclature

Since their discovery in the 1970s, many restriction enzymes have been identified; for example, more than 3500 different Type II restriction enzymes have been characterized. Each enzyme is named after the bacterium from which it was isolated, using a naming system based on bacterial genus, species and strain. For example, the name of the EcoRI restriction enzyme was derived as shown in the box.

Derivation of the EcoRI name		
Abbreviation	**Meaning**	**Description**
E	*Escherichia*	genus
co	*coli*	specific species
R	RY13	strain
I	First identified	order of identification in the bacterium

Examples:

Haemophilus influenzae D III = Hind III

Serratia marcescens I= SmaI

Types of restriction enzyme

Naturally occurring restriction endonucleases are categorized into four groups (Types I, II III, and IV) based on their composition and enzyme cofactor requirements, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence. DNA sequence analyses of restriction enzymes however show great variations, indicating that there are more than four types. All types of enzymes recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific fragments with terminal 5'-phosphates. They differ in their recognition sequence, subunit composition, cleavage position, and cofactor requirements as summarised below:

- Type I enzymes cleave at sites distance from a recognition site; require both ATP and S-adenosyl-L-methionine to function.
- Type II enzymes cleave within or at short specific distances from a recognition site; most require magnesium.
- Type III cleave at sites a short distance from a recognition site; require ATP , S-adenosyl-L-methionine stimulates the reaction but is not required.
- Type IV enzymes target modified DNA, e.g. methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA
- Type V enzymes utilize guide RNAs (gRNAs: non coding short RNA sequences which bind to complementary target DNA sequences)

DNA ligase

DNA ligase is a specific type of enzyme that facilitates the joining of DNA strands together by catalysing the formation of a phosphodiester bond. It plays a role in repairing single-strand breaks in duplex DNA in living organisms. Single-strand breaks are repaired by DNA ligase using the complementary strand of the double helix as a template, with DNA ligase creating the final phosphodiester bond to fully repair the DNA.

DNA ligase is used in both (DNA repair and DNA replication). In addition, DNA ligase has extensive use in molecular biology laboratories for recombinant DNA experiments.

Purified DNA ligase is used in gene cloning to join DNA molecules together to form recombinant DNA.

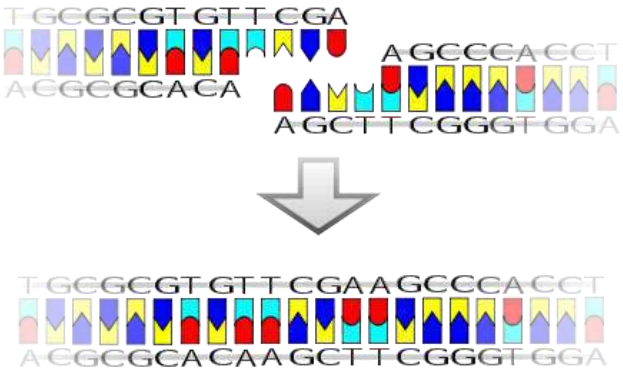
Ligase mechanism

The mechanism of DNA ligase is to form two covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide ("acceptor"), with the 5' phosphate end of another ("donor"). Two ATP molecules are consumed for each phosphodiester bond formed. AMP is required for the ligase reaction, which proceeds in three steps:

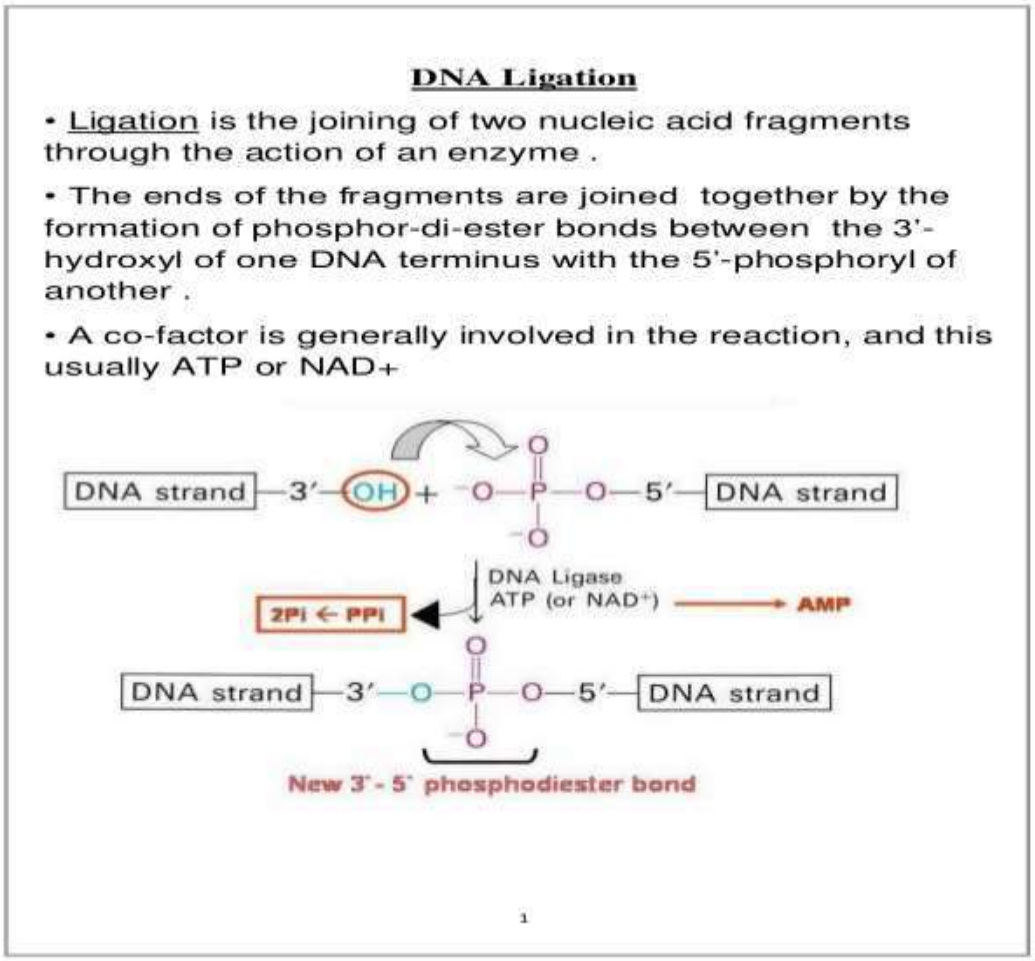
1- Adenylation (addition of AMP) of a lysine residue in the active centre of the enzyme, pyrophosphate is released.

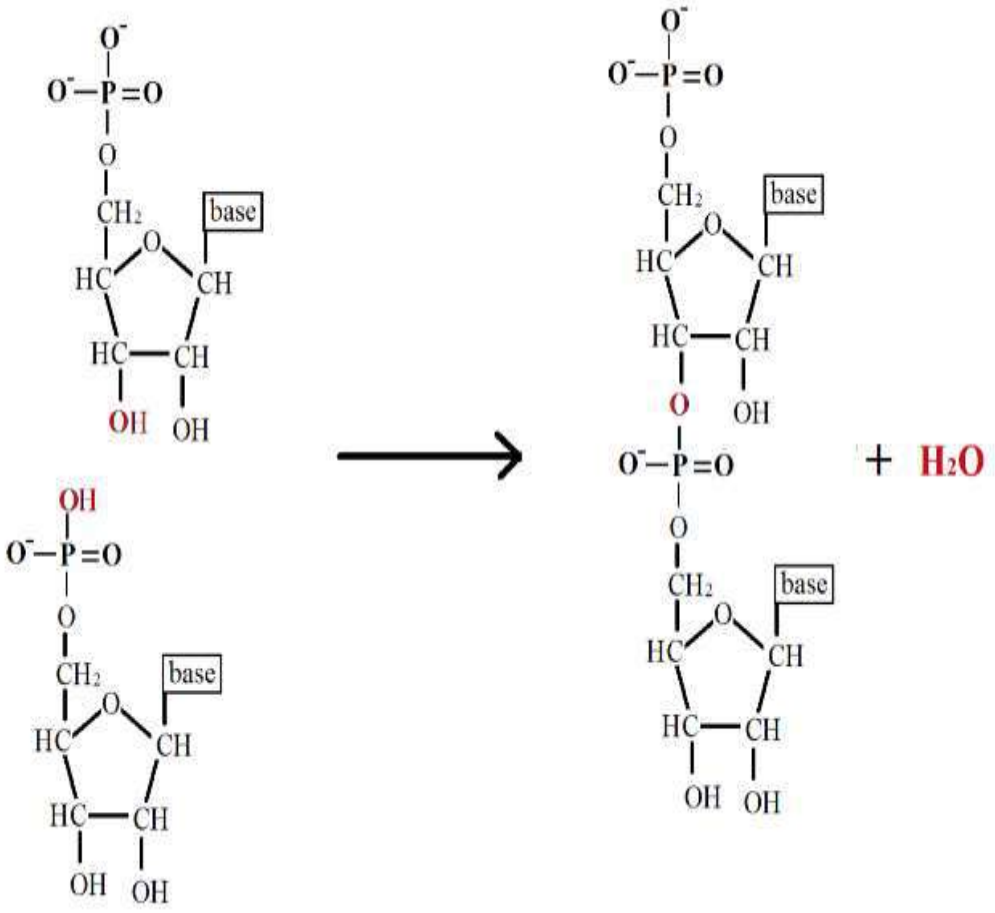
2- Transfer of the AMP to the 5' phosphate of the so-called donor, formation of a pyrophosphate bond.

3- Formation of a phosphodiester bond between the 5' phosphate of the donor and the 3' hydroxyl of the acceptor.



A pictorial example of how a ligase works (with sticky ends)





The formation of a phosphodiester bond

Types of ligases

1- Bacteriophage T4 DNA Ligase

2- E.coli DNA ligase

Differences between Bacteriophage T4 DNA Ligase and E.coli DNA ligase

Bacteriophage T4 DNA Ligase	***E.coli DNA ligase***
1- The most widely used DNA ligase is derived from the T4 bacteriophage.	1- It is derived from E.coli cell.
2- Require ATP as cofactor	2- Require NAD+ as cofactor
3- - It is a monomeric enzyme of MW 68KDa	3- It is a monomeric enzyme of MW 74KDa
4- encoded by bacteriophage gene30	4-encoded by ligA gene of E.coli
5- Repairs single stranded Nicks in duplex DNA, RNA or DNA: RNA hybrids by forming phosphodiester bond.	5-Catalysis the formation of the phosphodiester bond in duplex DNA containing cohesive (sticky) ends.
6- Ligation of sticky and blunt ends. Ligation of blunt end is improved by addition of monovalent cation (Na^+) and low concentration of PEG.	6- Ligation of cohesive (sticky) ends only.
7- Ligation of synthetic linkers or adapter to DNA	7- Using in the cloning of full length cDNA.

Factors affecting ligation

1- DNA concentration

The concentration of DNA can affect the rate of ligation, and whether the ligation is an inter-molecular or intra-molecular reaction. Ligation involves joining up the ends of a DNA with other ends, however, each DNA fragment has two ends, and if the ends are compatible, a DNA molecule can circularize by joining its own ends. At high DNA concentration, there is a greater chance of one end of a DNA molecule meeting the end of another DNA, thereby forming intermolecular ligation. At a lower DNA concentration, the chance that one end of a DNA molecule would meet the other end of the same molecule increases, therefore intramolecular reaction that circularizes the DNA is more likely. The transformation efficiency of linear DNA is also much lower than circular DNA, and for the DNA to circularize, the DNA concentration should not be too high. As a general rule, the total DNA concentration should be less than 10 μg/ml.

The concentration of DNA can be artificially increased by adding condensing agents such as cobalt hexamine and spermidine, or by using crowding agents such as polyethylene glycol (PEG) which also increase the effective concentration of enzymes.

2- Ligase concentration

The higher the ligase concentration, the faster the rate of ligation. Blunt-end ligation is much less efficient than sticky end ligation, so a higher concentration of ligase is used in blunt-end ligations. High DNA ligase concentration may be used in conjunction with PEG for a faster ligation, and they are the components often found in commercial kits designed for rapid ligation.

3- Temperature

Two issues are involved when considering the temperature of a ligation reaction. First, the optimum temperature for DNA ligase activity which is 37˚C, and second, the melting temperature (T_m) of the DNA ends to be ligated. The melting temperature is dependent on length and base composition of the DNA overhang—the greater the number of G and C, the higher the T_m since there are three hydrogen bonds formed between G-C base pair compared to two for A-T base pair—with some contribution from the stacking of the bases between fragments. For the ligation reaction to proceed efficiently, the ends should be stably annealed, and in ligation experiments, the T_m of the DNA ends is generally much lower than 37˚C. The optimal temperature for ligating cohesive ends is therefore a compromise between the best temperature for DNA ligase activity and the T_m where the ends can associate.

4- Buffer composition

The ionic strength of the buffer used can affect the ligation. The kinds of cations presence can also influence the ligation reaction, for example, excess amount of Na^+ can cause the DNA to become more rigid and increase the likelihood of intermolecular ligation. At high concentration of monovalent cation (Na^+) (>200 mM) ligation can also be almost completely inhibited. The standard buffer used for ligation is designed to minimize ionic effects.

Cloning Vectors

In molecular biology, a vector is a DNA molecule used as a vehicle to transfer foreign genetic material into another cell. The three major types of vectors are studying in this course (plasmids, viral vectors (lambda phage and M13) and cosmids . All engineered vectors have an origin of replication, a multi-cloning site, and a selectable marker. The purpose of a vector that transfers genetic information to another cell is typically to isolate, multiply, or express the insert in the target cell.

General Characteristics of a cloning vectors

- Cloning vector is a small DNA molecule capable of self-replication inside the host cell. Cloning vector is used for replicating donor DNA fragment within host cell.

1. it must be small in size
2. It must be self-replicating inside host cell
3. It must possess restriction site for Restriction Endonuclease enzymes
4. It must possess some marker gene such that it can be used for later identification of recombinant cell
5. it must possess multiple cloning site

What Are Some Distinct Features of ideal Cloning Vectors?

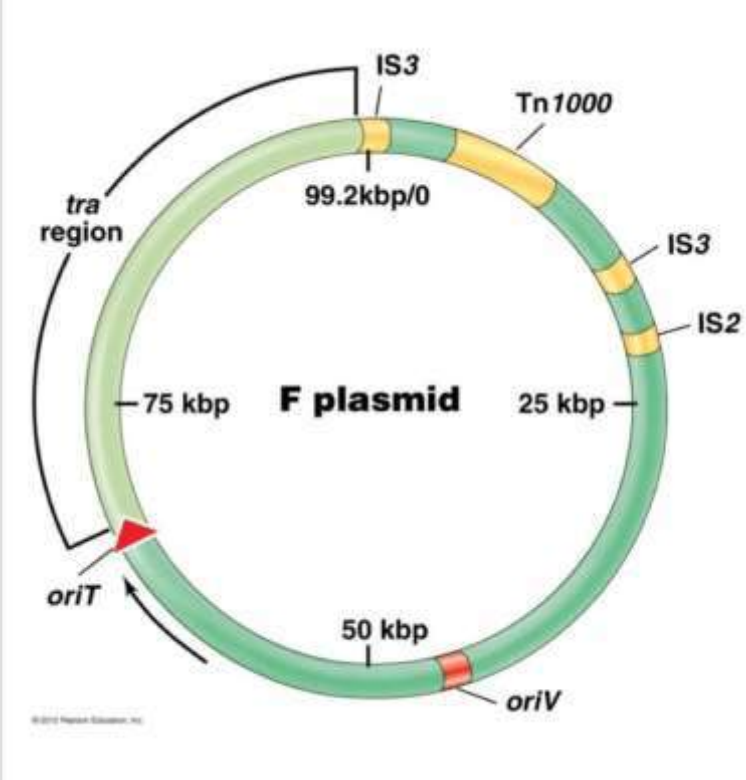
Some of these key features are extremely necessary for their functions. This includes the presence of an appropriate cloning site and selectable markers. Some other features can be present but these are only limited to their usage. The process of cloning is usually performed using E. Coli and hence the cloning vectors usually have systems to enable the maintenance and functioning in E.Coli.

Sometimes, there are other features too that enable them to maintain themselves in other organisms in addition to E. Coli.

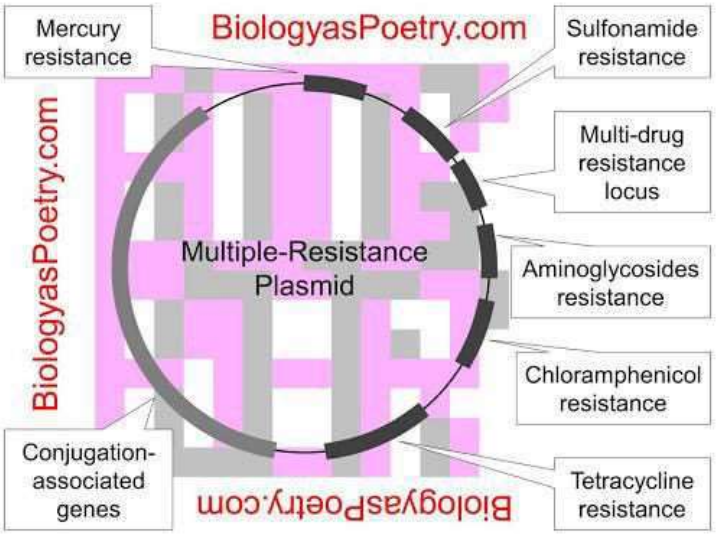
1. Origin of Replication – The specific sequence of nucleotide in a DNA, which acts, as the origin of the replication process is known as ORI. The foreign DNA starts replicating along with the host cell when it is integrated or connected to this sequence.
2. Selectable marker gene – The cloning vector must possess a selectable marker gene as it allows the selection of the host cells, which carry the recombinant DNA, and separates them from those that do not
3. Presence of restriction sites – It should have restriction sites to enable breakup of certain sequences with respect to restriction endonuclease.
4. It must be not too big in size (small size)
5. The insertion of donor DNA should not hamper the replication process and property of the cloning vector.
6. There must be multiple sites for cloning.
7. The vector and the sample DNA are both digested with the same restriction enzyme. It is then recombined so as to enable them to grow in a host. Vectors contain selectable markers that aid in determining which recombinant has to be inserted.
8. Conformation: the vectors prefer circular than linear because the circular is small in size and can enter easily to the bacteria via its pores because circular vectors have small surface area.

Another way to classify plasmids is by function. There are four main classes:

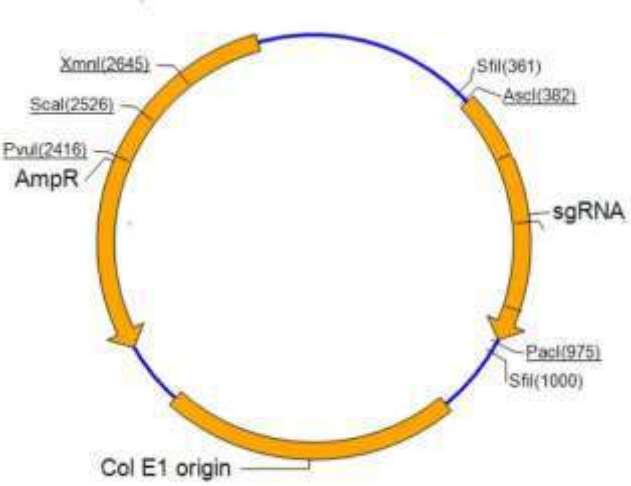
- Fertility F-plasmids, which contain tra genes. They are capable of conjugation and result in the expression of sex pilli.



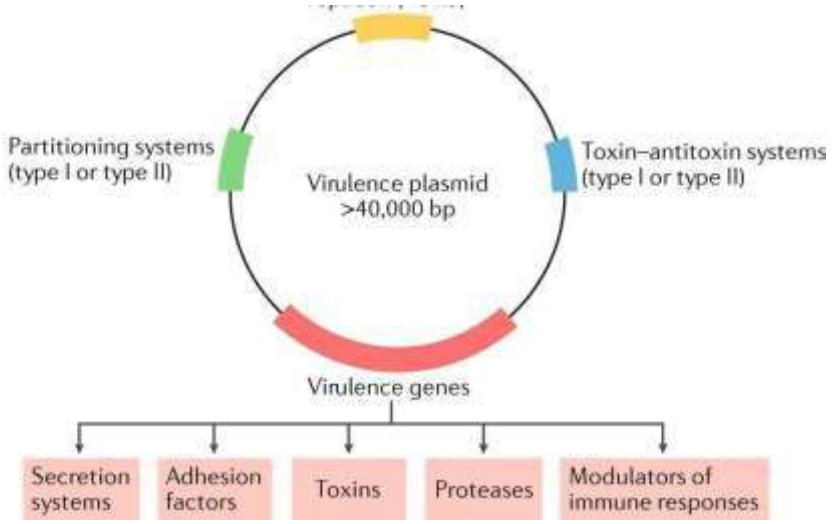
- Resistance plasmids, which contain genes that provide resistance against antibiotics or poisons. They were historically known as R-factors, before the nature of plasmids was understood.



- Col plasmids, which contain genes that code for bacteriocins, proteins that can kill other bacteria.



- Virulence plasmids, which turn the bacterium into a pathogen.



Principle of genetic engineering

د.عبد الكريم القزاز
د.ريما محمد العبيدي

Lec.10

Classified the plasmids according to their function.

1- Fertility F-plasmids

Fertility plasmids, also known as F-plasmids, contain transfer genes that allow genes to be transferred from one bacteria to another through conjugation. Bacteria that have the F-plasmid are known as F positive (F^+), and bacteria without it are F negative (F^-). When an F^+ bacterium conjugates with an F^- bacterium, two F^+ bacterium result.

2-Resistance Plasmids

Resistance or R plasmids contain genes that help a bacterial cell defend against environmental factors such as poisons or antibiotics. Some resistance plasmids can transfer themselves through conjugation. When this happens, a strain of bacteria can become resistant to antibiotics.

3-Virulence Plasmids

When a virulence plasmid is inside a bacterium, it turns that bacterium into a pathogen, which is an agent of disease. Bacteria that cause disease can be easily spread and replicated among affected individuals. The bacterium *Escherichia coli (E. coli)* has several virulence plasmids

4- Col Plasmids: Col plasmids contain genes that make bacteriocins (also known as colicins), which are proteins that kill other bacteria and thus defend the host bacterium. Bacteriocins are found in many types of bacteria including *E. coli.*

Classified the plasmids according to their number of copies:

1-relaxed plasmids :exist in large number of copies with small in sizes (50-1000)copy

2-stringent plasmids: exist in little number of copies with large in size

Classified the plasmids according to conjugation:

1-Conjugative plasmids have (tra genes) : Conjugative plasmids mediate DNA transfer through conjugation and therefore spread rapidly among the bacterial cells of a population (high transformation efficiency)

2-Non- conjugative plasmids: Non-conjugative plasmids do not mediate DNA through conjugation.

The cloning vector must have the following genes and sequences to be successful cloning vector:

1- Ori sequence (original sequence)
2- Tra genes (transfer gene)
3- Par sequence (partition sequence)
4- Inc genes (in compatibility genes)

Plasmid vector

• Plasmid vectors are double-stranded, extra-chromosomal DNA molecules, circular, self-replicating.

• *Advantages:*

– Small, easy to handle

– Easy purification

– Useful for cloning small DNA fragments (< 10kbp)

• *Disadvantages:*

– Less useful for cloning large DNA fragments (> 10kbp)

A plasmid vector for cloning

1. Contains an origin of replication, allowing for replication independent of host's genome.

2. Contains Selective markers: Selection of cells containing a plasmid

- two antibiotic resistance

- blue-white screening

3. Contains a multiple cloning site (MCS)

4. Easy to be isolated from the host cell.

5. Plasmids range in size from 1.0kb to 250kb e.g. pUC8 is 2.1 kb.

6. Plasmid can cloned foreign DNA up to 10kbp

The best plasmid is pBR322

• It was one of the first vectors to be developed in 1977.

• The 'p' indicates that it is plasmid, 'BR' indicates Bolivar and Rodriguez

• '322' distinguishes it from the other plasmids produced in the same laboratory e.g. pBR325, pBR327, pBR328.

• It is 4361bp (4.361 kbp) in size i.e. less than 10kb

• It carries two sets of antibiotic resistance genes i.e. either ampicillin or tetracycline can be used as a selectable marker.

• Each of the marker genes carries unique restriction sites and insertion of DNA into these sites inactivates the specific marker site. e.g. insertion of new DNA with Pst1, Puv1, Ppa1 or Sca1 inactivates the amp^R gene.

• It has a high copy number. They are about 15 molecules present in ransformed cells but it can be increased to 1000 to 3000 by plasmid amplification in the presence of protein synthesis inhibitor i.e. chloramphenicol.

• The vector comprises DNA derived from three different naturally occurring plasmids: the amp^R gene is from R1 plasmid, tet^R from R6-5 plasmid and the ori gene from pMB1 plasmid.

The constructed *E. coli* plasmid pBR322

Features of pBR322

1.An origin of replication (ori)
2.Two genes that confer resistance to different antibiotics (tet^R, amp^R)
3. Several unique recognition sequences (EcoRI, BamH1)
4. Small size (4,361 bp)