Sengamala Thayaar Educational Trust Women's College
(Affiliated to Bharathidasan University)
(Accredited with 'A' Grade {3.45/4.00} By NAAC)
(An ISO 9001: 2015 Certified Institution)
Sundarakkottai, Mannargudi-614 016.
Thiruvarur (Dt.), Tamil Nadu, India.

# GENETIC ENGINEERING

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**PG & RESEARCH DEPARTMENT OF BIOCHEMISTRY**

## II M.Sc., BIOCHEMISTRY

**Semester : III**

## ELECTIVE III -GENETIC ENGINEERING-P16BCE3

**Inst. Hours/Week : 5** **Credit : 5**

### Objective:

To understand and learn the emergence and early development and application of technology.

### UNIT I

Introduction to genetic engineering and rDNA technology, gene cloning, specialized tools and techniques, benefits of gene cloning. Isolation and purification of DNA: Preparation of total Cellular DNA, plasmid DNA, bacteriophage DNA, plant cell DNA, isolation of mRNA from mammalian cells.

### UNIT II

Vectors and enzymes in cloning: Cloning and Expression vectors- Plasmids pBR, pUC, phages (M3, λ), yeast vectors, cosmids, phagemids, agrobacterium, PAC, BAC, YAC, MAC, HAC vectors, Plant and Animal viruses as vector, binary and shuttle vectors, expression vectors for prokaryotes and eukaryotes, expression cassettes. Restriction endonucleases, ligases, S1 nuclease, reverse transcriptase, polymerase, alkaline phosphatase, terminal transferase, methods of ligation.

### UNIT III

Construction of genomic and cDNA libraries, selection and screening of recombinants, probes- types, synthesis and uses of probes. Blotting techniques (Southern, Northern and Western), PCR- types and applications, Sequencing: DNA and RNA, site directed mutagenesis. Chromosome walking, jumping, DNA finger printing and foot printing.

## UNIT IV

Methods of gene transfer: Microinjection, electroporation, particle bombardment gun (biolistic), ultrasonication, liposome mediated and direct transfer. Restriction analysis of DNA, molecular markers- RFLP, RAPD, VNTR, SSR, AFLP, STS, SCAR, SNP. Microarrays. Genomics ( human genomic project) and proteomics – types and applications.

## UNIT V

Applications of Genetic Engineering: Recombinant insulin, somatotropin, vaccines, role of genetic engineering in diagnosis and cure of diseases, gene therapy, transgenic plants (herbicide resistant, pesticide resistant, and antisense RNA technology and its application). Transgenic animals. IPR, Patenting, Ethical, legal issues and hazards of genetic engineering.

## Reference Books:


1. Principles of Gene Manipulation and Genomics, Seventh edition, S.B. Primrose and R.M. Twyman, 2006 Blackwell Publishing, USA.
2. Molecular Biotechnology- Principles and applications of Recombinant DNA, Bernard R. Glick, Jack J. Pasternak, and Cheryl L. Patten. — 4th ed., ASM Press, Washington, DC , USA
3. Gene cloning and DNA analysis : an introduction / T.A. Brown.—6th ed- Brown, T.A. (Terence A.) , Wiley-Blackwell. 2010.
4. Elements of Biotechnology, P.K. Gupta, Rastogi Publications, 2nd edition 3rd reprint, 2015-2016.
5. A text book of Biotechnology, R.C.Dubey, S.Chand Publications, 2014
6. An Introduction to Genetic Engineering, Third Edition, Desmond S. T. Nicholl, Cambridge University Press, USA
7. Genetic Engineering – Basics, New Applications and Responsibilities, Edited by Hugo A. Barrera-Saldaña, Published by InTech, Croatia, 2011.


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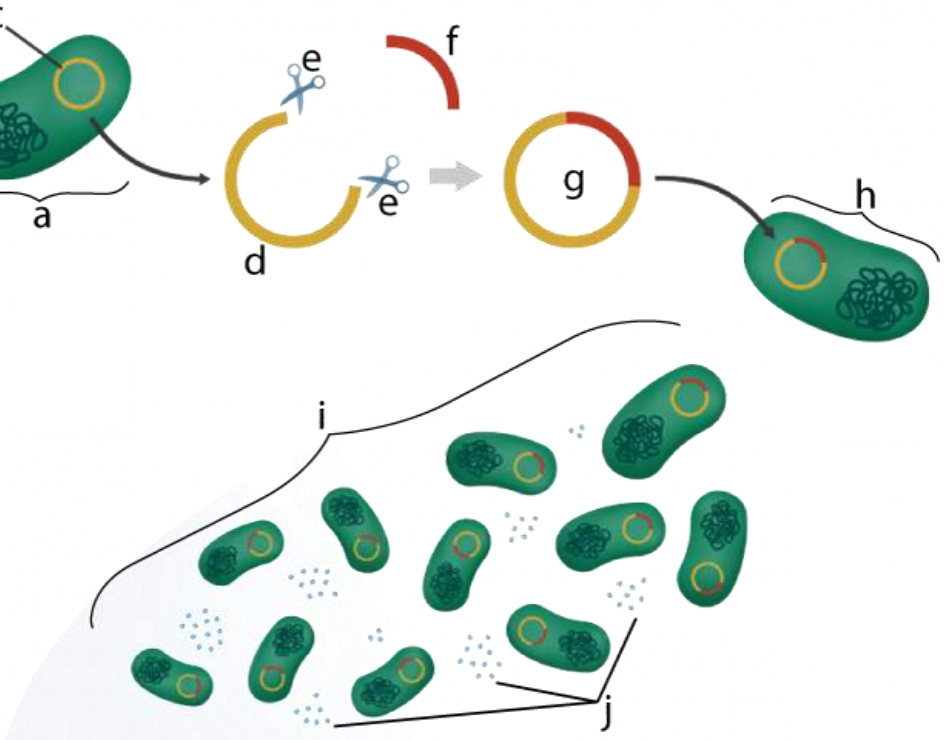
# Genetic Engineering

## Introduction

Genetic engineering is the process of altering the DNA in an organism's genome. While it might be argued that humans have been genetically engineering plants and animals for thousands of years through plant and animal breeding, the kind of genetic engineering we'll be discussing here is different: it involves direct manipulation of DNA, usually by introducing genes from one species into another.

An example of genetic engineering that touches millions of lives each year: the synthesis of the hormone insulin to treat diabetes.




In the diagram at right, letter "a" is a bacterial cell. In addition to this cell's main chromosome (b) it also has a small circle of DNA that's called a plasmid (shown at "c"). Plasmids are natural parts of the bacterial genome, and they're often used to transfer genes between bacterial cells (a kind of genetic exchange that does not happen in animals or plants).

Using techniques of genetic engineering (some of which I'll expand upon below), a plasmid can be extracted from a bacterial cell, and then, using enzymes (e) that act as a kind of molecular scissors, the plasmid can be cut open (as shown at "d"). This plasmid DNA can then be combined with DNA from another species

(such as the human insulin gene, shown at “f”). This creates a piece of **recombinant DNA** (g): DNA that is combined from two sources. In this case, it’s a plasmid that contains both bacterial DNA and the human insulin gene. Then, through a technique called transformation, the genetically engineered plasmid is re-inserted into a bacterial cell (h). Every time the bacterial cell reproduces, it will copy the recombinant plasmid (i). The plasmid’s DNA will also be transcribed and translated, producing human insulin (j).

# Recombinant DNA Technology

## Introduction

### Followings are some definitions of recombinant DNA technology:

1. Recombinant DNA technology or Genetic engineering is the deliberate, controlled manipulation of the genes in an organism with the intent of making that organism better in some way.
2. Recombinant DNA technology covers all various experimental techniques that manipulate the genes of the organism. It uses recombinant DNA, molecular cloning and transformation.
3. Recombinant DNA technology involves the scientific alteration of the structure of genetic material in a living organism. It involves the production and use of recombinant DNA and has been employed to create bacteria that synthesize insulin and other human proteins.
4. Recombinant DNA technology is the branch of biology dealing with the splicing and recombining of specific genetic units from the DNA of living organisms as in order to produce new species or bio-chemicals.
5. Recombinant DNA technology is defined as the development and application of scientific methods, procedures, and technologies that permit direct manipulation of genetic material in order to alter the hereditary traits of a cell, organism, or population.
6. Recombinant DNA technology includes a bunch of techniques that produce unlimited amounts of otherwise unavailable or scarce biological product by introducing DNA isolated from animals or plants into bacteria and then harvesting the product from a bacterial colony, as human insulin produced in bacteria by the human insulin gene.

## Basic Principle of Recombinant DNA Technology

Although there are many diverse and complex techniques involved, the basic principles of genetic manipulation are reasonably simple. The premise on which the technology is based is that genetic information, encoded by DNA and arranged in the form of genes, is a resource that can be manipulated in various ways to achieve certain goals in both pure and applied science and medicine.

Every living entity consists of one or more cells with DNA-molecules that contain the blueprint for thousands of cellular proteins.

A piece of DNA with the code for a particular protein (called gene of interest), together with appropriate control codes (like promoter, operator and

regulator), can be inserted into a host cell, where it becomes integrated into the latter's genome.

The recombinant cell is then grown in large quantities to produce the protein of interest. This recombinant protein, which is stored inside the cell or secreted into the culture medium, can be recovered, purified and formulated into a product used in healthcare, industry or agriculture.

The host cell can be a bacterium, fungus, yeast or animal-derived cell. In some cases we just want to make multiple copies of the inserted gene of interest and don't want it be translated into proteins. In this case we collect these replicated copies of the gene, purify it and store it.

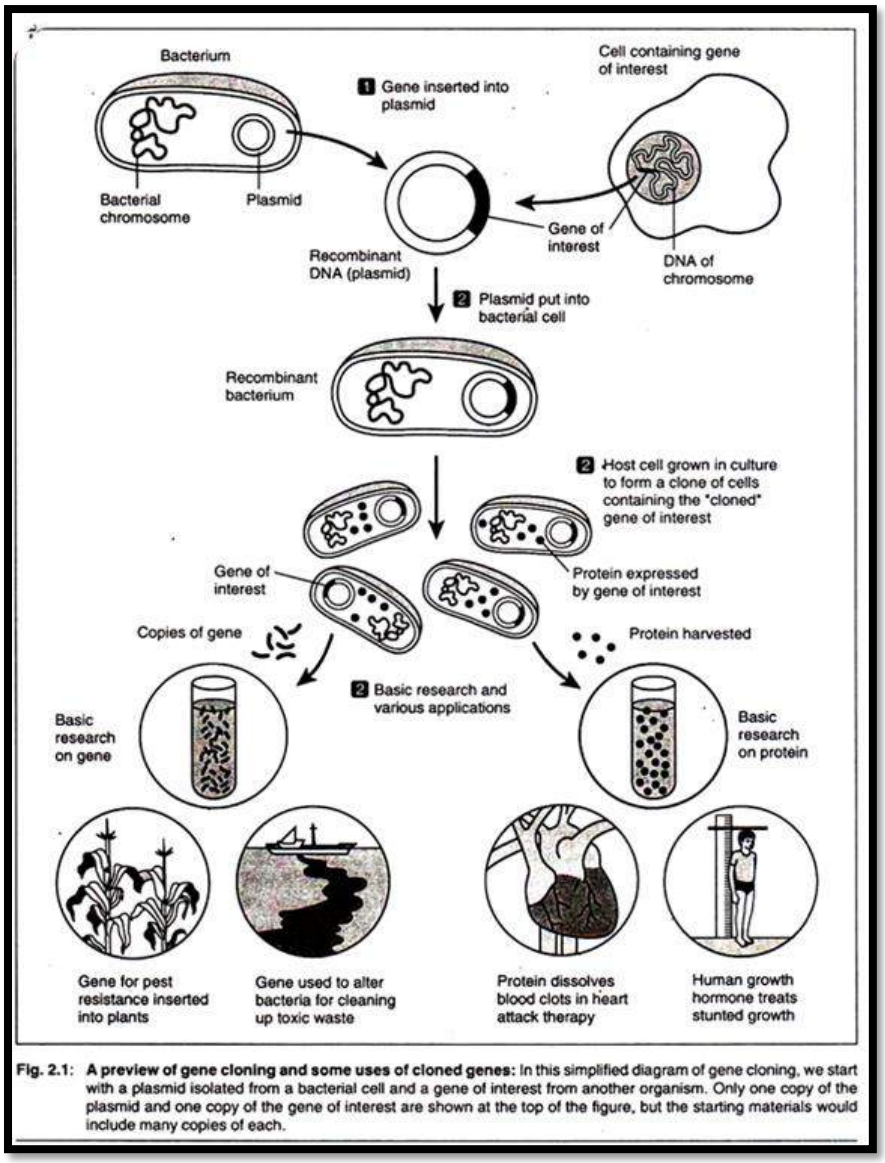



**Fig. 2.1: A preview of gene cloning and some uses of cloned genes:** In this simplified diagram of gene cloning, we start with a plasmid isolated from a bacterial cell and a gene of interest from another organism. Only one copy of the plasmid and one copy of the gene of interest are shown at the top of the figure, but the starting materials would include many copies of each.

## Gene Cloning, Specialized Tools and Techniques, Benefits of Gene Cloning

## Gene Cloning- Requirements, Principle, Steps, Applications

- The production of exact copies of a particular gene or **DNA** sequence using genetic engineering techniques is called gene cloning.
- The term "gene cloning," "DNA cloning," "molecular cloning," and "recombinant DNA technology" all refer to same technique.
- When DNA is extracted from an organism, all its genes are obtained. In gene (DNA) cloning a particular gene is copied forming "clones".
- Cloning is one method used for isolation and amplification of gene of interest.

**DNA cloning can be achieved by two different methods**:

1. Cell based DNA cloning
2. Cell-free DNA cloning (PCR)

### Requirements for Gene Cloning (Cell-based)

1. **DNA fragment** containing the desired genes to be cloned.
2. **Restriction enzymes** and **ligase enzymes**.
3. **Vectors** – to carry, maintain and replicate cloned gene in host cell.
4. **Host cell**– in which recombinant DNA can replicate

### Principle of Gene Cloning

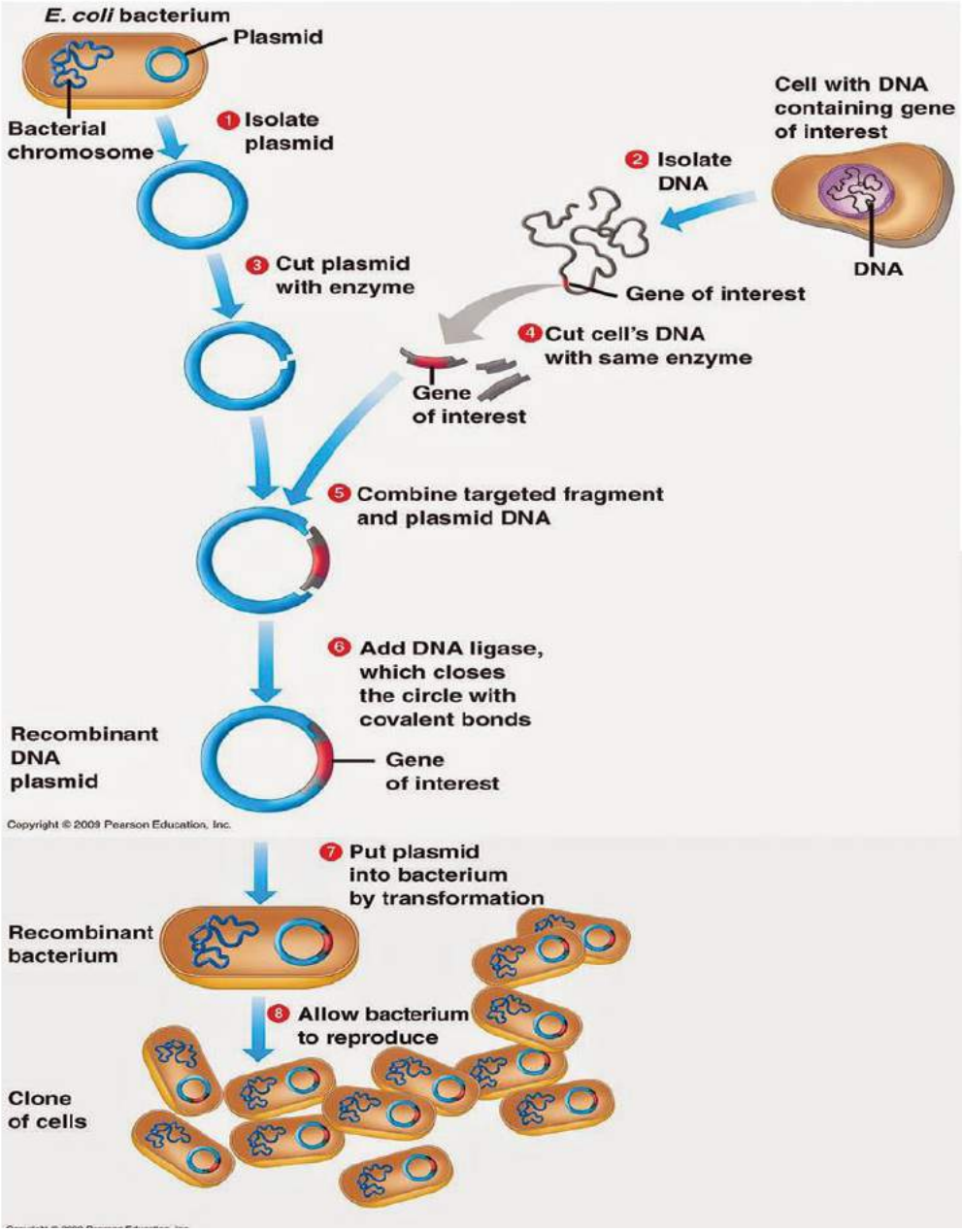
A fragment of DNA, containing the gene to be cloned, is inserted into a suitable vector, to produce a recombinant DNA molecule. The vector acts as a vehicle that transports the gene into a host cell usually a bacterium, although other types of living cell can be used. Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place. After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.

### Steps in Gene Cloning

The basic 7 steps involved in gene cloning are:

1. Isolation of DNA [gene of interest] fragments to be cloned.
2. Insertion of isolated DNA into a suitable vector to form recombinant DNA.

3. Introduction of recombinant DNA into a suitable organism known as host.
4. Selection of transformed host cells and identification of the clone containing the gene of interest.
5. Multiplication/Expression of the introduced Gene in the host.
6. Isolation of multiple gene copies/Protein expressed by the gene.
7. Purification of the isolated gene copy/protein



**A. Isolation of the DNA fragment or gene**

- The target DNA or gene to be cloned must be first isolated. A gene of interest is a fragment of gene whose product (a protein, enzyme or a hormone) interests us. For example, gene encoding for the hormone insulin.
- The desired gene may be isolated by using restriction endonuclease (RE) enzyme, which cut DNA at specific recognition nucleotide sequences known as restriction sites towards the inner region (hence endonuclease) producing blunt or sticky ends.
- Sometimes, reverse transcriptase enzyme may also be used which synthesizes complementary DNA strand of the desired gene using its mRNA.

**B. Selection of suitable cloning vector**

- The vector is a carrier molecule which can carry the gene of interest (GI) into a host, replicate there along with the GI making its multiple copies.
- The cloning vectors are limited to the size of insert that they can carry. Depending on the size and the application of the insert the suitable vector is selected.
- The different types of vectors available for cloning **are plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs)** and **mammalian artificial chromosomes (MACs).**
- However, the most commonly used cloning vectors include plasmids and bacteriophages (phage λ) beside all the other available vectors.

**C. Essential Characteristics of Cloning Vectors**

All cloning vectors are carrier DNA molecules. These carrier molecules should have few common features in general such as:

- It must be self-replicating inside host cell.
- It must possess a unique restriction site for RE enzymes.
- Introduction of donor DNA fragment must not interfere with replication property of the vector.
- It must possess some marker gene such that it can be used for later identification of recombinant cell (usually an antibiotic resistance gene that is absent in the host cell).
- They should be easily isolated from host cell.

**D. Formation of Recombinant DNA**

- The plasmid vector is cut open by the same RE enzyme used for isolation of donor DNA fragment.
- The mixture of donor DNA fragment and plasmid vector are mixed together.
- In the presence of DNA ligase, base pairing of donor DNA fragment and plasmid vector occurs.
- The resulting DNA molecule is a hybrid of two DNA molecules – the GI and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination.
- Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the **recombinant DNA technology**.

**E. Transformation of recombinant vector into suitable host**

- The recombinant vector is transformed into suitable host cell mostly, a bacterial cell.
- This is done either for one or both of the following reasons:
    - To replicate the recombinant DNA molecule in order to get the multiple copies of the GI.
    - To allow the expression of the GI such that it produces its needed protein product.
- Some bacteria are naturally transformable; they take up the recombinant vector automatically.

For example: Bacillus, Haemophillus, Helicobacter pylori, which are naturally competent.

- Some other bacteria, on the other hand require the incorporation by artificial methods such as $Ca^{++}$ ion treatment, electroporation, etc.

**F. Isolation of Recombinant Cells**

- The transformation process generates a mixed population of transformed and non-trans- formed host cells.
- The selection process involves filtering the transformed host cells only.
- For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.
- For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is

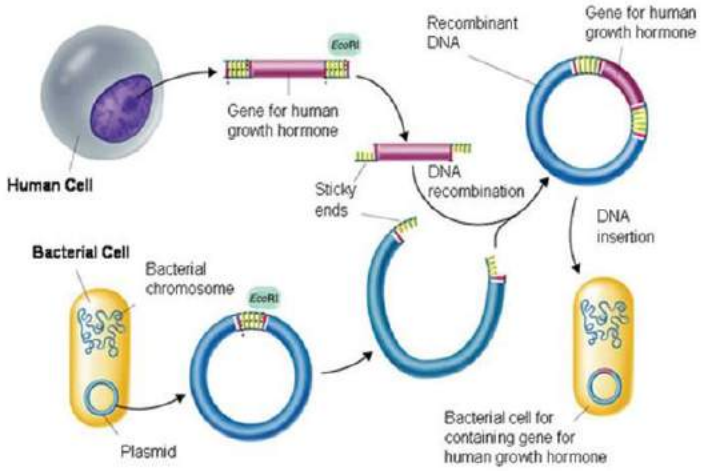
used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.

**G. Multiplication of Selected Host Cells**

- Once transformed host cells are separated by the screening process; becomes necessary to provide them optimum parameters to grow and multiply.
- In this step the transformed host cells are introduced into fresh culture media .
- At this stage the host cells divide and re-divide along with the replication of the recombinant DNA carried by them.
- If the aim is obtaining numerous copies of GI, then simply replication of the host cell is allowed. But for obtaining the product of interest, favourable conditions must be provided such that the GI in the vector expresses the product of interest.

**H. Isolation and Purification of the Product**

- The next step involves isolation of the multiplied GI attached with the vector or of the protein encoded by it.
- This is followed by purification of the isolated gene copy/protein.




## Applications of Gene Cloning

- A particular gene can be isolated and its nucleotide sequence determined
- Control sequences of DNA can be identified & analyzed
- Protein/enzyme/RNA function can be investigated
- Mutations can be identified, e.g. gene defects related to specific diseases Organisms can be 'engineered' for specific purposes, e.g. insulin production, insect resistance, etc.

# ISOLATION AND PURIFICATION OF NUCLEIC ACIDS (GENOMIC/PLASMID DNA AND RNA)

## 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 remaining unchanged (helical ribbon). 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.

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

## Purification of DNA

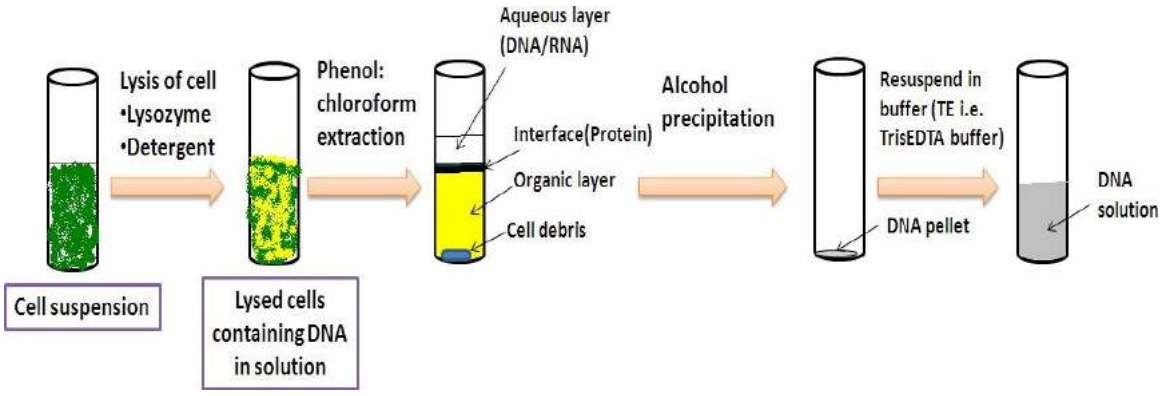
In addition to DNA, a cell extract contains significant quantities of protein and RNA which can be further purified by following methods-

### 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.

### 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.

### Advantage

It leaves short-chain and monomeric nucleic acid components in solution. Ribonucleotides produced by the ribonuclease treatment are separated from 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.

## Growth of the bacterial cell

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

## 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.

## Purification of Plasmid DNA

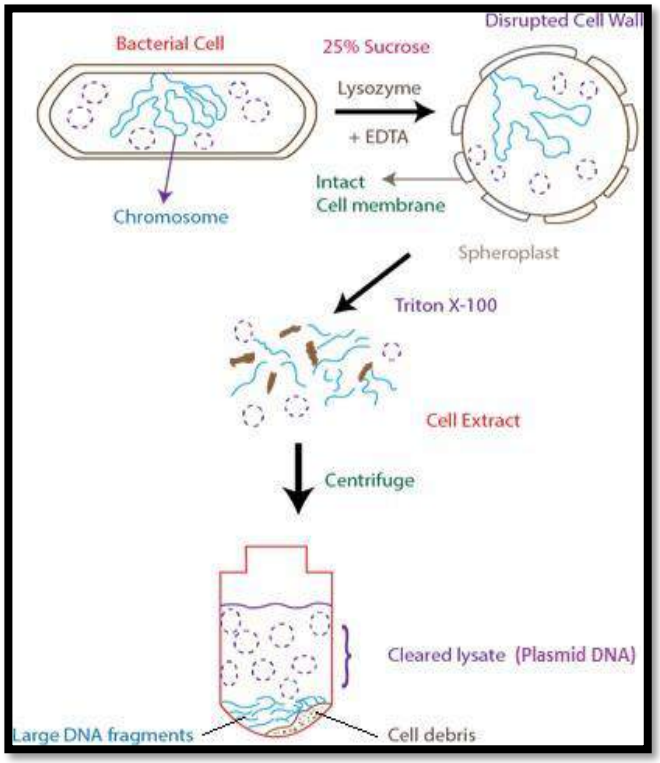
This step is same for both plasmid and genomic but former involves an additional step i.e. the separation of plasmid DNA from the large bacterial chromosomal DNA.

## 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-

### Separation based on size difference

- It involves lysis of cells with lysozyme and EDTA in the presence of sucrose (prevents the immediate bursting of cell).
- Cells with partially degraded cell walls are formed that retain an intact cytoplasmic membrane called as sphaeroplasts.
- 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.**

## 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-

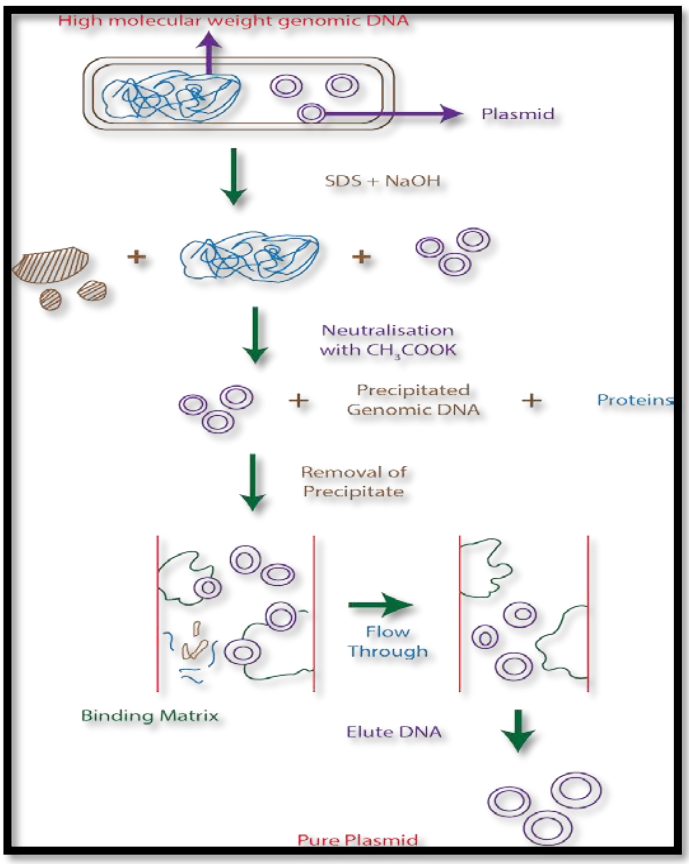
### (a). Alkaline denaturation method

- This method is based on maintaining a very narrow pH range for the denaturation of non-supercoiled DNA but not the supercoiled plasmid (Figure 4-1.3.3.1.2(a).).

- 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.




**(a). 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.

## Phage DNA Extraction with Phenol:Chloroform

In this protocol you extract the genomic DNA from the phages in a lysate. The lysates are "dirty" in that they contain spent media components, cell wall debris, flagella, nucleic acids, bacterial proteins and unassembled phage proteins in addition to the phage themselves. There are many methods for purifying phage DNA from the rest of the lysate, This protocol is recommended specifically for phages that have not resulting in high-yield or high-quality DNA from a kit (like the Promega Wizard DNA Clean-up Kit used in the DNA Extraction Protocol). For best results, adhere to the recommendations for incubation times listed in each step.

### Day 1: PEG Precipitation

1. Take 1 volume of phage high titer lysate (see below) and add PEG (10% PEG- 8000, 1 M NaCl final concentration) at a ratio of 1:2 precipitant:lysate. Mix gently by inversion.
    a. As a rule of thumb, 1 ml of phage lysate with a titer of 1 x 1010

contains about 0.5 µg of phage DNA, assuming the phage has a 50 kb genome. Typically 10 ml of a high-titer phage lysate is used for DNA extraction. More or less lysate (up to 20 ml) may be used depending on the phage stock titer and the expected genome size of the phage. For low-titer phage stocks, up to 20 ml of lysate may be used. With high titer stocks of large- genome phages, use 10 ml of lysate or less.

2. Incubate at 4°C overnight (this is ideal, can also do 60 minutes on ice if you need to work faster). Most phages are stable like this for several days.

### Day 2: DNA Extraction

1. Centrifuge the PEG-precipitated sample at 10,000 *xg* for 30 minutes.
2. Use 5mM $MgSO_4$ to re-suspend the pellet, pipetting gently up and down. AVOID introducing BUBBLES during the resuspension. Be sure to rinse down the sides of the tube to obtain all of the pellet. Transfer to a labeled 2 mL epi tube.
3. Using 500 µl of the concentrated sample: add 1.25 ul of DNaseI and RNase (20 mg/ml) and incubate at 37ºC for 1hour.
    a. (You can scale all volumes up if more than 500 uL are needed for resuspension).
4. Add 1.25 µl of Proteinase K 20 mg/ml stock (20 g total) and 25 l 10% SDS stock (0.5% final concentration) and 20 ul of 0.5 M EDTA pH 8.0 (20 mM final). Mix and incubate 1 hour at 60C.
5. Allow to cool to room temperature.
6. Add an equal volume of **phenol:chloroform** (1:1) and invert several times.
7. Spin 3000 *xg* (6000 rpm on microfuge), 5 minutes room temperature.
8. Carefully transfer the supernatant with a wide-bore pipette tip to a fresh, labeled 2 mL epi tube.
9. Add an equal volume of **phenol:choroform** (1:1), invert.
10. Centrifuge as above and transfer the supernatant again.
11. Add an equal volume of **chloroform**, invert.
12. Centrifuge as above and transfer the supernatant to a fresh, labeled 2mL epi tube.
    a. Depending on volume here you may need to use a 15mL falcon tube
13. Add 1/10 volume of 3 M NaOAc (pH 7.5), and 2.5 volumes of ice cold ethanol (100%). Mix well and incubate at -20°C overnight.
    a. A fast method allows incubation on ice for 15-30 minutes.

## Day: 3 DNA Precipitation

1. Centrifuge in a benchtop microfuge at max speed 20 minutes (10000 rpm for 15ml tubes as tube rating allows, 15000 rpm on the microfuge).
2. Carefully remove supernatant and fill tube halfway with 70% ethanol (made from 100% with *purified nuclease free water*), spin at max speed for 2 minutes.
3. Repeat the above step one time (2nd 70% wash).
4. Remove as much ethanol as possible without disturbing the pellet – good idea to hold onto the supernatant until DNA recovery has been confirmed.
5. Leave tube open on bench ~ 15-30 minutes to let ethanol disperse.
6. Dissolve in TE buffer (~ pH 7.6).

# ISOLATION OF PLANT DNA BY CTAB METHOD

### EXTRACTION PROCEDURE

- Grind −80◦C stored leaves (1 g) to fine powder in ice cold condition in the presence of 250 mg PVP (PolyVinyl Pyrrolidone, Mr 10,000) by using pre chilled mortar and pestle (−40◦C/−80◦C).
- Transfer the content in 2 mL microcentrifuge tubes and suspend in two volumes of suspension buffer. (Suspension buffer (pH 8) containing 50 mM EDTA, 120 mM Tris-HCl, 1 M NaCl, 0.5 M sucrose, 2% Triton-X 100, and 0.2% β-mercaptoethanol (to be freshly added just before use).
- Invert and mix gently and incubate at 60◦C for 40 min.
- Centrifuge the suspension at 10,000 rpm for 15 min at room temperature.
- Add 1.5 mL of extraction buffer containing 20 mM EDTA, 100 mM Tris-HCl, 1.5 M NaCl, 2% CTAB, 1% β-mercaptoethanol and incubate at 60◦C for 30 min.
- Centrifuge at 12,000 rpm for 15 min at room temperature.
- Carefully transfer the aqueous phase into a new tube.

  Note: Use wide-bore tips for transferring the aqueous phase to avoid mechanical damage to DNA.
- Add double volume of Chloroform: Isoamyl alcohol (24 : 1), and invert gently 15 to 20 times and centrifuge at 12,500 rpm for 15 min.

  Note: If the aqueous layer appears translucent, repeat the step until the solution is transparent.

- Add double volume of chilled isopropanol and keep at −20◦C for one hour to precipitate the DNA.

  Note: The longer the chilled incubation, the more the precipitation.
- Centrifuge at 12,000 rpm for 15 min and discard the supernatant.
- To the pellet, add 70% chilled ethanol and spool out the pellet carefully and centrifuge again at 12,000 rpm for 15 min.
- Discard the supernatant and vacuum dry or air dry the pellet at room temperature.

- Add 100 µL of high salt TE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 8).
- Add 3 µL RNase (10 mg/mL) and keep at 37◦C for 30 min followed by chloroform: isoamyl alcohol extraction and ethanol precipitation in the presence of 3 M sodium acetate (pH 5.2).
- Spool out the DNA, wash in 70% ethanol, air or vacuum dry.
- Add 30 to 50 µL (depending upon the pellet) of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) to dissolve the precipitate.
- The isolated DNA was subjected to Agarose Gel electrophoresis

## Isolation and Purification of RNA

RNA (Ribonucleic acid) is a polymeric substance consisting of a long single-stranded chain of phosphate and ribose units with the nitrogen bases adenine, guanine, cytosine and uracil bonded to the ribose sugar present in living cells and many viruses. The steps for preparation of RNA involve homogenization, phase separation, RNA precipitation, washing and re-dissolving RNA.

The method for isolation and purification of RNA are as follows-

1) Organic extraction method
2) Filter-based, spin basket formats
3) Magnetic particle methods
4) Direct lysis method.

## Organic extraction method

This method involves phase separation by addition and centrifugation of a mixture of a solution containing phenol, chloroform and a chaotropic agent (guanidinium thiocyanate) and aqueous sample. Guanidium thiocyanate results in the denaturation of proteins and RNases, separating rRNA from ribosomes. Addition of chloroform forms a colorless upper aqueous phase containing RNA, an interphase containing DNA and a lower phenol-chloroform phase containing protein. RNA is collected from the upper aqueous phase by alcohol (2-propanol or ethanol) precipitation followed by rehydration.

One of the advantages of this method is the stabilization of RNA and rapid denaturation of nucleases. Besides advantages, it has several drawbacks such as it is difficult to automate, needs labor and manual intensive processing, and use of chlorinated organic reagents

## Direct lysis methods

This method involves use of lysis buffer under specified conditions for the disruption of sample and stabilization of nucleic acids. If desired, samples can also be purified from stabilized lysates. This method eliminates the need of binding and elution from solid surfaces and thus avoids bias and recovery efficiency effects.

### Advantages

- Extremely fast and easy.
- Highest ability for precise RNA representation.
- Easy to work on very small samples.
- Amenable to simple automation.

### Drawbacks

- Unable to perform traditional analytical methods (e.g. spectrophotometric method).
- Dilution-based (most useful with concentrated samples).
- Potential for suboptimal performance unless developed/optimized with downstream analysis.
- Potential for residual RNase activity if lysates are not handled properly.

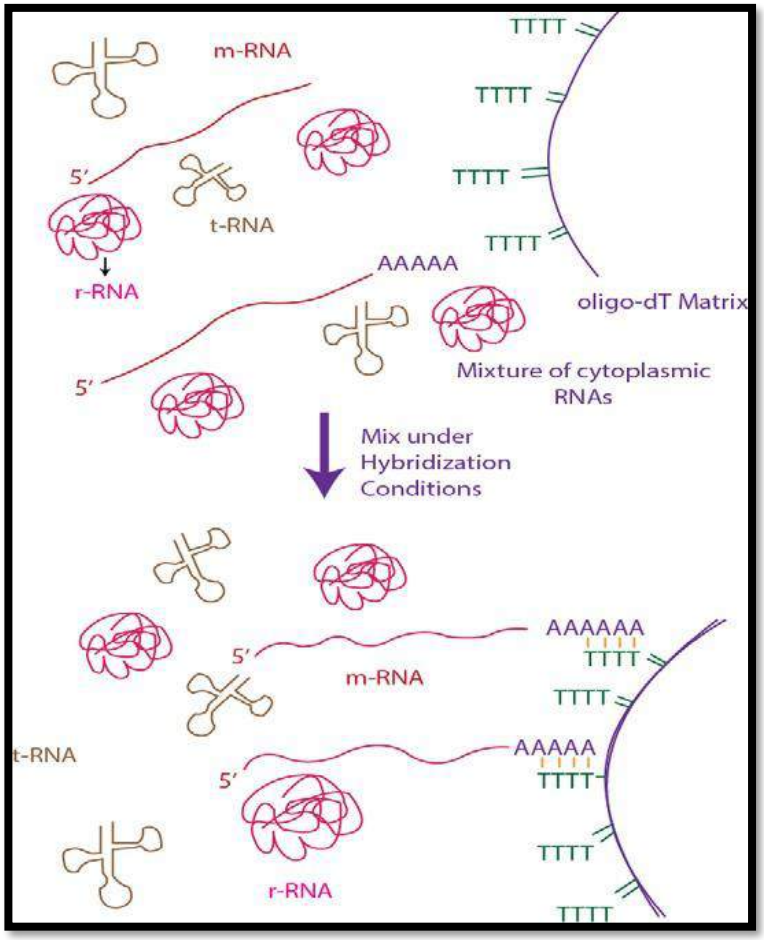
## Isolation of mRNA

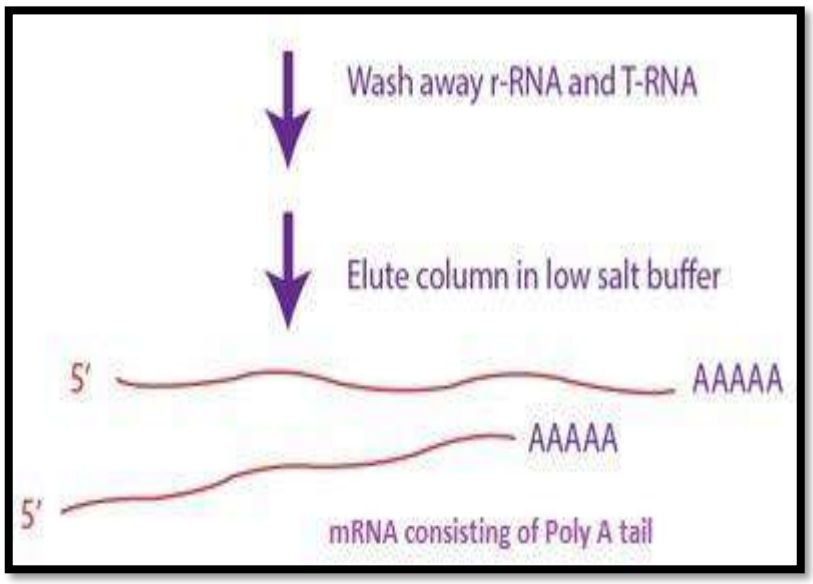
It involves the isolation of total mRNA from a cell type or tissue of interest. The amount of desired mRNA can be increased by following ways-

- Chromatographic purification of mRNA using oligo-dT column, which retains mRNA molecules, resulting in their enrichment.
- Spinning down mRNA by density gradient centrifugation.
- mRNA preparation from specialized cell types, e.g. developing seeds, chicken oviduct, erythrocytes, β cells of pancreas etc.

The 3′ ends of eukaryotic mRNA consist of a string of 50 – 250 adenylate residues (poly A Tail) which makes the separation easy from the much more prevalent rRNAs and tRNAs in a cell extract using a column containing oligo-dTs tagged onto its matrix.

When a cell extract is passed through an oligo-dT column, the mRNAs bind to the column due to the complementary base-pairing between poly (A) tail and oligo-dT. Other RNAs (ribosomal RNAs and transfer RNAs) flow through as unbound fraction. The bound mRNAs can then be eluted using a low-salt buffer.




**Isolation of mRNA using oligo-dT column chromatography.**