

5. **Concentration of DNA samples:** The most frequently used method is ethanol precipitation. Absolute ethanol is layered on top of a concentrated solution of DNA. Fibers of DNA can be withdrawn with a glass rod. For less concentrated solutions, ethanol is added at a ratio of 2.5 volumes of absolute ethanol to 1 volume of DNA solution. Precipitated DNA is collected by centrifugation.

Measurement of DNA concentration: DNA concentration can be accurately measured by ultraviolet absorbance spectrophotometry at 260 nm. The amount of UV radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample.

Methods for the preparation of total cell DNA from plants: Plant tissues are particularly difficult in this respect as they often contain large amounts of carbohydrates that are not removed by phenol extraction. Instead, a different approach must be used. One method makes use of a detergent called cetyltrimethyl-ammonium bromide (CTAB), which forms an insoluble complex with nucleic acids.

When CTAB is added to a plant cell extract, the nucleic acid-CTAB complex precipitates, leaving carbohydrate, protein and other contaminants in the supernatant.

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The precipitate is then collected by centrifugation and re-suspended in 1M solution of chloride, which causes the complex to break down.

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The nucleic acids can now be concentrated by ethanol precipitation and the RNA removed by ribonuclease treatment.

Preparation of plasmid DNA: There is an important distinction between plasmid purification and preparation of total cell DNA. In a plasmid preparation, it is always necessary to separate the plasmid DNA from the large amount of bacterial chromosomal DNA that is also present in the cells. The methods are based on the several physical differences between plasmid DNA and bacterial DNA.

- Plasmids and bacterial DNA differ in size.
- Plasmids and bacterial DNA differ in conformation.

A. Separation on the basis of size:

Principle: The largest plasmids are only 3% of the size of the *E. coli* chromosome and most are much smaller than this. Techniques that can separate small DNA molecules from large ones should therefore effectively purify plasmid DNA.

Procedure:

If the cells are lysed under very carefully controlled conditions, only a minimal amount of chromosomal DNA breakage occurs. The resulting DNA fragments are still very large; much larger than the plasmids and can be removed with the cell debris by centrifugation.

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Cell disruption is carried out very gently to prevent wholesale breakage of the bacterial DNA.

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Treatment with EDTA and lysozyme in the presence of sucrose, prevents the cells from bursting immediately.

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Spheroplasts are formed (cells with partially degraded cell walls that retain an intact cytoplasmic membrane)

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Cell lysis is now induced by adding a non-ionic detergent such as Triton X – 100 (ionic detergents such as SDS causes chromosomal breakage). This method causes very little breakage of the bacterial DNA, so centrifugation leaves a cleared, consisting almost entirely of plasmid DNA.

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If the plasmids themselves are large molecules, they may also sediment with the cell debris.

- B. **Separation on the basis of conformation:** The term conformation refers to the overall spatial configuration of the molecules, with the two simplest conformations being linear and circular. Most plasmids exist in the cell as supercoiled. Molecule supercoiling occurs because the double helix of the plasmid DNA is partially unwound during the plasmid replication process by enzymes called

topoisomerases. The supercoiled conformation can be maintained only if both polynucleotide strands are intact, hence the technical name of covalently closed circular DNA (ccc DNA). If one of the polynucleotide strands is broken, the double helix reverts to its normal relaxed state, and the plasmid takes on the alternative conformation, called open circular. Supercoiling is important in plasmid preparation because supercoiled molecules can be fairly easily separated from non-supercoiled DNA. It can be done by –

1. Alkaline denaturation and
2. Ethidium bromide – caesium chloride density gradient centrifugation.

They are discussed below:

1. Alkaline denaturation:

The basic of the technique is the use of narrow pH range at which non-supercoiled DNA is denatured, whereas supercoiled plasmids are not.



NaOH is added to a cell extract or cleared lysate, so that the pH is adjusted to 12 – 12.5.



Then the H bonding in non-supercoiled DNA molecule is broken, causing the double helix to unwind and the two polynucleotide chains to separate.



Acid is now added so that the denatured bacterial DNA strands re-aggregate into a tangled mass.



The insoluble network can be pelleted by centrifugation, leaving plasmid DNA in the supernatant.

2. Ethidium bromide – cesium chloride density gradient centrifugation:

A density gradient is produced by centrifuging a solution of CsCl₂ at a very high speed which pulls the CsCl₂ 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 CsCl₂ i.e. 1.7 g/cm³ 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.



EtBr binds to DNA molecules by intercalating between adjacent base pairs, causing partial unwinding of the double helix.



The unwinding results in a decrease in the buoyant density, by as much as 0.125 g/cm³ for linear DNA.



Supercoiled DNA with no free ends, has very little freedom to unwind and can only bind to a limited amount of EtBr.



The decrease in buoyant density of a supercoiled DNA is much less only about 0.085 g/cm³. As a consequence, supercoiled DNA form a band in an EtBr-CsCl₂ gradient at a different position to linear and open-circular DNA.



Plasmids band at a distinct point, separated from the linear bacterial DNA, with the protein floating on the top of the gradient and RNA pelleted at the bottom.



The position of the DNA bands can be seen by shining UV radiation on the tube, which causes the bound EtBr to fluoresce.



The pure plasmid DNA is removed by puncturing the side of the tube and withdrawing a sample with a syringe.



The EtBr bound to the plasmid DNA is extracted with n-butanol and the CsCl₂ removed by dialysis.

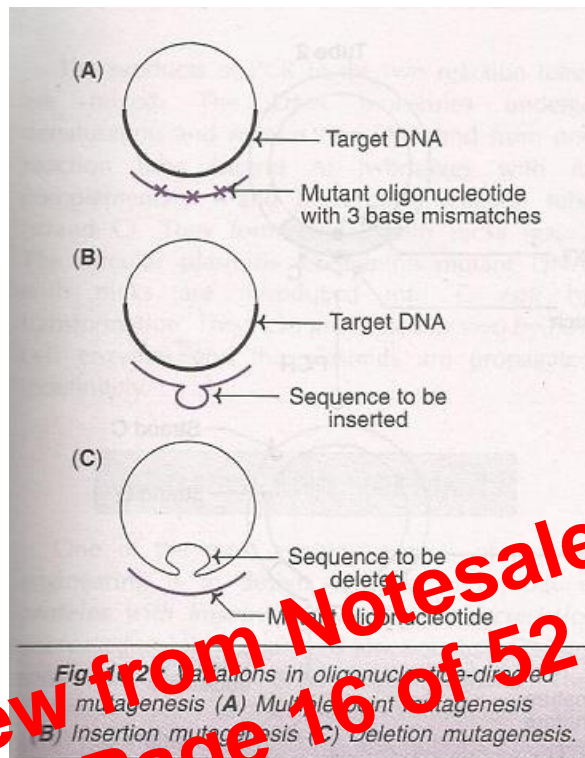


The resulting plasmid preparation is virtually 100% pure and ready for use as a cloning vector.

Density gradient centrifugation in the presence of ethidium bromide (EtBr) can be used to separate supercoiled DNA from non-supercoiled DNA.

Variations in oligonucleotide directed mutagenesis: There are some variations in use in the oligonucleotide directed mutagenesis, as the situation demands:

1. **Multiple point mutagenesis:** Oligonucleotide directed mutagenesis can be used to create DNAs with multiple point mutations with the requisite number of base mismatches.
2. **Insertion mutagenesis:** In this case, the mutant oligonucleotide carries a sequence to be inserted (sandwiched between two sites with complementary sequences). This can bind with target DNA on either side.
3. **Deletion mutagenesis:** The mutant oligonucleotide binds to two separate sites on either side of the target DNA. This enables a small position of target DNA to be deleted.



DNA fingerprinting or DNA proofing: DNA fingerprinting is an analysis of the nitrogenous base sequence in the DNA of an individual. DNA fingerprinting is the present day genetic detective in the practice of modern medical forensics.

Steps:

Isolation of DNA



Isolated DNA is cut by special enzymes called restriction enzymes.



The DNA is transferred to nylon. The nylon sheet is placed on the gel and soaked overnight.



Adding radioactive or colored probes to the nylon sheet produces a pattern called the DNA fingerprint.



The final DNA fingerprint is built by using several probes (5-10 or more) simultaneously.

Applications:

1. **Setting up of genetic databank:** DNA fingerprinting has a lot of importance in setting up of genetic databank all over the world.
2. **Reuniting the lost children:** DNA profiling technology helps in reuniting the lost children with their respective parents or vice versa.
3. Verification of paternity.

2. They contain Ori genes which encode Ori binding proteins. These proteins are critical for BAC replication.
3. It is derived from naturally occurring F plasmid.
4. The DNA inset size varies between 150 to 350 kb.

Advantages:

1. They are capable of accommodating large sequences without any risk of rearrangement.
2. BACs are frequently used for studies of genetic or infectious disorders.
3. High yield of DNA clones is obtained.

Disadvantages:

1. They are present in low copy number.
2. The eukaryotic DNA inserts with repetitive sequences are structurally unstable in BACs often resulting in deletion or rearrangement.

Yeast artificial chromosome:

Characteristics:

1. A large DNA inset of up to 200 kb can be cloned.
2. They are used for cloning inside eukaryotic cells. These act as eukaryotic chromosomes inside the host eukaryotic cell.
3. It possesses the yeast telomere at each end.
4. A yeast chromosome sequence (CEN) is present which allows proper segregation during meiosis.
5. The Ori is bacterial in origin.
6. Both yeast and bacterial cells can be used as hosts.

Advantages:

1. A large amount of DNA can be cloned.
2. Physical maps of large genomes like the human genome can be constructed.

Disadvantages:

1. Overall transformation efficiency is low.
2. The yield of cloned DNA is also low.

Advantages of BACs over YACs:

1. Comparatively stable.
2. Easy to transform.
3. Simple purification is required.
4. User friendly.
5. Aid in the development of vaccines.

Human artificial chromosome (HAC)

Characteristics:

1. Human artificial chromosomes are artificially synthesized.
2. They are utilized for gene transfer or gene delivery into human cells.
3. It can carry large amounts of DNA inserts.
4. They are used extensively in expression studies and determining the function of the human chromosome.

Advantages:

of new protein-based drugs, improving their half-life, reducing their toxicity and preventing multidrug resistance development.

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Ti plasmid

Ti plasmid is the large sized tumor inducing plasmid found in *Agrobacterium tumefaciens*. It directs crown gall formation in certain plant species. *A. tumefaciens* is a Gram negative soil bacterium which naturally transforms plant cells, resulting in crown gall tumors. They infect plants through breaks or wounds. Tumor formation is the result of integration of T DNA (transfer DNA) in plant genome.

Characteristics:

1. A Ti or tumor inducing plasmid is a plasmid that often, but not always, is a part of the genetic equipment that *A. tumefaciens* and *A. rhizogenes* use to transduce its genetic material to plants.
2. The Ti plasmid is lost when *Agrobacterium* is grown above 28° C.
3. The plasmid has 196 genes that code for 195 proteins. There is one structural RNA.
4. The modification of this plasmid is very important in the creation of transgenic plants.

Regions: The Ti plasmid has three important regions:

1. **T DNA region:** This region has the genes for the biosynthesis of auxin (aux), cytokinin (cyt) and opine (ocs) and is flanked by left and right borders.
2. **Virulence region:** The genes responsible for the transfer of T DNA into host plant are located outside T DNA and the region is referred to as Vir or virulence region.
3. **Opine catabolism region:** This region codes for proteins in the uptake and metabolism of opiens.

Besides the above three regions, there is Ori region that is responsible for origin of DNA replication which permits the Ti plasmid to be stably maintain *A. tumefaciens*.

Limitations of Ti plasmid as vector:

1. It is large in size. Smaller vectors are preferred for recombinant experiment.
2. Absence of unique restriction enzyme sites on Ti plasmids.
3. The phytohormones (auxin and cytokinin) produced by the plant cells, so auxin and cytokinin must be removed.
4. Opine production in transformed plant cells lowers the plant yield, so opine synthesizing genes should be removed.

Chromosome Walking

Chromosome walking. Chromosome walking is sequential isolation of overlapping DNA sequences in order to find a gene on the chromosome studied.

Steps of chromosome walking:

From the genomic library select a clone of interest and subclone a small fragment from one end of the clone

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The subcloned fragment of the selected clone may be hybridized with other clones in the library and a second clone hybridizing with the subclone of the first clone is identified due to presence of overlapping region

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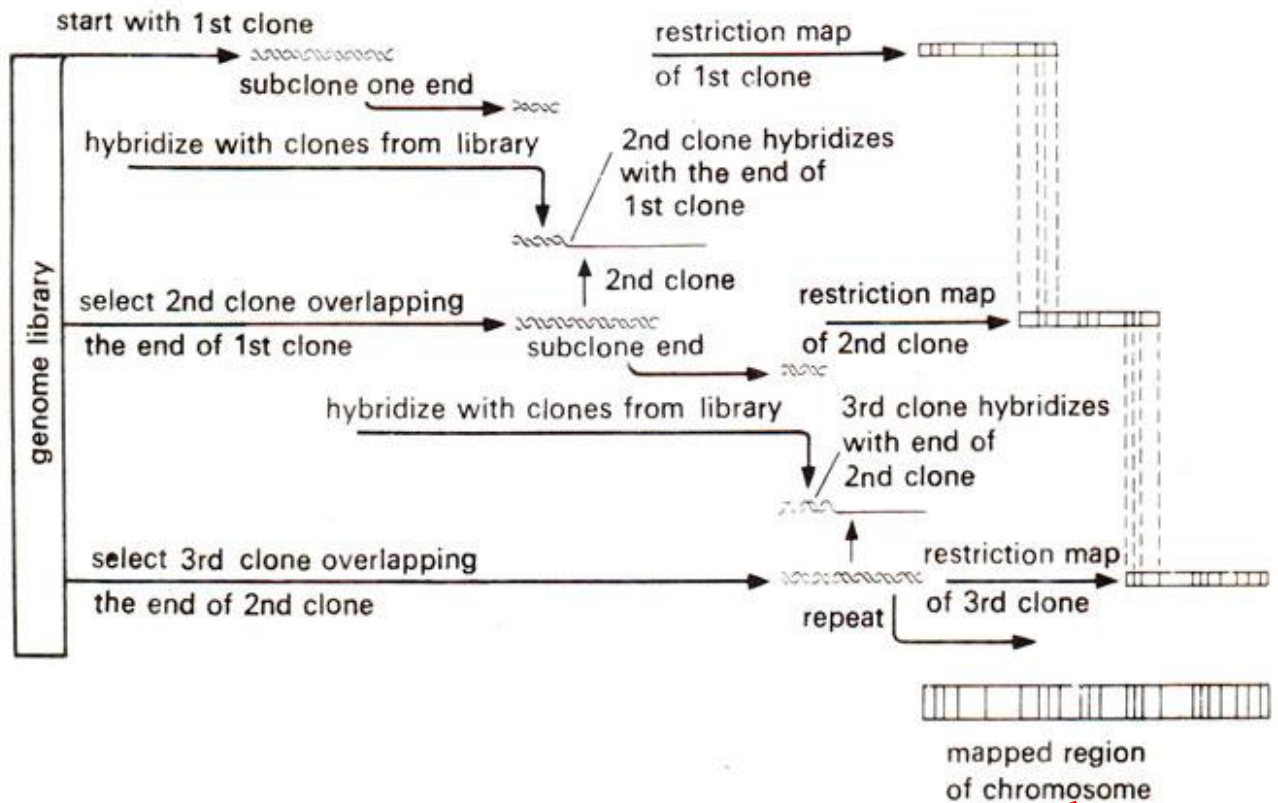
The end of the second clone is then subcloned and used for hybridization with other clones to identify a third clone having overlapping region with the subcloned end of the second clone

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Third clone identified as above is also subcloned and hybridized with clones in the same manner and the procedure may continued

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Restriction map of each selected clone may be prepared and compared to know the regions of overlapping so that the identification of new overlapping restriction sites will amount to walking along the chromosome or along a long chromosome segment.



Nucleotide

Nucleotides are the monomer units or building blocks of nucleic acids.

Basic chemistry:

Nucleotide = Nitrogenous base + Pentose sugar + Phosphate

Nucleoside = Nitrogenous base + Pentose sugar

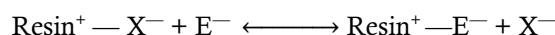
Types:

1. Purine e.g. adenine and guanine.
2. Pyrimidine e.g. cytosine, thymine and uracil.

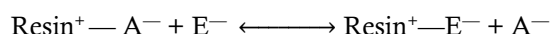
Functions or biomedical importance:

1. They are building blocks or monomers of nucleic acids.
2. They participate in the synthesis of some carbohydrate, proteins and lipids.
3. Many coenzymes are derivatives of nucleotides e.g. NAD⁺, NADP⁺ etc.
4. They can act as intracellular second messenger e.g. cAMP, cGMP etc.
5. They play an important role as energy currency in the cell.
6. They participate in different phosphorylation processes e.g. Glucose + ATP → Glucose 6-phosphate + ADP.
7. ADP level regulates oxidative phosphorylation.
8. Synthetic nucleotide analogues are used in chemotherapy of cancer, AIDS and as suppressors of immune response during organ transplantation.

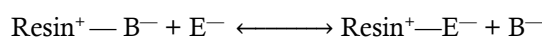
1. The eluent loaded onto the column displaces any anions bonded to the resin and saturates the resin surface with the eluent anion. This process of eluent ion (E^-) displacing an anion (X^-) bonded to the resin can be expressed by the following chemical interaction:



2. A sample containing anion A and anion B are injected into the column. This sample could contain many different ions, but for simplicity this example uses just two different ions as analytes in the sample.
3. After the sample has been injected, the continued addition of eluent causes a flow through the column. As the sample elutes (or moves through the column), anion A and anion B adhere to the column surface differently. The sample zones move through the column as eluent gradually displaces the analytes.
4. As the eluent continues to be added, the anion A moves through the column in a band and ultimately is eluted first. This process can be represented by the chemical interaction showing the displacement of the bound anion (A^-) by the eluent anion (E^-).



5. The eluent displaces anion B and anion B is eluted off the column.



[Elution is the process where the compound of interest is moved through the column. This happens, because the eluent, the solution used as the solvent in chromatography, is constantly pumped through the column].

Uses:

1. Separation of proteins from foods, for example, to investigate the effects of individual food components on health – this type of analysis is used in nutrition research
2. Separation of high value proteins from substances
3. Drinking water analysis for pollution and other constituents
4. Determination of water chemistries in aquatic ecosystems
5. Determination of sugar and salt content in foods.

Operon

According to operon model proposed by F. Jacob and I. Monod, an operon was defined as a unit of coordinated control of protein synthesis, which consisted of (i) an operator gene which controlled the activity of (ii) a number of structural genes which took part in the synthesis of protein(s). This means that the structural genes will synthesize mRNA under the operational control of an operator gene. The operator gene, in turn, is under the control of a repressor molecule synthesized by a regulator gene, which is not a part of the operon. Thus, the members of an operon are transcribed coordinately a single, long, polycistronic mRNA molecule. One such an operon in *E. coli*, called the lactose or lac operon, has provided a model system for the study of gene regulation.

The lac operon system is describe hereunder:

Structure: A lac operon contains three structural genes or cistrons namely z, y and a, whose products (enzymes) are involved in the breakdown of the sugar lactose. Gene z codes for β galactosidase, which converts lactose into glucose and galactose; gene y codes for galactoside permease which is a plasma membrane bound protein and facilitates the entrance of lactose into the cell. Gene a codes for thiogalactoside acetylase, which transfers an acyl group from acetyl Co A to β galactoside (i.e. this enzyme is indirectly involved in lactose utilization). Mapping experiments employing mutations of these three genes have demonstrated that the gene order is z-y-a.

Mechanism:

In the absence of lactose (inducer), the regulator gene produces a repressor protein, which binds to the operator site and prevents the transcription. As a result, the structural gene do not produce mRNA and the proteins are not produced.

In the presence of lactose, it binds to the repressor protein; the repressor now fails to bind to the operator. Therefore, the operator is made free and induces the mRNA polymerase to bind to the initiation site on promoter which results in the synthesis of the lac mRNA. This mRNA codes for three enzymes necessary for lactose catabolism.