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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – GENETIC ENGINEERING – SBB2102

rDNA TECHNOLOGY AND TOOLS INVOLVED IN GENETIC MANIPULATIONS

1. Recombinant DNA Technology:

Through the years, scientists have studied the life and how it changes and adapts to the changes in the surroundings. One of their studies to cope up with the changes is through genetic modification. This process has been done indirectly to plants and animals to control their characteristics. Genetic modification is the process of changing the genetic makeup of an organism to express new traits. Modern biotechnology has made it much easier and faster in targeting specific genes to alter through genetic engineering. One of the most known technologies used in genetic engineering is Recombinant DNA Technology.

DNA exists in the cells of all living things. These long chains of amino acids serve as the genetic blueprints for living organisms. DNA controls how they form before birth and which traits they pass on to the next generation. Recombinant DNA exists in a laboratory by combining genetic material from multiple sources. Recombinant DNA technology can create new kinds of living organisms or alter the genetic code of existing organisms. As with most technology, there are great benefits and notable downsides to the use of recombinant DNA technology.

1.1.Pros of Recombinant DNA Technology

Recombinant DNA technology, sometimes referred to as "genetic engineering," can benefit people in several ways. For example, scientists made artificial human insulin with the help of recombinant DNA technology. Diabetic people cannot produce their own insulin, which they need in order to process sugar. Animal insulin is not a suitable replacement, since it causes severe allergic reactions in most people. Thus, scientists used recombinant DNA technology to isolate the gene for human insulin and insert it into plasmids (cellular structures that can replicate independently of chromosomes). These plasmids were then inserted into bacterial cells, which created insulin based on the human genetic code inside of them. The resulting insulin was safe for humans to use. Thus, people with diabetes went from having a life expectancy of around 4 years after diagnosis to having a normal human life expectancy.

Recombinant DNA technology helped improve food production. Fruits and vegetables, which were prone to attacks from pests, now have genetic modifications to be more resistant. Some foods have modifications for longer shelf lives or higher nutritional content. These advancements greatly increased crop yields, which means that more food is available to the public at the end of each growing cycle.

Scientists have been working to improve vaccines and produce new ones using recombinant DNA technology. These "DNA vaccines," which utilize recombinant DNA, are in the testing stages. Most modern vaccines introduce a small "piece" of a disease into the body, so the body can develop ways to fight that particular disease. DNA vaccines would directly introduce the antigen itself and lead to more immediate and permanent immunity. Such vaccines could potentially protect people against diseases such as diabetes and even cancer.

1.2.Cons of Recombinant DNA Technology

Most of the downsides of recombinant DNA technology are ethical in nature. Some people feel that recombinant DNA technology goes against the laws of nature, or against their religious beliefs, due to how much control this technology gives humans over the most basic buildings blocks of life.

Other ethical concerns also exist. Some people worry that if companies can pay scientists to patent, buy and sell genetic material, then genetic material could become an expensive commodity. Such a system might lead to people having their genetic information stolen and used without permission. It may sound odd, but such cases have already happened. In 1951, a scientist used unique cells stolen from a woman named Henrietta Lacks to create an important cell line (the HeLa cell line) which is still used in medical research today. Her family did not know about her involuntary donation until after her death, and never received compensation, but others have profited from the use of HeLa cells.

Many people worry about the safety of modifying food and medicines using recombinant DNA technology. Although genetically modified foods seem safe in multiple studies, it is easy to see why such fears exist.

What might happen if a crop of tomatoes with modified jellyfish genes to make them more robust became common? What would happen to an unsuspecting person, who is allergic to jellyfish, after eating one of these tomatoes? Would the person have a reaction? Some people fear that such questions will not come up until it is too late.

Other people worry that humans may begin tampering too much with their own genetic material and create societal problems. What if people use recombinant DNA technology to live

longer, become stronger or handpick certain traits for their offspring? Will societal division swell between genetically modified people and "normal" people? These are questions that scientists and the public will likely continue to consider as humanity moves toward a future where manipulating DNA is easier than ever before.

2. Restriction enzymes

One of the most important steps in molecular biology, especially molecular genetics and analysis, is the isolation of DNA from the human genome and make many copies of it. A restriction enzyme is a kind of nuclease enzyme which is capable of cleaving double-stranded DNA. The enzymes may cleave DNA at random or specific sequences which are referred to as restriction sites (Fig 1). The recognition sites are palindromic in origin, that is, they are the sequences which are read the same forward and backward. These restriction enzymes are produced naturally by bacteria. The bacterial species use it as a form of defense mechanism against viruses. However, in bacteria, restriction enzymes are present as a part of a combined system called the restriction modification system. The bacterial species modify their own DNA with the help of enzymes which

methylate it. This particular process of methylation of bacterial DNA protects it from cleavage from its own restriction endonucleases.

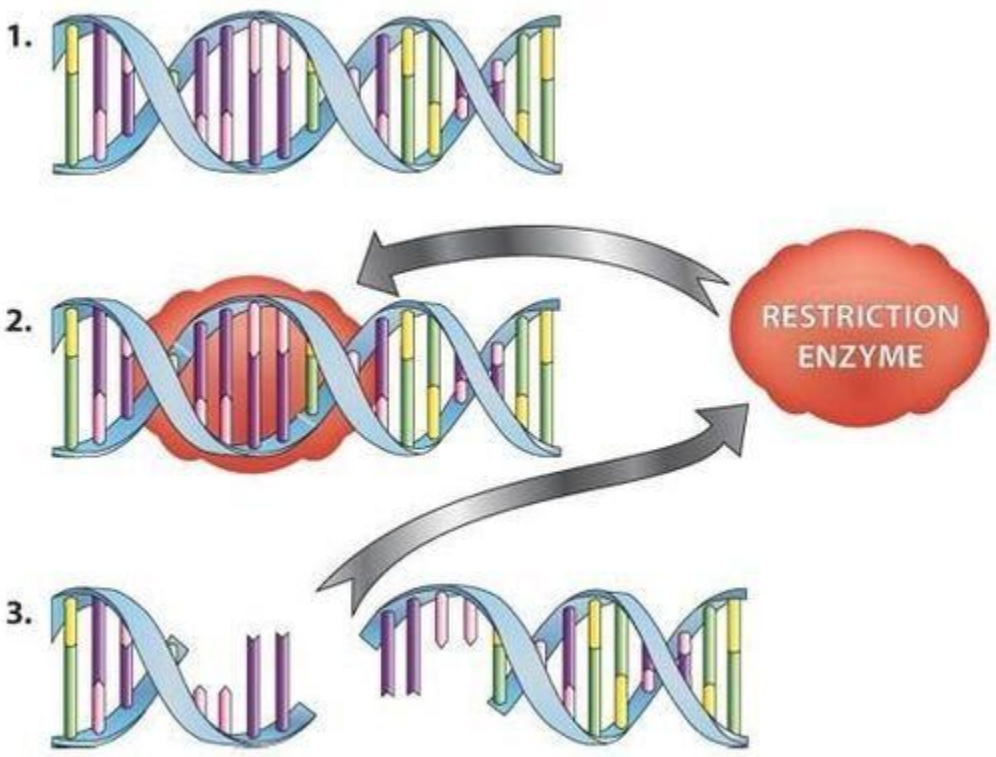


Fig 1: The Restriction enzymes cleave DNA at random or specific sequences

Restriction enzymes are DNA-cutting enzymes found in bacteria (and harvested from them for use). Because they cut within the molecule, they are often called restriction endonucleases.

A restriction enzyme recognizes and cuts DNA only at a particular sequence of nucleotides. For example, the bacterium Hemophilus aegypticus produces an enzyme named Haelll that cuts DNA wherever it encounters the sequence

5’GGCC3’

3’CCGG5’

The cut is made between the adjacent G and C. This particular sequence occurs at 11 places in the circular DNA molecule of the virus phiX174. Thus treatment of this DNA with the enzyme produces 11 fragments, each with a precise length and nucleotide sequence. These fragments can be separated from one another and the sequence of each determined. Haelll and Alul cut straight across the double helix producing “blunt” ends. However, many restriction enzymes cut in an offset fashion.

The ends of the cut have an overhanging piece of single-stranded DNA. These are called “sticky ends” because they are able to form base pairs with any DNA molecule that contains the

complementary sticky end (Fig.2). Any other source of DNA treated with the same enzyme will produce such molecules. Mixed together, these molecules can join with each other by the base pairing between their sticky ends. The union can be made permanent by another enzyme, DNA ligase that forms covalent bonds along the backbone of each strand. The result is a molecule of recombinant DNA (rDNA). Because recognition sequences and cleavage sites differ between restriction enzymes, the length and the exact sequence of a sticky-end "overhang", as well as whether it is the 5′ end or the 3′ end strand that overhangs, depends on which enzyme produced it. Base-pairing between overhangs with complementary sequences enables two fragments to be joined or "spliced" by a DNA ligase.

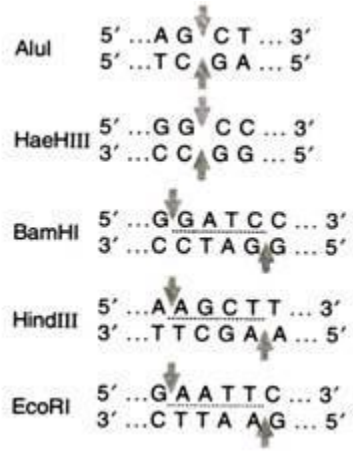


Fig.2 Alu1 and HaeIII produce blunt ends. BamH1, HindIII and EcoR1 produce "sticky" ends

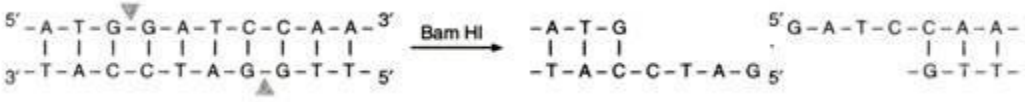
A sticky-end fragment can be ligated not only to the fragment from which it was originally cleaved, but also to any other fragment with a compatible sticky end. The sticky end is also called a cohesive end or complementary end in some reference.

If a restriction enzyme has a non- degenerate palindromic (the sequence on one strand reads the same in the same direction on the complementary strand e.g. GTAATG is not a palindromic DNA sequence, but GTATAC is, GTATAC is complementary to CATATG) cleavage site, all ends that it produces are compatible. Ends produced by different enzymes may also be compatible.

Patterns of DNA Cutting by Restriction Enzymes:

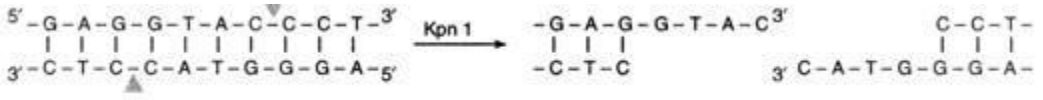
(i) 5′ overhangs:

The enzyme cuts asymmetrically within the recognition site such that a short single-stranded segment extends from the 5′ ends. BamHI cuts in this manner.



(ii) 3′ overhangs:

Again, we see asymmetrical cutting within the recognition site, but the result is a single-stranded overhang from the two 3′ ends. Kpnl cuts in this manner.

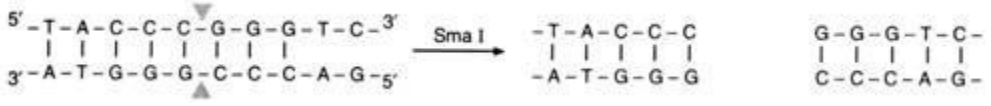


Enzyme	Source	Recognition Sequence	Cut
*Eco*RI	*Escherichia coli*	5′GAATTC 3′CTTAAG	5′---G AATTC---3′ 3′---CTTAA G---5′
*Bam*HI	*Bacillus amyloliquefaciens*	5′GGATCC 3′CCTAGG	5′---G GATCC---3′ 3′—CCTAG G---5′
*Hind*III	*Haemophilus influenzae*	5′AAGCTT 3′TTCGAA	5′---A AGCTT---3′ 3′---TTCGA A---5′
*Taq*I	*Thermus aquaticus*	5′TCGA 3′AGCT	5′---T CGA---3′ 3′---AGC T---5′
*Hinf*I	*Haemophilus influenzae*	5′GANTC 3′CTNAG	5′—G ANTC---3′ 3′---CTNA G---5′
*Sau*3A	*Staphylococcus aureus*	5′GATC 3′CTAG	5′--- GATC---3′ 3′---CTAG ---3′
*Pov*II	*Proteus vulgaris*	5′CAGCTG 3′GTCGAC	5′---CAG CTG---3′ 3′---GTC GAC---5′
*Hae*III	*Haemophilus egytius*	5′GGCC 3′CCGG	5′---GG CC---3′ 3′---CC GG---5′
*Alu*I	*Arthrobacter luteus*	5′AGCT 3′TCGA	5′---AG CT---3′ 3′---TC GA---5′
*Eco*RV	*Escherichia coli*	5′GATATC 3′CTATAG	5′---GAT ATC---3′ 3′---CTA TAG---5′
*Sal*I	*Streptomyces albue*	5′GTCGAC 3′CAGCTG	5′---G TCGAC---3′ 3′---CAGCT G---5′
*Sca*I	*Streptomyces caespitosus*	5′AGTACT 3′TCATGA	5′---AGT ACT---3′ 3′---TCA TGA---5′

Table: 1 Restriction ennzymes their sources and recognition sites.

(iii) Blunts:

Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs. Smal is an example of an enzyme that generates blunt ends.



The 5′ or 3′ overhangs generated by enzymes that cut asymmetrically are called sticky ends or cohesive ends, because they will readily stick or anneal with their partner by base pairing.

Types

There are two different kinds of restriction enzymes:

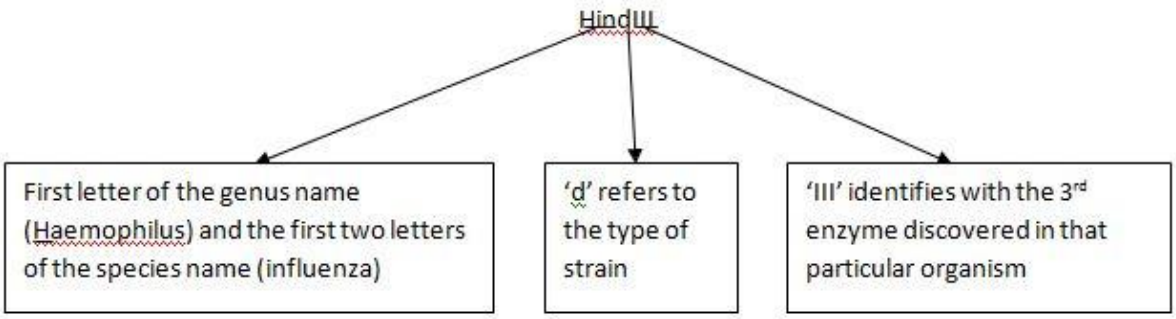
1. **Exonucleases:** restriction exonucleases are primarily responsible for hydrolysis of the terminal nucleotides from the end of DNA or RNA molecule either from 5' to 3' direction or 3' to 5' direction; for example- exonuclease I, exonuclease II, etc.
2. **Endonuclease:** restriction endonucleases recognize particular base sequences (restriction sites) within DNA or RNA molecule and catalyze the cleavage of internal phosphodiester bond; for exEcoRI, Hind III, BamHI, etc.

The first restriction enzyme to be discovered was Hind II in the year 1970. In 1978, Daniel Nathans, Werner Arber, and Hamilton O. Smith were awarded the Nobel Prize for Physiology or Medicine.

Restriction Enzyme Nomenclature

The very name of the restriction enzymes consists of three parts:

1. An abbreviation of the genus and the species of the organism to 3 letters, for example- Eco for Escherichia coli identified by the first letter, E, of the genus and the first two letters, co, of the species.
2. It is followed by a letter, number or combination of both of them to signify the strain of the species.
3. A Roman numeral to indicate the order in which the different restriction-modification systems were found in the same organism or strain per se.



Classification of Restriction Endonucleases

Based on the types of sequences identified, the nature of cuts made in the DNA, and the enzyme structure, there are three classes:

1. Type I restriction enzymes,
2. Type II restriction enzymes, and
3. Type III restriction enzymes.

Type I Restriction Enzymes

- Type I restriction enzymes possess both restriction and modification activities. In this case, the restriction will depend upon the methylation status of the target DNA sequence.
- Cleavage takes place nearly 1000 base pairs away from the restriction site. The structure of the recognition site is asymmetrical. It is composed of 2 parts. One part of the recognition site is composed of 3-4 nucleotides while the other one contains 4-5 nucleotides. The two parts are separated by a non-specific spacer of about 6-8 nucleotides.
- For their function, the type I restriction enzymes require S- adenosylmethionine (SAM), ATP, and Mg^{2+} .
- They are composed of 3 subunits, a specificity subunit which determines the recognition site, a restriction subunit, and a modification subunit.

Type II Restriction Enzymes

- Two separate enzymes mediate restriction and modification. Henceforth, DNA can be cleaved in the absence of modifying enzymes. Although the target sequence identified by the two enzymes is the same, they can be separately purified from each other.
- The nucleotides are cleaved at the restriction site only. The recognition sequence is rotationally symmetrical, called palindromic sequence. The specific palindromic site can either be continuous (e.g., KpnI identifies the sequence 5´-GGTACC-3´) or non-continuous (e.g., BstEII recognizes the sequence 5´-GGTNACC-3´, where N can be any nucleotide).
- These require Mg^{2+} as a cofactor but not ATP.
- They are required in genetic mapping and reconstruction of the DNA in vitro only because they identify particular sites and cleave at those sites only.
- The type II restriction enzymes first establish non-specific contact with DNA and bind to them in the form of dimmers.
- The target sequence is then detected by a combination of two processes. Either the enzyme diffuses linearly/ slides along the DNA sequence over short distances or hops/ jumps over long distances.
- Once the target sequence is located, various conformational changes occur in the enzyme as well as the DNA. These conformational changes, in turn, activate catalytic center.
- The phosphodiester bond is hydrolyzed, and the product is released.

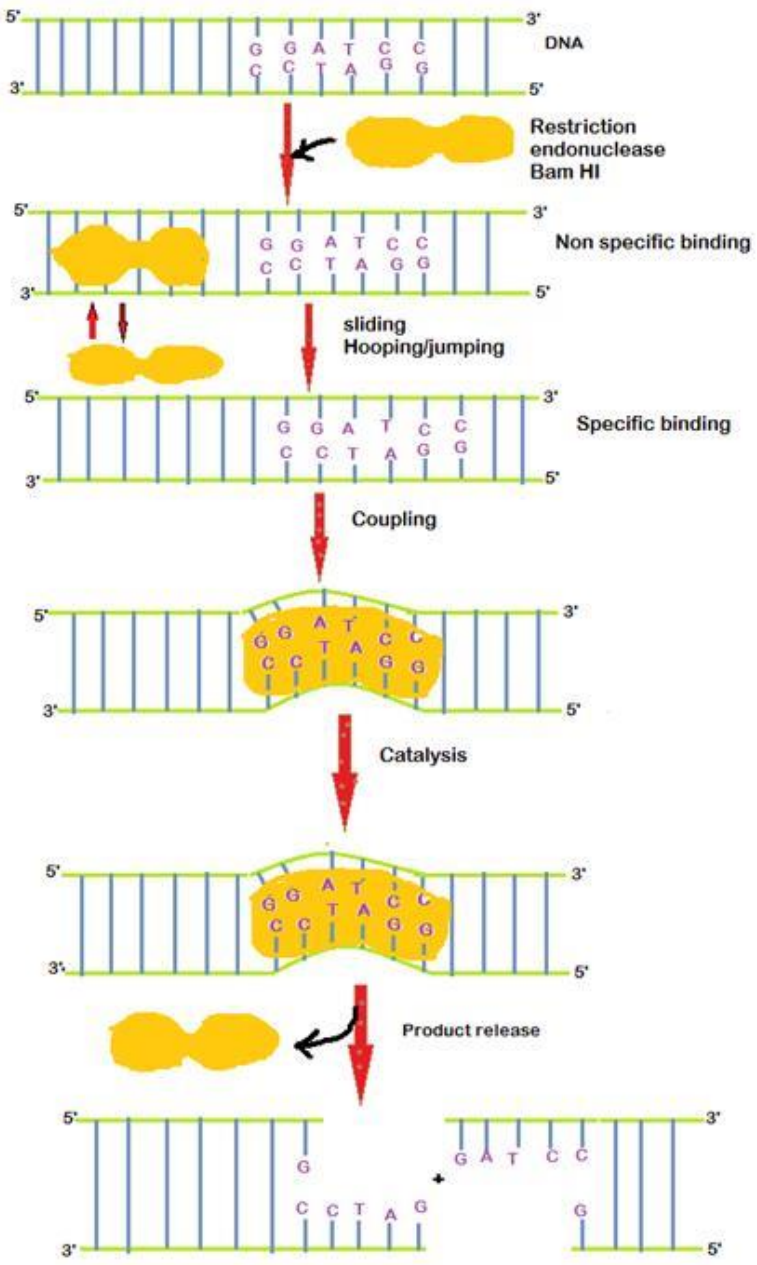


Fig 3: Structures of free, nonspecific, and specific DNA-bound forms of BamHI

Type III Restriction Enzyme

- The type III enzymes recognize and methylate the same DNA sequence. However, they cleave nearly 24-26 base pairs away.
- They are composed of two different subunits. The recognition and modification of DNA are carried out by the first subunit- 'M' and the nuclease activity is rendered by the other subunit 'R'.
- DNA cleavage is aided by ATP as well as Mg2+ whereas SAM is responsible for stimulating cleavage.

- Only one of the DNA strand is cleaved. However, to break the double-stranded DNA, two recognition sites in opposite directions are required.

Table 2: Types of Restriction enzymes

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non- specific location > 1000 bp away from recognition site	Cut both strands at a specific, usually palindromic recognition site (4-8 bp)	Cleavage of one strand, only 24-26 bp downstream of the 3′ recognition site
Restriction and modification	Single multifunctional enzyme	Separate nuclease and methylase	Separate enzymes sharing a common subunit
Nuclease subunit structure	Heterotrimer	Homodimer	Heterodimer
Cofactors	ATP, Mg2+, SAM	Mg2+	Mg2+ (SAM)
DNA cleavage requirements	Two recognition sites in any orientation	Single recognition site	Two recognition sites in a head-to-head orientation
Enzymatic turnover	No	Yes	Yes
DNA translocation	Yes	No	No
Site of methylation	At recognition site	At recognition site	At recognition site

Applications:

In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.

- They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.

- Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restrictionsite present in the allele.
- Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.

Polynucleotide phosphorylase:

- Polynucleotide phosphorylase was first discovered from extracts of Azotobacter agile by Grunberg-Manago and Ochoa.
- Polynucleotide phosphorylase (PNPase) catalyzes the synthesis of long chain polyribonucleotides (RNA) in 5' to 3' direction from nucleotide diphosphates as precursors and reversibly catalyzes phosphorolytic cleavage of polyribonucleotides in 3' to 5' direction with a release of orthophosphate in presence of inorganic phosphate.
- PNPase is a bifunctional enzyme and functions in mRNA processing and degradation inside the cell.
- Structural and physiochemical studies in enzymes showed that it is formed of subunits. The arrangements of the subunits may vary from species to species which would alter their properties.
- These enzyme can catalyze not only the synthesis of RNA from the mixtures of naturally occurring ribonucleoside diphosphates, but also that of non-naturally occurring polyribonucleotides

Mechanism of action:

As mentioned earlier, polynucleotide phosphorylase is a bifunctional enzyme. The mechanism of action of this enzyme can be represented by following reactions:

In E.coli, polynucleotide phosphorylase regulates mRNA processing either by adding ribonucleotides to the 3' end or by cleaving bases in 3' to 5' direction. The function of PNPase depends upon inorganic phosphate (Pi) concentration inside the cell. The transcripts are polyadenylated using enzyme polyadenylate polymerase I (PAPI). After primary polyadenylylation of the transcript by PAP I, PNPase may bind to the 3′ end of the poly(A) tail. PNPase works either degradatively or biosynthetically inside the cell depending on the Pi concentration. Under high Pi concentration, it degrades the poly(A) tail releasing adenine diphosphates. If the Pi concentration is low, PAP I initiates addition of one or more nucleotides to the existing poly (A) tail and in the process generates inorganic phosphate. On dissociation of PNPase, the 3′ end again is available to PAP I for further polymerization.

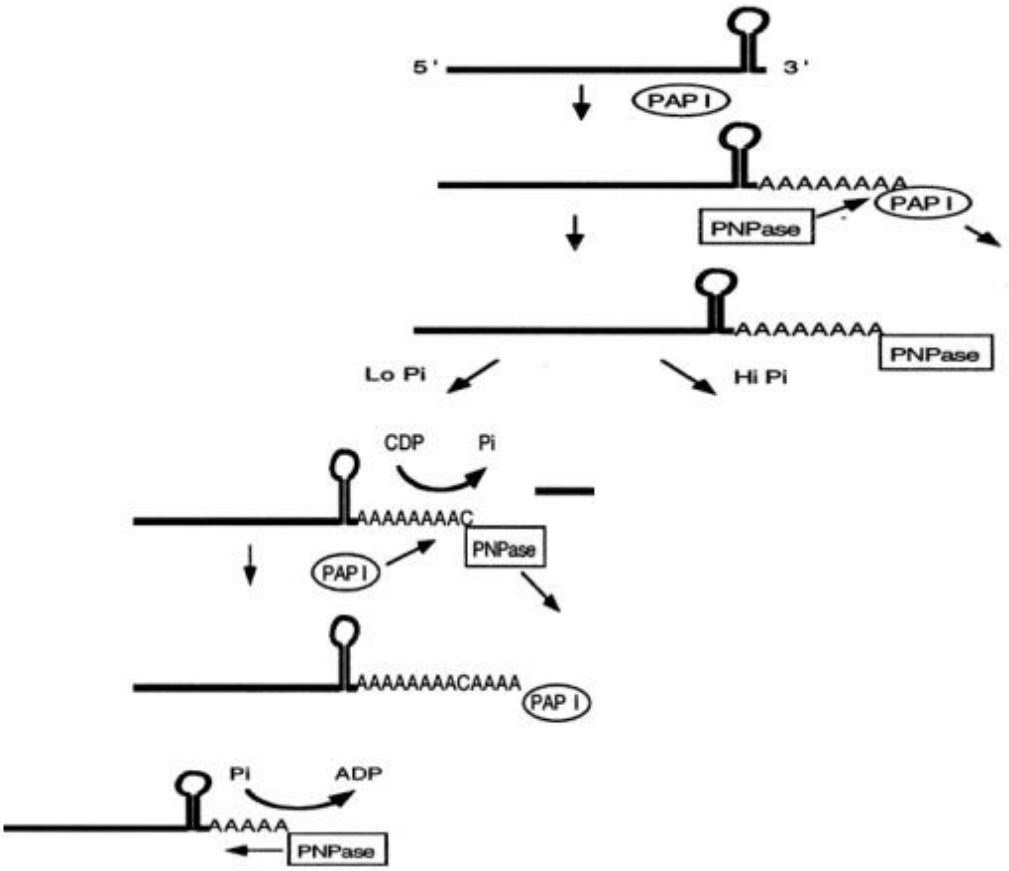


Fig 4: Schematic representation of the role of PNPase in poly(A) tail metabolism in E. coli.

Function:

Different functions of Polynucleotide phosphorylase are:

- It is involved in mRNA processing and degradation in bacteria, plants, and in humans.
- It synthesizes long, highly heteropolymeric tails in vivo as well as accounts for all of the observed residual polyadenylation in poly(A) polymerase I deficient strains.
- PNPase function as a part of RNA degradosome in E.coli cell. RNA degradosome is a multicomponent enzyme complex that includes RNaseE (endoribinuclease), polynucleotide phosphorylase (3' to 5' exonuclease), RhlB helicase (a DEAD box helicase) and a glycolytic enzyme enolase. This complex catalyzes 3' to 5' exonuclease activity in presence of ATP. In eukaryotes, the exosomes are located in nucleus and cytoplasm. Degradsomes in bacteria and exosomes in eukaryotes are associated with processing, control and turnover of RNA transcripts.
- In rDNA cloning technology, it has been used to synthesize radiolabelled polyribonucleotides from nucleoside diphosphate monomers.

Deoxyribonuclease (DNaseI):

- A nuclease enzyme that can catalyze the hydrolytic cleavage of phosphodiester bonds in the DNA backbone are known as deoxyribonuclease (DNase).
- Based on the position of action, these enzymes are broadly classified as endodeoxyribonuclease (cleave DNA sequence internally) and exodeoxyribonuclease (cleave the terminal nucleotides)
- Unlike restriction enzymes, DNase does not have any specific recognition/restriction site and cleave DNA sequence at random locations.
- There is a wide variety of deoxyribonucleases known which have different substrate specificities, chemical mechanisms, and biological functions. They are:

1. **Deoxyribonuclease I (DNaseI):**

An endonuclease which cleaves double-stranded DNA or single stranded DNA. The cleavage preferentially occurs adjacent to pyrimidine (C or T) residues. The major products are 5'-phosphorylated bi-, tri- and tetranucleotides. It requires divalent ions (Ca^{2+} and Mn^{2+}/Mg^{2+}) for its activity and creates blunt ends or 1-2 overhang sequences.

DNaseI is the most widely used enzyme in cloning experiments to remove DNA contamination from mRNA preparation (to be used for cDNA library preparation, northern hybridization, RT-PCR etc). The mode of action of DNaseI varies according to the divalent cation used.

In the presence of magnesium ions (Mg^{+2}), DNaseI hydrolyzes each strand of duplex DNA producing single stranded nicks in the DNA backbone, generating various random cleavages. On the other hand, in the presence of manganese ions (Mn+2), DNaseI cleaves both strands of a double stranded DNA at approximately the same site, producing blunt ended DNA fragments or with 1-2 base overhangs. The two major DNases found in metazoans are: deoxyribonuclease I and deoxyribonuclease II.

Some of the common applications of DNase I in rDNA technology have been mentioned below:

- Eliminating DNA contamination (e.g. plasmid) from preparations of RNA.
- Analyzing the DNA-protein interactions via DNA footprinting.
- Nicking DNA prior to radio-labeling by nick translation.

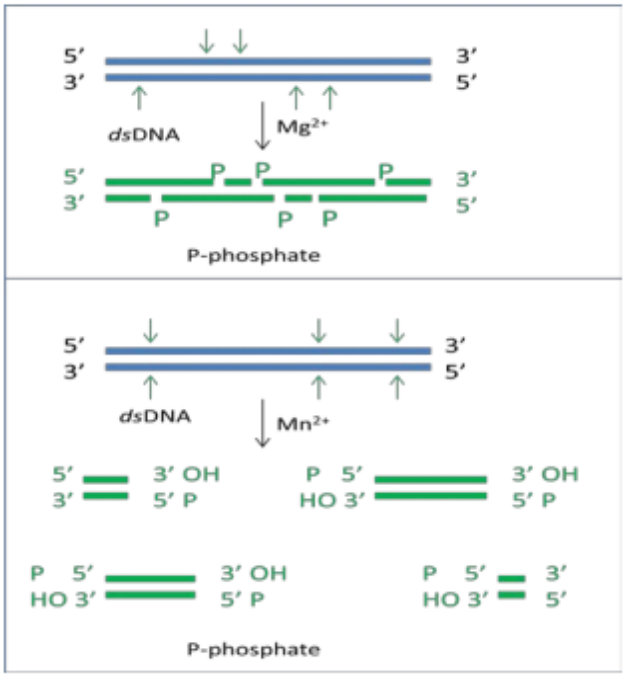


Fig 5: Action of DNase I in the presence of Mg^{+2} and Mn^{+2} ions. (Arrowhead denoting random site of cleavage in double stranded DNA by DNase I)

2. **DeoxyribonucleaseII (DNaseII):**

It is a non-specific endonuclease with optimal activity at acidic pH (4.5-5.5) and conserved from human to C.elegans.. It does not require any divalent cation for its activity. DNaseII initially introduces multiple single stranded nicks in DNA backbone and finally generates 3' phosphate groups by hydrolyzing phosphodiester linkages.

This enzyme releases 3'phosphate groups by hydrolyzing phosphodiester linkage and creating nicks in the DNA backbone. DNaseII acts by generating multiple single stranded nicks followed by production of acid soluble nucleotides and oligonucleotides. The catalytic site of the enzyme contains three histidine residues which are essential for enzyme activity.

Some of the common applications of DNase II are as follows:

- DNA fragmentation
- Molecular weight marker
- Cell apoptosis assays etc.

3. **Exonuclease III:**

Exonuclease III is a globular enzyme which has 3'→5' exonuclease activity in a double stranded DNA. The template DNA should be double stranded and the enzyme does not cleave single stranded DNA. The enzyme shows optimal activity with blunt ended sequences or sequences with 5' overhang. Exonuclease III enzyme has a bound divalent cation which is essential for enzyme activity. The mechanism of the enzyme can be affected by variation in temperature, monovalent

ion concentration in the reaction buffer, and structure and concentration of 3'termini. The enzyme shows optimal activity at 37°C at pH 8.0. Various application of exonuclease III in molecular cloning experiments are:

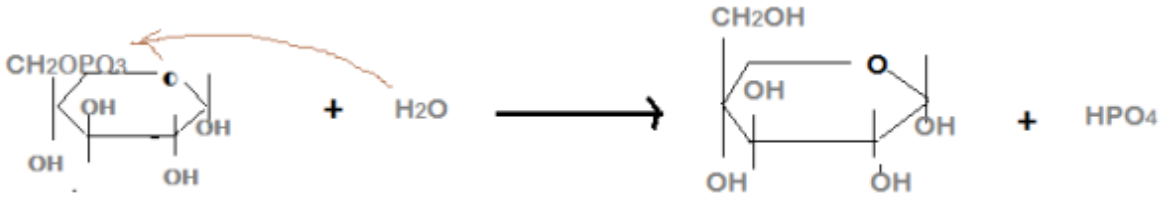
- To generate template for DNA sequencing
- To generate substrate for DNA labeling experiments
- Directed mutagenesis
- DNA-protein interaction assays (to find blockage of exonuclease III activity byprotein-DNA binding) etc.

4. Mung bean nuclease:

As the name suggest, this nuclease enzyme is isolated from mung bean sprouts (Vigna radiata). Mung bean nuclease enzymes can degrade single stranded DNA as well RNA. Under high enzyme concentration, they can degrade double stranded DNA, RNA or even DNA/RNA hybrids. Mung bean nuclease can cleave single stranded DNA or RNA to produce 5'-phosphoryl mono and oligonucleotides. It requires Zn^{2+} ion for its activity and shows optimal activity at 37°C. The enzyme works in low salt concentration (25mM ammonium acetate) and acidic pH (pH 5.0). Treatment with EDTA or SDS results in irreversible inactivation of the enzyme. Mung bean nuclease is less robust than S1 nuclease and easier to handle. It has been used to create blunt end DNA by cleaving protruding ends from 5' ends. This enzyme cannot produce nicks in a double stranded DNA but at higher concentration, it can generate nicks and cleave double stranded DNA.

5. Phosphatase:

- Phosphatase catalyses the cleavage of a phosphate (PO4 -2) group from substrate by using a water molecule (hydrolytic cleavage).
- This reaction is not reversible.
- This shows totally opposite activity from enzyme like kinase and phosphorylase that add a phosphate group to their substrate. On the basis of their activity there are two types of phosphatase i.e acid phosphatase and alkaline phosphatase. In both forms the alkaline phosphatase are most common.
- Special class of phosphatase that remove a phosphate group from protein, called "Phosphoprotein phosphatase".



i. Acid phosphatase:

It shows its optimal activity at pH between 3 and 6, e.g. a lysosomal enzyme that hydrolyze organic phosphates liberating one or more phosphate groups. They are found in prostatic epithelial cells, erythrocyte, prostatic tissue, spleen, kidney etc.

ii. Alkaline phosphatase:

- Homodimeric enzyme which catalyzes reactions like hydrolysis and transphosphophorylation of phosphate monoester.

- They show their optimal activity at pH of about 10**.**
- Alkaline phosphatase was the first zinc enzyme discovered having three closed spaced metal ion. Two Zn+2 ions and one Mg+2 ion, in which Zn+2 ions are bridges by Asp 51. The mechanism of action is based on reaction where a covalent serine – phosphate intermediate is formed to produce inorganic phosphate and an alcohol.
- In human body it is present in four isoforms, in which three are tissue specific isoform i.e. placental, germ cell, intestinal and one is non tissue specific isoform. The genes that encode for tissue specific isoforms are present on chromosome -2 p37-q37, while the genes for one non tissue specific are present on chromosome -1 p34- p36.1.
- During post-translational modification, alkaline phosphatase is modified by Nglycosylation. It undergoes a modification through which uptake of two Zn+2 ion and one Mg+2 ion occurs which is important in forming active site of that enzyme. Alkaline phosphatases are isolated from various sources like microorganisms, tissue of different organs, connective tissue of invertebrate and vertebrate, and human body.

Fig6: Action of alkaline phosphatase

- **Bacterial alkaline phosphatase (BAP**) - Bacterial alkaline phosphate is a phosphomonoester that hydrolyzes 3' and 5' phosphate from nucleic acid (DNA/ RNA). It more suitably removes phosphate group before end labeling and remove phosphate from vector prior to insert ligation. BAP generally shows optimum activity at temperature 65°C. BAP is sensitive to inorganic phosphate so in presence of inorganic phosphates activity may reduce.
- **Calf intestinal alkaline phosphatase (CIP)** – It is isolated from calf intestine, which catalyzes the removal of phosphate group from 5' end of DNA as well as RNA. This enzyme is highly used in gene cloning experiments, as to make a construct that could not undergo self-ligation. Hence after the treatment with CIP, without having a phosphate group at 5' ends a vector cannot self ligate and recircularise. This step improves the efficiency of vector containing desired insert.
- **Shrimp alkaline phosphatase (SAP)** - Shrimp alkaline phosphatase is highly specific, heat labile phosphatase enzyme isolated from arctic shrimp (Pandalus borealis). It removes 5' phosphate group from DNA, RNA, dNTPs and proteins. SAP has similar specificity as CIP but unlike CIP, it can be irreversibly inactivated by heat treatment at 65°C for 15mins. SAP is used for 5' dephosphorylation during cloning experiments for various application as follows:
    a. Dephosphorylate 5'-phosphate group of DNA/RNA for subsequent
    b. labeling of the ends.
    c. linearized plasmid.

d. To prepare PCR product for sequencing.
e. To inactivate remaining dNTPs from PCR product (for downstream sequencing appication).

Two primary uses for alkaline phosphatase in DNA modification:

Removing 5' phosphate from different vector like plasmid, bacteriophage after treating with restriction enzyme. This treatment prevents self ligation because unavailability of phosphate group at end. So, this treatment greatly enhances the ligation of desired insert. During ligation of desired insert, the complementary ends of the insert and vector will come to proximity of each other (only for sticky ends but not for blunt ends). One strand of the insert having 5'-phosphate will ligate with the 3'OH of the vector and the remaining strand will have a nick. This nick will be sealed in the next step by ligase enzyme in the presence of ATP. It is used to remove 5' phosphate from fragment of DNA prior to labeling with radioactive phosphate.

6. Methylase:

- Methyltransferase or methylase catalyzes the transfer of methyl group (-CH3) to its substrate. The process of transfer of methyl group to its substrate is called methylation.
- Methylation is a common phenomenon in DNA and protein structure.
- Methyltransferase uses a reactive methyl group that is bound to sulfur in Sadenosyl methionine (SAM) which acts as the methyl donor.
- Methylation normally occurs on cytosine (C) residue in DNA sequence. In protein, methylation occurs on nitrogen atom either on N-terminus or on the side chain of protein.
- DNA methylation regulates gene or silence gene without changing DNA sequences, as a part of epigenetic regulation.
- In bacterial system, methylation plays a major role in preventing their genome from degradation by restriction enzymes. It is a part of restriction – modification system in bacteria.
- In bacterial system, methylation plays a major role in preventing their genome

Methyltransferase can be classified in three groups:

a) m6A-generates N6 methyladenosine,

b) m4C-generates N4 methylcytosine,

c) m5C-generatesN5 methylcytosine.

m6A and m4C methyltransferase are primarily found in prokaryotes. These enzymes are responsible for methylation of DNA sequences in order to prevent the host from digesting its own genome via its restriction enzyme.

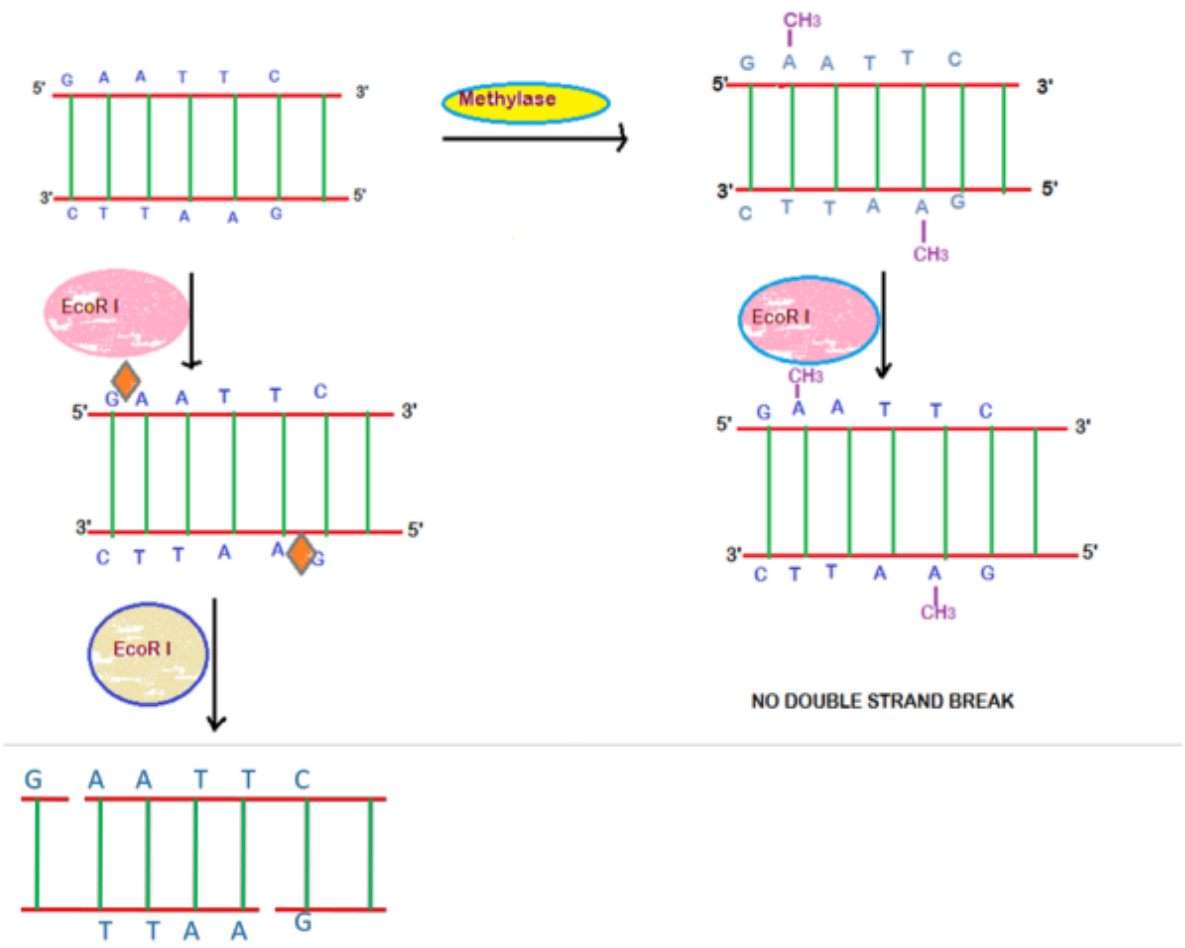


Fig 7: Activity of restriction and methylase enzymes

Restriction enzyme EcoRI cleaves within the recognition sequence if the DNA is unmethylated. On methylation by methylases, the restriction enzyme EcoRI is inhibited from cleaving within the restriction site.

Some common examples of methytransferases are: DNA adenyl methytransferase (DAM), histone methyltransferase, O-methyltransferase etc. DAM methylase is generally used in recombinant DNA technology which can methylate adenine (A) in the sequence 5'GATC3'. This enzyme can methylate a newly synthesized DNA strand on specific sites.

7. **Ligases:**
    - DNA ligase catalyses the formation of phosphodiester bond between two deoxynucleotide residues of two DNA strands.
    - DNA ligase enzyme requires a free hydroxyl group at the 3´ -end of one DNA chain and a phosphate group at the 5´-end of the other and requires energy in the process.
    - E.coli and other bacterial DNA ligase utilizes NAD+ as energy donor, whereas in T4 bacteriophage, T4 DNA ligase uses ATP as cofactor.

- The role of DNA ligase is to seal nicks in the backbone of double-stranded DNA after lagging strand formation to join the okazaki fragments.
- This joining process is essential for the normal synthesis of DNA and for repairing damaged DNA. It has been exploited by genetic engineers to join DNA chains to form recombinant DNA molecules. Usually single stranded break are repaired using the complimentary strand as the template but sometimes double stranded breaks can also be repaired with the help of DNA ligase IV.
- The most widely used DNA ligase is isolated from T4 bacteriophage. T4 DNA ligase needs ATP as a cofactor. The enzyme from E. coli uses cofactor NAD. Except this, the catalysis mechanism is somewhat similar for both the ligases. The role of cofactor is splitting and forming an enzyme-AMP complex which further aids in formation of phosphodiester bonds between hydroxyl and phosphate groups by exposing them.

i. Mechanism of Action of DNA Ligases:

- ATP, or NAD+, reacts with the ligase enzyme to form a covalent enzyme–AMP complex in which the AMP is linked to ε-amino group of a lysine residue in the active site of the enzyme through a phosphoamide bond.
- The AMP moiety activates the phosphate group at the 5´-end of the DNA molecule to be joined. It is called as the donor.
- The final step is a nucleophilic attack by the 3´-hydroxyl group on this activated phosphorus atom which acts as the acceptor. A phosphodiester bond is formed and AMP is released.
- The reaction is driven by the hydrolysis of the pyrophosphate released during the formation of the enzyme–adenylate complex. Two high-energy phosphate bonds are spent in forming a phosphodiester bond in the DNA backbone with ATP serving as energy source.
- The temperature optimum for T4 DNA ligase mediated ligation in vitro is 16˚C. However ligation is also achieved by incubation at 4˚C by incubating over night or at room temperature condition by incubating for 30 minutes.
- Adenylate and DNA-adenylate are the important intermediates of the phosphodiester bond forming pathway.

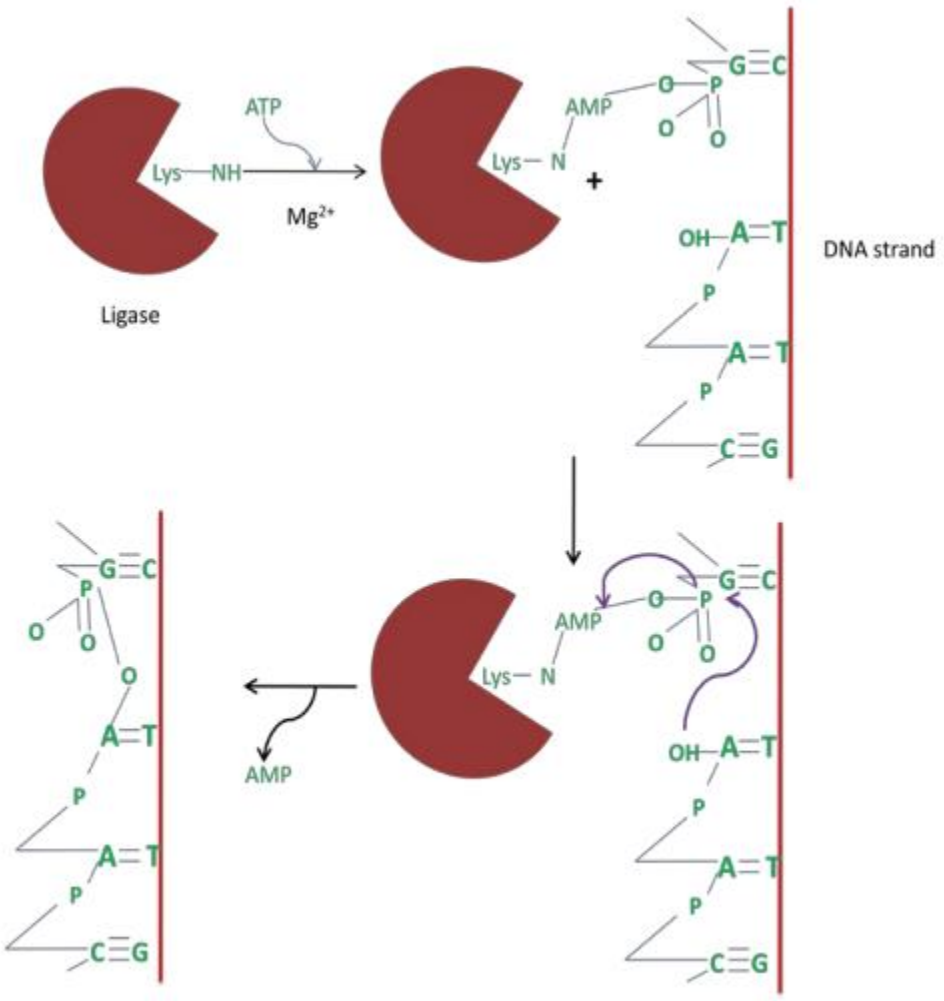


Fig 8: The mechanism of DNA joining by DNA ligase

ii. Application:

- DNA ligase enzyme is used by cells to join the "okazaki fragments" during DNA replication process. In molecular cloning, ligase enzyme has been routinely used to construct a recombinant DNA. Followings are some of the examples of application of ligase enzyme in molecular cloning.Joining of adapters and linkers to blunt end DNA molecule.
- Cloning of restricted DNA to vector to construct recombinant vector.

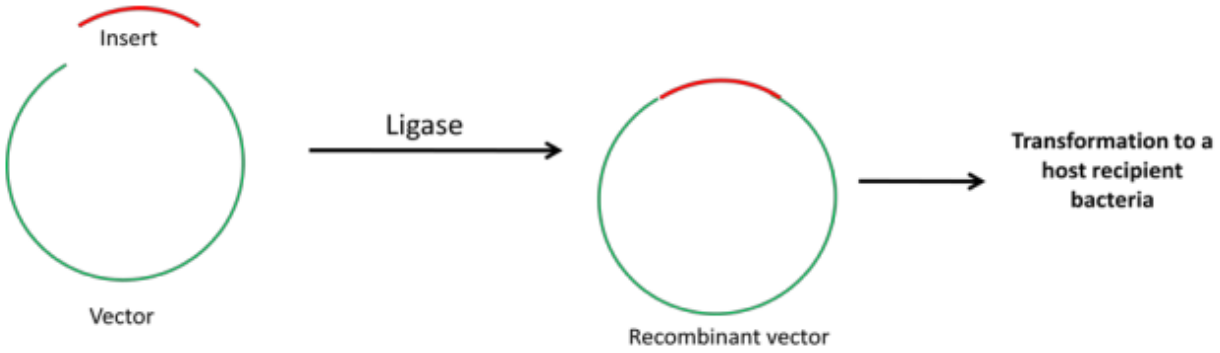


Fig 9: Ligation of a gene fragment into the vector and transformation of the cell

8. **Polynucleotide Kinase:**
    - PNK is a homotetramer with phosphatase activity at 3’ end and kinase activity at 5’ end with a tunnel like active site. The active site has side chains which interact with NTP donor’s beta-phosphate and 3’ phosphate of acceptor with an acid which activated 5’ –OH. Lys-15 and Ser-16 are important for the kinase activity of the enzyme.
    - The basic residues of active site of PNK interact with the negatively charged phosphates of the DNA.
    - Polynucleotide kinase (PNK) catalyzes the transfer of a phosphate group (PO4 -2) from γ position of ATP to the 5' end of either DNA or RNA and nucleoside monophosphate.
    - PNK can convert 3' PO4/5' OH ends into 3' PO4/5' PO4 ends which blocks further ligation by ligase enzyme.
    - PNK is used to label the ends of DNA or RNA with radioactive phosphate group.
    - T4 polynucleotide kinase is the most widely used PNK in molecular cloning experiments, which was isolated from T4 bacteriophage infected E.coli.

PNK carries out two types of enzymatic activity:

- **Forward reaction:** γ-phosphate is transferred from ATP to the 5' end of a polynucleotide (DNA or RNA). 5’ phosphate is not present either due to chemical synthesis or dephosphorylation. The 5’ OH nucleophile is activated by abstraction of the proton. Asp35 of PNK forms the co-ordinate bond with 5’ OH and attacks γ phosphorus forming an intermediate.
- **Exchange reaction**: target DNA or RNA having a 5' phosphate is incubated with an excess of ADP - where PNK transfers the phosphate from the nucleic acid to an ADP, forming ATP. PNK then performs a forward reaction and transfer a phosphate from ATP to the target nucleic acid. Exchange reaction is used to label with radioactive phosphate group.

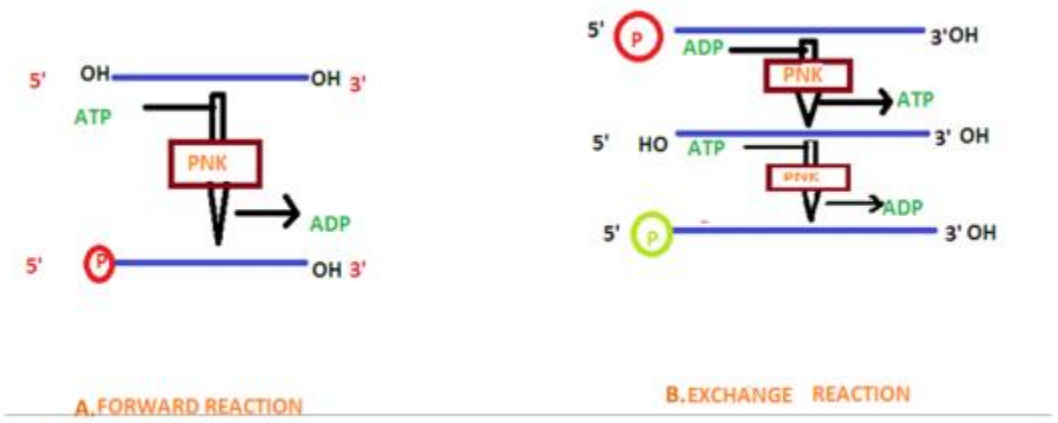


Fig 10: Polynucleotide kinase reaction (A) forward (B) exchange

9. Ribonuclease (RNase):

- Nuclease that can catalyze hydrolysis of ribonucleotides from either single stranded or double stranded RNA sequence are called ribonucleotides (RNase).
- RNase are classified into two types depending on position of cleavage, i.e. endoribonuclease (cleave internal bond) and exoribonuclease (cleave terminal bond).
- RNase is important for RNA maturation and processing.
- RNaseA and RNaseH play important role in initial defence mechanism against RNA viral infection. Two common types of ribonucleases are discussed below:

9.1. RibonucleaseA (RNaseA):

- An endo-ribonuclease that cleaves specifically single-stranded RNA at the 3' end of pyrimidine residues.
- The RNA is degraded into 3'-phosphorylated mononucleotides C and U residues and oligonucleotides in the form of 2', 3'-cyclic monophosphate intermediates.
- Optimal temperature for RNaseA is 60˚C (activity range 15-70˚C) and optimal pH is 7.6.
- RNaseA has two histidine residues in its active site (His12 and His119). In the first step, His12 acts as a base; accepting proton forming a nucleophile which then attacks positively charged phosphorus atom. His119 acts as an acid in this case, donating a proton to oxygenated P-O-R' bond. The imidazole side chain acts as base in His 12 here.
- The side chain of Lys41 and Phe120 further stabilize the transition state. Nitrogen of the main chain of Phe120 donates hydrogen, thus bonding with the unbound oxygen atom.
- In the second step the acid base activities get reversed and His119 accepts proton from water causing hydroxyl attack on cyclic intermediate.

- Activity of RNaseA can be inhibited by alkylation of His12 and His119 residue essential for activity of the enzyme.

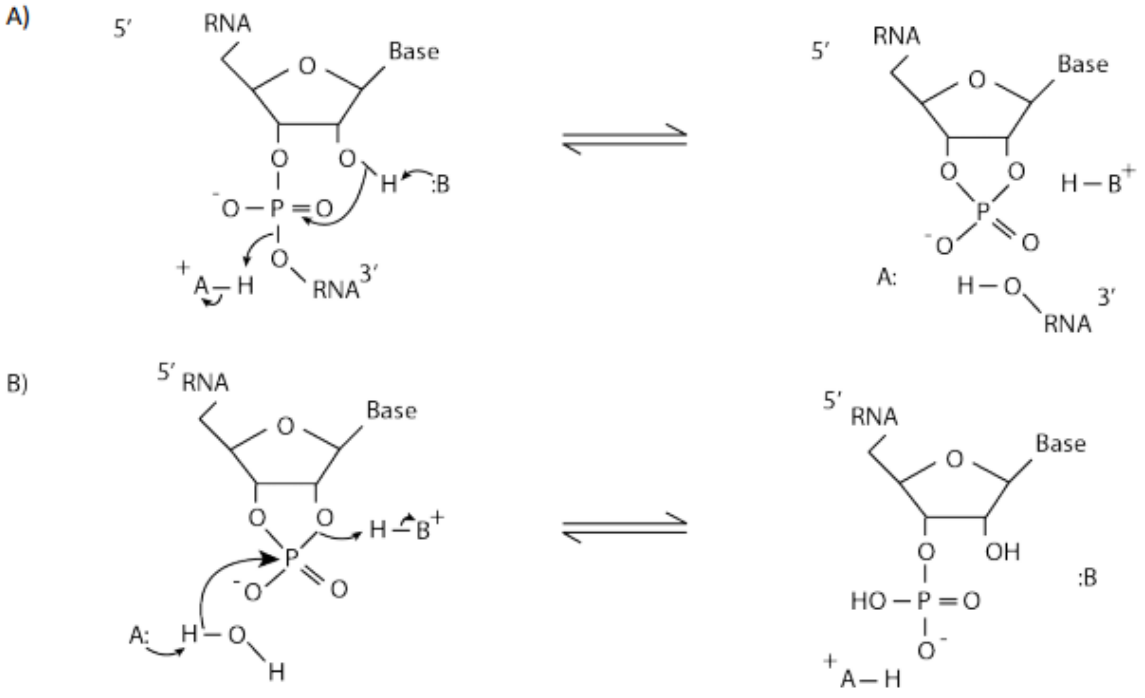


Fig : (A) Transphosphorylation reaction by RNase A
(B) Hydrolysis reaction catalyzed by RNase A

"B" → His 12
"A" → His 119

Fig 11: Mechanism of action of RNase A

Application:

- It is used to remove RNA contamination from DNA sample.

9.2 RibonucleaseH:

- Non-specific endoribonuclease that degrades RNA by hydrolytic mechanism from DNA/RNA duplex resulting in single stranded DNA.
- Enzyme bound divalent metal ion is a cofactor here. The product formed is 5' phosphorylated ssDNA.
- During cDNA library preparation from RNA sample, RNaseH enzyme is used to cleave RNA strand of DNA-RNA duplex.

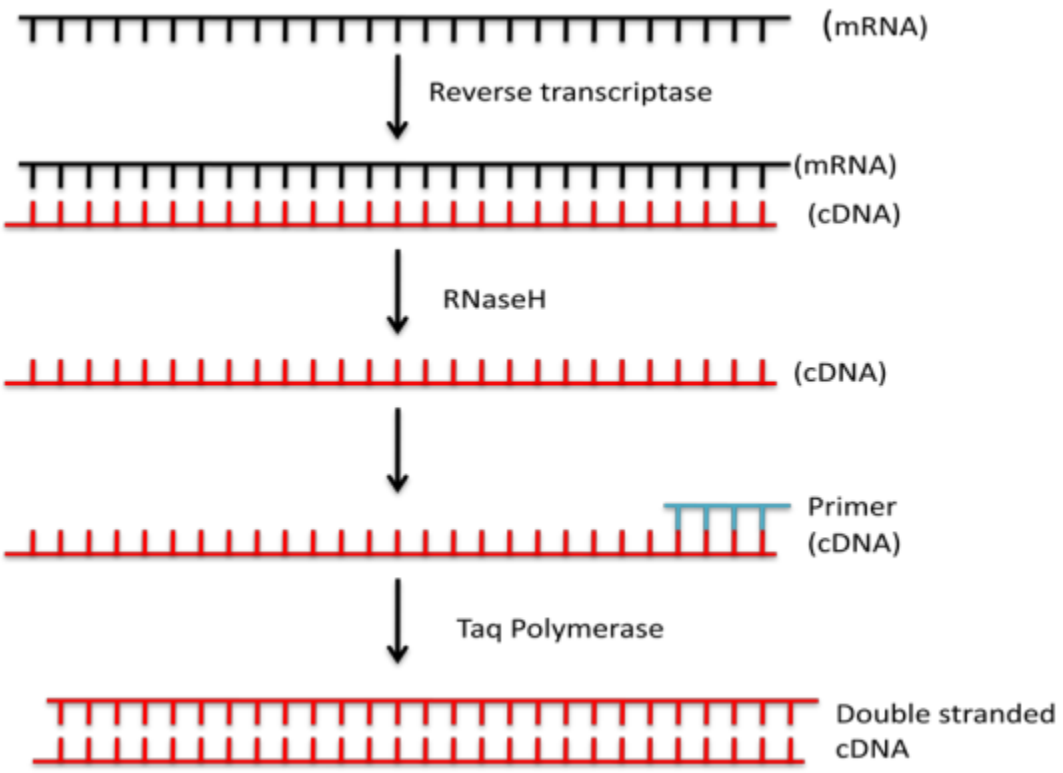


Fig 12: Schematic representation of cDNA preparation from mRNA

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – 2– GENETIC ENGINEERING – SBB2102

BIOLOGY OF CLONING VECTORS

1. Plasmids

A plasmid is a small, circular piece of DNA that is different than the chromosomal DNA, which is all the genetic material found in an organism's chromosomes. It replicates independently of chromosomal DNA. Plasmids are mainly found in bacteria, but they can also be found in archaea and multicellular organisms. Plasmids usually carry at least one gene, and many of the genes that plasmids carry are beneficial to their host organisms. Although they have separate genes from their hosts, they are not considered to be independent life.

In addition to bacterial chromosome (nucleoid), bacterial cells normally contain genetic elements in their cytoplasm. These genetic elements exist and replicate separately from the chromosome and are called plasmids. The very existence of plasmids in bacterial cytoplasm was revealed by Lederberg in 1952 while working on conjugation process in bacteria.

Lederberg coined the term 'plasmid' to refer to the transmissible genetic elements that were transferred from one bacterial cell to another and determined the maleness in bacteria. Literally, thousands of plasmids are now known; over 300 different naturally occurring plasmids have been isolated from strains of Escherichia coli alone. Besides naturally occurring plasmids, many artificially modified plasmids have been developed and used as vectors in the process of gene cloning (genetic engineering).

2.1. Physical Nature and Copy Number of Plasmids

The physical nature of plasmids is quite simple. They are small double-stranded DNA molecules. Majority of the plasmids are circular, but many linear plasmids are also known. Naturally occurring plasmids vary in size from approximately 1 kilobase to more than 1 megabase, and a typical plasmid DNA is considered to be less than 5% the size of the bacterial chromosome. Most of the plasmid DNA isolated from bacterial cells exist in the supercoil configuration, which is the most compact form for DNA to exist within the cell.

The copy number refers to the fact that different plasmids occur in cells in different numbers. Some plasmids are present in the cell in only 1-3 copies, whereas others may be present in over 100 copies. Copy number is controlled by genes on the plasmid and by interactions between the host and the plasmid.

2.2. Properties of Plasmids:

1. They are specific to one or a few particular bacteria.
2. They replicate independently of the bacterial chromosome.
3. They code for their own transfer.
4. They act as episomes and reversibly integrate into bacterial chromosome.
5. They may pick-up and transfer certain genes of bacterial chromosome,
6. They may affect certain characteristics of the bacterial cell,
7. Plasmids differ from viruses in following two ways.

8. They do not cause damage to cells and generally are beneficial.
9. They do not have extracellular forms and exist inside cells simply as free and typically circular DNA.

2.3. Incompatibility of Plasmids:

In some cases, a single bacterial cell contains several different types of plasmids. Borrelia burgdorferi that causes Lyme disease, for convenience, possesses 17 different circular and linear plasmids. In a condition when a plasmid is transferred to a new bacterial cell that already possesses another plasmid, it is commonly observed that the second (transferred) plasmid is not accommodated and is lost during subsequent replication. This condition is called plasmid incompatibility and the two plasmids are said to be incompatible. A number of incompatibility groups of plasmids have been recognised in bacteria. The plasmids of one incompatibility group exclude each other from replicating in the cell but generally coexist with plasmids from other groups.

Plasmids of an incompatibility group share a common mechanism of regulating their replication and are thus related' to one another. Therefore, although a bacterial cell may possess various types of plasmids, each is genetically distinct.

2.4. Types of Plasmids:

Various types of plasmids naturally occur in bacterial cells, and the most favoured classification of such plasmids is based on their main functions encoded by their own genes.

Following are the main type of plasmids recognised on the basis of above mentioned characteristic feature:

2.4.1. F-plasmid (or F-factor):

F-plasmid or F-factor ("F" stands for fertility) is the very well characterised plasmid. It plays a major role in conjugation in bacteria E. coli and was the first to be described. It is this plasmid that confers 'maleness' on the bacterial cells; the term 'sex-factor' is also used to refer to F-plasmid because of its this property. F-plasmid is a circular dsDNA molecule of 99,159 base pairs.

The genetic map of the F-plasmid is shown in Fig. 5.31. One region of the plasmid contains genes involved in regulation of the DNA replication (rep genes), the other region contains transposable elements (IS3, Tn 1000, IS3 and IS2 genes) involved in its ability to function as an episome, and the third large region, the tra region, consists of tra genes and possesses ability to promote transfer of plasmids during conjugation. Example F-plasmid of E. coli.

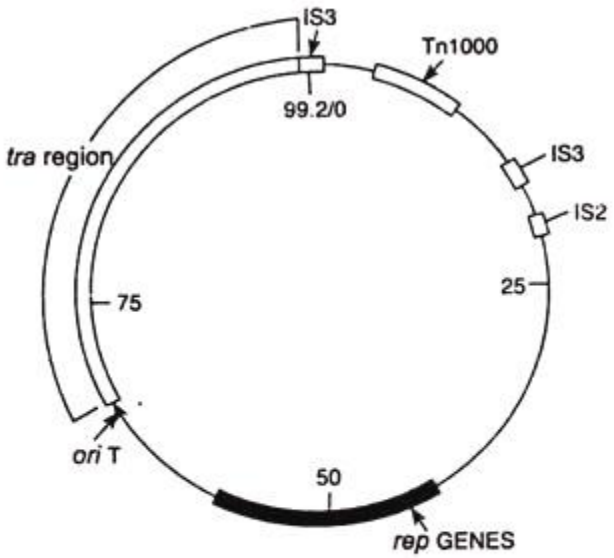


FIG. 5.31. Genetic map of the F (fertility) plasmid of *Escherichia coli*. *tra* region contains *tra* genes involved in conjugative transfer; *Ori* T sequence is the origin of transfer during conjugation; transposable element region responsible for functioning as episome, and; the *rep* genes regulate DNA replication.

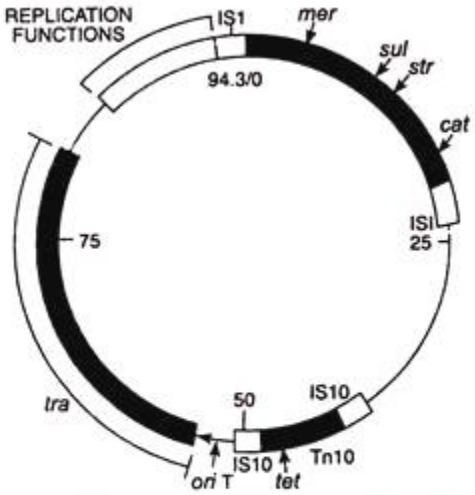


FIG. 5.32. Genetic map of the resistance plasmid R100. cat = chloramphenicol resistance gene; str=streptomycin resistance gene; sul = sulfonamide resistance gene; mer = mercury ion resistance gene; IS = insertion sequences.

Fig 1: Genetic map of the F plasmid of *E.coli* and Resistance plasmid R100

2.4.2. R-plasmids:

R-plasmids are the most widespread and well-studied group of plasmids conferring resistance (hence called resistant plasmids) to antibiotics and various other growth inhibitors. R- plasmids typically have genes that code for enzymes able to destroy and modify antibiotics. They are not usually integrated into the host chromosome. Some R-plasmids possess only a single resistant gene whereas others can have as many as eight.

Plasmid R 100, for example, is a 94.3 kilobase-pair plasmid (Fig. 5.32) that carries resistant genes for sulfonamides, streptomycin and spectinomycin, chloramphenicol, tetracyclin etc. It also carries genes conferring resistance to mercury. Many R-plasmids are conjugative and possess drug- resistant genes as transposable elements, they play an important role in medical microbiology as their spread through natural populations can have profound consequences in the treatment of bacterial infections.

2.4.3. Virulence-plasmids:

Virulence-plasmids confer pathogenesity on the host bacterium. They make the bacterium more pathogenic as the bacterium is better able to resist host defence or to produce toxins. For example, Ti-plasmids of Agrobacterium tumefaciens induce crown gall disease of angiospermic plants; entertoxigenic strains of E. coli cause traveller's diarrhoea because of a plasmid that codes for an enterotoxin which induces extensive secretion of water and salts into the bowel.

2.4.4. Col-plasmids:

Col-plasmids carry genes that confer ability to the host bacterium to kill other bacteria by secreting bacteriocins, a type of proteins. Bacteriocins often kill cells by creating channels in the plasma membrane thus increasing its permeability. They also may degrade DNA or RNA or attack peptidoglycan and weaken the cell-wall. Bacteriocins act only against closely related strains. Col E1 plasmid of E. coli code for the synthesis of bacterioein called colicins which kill other susceptible strains of E. coli. Col plasmids of some E.coli code for the synthesis of bacteriocin, namely cloacins that kill Enterobacter species. Lactic acid bacteria produce bacteriocin NisinA which strongly inhibits the growth of a wide variety of gram-positive bacteria and is used as a preservative in the food industry.

2.4.5. Metabolic plasmids:

Metabolic plasmids (also called degradative plasmids) possess genes to code enzymes that degrade unusual substances such as toluene (aromatic compounds), pesticides (2, 4-dichloro-phenoxyacetic acid), and sugars (lactose).

TOL (= pWWO) plasmid of Pseudomonas putida is an example. However, some metabolic plasmids occurring in certain strains of Rhizobium induce nodule formation in legumes and carry out fixation of atmospheric nitrogen.

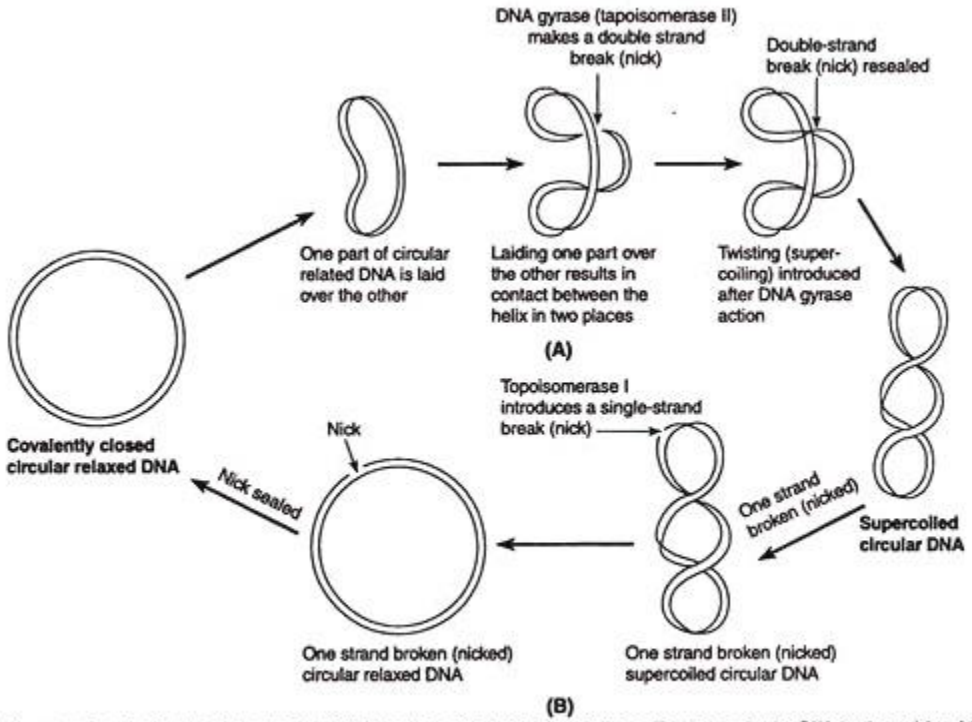


FIG. 5.35. Interconversion of relaxed and supercoiled DNA in bacteria. (A) Introduction of supercoiling into a circular DNA by the activity of DNA gyrase (topoisomerase II), which makes double-strand breaks (nicks). (B) Conversion of supercoiled circular DNA into relaxed circular DNA by the activity of topoisomerase I.

Fig 2: Interconversion of relaxed and supercoiled DNA in bacteria

2.5. Replication of Plasmids:

Plasmids replicate autonomously because they have their own replication origins. The enzymes involved in plasmid replication are normal cell enzymes particularly in case of small plasmids. But, some large plasmids carry genes that code for enzymes that are specific for plasmid replication.

Plasmids possess relatively few genes, generally less than 30, and the genes are concerned primarily with control of the replication initiation process and with apportionment of the replicated plasmids between daughter cells; the genetic information carried in plasmid genes is not essential to the host because the bacteria that lack them usually function normally.

Since the plasmid DNA is of small size, the whole process of its replication takes place very quickly, perhaps in 1/10 or less of the total time of cell division cycle.

Most plasmids in gram-negative bacteria replicate in a manner similar to the replication of bacterial chromosome involving initiation at the replication origin site and bidirectional replication around the DNA circle giving a theta (Θ) intermediate.

However, some plasmids of gram-negative bacteria replicate by unidirectional method. Most plasmids of gram-positive bacteria replicate by a rolling circle mechanism similar to that used by phage φx174. Most linear plasmids replicate by means of a mechanism that involves a protein bound to the 5′-end of each DNA strand that is used in priming DNA synthesis.

2.6. Plasmid Curing:

Plasmids can be eliminated from bacterial cells, and this process is called curing. Curing may take place spontaneously or it may be induced by various treatments, which inhibit plasmid replication but do not affect bacterial chromosome replication and cell reproduction. The inhibited plasmids are slowly diluted out of the growing bacterial population.

Some commonly used curing treatment agents are acridine dyes, ultraviolet (UV) and ionizing radiation, thymine starvation and growth above optimal temperatures. These curing treatment agents interfere with plasmid replication than with bacterial chromosome replication.

2.7 Use of Plasmids as Cloning Vectors:

Significance of plasmids dramatically increased with the advent of recombinant DNA technology as they became the first cloning vectors, and even today they are the most widely used cloning vectors especially in gene cloning in bacteria.

They enjoy this status because they have very useful properties as cloning vectors that include:

(i) Small size, which makes the plasmid easy to isolate and manipulate;

(ii) Independent origin of replication, which allows plasmid replication in the cell to proceed independently from direct chromosomal control;

(iii) Multiple copy number, which makes them to be present in the cell in several copies so that amplification of the plasmid DNA becomes easy; and

(iv) Presence of selectable markers such as antibiotic resistance genes, which make detection and selection of plasmid-containing clones easier.

The plasmid vector is isolated from the bacterial cell and at one site by restriction enzyme. The cleavage converts the circular plasmid DNA into a linear DNA molecule.

Now the two open ends of linear plasmid are joined to the ends of the foreign DNA to be inserted with the help of enzyme DNA ligase. This regenerates a circular hybrid or chimeric plasmid, which is transferred to a bacterium wherein it replicates and perpetuates indefinitely.

One of the most widely used plasmids in gene cloning in bacteria is pBR322, which has both resistance genes for ampicillin and tetracycline and many restriction sites. When a foreign DNA is inserted into the ampicillin resistance gene of pBR322, the plasmid is no longer able to confer resistance to ampicillin.

2.8. Applications of Plasmids

Humans have developed many uses for plasmids and have created software to record the DNA sequences of plasmids for use in many different techniques. Plasmids are used in genetic engineering to amplify, or produce many copies of, certain genes. In molecular cloning, a plasmid is a type of vector. A vector is a DNA sequence that can transport foreign genetic material from one cell to another cell, where the genes can be further expressed and replicated. Plasmids are useful in cloning short segments of DNA. Also, plasmids can be used to replicate proteins, such as the protein that codes for insulin, in large amounts. Additionally, plasmids are being investigated as a way to transfer genes into human cells as part of gene therapy. Cells may lack a specific protein if the patient has a hereditary disorder involving a gene mutation. Inserting a plasmid into DNA would allow cells to express a protein that they are lacking.

2.9. Origin of replication.

Required for automous replication of the plasmid using the host's replication machinery. Almost all commonly used plasmids are based on the ColE1 origin of replication (ori). It is worth noting that bacterial origins of replication are tightly regulated. While R factors are smaller than the host genome (105 bp compared to 5x106 bp), replication of these factors to high copy number in the host places a considerable load on the host replication machinery. Naturally occuring origins of replication are therefore negatively regulated to keep copy number down (typically 5 to 10 copies per cell). While high copy number is disadvantageous in a natural system, it is a desirable feature in a cloning vector - since the whole idea is to be able to easily isolate substantial quantities of particular DNA sequences. Therefore considerable work has gone into engineering the ColE1 ori such that the negative regualtory mechanisms that limit episome copy number are disabled. Modern plasmid vectors are therefore often called 'runaway replicons' and are present at 100 to 1000 copies per cell.

2.10 Selectable Markers

Selectable markers are essential for the identification of bacteria containing recombinant plasmids. Selection can be divided into two types:

Positive Selection

Positive selection is used to identify bacteria that contain a plasmid. The most common markers used for positive selection are the antibiotic resistance genes carried by the original R factors.

While many antibiotics and resistance genes are available, the commonly used ones fall into two general classes:

Antibiotics affecting cell wall synthesis

Ampicillin is a beta-lactam based antibiotic that acts by inhibiting the synthesis of the bacterial peptidoglycan cell wall. Sensitive bacteria are not actively 'killed', but on cell division are unable to synthesize the cell wall and suffer from osmotic lysis.

The enzyme beta-lactamase is secreted into the periplasmic space where it breaks down the antibiotic, allowing cell wall synthesis to proceed.

Antibiotics affecting translation:

The antibiotics tetracycline, kanamycin and chloramphenicol all act by inhibiting translation. The covalent modification (phosphorylation, acetylation) of these antibiotics blocks their interaction with the translation apparatus.

Positive selection is particularly important when introducing plasmids into bacteria by transformation. At best, only about 1 in 10,000 bacteria picks up a plasmid that carries the antibiotic resistance. A strong positive selection system is essential to eliminate the 9,999 bacteria that didn't pick up a plasmid from the one that did. By plating the transformation products directly on antibiotic plates, all untransformed bacteria die and only those containing the plasmid (and antibiotic resistance marker) grow to form colonies.

Negative Selection

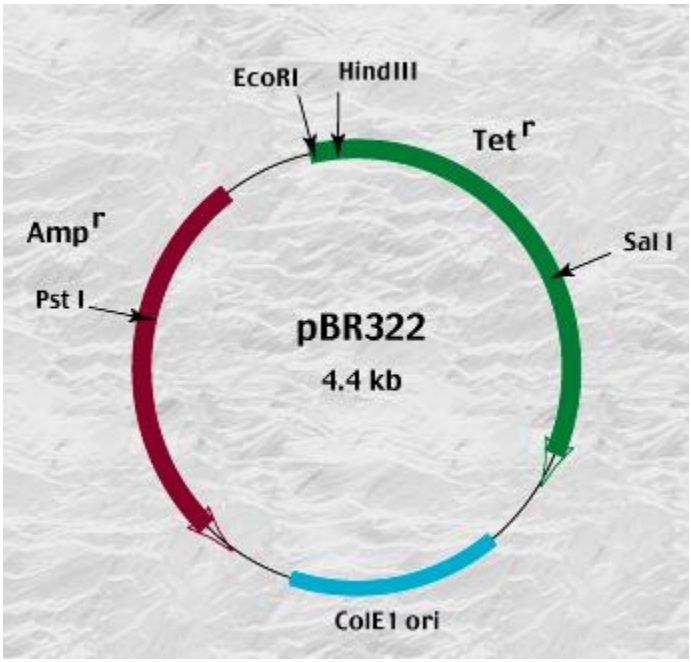
The ligation of foreign DNA fragments into plasmid vectors is a relatively inefficient process - ligation can either produce recircularized plasmid with no insert, or plasmids containing a foreign DNA insert. These products are then transformed into an antibiotic sensitive bacterial host, an positive selection applied to identify bacteria that contain a plasmid. A second selection system is necessary to distinguish between plasmids that merely recircularized from those that carry a foreign DNA insert.

In order to identify those plasmids carrying a foreign DNA fragment, the site of insertion is chosen such that insertion disrupts a selectable marker - a phenomenon we call insertional inactivation.

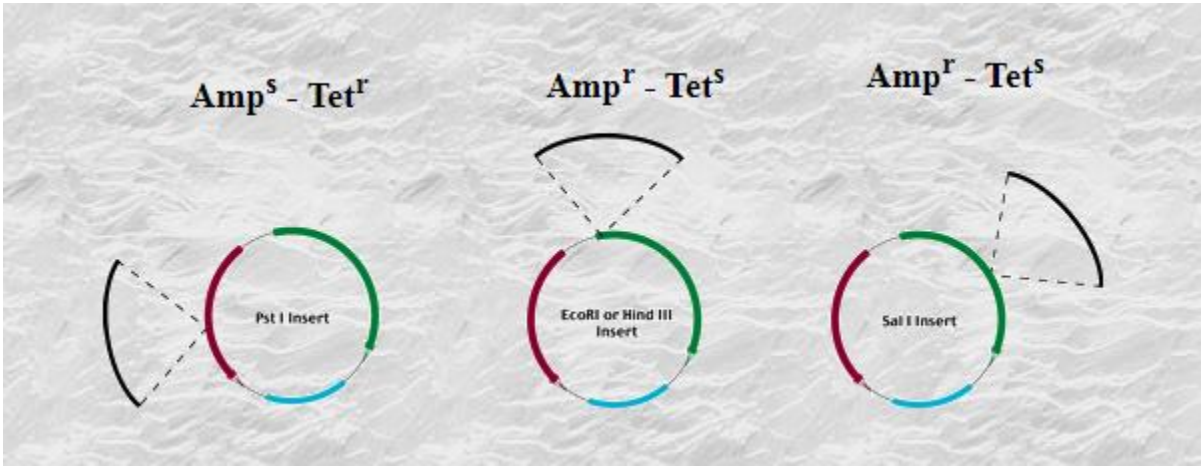
Two types of selectable markers are used for negative selection

Insertional inactivation of antibiotic resistance.

In order to use antibiotic resistance as a negative as well as a positive selection system, the plasmid vector must carry two different antibiotic resistance genes. An example of such a vector is pBR322.



- pBR322 carries both an ampicllin and a tetracycline resistance gene.
- The phenotype of bacteria containing the intact plasmid is Ampr Tetr
- Insertion of foreign DNA into
- the Pst I site located in the Ampr gene results in an Amps Tetr phenotype.
- Conversely, insertion of foreign DNA into the EcoRI, Hind III or Sal I sites located in the Tetr gene results in an
- Ampr Tets phenotype.
- In order to reveal these phenotypes, transformants are first plated on positive selective media Tet or Amp respectively).
- Positively selected colonies are then restreaked on positive selective media (master plate from which we recover our desired vector + insert) and on the negative selection media. (Amp or Tet media respectively)



Insertional Inactivation of Enzymatic Activity

While the insertional inactivation of antibiotic resistance works, it requires a lot of manipulation - picking the positively selected bacteria and replating on negative selection media etc. In addition to the tedium of picking colonies, vectors like pBR322 also suffer from a paucity of convenient restriction sites at which to insert the foreign DNA fragemnts.

These limitations promted the development of a set of host-vector systems in which it is possible to positively select for bacteria carrying a plasmid and simultaneously select for insertional inactivation of enzymatic activity. This system is based on our old friend, the beta-galactosidase gene of the E coli lac operon.

2.11. Examples of Cloning Vector:
a pBR322

- pBR322 is a widely-used E. coli cloning vector. It was created in 1977 in thelaboratory of Herbert Boyer at the University of California San Francisco. The pstands for "plasmid" and BR for "Bolivar" and "Rodriguez", researchers who constructed it.
- pBR322 is 4361 base pairs in length.
- pBR322 plasmid has the following elements
- rep" replicon from plasmid pMB1 which is responsible for replication of the plasmid.
- "rop" gene encoding Rop protein. Rop proteins are associated with stability of RNAI-RNAII complex and also decrease copy number. The source of "rop" gene is pMB1plasmid.
- "tet" gene encoding tetracycline resistance derived from pSC101 plasmid.
- "bla" gene encoding β lactamase which provide ampicillin resistance (source: transposon Tn3).

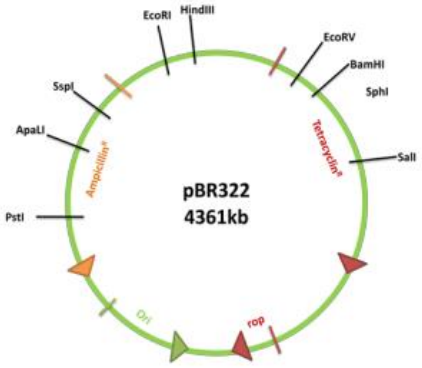


Fig 3: PlasmidPBR322

b. pUC plasmids:

- pUC plasmids are small, high copy number plasmids of size 2686bp.
- This series of cloning vectors were developed by Messing and co-workers in the University of California. The p in its name stands for plasmid and UC represents the University of California.
- pUC vectors contain a lacZ sequence and multiple cloning site (MCS) within lacZ. This helps in use of broad spectrum of restriction endonucleases and permits rapid visual detection of an insert.
- pUC18 and pUC19 vectors are identical apart from the fact that the MCS is arranged in opposite orientation. pUC vectors consists of following elements:

  - ✓ pMB1 "rep" replicon region derived from plasmid pBR322 with single point mutation (to increase copy number).
  - ✓ "bla" gene encoding β lactamase which provide ampicillin resistance which is derived from pBR322. This site is different from pBR322 by two point mutations.
  - ✓ E.coli lac operon system.

"rop" gene is removed from this vector which leads to an increase in copy number

An MCS is a short DNA sequence consisting of restriction sites for many different restriction endonucleases. MCS escalates the number of potential cloning strategies available by extending the range of enzymes that can be used to generate a restriction fragment suitable for cloning. By combining them within a MCS, the sites are made contiguous, so that any two sites within it can be cleaved simultaneously without excising vector sequences. The MCS is inserted into the lacZ sequence, which encodes the promoter and the α-peptide of β-galactosidase. Insertion of the MCS into the lacZ fragment does not affect the ability of the α-peptide to mediate complementation, while cloning DNA fragments into the MCS does. Therefore, recombinants can be detected by blue/white screening on growth medium containing X gal in presence of IPTG as an inducer.

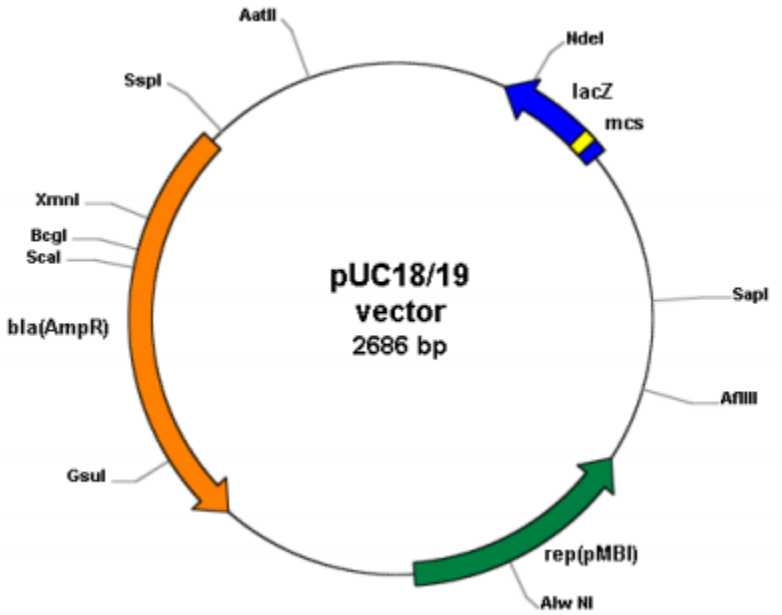


Fig 4: pUC plasmid

2.12. Bacteriophage

Bacteriophage, also called phage or bacterial virus, any of a group of viruses that infect bacteria. Bacteriophages were discovered independently by Frederick W. Twort in Great Britain (1915) and Félix d'Hérelle in France (1917). D'Hérelle coined the term bacteriophage, meaning "bacteria eater," to describe the agent's bacteriocidal ability. Bacteriophages also infect the single-celled prokaryotic organisms known as archaea.

Characteristics of Bacteriophages

Thousands of varieties of phages exist, each of which may infect only one type or a few types of bacteria or archaea. Phages are classified in a number of virus families; some examples include Inoviridae, Microviridae, Rudiviridae, and Tectiviridae. Like all viruses, phages are simple organisms that consist of a core of genetic material (nucleic acid) surrounded by a protein capsid. The nucleic acid may be either DNA or RNA and may be double-stranded or single-stranded. There are three basic structural forms of phage: an icosahedral (20-sided) head with a tail, an icosahedral head without a tail, and a filamentous form.

All viruses depend on cells for reproduction and metabolic processes. By themselves, viruses do not encode for all of the enzymes necessary for viral replication. But within a host cell, a virus can commandeer cellular machinery to produce more viral particles. Bacteriophages replicate only in the cytoplasm, since prokaryotic cells do not have a nucleus or organelles. In eukaryotic cells, most DNA viruses can replicate inside the nucleus, with an exception observed in the large DNA viruses, such as the poxviruses, that can replicate in the cytoplasm. RNA viruses that infect animal cells often replicate in the cytoplasm.

The Life Cycle of Viruses with Prokaryote Hosts

The life cycle of bacteriophages has been a good model for understanding how viruses affect the cells they infect, since similar processes have been observed for eukaryotic viruses, which can cause immediate death of the cell or establish a latent or chronic infection. Virulent phages typically lead to the death of the cell through cell lysis. Temperate phages, on the other hand, can become part of a host chromosome and are replicated with the cell genome until such time as they are induced to make newly assembled viruses, or progeny viruses.

The Lytic Cycle

During the lytic cycle of virulent phage, the bacteriophage takes over the cell, reproduces new phages, and destroys the cell. T-even phage is a good example of a well-characterized class of virulent phages. There are five stages in the bacteriophage lytic cycle (see Figure 5). Attachment is the first stage in the infection process in which the phage interacts with specific bacterial surface receptors (e.g., lipopolysaccharides and OmpC protein on host surfaces). Most phages have a narrow host range and may infect one species of bacteria or one strain within a species. This unique recognition can be exploited for targeted treatment of bacterial infection by phage therapy or for phage typing to identify unique bacterial subspecies or strains. The second stage of infection is entry or penetration. This occurs through contraction of the tail sheath, which acts like a hypodermic needle to inject the viral genome through the cell wall and membrane. The phage head and remaining components remain outside the bacteria.

The third stage of infection is biosynthesis of new viral components. After entering the host cell, the virus synthesizes virus-encoded endonucleases to degrade the bacterial chromosome. It then hijacks the host cell to replicate, transcribe, and translate the necessary viral components (capsomeres, sheath, base plates, tail fibers, and viral enzymes) for the assembly of new viruses. Polymerase genes are usually expressed early in the cycle, while capsid and tail proteins are expressed later. During the maturation phase, new virions are created. To liberate free phages, the bacterial cell wall is disrupted by phage proteins such as holin or lysozyme. The final stage is release. Mature viruses burst out of the host cell in a process called lysis and the progeny viruses are liberated into the environment to infect new cells.

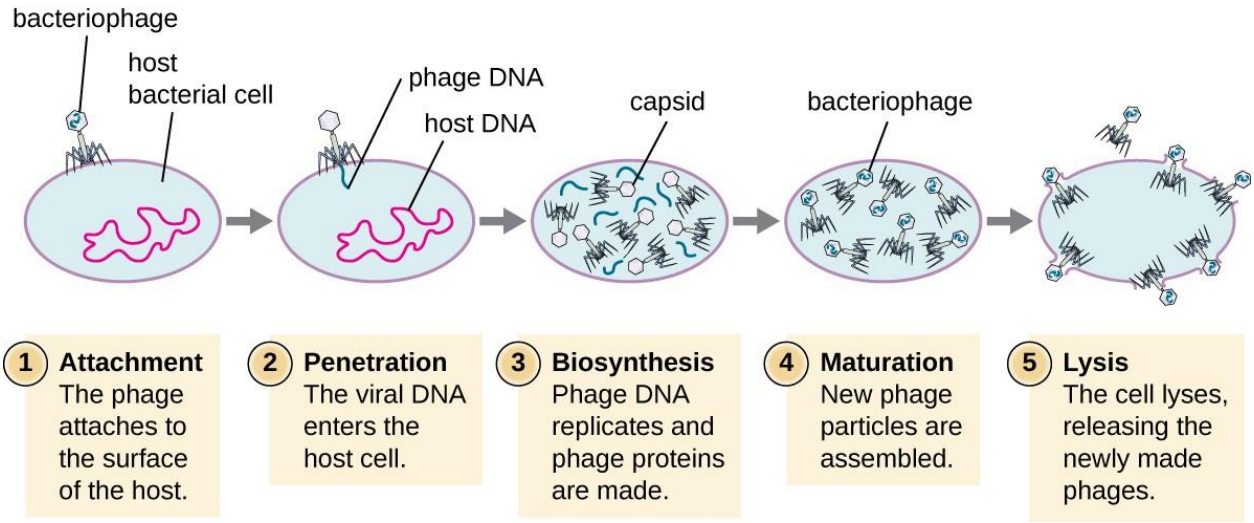


Figure 5. A virulent phage shows only the lytic cycle pictured here. In the lytic cycle, the phage replicates and lyses the host cell.

The Lysogenic Cycle

In a lysogenic cycle, the phage genome also enters the cell through attachment and penetration. A prime example of a phage with this type of life cycle is the lambda phage. During the lysogenic cycle, instead of killing the host, the phage genome integrates into the bacterial chromosome and becomes part of the host. The integrated phage genome is called a prophage. A bacterial host with a prophage is called a lysogen. The process in which a bacterium is infected by a temperate phage is called lysogeny. It is typical of temperate phages to be latent or inactive within the cell. As the bacterium replicates its chromosome, it also replicates the phage's DNA and passes it on to new daughter cells during reproduction. The presence of the phage may alter the phenotype of the bacterium, since it can bring in extra genes (e.g., toxin genes that can increase bacterial virulence). This change in the host phenotype is called lysogenic conversion or phage conversion. Some bacteria, such as Vibrio cholerae and Clostridium botulinum, are less virulent in the absence of the prophage. The phages infecting these bacteria carry the toxin genes in their genome and enhance the virulence of the host when the toxin genes are expressed. In the case of V. cholera, phage encoded toxin can cause severe diarrhea; in C. botulinum, the toxin can cause paralysis. During lysogeny, the prophage will persist in the host chromosome until induction, which results in the excision of the viral genome from the host chromosome. After induction has occurred the temperate phage can proceed through a lytic cycle and then undergo lysogeny in a newly infected cell (see Figure 6).

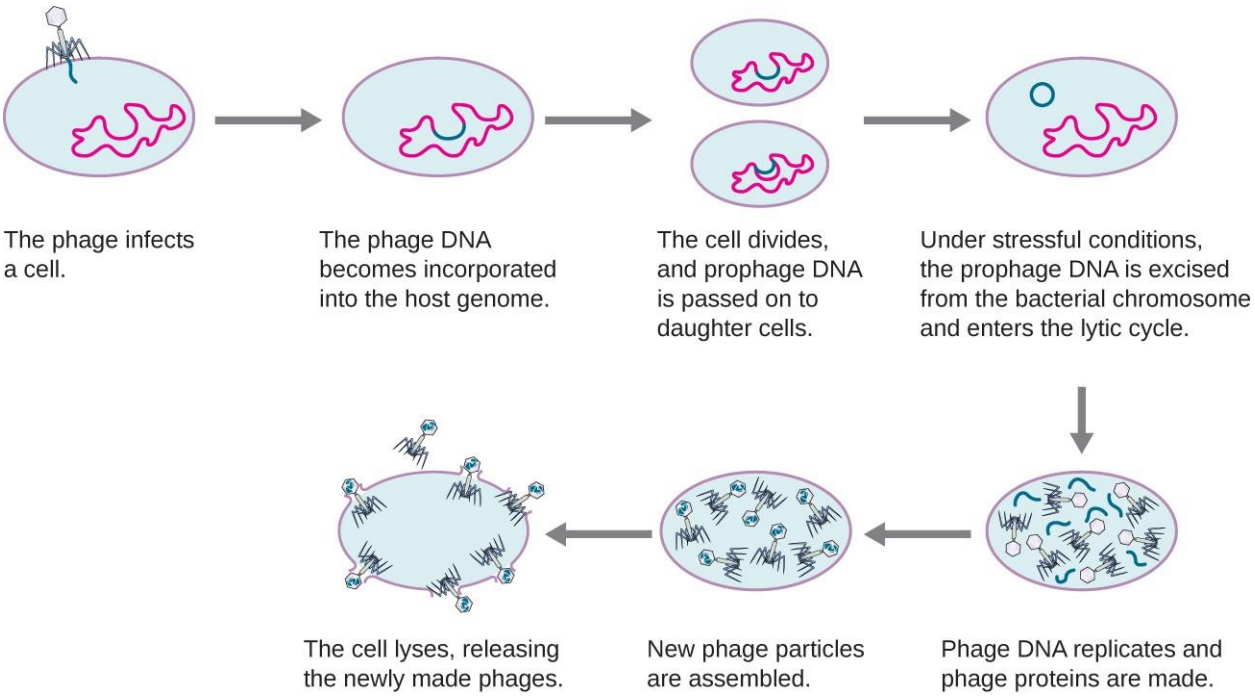


Figure 6. A temperate bacteriophage has both lytic and lysogenic cycles. In the lysogenic cycle, phage DNA is incorporated into the host genome, forming a prophage, which is passed on to subsequent generations of cells. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to be excised and enter the lytic cycle.

Morphology of Phage Lambda:

Morphological structure of phage λ is given in Fig. 18.11A. The head has 20 faces. A 20-faces 3-D picture is called an icosahedron. The head is made of protein of several types and contains a 46,500 bp long genomic (g) DNA. The phage λ contains double stranded circular DNA of about 17 µm in length packed in protein head of capsid. The head is 55 nm in diameter consisting of 300-600 capsomers (subunits) of 37,500 Daltons.

The capsomers are arranged in clusters of 5 and 6 subunits i.e. pentamers and hexamers. The head is joined to a non-contractile 180 µm long tail by a connector. The tail consists of 35 stacked discs. It ends in a fiber. There is a hole in capsid through which passes this narrow neck portion expanding into a knob like structure inside. The tail possesses a thin tail fibre (25 nm long) at its end which recognises the hosts. Also the tail consists of about 35 stacked discs or annuli. Unlike T-even phage, it is a simple structure devoid of the tail sheath. Bacteriophage A falls under the family Siphoviridae of the Group I (dsDNA viruses). Phage lambda is a virus of E. coli K12 which after entering inside host cell normally does not kill it in-spite of being capable of destroying the host.

Therefore, it leads its life cycle in two different ways, one as virulent virus and the second as non-virulent. The virulent phase is called lytic cycle and the non- virulent as temperate or lysogenic one, and the respective viruses as virulent phage and temperate phage, respectively. The other temperate lamboid phages are 21, Ø80, Ø81, 424, 434, etc.

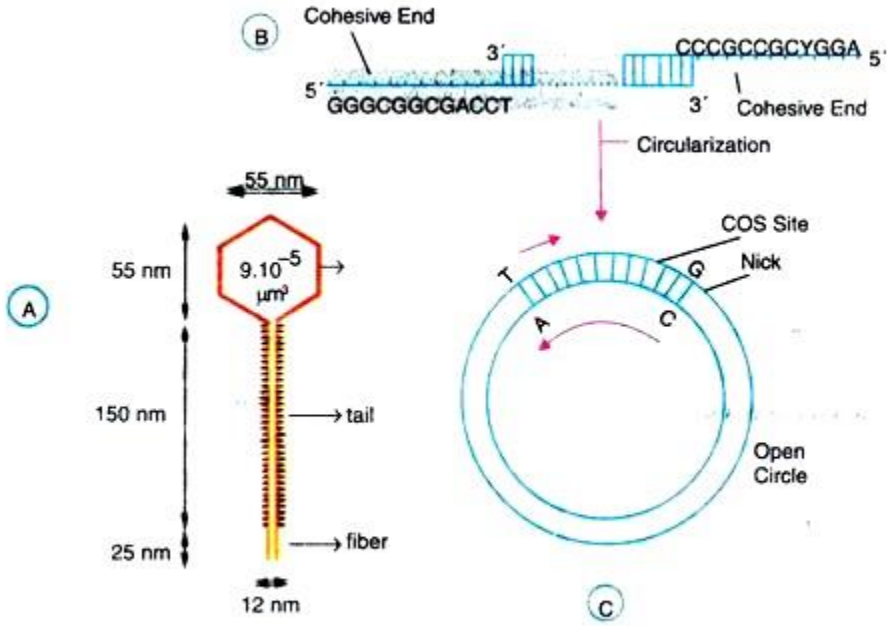


Fig. 18.11 : Structure of bacteriophage lambda (λ). (A), single standard cohesive ends of 12 bases (B), and circularization of complementary DNA to form *cos* site (C).

Fig 7: Structure of bacteriophage

DNA and Gene Organization of Phage Lambda:

Lambda DNA is a linear and double stranded duplex of about 17 μm in length. It consists of 48, 514 base pairs of known sequence. Both the ends of 5′ terminus consists of 12 bases which extend beyond the 3′ terminus nucleotide. This results in single stranded complementary region commonly called cohesive ends. The cohesive ends form base-pairs and can easily circularize. Consequently a circular DNA with two single strand breaks are formed. The double stranded region formed after base pairing of complementary nucleotides is designated as COS. The 12 nucleotides of cohesive ends and process of circularization are shown in Fig. 18.11B-C. The events of circularization occurs after injection of phage DNA into E.coli cell where the bacterial enzyme, i.e., E.coli DNA ligase, converts the molecule to a covalently sealed circle.

Life Cycles of Phage Lambda:

Upon adsorption on the lamb receptor of the host cell, lambda gDNA is injected through the tail which forms a hollow tube through which the DNA passes to the cell. The phage λ leads two life cycles, the lytic cycle and the lysogenic cycle after injecting its DNA into E.coli cell.

In the lytic cycle, phage genes are expressed and DNA is replicated resulting in production of several phage particles. The lytic cycle ends with lysis of E.coli cells and liberation of phage particles. This lytic cycle is a virulent or Sin-temperate where phage multiplies into several particle (Fig. 18.12).

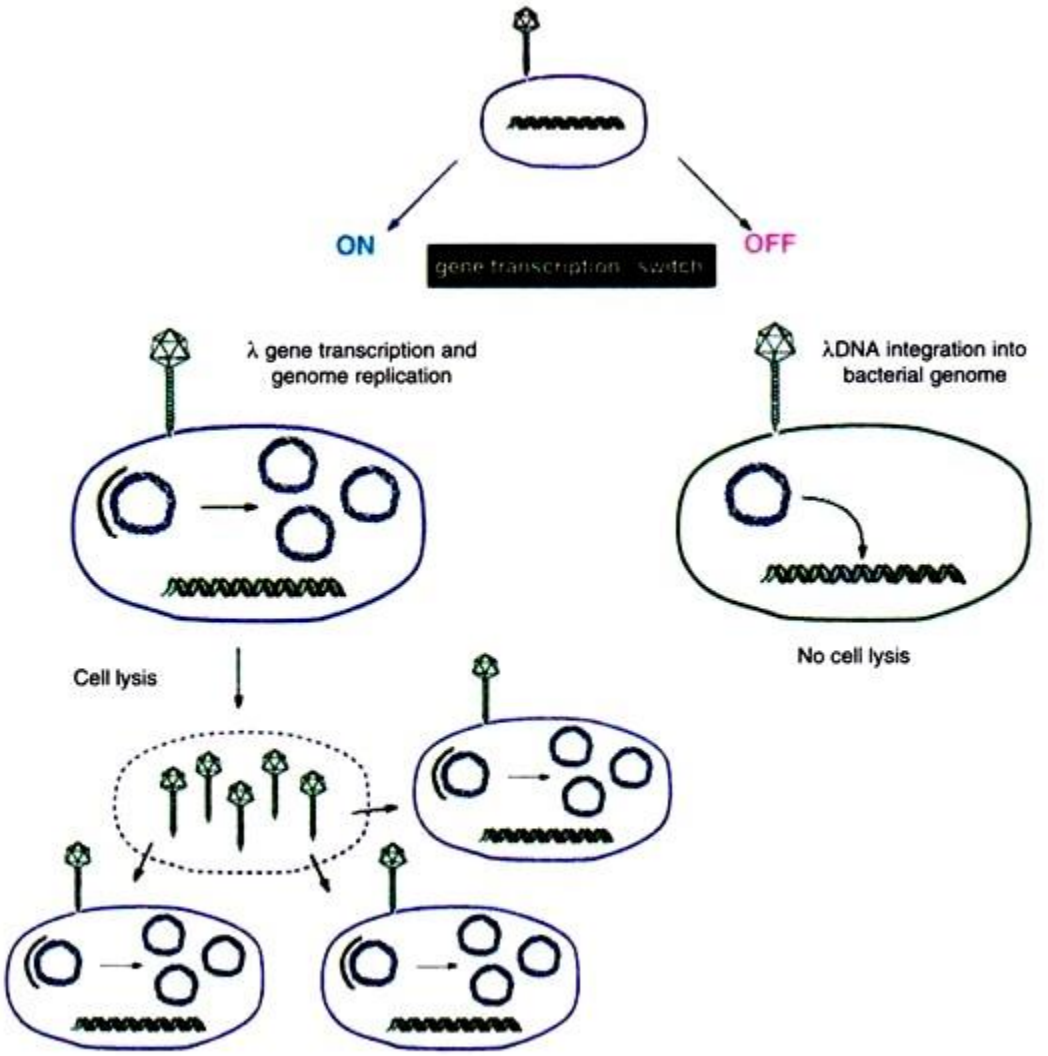


Fig. 18.12 : Multiplication cycle of phage λ; lytic cycle operates when genes are switch ON; lysogenic cycle starts when genes are switched off.

Fig 7: Multiplication of bacteriophage

In addition, the lysogenic cycle results in integration of phage DNA with bacterial chromosome and becomes a part of host DNA. It replicates along with bacterial chromosome and is inherited into progenies. The phage DNA integrated with bacterial chromosome is called prophage.

The prophage is non-virulent and termed as temperate phage. The bacteria containing prophage are called lysogenic bacteria, and the prophage stage of viruses as lysogenic viruses. After treatment of lysogenic bacteria with UV light. X-rays or mitomycin, the prophage can be separated from bacterial chromosomes and enter the lytic cycle. This process is known as induction (Fig. 18.12).

Genetic Map of Phage Lambda:

The genetic map of phage λ is given in Fig 18.13. The remarkable characteristics of the map is the clustering of genes according to their functions. For example the head and tail synthesis, replication and recombination genes are arranged in four distinct clusters. These genes can also be grouped into three major operons viz. right operon, left operon and immunity operon.

The right operon is involved in the vegetative function of the phage e.g. head synthesis, tail synthesis and DNA replication leading lytic cycle. The left operon is associated with integration and recombination events of lysognic cycle.

The immunity operon products interact with DNA and decide whether the phage will initiate lytic cycle or lysogenic cycle. Singer et al (1977) have given the nucleotide sequence of ØX174. Genetic map of bacteriophage has been given by Echols and Murialdo (1978).

Function of some important genes is summarized in Table 18.4 and briefly described below.

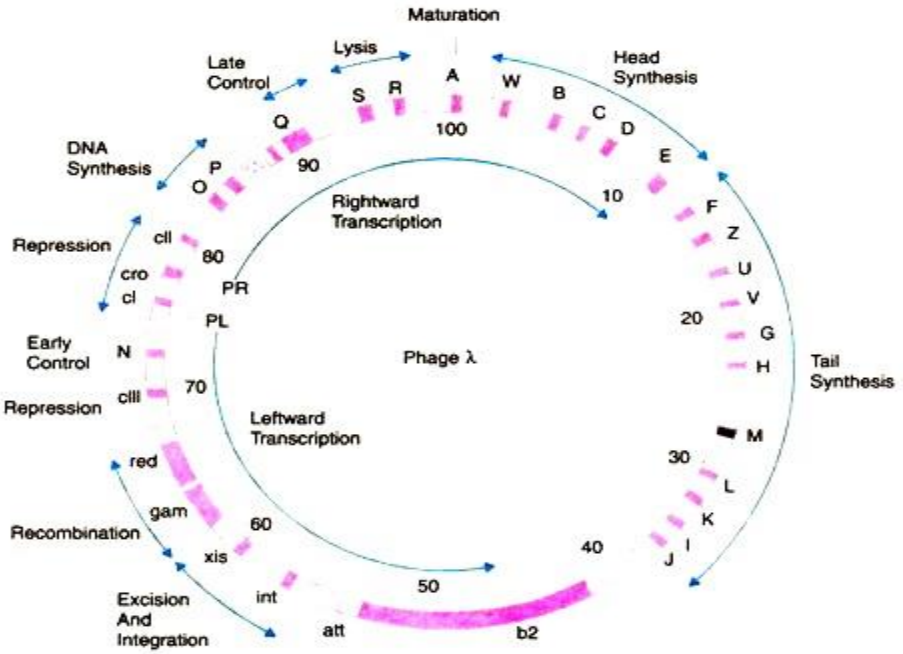


Fig. 18.13: Genetic map of phage λ showing some important genes and their function; PL, leftward promoter; PR, righward promotor.

Fig 9: Genetic map of bacteriophage

(i) Head Synthesis Genes:

At the left end of phage genome the head genes viz. A, W, B, C, D, E are located which are associated with phage DNA maturation and head proteins.

(ii) Tail Synthesis Genes:

The genes F, Z, U, V, G, H, M, L, K, I, J are clustered just right to head genes and code for tail proteins.

(iii) Excision and Integration Genes:

The gene xis codes protein that excises the phage DNA from the bacterial chromosomes, and int coded protein is involved in integration of phage DNA into the bacterial chromosome.

(iv) Recombination:

The two genes int and xis codes at att P for site-specific recombination. The three red genes code for three proteins at normal frequency for general recombination. The redL codes for exonuclease, red B for beta-protein and red V for gamma protein. The gamma protein inhibits exonuclease V.

(v) Positive Regulation Gene:

The genes N and R are the positive regulation genes. The proteins code4by these genes increase the rate of transcription of other genes. Protein coded by N gene induces the transcription of cll, Q, P, A, red, gam, xis and int, whereas the protein coded by Q gene stimulates the transcription of head, tail and lysis genes. The N and Q genes are also required in plaque formation, in the absence of which the number of phage particles would be less but not zero.

(vi) Negative Regulation Genes:

The cl gene acts as a repressor and its product maintains the prophage in the lysogenic form in bacterial host. Moreover, the cll and cIII assist the d gene in lysogeny. The proteins encoded by cro binds to PL and PR and reduce the expression of cl, N, red and xis genes. The interactions between Q proteins encoded by cro and phage repressor occur in host cell and the result decides the operation of lytic or lysogenic cycle. The choice between lysogeny and lysis has been discussed in the preceeding section.

Table 18.4 : Some important phage genes and their function.

	Genes	Functions
1.	*A, W, B, C, D, E*	Code for DNA maturation and phage head proteins
2.	*E, Z, U, V, G, T, M, L, K, I, J*	Synthesis of phage tail protein
3.	*b2*	Deletion for non-essential region of phage DNA
4.	*att*	Site for integration of phage DNA into bacterial chromosome
5.	*int, xis*	Code for integrase and excisionase required for integration and excision of phage DNA into and from bacterial chromosome, respectively
6.	*gam, red*	Codes for gamma proteins that inhibits exonuclease V, kills host
7.	*cIII*	Activates *cro*, codes for *cI* and *int* gene products.
8.	*N*	Product extends transcription from PL and PR, antiterminator for tL or tR
9.	*PL*	Leftward promoter that promotes transcription initiation from N through *int*
10.	*cI*	Codes for immunity repressor
11.	*PR*	Rightward promoter that initiates transcription from *cro* through O region
12.	*cro*	Depresses transcription from PL and PR, directly inhibits repressor synthesis
13.	*cII*	Helps in synthesis of *cI* and *int* gene products.
14.	*O,P*	Control replication of phage DNA
15.	*Q*	Positive regulation for transcription of phage late genes *S* to *J* from PR Promoter.
16.	*S*	Product shuts off synthesis of phage DNA and affects hosts membrane
17.	*R*	Codes for phage endolysin endopeptidase.
18.	*A*	Phage maturation.

(vii) DNA Synthesis Genes:

The two genes O and P are involved in synthesis of phage DNA. The origin of DNA replication lies within the coding sequence for gene Q which encodes a protein for initiation of DNA replication, and the gene that generates the cohesive ends is located adjacent to one of the ends. The function of gene N is required in transcriptional process of these genes.

(viii) Lysis Genes:

The S and R genes control the lysis of bacterial cell envelope which occurs at the end of lytic cycle.

Choice between Lytic and Lysogenic Cycles:

Soon after circularization of genome and start of transcription, the gpcll and gpcIII accumulate. The gpcll binds to PRE (promoter for repressor establishment) and stimulates binding of RNA polymerase (Fig 18.14A). The gpcIII protects gpcll from degradation by host nucleases.

Lambda repressor (gpcl) is rapidly synthesized (B), binds to OL and OR, and inhibits the synthesis of mRNA and production of gpcll and gpcIII (proteins) (C). The repressor activates the promoter for repressor maintenance (PRM) which induces c/gene to be transcribed continuously at a low rate. This process goes on continuously and ensures the stable lysogeny when it is established (C).

During the course of time the gpcro also accumulates. It binds to OL and OR, turns of the transcription repressor gene cl and represses PRM function (D). The repressor (gpcl) can block cro transcription. Therefore, there is a race between the production of gpcl and gpcro proteins.

The detail of this competition is not yet clear but the environmental factors influence the result of this race for choice of the two cycles. If the repressor wins the competitions, the circular DNA is inserted into the E. coli genome. The amount of gpcro and the outcome of competition with gpcl decide the establishment of lysogenic or lytic pathways.

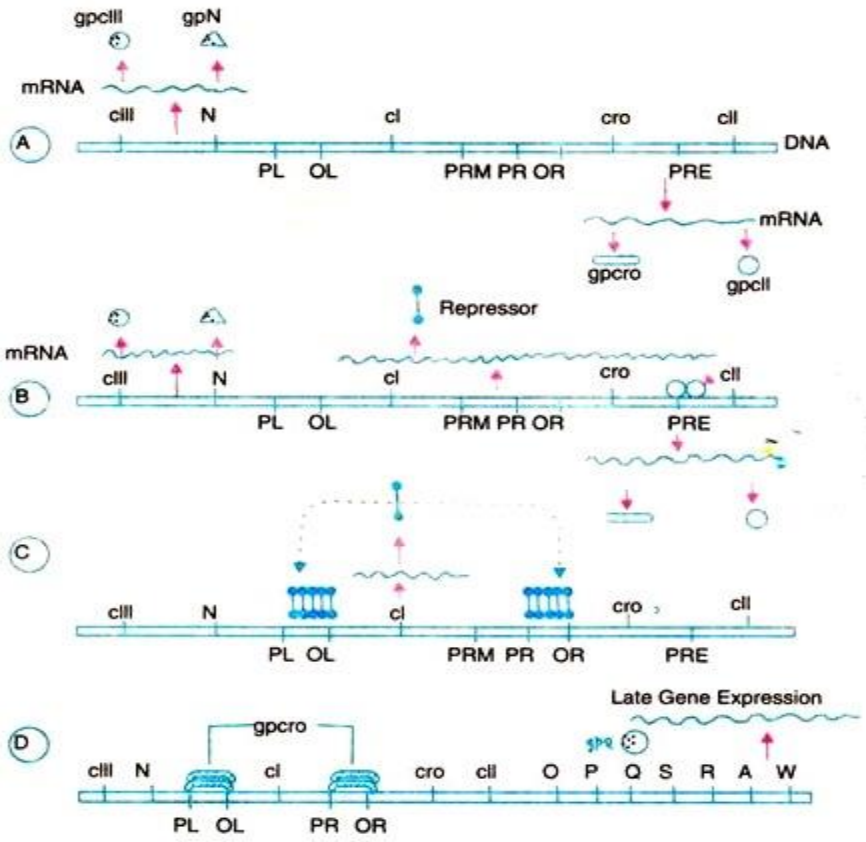


Fig. 10 : Choice between lysis and lysogeny.

2.13. Cloning Vectors: Types & Characteristics

A vector is a DNA molecule which is used for transporting exogenous DNA into the host cell. A vector is capable of self-replication and stable integration inside the host cell. The molecular analysis of DNA has been made possible only after the discovery of vectors. The whole process of molecular cloning involves the following steps:

- Digestion of DNA fragments of the target segment and the vector DNA with the help of restriction enzymes,
- Ligation of the target segment with the vector DNA with the help of DNA ligases, and
- Introduction of the ligated segment into the host cell for propagation.

General characteristics of a vector:

- It should have an Origin of Replication, known as ori, so that the vector is capable of autonomous replication inside the host organism.
- It must possess a compatible restriction site for insertion of DNA molecule.
- A vector should always harbour a selectable marker to screen the recombinant organism. This selectable marker can be an antibiotic resistance gene.
- For easy incorporation into the host machinery, a vector should itself be small in size and be able to integrate large size of the insert.

2.14. CLONING VECTOR

A cloning vector is also a fragment of DNA which is capable of self-replication and stable maintenance inside the host organism. It can be extracted from a virus, plasmid or cells of a higher organism. Most of the cloning vectors are genetically engineered. It is selected based upon the size and the kind of DNA segment to be cloned.

The cloning vectors must possess the following general characteristics:

- It should small in size.
- It must have an origin of replication.
- It must also be compatible with the host organism.
- It must possess a restriction site.
- The introduction of donor fragment must not intervene with the self-replicating property of the cloning vector.
- A selectable marker, possibly an antibiotic resistance gene, must be present to screen the recombinant cells.
- It should be capable of working under the prokaryotic as well as the eukaryotic system.
- Multiple cloning sites should be present.

Importance of Cloning Vectors

Cloning Vectors are used as the vehicle for transporting foreign genetic material into another cell. This foreign segment of DNA is replicated and expressed using the machinery of the host organism.

A cloning vector facilitates amplification of a single copy DNA molecule into many copies. Molecular gene cloning is difficult without the use of the cloning vectors.

History of Cloning Vectors

Herbert Boyer, Keiichi Itakura, and Arthur Riggs were three scientists working in the Boyer's lab, University of California, where they recognized a general cloning vector. This cloning vector had restriction sites for cloning foreign DNA and also, the expression of antibiotic resistance genes for the screening of recombinant/ transformed cells. The first vector used for cloning purposes was pBR322, a plasmid. It was small in size, nearly 4kB, and had two selectable markers.

Features of Cloning Vectors

1. **Origin of Replication (ori)**
    - A specific set/ sequence of nucleotides where replication initiates.
    - For autonomous replication inside the host cell.
    - Foreign DNA attached to ori also begins to replicate.
2. **Cloning Site**
    - Point of entry or analysis for genetic engineering.
    - Vector DNA at this site is digested and foreign DNA is inserted with the aid of restriction enzymes.
    - Recent works have discovered plasmids with multiple cloning sites (MCS) which harbour up to 20 restriction sites.
3. **Selectable Marker**
    - Gene that confers resistance to particular antibiotics or selective agent which, under normal conditions, is fatal for the host organism.
    - Confers the host cell the property to survive and propagate in culture medium containing the particular antibiotics.
4. **Marker or Reporter Gene**
    - Permits the screening of successful clones or recombinant cells.
    - Utilised extensively in blue-white selection.
5. **Inability to Transfer via Conjugation**
    - Vectors must not enable recombinant DNA to escape to the natural population of bacterial cells.

Essential Characteristics of Cloning Vectors

Regardless of the selection of a vector, all 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.

Plasmids

- Plasmids are extra chromosomal circular double stranded DNA replicating elements present in bacterial cells.
- Plasmids show the size ranging from 5.0 kb to 400 kb.
- Plasmids are inserted into bacterial calls by a process called transformation.
- Plasmids can accommodate an insert size of upto 10 kb DNA fragment.
- Generally plasmid vectors carry a marker gene which is mostly a gene for antibiotic resistance; thereby making any cell that contains the plasmid will grow in presence of the selectable corresponding antibiotic supplied in the media.

Phagemids or Phasmid

- They are prepared artificially.
- Phasmid contains the F1 origin of replication from F1 phage.
- They are generally used as a cloning vector in combination with M13 phage.
- It replicates as a plasmid and gets packaged in the form of single-stranded DNA in viral particles.

Advantages of using Phagemids

- They contain multiple cloning sites.
- An inducible lac gene promoter is present.
- Blue-white colony selection is observed.

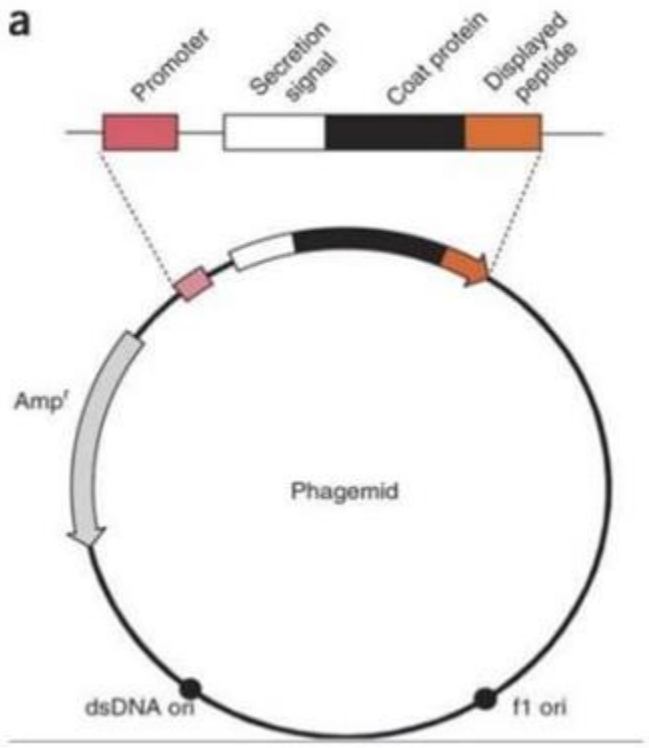


Fig 11: Phagemid

Cosmids

- Cosmids are plasmids.
- They are capable of incorporating the bacteriophage λ DNA segment. This DNA segment contains cohesive terminal sites (cos sites).
- Cos sites are necessary for efficient packaging of DNA into λ phage particles.
- Large DNA fragments of size varying from 25 to 45 kb can be cloned.
- They are also packaged into λ This permits the foreign DNA fragment or genes to be introduced into the host organism by the mechanism of transduction.

Advantages of using cosmids as vectors

- They have high transformation efficiency and are capable of producing a large number of clones from a small quantity of DNA.
- Also, they can carry up to 45 kb of insert compared to 25 kb carried by plasmids and λ.

Disadvantages of using cosmids as vectors:

- Cosmids cannot accept more than 50 kb of the insert.

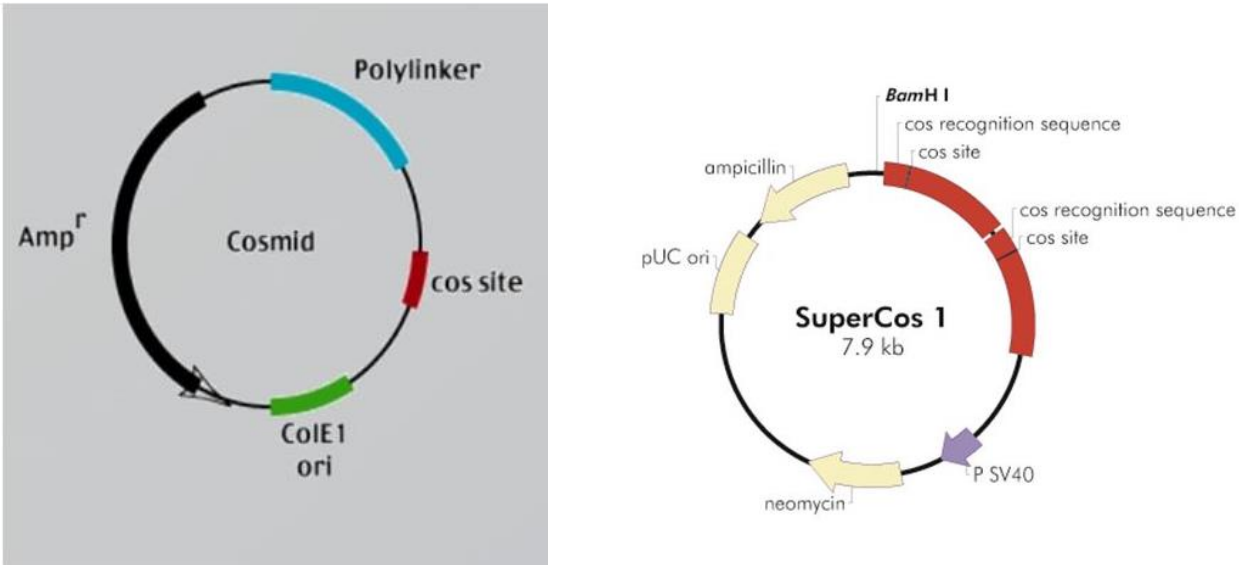


Fig 12: Cosmid Vector

Bacteriophage

- The viruses that infect bacteria are called bacteriophage. These are intracellular obligate parasites that multiply inside bacterial cell by making use of some or all of the host enzymes.

- Bacteriophages have a very high significant mechanism for delivering its genome into bacterial cell. Hence it can be used as a cloning vector to deliver larger DNA segments.
- Most of the bacteriophage genome is non-essential and can be replaced with foreign DNA.
- Using bacteriophage as a vector, a DNA fragment of size up to 20 kb can be transformed.

Bacterial artificial chromosomes (BACs)

- Bacterial artificial chromosomes (BACs) are simple plasmid which is designed to clone very large DNA fragments ranging in size from 75 to 300 kb.
- BACs basically have marker like sights such as antibiotic resistance genes and a very stable origin of replication (ori) that promotes the distribution of plasmid after bacterial cell division and maintaining the plasmid copy number to one or two per cell.
- BACs are basically used in sequencing the genome of organisms in genome projects (example: BACs were used in human genome project).
- Several hundred thousand base pair DNA fragments can be cloned using BACs.

Advantages of BACs:

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

Disadvantages of BACs:

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

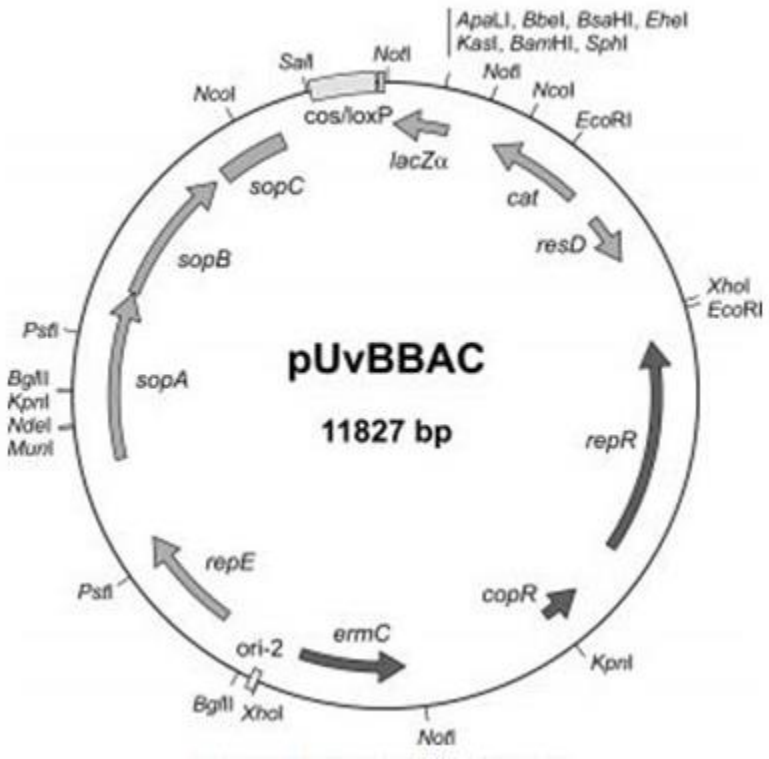


Fig 13; Bacterial artificial vector

Yeast artificial chromosomes (YACs)

- YACs are yeast expression vectors.
- A very large DNA fragments whose sizes ranging from 100 kb to 3000 kb can be cloned using YACs.
- Mostly YACs are used for cloning very large DNA fragments and for the physical mapping of complex genomes.
- YACs have an advantage over BACs in expressing eukaryotic proteins that require post translational modifications.
- But, YACs are known to produce chimeric effects which make them less stable compared to BACs.

Advantages of using YACs:

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

Disadvantages of using YACs:

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

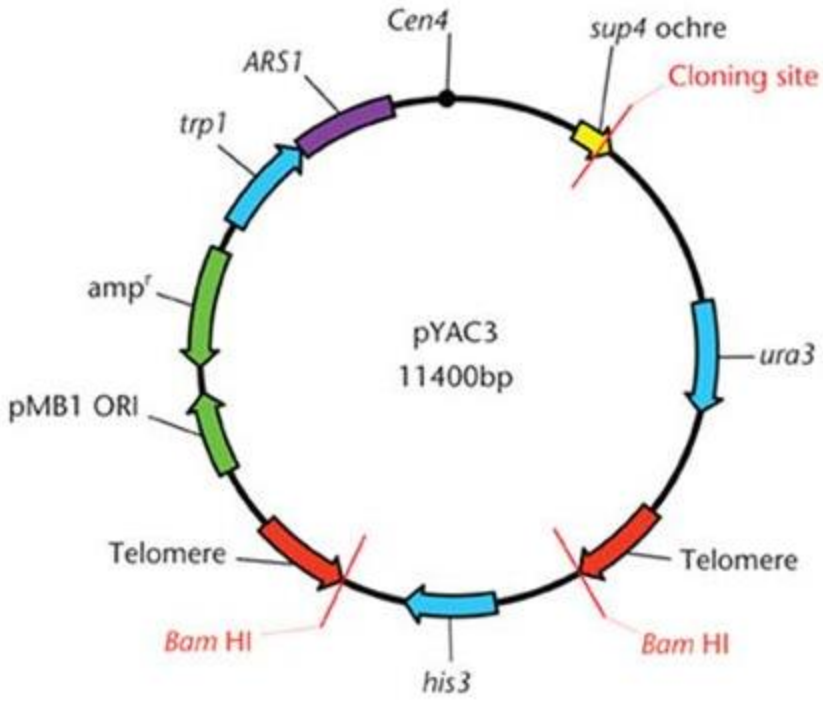


Fig 14: Yeast artificial Chromosome

Advantages of BACs over YACs

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

Human artificial chromosomes (HACs)

- Human artificial chromosomes (HACs) or mammalian artificial chromosomes (MACs) are still under development.
- HACs are microchromosomes that can act as a new chromosome in a population of human cells.
- HACs range in size from 6 to 10 Mb that carry new genes introduced by human researchers.
- HACs can be used as vectors in transfer of new genes, studying their expression and mammalian chromosomal function can also be elucidated using these microchrosomes in mammalian system.

Advantages of using HACs:

- No upper limit on DNA that can be cloned.
- It avoids the possibility of insertional mutagenesis.

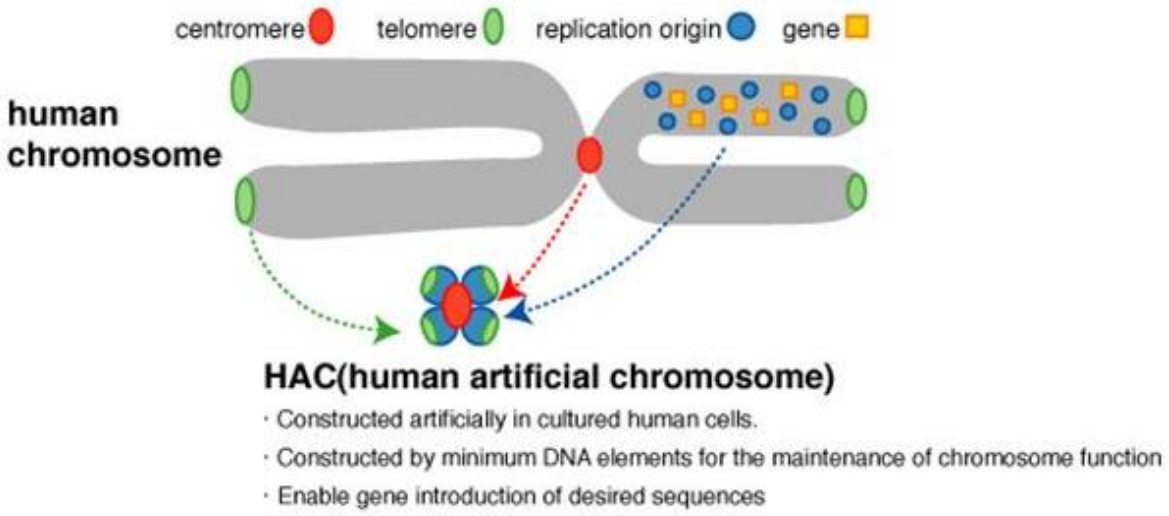


Fig 15: Human Artificial Chromosome

Other Types of Vectors

All vectors may be used for cloning and are therefore cloning vectors, but there are also vectors designed specially for cloning, while others may be designed specifically for other purposes, such as transcription and protein expression.

Expression Vectors

Vectors designed specifically for the expression of the transgene in the target cell are called expression vectors, and generally have a promoter sequence that drives expression of the transgene. Expression vectors produce proteins through the transcription of the vector's insert followed by translation of the mRNA produced.

Transcription Vectors

Simpler vectors called transcription vectors are only capable of being transcribed but not translated: they can be replicated in a target cell but not expressed, unlike expression vectors. Transcription vectors are used to amplify their insert.

Uses of Vectors

Vectors have been developed and adapted for a wide range of uses. Two primary uses are:

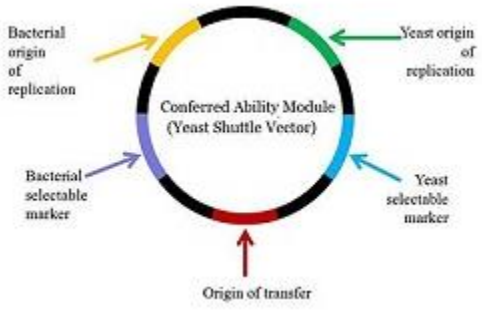
(1) To isolate, identify and archive fragments of a larger genome

(2) To selectively express proteins encoded by specific genes.

Vectors were the first DNA tools used in genetic engineering, and continue to be cornerstones of the technology.

Shuttle vectors:

Shuttle vectors are those which can multiply into two different unrelated species. Shuttle reactors are designed to replicate in the cells of two species, as they contain two origins of replication, one appropriate for each species as well as genes that are required for replication and not supplied by the host cell, i.e., it is self-sufficient with the process of its replication.



The shuttle vectors are of following types:

1. Eukaryotic – Prokaryotic Shuttle Vec-tors:

Vectors that can propagate in eukaryotes and prokaryotes. e.g., YEp vec-tors can be propagated in yeast (fungi) as well as in E. coli (bacteria).

2. Prokaryotic – Prokaryotic Shuttle Vec-tors:

Vectors that can be propagated in two unrelated prokaryotic host cells, e.g., RSF1010 vectors can be propagated both in bacteria as well in spirochetes.

The common features of such shuttle vec-tors or eukaryotic vectors are the following:

(a) They are capable of replicating into two or more types of hosts including prokaryotic and eukaryotic cells.

(b) They replicate autonomously, or integrate into host genome and replicate when the host cell multiplies.

(c) These vectors are commonly used for transporting genes from one organism to another.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – 3 – GENETIC ENGINEERING – SBB2102

INTRODUCTION TO R-DNA TECHNOLOGY

1. General strategies for isolation of genomic and plasmid DNA

1.1.DNA Extraction

History of DNA extraction

The first DNA extraction attempt had performed by Friedrich Miescher in 1869. He had isolated the cell material and named it as the “nuclei” later on his student named it as a “nucleic acid”. Although he accidentally developed a method for isolation of nucleic acid, he was not sure that what he isolated was DNA or not. Later on, in 1958 Meselson and Stahl developed a full-function protocol for DNA extraction. The density gradient centrifugation protocol was the first protocol described by isolating DNA from E.coli bacteria. The protocol of the proteinase K enzyme method of DNA extraction was developed by Lahiri and Nurenberger in 1991. They also modified the protocol by using the Nonidet P40 and SDS. However, the use of proteinase K in DNA extraction was reported earlier by Miller et al., in 1988.

The phenol-chloroform isoamyl alcohol (PCI) method which is most popular in recent days was developed by Joseph Sambrook and David W. Russell. The PCI method becomes so popular that most of the researcher using them in their daily extraction. The yield and consistency of PCI are much decent.

DNA extraction is required for a variety of molecular biology applications. Figure 1 lists the basic steps involved in all DNA extraction methods. Many commercial kits are available to isolate DNA from a variety of biological materials

The basic criteria that any method of DNA isolation from any sample type should meet include:

(1) efficient extraction of DNA from the sample ,

(2) production of a sufficient amount of DNA for use in downstream processes,

(3) successful removal of contaminants,

(4) isolation of high quality and high purity DNA.

There are three basic steps involved in the process of DNA extraction

1) lysis,

2) precipitation,

3) purification.

Step 1 Lysis

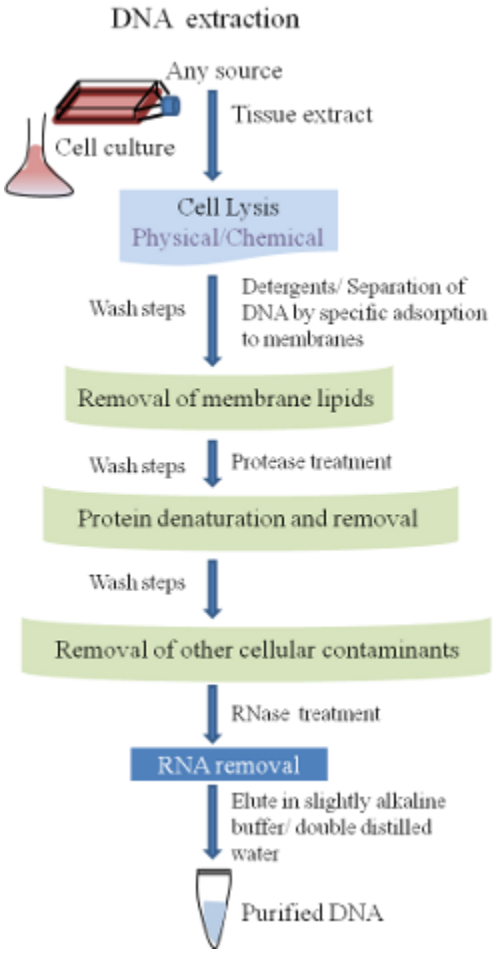
In this step, the cell and the nucleus are broken open to release the DNA inside and there are three ways to do this.

Chemical disruption

Chemicals such as SDS, CTAB, Tris and other detergents can lyse the cell wall/ cell membrane by solubilizing it. Later DNA dissolves along with cellular proteins.

Enzymatic disruption

Enzymes such as proteinase K, peptidase, protease disrupt proteins by digesting it. The enzyme works better than any other chemicals because it directly targets bonds between the amino acids and digests the protein. Some of the bacteria have a very smooth and soft cell wall. For example, M.tuberculosis has a smooth cell wall. By only heating the bacterial solution we can lyse cell wall.



Mechanical disruption

This can be done with a tissue homogenizer (like a small blender), with a mortar and pestle, or by cutting the tissue into small pieces. Mechanical disruption is particularly important when using plant cells because they have a tough cell wall. Plant cells have pectin and other polysaccharides present in their cell wall. This pectin protects the cell from mechanical damage. Therefore pectin provides additional strength to the cell wall of the plant.Some of the fungus, algae and bacteria also have hard cell walls for surviving in harsh conditions. For extracting DNA from this type of cells, we have to modify protocol with a combination of mechanical- chemical- enzymatic method.

Once the cell wall or cell membrane lysed, there are no compartments inside the cell hence all the cell organelles are mixed into the solution. By doing high-speed centrifugation DNA remains in the solution and the other cell debris settled into the bottom of the tube.

Step 2: Precipitation

When you complete the lysis step, the DNA has been freed from the nucleus, but it is now mixed with mashed up cell parts. Precipitation separates DNA from this cellular debris. First, Na+ ions (sodium) neutralize the negative charges on the DNA molecules, which makes them more stable and less water soluble. Next, alcohol (such as ethanol or isopropanol) is added and causes the DNA to precipitate out of the aqueous solution because it is not soluble in alcohol.

Step 3: Purification

Now that DNA has been separated from the aqueous phase, it can be rinsed with alcohol to remove any remaining unwanted material and cellular debris. At this point the purified DNA is usually re-dissolved in water for easy handling and storage.

Types of DNA extraction methods

Different extraction methods result in different yields and purity of DNA. Some of the extraction methods have been systematically evaluated for specific applications such as soil and sediment samples, human microbiome, and fecal samplesDNA extraction methods are broadly categorized into two categories:

1. Chemical-based DNA extraction method.
2. Solid-phase DNA extraction method.

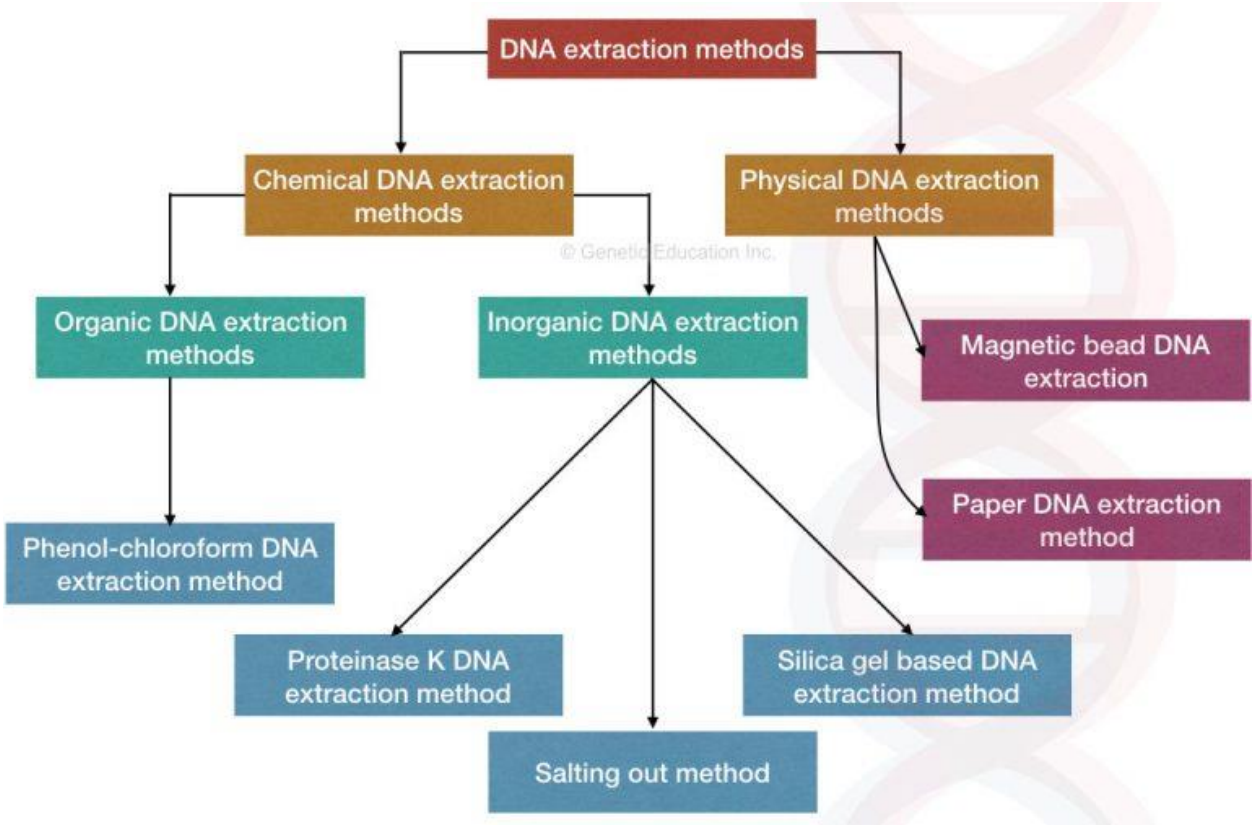


Fig:1 DNA extraction Methods

Different types of organic and inorganic solutions are used in the chemical or solution-based DNA extraction method.

The steps of the DNA extraction remain the same in all the types of DNA extraction methods.SDS, CTAB, phenol, chloroform, isoamyl alcohol, Triton X100, guanidium thiocyanate, Tris and EDTA are several common chemicals used in the solution based DNA extraction method.

Organic Extraction

In this conventional, widely used method, cells are lysed and cell debris is usually removed by centrifugation. Then, proteins are denatured/digested using a protease, and precipitated with organic solvents such as phenol, or 1:1 mixture of phenol and chloroform. The protein precipitate is removed following separation by centrifugation. Purified DNA is usually recovered by precipitation using ethanol or isopropanol. At some point in the process, RNAs are degraded through incubation with RNase. In the presence of monovalent cations such as Na+, and at -20°C, absolute ethanol efficiently precipitates polymeric nucleic acids and leaves behind short-chain and monomeric nucleic acid components, including the ribonucleotides from RNase treatment in solution. This method uses hazardous organic solvents, is relatively time-consuming, and residual phenol or chloroform may affect downstream applications such as PCR. An example of a commercially available kit that relies on this chemistry is the Easy-DNA® Kit from Thermo Fisher.

Phenol-chloroform method of DNA extraction:

This method is one of the best methods of DNA extraction. The yield and quality of DNA obtained by the PCI method is very good if we perform it well. The method is also called as a phenol-chloroform and isoamyl alcohol, PCI method of DNA extraction.

The major chemicals of PCI DNA extraction methods are lysis buffer, Phenol and chloroform.The lysis buffer contains Tris, EDTA, MgCl2, NaCl, SDS, and other salts. Here the components of the lysis buffer help in lysis of cell membrane as well as the nuclear envelope. The protein portion of the cell denatured with the help of chloroform and phenol which are organic in nature.

Silica-based technology

Silica-based technologies are widely employed in current kits. DNA adsorbs specifically to silica membranes/beads/particles in the presence of certain salts and at a defined pH [10]. The cellular contaminants are removed by wash steps. DNA is eluted in a low salt buffer or elution buffer. Chaotropic salts are included in the kit buffers to aid in protein denaturation and extraction of DNA. This method can be incorporated in spin columns and microchips, is cost-effective, has a simpler and faster procedure than the organic extraction, and is suitable for automation. Kits based on this method include Purelink Genomic DNA extraction kit from Thermo Fisher and DNeasy Blood and Tissue Kit from QIAGEN.

Magnetic separation

Magnetic separation is based on DNA reversibly binding to a magnetic solid surface/bead/particles that have been coated with a DNA binding antibody, or a functional group that interacts specifically with DNA. After DNA binding, beads are separated from other contaminating cellular components, washed, and the purified DNA is eluted using ethanol extraction. This method is rapid, simple to perform and can be automated. However, it can be more costly than other methodologies. Examples of commercially available kits include the Agencourt DNAdvance Kit from Beckman Coulter) and Magnetic Beads Genomic DNA Extraction Kit from Geneaid.

Anion exchange technology

DNA extraction by anion exchange chromatography is based on the specific interaction between negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. DNA binds specifically to the substrate in the presence of low salt, contaminants are removed by wash steps using a low or medium salt buffer, and purified DNA is eluted using a high salt buffer. This technology is most commonly employed in plasmid isolation kits such as PureLink® HiPure Plasmid DNA Purification Kits from Thermo Fisher, QIAGEN plasmid mini/midi kits and Genomic-tip, and NucleoBond® PC kits from Macherey Nagel.

Others

Other methods of DNA extraction include salting out, cesium chloride density gradients, and chelex 100 resin. DNA isolation methods are often modified and optimized for different cell types or sample sources. For example, cetyltrimethylammonium bromide (CTAB) and guanidium thiocyanate (GITC) are often included in protocols for DNA extraction from plant materials, and are discussed in more detail in "DNA extraction from plant tissue and cells".

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.

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.

Role of chemicals:

Tris: DNA is pH sensitive, Tris buffer maintains the pH of the solution. Also, it interacts with the lipopolysaccharides of the cell membrane and makes them permeable, this will help in lysis of the cell membrane.

EDTA: EDTA is a chelating agent and can be used to block DNase activity. DNase is an enzyme which lyses the DNA. However, every enzyme required cofactor to work properly. The chelator EDTA blocks the activity of DNase by blocking the cofactor binding site. It will work best in combination with Tris.

SDS: Sodium dodecyl sulphate is an anionic detergent which helps cell membrane and nuclear envelope to break open.The SDS removes the negative charges from the amino acid and disrupts the confirmation of a protein. Therefore, the protein loses its structure and stabilized by using the SDS.

NaCl: the Na+ ion of NaCl creates the ionic bond with the negative charge of DNA and neutralize it. It will help DNA comes together and protect from denaturation.

MgCl2: overall, it protects the DNA. $MgCl_2$ blocks the negative charge of the lipoproteins of the cell membrane. After the lysis of cells, there is no compartment in the cell hence it protects DNA by mixing with other cell organelles.

Phenol: it precipitates the protein impurities. The combination of phenol, chloroform and isoamyl alcohol helps in the removal of protein. DNA is insoluble in phenol because phenol is a nonpolar solution. On the other side, protein has both polar and nonpolar group present in it because of the long chain of different amino acids. Different amino acids have different groups present on their side chain. Also, the folding of the protein into the secondary, tertiary and quaternary structure depends on the polarity of the amino acids. The bonds between amino acids are broken by the addition of phenol and protein get denatured. Ultimately, we can say the protein becomes unfolded by addition of phenol. After centrifugation, the phenol settles in the bottom of the tube and DNA in the aqueous phase while the denatured protein remains between both layers as a whitish cloud.

Lysozyme

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

Chloroform

Chloroform increases the efficiency of phenol for denaturation of the protein. Here, chloroform allows proper separation of the organic phase and aqueous phase which keeps DNA protected into the aqueous phase.

Isoamyl alcohol

In the phenol-chloroform DNA extraction method, Isoamyl alcohol helps in reducing foaming between interphase. It prevents the emulsification of a solution. The liquid phase contains DNA and the organic phase contains lipid, proteins and other impurities. The precipitated protein denatured and coagulated between both these phases. This will create the cloudy, whitish- foam between interphase. The anti-foaming agent, isoamyl alcohol stabilized the interphase by removing the foaming. This will increase the purity of DNA.

1.2.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,

Separation based on size difference

- It involves lysis of cells with lysozyme and EDTA in the presence of sucrose (prevents the immediate bursting of cells).
- 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 X100) or ionic detergents (e.g. SDS) causing chromosomal breakage.
- Bacterial chromosomes attached to cell membranes, upon lysis, get removed with the cell debris.
- A cleared lysate consisting almost entirely of plasmid DNA is formed with very little breakage of the bacterial DNA.

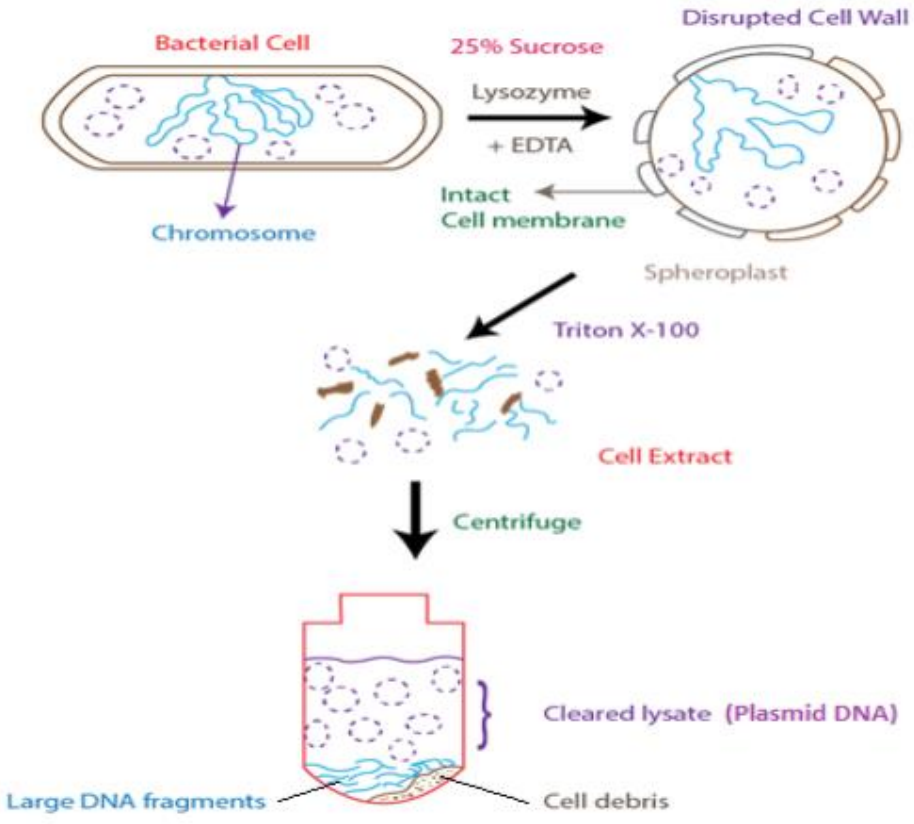


Fig 2: Separation of plasmid DNA on the basis of size.

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). Supercoiling 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 (below figure).
- 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.

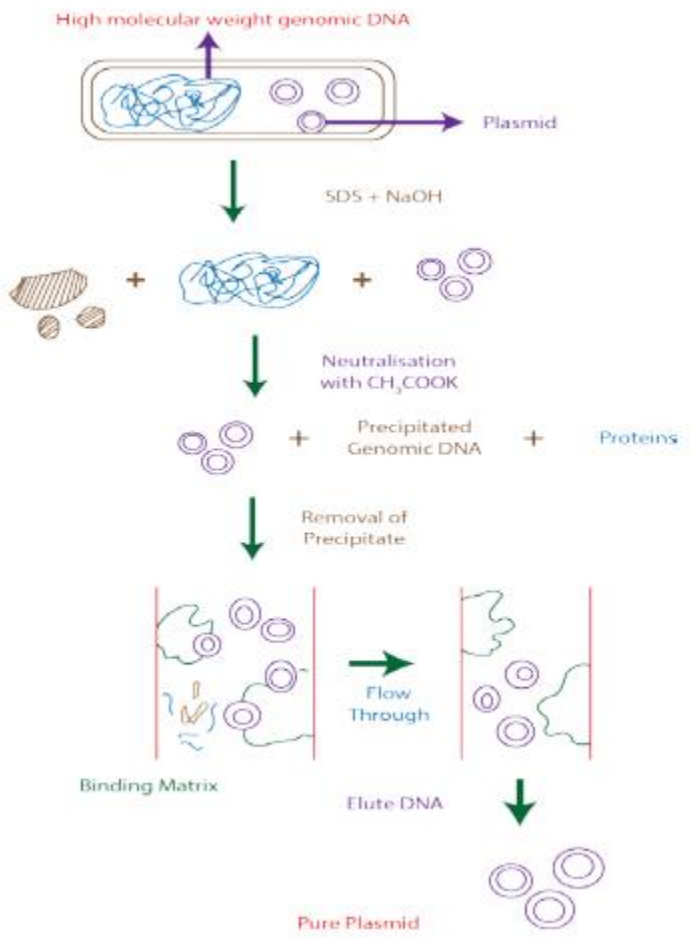


Fig 3: Separation of plasmid DNA by Alkaline denaturation method

(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 CsCli.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.

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/cm3) than that of linear DNA (0.125 g/cm3). 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.

QUANTIFICATION AND STORAGE OF NUCLEIC ACIDS

Quantification of nucleic acids is done to determine the average concentrations of DNA or RNA present in a mixture, as well as their purity. The accurate measurement is based on sensitivity, specificity and interference by contaminants. Various methds that can be employed to quantify the nucleic acid concentration are listed below,

1. Spectrophotometric analysis
2. Nanodrop
3. Fluorescence based method

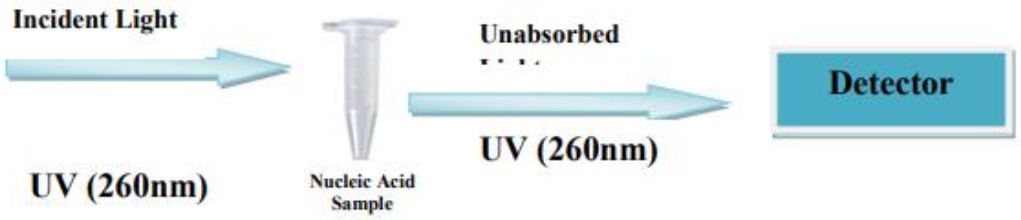
Spectrophotometric analysis

Majority of bio-molecules intrinsically absorb light in the ultraviolet and not in the visible range. This property of UV absorbance can be used to quickly estimate the concentration and purity of DNA and RNA (also proteins) in a analytical sample. The amount of DNA in a sample can be estimated by looking at its absorbance at a wavelength of 260nm or 280nm (in the UV region). Purines and pyrimidines have absorbance maxima slightly below and above 260 respectively. Thus the absorbance maxima of different fragments of DNA vary somewhat depending on their subunit.composition. Contaminants like proteins exhibit two absorbance peaks, one between 215-230 nm (due to peptide bonds absorption) and at about 280 nm (absorption by aromatic amino acids-tyrosine, tryptophan and phenylalanine). Remember that although proteins have little absorbance at 260 nm, both proteins and nucleic acids absorb light at 280 nm. That is the reason why, if nucleic acids and proteins are mixed in the same sample, their spectra interfere (overlap) with one another.

The relationship between concentration of DNA, RNA, Protein and absorptivity are as below:

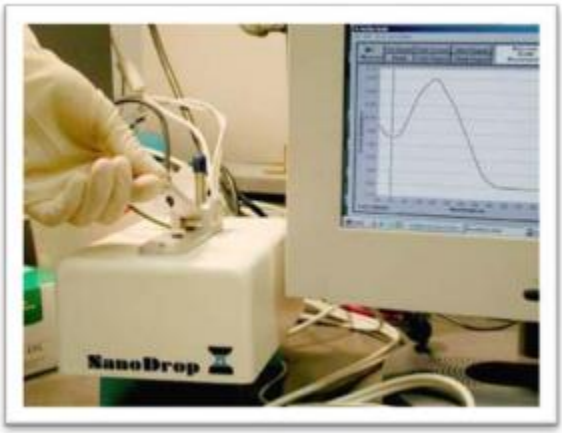
Sample	Absorbance value	*Quantity (*approximate*)*
Double-stranded DNA	1at **260 nm**	**50 µg/mL**
Pure single-stranded DNA	1 at **260 nm**	**33 µg/mL**
Pure RNA	1 at **260 nm**	**40 µg/mL**
Pure protein (vary in general) Antibodies[1]	**1 at 280 nm** **1.2-1.35**	**1 mg/mL** 1 mg/mL

The purity of a solution of nucleic acid is determined by measuring the absorbance of the solution at two wavelengths, usually 260 nm and 280 nm, and calculating the ratio of A_{260}/A_{280}. Value of this ratio is 2.0, 1.8and 0.6 for pure RNA, DNA and protein respectively. A ratio of less than 1.8 signifies that the sample is contaminated with protein or phenol and the preparation is not proper.



Nanodrop:

Detection assays are persistently being developed that use progressively smaller amounts of nucleic acid, often precluding the use of conventional cuvette-based instruments for nucleic acid quantitation for those that can perform micro-volume quantitation. The patented NanoDrop microvolume sample retention system functions by combining fiber optic technology and natural surface tension properties to capture and retain small amounts of sample . This is a novel technology which allows us to measure nano-liter volumes (pico concentration) of the nucleic acid (DNA or RNA) sample. It is a type of spectrophotometer with a smaller sample size (as much less as 1-2 microlitre) requirement and higher sensitivity (even upto pico molar level). This is also a time saving technology widely used in basic molecular biology research.



Fluorometric Quantification:

Fluorometric method applies fluorescence dyes to detect the presence and concentration of a class of nucleic acid (DNA or RNA). This method is more sensitive and less prone to contaminants than UV spectroscopy. An assay using Hoechst 33258 dye is specific for DNA because it is less sensitive to detect RNA. This assay is commonly used for rapid measurement of low quantities of DNA, with a detection limit of ~1 ng DNA. It is useful for the measurement of both small and large amounts of DNA (verifying DNA concentrations prior to performing electrophoretic separations and Southern blots) because this assay accurately quantifies a broad range of DNA concentrations from10 ng/ml to15 µg/ml. The Hoechst 33258 assay can also be employed for measuring products of the polymerase chain reaction (PCR) synthesis.

Hoechst 33258 is non-intercalating reagent and binds to the minor groove of the DNA with a preference for AT sequences (Portugal and Waring, 1988). The binding to the minor groove has is dependent upon a combination of structural preferences (eg., the minor groove with a series of contiguous AT base pairs is more narrow).(Neidle (2001) ,Like other minor groove binding ligands, Hoechst 33258 is positively charged and thus form electrostatic interaction with the negative potential of stretch of AT base pairs. Upon binding to the minor groove of the double helix DNA, the fluorescence characteristics of Hoechst 33258 change dramatically, showing a large increase in emission at ~458 nm.

According to Daxhelet et al, the fluorochrome 4',6-diamidino-2-phenylindole (DAPI) has similar characteristics to H33258 and binds to the minor groove as well. DAPI is also appropriate for DNA or RNA quantitation, although it is not as commonly used as Hoechst 33258. DAPI is excited with a peak at 344 nm. Emission is detected at around 466 nm for DNA, similar to Hoechst 33258 but for RNA the peak shifts to ~500 nm.

Ethidium Bromide Staining:

The IUPAC name for EtBr is 2, 7-diamino-10-ethyl-9-phenylphenanthridiniumbromide. It is commonly used as a fluorescent dye for nucleic acid staining. It binds as well as intercalates with nucleic acid (mainly with major and minor groove of DNA) and gives orange fluorescence under UV radiation from 500 – 590 nm. Usually EtBr may be added in warm agarose gel before solidification. When DNA or RNA samples are run in agarose gel electrophoresis EtBr molecules will bind with nucleic acids and help in detection under UV light. The post staining can also be done for nucleic acid detection.Ethidium bromide (EtBr) is a potent mutagen and carcinogen. Dyes to stain nucleic acids such as SYBR green, SYBR Safe etc are safer to use instead of EtBr.

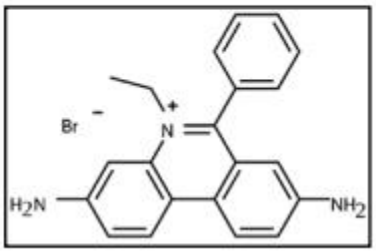


Fig4 Ethidium bromide

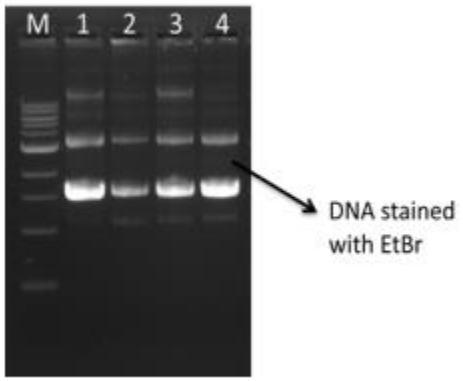


Fig 5 Agarose gel stained with EtBr

Silver Staining:

Silver staining based on reduction of silver nitrate is more sensitive than ethidium bromide for double stranded DNA, as well as detection of single stranded DNA or RNA with a good sensitivity (in picogram level). It is based on the reduction of silver cations to insoluble silver metal by nucleic acids. This chemical reaction is insensitive to the macrostructure of the DNA molecule. Reduced silver molecules deposit in the gel around the DNA bands, creating a dark black band like image (i.e. “latent image”). Then the latent image can be developed to visualize by soaking the gel in a solution of silver cations (Silver nitrate) and a reducing agent (eg. formaldehyde). The silver granules in the latent image catalyze the further reduction and deposition of silver from the solution. Bands manifest as dark brown or black regions which appear before significant background develops. Development is stopped by altering the pH of the gel to a point where silver reduction is no longer favored.

Fig 6: Representation of a silver staining of DNA Lane 1: DNA of lesser concentration Lane 2: DNA of higher concentration Lane 3: Low molecular weight ladder

Storage of Nucleic Acids

The purified DNA can be stored at -20°C or -70°C under slightly basic conditions (e.g., Tris- Cl, pH 8.0) as acidic conditions result in hydrolysis of DNA. Diluted solutions of nucleic acids can be stored in aliquots and thawed once only. RNA preservation under frozen conditions is helpful. Purified RNA can be stored at -20°C or -80°C in RNase-free solution such as-

- The RNA Storage Solution (1 mM sodium citrate, pH 6.4 ± 0.2): It is a buffer that delivers greater RNA stability than 0.1 mM EDTA or TE. The presence of sodium citrate and low pH minimizes base hydrolysis of RNA. Sodium citrate acts both as a chelating and buffering agent.
- 0.1 mM EDTA
- TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0)

This RNase-free solution is compatible with all RNA applications including in vitro translation, reverse transcription, nuclease protection assays and northern analysis.

3.2. Strategies for isolation of gene of interest (restriction digestion, PCR)

Gene Cloning: Gene cloning is a process by which large quantities of a specific, desired gene or section of DNA may be cloned or copied once the desired DNA has been isolated.

Method of Gene Cloning:

1. The gene or DNA that is desired is isolated using restriction enzymes.

2. Both the desired gene and a plasmid are treated with the same restriction enzyme to produce identical sticky ends.

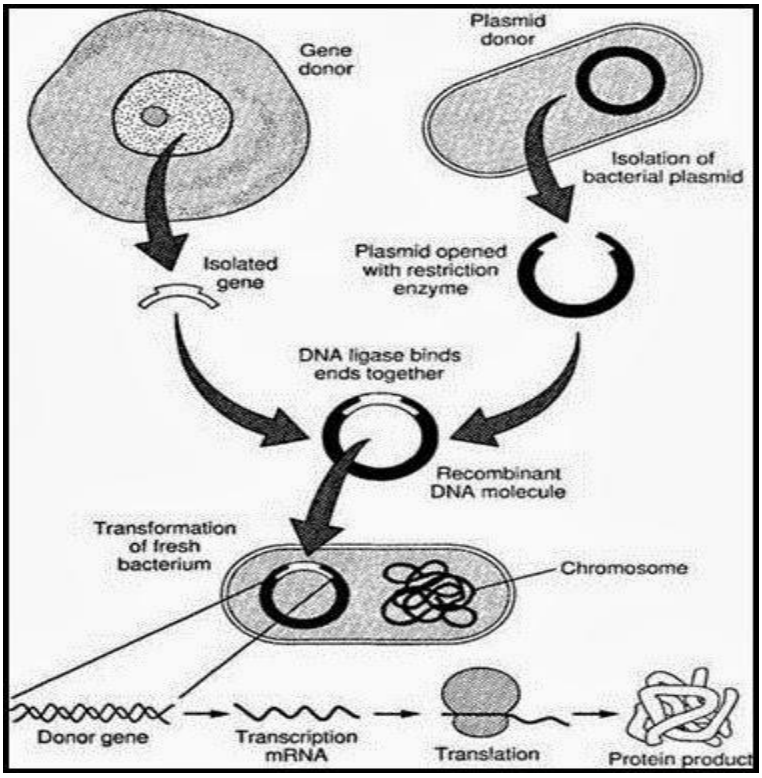
3. The DNAs from both sources are mixed together and treated with the enzyme DNA ligase to splice them together.

4. Recombinant DNA, with the plasmid containing the added DNA or gene has been formed.

5. The recombinant plasmids are added to a culture of bacterial cells. Under the right conditions, some of the bacteria will take in the plasmid from the solution during a process known as transformation.

6. As the bacterial cells reproduces (by mitosis), the recombinant plasmid is copied. Soon, there will be millions of bacteria containing the recombinant plasmid with its introduced gene.

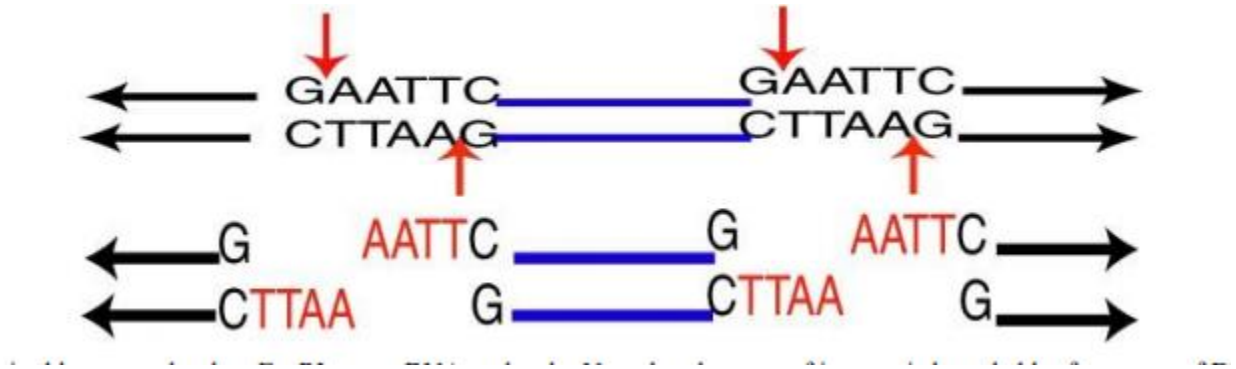
7. The introduced gene can begin producing its protein via transcription and translation.



Preparation of r-DNA

STEP 1: ISOLATION OF GENE OF INTEREST:

In order to clone a gene the first step is to isolate it using restriction enzymes. These enzymes recognize specific regions on the DNA molecule. The region of DNA shown below is from Rhodobacter sphaeroides. The gene of interest lies in the region of the chromosome indicated in blue. The base sequences are the ones that the restriction enzyme EcoRI recognizes. Note that reading from left to right in the top strand is the same as reading from right to left in the bottom strand. Use EcoRI to cut the sugar-phosphate backbone at the points indicated by the red arrows.

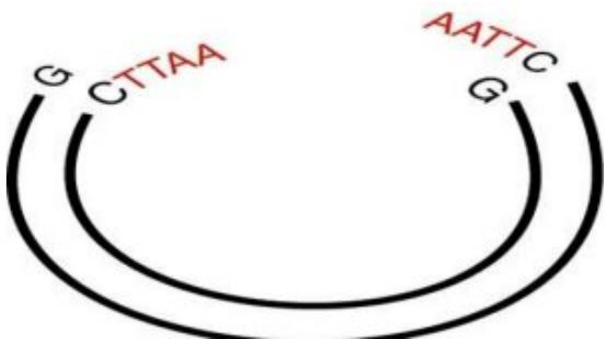


Unpaired bases result when EcoRI cuts a DNA molecule. Note that the gene of interest is bounded by fragments of DNA containing unpaired bases or "sticky ends". If the temperature is lowered and DNA ligase is added these unpaired bases can reanneal following the rules of base pairing.

Cut Molecule

Reannealed Molecule

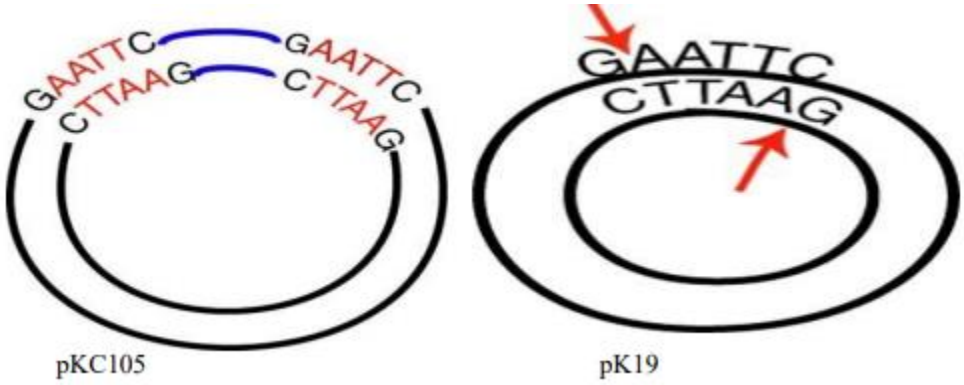
Compare the two molecules. Note the base pairing



When pK19 is cut by EcoRI it has "sticky ends" that are complementary to those made by cutting R. sphaeroides. Like R. sphaeroides the "sticky ends" can reanneal if DNA ligase is added. This would return the plasmid to it's original ring structure

STEP 2. TRANSFERRING GENE OF INTEREST INTO VECTORS

Cooled, added DNA ligase and the molecules can reanneal. Resulting in a variety of recombinant forms. One of interest is the plasmid containing the R. sphaeroides DNA.



Ligation strategies

In rDNA technology, sealing discontinuities in the sugar-phosphate chains, otherwise called as ligation, is vital step. This process is catalyzed by DNA ligase by repairing broken phophodiester bonds. During ligation, the enzyme's activity is influenced by factors such as 1) substrate specificity, 2) temperature and 3) salt concentration.

Ligation methods

Joining DNA fragments with cohesive ends by DNA ligase is a relatively efficient process, which has been extensively used to create artificial recombinants. If the termini of DNA fragments are not compatible, there are other methods to ligate the fragments.

Cohesive end ligation

The cohesive end ligation is possible when both the foreign DNA to be cloned and the vector DNA possess the same molecular ends. The compatible sticky ends have been generated by cleavage with the same enzyme on the same recognition sequences of both foreign DNA and vector DNA. Using DNA ligase, these molecules can be ligated without any problem. Very often it is necessary to ligate DNA fragments with different and non-compatible ends, or blunt ends with either staggered 3' or 5' ends. Incompatible DNA fragments with recessed ends can be ligated by modifying their ends by any one of the following methods viz., (i) filling in recessed 3' termini and (ii) renewal of 5' protruding termini.

Blunt end ligation

The E. coli DNA ligase will not catalyze blunt end ligation except under special reaction conditions of macromolecular crowding. The unique property of T4 DNA ligase was used to ligate DNA fragments with blunt ends involving short decameric oligonucleotides called linkers.

When same sticky end creating enzyme used for cleavage of vector and gene of interest, then DNA ligase seals the nick between gene of interest and vector and creates recombinant vector. Whereas when blunt end creating enzyme used then recoiling become difficult. Moreover, in both cases of using sticky end and blunt end enzymes, self coiling of vector also occur in high rate rather than the recombination of vector and genes. These situations are overcome by using

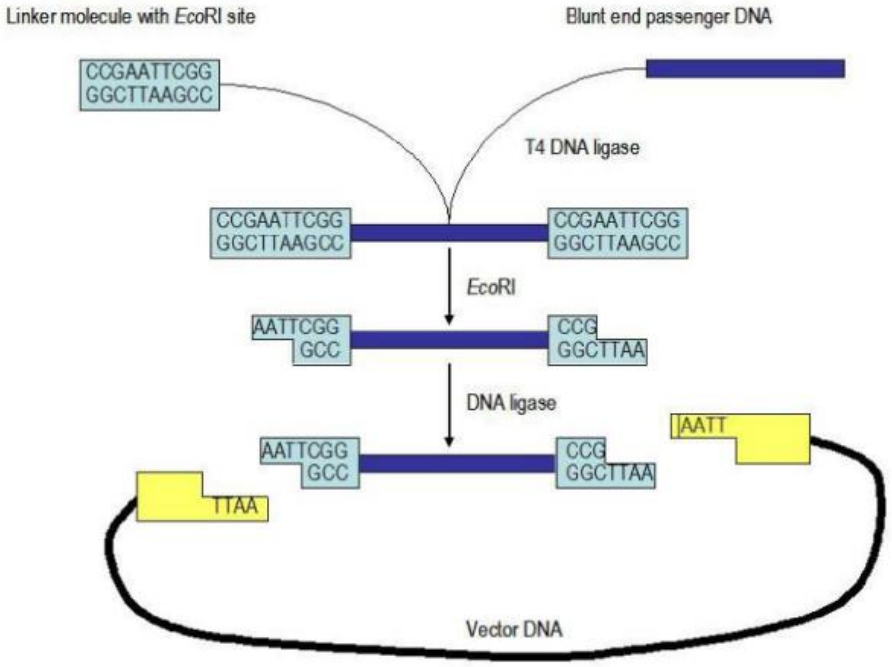
i) Linkers

ii) Adaptors

iii) Homopolymer tailing

linkers

Short oligonucleotides (decamers) which contain sites for one or more restriction enzymes are used to facilitate the ligation process among the DNA fragments with blunt ends.



Joining of blunt end DNA to a vector using linkers

The linker molecules can be ligated to both ends of the foreign DNA to be cloned and then treated with restriction endonuclease to produce sticky end fragments which can be incorporated into a vector molecule that has been cut with the same restriction endonuclease. Insertion by means of the linker creates restriction sites at each end of the foreign DNA, and thus enables the foreign DNA excised and recovered after cloning and amplification in the host bacterium.

Adaptors

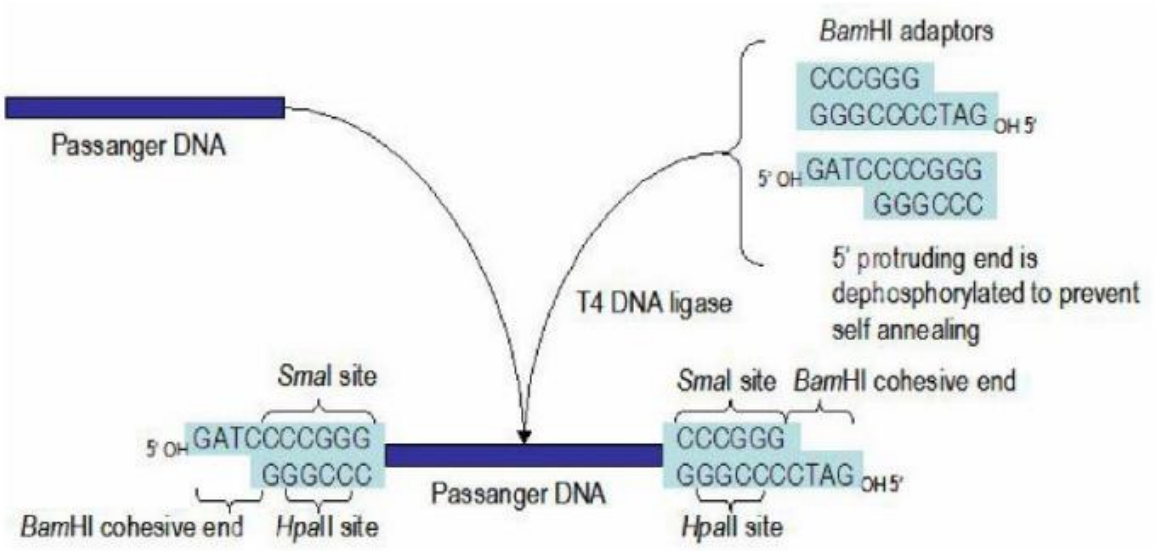
When linkers added to link at the end of blunt end of gene interest, then there is an possibility of joining of multiple linkers at the end. This makes some time larger genes and waste

of linker molecules. This problem is overcome by using adapters. Since adapters contain only one end suitable for joining this prevents multiple coiling of adapters. Adapter is a synthetic, double stranded oligonucleotide used to attach sticky ends to a blunt ended molecule. It contain normal 5' and 3' end at blunt end and the sticky end of adapter molecule is modified in such manner that it contain OH group on both 5' and 3' ends. This is achieved by using alkaline phosphatases. In contrast to linkers, adapters contain preformed sticky ends and joining blunt ends. Because of lack of 5' phosphate group on sticky end prevents adapter polymer formation. After the adaptors have been attached the abnormal 5'OH terminus is converted to the natural 5'P form by treatment with the enzyme polynucleotide kinase, producing sticky ended fragment that can be inserted into an appropriate vector.

The other strategy adopted for ligating DNA fragments with blunt ends is using adaptors. The adaptor molecules are synthetic deoxynucleotides that can be used to join two incompatible cohesive ends, two blunt ends or a combination of both. Such adaptors are of several types viz., preformed, conversion and single stranded adaptors.

Preformed adaptors

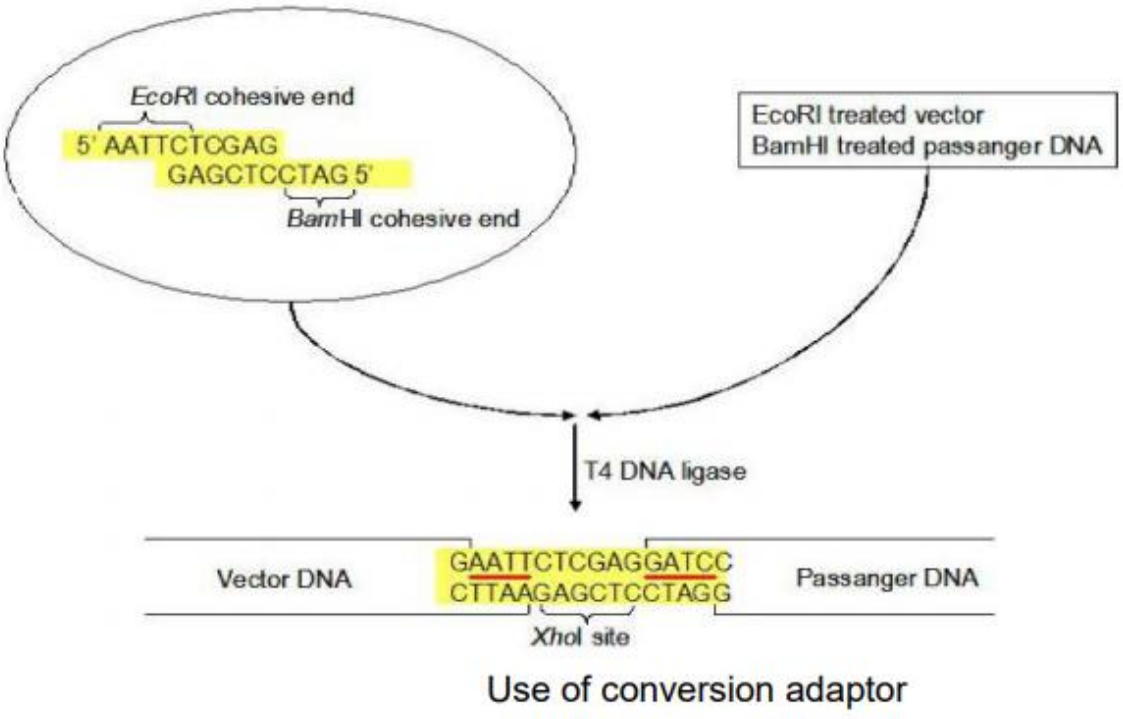
Preformed adaptors are short DNA duplexes with at least one cohesive end. The problem of internal cleavage of the insert DNA can be overcome by using a preformed adaptor that will introduce a new restriction site. For example, an adaptor having BamHI cohesive ends and sites HpaII and SmaI can be attached to passenger DNA and inserted into a BamHI in vector. After cloning, passenger DNA can be excised from the hybrid by using any one of the enzymes that recognize the restriction sites within the adaptor region.



Use of preformed adaptors

Conversion adaptors

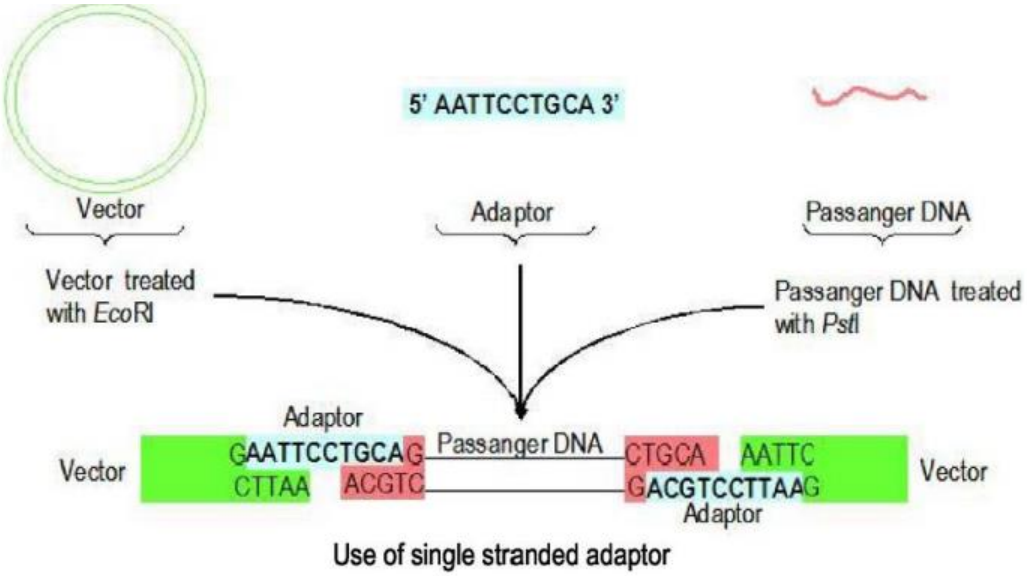
Conversion adaptors are synthetic oligonucleotides bearing different cohesive restriction termini. Such adaptors enable vector molecules that have been cleaved with one endonuclease to be joined to passenger fragments that have been cleaved with another. Often these adaptors contain internal restriction sites that permit recovery of the passenger fragment, for example, the EcoRI-BamHI adaptor contains a site for XhoI.



Use of conversion adaptor

Single stranded

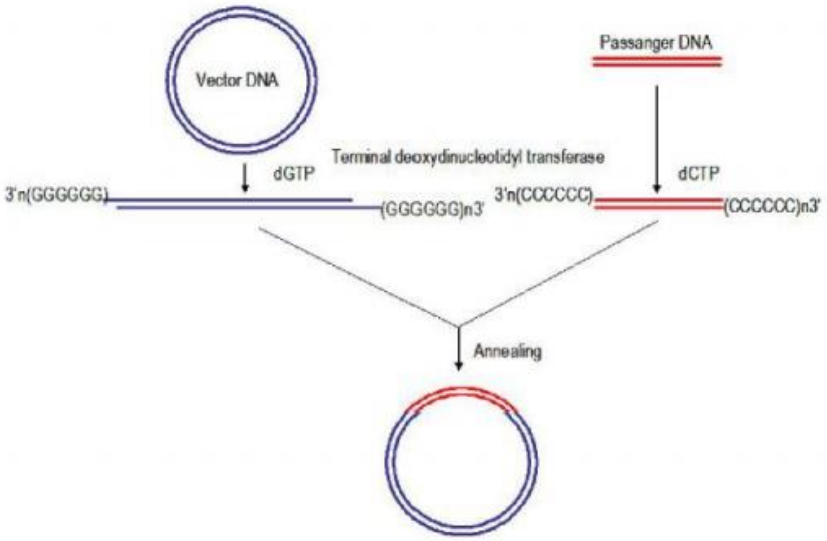
adaptors Single stranded adaptors can be used to make 3'-protruding cohesive ends compatible with 5' protruding ends. Such adaptors permit the insertion of passenger fragments into sites on vectors from which they would otherwise be precluded because of incompatible cohesive ends.



Use of single stranded adaptor

Homopolymer tailing

Homopolymer tailing is the other method adopted to clone blunt DNA molecules, especially cDNA molecules.



Homopolymer tailing

The addition of several nucleotides of single type to the 3' blunt end of DNA molecule is catalyzed by the enzyme terminal deoxynucleotidyl transferase. The terminal transferase permits the addition of complementary homopolymer tails (50 to 150 dA or dT long and about 20 dG or dC long) to 3' end of plasmid vector and passenger DNA. These tails can reanneal to form open circular hybrid molecules, which can be ligated in vitro or more commonly in vivo following transformations to produce functional recombinant molecules.

Cloning a Gene (Polymerase Chain Reaction): Clone:

Making exact genetic copies of whole organisms, cells or pieces of DNA are called clones. A clone is a copy of a plant, animal or micro-organism derived from a single common ancestor cell or organism. Clones are genetically identical. A gene is said to be cloned when its sequence is multiplied many times in a common laboratory procedure called polymerase chain reaction (PCR). PCR copies the cell's natural ability to replicate its DNA and can generate billions of copies within a couple of hours.

There are four main stages:

1. The DNA to be copied is heated, which causes the paired strands to separate. The resulting single strands are now accessible to primers (short lengths of DNA).

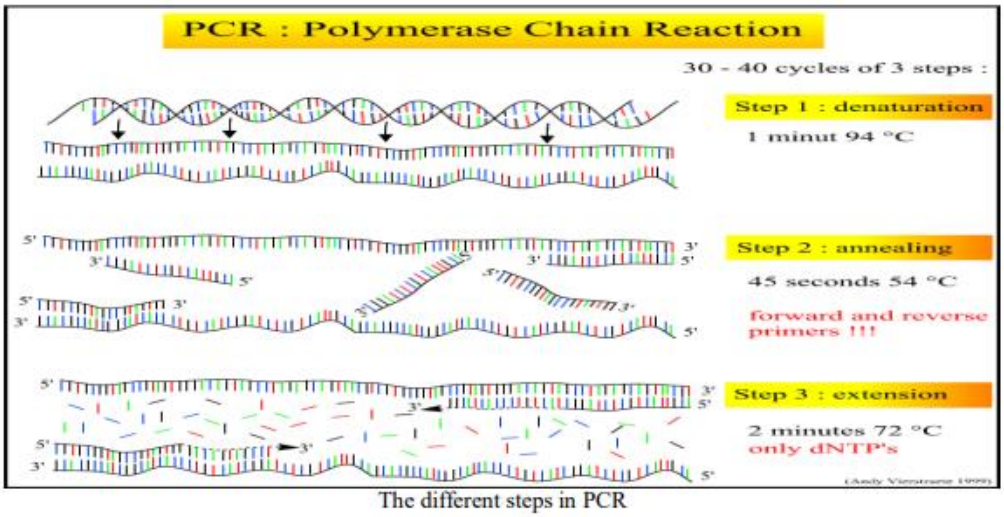
2. Large amounts of primers were added to the single strands of DNA. The primers bind to matching sequences along the DNA sequence, in front of the gene that is to be copied. The reaction mixture is then cooled which allows double-stranded DNA to form again. Because of the large amounts of primers, the two strands will always bind to primers, instead of to each other.

3. DNA polymerase was added to the mixture. This is an enzyme that makes DNA strands. It can synthesise strands from all the DNA primer combinations and dramatically increases the amount of DNA present. One enzyme used in PCR is called Taq polymerase which originally came from a bacterium that lives in hot springs. It can withstand the high temperature necessary for DNA strand separation and therefore, can be left in the reaction and still functions.

4. The above steps were repeated until enough DNA is obtained. This whole process is automated and happens very quickly. The reaction occurs in a small tube which is placed inside a specialised machine which can make the big temperature adjustments quickly.

Principle of the PCR: The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing.

The cycling reactions: There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler7, 8, which can heat and cool the tubes with the reaction mixture in a very short time. Denaturation at 94°C, Annealing at 54°C, Extension at 72°C.



The different steps in PCR

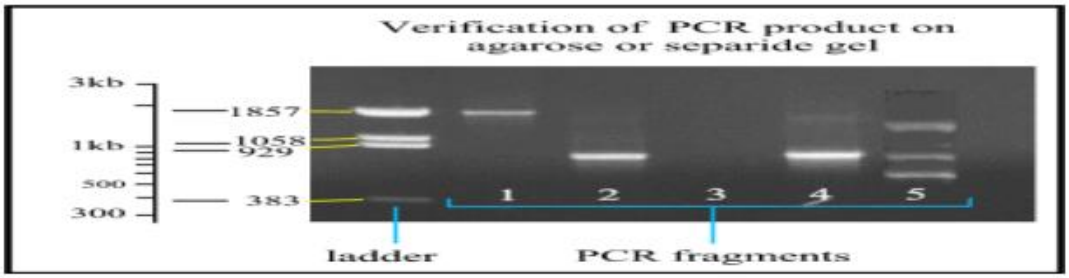


Fig 7: PCR

Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. Let us suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, and three cycles will result in 8 copies and so on3

To check whether gene is copied during PCR and to check its right size: Before the PCR product is used in further applications, it has to be checked if: There is a product formed, the product is of right size, only one band is formed.

3.3. Selectable Marker Genes and Reporter Genes

The marker genes are of two types:

I. Selectable marker genes.

II. Reporter genes.

Type # I. Selectable Marker Genes:

The selectable marker genes are usually an integral part of plant transformation system. They are present in the vector along with the target gene. In a majority of cases, the selection is based on the survival of the transformed cells when grown on a medium containing a toxic substance (antibiotic, herbicide, antimetabolite). This is due to the fact that the selectable marker gene confers resistance to toxicity in the transformed cells, while the non- transformed cells get killed.

A large number of selectable marker genes are available and they are grouped into three categories— antibiotic resistance genes, antimetabolite marker genes, and herbicide resistance genes (Table 1).

Selectable marker gene (encoded enzyme)	*Abbreviation*	*Source of gene*	*Substrate(s) used for selection*
Antibiotic resistance			
Neomycin phosphotransferase II	*nptII*	*E. coli*	Kanamycin, geneticin (G418)
Neomycin phosphotransferase III	*nptIII*	*Streptococcus faecalis*	Kanamycin, geneticin (G418)
Hygromycin phosphotransferase	*hpt/hyg*	*E. coli*	Hygromycin
Bleomycin resistance	*ble*	*E. coli*	Bleomycin
Aminoglycoside adenyltransferase	*aadA*	*Shigella flexneri*	Streptomycin, spectinomycin
Antimetabolite markers			
Dihydrofolate reductase	*dhfr*	Mouse	Methotrexate
Dihydropteroate synthase	*dhps/sul*	*E. coli*	Sulfonamides
Herbicide resistance			
Phosphinothricin acetyltransferase	*bar/pat*	*Streptomyces hygroscopicus/ S. viridochromogenes*	Glufosinate, L-phosphinothricin, Bialophos
Enolpyruvyl shikimate phosphate synthase	*epsps/aroA*	*Agrobacterium* sp/ *Petunia hybrida*	Glyphosate
Acetolactase synthase	*als*	*Arabidopsis* sp/maize/tobacco	Sulfonylureas
Glyphosate oxidoreductase	*gox*	*Achromobacter LBAA*	Glyphosate
Bromoxynil nitrilase	*bxn*	*Klebsiella pneumoniae*	Bromoxynil
Others			
β-Glucuronidase	*gus/uidA*	*E. coli*	Cytokinin glucuronide
Xylose isomerase	*xylA*	*Thermoanaerobcterium thermosulfurogenes*	Xylose
Mannose 6-phosphate isomerase	*pmi/manA*	*E. coli*	Mannose
Betaine aldehyde dehydrogenase	*badh*	Spinach	Betaine aldehyde

Table 1: List of Selectable Marker Genes

(a) Antibiotic Resistance Genes:

In many plant transformation systems, antibiotic resistance genes (particularly of E. coli) are used as selectable markers. Despite the plants being eukaryotic in nature, antibiotics can effectively inhibit the protein biosynthesis in the cellular organelles, particularly in chloroplasts. Some of the antibiotic resistance selectable marker genes are briefly described.

Neomycin phosphotransferase II (npt II gene):

The most widely used selectable marker is npt II gene encoding the enzyme neomycin phospho-transferase II (NPT II). This marker gene confers resistance to the antibiotic kanamycin. The trans-formants and the plants derived from them can be checked by applying kanamycin solution and the resistant progeny can be selected.

Hygromycin phosphotransferase (hpt gene):

The antibiotic hygromycin is more toxic than neomycin and therefore can kill non-transformed plant cells much faster. Hygromycin phospho-transferase (hpt) gene thus provides resistance to transformed cells.

Aminoglycoside adenyltransferase (aadA gene):

Aminoglycoside 3′-adenyltransferase (aadA) gene confers resistance to transformed plant cells against the antibiotics streptomycin and spectionomycin.

(b) Antimetabolite Marker Genes:

Dihydrofolate reductase (dhfr gene):

The enzyme dihydrofolate reductase, produced by dhfr gene is inhibited by the antimetabolite methotrexate. A mutant dhfr gene in mouse that codes for this enzyme which has a low affinity to methotrexate has been identified. This dhfr gene fused with CaMV promoter results in a methotrexate resistant marker which can be used for the selection of transformed plants.

(c) Herbicide Resistance Markers:

Genes that confer resistance to herbicides are in use as markers for the selection of transgenic plants.

Phosphinothricin acetytransferase (pat/bar gene):

Bialophos, phosphinothricin and glufosinate are commonly used herbicides. The pat/bar genes code for phosphinothricin acetyltransferase which converts these herbicides into acetylated forms that are non-herbicidal. Thus, pat/bar genes confer resistance to the transformed plants.

Enolpyruvylshikimate phosphate synthase (epsps/aroA genes):

The herbicide glyphosate inhibits photosynthesis. It blocks the activity of enolpyruvylshikimate phosphate (EPSP) synthase, a key enzyme involved in the biosynthesis of phenylalanine, tyrosine and tryptophan. Mutant strains of Agrobacterium and Petunia hybrida that are resistant to glyphosate have been identified. The genes epsps/aroA confer resistance to transgenic plants which can be selected.

Bromoxynil nitrilase (bxn gene):

The herbicide bromoxynil inhibits photosynthesis (photosystem II). Bromoxynil nitrilase enzyme coded by the gene bxn inactivates this herbicide. The gene bxn can be successfully used as a selectable marker for the selection of transformed plants.

Production of Marker-Free Transgenic Plants:

There is a growing concern among the public regarding the use of antibiotic or herbicide resistance genes as selectable markers of plant transformation:

i. The products of some marker genes may be toxic or allergic.

ii. The antibiotic resistance might be transferred to pathogenic microorganisms in the soil.

iii. There is a possibility of creation of super weeds that are resistant to normally used herbicides.

iv. A transgenic plant with selectable marker genes cannot be transformed again by using the same selectable markers.

In light of the apprehensions listed above, the public is concerned about the safety of transgenic technology, particularly related to the selectable marker genes (antibiotic/herbicide resistance genes). There are fears about the safety of consumption of foodstuffs derived from genetically engineered plants. This is despite the fact that so far none of the marker genes have been shown to adversely affect human, animal or environmental safety.

Clean Gene Technology:

The process of developing transgenic plants without the presence of selectable marker genes or by use of more acceptable marker genes is regarded as clean gene technology. And this will result in the production of many marker-free transgenic plants that will be readily acceptable by the public. Some of the approaches for clean gene technology are given.

Avoiding selectable marker genes:

Theoretically, it is possible to totally avoid marker genes and introduce only the transgene of interest. The transformed paints can then be screened by an advanced technique like polymerase chain reaction and the desirable plants selected. This approach is not practicable due to cost factor.

Co-transformation with two DNAs:

The transgenic plants can be produced by employing two separate DNAs — one carrying the desired target gene and the other the marker gene. The transformed plants contain both the genes, but at different sites on the chromosomal DNA. Traditional breeding techniques (a few rounds) can be used to get rid of the transgenic plants with selectable markers.

Removal of selectable markers:

It is possible to selectively remove the selectable marker genes from the plant genome. For this purpose, site-specific recombinase systems are utilized. Several recombinase systems are in fact available which can be used to selectively excise the marker genes from the plant genome.

Cloning of selectable markers between transposable elements:

A selectable marker gene can be cloned between plant transposable elements (Ds elements) and then inserted. The selectable marker is planked by the sequences that increase the intra-chromosomal recombination. This results in the excision of the marker gene.

Type # II. Reporter Genes:

A reporter gene may be regarded as the test gene whose expression can be quantified. The plant transformation can be assessed by the expression of reporter genes (also called as screenable or scoreable genes). In general, an assay for the reporter gene is carried out by estimating the quantity of the protein it produces or the final products formed. A selected list of the reporter genes along with the detection assays is given in Table 49.4, some of the important ones are discussed below.

TABLE 49.4 A selected list of reporter genes used for gene transfer in plants, their sources, and detection assays

Reporter gene (enzyme/protein encoded)	*Abbreviation*	*Source of gene*	*Detection assay*
Octopine synthase	*ocs*	*Agrobacterium tumefaciens*	Electrophoresis, chromatography
Nopaline synthase	*nos*	*Agrobacterium tumefaciens*	Electrophoresis, chromatography
β-Glucuronidase	*gus/uidA*	*E. coli*	Fluorometric or histochemical or colorimetric
Green fluorescent protein	*gfp*	*Aequorea victoria* (jelly fish)	Fluorescence
Luciferase (bacterial)	*luxA/luxB*	*Vibrio harveyi*	Bioluminescence
Luciferase (firefly)	*luc*	*Photonus pyralis*	Bioluminescence
Chloramphenicol acetyltransferase	*cat*	*E. coli*	Autoradiography

Table 2: List of Reporter Genes

Opine synthase (ocs, nos genes):

The common opines present in T-DNA of Ti or Ri plasmids of Agrobacterium are octopine and nopaline, respectively produced by the synthase genes ocs and nos. The transformed status of the plant cells can be easily detected by the presence of these opines. Opines can be separated by electrophoresis and identified. Alternately, the enzyme activities responsible for the production of opines can also be assayed.

β-Glucuronidase (gusluidA gene):

β-Glucuronidase producing gene (gusluidA) is the most commonly used reporter gene in assessing plant transformation for the following reasons:

i. β-Glucuronidase assays are very sensitive.

ii. Quantitative estimation of the enzyme can be done by fluorometric method (using substrate 4-methylumbelliferryl P-D-glucuronide which is hydrolysed to 4-methylumbelliferone).

iii. Qualitative data on the enzyme can be obtained by histochemical means (enzyme localization can be detected by chromogenic substance such as substrate X-gluc).

iv. No need to extract and identify DNA.

Green fluorescent protein (gfp gene):

Green fluorescent protein (GFP), coded by gfp gene, is being widely used in recent years. In fact, in many instances, GFP has replaced GUS since assays of GFP are easier and non-destructive. Thus, screening of even the primary transplants can be done by GFP which is not possible with other reporter genes.

Gene for GFP has been isolated from jelly fish Aequorea victoria which is a luminescent organism. The original gfp gene has been significantly modified to make it more useful as a reporter gene. GFP emits fluorescence which can be detected under a fluorescent microscope.

Bacterial luciferase (luxA/luxB genes):

The bacterial luciferase genes (luxA and luxB) have originated from Vibrio harveyi. They can be detected in some plant transformation vectors. The detection assay of the enzyme is based on the

principle of bioluminescence. Bacterial luciferase catalyses the oxidation of long-chain fatty aldehydes that results in the emission of light which can be measured.

Firefly luciferase (luc gene):

The enzyme firefly luciferase, encoded by the gene luc, catalyses the oxidation of D-luciferin (ATP dependent) which results in the emission of light that can be detected by sensitive luminometers. The firefly luciferase gene, however, is not widely used as a marker gene since the assay of the enzyme is rather cumbersome.

Chloramphenicol acetyl transferase (cat gene):

The cat gene producing chloramphenicol acetyl transferase (CAT) is a widely used reporter gene in mammalian cells. Due to the availability of GUS and GFP reporter systems for plant transformants, CAT is not commonly used. However, some workers continue to use CAT by a sensitive radioactive assay, for the detection of the reporter gene cat.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – 4 – GENETIC ENGINEERING – SBB2102

GENE TRANSFER TECHNIQUES

1. Choice of host organism:

A good host should have the following properties:

- Easy to grow and transform.
- Do not hinder replication of recombinant vector.
- Do not have restriction and methylase activities.
- Deficient in recombination function so that the introduced recombinant vector is not altered.
- Easily retrievable from the transformed host.

Various hosts are used in rDNA technology depending on the goal: For example bacteria, yeast, plant cells, animal cells, whole plants and animals.

Prokaryotic systems such as E. coli are commonly used due to various advantages like, They have-

- Well studied expression system,
- Compact genome,
- Versatile,
- Easy to transform,
- Widely available, and
- Rapid growth of recombinant organisms with minimal equipment.

Only disadvantage is that they lack post-translational modification (PTMs) machinery required for eukaryotic proteins.

Eukaryotic systems are difficult to handle in contrast to bacterial hosts. They are favoured for expression of recombinant proteins which require post translational modification and only if they can grow easily in continuous culture.

2. Choice of vector:

Vector is an autonomously replicating (inside a host cell) DNA molecule designed from a plasmid or phage DNA to carry a foreign DNA inside the host cell. Transformation vectors are of two types:

- Cloning vector is used increasing the number of copies of a cloned DNA fragment.
- Expression vector is used for expression of foreign gene into a protein.
- If a vector is designed to perform equally in two different hosts, it is called a shuttle vector.

Properties of an ideal vector: A good vector should have the following characteristics:

- Autonomously replicating i.e. should have ori (origin of replication) region.
- Contain at least one selectable marker e. g. gene for antibiotic resistance
- May contain a scorable marker (β-galactosidase, green fluorescent protein etc.)
- Presence of unique restriction enzyme site.
- Have multiple cloning sites.
- Preferably small in size and easy to handle.
- Relaxed control of replication to obtain multiple copies.
- Presence of appropriate regulatory elements for expression of foreign gene.
- High copy number

The selection of a suitable vector system depends mainly on the size limit of insert DNA and the type of host intended for cloning or expression of foreign DNA

3. Gene Transfer

Introduction

Gene transfer technique is used very widely both in basic research and applied biology. The delivery of DNA into animal cells is a fundamental and established procedure. It has become an indispensable tool for gene cloning, the study of gene function and regulation and the production of small amounts of recombinant proteins for analysis and verification. Gene transfer experiment helps to express the introduced genetic construct (or transgene) in the recipient cells or to disrupt or inactivate particular endogenous genes (resulting in a loss of function. There are many applications of gene transfer like large-scale commercial production of recombinant antibodies and vaccines and gene medicine or gene therapy. They range from the use of mammalian and insect cell cultures to transfer of DNA into human patients for the correction or prevention of disease.

In the organisms or genetically modified whole animals created by Gene transfer every cell or a specific target population of cells carries a particular alteration. Such animals are used to study gene function and expression, model human diseases, produce recombinant proteins in their milk and other fluids, and to improve the quality of livestock herds and other domestic species. The technology has contributed to understand the functions of the many genes discovered in the genome projects (functional genomics). Examples of such experiments include systematic DNA-mediated mutagenesis and gene trap programs in the mouse and in the fruit fly, Drosophila melanogaster, genome-wide RNA interference experiments in the nematode, Caenorhabditis elegans, and novel protein interaction screens based on the yeast two hybrid system but performed using mammalian cells.

Genetic engineering of food is the science which involves deliberate modification of the genetic material of plants or animals. Introduction of DNA into plants is of great agricultural potential and medical importance. The gene transfer results can be transient and stable transfection.

Gene therapy can be defined as the deliberate transfer of DNA for therapeutic purposes. Gene transfer is one of the key factors in gene therapy, and it is one of the key purposes of the clone. Gene transfer can be targeted to somatic (body) or germ (egg and sperm) cells. In somatic gene transfer the recipient's genome is changed, but the change will not be passed on to the next generation. In germline gene transfer, the parents' egg and sperm cells are changed with the goal of passing on the changes to their offspring.

A genetic engineering technique to transfer genes using vectors from one organism to another or from one cell to another in order to treat disease, construct GMO and economically important organisms are known as gene transfer. The first evidence of gene transfer was reported in bacteria by Frederick Griffith in 1928. He had named it a transformation. In 1944, Avery had demonstrated that the material transfer between bacteria during transformation is the nucleic acid (DNA) which was later validated by Hershey and Chase in 1952. Although the exogenous gene transfer in a eukaryotic cell (in vitro) was demonstrated by Rogers in 1970.

The concept of gene transfer between cells was first demonstrated in bacteria, which are capable of at least four natural forms of genetic exchange. The first mechanism was discovered in 1928 by Frederick Griffith named as transformation in bacterium Streptococcus pneumoniae. However, he was unable to determine the nature of the transforming principle. In 1944 Oswald Avery established that the substance transferred between cells was DNA. A second form of gene transfer

was named conjugation discovered by Joshua Lederberg and Edward Tatum in 1946 in Escherichia coli. This process involved the transfer of DNA through a direct link between the bacterial cells. In E. coli this conduit between cells took the form of a proteinaceous tube known as a pilus. The ability of the cells to construct the pilus and pass DNA through it was encoded on a large plasmid known as the F (for fertility) factor.

In most cases, the act of conjugation involved transfer of the plasmid alone, which became established in the recipient cell thereby converting it from an F− to an F+ phenotype. In some cases, however, the F plasmid could integrate into the bacterial chromosome, and conjugation could result in the transfer of chromosomal genes. This process, which was used to construct the first genetic map of E. coli, was termed sexduction. Then in 1951, transduction, a new type of gene transfer mediated by bacteriophage was discovered by Joshua Lederberg and Norton Zinder in Salmonella. They found that newly formed phage could occasionally package some of the host cell's DNA and then transfer it to a second host cell in a subsequent infection.

Two forms of transduction were identified – generalized and specialized transduction. In generalized transduction the phage head was mistakenly stuffed completely with host cell DNA. Whereas in specialized transduction the phage genome integrated into the bacterial chromosome and became linked to host DNA. The fourth mechanism of gene transfer in bacteria is mediated by complete cell fusion, and occurs in several genera of bacteria including Bacillus and Streptomyces. Under natural conditions, the viral capsid is needed to introduce the oncogene and the rest of the genome into an animal host cell. The unusual process of gene transfer without the viral capsid was named transfection to distinguish it from normal infection.

In bacterial genetics, the term transformation continued to be used to describe the uptake of naked plasmid or genomic DNA (essentially any DNA which had the potential to transform the phenotype of the recipient cell) while transfection was used specifically to describe the uptake of naked phage DNA (or RNA), i.e. nucleic acid which had the potential to initiate a phage replication cycle. The term transfection became generally accepted to mean the introduction of any sort of DNA— phage, plasmid, genomic or otherwise—into an animal cell, in the absence of a biological vector.

3.1. Different Gene Transfection Techniques

There are two types of transfection transient and stable. In transient transfection, the transfected DNA is not integrated into host chromosome. DNA is transferred into a recipient cell in order to obtain a temporary but high level of expression of the target gene. Stable transfection is also called permanent transfection. By the stable transfection, the transferred DNA is integrated (inserted) into chromosomal DNA and the genetics of recipient cells is permanent changed.

Regardless of the delivery method, gene transfer into animal cells must accomplish three distinct goals. First, the exogenous genetic must be transported across the cell membrane. In physical transfection methods, transport across the membrane is achieved by direct transfer, where the membrane is breached during delivery through which DNA and RNA can diffuse. In other delivery methods, the nucleic acid must form some sort of complex which binds to the cell surface before internalization. For example, in chemical transfection methods the complex is formed between nucleic acid and a synthetic compound, while in transduction methods the complex comprises nucleic acid packaged inside a viral capsid.

Once across the cell membrane, the genetic material must be released in the cell and transported to its site of expression or activity. Again, the nucleic acid is passive at this stage. In most transfection methods, DNA or RNA complexes are deposited in the cytoplasm, following escape from the endosomal vesicle. DNA must be transported to the nucleus, while RNA can function directly in the cytoplasm. In methods such as particle bombardment and microinjection, it is possible to deliver DNA directly into the nucleus, so intrinsic transport pathways are not required. Many viruses also deliver their nucleic acid cargo to the nucleus as part of the infection cycle, often after interaction with cell surface receptors and either internalization within endosomes or direct fusion with the plasma membrane. However, few exceptions are there like poxviruses (e.g. Vaccinia virus) and alphaviruses (e.g. Sindbis virus) which replicate in the cytoplasm. In the final stage of gene transfer, the exogenous genetic material must be activated. It must be released from its complex and rendered competent for expression and/or interaction with the host genome. Exogenous RNA exist only transiently in the host, whereas exogenous DNA can exist transiently or permanently.

The gene transfer methods normally include three categories: 1. transfection by biochemical methods; 2. transfection by physical methods; 3. virus-mediately transduction. The first gene transfer protocols used naked DNA which was mixed with particular chemicals to form

synthetic complexes. These synthetic complexes either interact with the cell membrane and promote uptake by endocytosis, or fuse with the membrane and deliver the DNA directly into the cytoplasm. Such chemical transfection methods have been widely used. However they are generally inefficient for gene transfer in vivo. In contrast, physical transfection methods are efficient for both in vitro and in vivo gene transfer.

Physical transfection methods involve breaching the cell membrane and introducing the nucleic acid directly into the cell or nucleus. Although there are advantages and disadvantages to both sets of procedures, some of the most efficient transfection methods in use today involve a combination of chemical and physical processes. Chemical and physical transfection methods were first used for the transfer of naked, wild-type viral DNA into animal cells. Now both techniques are more widely used for the introduction of plasmid vectors and recombinant viral genomes carrying specific transgenes of interest.

The first transgenic plant was produced via Agrobacterium mediated modified transformation of Nicotiana tabacum protoplasts by Horsch and co-workers in 1984. Since then several dozen plant species have been genetically engineered using different techniques. Simultaneous development of other techniques such as selectable markers facilitated the development in genetic engineering for obtaining transformed plants. But this technique is not suitable for monocotyledon plants as they are not natural host of Agrobacterium (there is evidence that limited gene transfer is possible in monocots by this system). Therefore, other methods of direct gene transfer have been developed for use with monocots and other species. These can be categorized.on the basis of the use of protoplasts or cell and tissue as the target materials. The isolation and purification of protolasts have been presented in earlier Lessons. Freshly isolated protoplasts are used for genetic transformation using direct gene transfer methods. Let us understand the procedures of DNA transfer

1. **Microinjection**

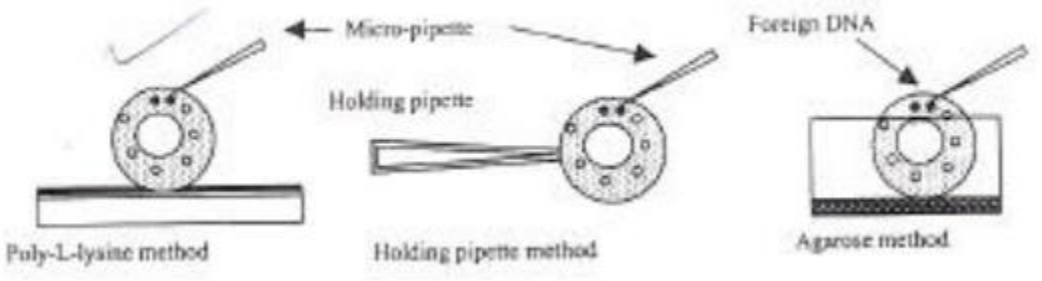
Delivery of nucleic acids to protoplasts or intact cells via microinjection is a labour intensive procedure that requires special capillary needles, pumps, micromanipulators, inverted microscope and other equipment. However, injection into the nucleus or cytoplasm is possible and cells can be cultured individually to produce callus or plants. In this way selection of transformants by drug resistance or marker genes may be avoided. This method involves skill of the worker to insert needle into the cytoplasm or in the nucleus. The basic technique is similar to that used for animal

cell microinjection. In order to microinject protoplasts or other plant cells, the cells need to be immobilized (Fig.).

The cells are immobilized by:

1. The use of a holding pipette which holds the cells by vacuum.

2.



Attachment of cells to poly-L-lysine coated cover slips. 3. Embedding the cells in agarose, agar or sodium alginate.

Glass micropipette are prepared to have openings of about 0.3 uM in diameter and are inserted into plant cell cytoplasm and nuclei with the aid of a micro manipulators device. A syringe like device is used for the controlled delivery of volume (10-11 - 10-4 ul) into the plant cell. Most plant cells are injected while keeping inside microdroplets (2-50 ul) of medium using a chamber which is sterile, vibration free and permits temperature and humidity regulation. A maximum of 100-200 cells per hour can be microinjected by this method. The recovery of transformants is dependent upon the regeneration ability of the microinjected cells. Different methods have been used to grow injured (microinjected) single cells or protoplasts. Hanging droplets, covered under thin layer of agar or agarose, and micro culture have been used (Fig). Attempts have been made to inject linear, or super coiled DNA, in cytoplasm or in nucleus. Nuclear injections are found better for transformations.

Advantages

- No requirement of a marker gene.
- Introduction of the target gene directly into a single cell.
- Easy identification of transformed cells upon injection of dye along with the DNA.

• No requirement of selection of the transformed cells using antibiotic resistance or herbicide resistance markers.

• It can be used for creating transgenic organisms, particularly mammals.

Disadvantages:

- Only one cell receives DNA per injection.
- Handling of protoplast for microinjection requires skilled persons.
- Sophisticated equipment.
- Requirement of regeneration process from microinjected cells.

2. Particle Gun or Biolistic Method

This is latest technology to transfer DNA into intact tissues. Several devices are developed using different methods. All to achieve the transfer of micro-sized particles (microprojectiles) coated with DNA to penetrate the cells. In this procedure micron size tungsten or gold particles are accelerated in a gun barrel to velocities sufficient for non-lethal penetration of cell walls and membranes. Klein and co-workers in 1987 developed and used for the first time the particle gun to transfer chimeric DNA and viral RNA molecules into intact onion cells. Tungsten acted as a carrier of nucleic acids because it was available in micro-size balls, non-toxic to cells and dense enough (high density) for rapid penetration of target material. Microprojectile mediated transformation is a mechanical method of introducing DNA in to any plant species. This method can be successfully used where plasmids or protoplasts mediated transformation cannot be used. An acceleration device used to propel particles (micro projectiles) carrying plasmid DNA is called by various names based on machine or technique used to accelerate the particles such as ‘particle gun technology’, ‘biolistic method’, ‘DNA bombardment’, ‘particle acceleration of DNA method’ and ‘electric discharge particle acceleration method’.

This is a quick method of stable transformation and testing a gene for cell and organelle specific expression. This technique has three components –

1. The basic equipment to generate particle acceleration.

2. Metal particles coated with precipitated DNA (desired gene).

3. Plant tissues to be used for particle penetration.

The method for regeneration should be previously standardized and proper tissues be selected for bombardment. (a) Instrument: The instrument is commercially available. Prototype was designed by Klein and co-workers. It uses the explosive force of gun powder (0.22 caliber gun cartridge) to accelerate a polypropylene cylindrical macroprojectile. Thin piece of polypropylene macroprojectile is loaded with microprojectiles coated with DNA. Gun powder explosion forces this macroprojectile to move with high speed toward another end of barrel, where it is blocked by a polycarbonate disc having an aperture. Macroprojectile is stopped but microprojectiles move fast through the aperture towards tissue placed in the same direction. For each transfer, 50 mg tungsten is accelerated upto 2000 ft per second in a partial vacuum. With this speed, particles reach upto lower layers of cells in target tissues (Fig). The other devices are similar in basic design concept but use different methods to accelerate particles like use of compressed air or gas. Compressed air (130 kg/cm2 pressure) has been used to accelerate microprojectiles at velocities (approximately 440 m/ sec) necessary to achieve DNA delivery to plant cells (Fig). An electric discharge particle acceleration device differs in basic design from the above described devices. In this device, a high voltage discharge (14 KV current) delivered to a small water droplet which quickly vaporizes and releases energy to propel DNA coated gold spheres into target cells (Fig.). In a similar way to above devices, a DNA carrier is attracted (accelerated) due to potential differences, stopped in-between by a screen, DNA coated particles cross the screen and fly towards target tissue and deliver the DNA into cells.

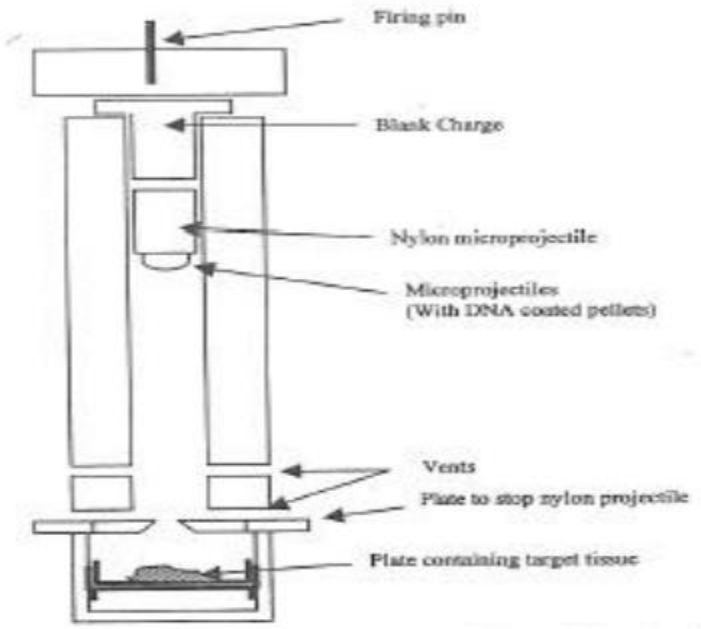


Fig 1: Particle gun or shotgun for delivering DNA coated microprojectiles into plant cells

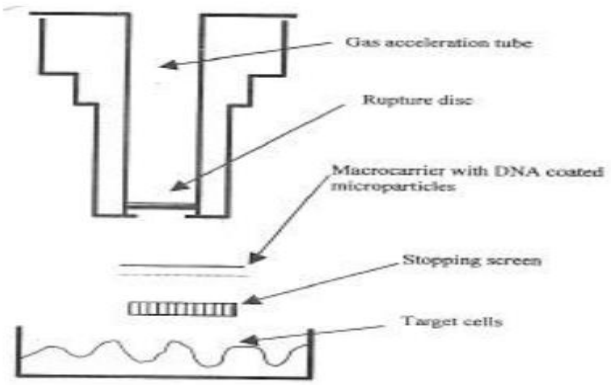


Fig 2. Compressed air particle acceleration device for delivering DNA coated microprojectiles into plant cells

Advantages

- Simple and convenient method involving coating DNA or RNA on to gold microcarrier, loading sample cartridges, pointing the nozzle and firing the device.
- No need to obtain protoplast as the intact cell wall can be penetrated.
- Manipulation of genome of sub-cellular organelles can be done.

- Eliminates the use of potentially harmful viruses or toxic chemical treatment as gene delivery vehicle.
- This device offers to place DNA or RNA exactly where it is needed into any organism.

Disadvantages

- The transformation efficiency may be lower than *Agrobacterium- mediated transformation*.
- Specialized equipment is needed. Moreover the device and consumables are costly.
- Associated cell damage can occur.
- The target tissue should have regeneration capacity.
- Random integration is also a concern.
- Chances of multiple copy insertions could cause gene silencing.

3. Electroporation

Electroporation is a mechanical method used for the introduction of polar molecules into a host cell through the cell membrane. This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer in mouse cells. It is now a widely used method for the introduction of transgene either stably or transiently into bacterial, fungal, plant and animal cells. It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA.

This method is based on the use of the short electrical pulses of high field strength. Electroporation causes the uptake of DNA into protoplasts by temporary permeabilization of the plasma membrane to macromolecules. Protoplasts and foreign DNA are placed in a buffer between two electrodes and a high intensity electric current is passed (Fig). Electric field damages membranes and creates pores in membranes. DNA diffuses through these pores immediately after electric field is applied, until the pore are resealed. The technique is optimized by using appropriate electric field strength (defined as the applied voltage divided by the distance between two electrodes).

The optimum field strength is dependent on the following:

1. The pulse length of electric current

2. Composition and temperature of the buffer solution

3. Concentration of foreign DNA in the suspension

4. Protoplasts density, and

5. Size of the protoplasts.

It has been demonstrated that the removal of pectin from the plant wall increases the amount of DNAwhich can be introduced by electroporation. Tobacco mosaic virus was introduced in tobacco protoplasts by this method. Electroporation has been used successfully for transient and stable transformation of protoplasts from a wide range of species. Plating efficiency (i.e. number of colonies recovered out of number of cells transferred on plates) of electroporated protoplasts grown on selection medium (containing selective marker) can be as high as 0.5%. The highest plant transformation efficiencies have been reported for tobacco, with 0.2% of electroporated leaf mesophyll protoplasts giving rise to transgenic calli. Low transformation efficiency is common in cereals, e.g. in rice 0.002% efficiency was recorded.

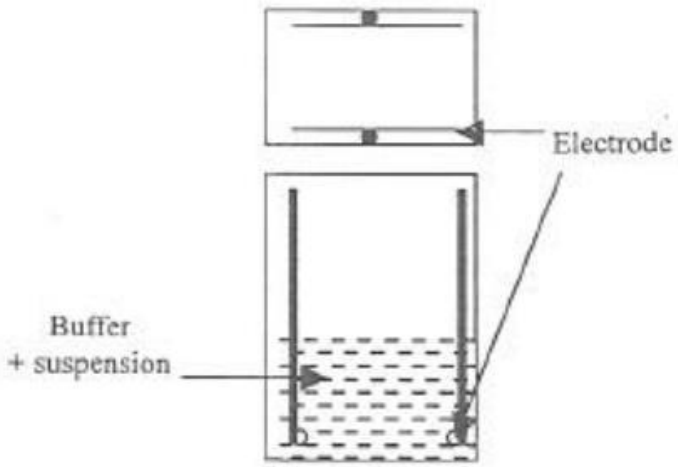


Fig 3. Top (above) and side view (below) of glass cell with electrodes used for electroporation.

Applications

Electroporation is widely used in many areas of molecular biology and in medical field. Some applications of electroporation include:

- **DNA transfection or transformation**

Electroporation is mainly used in DNA transfection/transformation which involves introduction of foreign DNA into the host cell (animal, bacterial or plant cell).

- **Direct transfer of plasmids between cells**

It involves the incubation of bacterial cells containing a plasmid with another strain lacking plasmids but containing some other desirable features. The voltage of electroporation creates pores, allowing the transfer of plasmids from one cell to another. This type of transfer may also be performed between species. As a result, a large number of plasmids may be grown in rapidly dividing bacterial colonies and transferred to yeast cells by electroporation.

- **Gene transfer to a wide range of tissues**

Electroporation can be performed in vivo for more efficient gene transfer in a wide range of tissues like skin, muscle, lung, kidney, liver, artery, brain, cornea etc. It avoids the vector-specific immune-responses that are achieved with recombinant viral vectors and thus are promising in clinical applications.

Advantages

- It is highly versatile and effective for nearly all cell types and species.
- It is highly efficient method as majority of cells take in the target DNA molecule.
- It can be performed at a small scale and only a small amount of DNA is required as compared to other methods.

Disadvantages

- Cell damage is one of the limitations of this method caused by irregular intensity pulses resulting in too large pores which fail to close after membrane discharge.
- Another limitation is the non-specific transport which may result in an ion imbalance causing improper cell function and cell death.

3.2. GENE TRANSFER TECHNIQUES: CHEMICAL METHODS

Introduction

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compounds are introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell.

An ideal chemical used for DNA transfer should have the ability to-

- Protect DNA against nuclease degradation.
- Transport DNA to the target cells.
- Facilitate transport of DNA across the plasma membrane.
- Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

1. Calcium phosphate
2. DEAE dextran

1. Calcium phosphate mediated DNA transfer

Historical perspective

The ability of mammalian cells to take up exogenously supplied DNA from their culture medium was first reported by Szybalska and Szybalski (1962).

They used total uncloned genomic DNA to transfect human cells deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT). Rare HPRT-positive cells with fragments of DNA containing the functional gene were identified by selection on HAT medium. Till then, the actual mechanism of DNA uptake was not understood. It was later found that successful DNA transfer takes place by the formation of a fine DNA/calcium phosphate co-

precipitate, which first settles onto the cells and is then internalized. This technique was first applied by Graham and Van Der Eb in 1973 for the analysis of the infectivity of adenoviral DNA.

Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction.

Inthis method, the precipitates of calcium phosphate and DNA being small and insoluble can be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis and the DNA gets integrated into the cell genome resulting in stable or permanent transfection.

Uses

- This method is mainly used in the production of recombinant viral vectors.
- It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.

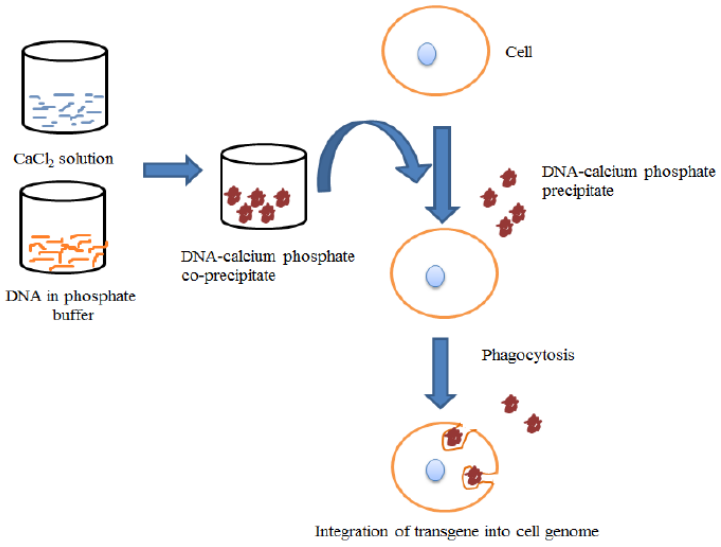


Fig 4: schematic representation of transfection by Calcium Phosphate Precipitation.

Advantages

- Simple and inexpensive
- Applicability to generate stably transfected cell lines
- Highly efficient (cell type dependent) and can be applied to a wide range of cell types.
- Can be used for stable or transient transfection

- Toxic especially to primary cells
- Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
- Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
- Limited by the composition and size of the precipitate.
- Random integration into host cell.
-

Optimal factors (amount of DNA in the precipitate, the length of time for precipitation reaction and exposure of cells to the precipitate) need to be determined for efficient transfection of the cells. This technique is simple, expensive and has minimal cytotoxic effect but the low level of transgene expression provoked development of several other methods of transfection.

2. DEAE-Dextran (Diethylaminoethyl Dextran) mediated DNA transfer

- This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer.
- Diethylaminoethyl dextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell.
- In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE – dextran in mixture results in net positive charge in the DEAE – dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.
- Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.

Advantages

- Simple and inexpensive
- More sensitive
- Can be applied to a wide range of cell types
- Can be used for transient transfection.

Disadvantages

- Toxic to cells at high concentrations
- Transfection efficiency varies with cell type
- Can only be used for transient transfection but not for stable transfection
- Typically produces less than 10% delivery in primary cells.

Another polycationic chemical, the detergent Polybrene, has been used for the transfection of Chinese hamster ovary (CHO) cells, which are not amenable to calcium phosphate transfection.

3. Lipofection

- Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.
- Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of0.1 to 10 micrometer or 20-25 nanometers respectively.
- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.
- Liposomes can be classified as either cationic liposome or pH-sensitive.

Cationic liposomes

- Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum. A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

- The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.
- The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.
- The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

Negatively charged liposomes

- Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.
- However, formation of lipoplex, a complex between DNA and anionic lipid scan occur by using divalent cations (e.g. Ca2+, Mg2+, Mn2+, and Ba2+) which can neutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.
- They are termed as **pH sensitive** due to destabilization at low pH.
- The efficiency of both in vivo and in vitro gene delivery using cationic liposomes is higher than that of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.

Liposome Action

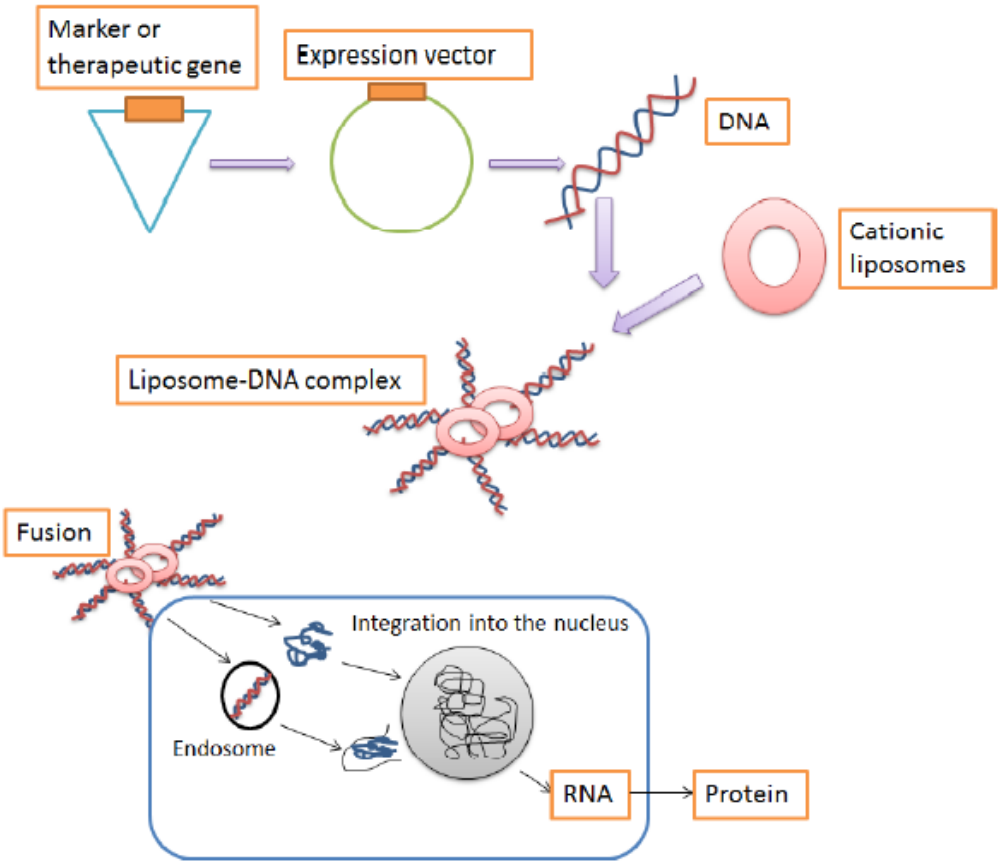


Fig 5: Schematic representation of liposome action in gene transfer. (Source: Pleyer U, Dannowski H. 2002. Delivery of genes via liposomes to corneal endothelial cells. Drug News Perspect, 15(5): 283)

In addition, liposomes can be directed to cells using monoclonal antibodies which recognize and bind to the specific surface antigens of cells along with the liposomes. Liposomes can be prevented from destruction by the cell's lysosomes by pre- treating the cells with chemicals such as chloroquine, cytochalasin B, colchicine etc. Liposome mediated transfer into the nucleusis still not completely understood.

Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.
- Delivery of the nucleic acids with minimal toxicity.

- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

AGRO- BACTERIUM MEDIATED GENE TRANSFER IN PLANTS

Cloning vectors for higher plants were developed in the 1980s and their use has led to the genetically modified (GM) crops that are in the headlines today. We will examine the genetic modification of crops and other plants in Chapter 15. Here we look at the cloning vectors and how they are used. Three types of vector system have been used with varying degrees of success with Higher plants:

- Vectors based on naturally occurring plasmids of Agrobacterium;
- Direct gene transfer using various types of plasmid DNA;
- Vectors based on plant viruses.

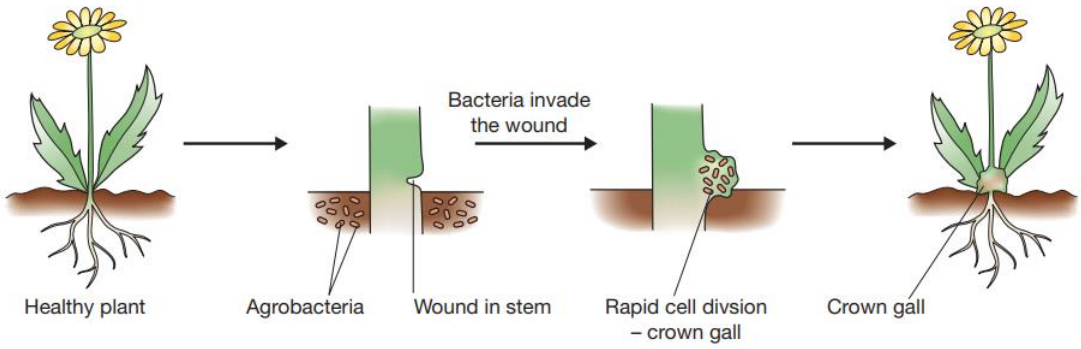


Fig6; Crown gall disease.

Agrobacterium tumefaciens—nature's smallest genetic engineer

Although no naturally occurring plasmids are known in higher plants, one bacterial plasmid, the Ti plasmid of Agrobacterium tumefaciens, is of great importance. A. tumefaciens is a soil microorganism that causes crown gall disease in many species of dicotyledonous plants. Crown gall occurs when a wound on the stem allows A. tumefaciens bacteria to invade the plant. After infection the bacteria cause a cancerous proliferation of the stem tissue in the region of the crown (Figure 7.9). The ability to cause crown gall disease is associated with the presence of the Ti (tumor inducing) plasmid within the bacterial cell. This is a large (greater than 200 kb) plasmid that carries numerous genes involved in the infective process (Figure 7.10a). A remarkable feature of the Ti plasmid is that, after infection, part of the molecule is integrated into the plant chromosomal DNA (Figure 7.10b). This segment, called the T-DNA, is between 15 and 30 kb in size, depending on the strain. It is maintained in a stable form in the plant cell and is passed on to daughter cells as an integral part of the chromosomes. But the most remarkable feature of the Ti plasmid is that the T-DNA contains eight or so genes that are expressed in the plant cell and are responsible for the cancerous properties of the transformed cells. These genes also direct synthesis of unusual compounds, called opines, that the bacteria use as nutrients (Figure 7.10c). In short, A. tumefaciens genetically engineers the plant cell for its own purposes.

Using the Ti plasmid to introduce new genes into a plant cell

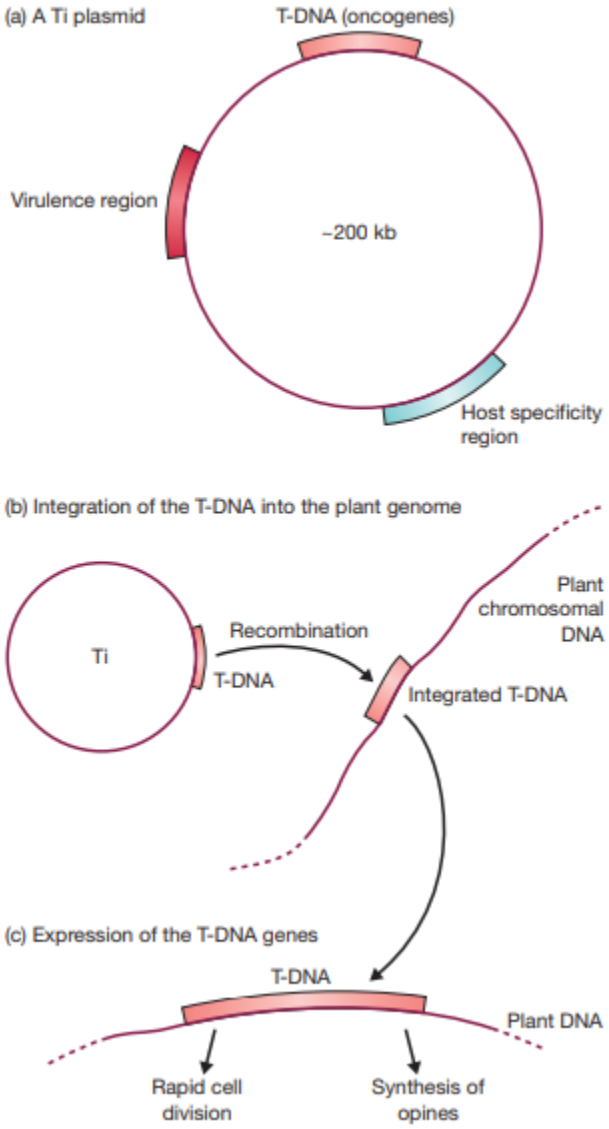
It was realized very quickly that the Ti plasmid could be used to transport new genes into plant cells. All that would be necessary would be to insert the new genes into the T-DNA and then the bacterium could do the hard work of integrating them into the plant chromosomal DNA. In practice this has proved a tricky proposition, mainly because the large size of the Ti plasmid makes manipulation of the molecule very difficult.

The main problem is, of course, that a unique restriction site is an impossibility with a plasmid 200 kb in size. Novel strategies have to be developed for inserting new DNA into the plasmid. Two are in general use:

- **The binary vector strategy** (Figure 7.11) is based on the observation that the T-DNA does not need to be physically attached to the rest of the Ti plasmid. A two-plasmid system, with the T-DNA on a relatively small molecule, and the rest of the plasmid in normal form, is just as effective at transforming plant cells. In fact, some strains of A. tumefaciens, and related agrobacteria, have natural binary plasmid systems. The T-DNA plasmid is small enough to have a unique restriction site and to be manipulated using standard techniques.

Figure 7.10

The Ti plasmid and its integration into the plant chromosomal DNA after *A. tumefaciens* infection.



- **The co-integration strategy (Figure 7.12)** uses an entirely new plasmid, based on an E. coli vector, but carrying a small portion of the T-DNA. The homology between the new molecule and the Ti plasmid means that if both are present in the same A. tumefaciens cell, recombination can integrate the E. coli plasmid into the T-DNA region. The gene to be cloned is therefore inserted into a unique restriction site on the small E. coli plasmid, introduced into A. tumefaciens cells carrying a Ti plasmid, and the natural recombination

process left to integrate the new gene into the T-DNA. Infection of the plant leads to insertion of the new gene, along with the rest of the T-DNA, into the plant chromosomes.

Production of transformed plants with the Ti plasmid

If A. tumefaciens bacteria that contain an engineered Ti plasmid are introduced into a plant in the natural way, by infection of a wound in the stem, then only the cells in the

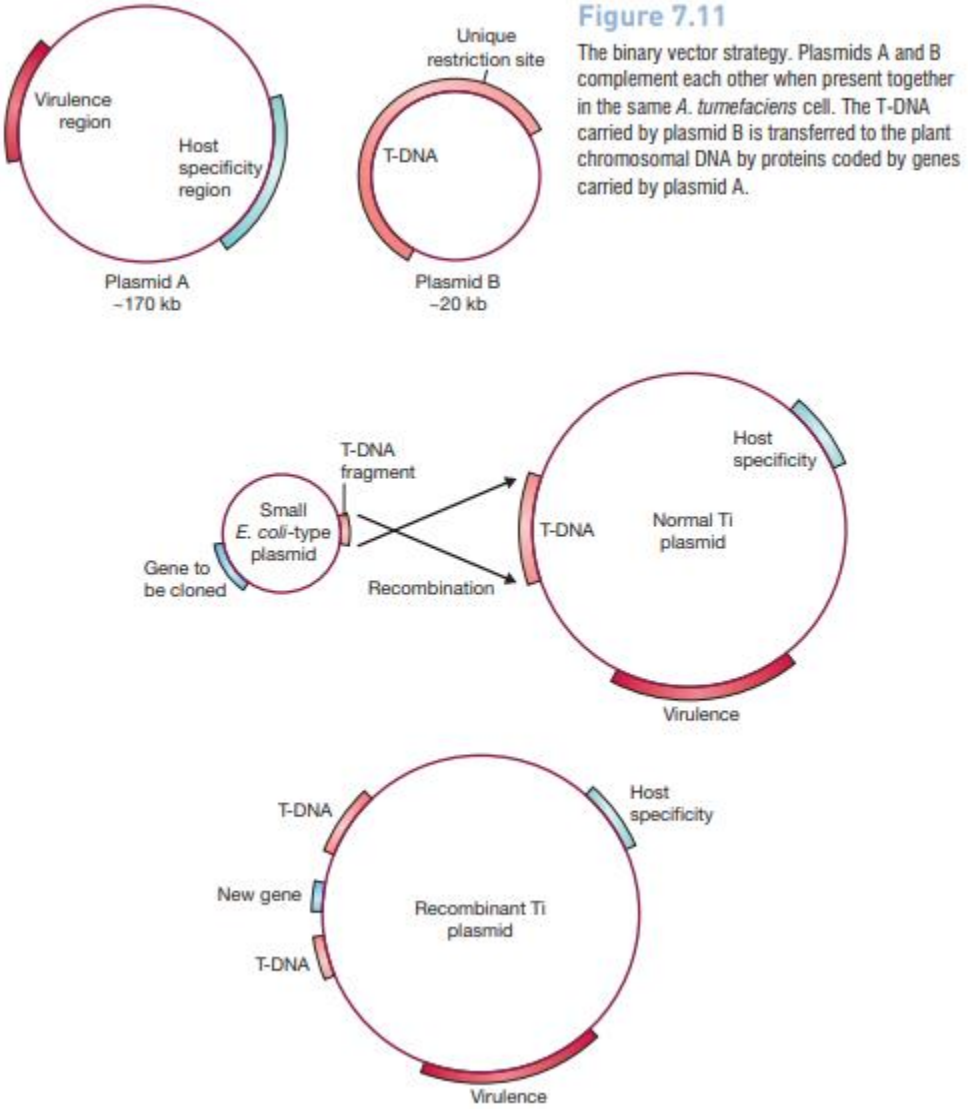


Figure 7.11
The binary vector strategy. Plasmids A and B complement each other when present together in the same *A. tumefaciens* cell. The T-DNA carried by plasmid B is transferred to the plant chromosomal DNA by proteins coded by genes carried by plasmid A.

resulting crown gall will possess the cloned gene (Figure 7.13a). This is obviously of little value to the biotechnologist. Instead a way of introducing the new gene into every cell in the plant is needed.

There are several solutions, the simplest being to infect not the mature plant but a culture of plant cells or protoplasts (p. 85) in liquid medium (Figure 7.13b). Plant cells and protoplasts whose cell walls have re-formed can be treated in the same way as microorganisms: for example, they can be plated onto a selective medium in order to

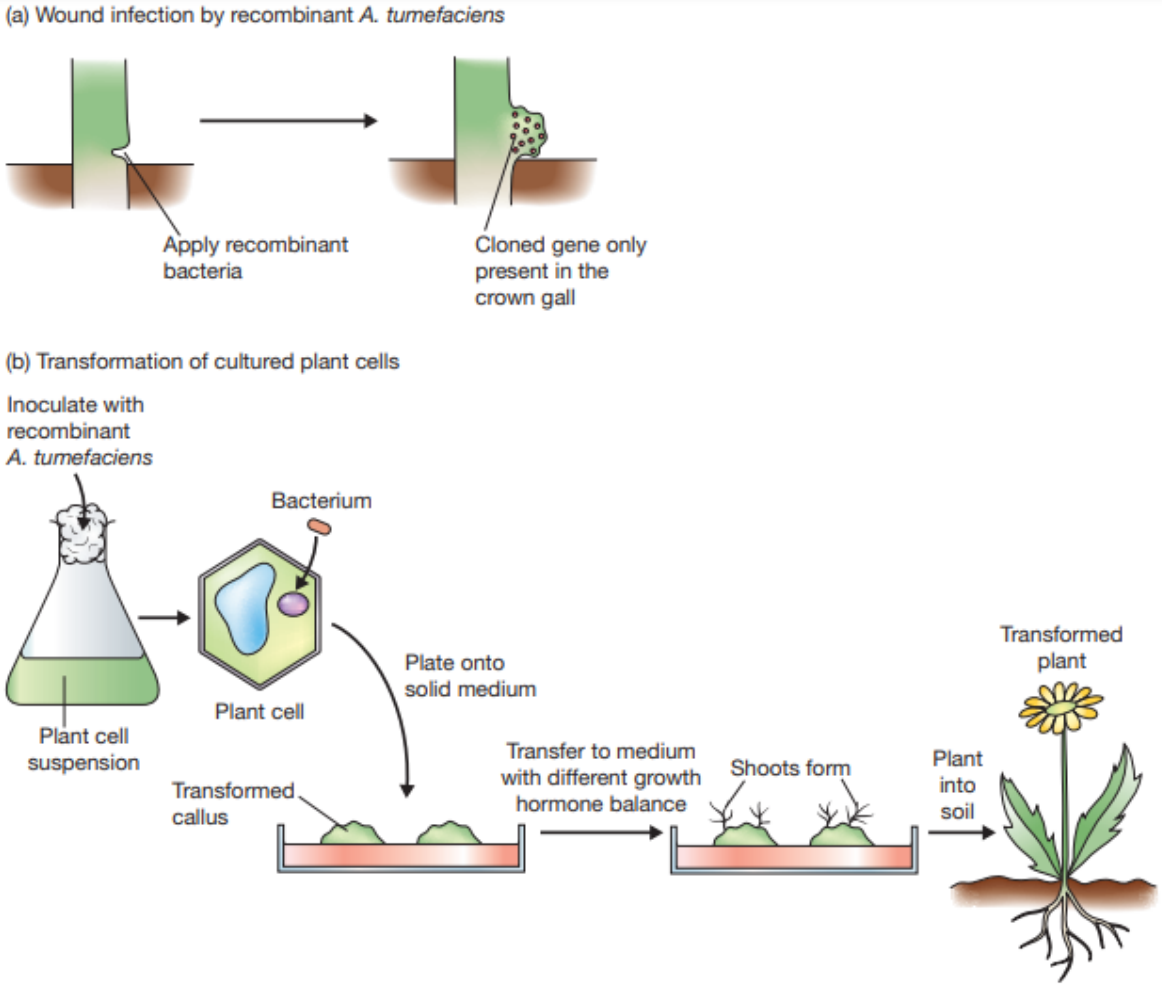


Figure 7.13

Transformation of plant cells by recombinant *A. tumefaciens*. (a) Infection of a wound: transformed plant cells are present only in the crown gall. (b) Transformation of a cell suspension: all the cells in the resulting plant are transformed.

isolate transformants. A mature plant regenerated from transformed cells will contain the cloned gene in every cell and will pass the cloned gene to its offspring. However, regeneration of a transformed plant can occur only if the Ti vector has been "disarmed" so that the transformed cells do not display cancerous properties. Disarming is possible because the cancer genes, all of which lie in the T-DNA, are not needed for the infection process, infectivity being controlled mainly by the virulence region of the Ti plasmid. In fact, the only parts of the T-DNA that are involved in infection are two 25 bp repeat sequences found at the left and right borders of the region integrated into the plant DNA.

Any DNA placed between these two repeat sequences will be treated as "T-DNA" and transferred to the plant. It is therefore possible to remove all the cancer genes from the normal T-DNA, and replace them with an entirely new set of genes, without disturbing the infection process.

A number of disarmed Ti cloning vectors are now available, a typical example being the binary vector pBIN19 (Figure 7.14). The left and right T-DNA borders present in this vector flank a copy of the lacZ′ gene, containing a number of cloning sites, and a kanamycin resistance gene that functions after integration of the vector sequences into the plant chromosome. As with a yeast shuttle vector, the initial manipulations

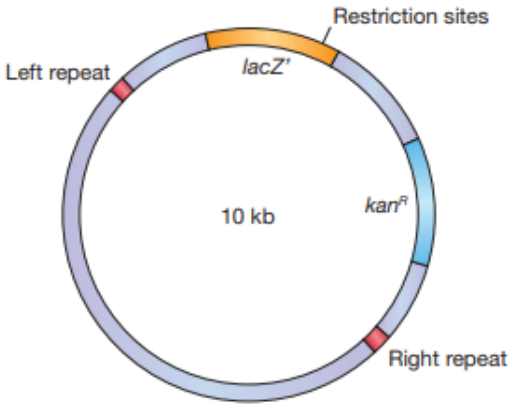


Figure 7.14
The binary Ti vector pBIN19. *kan*^{R} = kanamycin resistance gene.

that result in insertion of the gene to be cloned into pBIN19 are carried out in E. coli, the correct recombinant pBIN19 molecule then being transferred to A. tumefaciens and thence into the plant. Transformed plant cells are selected by plating onto agar medium containing kanamycin.

The Ri plasmid

Over the years there has also been interest in developing plant cloning vectors based on the Ri plasmid of Agrobacterium rhizogenes. Ri and Ti plasmids are very similar, the main difference being that transfer of the T-DNA from an Ri plasmid to a plant results not in a crown gall but in hairy root disease, typified by a massive proliferation of a highly branched root system. The possibility of growing transformed roots at high density in liquid culture has been explored by biotechnologists as a potential means of obtaining large amounts of protein from genes cloned in plants.

Limitations of cloning with Agrobacterium plasmids

Higher plants are divided into two broad categories, the monocots and the dicots. Several factors have combined to make it much easier to clone genes in dicots such as tomato, tobacco, potato, peas, and beans, but much more difficult to obtain the same results with monocots. This has been frustrating because monocots include wheat, barley, rice, and maize, which are the most important crop plants and hence the most desirable targets for genetic engineering projects.

The main difficulty stems from the fact that in nature A. tumefaciens and A. rhizogenes infect only dicotyledonous plants; monocots are outside of the normal host range. For some time it was thought that this natural barrier was insurmountable and that monocots were totally resistant to transformation with Ti and Ri vectors, but eventually artificial techniques for achieving T-DNA transfer were devised. However, this was not the end of the story. Transformation with an Agrobacterium vector normally involves regeneration of an intact plant from a transformed protoplast, cell, or callus culture. The ease with which a plant can be regenerated depends very much on the particular species involved and, once again, the most difficult plants are the monocots. Attempts to circumvent this problem have centered on the use of biolistics— bombardment with microprojectiles (p. 85)—to introduce plasmid DNA directly into plant embryos. Although this is a fairly violent transformation procedure it does not appear to be too damaging for the embryos, which still continue their normal development program to produce mature plants. The approach has been successful with maize and several other important monocots.

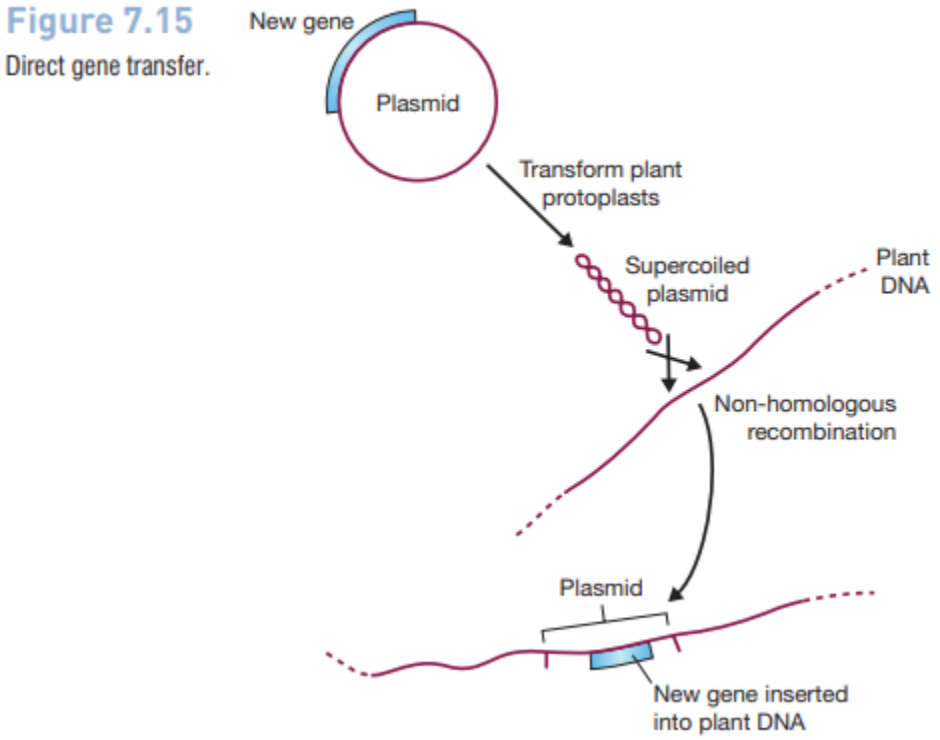


Fig 8 Cloning genes in plants by direct gene transfer

Biolistics circumvents the need to use Agrobacterium as the means of transferring DNA into the plant cells. Direct gene transfer takes the process one step further and dispenses with the Ti plasmid altogether.

Direct gene transfer into the nucleus Direct gene transfer is based on the observation, first made in 1984, that a supercoiled bacterial plasmid, although unable to replicate in a plant cell on its own, can become integrated by recombination into one of the plant chromosomes. The recombination event is poorly understood but is almost certainly distinct from the processes responsible for T-DNA integration. It is also distinct from the chromosomal integration of a yeast vector, as there is no requirement for a region of similarity between the bacterial plasmid and the plant DNA. In fact, integration appears to occur randomly at any position in any of the plant chromosomes (Figure 7.15). Direct gene transfer therefore makes use of supercoiled plasmid DNA, possibly a simple bacterial plasmid, into which an appropriate selectable marker (e.g., a kanamycin resistance gene) and the gene to be cloned have been inserted. Biolistics is frequently used to introduce the plasmid DNA into plant embryos, but if the species being engineered can be regenerated from protoplasts or single cells, then other strategies, possibly more efficient than biolistics,

are possible. One method involves resuspending protoplasts in a viscous solution of polyethylene glycol, a polymeric, negatively charged compound that is thought to precipitate DNA onto the surfaces of the protoplasts and to induce uptake by endocytosis (Figure 7.16). Electroporation is also sometimes used to increase transformation frequency. After treatment, protoplasts are left for a few days in a solution that encourages regeneration of the cell walls. The cells are then spread onto selective medium to identify transformants and to provide callus cultures from which intact plants can be grown (exactly as described for the Agrobacterium system, Figure 7.13b).

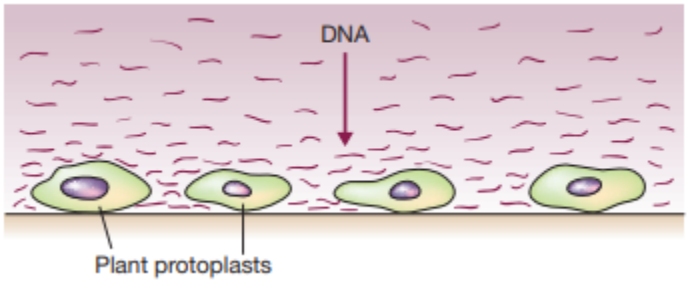


Figure 7.16
Direct gene transfer by precipitation of DNA onto the surfaces of protoplasts.

Transfer of genes into the chloroplast genome

If biolistics is used to introduce DNA in a plant embryo, then some particles may penetrate one or more of the chloroplasts present in the cells. Chloroplasts contain their own genomes, distinct from (and much shorter) than the DNA molecules in the nucleus, and under some circumstances plasmid DNA can become integrated into this chloroplast genome. Unlike the integration of DNA into nuclear chromosomes, integration into the chloroplast genome will not occur randomly. Instead the DNA to be cloned must be flanked by sequences similar to the region of the chloroplast genome into which the DNA is to be inserted, so that insertion can take place by homologous recombination (see p. 107). Each of these flanking sequences must be 500 bp or so in length. A low level of chloroplast transformation can also be achieved after PEG-induced DNA delivery into protoplasts if the plasmid that is taken up carries these flanking sequences.

A plant cell contains tens of chloroplasts, and probably only one per cell becomes transformed, so the inserted DNA must carry a selectable marker such as the kanamycin resistance gene, and the embryos must be treated with the antibiotic for a considerable period to ensure that the transformed genomes propagate within the cell. Although this

means that chloroplast transformation is a difficult method to carry out successfully, it could become an important adjunct to the more traditional methods for obtaining GM crops. As each cell has many chloroplasts, but only one nucleus, a gene inserted into the chloroplast genome is likely to be expressed at a higher level than one placed in the nucleus. This is particularly important when the engineered plants are to be used for production of pharmaceutical proteins (Chapter 13). So far the approach has been most successful with tobacco but chloroplast transformation has also been achieved with more useful crops such as soybean and cotton.'

Attempts to use plant viruses as cloning vectors

Modified versions of e and M13 bacteriophages are important cloning vectors for E. coli (Chapter 6). Most plants are subject to viral infection, so could viruses be used to clone genes in plants? If they could, then they would be much more convenient to use than other types of vector, because with many viruses transformation can be achieved simply by rubbing the virus DNA onto the surface of a leaf. The natural infection process then spreads the virus throughout the plant. The potential of plant viruses as cloning vectors has been explored for several years but without great success. One problem is that the vast majority of plant viruses have genomes not of DNA but of RNA. RNA viruses are not so useful as potential cloning vectors because manipulations with RNA are more difficult to carry out. Only two classes of DNA virus are known to infect higher plants, the caulimoviruses and geminiviruses, and neither is ideally suited for gene cloning.

Caulimovirus

vectors Although one of the first successful plant genetic engineering experiments, back in 1984, used a caulimovirus vector to clone a new gene into turnip plants, two general difficulties with these viruses have limited their usefulness. The first is that the total size of a caulimovirus genome is, like that of e, constrained by the need to package it into its protein coat. Even after deletion of non-essential sections of the virus genome, the capacity for carrying inserted DNA is still very limited. Recent research has shown that it might be possible to circumvent this problem by adopting a helper virus strategy, similar to that used with phagemids (p. 96). In this strategy, the cloning vector is a cauliflower mosaic virus (CaMV) genome that lacks several of the essential genes, which means that it can carry a

large DNA insert but cannot by itself direct infection. Plants are inoculated with the vector DNA along with a normal CaMV genome. The normal viral genome provides the genes needed for the cloning vector to be packaged into virus proteins and spread through the plant. This approach has considerable potential, but does not solve the second problem, which is the extremely narrow host range of caulimoviruses. This restricts cloning experiments to just a few plants, mainly brassicas such as turnips, cabbages, and cauliflowers. Caulimoviruses have, however, been important in genetic engineering as the source of highly active promoters that work in all plants and that are used to obtain expression of genes introduced by Ti plasmid cloning or direct gene transfer.

Geminivirus vectors

What of the geminiviruses? These are particularly interesting because their natural hosts include plants such as maize and wheat, and they could therefore be potential vectors for these and other monocots. But geminiviruses have presented their own set of difficulties, one problem being that during the infection cycle the genomes of some geminiviruses undergo rearrangements and deletions, which would scramble up any additional DNA that has been inserted, an obvious disadvantage for a cloning vector. Research over the years has addressed these problems, and geminiviruses are beginning to find some specialist applications in plant gene cloning. One of these is in virusinduced gene silencing (VIGS), a technique used to investigate the functions of individual plant genes. This method exploits one of the natural defence mechanisms that plants use to protect themselves against viral attack. This method, called RNA silencing, results in degradation of viral mRNAs. If one of the viral RNAs is transcribed from a cloned gene contained within a geminivirus genome, then not only the viral transcripts but also the cellular mRNAs derived from the plant's copy of the gene are degraded (Figure 7.17). The plant gene therefore becomes silenced and the effect of its inactivation on the phenotype of the plant can be studied.

Viruses as cloning vectors for mammals

For many years it was thought that viruses would prove to be the key to cloning in mammals. This expectation has only partially been realized. The first cloning experiment involving mammalian cells was carried out in 1979 with a vector based on simian virus 40 (SV40). This virus is capable of infecting several mammalian species, following a lytic cycle in some hosts and a lysogenic cycle in others. The genome is 5.2 kb in size (Figure

7.19a) and contains two sets of genes, the “early” genes, expressed early in the infection cycle and coding for proteins involved in viral DNA replication, and the “late” genes, coding for viral capsid proteins. SV40 suffers from the same problem as e and the plant caulimoviruses, in that packaging constraints limit the amount of new DNA that can be inserted into the genome. Cloning with SV40 therefore involves replacing one or more of the existing genes with the DNA to be cloned. In the original experiment a segment of the late gene region was replaced (Figure 7.19b), but early gene replacement is also an option. In the years since 1979, a number of other types of virus have been used to clone genes in mammals. These include:

- Adenoviruses, which enable DNA fragments of up to 8 kb to be cloned, longer than is possible with an SV40 vector, though adenoviruses are more difficult to handle because their genomes are bigger.
- Papillomaviruses, which also have a relatively high capacity for inserted DNA. Bovine papillomavirus (BPV), which causes warts on cattle, is particularly attractive because it has an unusual infection cycle in mouse cells, taking the form of a multicopy plasmid with about 100 molecules present per cell. It does notcause the death of the mouse cell, and BPV molecules are passed to daughter cells on cell division, giving rise to a permanently transformed cell line. Shuttle vectors consisting of BPV and E. coli sequences, and capable of replication in both mouse and bacterial cells, have been used for the production of recombinant proteins in mouse cell lines.

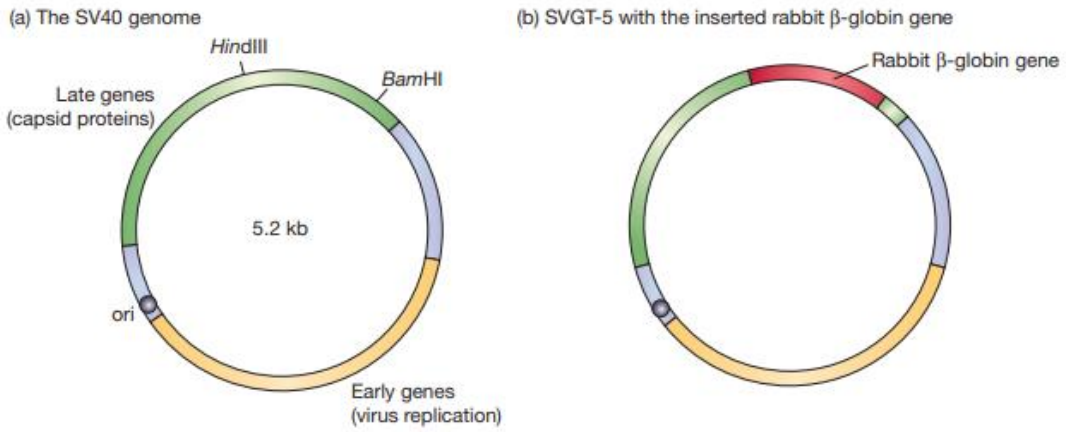


Figure 7.19

SV40 and an example of its use as a cloning vector. To clone the rabbit β-globin gene the *Hin*dIII to *Bam*HI restriction fragment was deleted (resulting in SVGT-5) and replaced with the rabbit gene.

- **Adeno-associated virus (AAV),** which is unrelated to adenovirus but often found in the same infected tissues, because AAV makes use of some of the proteins synthesized by adenovirus in order to complete its replication cycle. In the absence of this helper virus, the AAV genome inserts into its host's DNA. With most integrative viruses this is a random event, but AAV has the unusual property of always inserting at the same position, within human chromosome 19. Knowing exactly where the cloned gene will be in the host genome is important if the outcome of the cloning experiment must be checked rigorously, as is the case in applications such as gene therapy. AAV vectors are therefore looked on as having major potential in this area.
- **Retroviruses**, which are the most commonly-used vectors for gene therapy. Although they insert at random positions, the resulting integrants are very stable, which means that the therapeutic effects of the cloned gene will persist for some time

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – 5 – GENETIC ENGINEERING – SBB2102

IDENTIFICATION OF GENETIC TRANSFORMANTS AND THEIR APPLICATIONS

1. Selection of recombinants

The need to identify the cells that contain the desired insert at the appropriate and right orientation and isolate these from those not successfully transformed is of utmost importance to researchers. Modern cloning vectors include selectable markers (most frequently antibiotic resistance markers) that allow only cells in which the vector, but not necessarily the insert, has been transformed to grow. Additionally, the cloning vectors may contain color selection markers which provide blue/white screening (via alpha-factor complementation) on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present in the cells. Further investigation of the resulting colonies is required to confirm that cloning was successful. This may be accomplished by means of PCR, restriction fragment analysis and/or DNA sequencing.

A host cell having foreign DNA introduced in it is called a transformant. At the end of the transformation experiment, we get bacterial cells that may contain non-recombinant vector, desired recombinant vector or undesired recombinant vector or may not contain any vector i.e. non-transformants. To identify the clone containing desired piece of DNA from among several others, screening is carried out in two steps:

Selection of clones carrying recombinants

The selection of recombinants is generally done on the basis of marker genes present in the vector. There are two types of marker genes, selectable marker and a reporter gene or scorable marker.

Selectable markers

A selectable marker gene codes for a function which enables only those cells which possess it to survive under suitable conditions. For example, genes conferring resistance to antibiotics like ampicillin, tetracycline and kanamycin are good selectable markers. When a population of bacterial cells is plated on an ampicillin containing medium, only those cells that have ampicillin resistance genes survive and form colonies.

Reporter genes

A reporter gene produces a protein product whose activity can be assayed and permits either an easy selection or quick identification of cells in which it is present. Therefore it is also called a scorable marker. Among the more commonly used reporter genes are gus (codes for β glucuronidase which produces blue colour in the presence of suitable substrate), lux (luciferase, produces phosphorescence, gfp (green fluorescence protein, fluoresces on irradiation with U.V.). Some examples making use of selectable and or scorable markers to identify recombinant clones are listed below:

i) Insertional inactivation of antibiotic resistance gene This can be explained with the help of pBR322 which has two selectable markers i.e. Apr and Tcr . Both these genes have unique cloning sites in them. Insertion of foreign DNA in any of the sites causes inactivation of that gene. The recombinants thus become susceptible to one of the antibiotics while non-recombinants are resistant to both.

ii) Insertional inactivation of lacZ gene Some vectors contain a gene or sometimes only part of a gene, which complements a function missing in their host cells, e.g. lacZ gene (encodes fragment of β-galactosidase) in the pUC vectors, M13 and some λ phage vectors which complements defective lacZ gene (encodes part of β- galactosidase) in E. coli host strains. Insertion of foreign gene in the vector causes inactivation of lacZ gene. The recombinants are identified by formation of white colonies /plaques while nonrecombinants form blue colonies /plaques (Fig.11).

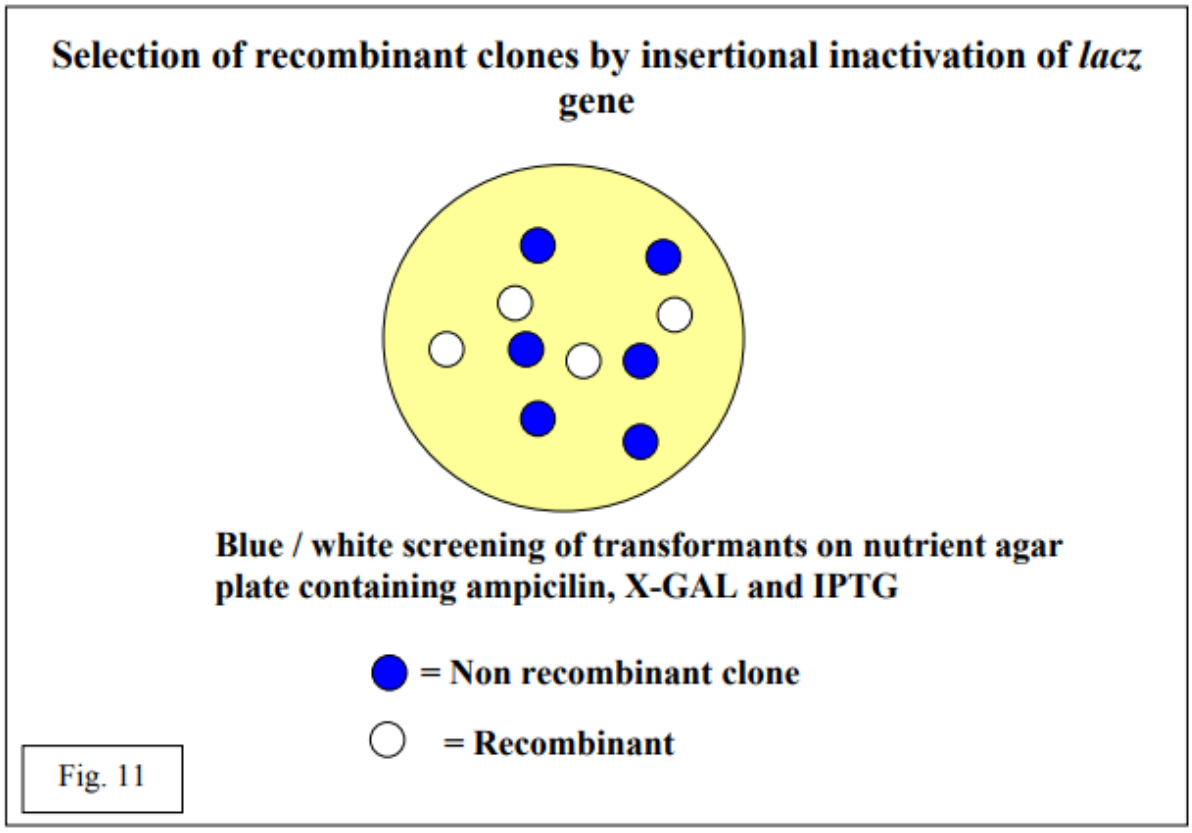


Fig 1

iv) Selection on the basis of λ genome size The λ packaging system can only insert DNA molecules between 38 and 52 kb size into the phage head. Anything less than 38 kb is not packaged. In some vectors e.g. λ replacement vectors, the size of the vector is less than 38 kb. The length of DNA insert can be so adjusted as to allow the packaging of only the recombinant DNA.

v) Selection based on Spi (sensitive to phage infection) phenotype Phage λ cannot infect E.coli cells that have P2 phage integrated in their genome and is said to be Spi+ . Insertion of foreign DNA causes change in phenotype from Spi+ to Spi− . The recombinants can infect E.coli having P2 phage while non-recombinants cannot.

vi) Selection based on growth on minimal medium In yeast, an auxotrophic mutant that has non-functional leu2 gene is used as a host. Such a mutant is able to survive only if leucine is supplied in the growth medium. However, transformants are able to grow on a minimal medium (contains no added leucine) due to presence of leu2 gene in the vector (e.g. YEps).

Visual screening

The blue-white screening The blue white screening is one of the most common molecular techniques that allow detecting the successful ligation of gene of interest in vector (Langley et al., 1975; Zamenhof & Villarejo 1972; Ausubel et al., 1988). The **β**-Complementation plasmids are among the most commonly used vectors for cloning and sequencing the DNA fragments, as they generally have a good multiple cloning site and an efficient blue-white screening system for identification of recombinants in presence of a histochemical dye, 5-bromo-4-chloro-3- indolyl-**β**-d-galactoside (X-gal), and binding sites for commercially available primers for direct sequencing of cloned fragments (Manjula 2004). The molecular mechanism for blue/white screening is based on a genetic engineering of the lac operon in the E. coli as a host cell combined with a subunit complementation achieved with the cloning vector. The lacZ product, a polypeptide of 1029 amino acids, gives rise to the functional enzyme after tetramerization (Jacobson et al., 1994) and is easily detected by chromogenic substrates either in cell lysates or directly on fixed cells in situ (Ko et al., 1990). The tetramerization is dependent on the presence of the N-terminal region spanning the first 50 residues. Deletions in the N-terminal sequence generate a so-called omega peptide that is unable to tetramerize and does not display enzymatic activity. The activity of the omega peptide can be fully restored either in bacteria or in vitro, if a small fragment (called alpha peptide) corresponding to the intact N-terminal portion is added in trans (Gallagher et al., 1994). The phenomenon is called α-complementation and the small N-terminal peptide is called alpha peptide. This effect has been widely exploited for studies in prokaryotes, where special strains that constitutively express omega peptide exist and allow the detection of expression of the small alpha peptide.

The vector (e.g. pBluescript) encodes the ⌟ subunit of LacZ protein with an internal multiple cloning site (MCS), while the chromosome of the host strain encodes the remaining omega subunit to form a functional **β**,-galactosidase enzyme upon complementation. The foreign DNA can be inserted within the MCS of lacZα gene, thus disrupting the formation of functional **β**,-galactosidase. The chemical required for this screen is X-gal, a colorless modified galactose sugar that is metabolized by **β**,-galactosidase to form 5-bromo-4-chloroindoxyl which is spontaneously oxidized to the bright blue insoluble pigment 5,5'-dibromo4,4'-dichloro-indigo and thus functions as an indicator. Isopropyl **β**,-D-1- thiogalactopyranoside (IPTG) which functions as the inducer of the Lac operon, can be used to enhance the phenotype. The hydrolysis of colorless X-gal by the **β**,galactosidase causes the characteristic blue colour in the colonies indicating that the colonies contain vector without insert. White colonies indicate insertion of foreign DNA and loss of the cells ability to hydrolyze the marker. Bacterial colonies in general, however, are white, and so a bacterial colony with no vector will also appear white. These are usually suppressed by the presence of an antibiotic in the growth medium. Blue white screening is thus a quick and easy technique that allows for the screening of successful cloning reactions through the color of the bacterial colony. However, the correct type of vector and competent cells are important considerations when planning a blue white screen. Although the lacZ and many other systems have been extensively used for gram negative bacteria like E. coli, there are limited options available

for screening recombinants transformed in Gram positive bacteria. Chaffin & Rubens (1998) have developed a gram positive cloning vector pJS3, that utilizes the interruption of an alkaline phosphatase gene, phoZ, to identify

recombinant plasmids. A multiple cloning site (MCS) was inserted distal to the region coding for the putative signal peptide of phoZ where the alkaline phosphatase protein expressed from the phoZ gene (phoZMCS) retained activity similar to that of the native protein and cells displayed a blue colonial phenotype on agar containing 5-bromo-4-chloro-3-indolyl phosphate (X-p). Introduction of any foreign DNA into the MCS of phoZ produced a white colonial phenotype on agar containing X-p and allowed discrimination between transformants containing recombinant plasmids versus those maintaining self-annealed or uncut vector. This cloning vector has improved the efficiency of recombinant DNA experiments in gram-positive bacteria. Cloning inserts into the multiple cloning region of the pGEMÂ®-Z Vectors disrupts the alpha-peptide coding sequences, and thus inactivates the beta-galactosidase enzyme resulting in white colonies. Recombinant plasmids are transformed into the appropriate strain of bacteria (i.e. JM109, DH5), and subsequently plated on indicator plates containing 0.5 mM IPTG and 40 µg/ml X-gal. A new version of TA cloning vector with directional enrichment and blue-white color screening has been reported by Horn (2005).

Limitations of blue-white screening

The "blue screen" technique described above suffers from the disadvantage of using a screening procedure (discrimination) rather than a procedure for selecting the clones. Discrimination is based on visually identifying the recombinant within the population of clones on the basis of a color. The LacZ gene, in the vector used for generating recombinants, may be non-functional and may not produce β-galactosidase. As a result, these cells cannot convert X-gal to the blue substance so the white colonies seen on the plate may not be recombinants but just the background vector.

A few white colonies might not contain the desired recombinant but a small piece of DNA to be ligated into the vector's MCS might change the reading frame for LacZα, and thus prevent its expression giving rise to false positive clones. Furthermore, a few linearized vectors may get transformed into the bacteria, the ends "repaired" and ligated together such that no Lac α is produced as a result, these cells cannot convert X-gal to the blue substance. On the other hand, in some cases, blue colonies may contain the insert, when the insert is "in frame" with the LacZα gene and is devoid of stop codon. This could sometimes lead to the expression of a fusion protein that is still functional as LacZα. Small inserts which happen to be in frame with the alpha-peptide coding region may produce light blue colonies, as beta galactosidase activity is only partially inactivated. Last but not the least, this complex procedure requires the use of the substrate X-gal which is very expensive, unstable and is cumbersome to use.

Reporter gene based screening

Another method for screening and identification of recombinant clones is by using the green fluorescent protein (GFP) obtained from jellyfish Aequorea victoria. It is a reporter molecule for monitoring gene expression, protein localization, protein-protein interaction etc. GFP has been expressed in bacteria, yeast, slime mold, plants, drosophila, zebrafish and in mammalian cells. Inouye et al., (1997) have described a bacterial cloning vector with mutated Aequorea GFP protein as an indicator for screening recombinant plasmids. The pGREENscript A when expressed in E. coli produced colonies showing yellow color in day light and strong green fluorescence under long-UV. Inserted foreign genes are selected on the basis of loss of the fluorescence caused by inactivation of the GFP production. The vector used in the study is a derivative of pBluescript SK(+) (Short et al., 1998) and encodes for the same MCS flanked by T3 and T7 promoters, but lacks the lacZ gene and the f1 origin region (for single strand DNA production). Instead, the GFP-S65A cDNA is substituted in its place and is under the control of the lac promoter/operator. The insertion of foreign DNAs into MCS of pGreenscript A interferes with the production of GFPS65A and causes a loss in the green fluorescence and yellow color of E. coli colonies. While GFP solubility appears to be one of the limiting factors in whole cell fluorescence, Davis & Vierstra (1998) have reported about soluble derivatives of GFP for use in Arabidopsis thaliana.

A system for direct screening of recombinant clones in Lactococcus lactis, based on secretion of the staphylococcal nuclease (SNase) in the organism, was developed by Loir and co-workers (Loir et al, 1994). L. lactis strains containing the nuc' plasmids secrete SNase and are readily detectable by a simple plate test. An MCS was introduced just after the cleavage site between leader peptide and the mature SNase, without affecting the nuclease activity. Cloning foreign DNA fragments into any site of the MCS interrupts nuc gene and thus results in nuc mutant clones which are easily distinguished from nuc' clones on plates. The biggest advantage of this vector is the possibility of assessing activity of the fusion protein since the nuclease activity is not diminished by its N-terminal tail and is also reported to be unaffected by denaturing agents such as sodium dodecyl sulfate (SDS) or trichloroacetic acid.

Limitations of the reporter gene based screening

All the above described plasmids could also result in false positive clones, which is a major concern for researchers. Loss of GFP fluorescence due to medium composition is also known to lead to false positive results. Although the SNase based screening would give absolute 100% recombinants, the active nuc fusion protein expression might render the cell fragile and enhance its susceptible to the lethal action of the fusion protein upon hyper expression. Also, for all the above cases, there is a requirement of transfer the genes of interest from the cloning vector to the expression vectors which calls for fresh cloning followed by screening for recombinants. Hence, it is evident that the commonly used method for screening and identification of recombinant clones are associated with problems of false positive results forcing researchers to look for alternate methods of screening bacterial recombinants and availability of vectors that would act both as cloning vectors and expression vector are user friendly and advantageous.

Indirect methods for clone identification

PCR

Reverse transcription followed by polymerase chain reaction (RT-PCR) can be used for amplification of RNA sequences in cDNA form. Using gene specific primers, it is possible to clone specific cDNA molecules from total cellular RNA without need to purify mRNA. Under certain situations such as when starting DNA material is very small, for example, single cells, it is difficult to prepare genomic libraries by cell based cloning. In these cases, PCR is the only available alternative for gene isolation if specific primers are available. However, besides amplifying specific fragments, PCR can also be used to generate random genomic libraries with the help of random primers. One limitation of PCR is that the enzyme Taq polymerase can amplify only small fragments (1-2 kb). In recent years this has been overcome to some extent and it has been possible to amplify DNA fragments up to 22 kb by method called long PCR.

Screening of transformants for presence of desired gene/gene product

A number of methods have been devised for screening of desired transformants. These include the following:-

1. Direct selection for the desired gene
2. Identification of the clone from a gene library

Direct selection for the desired gene

In this method, the cloning experiment is designed in such a way that only the desired recombinant clones are obtained. Selection here occurs at the plating out stage. An example of direct selection is cloning of genes that specify antibiotic resistance such as kanamycin, tetracycline or ampicillin resistance. For example, let us consider selection of recombinants that contain gene for kanamycin resistance. The transformants are plated on agar medium containing kanamycin. Only the cells that contain the cloned kanamycin resistance gene are able to survive and form colonies. Hence the technique is called direct selection.

Another situation where direct selection of recombinant clones is done is the marker rescue technique (Fig. 19). It makes use of auxotrophic mutant strains that have nutritional defects as the hosts for transformation. For example, suppose an E.coli strain is available which has a mutation in a gene encoding an enzyme involved in the biosynthesis of amino acid leucine. Such a mutant strain will only grow in a medium supplemented with leucine. By cloning DNA from a normal strain i.e. one that can synthesize its own leucine, in the mutant strain and selecting those transformants that can grow in absence of leucine it is possible to isolate the gene of interest.

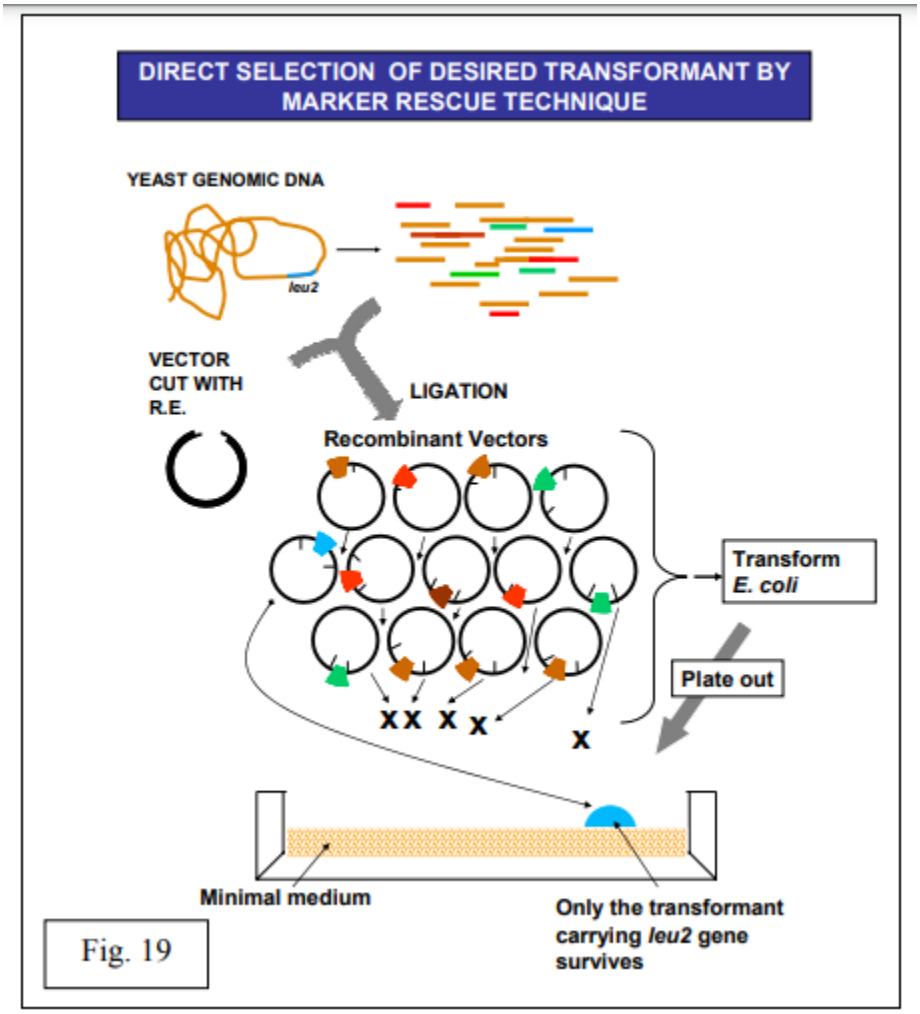


Fig 2

Marker rescue is applicable for most genes that encode for biosynthetic enzymes as clones of these genes can be studied on minimal medium as described for leucine. Auxotrophic strains of yeast and filamentous fungi are also available and marker rescue has been used to select genes cloned in these organisms. However, the technique has two limitations: i) a mutant strain must be available for the gene in question ii) a medium on which only the wild type can survive is needed.

Identification of the clones from a gene library

A library has to be screened in order to find a clone. The identification of a specific clone from a DNA library can be carried out by exploiting either the sequence of the clone or the structure/ function of its expressed product. The strategy for screening depends upon information about the gene of interest, the availability of probe and the cloning method used. One of the key elements required to identify a gene during screening is a probe. A probe is a piece of DNA or RNA that contains a portion of the sequence complementary to the desired gene for which we are

searching. It is used to detect specific nucleic acid sequences by hybridization (based on complementarity). The probe can be labeled radioactively (with P32) or nonradioactively (biotin, digoxigenin and fluorescent dyes etc). Probes can be chemically synthesized based on the amino acid sequence of the protein coded by the gene. Probes can be homologous or heterologous.

Homologous probe - a probe that is exactly complementary to the nucleic acid sequence for which we are searching; e. g. a human cDNA used for searching a human genomic library.

Heterologous probe - a probe that is similar to, but not exactly complementary to the nucleic acid sequence for which we are searching; e.g., a mouse cDNA probe used to search a human genomic library.

There are several methods for screening DNA libraries. Some of the commonly used methods are described below:

1. Methods based on nucleic acid hybridization
2. Immunochemical methods
3. Screening DNA libraries using PCR

Methods based on nucleic acid hybridization

- Nucleic acid hybridization is the most commonly used method of library screening first developed by Grunstein and Hogness in1975 to detect DNA sequences in transformed colonies using radioactive RNA probes.
- It relies on the fact that a single-stranded DNA molecule, used as a probe can hybridize to its complementary sequence and identify the specific sequences.
- This method is quick, can handle a very large number of clones and used in the identification of cDNA clones which are not full-length (and therefore cannot be expressed).

a) Colony/ plaque hybridization

This method was given by Grunstein and Hogness (1975). It is used for screening both genomic as well as cDNA libraries and is the most common method of library screening. The procedure has following steps:

1. The recombinant bacterial colonies or phage plaques to be screened are transferred from the culture plate on to a nitrocellulose filter paper by replica plating (Fig. 20).
2. The filter with colony replicas is treated with NaOH to lyse the cells/ phages and to denature DNA.
3. The filter is then baked at 800 C for two hours in vacuum oven to fix the DNA.
4. The filter is allowed to hybridize with a labeled probe.
5. The filter is washed to remove the unbound excess probe, dried and then subjected to autoradiography if the probe is radioactively labeled. The washing conditions are kept stringent (high temperature and low salt concentration) when the probe is homologous and non-stringent (lower temperature and high salt concentration) in case of heterologous probe.

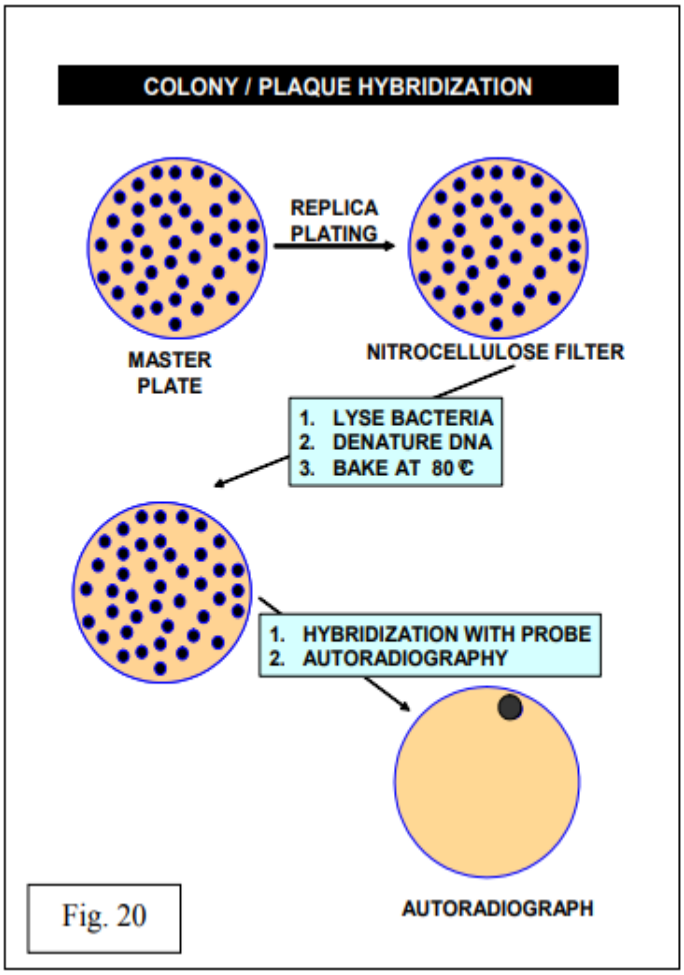


Fig 3

Advantages

- This method results in a 'cleaner' background and distinct signal (less background probe hybridization) for λ plaque screening due to less DNA transfer from the bacterial host to the nitrocellulose membrane while lifting plaques rather than bacterial colonies.
- Multiple screens can be performed from the same plate as plaques can be lifted several times.
- Screening can be performed at very high density by screening small plaques. High-density screening has the advantage that a large number of recombinant clones can be screened for the presence of sequences homologous to the probe in a single experiment.

Probes used for hybridization

Cloned DNA fragments can be used as probes in hybridization reactions if a cDNA clone is available. DNA or synthetic oligonucleotide probes can be used for identification of a clone from

a genomic library instead of RNA probes, for example, to study the regulatory sequences which are not part of the cDNA clone. A common method of labeling probes is the incorporation of a radioactive or other marker into the molecule. A number of alternative labeling methods are also available that involve an amplification process to detect the presence of small quantities of bound probe and avoid the use of radioactivity. These methods involve the incorporation of chemical labels such as digoxigenin or biotin into the probe which can be detected with a specific antibody or the ligand streptavidin, respectively.

Screening by PCR

PCR screening is employed for the identification of rare DNA sequences in complex mixtures of molecular clones by increasing the abundance of a particular sequence. It is possible to identify any clone by PCR only if there is available information about its sequence to design suitable primers.

Preparation of a library for screening by PCR can be done by following ways-

- The library can be plated as plaques or colonies on agar plates and individually inoculated into the wells of the multi-well plate. However it is a labor intensive process and can lead to bias in favor of larger colonies or plaques.
- The alternative method involves diluting the library. It involves plating out a small part of the original library (the packaging mix for a phage library, transformation for a plasmid library) and calculating the titer of the library. A larger sample is diluted to give a titer of 100 colonies per mL. Dispensing 100 μL into each well theoretically gives 10 clones in each well. These are then pooled and PCR reactions are carried out with gene-specific primers flanking a unique sequence in the target to identify the wells containing the clone of interest. This method is often used for screening commercially available libraries.

Screening methods based on gene expression

Immunological screening

This involves the use of antibodies that specifically recognize antigenic determinants on the polypeptide. It does not rely upon any particular function of the expressed foreign protein, but requires an antibody specific to the protein

Earlier immunoscreening methods employed radio-labeled primary antibodies to detect antibody binding to the nitrocellulose sheet. It is now superseded by antibody sandwiches resulting in highly amplified signals. The secondary antibody recognizes the constant region of the primary antibody and is, additionally, conjugated to an easily assayable enzyme (e.g. horseradish peroxidase or alkaline phosphatase) which can be assayed using colorimetric change or emission of light using X-ray film.

- In this technique, the cells are grown as colonies on master plates and transferred to a solid matrix.
- These colonies are subjected to lysis releasing the proteins which bind to the matrix.
- These proteins are treated with a primary antibody which specifically binds to the protein (acts as antigen), encoded by the target DNA. The unbound antibodies are removed by washing.
- A secondary antibody is added which specifically binds to the primary antibody removing the unbound antibodies by washing.
- The secondary antibody carries an enzyme label (e.g., horse radishperoxidase or alkaline phosphatase) bound to it which converts colorless substrate to colored product. The colonies with positive results (i.e. colored spots) are identified and subcultured from the master plate.

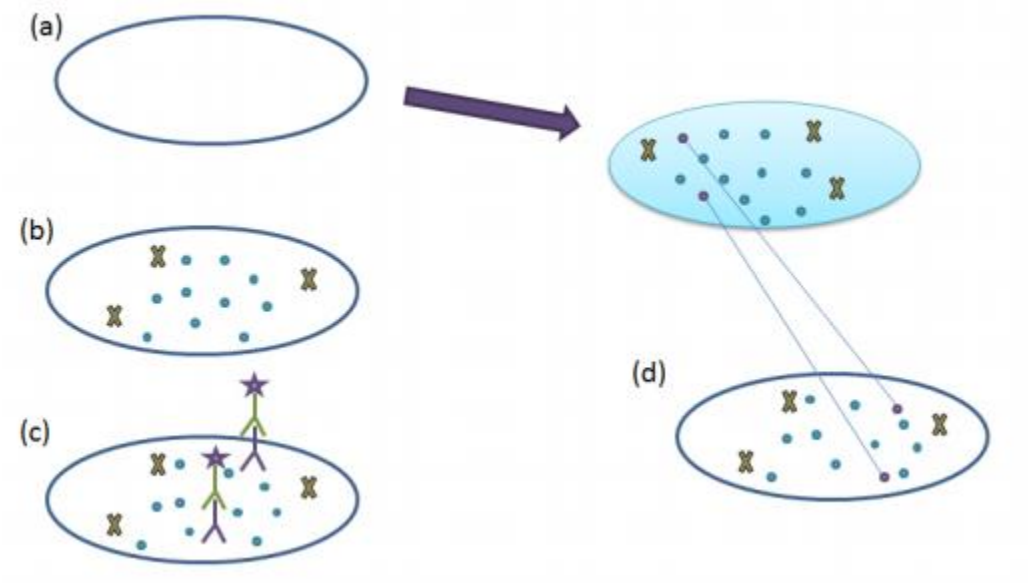


FIGURE 4:Schematic process of immunological screening (a) a nitrocellulose disk is placed onto the surface of an agar plate containing the phage library. Both agar plate and disk are marked so as to realign them later. (b) When the nitrocellulose disk is lifted off again, proteins released from the bacteria by phage lysis bind to the disk. (c) These proteins bind to specific antibody. (d) Plaques formed by bacteriophage that express the protein bound to the antibody will be detected by emission of light. The positive clones can be identified by realignment.

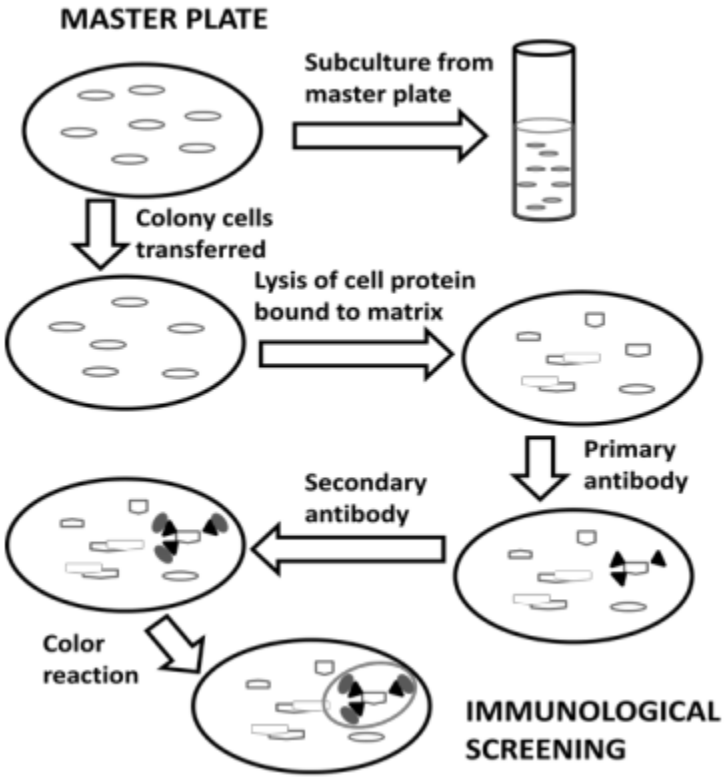


Figure 5: Schematic process of immunological screening using antibody sandwich.

The main difficulty with antibody-based screening is to raise a specific antibody for each protein to be detected by injecting a foreign protein or peptide into an animal. This is a lengthy and costly procedure and can only be carried out successfully with proteins produced in reasonably large amounts.

Screening by functional complementation

Functional complementation is the process of compensating a missing function in a mutant cell by a particular DNA sequence for restoring the wild-type phenotype. If the mutant cells are non-viable, the cells carrying the clone of interest can be positively selected and isolated. It is a very powerful method of expression cloning and also useful for identification of genes from an organism having same role as that of defective gene in another organism. The selection and

identification of positive clones is based on either the gain of function or a visible change in phenotype.

For example, the functional complementation in transgenic mice for the isolation of Shaker-2 gene applied by Probst et al in1988 shown in Figure

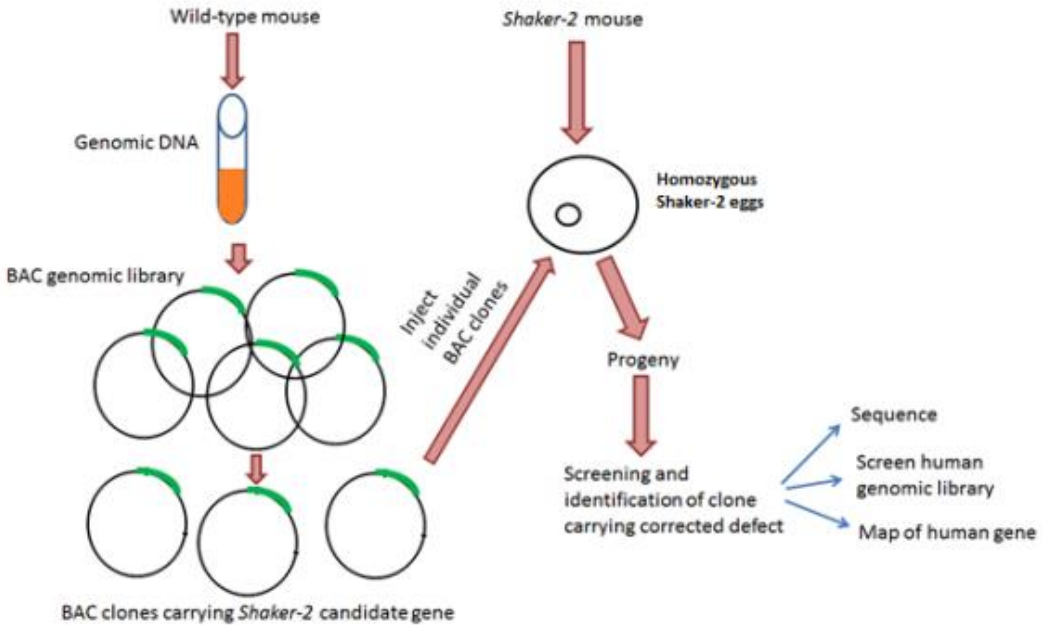


Figure 6. Functional complementation in transgenic mice for isolation of Shaker-2 gene

The Shaker-2 mutation is due to the defective gene associated with human deafness disorder. The BAC clone from the wild type mice are prepared and injected into the eggs of Shaker-2 mutants. The resulting mice are then screened for the presence of wild type phenotype. Thus the BAC clone carrying the functional Shaker-2 gene is identified which encodes a cytoskeletal myosin protein. This method can be used for screening human genomic libraries to identify equivalent human gene.

Drawbacks

- Presence of an assayable mutation within the host cell that can be compensated by the foreign gene expression which in most cases is not available. In addition, foreign genes may not fully compensate the mutations.

Applications

- This method can be used for the isolation of higher-eukaryotic genes (e.g. Drosophila topoisomerase II gene, a number of human RNA polymerase II transcription factors) from an organism.
- It can also be possible in transgenic animals and plants to clone a specific gene from its functional homologue.

Screening DNA libraries using PCR

PCR can be used as alternative to hybridization for screening of genomic and cDNA libraries. It is possible to identify any clone by PCR but only if there is sufficient information about its sequence to make suitable primers. To isolate a specific clone, PCR is carried out with gene specific primers that flank a unique sequence in the target. Pools of clones are maintained in multi-well plates. Each well is screened by PCR and positive wells are identified. The clones in each positive well are then diluted into a series in a secondary set of plates and screened again. The process is repeated until wells having homogenous clones corresponding to the gene of interest have been identified.

Some important techniques used in characterization of genes/gene products

A number of techniques are used for analyzing the genes/gene products. Some of these techniques are blotting and hybridization, DNA finger printing and Micro-array analysis. These are briefly discussed below.

Blotting and hybridization techniques

These techniques are used for detecting a specific gene sequence or its expression, number of copies of a gene, relatedness among organisms etc. There are three types of blotting techniques: Southern, Northern and Western blotting.

Southern Blotting

Principle:

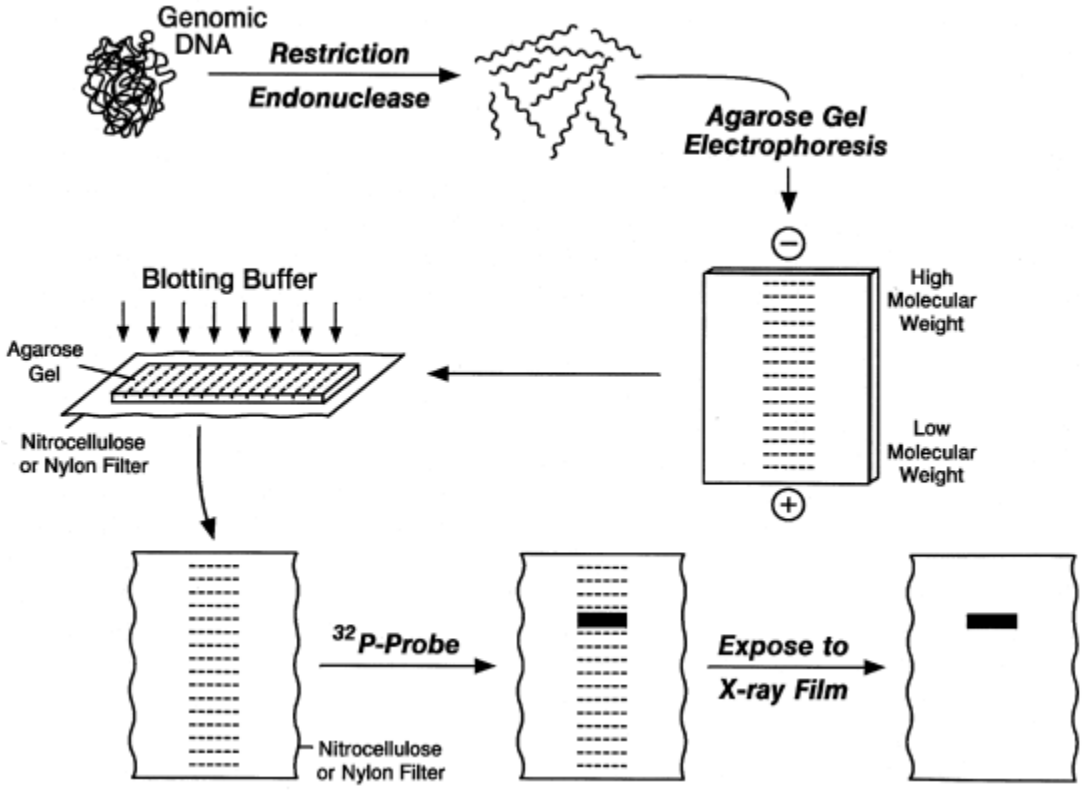
- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.

- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/ Steps

Restriction digest: by RE enzyme and amplification by PCR

1. Gel electrophoresis: SDS gel electrophoresis
2. Denaturation: Treating with HCl and NaOH
3. Blotting
4. Baking and Blocking with casein in BSA
5. Hybridization using labelled probes
6. Visualization by autoradiogram



Step I: Restriction digest

- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments

- The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

- The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.
- DNA strands get separated

Step IV: Blotting

- The separated strands of DNA is then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

- The DNA bound to membrane is then treated with labelled probe
- The labelled probe contains the complementary sequences to the gene of interest
- The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Application of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting
3. Used for paternity testing, criminal identification, victim identification
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism
6. To identify mutation or gene rearrangement in the sequence of DNA
7. Used in diagnosis of disease caused by genetic defects
8. Used to identify infectious agents

Northern Blotting Technique (Northern Analysis)

The term northern blot actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane. However, the entire process is commonly referred to as northern blotting. The northern blot technique was developed in 1977 by James Alwine, David Kemp, and George Stark at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot, named for biologist Edwin Southern. The major difference is that RNA, rather than DNA, is analyzed in the Northern blot.

Northern blotting involves:

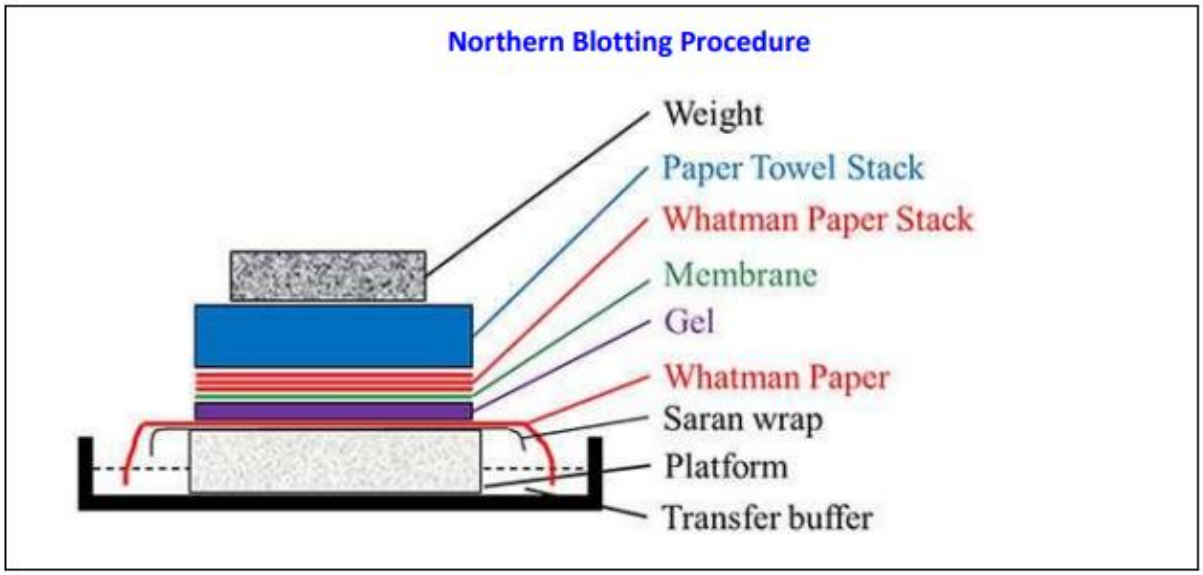
- The use of electrophoresis to separate RNA samples according to size and
- Detection of the target sequence using a hybridization probe, whose sequence is complementary to the part or to the entire target sequence.

Procedure

1. A general blotting procedure starts with extraction of total RNA from a homogenized tissue sample or from cells.

2.. RNA samples are then separated by gel electrophoresis.

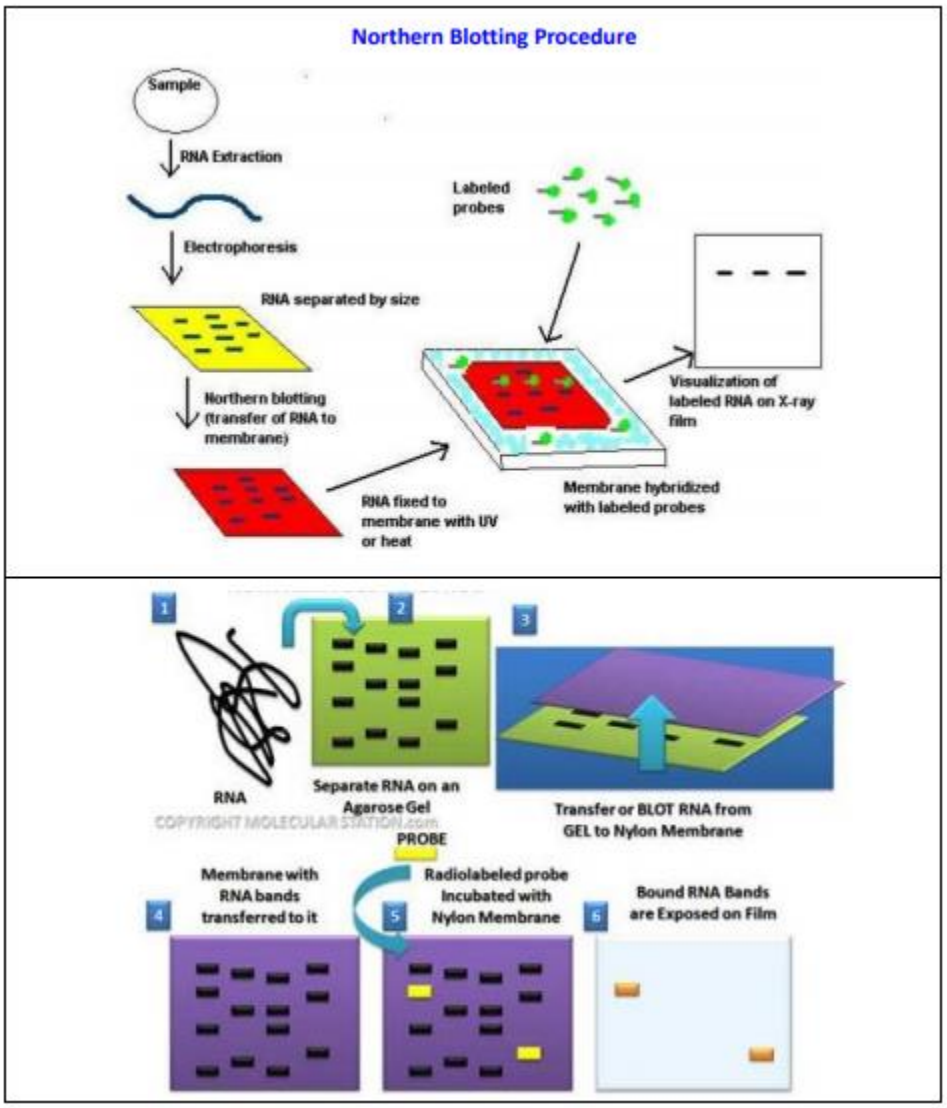
3. Since the gels are fragile and the probes are unable to enter the matrix, the RNA samples, now separated according to size, are transferred to a nylon membrane through a capillary or vacuum blotting system.



4. A sheet of nitrocellulose membrane is placed on top of the gel.

5. To ensure good and even contact between gel and membrane, pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel).

6. The transfer-buffer used for the Northern blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction, thus preventing RNA degradation by high temperatures.



Buffer-transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is then used to move the RNA from the gel on to the membrane; ion exchange interactions bind the RNA. to the membrane due to negative charge of the RNA and positive charge of the membrane. Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. Experimental conditions that can affect the efficiency and specificity of hybridization include ionic strength, viscosity, duplex length, mismatched base pairs, and base composition. The membrane is washed to ensure

that the probe has bound specifically and to avoid background signals from arising. The hybrid signals are then detected by X-ray film and can be quantified by densitometry.

Separation of RNA

The RNA samples are most commonly separated on agarose gels containing formaldehyde as a denaturing agent for the RNA to limit secondary structure. The gels can be stained with ethidium bromide (EtBr) and viewed under UV light to observe the quality and quantity of RNA before blotting. Polyacrylamide gel electrophoresis with urea can also be used in RNA separation but it is most commonly used for fragmented RNA or micro RNAs.

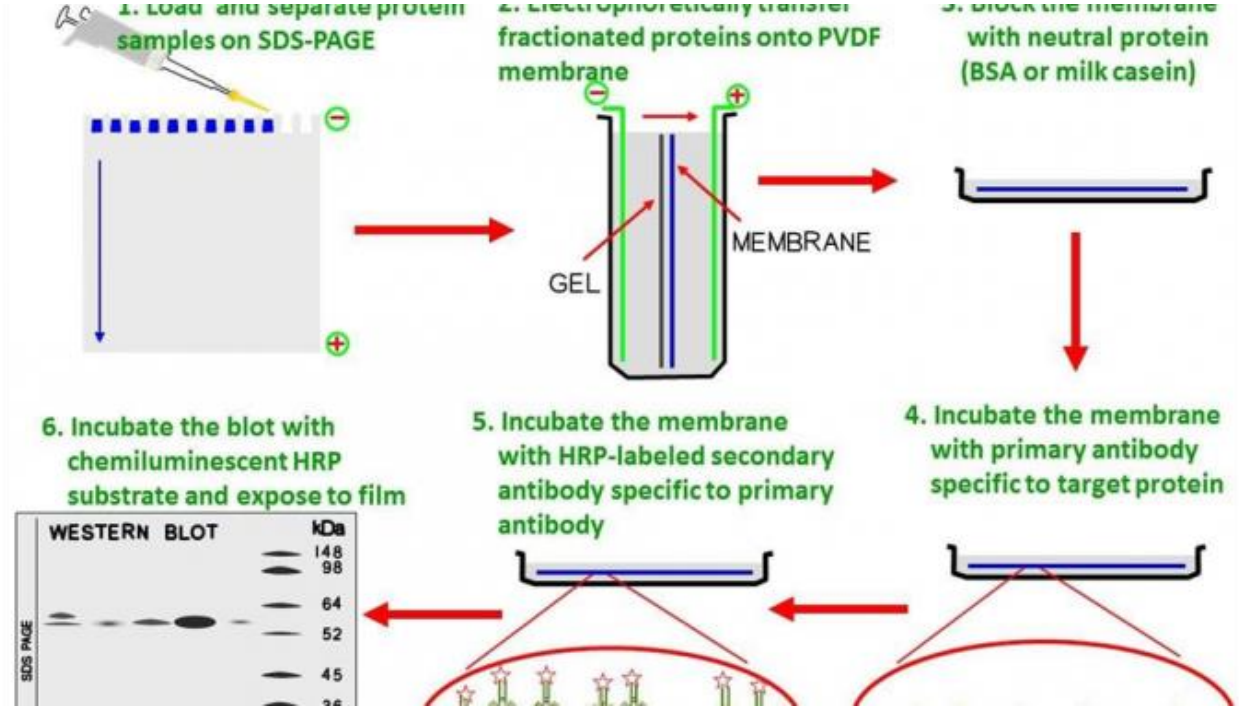
Detection with Probes

Probes for northern blotting are composed of nucleic acids with a complementary sequence to all or part of the RNA of interest, they can be DNA, RNA, or oligonucleotides with a minimum of 25 complementary bases to the target sequence. Commonly, cDNA is created with labelled primers for the RNA sequence of interest to act as the probe in the northern blot. The probes need to be labelled either with radioactive isotopes (^{32}P) or with chemiluminescence, which produces detectable emission of light. X-ray film can detect both the radioactive and chemiluminescent signals and many researchers prefer the chemiluminescent signals because they are faster, more sensitive, and reduce the health hazards that go along with radioactive labels. The same membrane can be probed up to five times without a significant loss of the target RNA.

Applications

1. Northern blotting allows one to observe expression pattern of a particular gene between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment.
2. The expression patterns obtained under given conditions can provide insight into the function of that gene.
3. Using northern blotting, RNA size can be detected. The quality and quantity of RNA can be measured on the gel prior to blotting, and the membranes can be stored and re-probed for years after blotting.

Western blotting



Principle:

- Western blotting technique is used for identification of particular protein from the mixture of protein.
- In this method labelled antibody against particular protein is used identify the desired protein, so it is a specific test. Western blotting is also known as immunoblotting because it uses antibodies to detect the protein.

Procedure/Steps:

1. Extraction of protein
2. Gel electrophoresis: SDS PAGE
3. Blotting: electrical or capillary blotting
4. Blocking: BSA
5. Treatment with primary antibody
6. Treatment with secondary antibody(enzyme labelled anti Ab)
7. Treatment with specific substrate; if enzyme is alkaline phosphatase, substrate is p-nitro phenyl phosphate which give color.

Step I: Extraction of Protein

- Cell lysate is most common sample for western blotting.
- Protein is extracted from cell by mechanical or chemical lysis of cell. This step is also known as tissue preparation.
- To prevent denaturing of protein protease inhibitor is used.
- The concentration of protein is determined by spectroscopy.
- When sufficient amount of protein sample is obtained, it is diluted in loading buffer containing glycerol which helps to sink the sample in well.
- Tracking dye (bromothymol blue) is also added in sample to monitor the movement of proteins.

Step II: Gel electrophoresis

- The sample is loaded in well of SDS-PAGE Sodium dodecyl sulfate- poly-acrylamide gel electrophoresis.
- The proteins are separated on the basis of electric charge, isoelectric point, molecular weight, or combination of these all.
- The small size protein moves faster than large size protein.
- Protein are negatively charged, so they move toward positive (anode) pole as electric current is applied.

Step III: Blotting

- The nitrocellulose membrane is placed on the gel. The separated protein from gel get transferred to nitrocellulose paper by capillary action. This type of blotting is time consuming and may take 1-2 days
- For fast and more efficient transfer of desired protein from the gel to nitrocellulose paper electro-blotting can be used.
- In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

Step IV: Blocking

- Blocking is very important step in western blotting.
- Antibodies are also protein so they are likely to bind the nitrocellulose paper. So before adding the primary antibody the membrane is non-specifically saturated or masked by using casein or Bovine serum albumin (BSA).

Step V: Treatment with Primary Antibody

- The primary antibody (1° Ab) is specific to desired protein so it form Ag-Ab complex

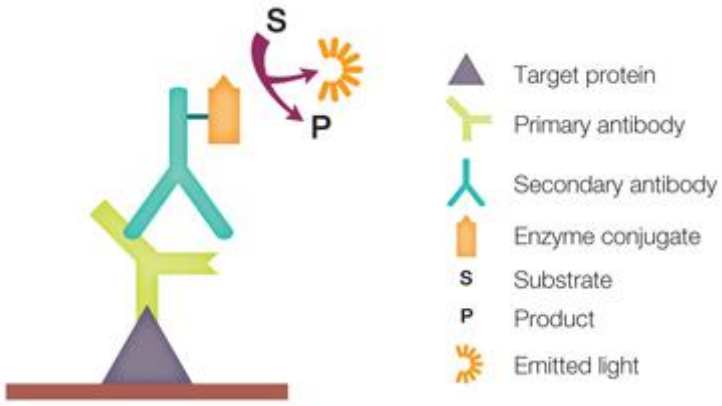
Step VI: Treatment with secondary antibody

- The secondary antibody is enzyme labelled. For eg. alkaline phosphatase or Horseradish peroxidase (HRP) is labelled with secondary antibody.

- Secondary antibody (2° Ab) is antibody against primary antibody (anti-antibody) so it can bind with Ag-Ab complex.

Step VII: Treatment with suitable substrate

- To visualize the enzyme action, the reaction mixture is incubated with specific substrate.
- The enzyme convert the substrate to give visible colored product, so band of color can be visualized in the membrane.
- Western blotting is also a quantitative test to determine the amount of protein in sample.



Application:

1. To determine the size and amount of protein in given sample.
2. Disease diagnosis: detects antibody against virus or bacteria in serum.
3. Western blotting technique is the confirmatory test for HIV. It detects anti HIV antibody in patient's serum.
4. Useful to detect defective proteins. For eg Prions disease.
5. Definitive test for Creutzfeldt-Jacob disease, Lyme disease, Hepatitis B and Herpes\

Dot Blot and Slot Blot Hybridization

These two techniques represents the simplification of Southern and Western blots saving the time involved in procedures of chromatography, electrophoresis, restriction digestion and blotting of DNA or proteins from the gel to membrane. Here nucleic acid mixture is directly applied (blotted) on to the nylon or nitrocellulose membrane where hybridization between probe and target takes place, denatured to single-stranded form and baked at 80°C to bind DNA target to membrane. In dot-blot, target is blotted as circular blots whereas in slot-blots, it is in the form of rectangular blots. Due to this, slot-blot offers greater precision in observing different hybridization signals. After blotting, membrane is allowed to dry and non-specific sites are blocked by soaking

in blocking buffer containing BSA. It is then followed by hybridization of labeled probe for detection of specific sequences or gene.

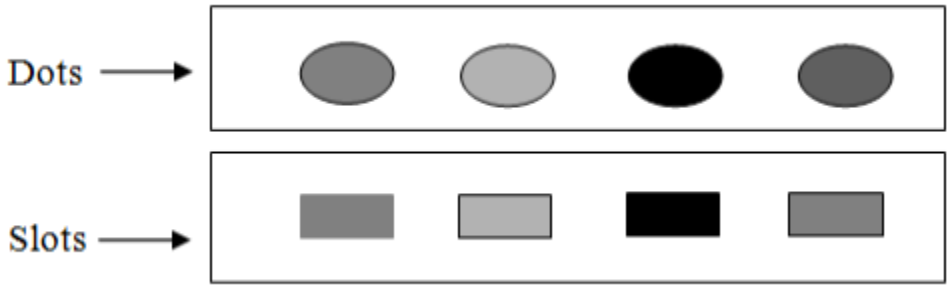


Figure:Dots and slots in dot and slot blot hybridization

These procedures can only detect presence and absence of particular sequence or gene. It cannot distinguish between two molecules of different sizes as they appear as single dot on membrane. It also has application in detecting alleles that differ in single nucleotide with the help of allele-specific oligonucleotides.

Important Examples of Transgenic Animal

The following points highlight the three important examples of transgenic animal. The examples are: 1. Cloning Dolly 2. Transgenic Mice 3. Reporter System.

1. Cloning Dolly:

In February 1996, Ian Wilmut and co-workers from the Roslin Institute and PPL Therapeutics, both in Edinburgh, Scotland, reported in Nature journal that they had successfully cloned a sheep from a cell taken from the udder of a six-year old ewe.

The cloned lamb was named Dolly. In the past, genetically identical animal embi70s had been created only with amphibian cells, and those created from adult nuclei had never successfully reached adulthood. Cloning in which the nuclei came from fetal cells or cells from cell lines had been successful before in mammals. The word clone means to create a genetically identical copy.

To clone an animal, it is necessary to begin with an egg, the only cell known to initiate and support development. In order to clone an individual, it is necessary to get an egg without a nucleus and then to transplant in it a nucleus of known origin.

Techniques for nuclear transplantation had been worked out with frogs and toads in the 1950s. The Scottish scientists succeeded in obtaining sheep eggs, enucleating them (removing their nuclei) and then transferring in donor nuclei by fusing the donor cells and the enucleated eggs with an electric pulse.

The electric pulse also initiated development of the egg. Although only one pregnancy of the twenty nine initiated was successful, the lamb that was born seemed normal in every respect; since it had produced offspring.

Others had tried this type of experiments with many types of animals, including mice. They were not successful for numerous reasons. The most likely explanation for the recent success, according to scientists, is that the donor cells were kept in a non-growth phase for several days, which may have synchronized them with the oocyte.

Thus the nucleus and the oocyte were at the same stage of cell cycle and thus compatible. Other reorganization that had to take place in the donor chromosomes are not really known for certain but one thing is clear: the nucleus of an adult cell in the sheep has all of the genetic material needed to support normal growth and development of the egg. The work has since been repeated with goats, cattle and mice.

There are various ramifications to the success of this work:

1. Mammal cloning could becomes a routine procedure. This would allow us to study mammalian development and to replicate genetically identical individuals, particularly transgenic animals that would have particular genome of value.

2. We can also use these techniques to study aging, since an “old” nucleus in initiating development of a new organism.

3. Also, of interest is the interaction of a particular genome with a particular cytoplasm, since the cytoplasm contains not only the material needed for early development, but also cell organelles, including mitochondria that have their own genetic material.

Transgenic Mice:

- Animal cells, like the protoplasts of plant cells, can take up foreign chromosomes or DNA directly from the environment with a very low efficiency (in the presence of calcium phosphate). Directly injecting the DNA greatly improves the efficiency.
- For example, transgenic mice are now routinely prepared by injecting DNA either into oocytes or one or two- celled embryos obtained from female mice after appropriate hormonal treatment.
- After injection of about 2 picoliters (2 X 10-12 liters) of cloned DNA, the cells are reimplanted into the uteruses of receptive female hosts. In about 15% of these injections, the foreign DNA incorporates into the embryo.
- Transgenic animals are used to study the expression and control of foreign eukaryotic genes. In 1988, a transgenic mouse prone to cancer was first genetically engineered animal to be patented. Thus mouse provides an excellent model for studying cancer. (A controversy arose as to whether engineered higher organisms should be patentable; currently they are).
- Mice have already been successfully transfected with a rat growth-hormone gene, and transgenic sheep have been produced that express the gene for a human clotting factor. The latest recombinant DNA dispute arose, from cloning of sheep in 1997.
- The transfection can also be mediated by retroviruses (RNA viruses containing the gene for reverse transcriptase). For example, a retroviral vector was ritrodused and repaired human white blood cell lacking the enzyme adenosine deaminase.
- A retrovirus responsible for a form of leukemia in rodents, the Moloney Murine leukemia viruses was engineered so that all the virus genes were removed and replaced with an antibiotic marker (neomycin resistance) and the human adenosine deaminase gene.
- The virus binds to the cell surface and is taken into the cell, its RNA is converted to DNA by reverse transcription and the DNA is incorporated into one of the cells, chromosomes. It is not possible for the highly modified virus to attack and damage the cells.

Reporter System:

- Two reporter systems are used to indicate that a transfection experiment was successful. Plants can be transfected with the Ti plasmids of Agrobacterium tumefaciens. When a plant is infected with A. tumefaciens containing the Ti plasmid, a crown gall tumor is induced transferring the T- DNA region.
- Those cells transfected with the T-DNA are induced to grow as well as to produce opines that the bacteria feed on. Much recent research has concentrated on engineering Ti plasmids to contain other genes that are also transferred to the host plants during infection, creating transgenic plants. One series of experiments have been especially charming.
- Tobacco plants have been transinfected by Ti plasmids containing the luciferase gene from fireflies. The product of this gene catalyzes the ATP- dependent oxidation of luciferin, which emits light. When a transfected plant is watered with luciferin, it glows like a firefly. The value of these experiments is not the production of glowing plants but rather the use of the glow to "report" the action of specific genes.
- In further experiments, the promoters and enhancers of certain genes were attached to the luciferase gene. As a result, luciferase would only be produced when these promoters were activated; thus, the glowing areas of the plant show where the transfected gene is active.
- One of the more recent reported systems developed uses a gene from jellyfish that produces green fluorescent protein. The value of this system is that it "reports" when ultra-violet light falls on it, rather than it requiring an addition, as in the luciferase system (see Tamarin, 2002).

Examples of Transgenic Plants

The following points highlight the top six examples of transgenic plants. The examples are: 1. "High Lysine" Corn 2. Enhanced Nitrogen Fixation 3. Herbicide-Tolerant Plants 4. Disease-Insect-Resistant Varieties 5. Male Sterility 6. Transgenic Plants as Bioreactors (Molecular Farming).

1. "High Lysine" Corn:

    - The proteins stored in plant seeds function as reserves of amino acids used during seed germination and pre- emergence growth of the young seedling. Plant seed

storage proteins also provide the major source of proteins in the diets of most humans and herbivorous higher animals.

  - Worldwide, the seeds of legumes and cereal grains are estimated to provide humans with 70 per cent of their dietary requirements. Unfortunately, the major seed storage proteins of cereals, called prolamines (Zeins in com or maize), are virtually lacking in the amino acid lysine.

- Since prolamines account for about half of the total protein content of cereal seeds, diets based largely on cereal grains will be deficient in lysine (an essential amino acid). In the case of com, the seed proteins are also deficient in tryptophan (another essential amino acid) and to a lesser extend, methionine (an essential amino acid).
- Because of the importance of cereal seeds as human and animal foods, plant breeders have attempted for several decades to develop varieties with increased lysine, tryptophan and methionine content.
- In com, mutants such as opaque-2, sugary-1 and floury-2 have increased amounts of lysine and/or methionine in seeds, but these mutant strains have undesirable soft kernels and produce lower yields. These "high lysine" mutant strains all result from mutations that alter the relative proportions of different seed storage proteins.
- In general, they lower the prolamine (zein) content so that other seed proteins account for a larger proportion of the total seeds proteins. This, in turn, increases the relative amounts of lysine and/or methionine in the seeds.
- Several genes of corn encoding zeins have now been cloned and sequenced. With this information in hand, researchers have suggested that it might be possible to produce **"high lysine"** corn by genetic engineering. Since the zeins have no known enzymatic functions, one might be able to modify zein genes by mutagenesis without inflicting any deleterious effects on function(s).
- Specifically site-specific mutagenesis could be used to introduce more lysine codons into zein sequences. Then, these "high lysine" zein coding sequences could be joined to strong promoters such as the CaMV35S promoter and reintroduced into com plants by transformation by means of electroporation or a microjectile gun.
- However, a possible difficulty in engineering **"high lysine"** com by this method is that the modified zein proteins might not package properly in seed storage structures.

- The zein proteins are synthesized on rough endoplasmic reticulum and they aggregate within this membranous structure into dense deposits called protein bodies. The formation of protein bodies is thought to involve hydrophobic and weak polar interactions between the zein monomers.
- If so, charged amino acids such as lysine might interfere with proper packaging of zeins during protein body formation. In 1988, B.A. Larkin and colleagues have introduced new lysine and tryptophan codon into a zein cDNA by oligonucleotide-directed site-specific mutagenesis.
- When BNA transcripts of these modified cDNAs were translated efficiently and the "high lysine" zein products were found to self-aggregate into dense structures similar to those present during polar body formation in com. These results offer encouragements that "high lysine" com might indeed be produced by means of genetic engineering.

2. Enhanced Nitrogen Fixation:

Plants are only able to utilize nitrogen that has been incorporated into chemical compounds such as ammonia, urea, or nitrates. No green plant is capable of extracting diatomic nitrogen (N2) molecules directly from the atmosphere. Although plants use only a small fraction of the total nitrogen pool, they are dependent on a continuous supply of nitrogen in usable form (most often called "fixed nitrogen").

- On-going fixation of atmospheric nitrogen is required because the fixed nitrogen in soil is constantly being depleted by leaching, by utilization for the growth of plants and microorganisms and by denitrifying bacteria that converts fixed nitrogen back to N2. As a result, millions of dollars/rupees is spent each year on nitrogen fertilizers in order to obtain optimal yields of major crops such as com and the cereal grains.
- Biological nitrogen fixation is the alternative to the use of the industrially fixed nitrogen provided in fertilizers. Several species of bacteria and lower algae are capable of converting N2 to fixed forms of nitrogen that can be utilized by plants.
- Because the purchase of nitrogen fertilizers represent one of the major expenses incurred with current agricultural production methods, a major effort has been made and continues to be devoted to the development of enhanced methods of biological nitrogen fixation.

- Certain free-living soil bacteria such as Azotobacter vinelandii and Klebsiella pneumoniae directly convert atmospheric nitrogen to ammonia. These bacteria are an important source of fixed nitrogen and in addition, have proven to be extremely valuable subjects for studies on the mechanism of nitrogen fixation.
- In Klebsiella, there are 17 nif (nitrogen fixation) genes organised in seven operons. The complexity of the nitrogen fixation metabolic machinery in these bacteria has important implications for anyone who might aspire to engineer nitrogen-fixing plants.
- The situation with nitrogen fixation is very different from that of herbicide tolerance. It is one thing to construct a single chimeric gene and transfer that gene to plants, but it is far more difficult to engineer 17 different chimeric genes, to transfer all of them to the same recipient plant, and to coordinate their expression in the plant so that all the components of the complex nitrogen-fixing enzymatic machinery are synthesized in proper amounts and in the appropriate cells of the plant.
- At present, the possibility of engineering nitrogen-fixing plants is largely fantasy, but remember that travelling to the moon was pure science fiction not too many years ago.

Some facts about nitrogen fixation:

The phenomenon of fixation of atmospheric nitrogen by biological means is known as diazotrophy or biological nitrogen fixation and these prokaryotes as diazotrophs or nitrogen fixers. For the first time, Beijerinck (1888) isolated Rhizobium from root nodules of leguminous plants. Thereafter, S .Winogradsky discovered a free-living nitrogen fixing bacterium, Clostridium pasteurianum.

- Then a large number of nitrogen fixers were discovered from different sources and associations. For example, Frankia from nodules of non-legumes (e.g., alder, Casuarina, etc.), Nostoc from lichens, Anabaena from Azolla leaves, and coralloid roots of Cycas. The diazotrophs may be free living or in symbiotic form.
- Heterocysts are the sites of nitrogen fixation in some cyanobacteria, e.g., Anabaena, Nostoc, etc. Heterocysts are formed in the absence of utilizable combined nitrogen, such as ammonia because it inhibits heterocyst differentiation and N2-fixing enzyme, the nitrogenase. Heterocysts lack oxygen evolving photosystem II, ribulose biphosphate and may lock photosynthetic biliproteins.

- Chlorophyll-a is present in heterocysts. Wall of heterocyst contains O2 binding glycolipids which together with respiratory consumption maintain the anaerobic conditions (i.e., highly reduced atmosphere) necessary for N2 fixation. In contrast, vegetative cells adjacent to heterocysts, both photosystem I and II are present; therefore, oxygen evolution takes place by these cells.
- Other cyanobacteria, that lack heterocyst also do N2-fixation, e.g., Oscillatoria (see Dubey 2006).
- Another very important source of biologically fixed nitrogen is the symbiotic relationship between bacteria of the genus Rhizobium and plants of family Leguminosae (the alfalfa, clovers, soyabeans, peanuts, peas, etc.).
- This symbiotic nitrogen fixation occurs in highly differentiated root nodules that develop when Rhizobium bacteria interacts with the roots of legumes. Nodule formation is dependent on genetic information of both the plants and the bacterium. The nitrogenase that catalyzes N2 reduction is encoded by the bacterial genome, but the fixed nitrogen is utilised for growth of both bacteria and the host plants.
- Once the mechanisms responsible for establishing this symbiotic relationship and for nodule formation are known, and the genes that control these processes have been identified, it might be possible to use genetic engineering to modify non-legume plants (e.g., com, rice and wheat, such that they will participate in similar symbiotic relationships with nitrogen-fixing bacteria.
- However, once again, this will undoubtedly be a challenging task because the genetic control of nodule formation is clearly complex. Nevertheless, experiments are in progress with goals of modifying bacteria so as to enhance their nitrogen-fixing capacity and to broaden their host range to include additional plant species.

3. Herbicide-Tolerant Plants:

The development of herbicide-tolerant varieties of agronomically important plants such as com, soybeans and the cereas promises to have a major impact on agriculture, both economically and on production practices. Weeds compete with crops for soil nutrients and routinely lead to significant losses in yield.

- Modern agriculture makes use of herbcides to control weeds and minimize the losses. Unfortunately, the available herbicides seldom provide the degree of specifications that is desired, and most herbicides will control only certain classes and not others.
- Broad-spectrum herbicides may give good weed control, but in doing, so usually have deleterious effects on the growth of crop plants as well. As a result, scientists are now considering alternate approaches to weed control.
- The most promising of the alternate approaches is the development of herbicide-tolerant plant varieties for use with broad-spectrum or totally nonspecific herbicides. Obviously, the potential economic value of herbicide-tolerant plant varieties is significant.
- Herbicides are simple chemical compounds that kill or inhibit the growth of plants without deleterious effects on animals. Herbicides usually inhibit the processes that are unique to plants, for example, photosynthesis.
- Most frequently, herbicides act as inhibitors of essential enzyme reactions. Thus, anything that diminishes the level of inhibition will provide increased herbicide tolerance.

The two most common sources of herbicide tolerance are:

(1) Over-production of the target enzyme and

(2) Mutations resulting in enzymes that are less sensitive to the inhibitor (usually due to a lower affinity of the enzyme for the inhibitor).

- It seems likely that the most successful strategy for developing herbicide- tolerant plants will be to combine both sources of tolerance, that is, to engineer plants that overproduce herbicide-tolerant mutant enzymes. We can consider, here, the example of glyphosate.
- Glyphosate is one of the most potent broad-spectrum herbicide known; it is marketed under the trade name Roundup Glyphosate acts by inhibiting the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase), an essential compound in the biosynthesis of the aromatic amino acids tyrosine, phenylalanine and tryptophan.
- These aromatic amino acids are essential components in the diets of higher animals since the enzymes that catalyse the biosynthesis of these amino acids are not present in higher animals. Therefore, since higher animals contain EPSP synthase, glyphosate has no toxic effects on animal systems. In this respect, glyphosate is an ideal herbicide.

- Glyphosate of herbicide, does inhibit the EPSP synthases of microorganisms as well as those of plants. By selecting for growth in the presence of glyphosate concentrations that inhibit the growth of wild-type bacteria, researchers have been able to isolate glyphosate-tolerant mutants of Salmonella typhimurium, Aerobacter aerogenes and Escherichia coli.
- In bacteria, EPSP synthase is encoded by the aroA gene. When the mutant bacterial aroA genes were provided with plant promoters and polyadenylation signals (producing, chimeric genes) and were introduced into plants, the transgenic plants exhibited increased tolerance to glyphosate (herbicide).
- In plants, synthesis of aromatic amino acids takes place in chloroplasts, but the genes encoding the biosynthetic enzymes such as EPSP synthase are nuclear genes. The translation products contain a transit peptide that targets the protein to the chloroplasts.
- This transit peptide is then cleaved off proteolytically upon entering the chloroplasts to yield the active enzyme. Experiments have now shown that the petunia transit peptide will target the E. coli aroA gene product into tobacco chloroplasts and will produce glyphosate tolerance in the recipient cell lines.

4. Disease-Insect-Resistant Varieties:

- Several microorganisms and certain native plants produce proteins that are toxic to specific plant pathogens, both microbial pathogens and insects that feed on plants. One goal of plant genetic engineering is to transfer the genes encoding these protein toxins to agronomically important plants with the hope that expressing the toxin genes in these plants will provide biological control Disease-insect of at least some plant diseases and insect pests.
- Currently, plant diseases resistant plants and insect pests are controlled almost exclusively by the use of broad- spectrum chemical bacteriocides, fungicides and insecticides. However, there is reason for concern about the potential damage to ecosystems and pollution of groundwater that might result from the widespread use of these chemicals on agricultural crops. Thus, scientists are searching for alternate methods for controlling these pathogens.
- The best-known example of the use of natural gene products to control plant pests are the insect toxins of Bacillus thuringiensis. Each of the toxin genes of B. thuringiensis encodes a large protein that aggregate, to form protein crystals in spores and these protein crystals are highly toxic to certain insects.

- Some of the insects that are killed by these protein toxins are plant pests of major economic importance. Different subspecies of B. thuringiensis produce toxins that kill different insects. For example, the toxin produced by B. thuringiensis subspecies kurstaki kills lepidopteran larvae such as the tobacco hornworm.
- The gene that encodes this toxin has been isolated and shown to synthesize a functional toxin in E. coli. A chimeric gene with the structure CaMV35S promoter/B. thuringiensis subspecies kurstaki toxin coding sequence/Ti nos 3′ termination sequence was constructed.
- This chimeric gene was placed in a Ti vector, and tomato leaf disc cells were transformed by co-cultivation with A .tumefaciens harboring the engineered Ti vector-chimeric gene construct. Transgenic tomato plants were regenerated and shown to express the chimeric gene. The toxicity of the gene-product synthesized in the transgenic plants was tested by allowing tobacco hornworm larvae to feed on the transgenic plants and on control plants.
- All the larvae applied to the transgenic plants died within a few days; larvae applied to the control plants remained healthy and eventually consumed the entire plants. These results support the feasibility of using genetic engineering to produce transgenic tomato. Pathogen resistant plant varieties.

BT Cotton:

- The transgenic technology provides alternative and innovative method to improve pest control management which are ecofriendly, effective, sustainable and beneficial in terms of yield. The first genes available for genetic engineering of crop plants for pest resistance were cry genes (popularly known as Bt genes) from bacterium Bacillus thuringiensis. These genes are specific to particular group of insect pests and are not harmful to other useful insects such as butterfly, silk worms and honeybee.
- Transgenic crops (e.g., cotton, rice, maize, potato, tomato, brinjal, cauliflowers, cabbage, etc.) with Bt genes have been developed and such transgenetic variety proved effective in controlling insect pests and it has been claimed worldwide that it has led to significant increase in yield along with dramatic reduction in pesticide use.
- The most notable example is Bt cotton (which contain cry/Ac gene) that is resistant to a notorious insect pest bollworm (Helicoperpa armigera). Bt cotton was adopted for mass cultivation in India in year 2002.

5. Male Sterility:

- Male sterile plants are very important to prevent unnecessary pollination and to eliminate the process of emasculation during the production of hybrid plants. Such sterile male plants are created by introducing a gene coding for an enzyme (barnase), which is an RNA hydrolyzing enzyme) that inhibits pollen formation. This gene is expressed specifically in the tapetal cells of anther using tapetal specific promoter TA29 to restrict its activity only to the cells involved in pollen production.

6. Transgenic Plants as Bioreactors (Molecular Farming):

- Plants are amazing and cheap chemical factories that need only water, minerals, sunlight and carbon dioxide to produce thousand types of chemical molecules (see Dubey 2006). Given the right genes, plants can serve as bioreactors to new compounds such as amino acids, proteins, vitamins, plastics, pharmaceuticals (peptides and proteins), drugs, and enzymes for food industries and so on.

Thus, transgenic plants can be used for the following purposes:

(i) Nutrient quality:

- In section 58.2, under the heading of 'High-lysine com', we have described how cereals rich in certain essential amino acids such as lysine, methionine and tryptophan can be developed by genetic engineering. Likewise, rice is being modified into Golden rice by Prof. Inge Potrykus and Dr. Peter Beyer.
- This is done so that vitamin A potential is maintained even after the husks are removed, a procedure adopted to allow for storage since the husks become rancid. This change may improve health of millions of people throughout the world.

(ii) Diagnostic and therapeutic proteins:

- Transgenic plants can also produce a variety of proteins used in diagnosis for detecting human diseases and therapeutics for curing human and animal diseases in large-scale with low-cost. The monoclonal antibodies, blood plasma proteins, peptide hormones and

cytokinins are being produced in trangenic plants and their parts such as tobacco (in leaves), potato (in tubers), sugarcane (in stems) and maize (in seed endosperm).

(iii) Edible vaccines:

- Crop plants offer cost-effective bioreactors to express antigens which can be used as edible vaccines. The genes encoding antigenic proteins can be isolated from the pathogens and expressed in plants and such transgenic plants or their tissues producing antigens can be eaten for immunization (edible vaccines).
- The expression of such antigenic proteins in crops such as banana and tomato are useful for immunization of humans since both of these fruits can be eaten raw. Such edible vaccines of transgenic plants have the following advantages: lessening of their storage problems, their easy delivery system by feeding and low cost as compared to the recombinant vaccines produced by bacteria.

(iv) Biodegradable plastics:

- Transgenic plants can be used as factories to produce polyhydroxy butyrate (PHB, biodegradable plastics). Genetically engineered Arabidopsis plants produced PHB globules exclusively in their chloroplasts without effecting plant growth and development. The large-scale production of PHB may be easily achieved in tree plants such as populus, where PHB can be extracted from leaves.

Bio-safety measures and regulations for rDNA work

Introduction:

During early era of life sciences, biosafety principles and guidelines were mostly applied in the field of microbiology and medical practices. In recent time, biosafety guidelines are designed and applied to research involving recombinant DNA (rDNA) techniques. Handling, production, storing and transportation of genetically modified organism (GMOs) involve different biosafety issues under different category. Biosafety practices deal with the application of standard safety principles handling hazardous material/agents to minimize potential harmful effect on human health and environment.

- The definition of biosafety corresponds to recombinant DNA technology can be described as: “Application of safety principles to laboratory practices in which potentially hazardous materials or organism are manipulated or handled.”
- National Institutes of Health (NIH) developed certain guidelines on rDNA research in May, 1976. Similarly, Department of Biotechnology (Govt. of India) has also come out with its set of guidelines which is available at http://dbtindia.nic.in/index.asp . Scope of the guidelines suggested by DBT encompasses research, large scale operations and environmental risks. The guidelines prescribe specific actions that include establishing safety procedures for rDNA research, production and release to the environment and setting up containment conditions for certain experiments.
- Biosafety regulatory principles and protocols regulates the potential risk and allow access to the benefits of rDNA technology. Risk assessment and risk management are two important component of biosafety.
- Biosafety Levels (BSL): All the facilities handling microorganisms and materials containing recombinant DNA molecules have risk assessment program. Depending on the risk possessed by the samples, four biosafety levels have been assigned to rDNA research facilities. Each BSL facility has requirement of unique design features and safety equipments.
- Biosafety level-I (BSL-I):
    - Agents: Characterized strains of microorganisms known to cause no disease in healthy adults. eg. E. coli, S. cerevesiae, B. subtilis etc.
    - Recombinant DNA based research activities involving non-pathogenic micro-organisms for expression of genes using plasmid vectors or low risk viral vectors.
    - Work practice: Standard aseptic microbiological techniques.
    - Safety equipment requirement: Lab coats and eye protection recommended.
    - Facilities: Bench top, sink etc.
- Biosafety level-II (BSL-II):
    - Agents: Handling of micro-organisms which possess moderate hazard to personal and environment.
    - rDNA based research activities in micro-organisms using non-viral or viral vectors.

- Work practice: Standard BSL-I practices with addition of limited access, biohazard sign, defined procedure for disposal of "Regulated Medical Waste", proper training to lab personal and medical surveillance.
- Safety equipment: Class-II biological safety cabinet, lab coats, gloves, eye/face protection, physical containment equipment to reduce infectious aerosol exposure or splashes.
- Facility: BSL-I facility with addition of autoclave, decontamination facility and proper airflow.

- Biosafety level-III (BSL-III):
  - Agents: Handling of micro-organisms which are designated as hazardous or potentially lethal agents to personal and environment.
  - Laboratory personnel must have specific training in handling infectious micro-organisms and should be supervised by scientist competent in handling infectious agents.
  - Work practices: BSL-2 practices, with the addition of: controlled access, on-site decontamination of all waste and lab clothing and medical surveillance.
  - Safety equipment: Class-III biological safety cabinet, lab coats, gloves, eye/face protection, respiratory protection, physical containment equipment to reduce infectious aerosol exposure or splashes.
  - Facility: BSL-III facility has specific criteria to meet. Lab should have double door entry with physical separation of working area from the access corridors, directional airflow in lab, and no recirculation of exhaust air in the lab, sufficient decontamination facility, in lab autoclave etc.
- Biosafety level-IV (BSL-IV):
  - Agents: Hazardous and potentially lethal organisms that posses high individual risk of laboratory transmitted disease for which there is no vaccine or treatment, or a related agent with unknown risk of transmission.
  - Laboratory personnel must have specialized training in handling BSL-IV agents and should be supervised by scientist competent in handling infectious agents.
  - Safety equipment: Class-IV biological safety cabinet, lab coats, gloves, eye/face protection, respiratory protection, physical and containment equipment to reduce infectious aerosol exposure or splashes.

- Facility: BSL-IV facility requires specialized design to minimize the exposure to risk and only the authorized entry should be permitted in laboratory area in BSL-IV labs.

- Risk Analysis: The foundation of any safety program is the use of control measures appropriate for the risk posed by the activities and the agents in use. The process of analyzing and determining the risk associated with recombinant DNA work is called as Risk analysis. The principle behind biosafety regulations is to minimize the risk to human health and safety, and the conservation of environment including safe handling of hazardous material. Risk analysis consists of three components: risk assessment, risk management and risk communication. Risk Assessment: Estimation and determination of risk associated with the handling and production of a recombinant DNA molecule. Risk Management: The process of analyzing possible prevention measures to minimize the risk and designing policies accordingly including implementation of them. Risk Communication: The exchange of information and opinions on risk management between academic parties, industry, consumers and policy makers.
- Risk Assessment: The biosafety level is determined based on the risk associated with the work. The principle investigator is responsible for implementing the necessary safety requirements in his/her laboratory. Risk assessment process accounts the following criteria to determine biosafety level:
    - i. Pathogenicity – The ability of an organism to cause disease in human system.
    - ii. Virulence – The severity of the disease (lethal/non lethal, availability of cure etc) in a healthy adult.
    - iii. Proliferation – the subsequent multiplication, genetic reconstruction, growth, transport, modification and die-off of these micro-organisms in the environment, including possible transfer of genetic material to other micro- organisms.
    - iv. Transmission route – The possible route of transmission (mucous membrane, inhalation etc) to establish the disease in human or other organism.
    - v. Infectious dose (ID) – The amount of infectious agent required to cause disease in healthy human.
    - vi. Antibiotic/disinfectant resistance – The resistance acquired by the infectious agent to available antibiotic/disinfectant.

- The risk associated with recombinant DNA technology can be categorized under different headings based on their implication on different platforms.
- General Scientific Considerations-
    - A. Characteristics of Donor and Recipient Organisms
        - Taxonomy, identification, source and culture
        - Genetic characteristics of donor and recipient organisms
        - Pathogenic and physiological traits of donor and recipient organisms
    - B. Properties of the modified/engineered organism
    - C. Description of
        - (a) modification,
        - (b) nature, function and source of the insert,
        - (c) vector construction,
        - (d) transfer into host,
        - (e) stability of insert,
        - (f) frequency of mobilization,
        - (g) rate and level of expression, and
        - (h) Influence of the recipient organism on the activity of the foreign protein.
- Human Health Considerations:
    A. Characteristics of the modified/engineered organism.
    - Comparison of the recombinant organism to the wildtype organism regarding pathogenicity.
    - Transmission route to human.
    - Pathogenicity to humans (or to animals if appropriate).

    B. Health considerations generally associated with the presence of non-viable organisms or with the products of rDNA processes. C. Management of personnel exposure, including biological measures and physical and organizational measures.

- Environmental and Agricultural Considerations:
    - A. Ecological traits relating to the donor and recipient environment.
    - B. Properties of environment where the engineered organism are being applied.

- C. Survival, multiplication and dissemination of the engineered organism in the environment.
- D. Interactions of engineered organism(s) with biological systems (target and non-target populations, stability, and routes of dissemination).
- E. Potential environmental impacts (Effect on target and non-target organisms and ecosystems).

- Risk Management: Risk management in biosafety issues is related to the target site where the practice is conducting (laboratory, industry, agriculture field etc.). Recommendations: General i. Harmonization of approaches to rDNA techniques can be facilitated by exchanging principles or guidelines for national regulations; developments in risk analysis; and practical experience in risk management. Therefore, information should be shared as freely as possible. ii. There is no scientific basis for specific legislation for the implementation of rDNA techniques and applications. Member countries should examine their existing oversight and review mechanisms to ensure that adequate review and control may be applied while avoiding any undue burdens that may hamper technological developments in this field. iii. Any approach to implement guidelines should not impede future developments in rDNA techniques. International harmonization should recognize this need.
- iv. To facilitate data exchange and minimize trade barriers between countries, further developments such as testing methods, equipment design, and knowledge of microbial taxonomy should be considered at both national and international levels. Due account should be taken of ongoing work on standards within international organizations. v. Special efforts should be made to improve public understanding of the various aspects of rDNA techniques. vi. For rDNA applications in industry, agriculture and the environment, it will be important for member countries to watch the development of these techniques. For certain industrial applications and for environmental and agricultural applications of rDNA organisms, some countries may wish to have a notification scheme. vii. Recognizing the need for innovation, it is important to consider appropriate means to protect intellectual property and confidentiality interests while assuring safety.
- Recommendations: Specific for Industry
    - i. The large-scale industrial application of rDNA techniques wherever possible should utilize microorganisms that are intrinsically of low risk. Such

microorganisms can be handled under conditions of Good Industrial Large-Scale Practice (GILSP).

- ii. If a recombinant microorganism cannot be handled merely by GILSP, measures of containment corresponding to the risk assessment should be used in addition to GILSP.
- iii. Further, research to improve techniques for monitoring and controlling non-intentional release of rDNA organisms should be encouraged in large-scale industrial applications requiring physical containment.

- Containment levels: Biosafety containment levels have to be designated for a facility depending on the level of risk associated with the biological and chemical agents used and released from it. Following NIH (National Institute of Health, USA) and DBT (Department of Biotechnology, India) guidelines, different facilities for biological research have been classified under three containment levels.
- Containment Category 1:
  - Viable organisms should be handled in a production system which physically separates the process from the environment;
  - Exhaust gases should be treated to minimize (i.e. to reduce to the lowest practicable level consistent with safety) the release of viable organisms;
  - Sample collection, addition of materials to the system and the transfer of viable organisms to another system should be done in a manner which minimizes release;
  - Bulk quantities of culture fluids should not be removed from the system unless the viable organisms have been inactivated by validated means;
  - Effluent from the production facility should be inactivated by validated means prior to discharge.
- Containment Category 2:
  - Viable organisms should be handled in a production system which physically separates the process from the environment;
  - Exhaust gases should be treated to prevent the release of viable organisms;
  - Sample collection, addition of materials to a closed system and the transfer of viable organisms to another closed system should be done in a manner which prevents release;

- Culture fluids should not be removed from the closed system unless the viable organisms have been inactivated by validated chemical or physical means;
- Seals should be designed to prevent leakage or should be fully enclosed in ventilated housings;
- Closed systems should be located in an area controlled according to the requirements;
- Effluent from the production facility should be inactivated by validated chemical or physical means prior to discharge.

- Containment Category 3: •
  - Viable organisms should be handled in a production system which physically separates the process from the environment;
  - Exhaust gases should be treated to prevent the release of viable organisms;
  - Sample collection, addition of materials to a closed system and the transfer of viable organisms to another closed system should be done in a manner which prevents release.
  - Culture fluids should not be removed from the closed system unless the viable organisms have been inactivated by validated chemical or physical means; • Seals should be designed to prevent leakage or should be fully enclosed in ventilated housings;
  - Production systems should be located within a purpose built controlled area according to the requirements;
  - Entry should be restricted in the laboratory area and only persons with appropriate authority should be allowed access to the working area. Effluent from the production facility should be inactivated by validated chemical or physical means prior to discharge. Different containment levels have been assigned for rDNA GILSP (Good industrial large scale practice) micro-organisms. Examples of containment approaches for recombinant organisms are discussed in the following slide:
  - Containment levels for different facilities.
  - Recommendations: Specific for Environment and Agriculture

- Considerable data on the effects of different microorganisms on environmental and human health exist in literature and should be used to guide risk assessments.
- It is important to evaluate rDNA organisms for potential risk prior to applications in agriculture and the environment. However, the development of general international guidelines governing such applications is premature at this time. An independent review of potential risks should be conducted on a case-by-case basis prior to the application.
- Development of organisms for agricultural or environmental applications should be conducted in a stepwise fashion, moving, where appropriate, from the laboratory to the growth chamber and greenhouse, to limited field testing and finally, to large-scale field testing.
- Further research to improve the prediction, evaluation and monitoring of the outcome of applications of rDNA organisms should be encouraged.