SCOPE

To make the student to understand the concept of gene manipulation and gene transfer technologies.

OBJECTIVES

To understand the manipulation of genes, transfer techniques, expression systems and genetically modified foods.

UNIT I

Introduction to gene manipulation: Basic techniques- Isolation and purification of nucleic Acids, Agarose gel Electrophoresis. Hybridization of nucleic acids-probes and types. Hybridization techniques-Southern, Northern, Western blotting. DNA and RNA markers.

UNIT II

Gene cloning vectors: Plasmids, bacteriophages, phagemids, cosmids, Artificial chromosomes-BAC, YAC, HAC. Restriction mapping of DNA fragments, Map construction, Cloning in *E. coli*- Vector engineering and codon optimization. Gene expression in *E.coli.* Expression vector-PET vector. Genomic library.

UNIT III

Isolation and characterization of gene transcripts: Introduction, Converting mRNA transcripts into cDNA, Screening representative cDNA libraries, Functional sequencing of cDNA expression libraries. Expressed cDNAs compared with computer databases. Characterization of recombinant proteins- Processing, purification and refolding and stabilization-Insulin, hGH, tpA.

UNIT IV

Mutagenesis: Site-directed mutagenesis, *In vitro* mutagenesis-Linkers, synthetic oligonucleotides and transposons, Role of Tagging in gene analysis, Identification and isolation of genes through T-DNA or transposons.

Gene therapy- Different strategies for gene therapy, therapeutics based on targeted exhibition of gene expression and mutation correction *in vivo*, Gene therapy for inherited diseases, ADA, FH, Cystic fibrosis.

UNIT V

Transgenics: Gene transfer techniques- Microinjection, biolistic methods, vector based transfer.

Transgenic plants: Agrobacterium & Ti plasmids. Methods of engineering herbicide resistance plants, Stress resistance plants and modification of plant nutritional content (amino acids, β-carotene) Plants as bioreactors: edible vaccines.

Transgenic animals: Method of Engineering transgenic mice, transgenic cattle- applications

Biosafety- regularities and concerns. Societal impact of genetically modified food.

REFERENCES

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Watson, J.D., Gilamn, M., Witkowski, J., and Zotler, M., (2006). Recombinant DNA, 3rd Edition. W.H. Freeman Company, New York.

Kingsman, S .M., and Kingsman, A.J., (2001). Genetic Engineering: An Introduction to Gene Analysis and Exploitation in Eukaryotes, 6th Edition. Blackwell Scientific Publication, Oxford.

Kreuzer, H., and Massay, A., (2008). Molecular Biology and Biotechnology, 3rd Edition Aim Press, Washington,DC.

Primrose, S. B., (2003). Molecular Biotech, 2nd edition, Panima Publications, New Delhi.

Sambrook, J., Fritch, E.F., and Maniate, T., (2001). Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

Strachan, T., and Read, A.P., (2003). Human Molecular Genetics, 3rd edition. John Wiley and Sons,Toronto. Canada.

KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed to be University Established Under Section 3 of UGC Act 1956)
Coimbatore – 641 021.

LECTURE PLAN
DEPARTMENT OF BIOCHEMISTRY

STAFF NAME: Dr.S.PRIYANGA
SUBJECT NAME: RECOMBINANT DNA TECHNOLOGY
SUB.CODE: 17BCP205A SEMESTER: II CLASS: I M.Sc (BC)

S.No	Lecture Duration Period	Topics to be Covered	Support Material/Page Nos
		UNIT-I	
1	1	Introduction to gene manipulation	T1:41-46
2	2	Basic techniques- Isolation and purification of nucleic Acids	T4:94-96
3	1	Agarose gel Electrophoresis	T4:92-94
4	1	Hybridization of nucleic acids-probes and types	R1:192-193
5	1	Hybridization techniques-Southern blotting	T1:127-128
6	1	Northern blotting	T4:99-100
7	1	Western blotting	T1:128-129
8	1	DNA and RNA markers	T1:184-185
9	1	Class test 1	
	Total No of Hours Planned For Unit 1=10		
		UNIT-II	
1	1	Plasmids, bacteriophages	T1:86-102
2	1	Phagemids, cosmids	T1:102-104
3	1	Artificial chromosomes- BAC, YAC, HAC	T1:107-109
4	1	Restriction mapping of DNA fragments	T1:345-348
5	1	Map construction	T1:349-354

6	1	Cloning in *E. coli*- Vector engineering and codon optimization	T4:60-62
7	1	Gene expression in *E.coli*	T4:62-65
8	1	Expression vector- PET vector	T1:112-113
9	1	Genomic library	R1:35-36
10	1	Revision	
	Total No of Hours Planned For Unit II=10		
		UNIT-III	
1	1	Introduction	T4:120-121
2	1	Converting mRNA transcripts into cDNA	T4:123-125
3	1	Screening representative cDNA libraries	T4:125-127
4	1	Functional sequencing of cDNA expression libraries	W1
5	1	Expressed cDNAs compared with computer databases	W2
6	1	Characterization of recombinant proteins- Processing	T4:144-145
7	1	Purification and refolding	T4:145-146
8	1	Stabilization	T4:146-147
9	1	Insulin, hGH, tpA	T4:191-196
10	1	Class test 2	
	Total No of Hours Planned For Unit III=10		
		UNIT-IV	
1	1	Site-directed mutagenesis	T1:147-148
2	1	*In vitro* mutagenesis-Linkers	T4:129-130
3	1	Synthetic oligonucleotides	T4:130-133
4	1	Transposons	T4:32-33
5	1	Role of Tagging in gene analysis	T4:70-71
6	1	Identification and isolation of	T4:71-72

		genes through T-DNA or transposons	
7	2	Gene therapy- Different strategies for gene therapy	R1:438-439
8	1	Therapeutics based on targeted exhibition of gene expression	R1:413-414
9	1	Mutation correction *in vivo*	R1:414-415
10	1	Gene therapy for inherited diseases, ADA	R1:404-405
11	2	FH, Cystic fibrosis	R1:405-406
12	1	Revision	
	Total No of Hours Planned For Unit IV=14		
		UNIT-V	
1	1	Gene transfer techniques- Microinjection	T1:166-167
2	1	Biolistic methods	T4:584-586
3	1	Vector based transfer	T4:86-87
4	2	Transgenic plants: Agrobacterium & Ti plasmids	T3:276-279
5	1	Methods of engineering herbicide resistance plants	R1:348-349
6	2	Stress resistance plants and modification of plant nutritional content (amino acids, β- carotene)	R1:350-352
7	1	Plants as bioreactors: edible vaccines	R1:353-354
8	1	Transgenic animals: Method of Engineering transgenic mice	R1:365-370
9	1	Transgenic cattle- applications	R1:372-374
10	1	Biosafety- regularities and concerns	T1:601-603
11	1	Societal impact of genetically modified food	W3
12	1	Revision	
	Total No of Hours Planned for unit V=14		
1	1	Previous year ESE question paper Discussion	

2	1	Previous year ESE question paper Discussion	
	Total No of Hours Planned = 2		
Total Planned Hours	**60**		

TEXT BOOK

1. Dubey, R.C., (2012). A textbook of Biotechnology. S. Chand and Company Ltd, New Delhi.
2. Watson, J.D., Gilamn, M., Witkowski, J., and Zotler, M., (2006). Recombinant DNA, 3rd Edition. W.H. Freeman Company, New York.
3. Sambrook, J., Fritch, E.F., and Maniate, T., (2001). Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.
4. Sathyanarayana, U., (2009). Biotechnology, Books and allied Pvt. Ltd, Kolkatta.

REFERENCES

1. Glick, B.R., Pasternak, J.J., and Patten, C.L., (2009). Molecular Biotechnology, 4th edition, Panima Publishing Corporation, Delhi.

WEBSITES

W1: https://www.biotechniques.com/multimedia/.../BTN_A_04366RR03_O_36644a.pdf

W2: https://www.ncbi.nlm.nih.gov/guide/

W3: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3558185/

UNIT-I

SYLLABUS

Introduction to gene manipulation: Basic techniques- Isolation and purification of nucleic Acids, Agarose gel Electrophoresis. Hybridization of nucleic acids-probes and types. Hybridization techniques-Southern, Northern, Western blotting. DNA and RNA markers.

Recombinant DNA technology is one of the few techniques that made conventional biotechnology into "Modern Biotechnology." Paul Berg, Herbert Boyer, Annie Change, and Stanley Cohen are the team of scientists that made the first recombinant DNA molecule in 1973. Simply defined, it is the art of cutting and pasting genes. There are, however, many new applications of this technology invented each year, and it is impossible for any textbook to be completely up to date. This technique encompasses a number of methodologies or tools that enable us to construct new combinations of DNA (recombinant DNA or rDNA) in the laboratory for different purposes. The rDNA molecule thus constructed can be introduced into an appropriate host cell, where it can be multiplied and generate many copies. This forms the basic concept of the process known as gene cloning or DNA cloning. In this chapter we will examine the basic tools, methodologies, and applications of recombinant DNA techniques in various fields of biological research.

Gene cloning can generate unlimited copies of a DNA molecule (e.g., recombinant DNA) by replication in a host cell. It was first developed in 1970. The following are some of the major applications of rDNA technique:

_ Genetic mapping
_ DNA sequencing
_ Mutation studies
_ Transformation
_ Genetic engineering

_ Recombinant DNA libraries

_ Restriction enzyme site analysis

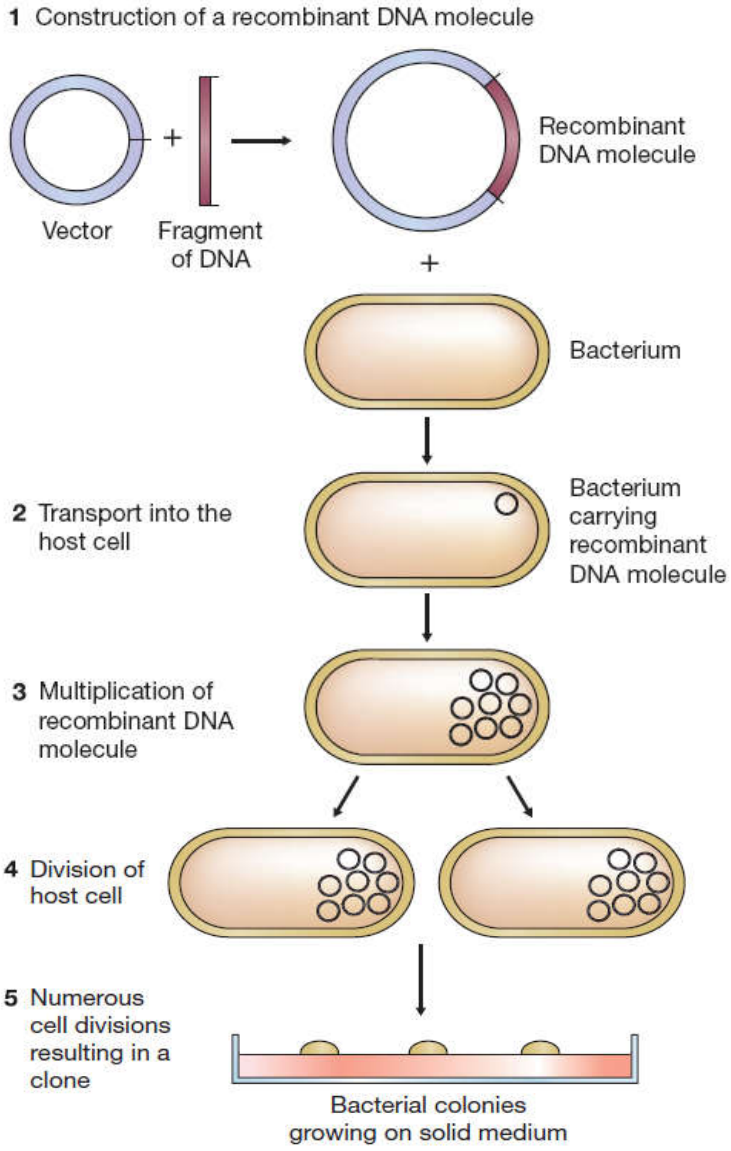
_ Analysis of gene transcripts

_ Polymerase chain reaction (PCR)

The goal of rDNA is gene cloning to generate large amounts of pure DNA that can be manipulated and studied. The following are the basic steps involved in the process of the rDNA technique for gene cloning:

The steps in a gene cloning experiment:

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



ISOLATION OF PURE DNA

The genetic engineer will, at different times, need to prepare at least three distinct kinds of DNA. First, **total cell DNA** will often be required as a source of material from which to obtain genes to be cloned. Total cell DNA may be DNA from a culture of bacteria, from a plant, from animal cells, or from any other type of organism that is being studied. It consists of the **genomic DNA** of the organism along with any additional DNA molecules, such as plasmids, that are present.

The second type of DNA that will be required is pure plasmid DNA. Preparation of plasmid DNA from a culture of bacteria follows the same basic steps as purification of total cell DNA,

with the crucial difference that at some stage the plasmid DNA must be separated from the main bulk of chromosomal DNA also present in the cell. Finally, phage DNA will be needed if a phage cloning vector is to be used. Phage DNA is generally prepared from bacteriophage particles rather than from infected cells, so there is no problem with contaminating bacterial DNA. However, special techniques are needed to remove the phage capsid. An exception is the double-stranded replicative form of M13, which is prepared from *E. coli* cells in the same way as a bacterial plasmid.

1. PREPARATION OF TOTAL CELL DNA

The fundamentals of DNA preparation are most easily understood by first considering the simplest type of DNA purification procedure, that where the entire DNA complement of a bacterial cell is required. The modifications needed for plasmid and phage DNA preparation can then be described later.

The procedure for total DNA preparation from a culture of bacterial cells can be divided into four stages (Figure):

1 A culture of bacteria is grown and then **harvested**.

2 The cells are broken open to release their contents.

3 This **cell extract** is treated to remove all components except the DNA.

4 The resulting DNA solution is concentrated.

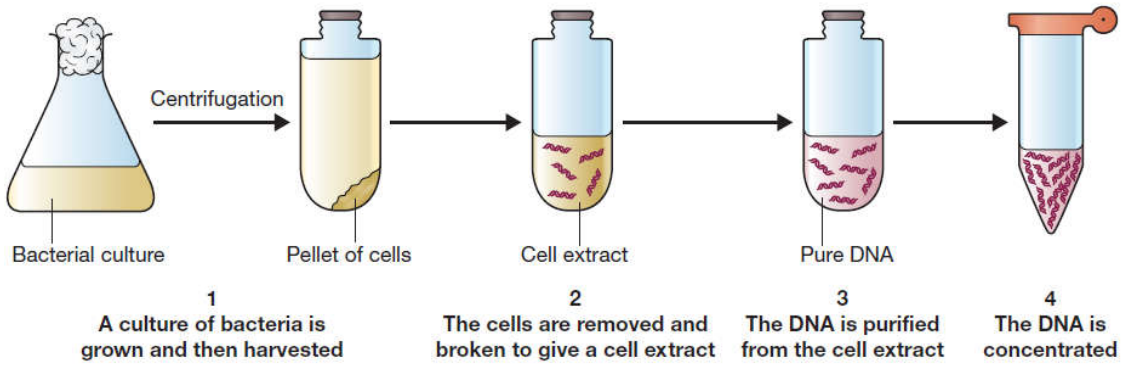


Fig: The basic steps in preparation of total cell DNA from a culture of bacteria

1.Growing and harvesting a bacterial culture

Most bacteria can be grown without too much difficulty in a liquid medium (**broth culture**). The culture medium must provide a balanced mixture of the essential nutrients at concentrations that will allow the bacteria to grow and divide efficiently.

M9 is an example of a defined medium in which all the components are known. This medium contains a mixture of inorganic nutrients to provide essential elements such as nitrogen, magnesium, and calcium, as well as glucose to supply carbon and energy. In practice, additional growth factors such as trace elements and vitamins must be added to M9 before it will support bacterial growth. Precisely which supplements are needed depends on the species concerned.

The second medium described in Table is rather different. Luria-Bertani (LB) is a complex or undefined medium, meaning that the precise identity and quantity of its components are not known. This is because two of the ingredients, tryptone and yeast extract, are complicated mixtures of unknown chemical compounds. Tryptone in fact supplies amino acids and small peptides, while yeast extract (a dried preparation of partially digested yeast cells) provides the nitrogen requirements, along with sugars and inorganic and organic nutrients. Complex media such as LB need no further supplementation and support the growth of a wide range of bacterial species.

Defined media must be used when the bacterial culture has to be grown under precisely controlled conditions. However, this is not necessary when the culture is being grown simply as a source of DNA, and under these circumstances a complex medium is appropriate. In LB medium at 37°C, aerated by shaking at 150–250 rpm on a rotary platform, *E. coli* cells divide once every 20 min The growth of the culture can be monitored by reading the optical density (OD) at 600 nm.

Preparation of a cell extract

The bacterial cell is enclosed in a cytoplasmic membrane and surrounded by a rigid cell wall. With some species, including *E. coli*, the cell wall may itself be enveloped by a second, outer membrane. All of these barriers have to be disrupted to release the cell components. Techniques for breaking open bacterial cells can be divided into physical methods, in which the cells are disrupted by mechanical forces, and chemical methods, where cell lysis is brought about by exposure to chemical agents that affect the integrity of the cellbarriers.

Chemical methods are most commonly used with bacterial cells when the object is DNA preparation. Chemical lysis generally involves one agent attacking the cell wall and another disrupting the cell membrane (Figure). The chemicals that are used depend on the species of bacterium involved, but with *E. coli* and related organisms, weakening of the cell wall is usually brought about by **lysozyme**, ethylenediamine tetraacetate (EDTA), or a combination of both. Lysozyme is an enzyme that is present in egg white and in secretions such as tears and saliva, and which digests the polymeric compounds that give the cell wall its rigidity. EDTA removes magnesium ions that are essential for preserving the overall structure of the cell envelope, and also inhibits cellular enzymes that could degrade DNA. Under some conditions, weakening the cell wall with lysozyme or EDTA is sufficient to cause bacterial cells to burst, but usually a detergent such as sodium dodecyl sulphate (SDS) is also added. Detergents aid the process of lysis by removing lipid molecules and thereby cause disruption of the cell membranes.

Having lysed the cells, the final step in preparation of a cell extract is removal of insoluble cell debris. Components such as partially digested cell wall fractions can be pelleted by centrifugation (Figure), leaving the cell extract as a reasonably clear supernatant.

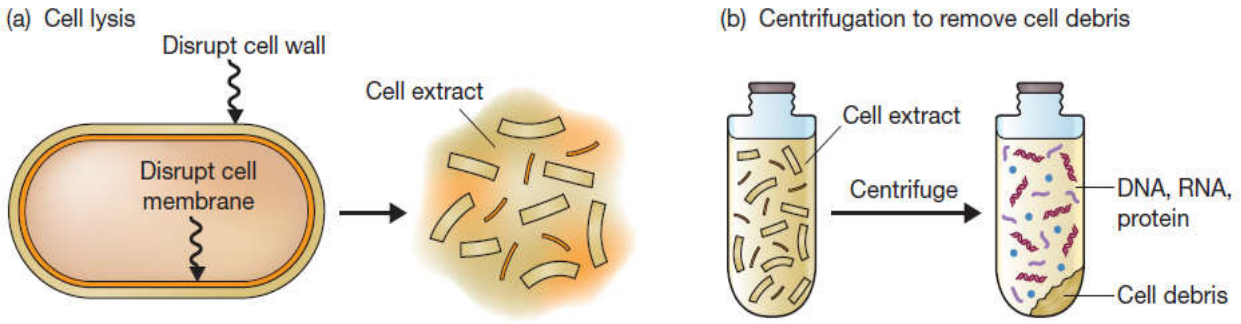


Fig: Preparation of a cell extract. (a) Cell lysis. (b) Centrifugation of the cell extract to remove insoluble debris.

3.Purification of DNA from a cell extract

In addition to DNA, a bacterial cell extract contains significant quantities of protein and RNA. A variety of methods can be used to purify the DNA from this mixture. One approach is to treat the

mixture with reagents which degrade the contaminants, leaving a pure solution of DNA (Figure 3.5a). Other methods use **ion-exchange chromatography** to separate the mixture into its various components, so the DNA is removed from the proteins and RNA in the extract (Figure).

Removing contaminants by organic extraction and enzyme digestion

The standard way to deproteinize a cell extract is to add phenol or a 1 : 1 mixture of phenol and chloroform. These organic solvents precipitate proteins but leave the nucleic acids (DNA and RNA) in aqueous solution. The result is that if the cell extract is mixed gently with the solvent, and the layers then separated by centrifugation, precipitated protein molecules are left as a white coagulated mass at the interface between the aqueous and organic layers (Figure). The aqueous solution of nucleic acids can then be removed with a pipette.

With some cell extracts the protein content is so great that a single phenol extraction is not sufficient to completely purify the nucleic acids. This problem could be solved by carrying out several phenol extractions one after the other, but this is undesirable as each mixing and centrifugation step results in a certain amount of breakage of the DNA molecules. The answer is to treat the cell extract with a **protease** such as pronase or proteinase K before phenol extraction. These enzymes break polypeptides down into smaller units, which are more easily removed by phenol. Some RNA molecules, especially **messenger RNA (mRNA)**, are removed by phenol treatment, but most remain with the DNA in the aqueous layer. The only effective way to remove the RNA is with the enzyme **ribonuclease**, which rapidly degrades these molecules into ribonucleotide subunits.

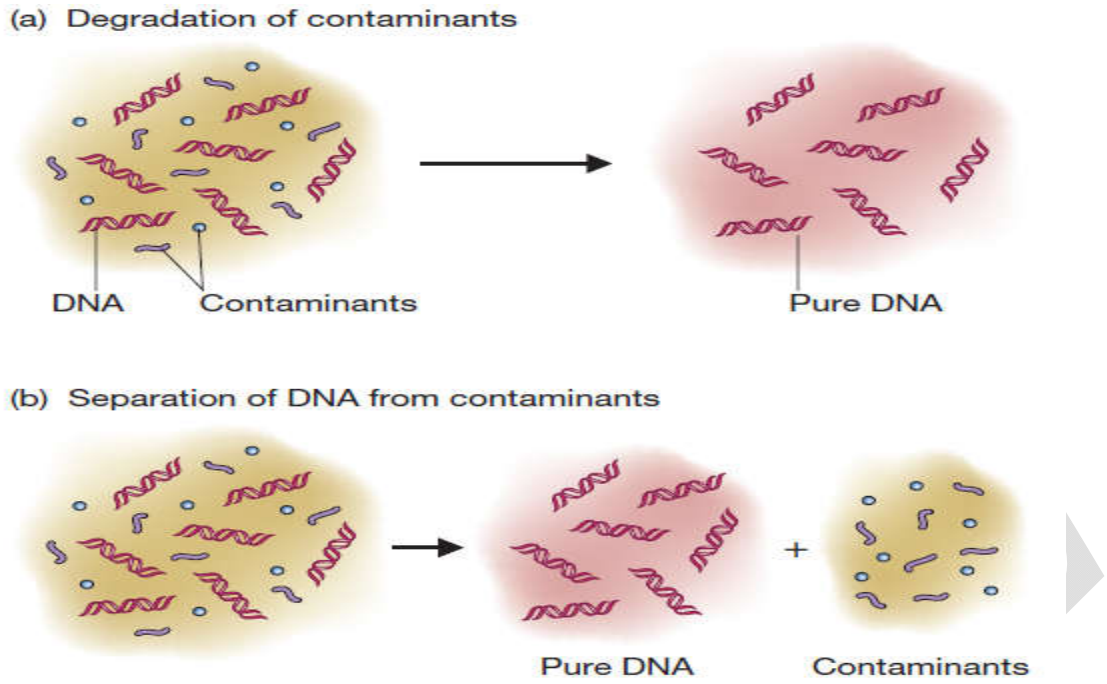


Fig: Two approaches to DNA purification. (a) Treating the mixture with reagents which degrade the contaminants, leaving a pure solution of DNA. (b) Separating the mixture into different fractions, one of which is pure DNA

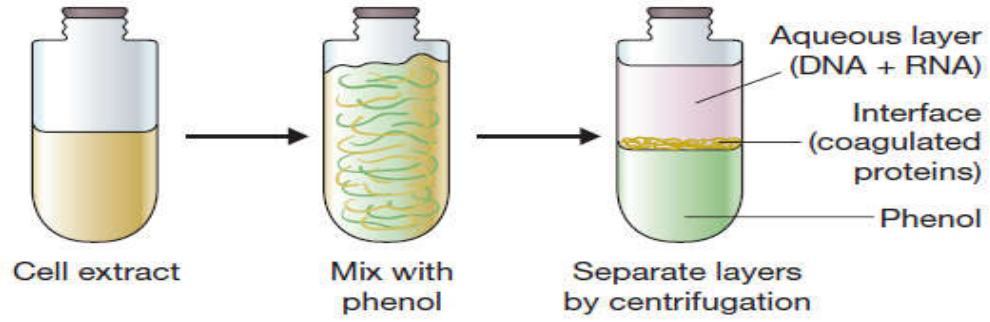


Fig: Removal of protein contaminants by phenol extraction

4. Concentration of DNA samples

Organic extraction often results in a very thick solution of DNA that does not need to be concentrated any further. Other purification methods give more dilute solutions and it is therefore important to consider methods for increasing the DNA concentration. The most frequently used method of concentration is **ethanol precipitation**. In the presence of salt (strictly

speaking, monovalent cations such as sodium ions and at a temperature of −20°C or less, absolute ethanol efficiently precipitates polymeric nucleic acids. With a thick solution of DNA the ethanol can be layered on top of the sample, causing molecules to precipitate at the interface. A spectacular trick is to push a glass rod through the ethanol into the DNA solution. When the rod is removed DNA molecules adhere and can be pulled out of the solution in the form of a long fiber (Figure).

Alternatively, if ethanol is mixed with a dilute DNA solution, the precipitate can be collected by centrifugation (Figure 3.8b), and then redissolved in an appropriate volume of water. Ethanol precipitation has the added advantage of leaving short-chain and monomeric nucleic acid components in solution. Ribonucleotides produced by ribonuclease treatment are therefore lost at this stage.

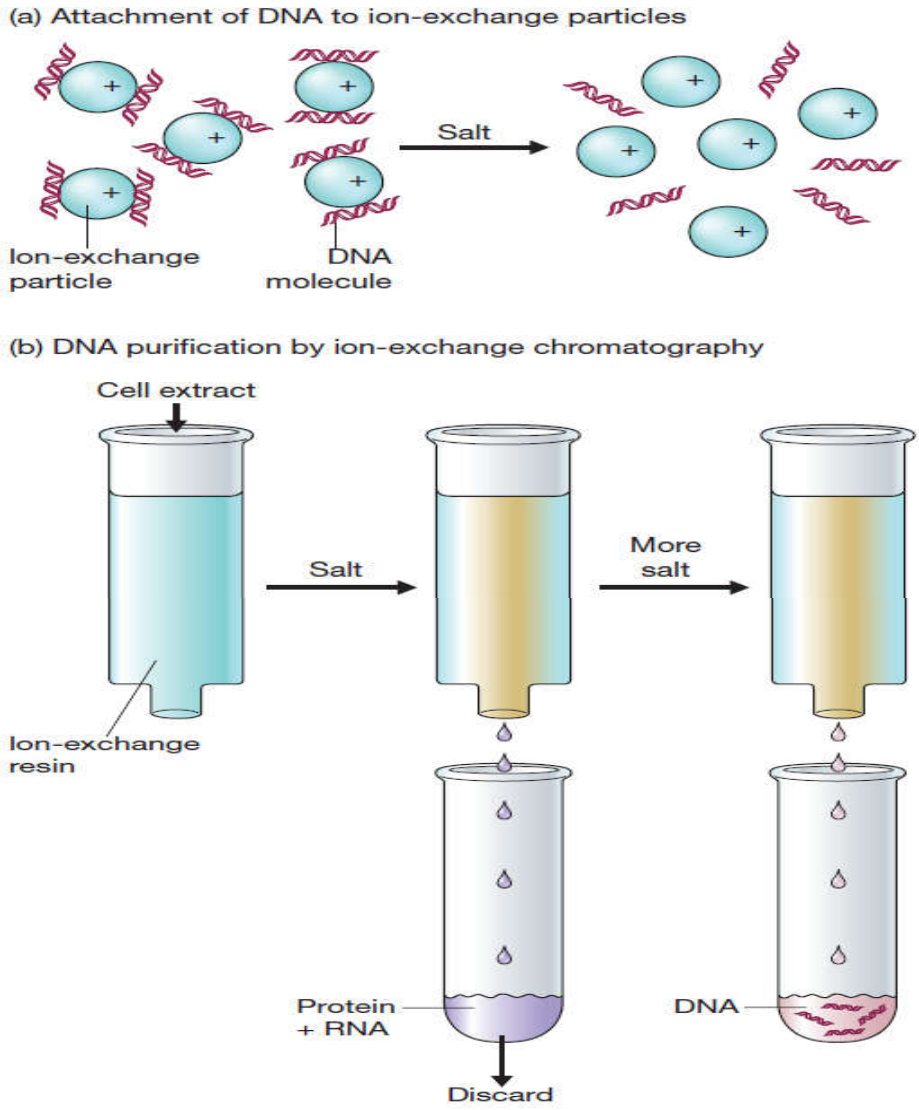


Fig:DNA purification by the guanidinium thiocyanate and silica method. (a) In the presence of guanidinium thiocyanate, DNA binds to silica particles. (b) DNA is purified by column chromatography.

5.Measurement of DNA concentration

It is crucial to know exactly how much DNA is present in a solution when carrying out a gene cloning experiment. Fortunately DNA concentrations can be accurately measured by **ultraviolet (UV) absorbance spectrophotometry**. The amount of UV radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Usually absorbance is measured at 260 nm, at which wavelength an absorbance (A260) of 1.0 corresponds to 50 mg of

double-stranded DNA per ml. Measurements of as little as 1 fl of a DNA solution can be carried out in spectrophotometers designed especially for this purpose. Ultraviolet absorbance can also be used to check the purity of a DNA preparation. With a pure sample of DNA, the ratio of the absorbances at 260 and 280 nm (A260/A280) is 1.8. Ratios of less than 1.8 indicate that the preparation is contaminated, either with protein or with phenol.

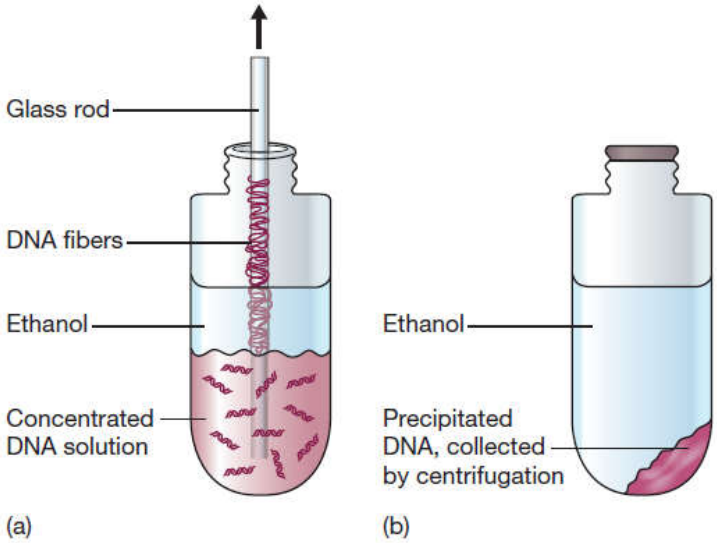


Fig: Collecting DNA by ethanol precipitation. (a) Absolute ethanol is layered on top of a concentrated solution of DNA. Fibers of DNA can be withdrawn with a glass rod. (b) For less concentrated solutions ethanol is added (at a ratio of 2.5 volumes of absolute ethanol to 1 volume of DNA solution) and precipitated DNA collected by centrifugation.

2.PREPARATION OF PLASMID DNA

Purification of plasmids from a culture of bacteria involves the same general strategy as preparation of total cell DNA. A culture of cells, containing plasmids, is grown in liquid medium, harvested, and a cell extract prepared. The protein and RNA are removed, and the DNA probably concentrated by ethanol precipitation. However, there is an

important distinction between plasmid purification and preparation of total cell DNA. In a plasmid preparation it is always necessary to separate the plasmid DNA from the large amount of bacterial chromosomal DNA that is also present in the cells. Separating the two types of DNA can be very difficult, but is nonetheless essential if the asmids are to be used as cloning vectors.

The presence of the smallest amount of contaminating bacterial DNA in a gene cloning experiment can easily lead to undesirable results. Fortunately several methods are available for removal of bacterial DNA during plasmid purification, and the use of these methods, individually or in combination, can
result in isolation of very pure plasmid DNA.

The methods are based on the several physical differences between plasmid DNA and bacterial DNA, the most obvious of which is size. The largest plasmids are only 8% of the size of the *E. coli* chromosome, and most are much smaller than this. Techniques that can separate small DNA molecules from large ones should therefore effectively
purify plasmid DNA.

In addition to size, plasmids and bacterial DNA differ in **conformation**. When applied to a polymer such as DNA, the term conformation refers to the overall spatial configuration of the molecule, with the two simplest conformations being linear and circular. Plasmids and the bacterial chromosome are circular, but during preparation of the cell extract the chromosome is always broken to give linear fragments. A method for separating circular from linear molecules will therefore result in pure plasmids.

a)Separation on the basis of size

The usual stage at which size fractionation is performed is during preparation of the cell extract. If the cells are lysed under very carefully controlled conditions, only a minimal amount of chromosomal DNA breakage occurs. The resulting DNA fragments are still very large—much larger than the plasmids—and can be removed with the cell debris by centrifugation. This process is aided by the fact that the bacterial chromosome is physically attached to the cell envelope, so fragments of the chromosome sediment with the cell debris if these attachments are not broken.

Cell disruption must therefore be carried out very gently to prevent wholesale breakage of the bacterial DNA. For *E. coli* and related species, controlled lysis is performed as shown in Figure 3.11. Treatment with EDTA and lysozyme is carried out in the presence of sucrose, which prevents the cells from bursting immediately. Instead, **sphaeroplasts** are formed, cells with

partially degraded cell walls that retain an intact cytoplasmic membrane. Cell lysis is now induced by adding a non-ionic detergent such as Triton X-100 (ionic detergents, such as SDS, cause chromosomal breakage). This method causes very little breakage of the bacterial DNA, so centrifugation leaves a **cleared lysate**, consisting almost entirely of plasmid DNA. A cleared lysate will, however, invariably retain some chromosomal DNA. Furthermore, if the plasmids themselves are large molecules, they may also sediment with the cell debris. Size fractionation is therefore rarely sufficient on its own, and we must consider alternative ways of removing the acterial DNA contaminants.

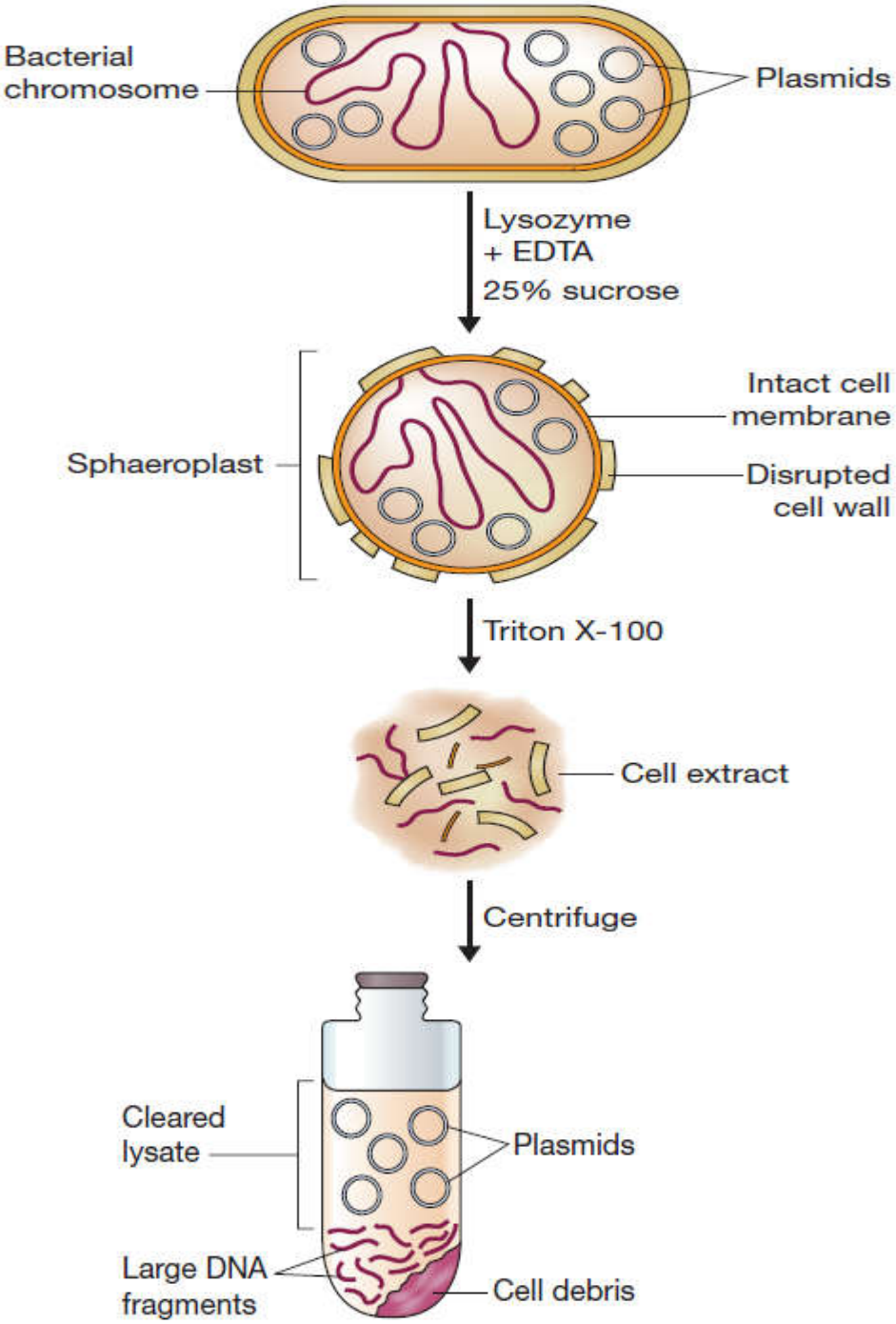


Fig: Preparation of a cleared lysate.

b) Separation on the basis of conformation

Before considering the ways in which conformational differences between plasmids and bacterial DNA can be used to separate the two types of DNA, we must look more closely at the overall structure of plasmid DNA. It is not trictly correct to say that plasmids have a circular conformation, because double-stranded DNA circles can take up one of two quite distinct configurations. Most plasmids exist in the cell as **supercoiled** molecules (Figure). Supercoiling occurs because the double helix of the plasmid DNA is partially unwound during the plasmid replication process by enzymes called topoisomerases (p. 69). The supercoiled conformation can be maintained only if both polynucleotide strands are intact, hence the more technical name of **covalently closedcircular (ccc) DNA**. If one of the polynucleotide strands is broken the double helix reverts to its normal **relaxed** state, and the plasmid takes on the alternative conformation, called **open-circular (oc)** (Figure).

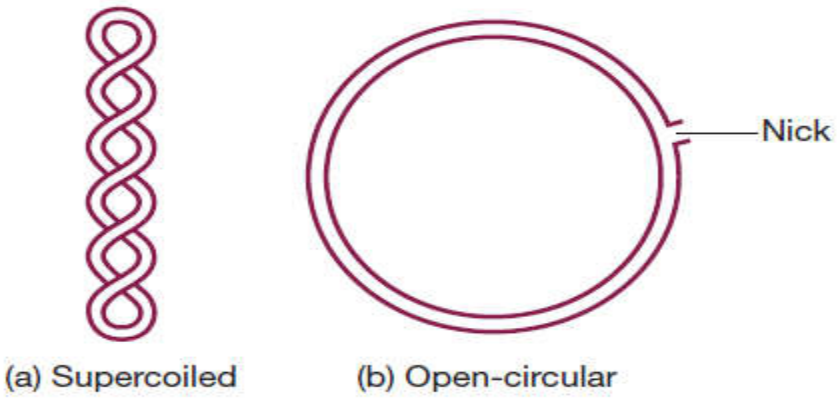


Fig: Two conformations of circular double-stranded DNA: (a) supercoiled—both strands are intact; (b) open-circular

—one or both strands are nicked

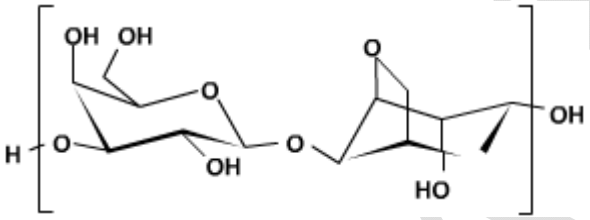
Supercoiling is important in plasmid preparation because supercoiled molecules can be fairly easily separated from non-supercoiled DNA. Two different methods are commonly used. Both can purify plasmid DNA from crude cell extracts, although in practice best results are obtained if a cleared lysate is first prepared.

Agarose gel electrophoresis

Agarose gel electrophoresis is the easiest and most popular way of separating and analyzing DNA. Here DNA molecules are separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter molecules migrate more easily and move faster than longer

molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide.

Agarose is a polysaccharide obtained from the red algae Porphyra umbilicalis. Its systematic name is (1 4)-3, 6-anhydro-a-L-galactopyranosyl-(1 3)-β-D-galactopyranan. Agarose makes an inert matrix. Most agarose gels are made between 0.7% and 2% of agarose. A 0.7% gel will show good separation for large DNA fragments (5-10kb) and a 2% gel will show good resolution for small fragments with size range of 0.2-1kb. Low percentage gels are very weak (Note:- it may break when you lift them) but high percentage gels are usually brittle and do not set evenly. The volume of agarose required for a minigel preparation is around 30-50ml and for a larger gel, it is around 250ml.



Voltage Applied

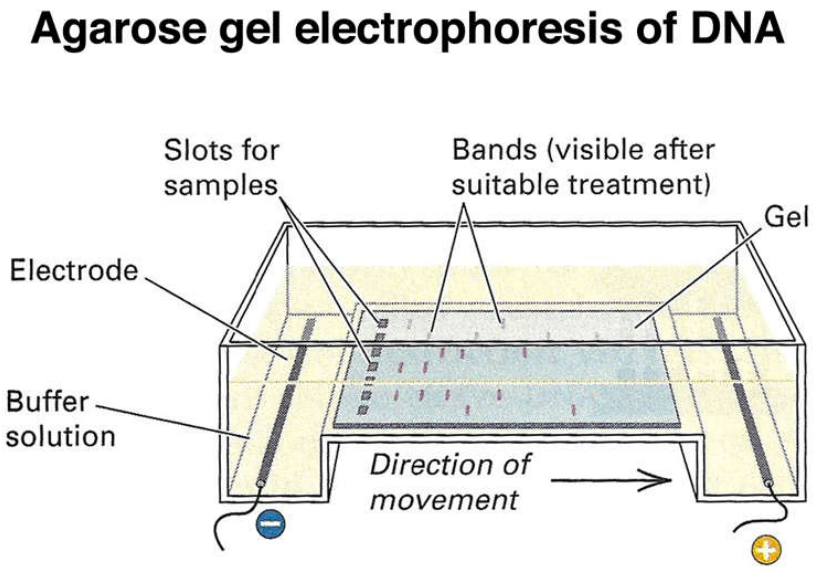
The migration rate of the linear DNA fragments through agarose gel is proportional to the voltage applied to the system. As voltage increases, the speed of DNA also increases. But voltage should be limited because it heats and finally causes the gel to melt.

Ethidium Bromide(EtBr)

It is an intercalating agent which intercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel. When exposed to UV light, it will fluoresce with an orange colour. After the running of DNA through an EtBr-treated gel, any band containing more than ~20 ng DNA becomes distinctly visible under UV light. EtBr is a known "mutagen", however, safer alternatives are available. It can be incorporated with agarose gels or DNA samples before loading, for visualization of the fragments. Binding of Ethidium bromide to DNA alters its mass and rigidity, and thereby its mobility.

Buffers

Several different buffers have been recommended for electrophoresis of DNA. The most commonly used buffers are Tris-acetate-EDTA (TAE) and Tris-borate-EDTA(TBE). The migration rate of DNA fragments in both of these buffers is somewhat different due to the differences in ionic strength. These buffers provide the ions for supporting conductivity.



Agarose gel electrophoresis

- The progress of the first experiments on cutting and joining of DNA molecules was monitored by velocity sedimentation in sucrose gradients. However, this has been entirely replaced by gel electrophoresis.
- Gel electrophoresis is not only used as an analytical method, it is routinely used preparatively for the purification of specific DNA fragments.
- The gel is composed of polyacrylamide or agarose.
- Agarose is convenient for separating DNA fragments ranging in size from a few hundred base pairs to about 20 kb.
- Polyacrylamide is preferred for smaller DNA fragments.
    - The migration of the DNA molecules through the pores of the matrix must play an important role in molecular-weight separations since the electrophoretic mobility of DNA in free solution is independent of molecular weight.

- An agarose gel is a complex network of polymeric molecules whose average pore size depends on the buffer composition and the type and concentration of agarose used.
- DNA movement through the gel was originally thought to resemble the motion of a snake (reptation).
- DNA molecules display elastic behaviour by stretching in the direction of the applied field and then contracting into dense balls.
- The larger the pore size of the gel, the greater the ball of DNA which can pass through and hence the larger the molecules which can be separated.
- Once the globular volume of the DNA molecule exceeds the pore size, the DNA molecule can only pass through by reptation.
- This occurs with molecules about 20 kb in size and it is difficult to separate molecules larger than this without recourse to pulsed electrical fields.
- The bands of DNA in the gel are stained with the intercalating dye ethidium bromide and as little as 0.05 μg of DNA in one band can be detected as visible fluorescence when the gel is illuminated with ultraviolet light.
- In addition to resolving DNA fragments of different lengths, gel electrophoresis can be used to separate different molecular configurations of a DNA molecule.
- Gel electrophoresis can also be used for investigating protein–nucleic acid interactions in the so-called *gel retardation* or *band shift* assay.
- It is based on the observation that binding of a protein to DNA fragments usually leads to a reduction in electrophoretic mobility.
- The assay typically involves the addition of protein to linear double-stranded DNA fragments, separation of complex and naked DNA by gel electrophoresis and visualization.
- Marker DNA fragments of known sizes are run in a separate lane; this permits an accurate determination of the size of an unknown DNA molecule by interpolation. The gels are stained with the intercalcating dye ethidium bromide which, gives visible fluorescence on illumination of the gel with UV light; as little as 0.05 μg of DNA in one band can be detected by using this dye.

HYBRIDISATION PROBES

A probe is a small, fluorescently or radioactively labeled DNA molecule that is used to locate similar or complementary sequences among a long stretch of DNA molecule or bacterial colonies such as genomic or cDNA libraries or in a genome. Such DNA probes are used in hybridization experiments such as Southern hybridization to detect certain specific sequences, which are complementary to the probes. Since the probe is labeled with a fluorescent dye or radioactive isotopes of phosphorous, its binding to specific sequences can be detected. DNA probes labeled with radioactive isotopes or fluorescent dyes can be used for the screening of transformed colonies having the correct recombinant plasmid by Southern hybridization.

HYBRIDISATION TECHNIQUES

Nucleic acid blotting

Nucleic acid labelling and hybridization on membranes have formed the basis for a range of experimental techniques central to recent advances in our understanding of the organization and expression of the genetic material.

These techniques may be applied in the isolation and quantification of specific nucleic acid sequences and in the study of their organization, intracellular localization, expression and regulation.

A variety of specific applications includes the diagnosis of infectious and inherited disease.

Blotting describes the immobilization of sample nucleic acids on to a solid support, generally nylon or nitrocellulose membranes.

The blotted nucleic acids are then used as 'targets' in subsequent hybridization experiments. The main blotting procedures are:

Southern Hybridization

The name of this technique is derived from the following:

(1) the name of its inventor, E.M. Southern, and

(2) the DNA-DNA hybridization that forms its basis. It is also called Southern blotting since the procedure for transfer of DNA from the gel to the nitrocellulose filter resembles blotting.

1. This technique has since been extended to the analysis of RNA (northern blotting) and proteins (western blotting); these names are only jargon terms, i.e., reverse of Southern being northern and so on, and do not reflect any functional or historical significance.
2. This approach is useful when few DNA fragments with considerable length differences are to be separated and studied. This approach also separates the closed circular (supercoiled), nicked (relaxed) and linear configurations of a single DNA molecule.
3. In many situations, it is critical to detect and identify DNA fragments in a sample that are complementary to a given DNA sequence, e.g., to demonstrate the presence of the gene in question in transgenics, to detect and stuty RFLP (restriction fragment length polymorphism), etc.

In Southern hybridization, a sample of DNA containing fragments of different sizes is subjected to electrophoresis using either polyacrylamide or agarose gel.
The DNA sample may either be subjected to mechanical shearing or to restriction endonuclease digestion in order to generate the fragments.

This is achieved by Southern hybridization in which the following steps are performed.
1. The restriction fragments of DNA present in agarose gel (after electrophoresis) are denatured into single-stranded form by alkali treatment.
2. They are then transferred onto a nitrocellulose filter membrane; this is done by placing the gel on top of a buffer saturated filter paper, then laying the nitrocellulose filter membrane on the top of gel, and finally placing some dry filter papers on top of this membrane. The buffer moves, due to capillary action, from the bottom filter paper through the gel carrying with it the denatured DNA present in the gel; the DNA becomes trapped in the nitrocellulose membrane as the buffer phases through it. This process is known as blotting and takes several hours to complete. The relative positions of the bands on the membrane remain the same as those in the gel and there is a minimal loss in their resolution (sharpness).
3. The nitrocellulose membrane is now removed from the blotting stack, and the DNA is permanently immobilized on the membrane by baking it at 80°C in vacuo.
4. Single-stranded DNA has a high affinity for nitrocellulose filter membrane. (Note that RNA

lacks this affinity). Therefore, the baked membrane is treated, with a solution containing 0.2% each of Ficoll (an artificial polymer of sucrose), polyvinylpyrrolidon and bovine serum albumin; this mixture is often supplemented with an irrelevant nucleic acid, e.g., tRNA (pretreatment). This treatment prevents nonspecific binding of the radioactive probe (to be used in the next step) probably by attaching macromolecules to all the free binding sites on the membrane. Often the above mixture is included in the hybridization reaction itself.

5. The pretreated membrane is placed in a solution of radioactive, single-stranded DNA or an oligodeoxynucleotide (a DNA segment having few to several nucleotides) called probe. The name probe signifies the fact that this DNA molecule is used to detect and identify the DNA fragment in the gel/membrane that is complementary to the probe.
The conditions during this step are chosen so that the probe hybridizes with the complementary DNA on the membrane to the greatest extent with a low nonspecific binding on the membrane; this step is known as hybridization reaction.

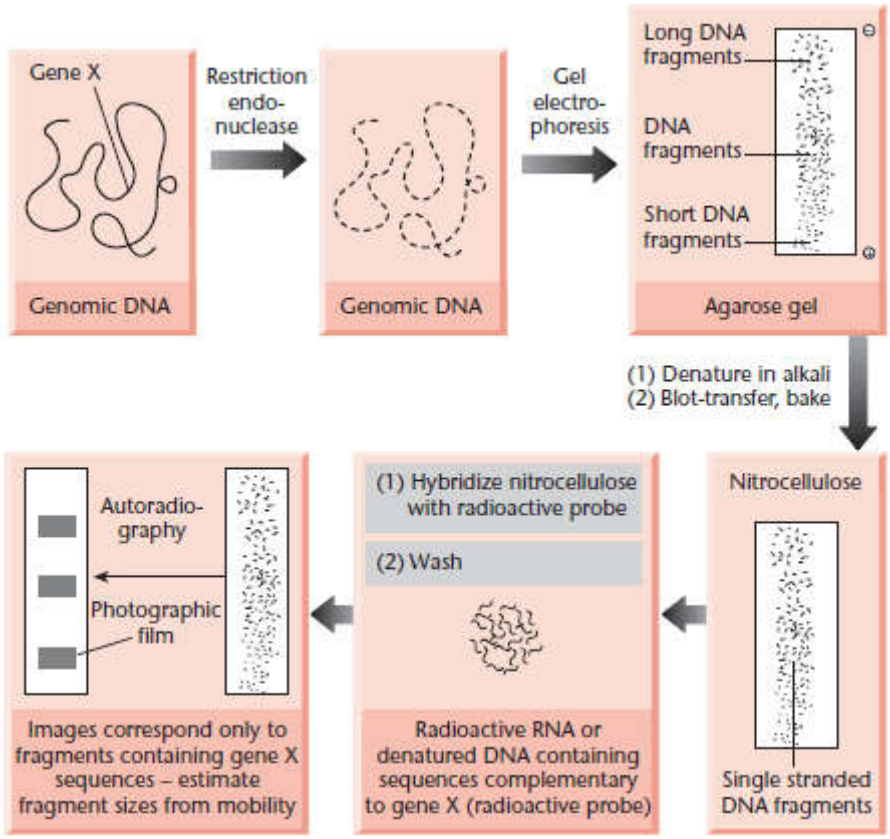
Usually, the initial hybridization reaction is carried out under conditions of relatively low stringency of hybridization to permit a high rate of hybridization; this is followed by a series of post hybridization washes of increasing stringency, i.e., higher temperature or, more commonly, lower ionic strength, with a view to eliminate the pairing of radioactive probe to related sequences and to allow only perfectly complementary pairing.

6. After the hybridization reaction, the membrane is washed to remove the unbound probes.

7. The membrane is now placed in close contact with an X-ray film and incubated for a desired period to allow images due to the radioactive probes to be formed on the film. The film is then developed to reveal distinct band(s) indicating positions in the gel of the DNA fragments that are complementary to the radioactive probe used in the study.

It should be kept in mind that electrophoresis of sheared or restricted DNA produces a smear in which the fragments are distributed in a continuum according to their size, and there are no distinct bands. The distinct bands are produced by the hybridization reaction of the selected probe with one or few fragment sequences present in the gel.

The Southern blotting technique is extremely sensitive. It can be used to map the restriction sites around a single copy gene sequence in any genome (even of man). It is used for DNA fingerprinting, preparation of RFLP maps, detection and identification of the transferred genes in transgenic individuals, etc.



Recently some new membrane materials, e.g., nylon membranes, have been developed which have the following advantageous features:

(1) They are physically robust in comparison to nitrocellulose filter membranes,

(2) both DNA and RNA become cross-linked to them by a brief exposure to UV light, which

(3) saves the time needed for baking in vacuo in the case of nitrocellulose membranes, and

(4) the same membrane blot, e.g., a membrane onto which DNA/RNA has been transferred from a gel and cross-linked by UV exposure, can be used for search with more than one probe after removing the earlier probe by high temperature washing or some other denaturing procedure; in other words, the nylon membranes are reusable.

Northern Hybridization - In this technique,
RNAs are separated by gel electrophoresis, the RNA bands are transferred onto a suitable membrane, e.g., diazobenzyloxymethyl (DBM) paper or nylon membranes, and immobilized; the bands are hybridized with radioactive single stranded DNA probes, and the bands showing hybridization are detected by autoradiography.

Clearly, northern blotting/hybridization is simply an extension of the Southern blotting technique. The two techniques, however, show the following differences:

(1) in Southern hybridization, DNA is separated by gel electrophoresis, while in northern blotting RNAs are separated;
(2) as a result, in Southern hybridization DNA has to be denatured before blotting, while this step is not needed in northern hybridization;
(3) nitrocellulose membrane is generally not used for northern, while it is often used for Southern hybridization; and finally
(4) hybridization with the probe produces DNA: DNA hybrid molecules in Southern hybridization but RNA : DNA molecules in northern hybridization.

Initially, specially prepared paper (diazobenzyloxymethyl, DBM, paper prepared by diazotization of aminobenzyloxymethyl paper) was used for northern blotting since RNA did not bind to nitrocellulose membrane. RNA becomes covalently bound to DBM paper due to which these blot transfers are reusable.
DBM is also equally effective in binding to denatured DNA, and is more efficient than nitrocellulose in binding to small DNA fragments. Recently developed nylon membranes have superceded the use of DBM paper as they are robust, reusable and bind (by cross linking) to RNA on a brief exposure to UV light.

Northern hybridization is useful in the identification and separation of the RNA that is

complementary to a specific DNA probe; this is a sensitive test for the detection of transcription of a DNA sequence that is used as probe.

3.2.3. Western Blotting - In western blotting,

proteins are electrophoresed in polyacrylamide gel, transferred onto a nitrocellulose or nylon membrane (to which they bind strongly), and the protein bands are detected by their specific interaction with antibodies, lectins or some other compounds. The various steps of this technique are briefly described below.

1. Protein bands are separated by polyacrylamide gel electrophoresis.
2. The protein bands are transferred onto a nitrocellulose or nylon membrane; initially this was achieved by a capillary movement of buffer similar to Southern blotting (capillary blotting), but nowadays it is usually done by electrophoresis (electrophoretic blotting).

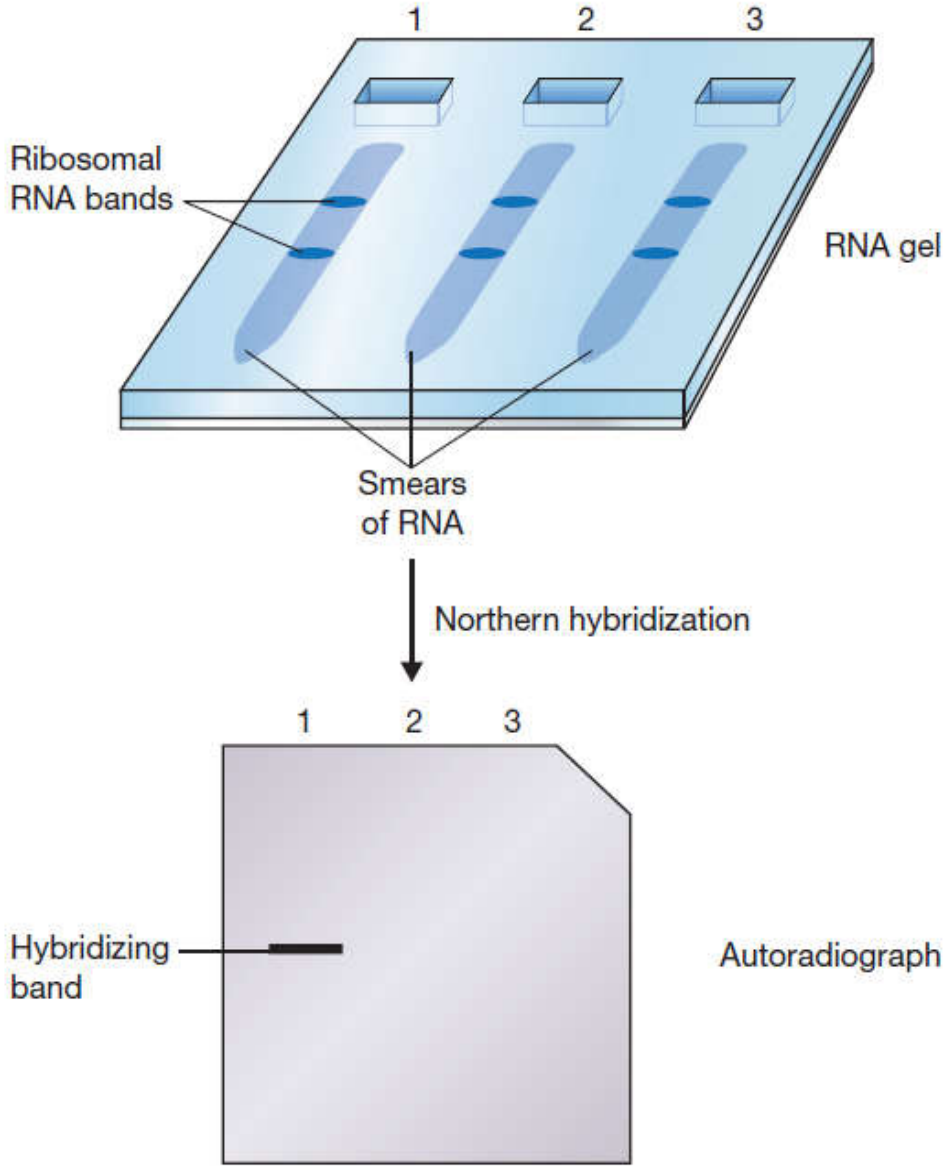
Electrophoresis has been applied for the blotting step in Southern and northern hybridizations as well; in such 'cases buffer of low ionic strength (to avoid overheating during electrophoresis) and nylon membranes (since nucleic acids bind to nitrocellulose membrane only under conditions of high ionic strength) are used. The electrophoretic blotting, both of proteins and nucleic acids is much faster and more efficient than capillary blotting.

3. The specific protein bands are identified in a variety of ways.

(i) Antibodies are the most commonly used as probes for detecting specific antigens.

(ii) Lectins are used as probes for the identification of glycoproteins.

- These probes may themselves be radioactive or a radioactive molecule may be tagged to them. Often the identification process is based on a 'sandwich' reaction.
- In such an approach, a species specific second antibody or protein A of Staphylococcus aureus (protein A binds to certain subclasses of IgG antibodies) or streptavidin (it binds to biotinylated antibodies) is used to bind to the antibodies bound to the protein bands.
- These second molecules may be labelled with radioactive, enzyme or fluorescent tags; a single preparation of these labelled molecules can be employed as a general detector for various probes.



Northern hybridization. Three RNA extracts from different tissues have been electrophoresed in an agarose gel. The extracts are made up of many RNAs of different lengths so each gives a smear of RNA, but two distinct bands are seen, one for each of the abundant ribosomal RNAs. The sizes of these rRNAs are known (e.g. 4718 and 1874 nucleotides in mammals), so they can be used as internal size markers. The gel is transferred to a membrane, probed with a cloned gene, and the results visualized, for example by autoradiography if the probe has been radioactively labeled. Only lane 1 gives a band, showing that the cloned gene is expressed only in the tissue from which this RNA extract was obtained.

Western blotting

The western blot (sometimes called the protein immunoblot) is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein. The gel electrophoresis step is included in western blot analysis to resolve the issue of the cross-reactivity of antibodies.

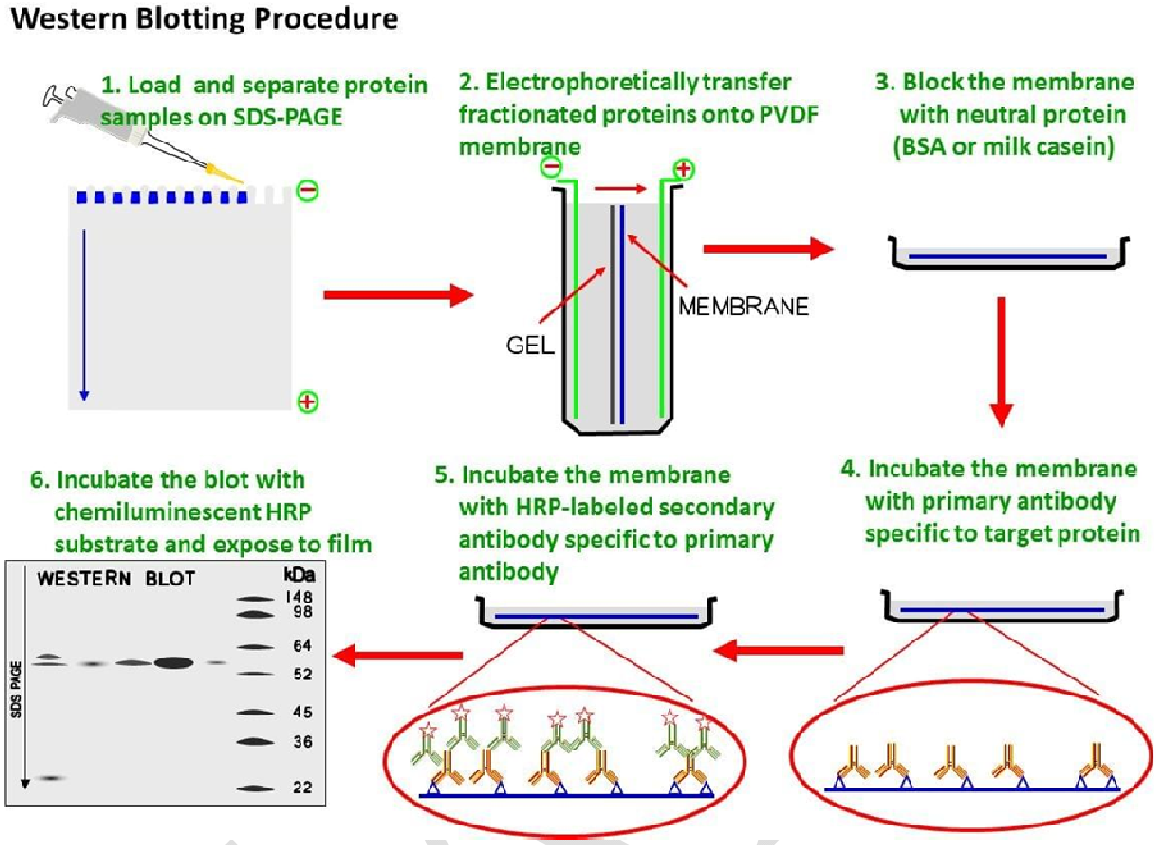
There are now many reagent companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against tens of thousands of different proteins.[3] Commercial antibodies can be expensive, although the unbound antibody can be reused between experiments. This method is used in the fields of molecular biology, immunogenetics and other molecular biology disciplines. A number of search engines, such as CiteAb, are available that can help researchers find suitable antibodies for use in western blotting.

Other related techniques include dot blot analysis, immunohistochemistry and immunocytochemistry where antibodies are used to detect proteins in tissues and cells by immunostaining, and enzyme-linked immunosorbent assay (ELISA).

The method originated in the laboratory of Harry Towbin at the Friedrich Miescher Institute.[1] The name *western blot* was given to the technique by W. Neal Burnett.

Applications

- Western blotting can be used to identify a specific antibody in a mixture. In this case, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotting onto nitrocellulose. The separated bands of known antigens are then probed with the sample suspected of containing antibody specific for one or more of these antigens. Reaction of an antibody with a band is detected by using either radiolabeled or enzyme linked secondary antibody that is specific for the species of the antibodies in the test sample. The most widely used application of this procedure is in confirmatory testing for HIV, where Western blotting is used to determine whether the patient has antibodies that react with one or more viral proteins.
- A western blot is also used as the definitive test for mad cow disease.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.



DNA and RNA markers

A genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. It can be described as a variation (which may arise due to mutation or alteration in the genomic loci) that can be observed.

DNA and RNA size markers contain a mixture of DNA (or RNA) fragments of known length, making them suitable for estimating the fragment length of concurrently run samples. They stain well with ethidium bromide and other common nucleic acid stains for visualization after gel electrophoresis.

POSSIBLE QUESTIONS

1. Detailed note on isolation and purification of nucleic acids.
2. Explain about agarose gel electrophoresis and its applications.
3. Discuss in detail about hybridization techniques.
4. Give an account on DNA and RNA markers.
5. Write about Southern blotting?
6. Discuss about the technique used for the detection of RNA.
7. Explain about Western blotting.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
I-M.Sc., BIOCHEMISTRY
RECOMBINANT DNA TECHNOLOGY (17BCP205A)
MULTIPLE CHOICE QUESTIONS

UNIT I

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	A recombinant DNA molecule is produced by	Joining of two DNA fragments	Joining of two or more DNA fragments	Joining of one or three DNA fragments	Joining of two or more DNA fragments originating from different organisms	Joining of two or more DNA fragments originating from different organisms
2	The gene formed by the joining of DNA segments from two different sources are called as	Recombinant gene	Joined gene	Gene	Chimeric gene	Chimeric gene
3	Which of the following enzyme is used to cut DNA molecule in rDNA technology	Ligase	Phosphatase	Ribonuclease	Restriction enzymes	Restriction enzymes
4	Restriction enzymes are also called as	Biological scissors	Molecular scalpels	Molecular knives	Biological cleavers	Biological scissors
5	The most important discovery that lead to the development of rDNA technology was	Double helix model of Watson and Crick	Discovery of restriction enzymes	Discovery of ligase enzyme	Discovery of plasmids	Discovery of restriction enzymes
6	Who discovered restriction enzymes	Nathan, Arber and Smith in 1970	Watson, Crick and Wilkins in 1970	Boyer and Cohen in 1975	Paul Berg in 1975	Nathan, Arber and Smith in 1970
7	Who created the first rDNA molecule	Nathan, Arber and Smith	Watson, Crick and Wilkins	Boyer and Cohen	Paul Berg	Paul Berg
8	Enzymes used to degrade nucleic acid is called as	Nucleases	Ligases	Polymerases	Modifying enzymes	Nucleases
9	The enzyme used to add a methyl group to the DNA is called	Amylase	Protease	Methylase	Ligases	Methylase
10	The enzyme used to joining nucleic acid molecules is	Topoisomerases	Exonuclease	Endonuclease	Ligases	Ligases
11	Which enzyme used to make copies of nucleic acid molecules	Nucleases	Ligases	Polymerases	Modifying enzymes	Polymerases
12	How many types of nucleases are present?	One	Two	Three	Four	Two
13	Which enzyme used to remove or add chemical groups?	Nucleases	Ligases	Polymerases	Modifying enzymes	Modifying enzymes
14	Which enzyme used to remove nucleotides from the ends of the DNA molecule?	Endonuclease	Exonuclease	Topoisomerases	Ligases	Exonuclease
15	The enzyme that introduce or remove supercoils from covalently closed circular DNA is	Topoisomerases	Exonuclease	Endonuclease	Ligases	Topoisomerases
16	The functions of Endonuclease is	Cleaving the DNA molecule	Add a methyl group to the DNA	Remove or add chemical groups to DNA	Introduce or remove supercoils from DNA	Cleaving the DNA molecule
17	The restriction endo nuclease that make cut on the recognition sequence itself is	Restriction endo nuclease I	Restriction endo nuclease II	Restriction endo nuclease III	Restriction endo nuclease IV	Restriction endo nuclease II
18	Which of the following is tracking dye	Methylene blue	Malachite green	Bromophenol blue	Xylene cyanol	Methylene blue
19	The crucial step in northern blotting is	Isolation of m RNA	Transfer of denatured RNA from gel to membrane	Denaturation of mRNA	Seperation through AGE	Isolation of m RNA
20	Southern blotting technique was identified by	Southern (1975)	Kary Mullis (1985)	Lederberg (1994)	Guray (1998)	Southern (1975)
21	DNA blotting technique is used to study about	DNA	RNA	Proteins	Lipids	DNA
22	The upward and backward transfer of DNA into membrane in southern blotting was performed by	Upward capillary transfer	Downward capillary transfer	Bi-directional transfer	Vacuum transfer	Bi-directional transfer
23	The method used when the target DNA fragment when present in higher concentration is	Capillary method	Vacuum blotting	Bi-directional blotting	Transfer in Alkaline buffer	Vacuum blotting
24	The polysaccharide contained in agarose is	Sucrose	Galactose	Fructose	Maltose	Galactose
25	Western blotting helps in the identification of_____	DNA	RNA	Protein	Aminoacid	Protein
26	Protein expression can be well studied with use of all except	Western blotting	ELISA	Flouresecence technique	Northern blotting	Western blotting
27	Which one is example for non radioactive labeling?	Biotin	RNA	Protein	Amino acid	Biotin
28	Useful method for mapping restriction sites around a single copy gene sequence	Western blotting	ELISA	Southern blotting	Northern blotting	Southern blotting
29	The southern hybridization results was read by	UV transilluminator	Autoradiography	Stainiing	Visible light	Autoradiography
30	Restriction DNA fragment lengths variation between individuals of a species is called	Restriction fragment length polymorphism	Random amplified polymorphic DNA	Amplified fragment length polymorphisms	Simple sequence repeats	Restriction fragment length polymorphism
31	Easily detectable differences showed by specific biomolecules among different strains of a species is termed as	DNA fingerprinting	Molecular marker	Molecular scissors	RFLP	Molecular marker
32	Quantitative genes location on chromosomes are called	Qualitative trait loci	Quantitative trait loci	Qualitative and quatitative trait	Trait loci	Qualitative trait loci
33	Which of the following statements about forensic analysis of DNA is incorrect?	A DNA profile using short tandem repeats is unique to an individual.	Forensic analysis makes use of SNPs in coding sequences to distinguish between individuals	PCR is used for DNA profiling (DNA fingerprinting).	DNA fingerprinting cannot be used for paternity testing	DNA fingerprinting cannot be used for paternity testing
34	Analysis of nitrogenous base sequence in DNA of an individual is	DNA foot printing	DNA finger printing	Protein profiling	RNA profiling	DNA finger printing
35	Settlement of paternity dispute are settled by	DNA foot printing	DNA finger printing	Protein profiling	RNA profiling	DNA finger printing
36	Pattern of RFLP and VNTR forms the basis of	DNA foot printing	DNA finger printing	Protein profiling	RNA profiling	DNA finger printing
37	DNA profiling is otherwise known as	DNA foot printing	DNA finger printing	Protein profiling	RNA profiling	DNA finger printing
38	---------- is the sample used for nuclec acid blotting	DNA	RNA	Protein	Both DNA and RNA	Both DNA and RNA
39	For nucleic acid hybridization nucleic acid is digested with------ -------- restriction enzyme	One	Two	More than two	Other than restriction enzymes	More than two
40	---------- refers to blotting of electrophoresed protein bands from SDS PAGE to membrane	Southern blotting	Northern blotting	Western blotting	Dot blotting	Western blotting

41	Which technique purified nucleic acid sample directlyapplied to nitrocellulose filter	Southern blotting	Northern blotting	Western blotting	Dot blotting	Dot blotting
42	Joining of donor DNA fragments and vector DNA fragments	Gene cloning	Splicing	Gene manipulation	Moleccular cloning	Gene cloning
43	Suitable host in genetic engineering to introduce DNA fragments of donor is	Bacillus subtilis	Escherechia coli	Bacteriophages	All of the above	All of the above
44	A southern blot is a technique used to detect	proteins	DNA	RNA	None	DNA
45	Type of restriction endonucleases most useful in molecular cloning is:	Type I	Type II	Type III	None	Type II
46	Northern blots probes are	DNA and RNA	DNA	RNA and protein	RNA	RNA
47	Which is the most specific recombinant library?	Genomic	protein	cDNA	cRNA	cDNA
48	Recombinant DNA research is dependent on……	Cloning	Hosts	Vectros	All of the above	All of the above
49	The polymerase chain reaction is a technique that selectively	RNA	DNA	Protein	None	DNA
50	Molecular Probe is a	Protein	Short piece of labeled DNA	Short piece of labeled protein	enzyme	Short piece of labeled DNA
51	The process somatic cell gene therapy involves	introduction of remedial gene into zygote	introduction of remedial gene into blood cells	introduction of remedial gene into sperm	introduction of remedial gene into egg	introduction of remedial gene into blood cells
52	In agarose gel electrophoresis, DNA is moved towards the	Cathode	Anode	DNA doesn't move	Both side	Cathode
53	In SDS electrophoresis, proteins are separated on basis of	Charge	Mass	Charge and mass	Structure	Mass
54	If proteins are separated according to their electrophoretic mobility then type of electrophoresis is	SDS page	Free flow electrophoresis	Electro focusing	Affinity electrophoresis	SDS page
55	A technique which separates charged particles using electric field is	Hydrolysis	Electrophoresis	Protein synthesis	Protein denaturing	Electrophoresis
56	Polyacrylamide gel is usually used for	Proteins	DNA	Proteins and DNA	Vitamins	Proteins and DNA
57	In genome southern blotting can be used to identify	Sequences	Number of sequences	DNA fragments	RNA sequence	Number of sequences
58	Arrange the following in correct order 1. Southern Blotting ----A)Alwine 2. Western blotting ----B)E.M.Southern 3. Northern Blotting----C) A. Jeffrey 4. DNA fingerprinting---- D) Towbin	1-A, 2-C, 3-D, 4-B	1-B, 2-D, 3-A, 4-C	1-B, 2-A, 3-D, 4-C	1-B, 2-C, 3-A, 4-C	1-B, 2-D, 3-A, 4-C
59	Arrange the following in correct order 1. Southern Blotting ----A)RNA-DNA hybrid 2. Western blotting -----B)DNA-DNA hybrid 3. Northern Blotting-----C)Southern blotting 4. DNA fingerprinting---D)antigen antibody reaction	1-A, 2-C, 3-D, 4-B	1-B, 2-D, 3-A, 4-C	1-B, 2-A, 3-D, 4-C	1-B, 2-C, 3-A, 4-C	1-B, 2-D, 3-A, 4-C
60	Which of the following technique is used in DNA finger printing?	Western blotting	Southern blotting	Northern blotting	Eastern blotting	Southern blotting

UNIT-II

SYLLABUS

Gene cloning vectors: Plasmids, bacteriophages, phagemids, cosmids, Artificial chromosomes-BAC, YAC, HAC. Restriction mapping of DNA fragments, Map construction, Cloning in *E. coli*- Vector engineering and codon optimization. Gene expression in *E.coli.* Expression vector-PET vector. Genomic library.

VECTORS - THE CLONING VEHICLES

Vectors are the DNA molecules, which can carry a foreign DNA fragment to be cloned. They are self-replicating in an appropriate host cell. The most important vectors are plasmids, bacteriophages, cosmids and artificial chromosome vectors.

Depending on the size of the insert DNA and the purpose of the experiment, many different types of cloning vectors can be used for the generation of recombinant molecules

A **cloning vector** is a small piece of DNA into which a foreign DNA fragment can be inserted. The insertion of the fragment into the cloning vector is carried out by treating the vehicle and the foreign DNA with a restriction enzyme that creates the same overhang, then ligating the fragments together. There are many types of cloning vectors. Genetically engineered plasmids and bacteriophages (such as phage λ) are perhaps most commonly used for this purpose. Other types of cloning vectors include bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs).

To act as cloning vector, a DNA molecule must be capable of entering a host and replicate itself inside the host. In addition, it must ideally be small in size (less than 10 kb) as larger molecules tend to break down during purification and are difficult to manipulate. Two naturally occurring types of DNA molecule satisfy these requirements: Plasmids and Viral Chromosomes.

1. Plasmid

Plasmids are extrachromosomal, doublestranded, circular, self-replicating DNA molecules. Almost all the bacteria have plasmids containing a low copy number (1-4 per cell) or a high copy

number (10-100 per cell). The size of the plasmids varies from 1 to 500 kb. Usually, plasmids contribute to about 0.5 to 5.0% of thetotal DNA of bacteria).Plasmids can carry one ore more genes. Most plasmids possess at least one DNA sequence that can act as an origin of replication, so they are able to multiply within the cell independently of the chromosome. A few types of plasmids insert themselves into the bacterial chromosome for replication. Such plasmids are called episomes.

The size and copy number of a plasmid is important. Copy number refers to the number of molecules of an individual plasmid that are normally found in a single bacterial cell. Plasmids range from 1 kb to 250 kb. Plasmid smaller than 10 kb are preferred for vectors.

Plasmids can be conjugative or non-conjugative. Conjugative plasmids are characterized by their ability to promote sexual conjugation between bacterial cells. Bacterial conjugation is the transfer of genetic material between bacteria through direct cell-to-cell contact. Conjugation and plasmid transfer are controlled by a set of transfer or tra genes which are present in conjugative genes but absent in non-conjugative genes.

To coexist in a cell, different plasmids must be compatible. If two plasmids are incompatible, one of them would quickly be lost from the cell. Thus plasmid can be assigned to different incompatibility groups. Naturally occuring plasmids are classified on the basis of the main characteristic code by the plasmid genes.

1. **Fertility or F plasmids:** carry only tra genes and have no characteristic beyond the ability to promote conjugation
2. **Resistance or R plasmids:** carry antibiotic resistance genes. Used as selectors in the lab
3. **Col plasmids:** code for colicins which are proteins that kill other bacteria
4. **Degradative plasmids:** allows the host bacteria to metabolize unusual molecules
5. **Virulence plasmids:** confer pathogenicity on the host bacteria

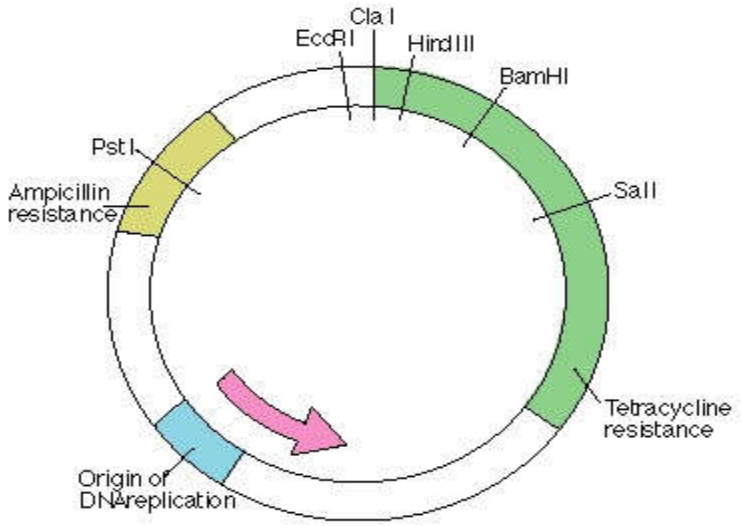
Nomenclature of plasmids : lt is a common practice to designate plasmid by a lower case p, followed by the first letter(s) of researcher(s) names and the numerical number given by the workers. Thus, pBR322 is a plasmid discovered by Bolivar and Rodriguezw ho designated it as 322.Some plasmids are given names of the places where they are discovered e.g. pUC is plasmid from University of California.

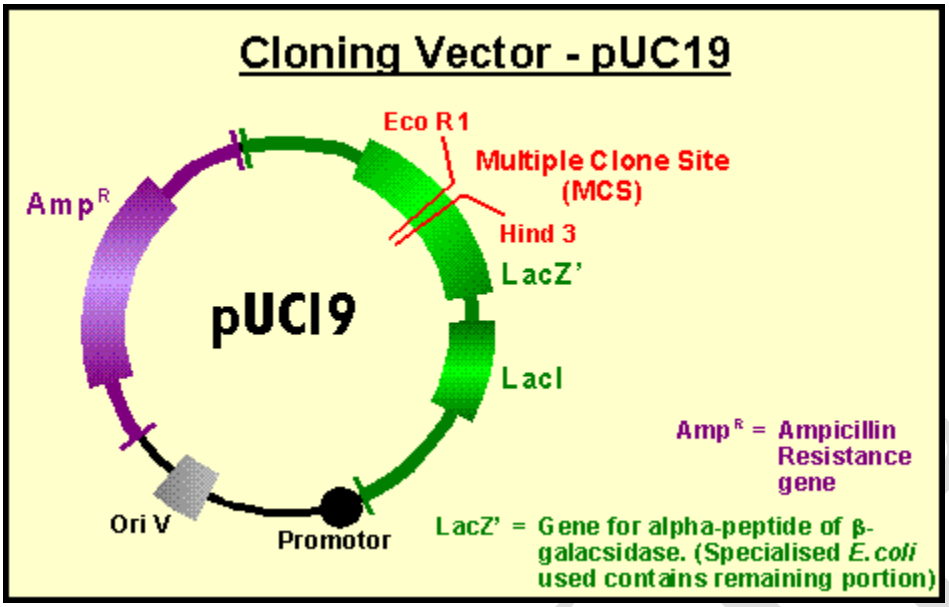
pBR322 - the most common plasmid vector :

pBR322 of *E.coli* is the most popular and widely used plasmid vector, and is appropriately regarded as the parent or grand parent of several other vectors. pBR322 has a DNA sequence of 4,361 bp. It carries genes resistance to ampicillin (Ampr) and tetracycline (Telr) that serve as markers for the identification of clones carrying plasmids. The plasmid has unique recognition sites for the action of restriction endonucleases such as *EcoRl, Hindlll, BamHl, Sall* and *Pstll.*

Other plasmid cloning vectors : The otherplasmids employed as cloning vectors include **pUC19** (2,686 bp, with ampicillin resistance gene), and derivatives of pBR322-p8R325, pBR328 and pBR329.

Cloning vector pBR322





2. Bacreriophages

Bacteriophages or phages are viruses that specifically infect bacteria. Viral chromosomes and chromosomes of bacteriophages, in particular, are ideal for inserting DNA into the host chromosome where they are replicated by the host chromosome.

Phages are very simple in structure. They consist DNA surrounded by a protective coat or capsid made up of proteins.

The general pattern of infection is a three-step process:

1.The phage attaches to the cell membrane of a bacteria and injects its DNA into the cell.

2. The inserted DNA is replicated by the bacteria's cell machinery

3. Protein components of the phage are assembled and the phages are released from the host

Lytic Cycle

The cell infection cycle is called a lytic as the release of phage particles is associated with lysis of bacterial cell. DNA is replicated immediately after insertion followed by capsid synthesis. The phage DNA is never maintained in a stable condition in the host cell.

Lysogenic Phages

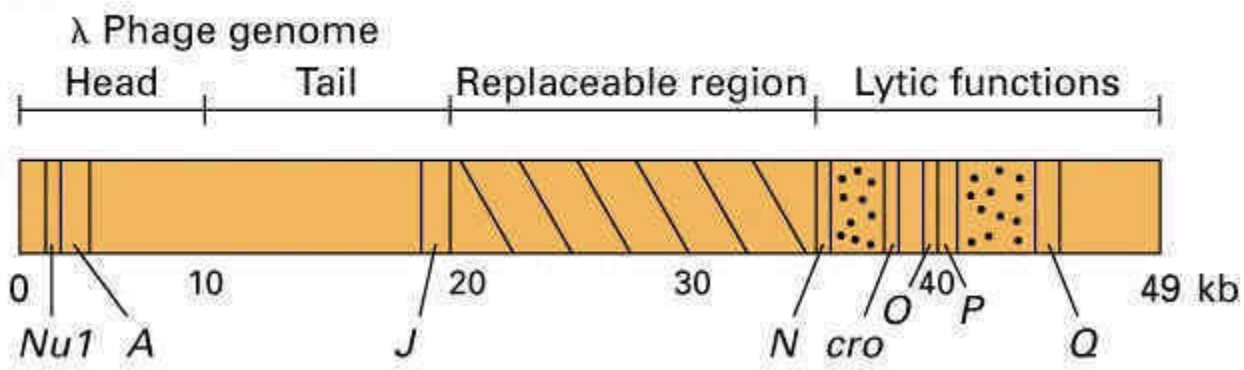
Lysogenic or temperate phages are those that can either multiply via the lytic cycle or enter a quiescent state in the cell. In this quiescent state most of the phage genes are not transcribed; the phage genome exists in a repressed state. The phage DNA in this repressed state is called a

prophage because it is not a phage but it has the potential to produce phage. In most cases the phage DNA actually integrates into the host chromosome and is replicated along with the host chromosome and passed on to the daughter cells. The cell harboring a prophage is not adversely affected by the presence of the prophage and the lysogenic state may persist indefinitely. The cell harboring a prophage is termed a lysogen.

Properties of λ DNA to act as bacteriophage vector

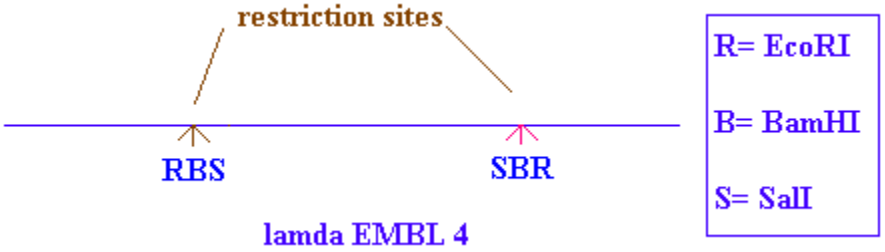
λ phage is a typical lysogenic phage. Bacteriophage λ vectors are frequently used for the initial isolation of either genomic or cDNA clones from eukaryotic cells. In λ DNA, genes coding for functionally related proteins (such as capsid proteins) are clustered together, so to make λ cloning vectors, sequences of the bacteriophage genome that are dispensable for virus replication have been removed and replaced with unique restriction sites for insertion of cloned DNA. The other property is that the linear molecule has a 12 nucleotide single strand stretch which is complementary to the other end. Thus the molecule can become circular. Such complementary ends are called cohesive ends or **cos sites**. Cos sites allows the DNA to be inserted into the bacterial genome**, circularization** is a necessary prerequisite. Cos sites also act as **recognition sequences for endonucleases** that cleave DNA at cos site, producing individual λ genomes.

Thery are found to have several features which are advantageous as vectors. First lamda phage head capable of possessing 50kb length and central part of the phage genome is not required for replication or packaging of virus. So the central part can be utilized to insert gene of interest. Secondly, the recombinant vector packaged into phage head naturally. Third, the presence of recombinant vector can be directly selected by the lawn formation due to lytic mechanism. Finally, transforming effiiciency of bacteriophage found to be greater. Bacteriophage is a virus whose host is a bacterium. Bacteriophage DNA molecules are often used as cloning vectors. These vectors can take upto 20 kb size of gene of interest.



The main disadvantage of phage vector are the lysis of culture and smaller inserting fragment size.

Example: Lamda EMBL4



Single stranded phage:

Some phages contain only single stranded DNA molecules. On infection of bacteria, the single infecting strand is converted into a double stranded replicative form, which can be isolated and used for cloning. The main advantage is that the sequence easily sequenced through Sanger method. Example for this type of vector is M13.

M13

M13 is filamentous has a completely different structure from λ. M13 genome is much smaller than λ genome, circular and single stranded. M13 DNA is inserted into E. coli during sexual conjugation via a pilus. Once inside the DNA is complemented and replicated. The DNA is never inserted in the bacterial genome.

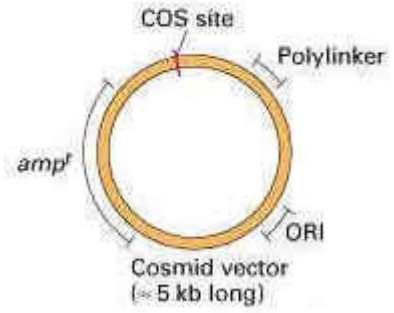
The small size of M13 genome is very desirable. The double-stranded replicative form (RF) of M13 genome behaves like a plasmid and can be treated as such for experimental purposes. It is easily prepared and reintroduced in the cell by transfection. Using M13 vector is an easy and

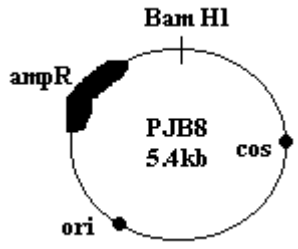
reliable way of obtaining single-stranded DNA. In an M13 phage, new phage particles are continuously assembled and released from the cell.

Both λ and M13 have found major roles as cloning vectors. Phage vectors can accept short fragments of foreign DNA into their genomes. The advantage with phages is that they can take up larger DNA segments than plasmids. Hence phage vectors are preferred for working with genomes of human cells.

3. Cosmids

A cloning vector consisting of the phage cos site inserted into a plasmid i.e. simply plasmid and cos sites. It is used to clone DNA fragments upto 40 kb in size. cos site is one of the cohesive, single stranded extensions present at the ends of the DNA molecules of certain strains of lamda phage. The main reason for the development of the cosmid vector is to irradiate the disadvantages of plasmids and phage vectors. Due to the formation of cosmids the low copy number property of plasmids overcome because of cos sites and rolling circle replication and the lysis of culture which is the disadvantage of phage virus overcome because the vector does not have sequence for the functional phage production. After the recombinant vector multiplied, they are packaged into virus hrough invitro packaging method because of the cos site in phage. Transforming efficiency increased by this package method.





Cosmids are the vectors possessing the properties of plasmid and bacteriophage. Ccosmid can be construted by mixing fragments of bacterio phage including cos sites with plasmids. A foreign DNA of about 40 kb can be inserted into cosmid DNA. The recombinant DNA so formed is packed as phages and injected into E.cili. Once injected into E.coli, cosmid behave just like cosmids and replicate. The advantage of plasmid is that they can take up larger fragments of foreign DNA compared to plasmid

Artificial chromosome vectors

1. Human artificial chromosome (HAC) : Developed in 1997 (by H. Willard), human artificiaf chromosome is a synthetically produced vector DNA, possessing the characteristics of human chromosome. HAC may be considered as a self-replicating microchromosome with a size ranging from 'l/ 10th to 1/5th of a human chromosome. The advantage with HAC is that it can carry human genes that are too long. Further, HAC can carry genes to be introduced into the cells in gene therapy.

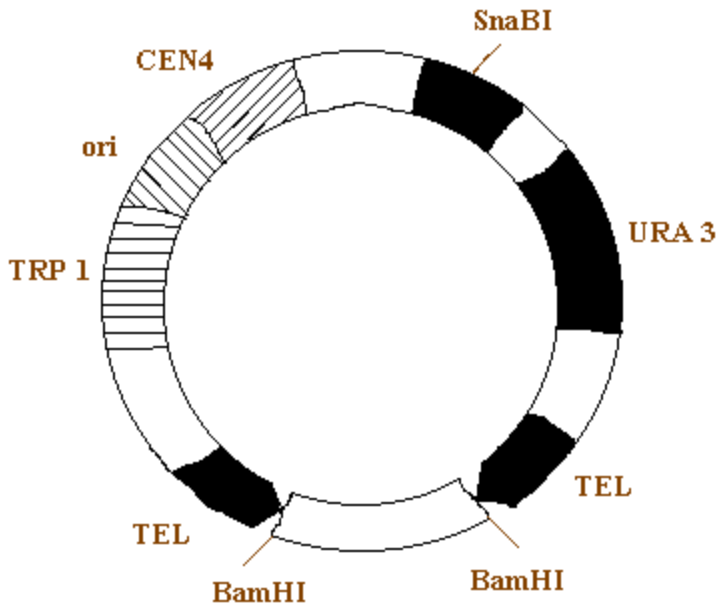
2. Yeast artificial chromosome

For some studies involving analysis of genomic DNA, it is desirable to clone even larger fragments of DNA than are accommodated by λ vectors. Introduced in'1987 (by M. Olson), yeast artificial chromosome (YAC) is a synthetic DNA that can accept lartge fragments of DNA (particularly human DNA). Cosmid and yeast artificial chromosome (**YAC**) vectors can be used for this purpose. Cosmid vectors accommodate inserts of approximately 45 kb. These vectors contain bacteriophage λ sequences that allow efficient packaging of the cloned DNA into phage particles. In addition, cosmids contain origins of replication and the genes for antibiotic resistance that are characteristic of plasmids, so they are able to replicate as plasmids in bacterial cells. Even larger fragments of DNA (hundreds of kilobases) can be cloned in YAC vectors, which replicate as chromosomes in yeast cells. These vectors are particularly useful for chromosome mapping studies.

Yeast artificial chromosomes (YACs) are the vectors that enable the formation of artificial chromosomes with the foreign DNA fragments and cloning into yeast. These are used for the cloning of very large DNA fragments in the range of **500 to 1,000 kb**. These vectors are constructed with yeast telomere sequences at each ends, yeast centromere sequences, and the

yeast **ARS (autonomously replicating sequences)** for replication. In addition to this there are the restriction sites for DNA insertion and selectable markers such as amino acid dependence and antibiotic resistance on each arm. The recombinant DNA is constructed by ligating highmolecular weight to the YAC arms using appropriate restriction enzymes. These recombinant vectors or the artificial chromosomes are used for the transformation of yeast cells by electroporation and the transformed cells are selected on selection growth medium.

Yeast artificial chromosomes



3. Bacterial artificial chromosomes (BACs) : The construction of BACs is based on one F-plasmid which is larger than the other plasmids used as cloning vectors. BACs can accept DNA inserts of around 300 kb.

Bacterial artificial chromosomes (BACs) are the cloning vectors based on the extrachromosomal plasmids of E.*coli* called F factor or fertility factor. These vectors enable the construction of artificial chromosomes, which can be cloned in *E.coli*. This vector is useful for cloning DNA fragments up to 350 kb, but can be handled like regular bacterial plasmid vectors, and is very useful for sequencing large stretches of chromosomal DNA. Like any other vector, BACs contain *ori* sequences derived from *e.coli* plasmid F factor, multiple cloning sites (MCS) having unique restriction sites, and suitable selectable markers.

Both YACs and BACs were very useful in genome-sequencing projects such as the Human Genome Project.

Choice of vector

Among the several factors, the size of the foreign DNA is very important in the choice of vectors. The efficiency of this process is often crucial for determining the success of cloning. The sizes of DNA insert that can be accepted by different vectors is shown in Table 27.4

TABLE 27.4 The different cloning vectors with the corresponding hosts and the sizes of foreign insert DNAs

Vector	Host	Foreign insert DNA size
Phage λ	*E. coli*	5–25 kb
Cosmid λ	*E. coli*	35–45 kb
Plasmid artifical chromosome (PAC)	*E. coli*	100–300 kb
Bacterial artificial chromosome (BAC)	*E. coli*	100–300 kb
Yeast chromosome	*S. cerevisiae*	200–2000 kb

Animal and Plant Vectors (Shuttle Vectors)

The genetic engineering of bacteria is possible because of the presence of special types of extra-chromosomal circular DNA that can replicate independently. Viruses that infect bacteria (M13 and lambda phage) have been used for constructing different types of vectors for the genetic transformation of bacteria. Similarly, there are some vectors developed for transforming plant and animal cells. Numerous vector systems have been developed for use in eukaryotic systems. The most common of these are often called **shuttle vectors** as they replicate in both prokaryotic and eukaryotic hosts. In general, DNA manipulations and characterizations are done in prokaryotic systems, and then the manipulated DNA is reintroduced to eukaryotic systems for functional analysis. The common features of such shuttle vectors or eukaryotic vectors are the following:

_ They are capable of replicating in two or more types of hosts including prokaryotic and eukaryotic cells.

_ They replicate autonomously, or integrate into the host genome and replicate when the host cell multiplies.

_ These vectors are commonly used for transporting genes from one organism to another (i.e., transforming animal and plant cells).

Mammalian Vectors

These are shuttle vectors developed for use in mammalian tissue culture. Like any other vector system, mammalian shuttle vectors require sequences enabling them to replicate in mammalian tissue culture. These eukaryotic origins of replication are typically derived from well-characterized mammalian viruses such as simian virus 40 (SV-40) with sv-40 ori and large T antigen system or Epstein-Barr virus (mononucleosis). SV-40 was the vector used in the first cloning experiment in mammalian cell cultures in 1979. Since then a number of mammalian vectors based on viruses such as adenovirus and pappilomavirus have been developed to transfer genes in mammals for various purposes including gene therapy. At present, retrovirus-based vectors are the most commonly used vectors for cloning genes in mammalian cells. Both systems, eukaryotic and prokaryotic, allow the vector to replicate within the host cell as a plasmid without integrating into the genome. In addition to the origin of replication, these shuttle vectors also carry antibiotic resistance genes, which function in eukaryotic cells (i.e., neomycin (G418) resistance, hygromycin resistance, methotrexate resistance, etc). Finally, artificial chromosome vectors have been developed for use in mammalian cells known as **mammalian artificial chromosomes (MACs)** such as the YACs and BACs. These eukaryotic vectors contain telomeric sequences (eukaryotic chromosomes are linear not circular molecules) and centromeres to ensure appropriate segregation of the artificial chromosomes as well as selectable markers. Such vector systems may gain in importance as we move toward manipulating the eukaryotic genome.

Plant Vectors

There are a number of shuttle vectors developed for the genetic transformation of plant systems. These shuttle vectors are based on certain viruses and bacteria, which are pathogenic to plants. Plant viruses such as the cauliflower mosaic virus (CMV), tobacco mosaic virus (TMV), and gemini viruses were used for developing vectors for plant cell transformations, but with limited success. The most successful shuttle vectors developed for the plant system are those that are based on the Ti plasmid of *Agrobacterium tumifaciens*, a bacterium that causes tumor formation

in plants. This has been successfully exploited for the development of *A.tumifaciens*-based vectors for the genetic engineering of plants. The success in plant genetic engineering and the development of a number of transgenic plants is mainly due to the development of vectors based on Ti plasmids and *A. tumifaciens*.

TABLE Different types of cloning vectors and the size of the insert DNA that can be accommodated.

Type of Vectors	Insert DNA Size (In kb)
Plasmid	0.5 to 8
Bacteriophage Lambda	9 to 25
Cosmid	30 to 45
YAC	250 to 1000
BAC	50 to 300

HOST CELLS

The ultimate aim of the development of recombinant DNA molecules or vectors is the multiplication of rDNA by cloning in a suitable host cell. There are many types of host cells available for the purpose of cloning. The kind of host cell used mainly depends on the aim of the cloning experiment. The host cell can be bacteria, yeast, plant or animal cells.

E. coli is the most extensively studied and widely used organism in rDNA experiments. It is very simple, easy to handle, grows rapidly and is able to accept and maintain a range of vectors. The doubling time of *E. coli* under ideal growth conditions is 20 minutes. As the cell undergoes multiplication, the rDNA within the cell also undergoes multiplication independent of its genome. Within a short time the number of cells and the quantity of the rDNA will be very high. If the recombinant plasmids have appropriate promoter signals for expression, a large quantity of protein can also be purified from these systems. There are a large number of genetically defined strains, which can be used freely for genetic engineering experiment and expression of recombinant proteins because they are disarmed and so are non-infective and harmless.

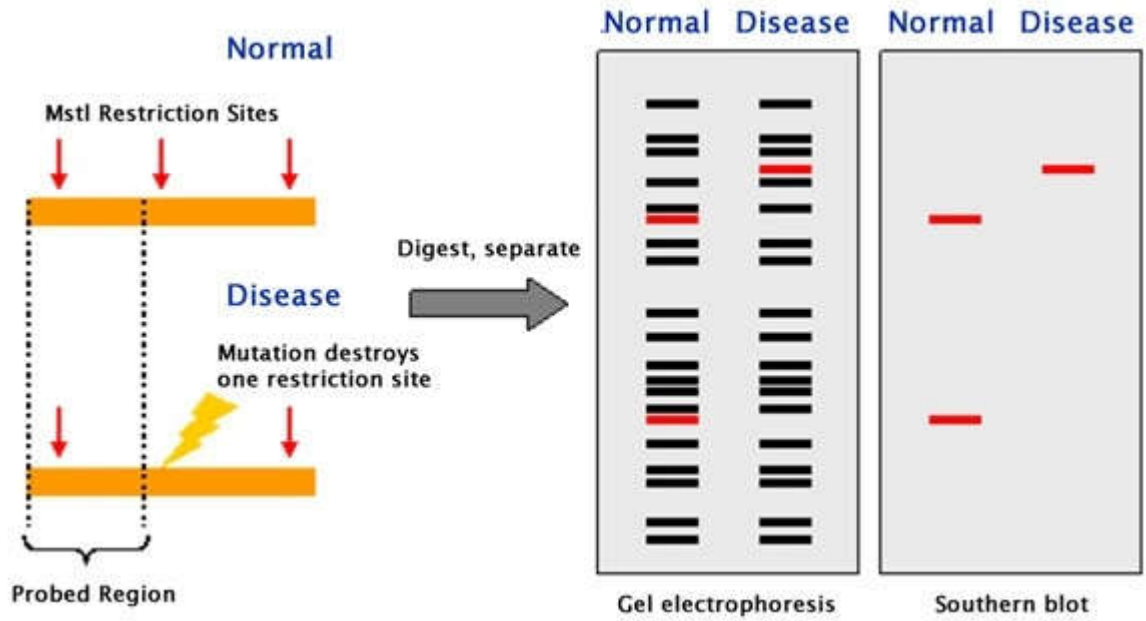
If the recombinant protein that we want to produce is of eukaryotic origin, it is better to opt for a eukaryotic host system such as yeast. There are several reasons for using a eukaryotic host system for the expression of a protein of eukaryotic origin. The polypeptide after its synthesis has to undergo folding in the correct manner to attain its functionally active three-dimensional structure. This may require the removal of some signal peptides or amino acids to initiate the process of folding to the correct three-dimensional structure. The correct three-dimensional structure is very essential for the production of functionally active protein. In addition to this, there may be removal or addition or chemical modification of amino acids or the addition of other macromolecules such as carbohydrates or lipids to make the protein functionally active. All these processes together are known as the post-translational modification of proteins. Prokaryotic systems such as *E coli* lack all these enzymes and other machinery essential for the post-translational modification of proteins. The eukaryotic protein produced in bacteria may fold incorrectly resulting in the production of a functionally inactive molecule.Prokaryotic systems do not have the molecular machinery necessary for the splicing of introns of eukaryotic genes resulting in the production of correct form of mRNA.

The most convenient eukaryotic system that can be used for the expression of eukaryotic genes is a species of yeast such as *Pichea pastoris*. There are several advantages in yeast as an expression system. Yeasts are the simplest of the eukaryotic systems, single-celled, genetically and physiologically well-characterized, and easy to grow and manipulate. Like bacteria it can be cultivated in different volumes, either in flasks, laboratory fermentors, or in industrial scales. Plant and animal cells can also be used as host cells for the expression of cloned genes either in tissue culture or as cultured cells in fermentors or as a genetically-engineered whole organisms—transgenic animals and plants.

Restriction Fragment Length Polymorphism (RFLP) Technique

Restriction fragment length polymorphism (RFLP) is a technique invented in 1984 by the English scientist Alec Jeffreys during research into hereditary diseases. It is used for the analysis of unique patterns in DNA fragments in order to genetically differentiate between organisms – these patterns are called Variable Number of Tandem Repeats (VNTRs).

Genetic polymorphism is defined as the inherited genetic differences among individuals in over 1% of normal population. The RFLP technique exploits these differences in DNA sequences to recognize and study both intraspecies and interspecies variation.



Principle

Restriction endonucleases are enzymes that cut lengthy DNA into short pieces. Each restriction endonuclease targets different nucleotide sequences in a DNA strand and therefore cuts at different sites.

The distance between the cleavage sites of a certain restriction endonuclease differs between individuals. Hence, the length of the DNA fragments produced by a restriction endonuclease will differ across both individual organisms and species.

How does it Work?

RFLP is performed using a series of steps briefly outlined below:

DNA Extraction

To begin with, DNA is extracted from blood, saliva or other samples and purified.

DNA Fragmentation

The purified DNA is digested using restriction endonucleases. The recognition sites of these enzymes are generally 4 to 6 base pairs in length. The shorter the sequence recognized, the greater the number of fragments generated from digestion.

For example, if there is a short sequence of GAGC that occurs repeatedly in a sample of DNA. The restriction endonuclease that recognizes the GAGC sequence cuts the DNA at every repetition of the GAGC pattern.

If one sample repeats the GAGC sequence 4 times whilst another sample repeats it 2 times, the length of the fragments generated by the enzyme for the two samples will be different.

Gel Electrophoresis

The restriction fragments produced during DNA fragmentation are analyzed using gel electrophoresis.

The fragments are negatively charged and can be easily separated by electrophoresis, which separates molecules based on their size and charge. The fragmented DNA samples are placed in the chamber containing the electrophoretic gel and two electrodes.

When an electric field is applied, the fragments migrate towards the positive electrode. Smaller fragments move faster through the gel leaving the larger ones behind and thus the DNA samples are separated into distinct bands on the gel.

Visualization of Bands

The gel is treated with luminescent dyes in order to make the DNA bands visible.

Applications of RFLP

RFLP has been used for several genetic analysis applications since its invention.

Some of these key applications of RFLP are listed below:

- To determine the status of genetic diseases such as Cystic Fibrosis in an individual.
- To determine or confirm the source of a DNA sample such as in paternity tests or criminal investigations.
- In genetic mapping to determine recombination rates that show the genetic distance between the loci.
- To identify a carrier of a disease-causing mutation in a family.

Disadvantages of RFLP

Since its invention, RFLP has been a widely used genome analysis techniques employed in forensic science, medicine, and genetic studies. However, it has become almost obsolete with the advent of relatively simple and less expensive DNA profiling technologies such as the polymerase chain reaction (PCR).

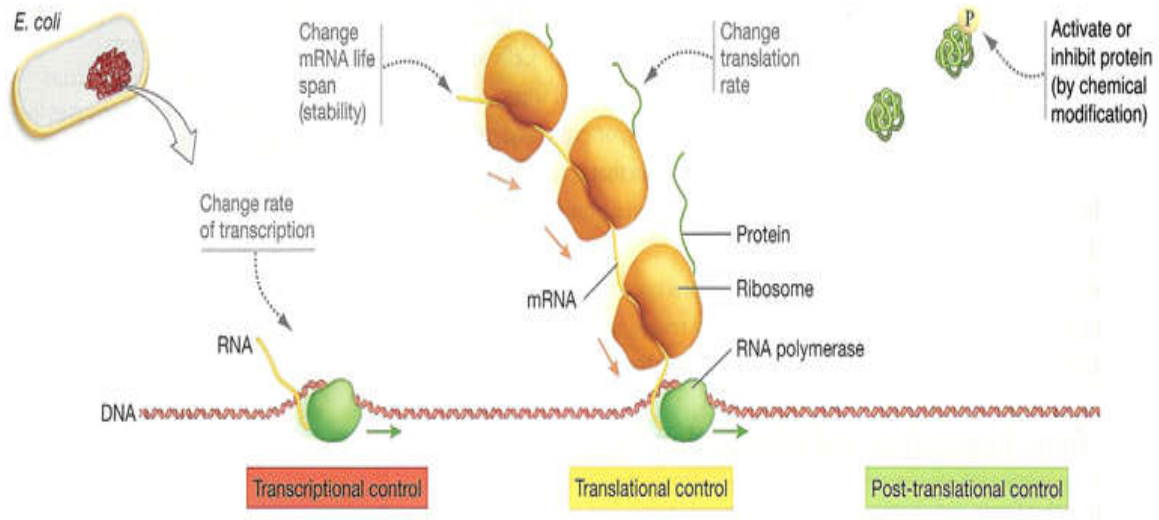
The RFLP procedure requires numerous steps and takes weeks to yield results, while techniques such as PCR can amplify target DNA sequences in a mere few hours.

Additionally, RFLP requires a large DNA sample, the isolation of which can be a laborious and time-consuming process. In contrast, PCR can amplify minute amounts of DNA in a matter of hours.

Due to numerous reasons such as these, the PCR technique has largely replaced RFLP in most applications requiring DNA sequencing such as paternity testing or forensic sample analysis.

Furthermore, the identification of single-nucleotide polymorphisms in the Human Genome Project has almost replaced the need for RFLP in disease status analysis.

Gene expression in *E. coli*



PET Expression

The PET expression system is one of the most widely used systems for the cloning and *in vivo* expression of recombinant proteins in *E. coli.* This is due to the high selectivity of the pET system's bacteriophage T7 RNA polymerase for its cognate promoter sequences, the high level

of activity of the polymerase and the high translation efficiency mediated by the T7 gene *10* translation initiation signals. In the pET system, the protein coding sequence of interest is cloned downstream of the T7 promoter and gene *10* leader sequences, and then transformed into *E. coli* strains.

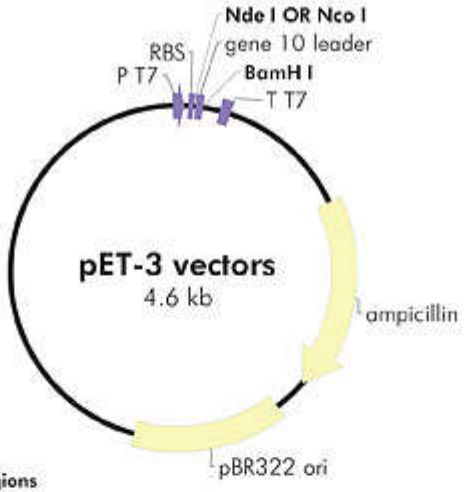
Protein expression is achieved either by IPTG induction of a chromosomally integrated cassette in which the T7 RNA polymerase is expressed from the *lacUV5* promoter, or by infection with the polymerase-expressing bacteriophage lambda CE6. Due to the specificity of the T7 promoter, basal expression of cloned target genes is extremely low in strains lacking a source of T7 RNA polymerase. Upon induction the highly active polymerase essentially out-competes transcription by the host RNA polymerase. This phenomenon, together with high-efficiency translation, achieves expression levels in which the target protein may constitute the majority of the cellular protein—after only a few hours.

PET Expression Vectors

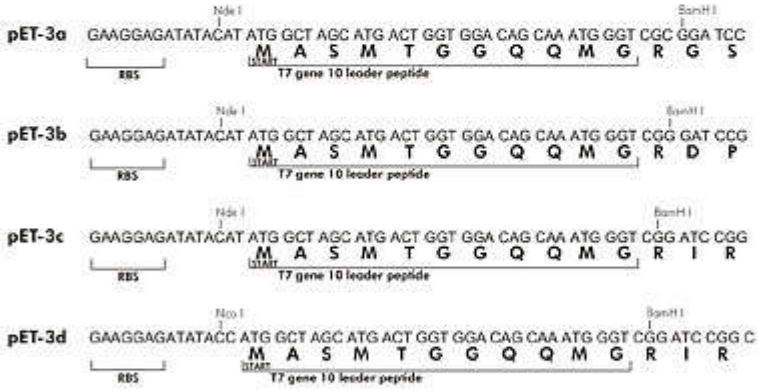
The PET expression vectors, derived from the pBR322 plasmid, are engineered to take advantage of the features of the T7 bacteriophage gene *10* that promote high-level transcription and translation. The bacteriophage-encoded RNA polymerase is highly specific for the T7 promoter sequences, which are rarely encountered in genomes other than T7 phage genome. First, this ensures that the T7 promoter will not be recognized by host cell RNA polymerase. Thus target genes are transcriptionally silent in the uninduced state—a feature that is very important if the gene to be expressed is toxic to the cell. Second, upon induction, the target gene is the only gene in the cell that will be transcribed by the highly active polymerase.

In addition to the T7 promoter, all the vectors contain the gene *10* 5′ leader, which facilitates highly efficient translation. The protein coding sequence of interest may be cloned directly after the gene *10* initiation codon using the *Nde* I (pET-3,-11, a, b and c) or *Nco* I sites (pET-3d, and -11d). Alternatively, the pET-3 and pET-11 vectors contain *Bam*H I cloning sites in all three reading frames relative to the gene *10* reading frame. Cloning the gene of interest using the *Bam*H I site results in a fusion protein containing 13 N-terminal amino acids from gene *10*. The gene *10* transcription terminator is also included downstream of the cloning sites to allow efficient termination of transcription, preventing transcriptional read-through of unwanted

plasmid sequences and increasing the RNA polymerase density on the sequence of interest—allowing high level accumulation of the specific protein-coding RNA transcripts



pET-3a–3d Cloning Site Regions
sequence shown (66–122)



Feature	Nucleotide Position			
	pET-3a	pET-3b	pET-3c	pET-3d
T7 promoter	1–19	1–19	1–19	1–19
ribosome binding site (RBS)	66–72	66–72	66–72	66–72
Nde I (pET-3a–c) or Nco I (pET-3d) cloning site	78–83	78–83	78–83	78–83
T7 gene 10 translated leader	81–113	81–113	81–113	80–112
BamH I cloning site	117–122	116–121	115–120	114–119
T7 terminator	191–237	190–236	189–235	188–234
ampicillin resistance (*bla*) ORF	840–1697	839–1696	838–1695	837–1694
pBR322 origin of replication	1848–2515	1847–2514	1846–2513	1845–2512

GENE LIBRARIES

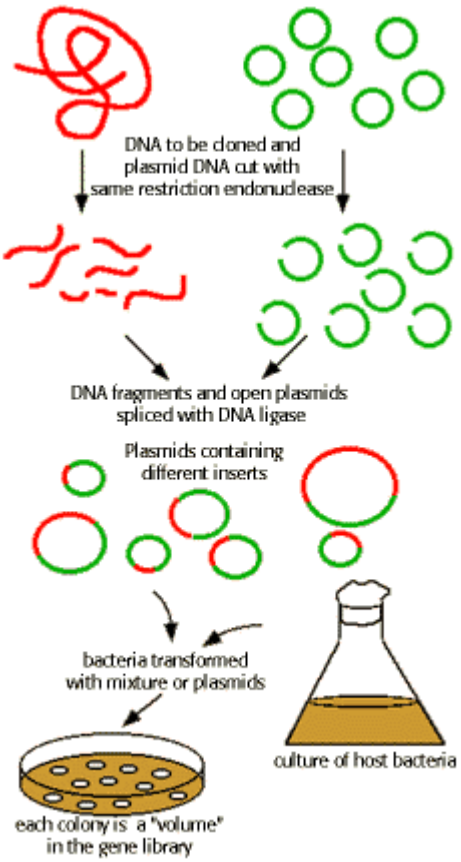
Libraries are repositories of DNA fragments cloned in their vectors. Libraries can be classified based on the cloning vector – e.g. plasmid, BAC, etc. Alternatively, the library can be described in terms of the source of the cloned DNA fragments.

GENOMIC LIBRARY

Genomic libraries (DNA LIBRARY)

It is a collection of cloned restriction fragments from a single organism's genome. If total genomic DNA is digested and the fragments are cloned into an appropriate vector, this is a genomic library. In principle, this library should consist of samples of the all the genomic DNA present in the organism, including both coding and non-coding sequences in the form of small DNA fragments (oligonucleotides) representing known genes.

Construction



Uses:

Libraries that are intended to serves as sources of probes for linkage map construction will consist of smaller inserts. Ideally, every copy of every gene (or a portion of every sequence) should be represented somewhere in the genomic library.

Only a genomic library can be used to study the function of regulatory sequences and the nature of how introns/exons affect the final protein product of a gene. Information about enhancers, introns, and other regulatory elements are found in a genomic DNA library

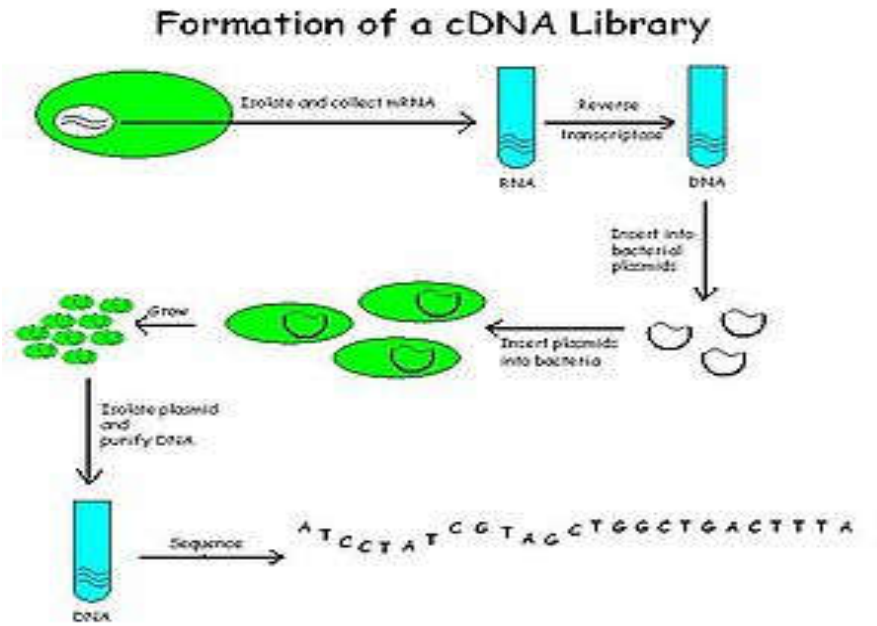
The new field of BIOINFORMATICS involves the use of computers to analyze and store genetic data, such as the DNA libraries of particular species

cDNA libraries:

A cDNA (complementary DNA) library is generated from mRNA transcripts, using the enzyme *reverse transcriptase*, which can create a DNA complement to a mRNA template. Since the cDNA library is based on mRNA, the library will represent only the genes that are expressed

Construction

cDNA is created from a mature mRNA from a eukaryotic cell with the use of an enzyme known as reverse transcriptase. The mRNA obtained is cleaved by restriction enzymes and used for th constrction of library.



Uses:

cDNA is produced from fully transcribed mRNA found in the nucleus and therefore contains only the expressed genes of an organism.

cDNA libraries are used to express eukaryotic genes in prokaryotes. cDNA do not have introns and therefore can be expressed in prokaryotic cells. The benefit of a cDNA clone is that it can be translated into functional protein if it's inserted into a bacterium. This way, the gene's function in a eukaryotic cell can actually be determined.

cDNA libraries are most useful in reverse genetics where the additional genomic information is of less use. Also, it is useful for subsequently isolating the gene that codes for that mRNA. It is good for studying which proteins are actually made by which genes.

Similarly, tissue specific cDNA libraries can be produced.

POSSIBLE QUESTIONS

1. Describe in detail about the plasmid vectors used in gene cloning.
2. Detailed note on PET vector.
3. Describe the transformation experiment and selection of recombinants.
4. Explain the following

   i) Cosmid ii) Bacterio phage
5. Give an account on restriction mapping of DNA fragments
6. Describe in detail about map construction.
7. Explain the following,

   i) BAC ii) YAC
8. List the feature of good vector and explain the structure of the vector pBR322.
9. Give an account on gene expression in *E.coli*.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
I-M.Sc., BIOCHEMISTRY
RECOMBINANT DNA TECHNOLOGY (17BCP205A)
MULTIPLE CHOICE QUESTIONS

UNIT II

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	The DNA molecule to which the gene of insert is integrated for cloning is called	Carrier	Transformer	Vector	Transducer	Vector
2	The DNA segment to be cloned is called	Gene segment	DNA fragment	DNA insert	Probe	DNA insert
3	The first successful transformation of rDNA molecule into a bacterium was carried out by	Nathan, Arber and Smith	Watson, Crick and Wilkins	Boyer and Cohen	Paul Berg	Boyer and Cohen
4	The plasmid used by Cohen and Boyer for their transformation experiment was	pSC 101	pUC 17	pBR 322	*E.coli* plasmids	pSC 101
5	The mechanism of intake of DNA fragments from the surrounding medium by a cell is called	Transformation	Transduction	Both a and b	Conjugation	Transformation
6	Gene cloning refers to the	Production of large number of copies of the gene being cloned	Production of asexual progeny from a single individual or a cell	Both a and b	Production of sexual progeny from a single individual or a cell	Production of large number of copies of the gene being cloned
7	Paul Berg's gene splicing experiment created the first rDNA molecule which was a	A T4 phage fragment incorporated into SV40 vector	A lambda phage fragment incorporated into SV40 vector	A T4 phage fragment incorporated into pSC 101 vector	A lambda phage fragment incorporated into pSC 101 vector	A lambda phage fragment incorporated into pSC 101 vector
8	In pUC vector, UC denotes	University of Calicut	University of California	University of Cambridge	University of Cornell	University of California
9	Polylinker site is present in _____ vector	pBR322	Lambda Phage	pUC8	Phagemid	pUC8
10	The largest cloned insert size of lambda vector is	5kb	15kb	40 kb	200kb	15kb
11	The size of DNA accommodated by cosmid is	5kb	15kb	40 kb	200kb	40 kb
12	Recombinant selection for pUC 8 vector is arrived by	Transformation	Blue white selection	Replica plating	Plaque formation	Blue white selection
13	pBR322 selection is based on	Insertional inactivation	Transformation	Blue white selection	Plaque formation	Insertional inactivation
14	The two protruding ends of lambda phage is called	Cosmid	Cos site	OriC	Restriction site	Cos site
15	The strain used for blue white selection is	*E.coli* lac Z^-	*E.coli* lac Z^+	*Staphylo cocci* lac Z^-	*Staphylo cocci* lac Z^+	*E.coli* lac Z^-
16	Lac Z code for _____ enzyme	Alpha galactosidase	Beta galactosidase	Permease	Lactose transferase	Beta galactosidase
17	Which of the following enzyme would you select for cutting a DNA strand with recognition sequence 'GAATTC'?	Alu I	Eco R1	Bam Hi	Hind III	Eco R1
18	Among the following which is not used for cloning purpose	Bacterio phage	Plasmid	Cosmid	*E.coli*	*E.coli*
19	Which of the technologies listed below is a valuable method for mass-producing drugs and other useful proteins?	Recombinant DNA technology	Transgenic technology	Biotechnology	Gene targeting	Recombinant DNA technology
20	Bacterio phages are	Bacteria	Virus	Fungi	Yeast	Virus
21	The length of cohesive end of lambda phage DNA is	18bp	15 bp	12 bp	8bp	12 bp
22	To be packed in lambda phage head the DNA must be larger than_____ and smaller than ____kb	38-42	20-45	25-50	30-60	38-42
23	What is the natural function of restriction enzymes?	Protecting bacteria by cleaving the DNA of infecting viruses.	Protecting bacteria by cleaving their own DNA.	Protecting bacteria by methylating their own DNA.	Protecting bacteria by methylating the DNA of infecting viruses	Protecting bacteria by cleaving the DNA of infecting viruses.

24	Which one of the following vector is used for obtaining the single strand DNA?	pBR322	Lambda phage	M13 phage	Cosmid	M13 phage
25	Among the following which is not used as viral vector	SV40	Adeno Virus	Retro Virus	Herpes virus	Herpes virus
26	Natural genetic engineering process is carried out by _____ bacterium	*E.coli*	*S. Cocci*	*A.tumifaciens*	*R.oryzae*	*A.tumifaciens*
27	Ti plasmid contain all the following portions except	T-DNA	Vir gene	Ori gene	Tra gene	Tra gene
28	The portion of Ti plasmid responsible for transfer of t-DNA is	T-DNA	Vir gene	Ori gene	Opine catabolism	Vir gene
29	The portion of Ti plasmid responsible for tumour formation is	T-DNA	Vir gene	Ori gene	Opine catabolism	T-DNA
30	Among the following which is not used in plant vector system	Gemini virus	Ti plasmid	CaMV virus	Adeno virus	Adeno virus
31	The vector helps to transfer gene between two different host	Viral vector	Plant vector	Shuttle vector	Plasmid vector	Shuttle vector
32	_____ is gene that helps in the identification of recombinants	Molecular marker	Selectable marker	Identification Marker	Suitable marker	Selectable marker
33	Recombinants using pBR322 plasmid vector are	Amp^R Tet^R	Amp^S Tet^S	Amp^R Tet^S	Amp^S Tet^R	Amp^R Tet^S
34	Addition of foreign insert result in insertional inactivation of _____ resistance in pBR322	Ampicillin	Tetracyclin	Ampicillin and Tetracyclin	No gene is inactivated	Tetracyclin
35	Selection of recombinants using pBR322 is achieved by	Blue white selection	Replica plating	Plaque formation	Colony hybridisation	Replica plating
36	Insertional inactivation redult in	Loss of tetracycline resistance	Disruption of Ori C	Change in restriction site	Loss of Ampicillin resistance	Loss of tetracycline resistance
37	The site of insertion a foreign DNA fragment disrupts a selectable marker in plasmid and that process is called as	Insertional inactivation	Insertional activation	Insertional inception	Insertional interruption	Insertional inactivation
38	Recombinants from pUC8 vector is selected by Insertional inactivation of _____gene	Ampicillin resistance	Tetracyclin resistance	Lac Z gene	Spi	Lac Z gene
39	Beta galatosidase gene is involved in breakdown of	Galactose	Glucose	Lactose	Laccose	Lactose
40	LacZ gene code for_____ enzyme	Beta galatosidase	Alpha galatosidase	Alpha amylase	Beta amylase	Beta galatosidase
41	Lacz- E.coli cell synthesis	Beta galatosidase	Alpha galatosidase	Partial Beta galatosidase	Partial Alpha galatosidase	Partial Beta galatosidase
42	pUC8 Recombinant are	$Amp^{R,}$ make Beta galatosidase	$Amp^{R,}$ unable to make Beta galatosidase	$Amp^{S,}$ make Beta galatosidase	$Amp^{S,}$ unable to make Beta galatosidase	$Amp^{R,}$ unable to make Beta galatosidase
43	Inducer in Blue white selection is	IPTG	ITPG	IGPT	IPGT	IPTG
44	Lactose analogue in Blue white selection is	G-gal	X-gal	I-gal	P-gal	X-gal
45	Method by which recombinants phage DNA introduced in bacterial cell	*In vitro* packaging	Tranduction	Transformation	Transdution	*In vitro* packaging
46	In Blue white selection is recombinants produce _____ colonies	Blue	White	Blue-White	Blue-Blue	White
47	The main difference between transformation and transfection is	Plasmid DNA	Phage DNA	Heat shock	Competent *E.coli* cells	Phage DNA
48	Phage infection in *E.coli* is visualized in agar medium through	Colony	Plaque	Blue colour	White colour	Plaque
49	Plaque formation is due to lysis of_____-	Bacteria	Bacterio phage	Virus	Bacterio phage and virus	Bacteria
50	Defective *cos* site is seen _____ strain of *E.coli*	SMR 10	MSR 20	BHB2688	BHB2690	SMR 10
51	Mutation in _____ site result in the formation of lambda DNA concatemer	Ori C	Amp^R	Tet^R	Cos	Cos
52	Replica plating is a process involved in the selection of recombinants from	pUC8	pBR322	pGEM	pUC19	pBR322
53	True plaque is produced by	*E.coli*	Bacterio phage	M143 phage	Yeast	Bacterio phage

54	Identification of recombinant phage is achieved by all except_____	LacZ	λ cI gene	Poly linker site	Spi phenotype	Poly linker site
55	Infection of *E.coli* by phage particle is	Transfection	Transformation	Transduction	Transdution	Transfection
56	------- gene produce a pheneotype that permits either an easy selection or quick identification of cell is	Scorable marker	Selectale marker	Marker gene	Readable gene	Marker gene
57	_____ marker enable only such cells that possessit ot survive under the selective condition	Scorable	Selectale	Reporter	Readable	Selectale
58	To maximize the gene expression the promoter should be	Strong and repressible	Weak and inducible	Strong and inducible	Weak and repressible	Strong and inducible
59	All the following promoters are used in expression vector except_____	lac	tac	trp	ara	ara
60	Except _____ all the others are important for gene expression in E.coli	Promoter	Ribosome binding site	Initiator	Terminator	Initiator

UNIT-III

SYLLABUS

Isolation and characterization of gene transcripts: Introduction, Converting mRNA transcripts into cDNA, Screening representative cDNA libraries, Functional sequencing of cDNA expression libraries. Expressed cDNAs compared with computer databases. Characterization of recombinant proteins- Processing, purification and refolding and stabilization-Insulin, hGH, tpA.

ISOLATION AND CHARACTERISATION OF GENE TRANSCRIPTS

Introduction

Converting mRNA transcripts into cDNA

With the advancement in the field of genetic engineering, gene expression analysis has become an indispensable tool. Researchers are always keen to find out whether their gene of interest is expressing (turned on) or not (turned off). For this, the mRNA (messenger RNA) is located and quantified in the given sample. mRNAs carry the information coded by DNA and, thus, further gets translated to produce respective proteins.

RNAs are very unstable and fragile, and are very likely to degrade by the omnipresent RNases. In order to combat this, the biological informations encoded in mRNA are stored in more stable form of nucleic acid, i.e. DNA. Therefore, cDNA is prepared from RNA, which stores entire sequence of the mRNA. It is more convenient to work with cDNA as compared to mRNA. This cDNA can be further used for various subsequent molecular biology and genetic studies.

What is cDNA?

cDNA means complementary DNA or copy DNA. According to the central dogma of the molecular biology, DNA is transcribed into mRNA. Then mRNA gets translated to produce protein. Therefore, the flow of biological information is from DNA to RNA to protein.

However, sometimes the flow of information is from RNA to DNA (as in the case of some viruses, e.g. HIV). This conversion of RNA to DNA is aided by an enzyme known as Reverse Transcriptase (i.e. RNA-dependent DNA polymerase). The cDNA prepared can be single stranded or double stranded. Therefore, molecular biologists make use of reverse transcriptase to prepare cDNA from mRNA for the sake of convenience in the molecular studies.

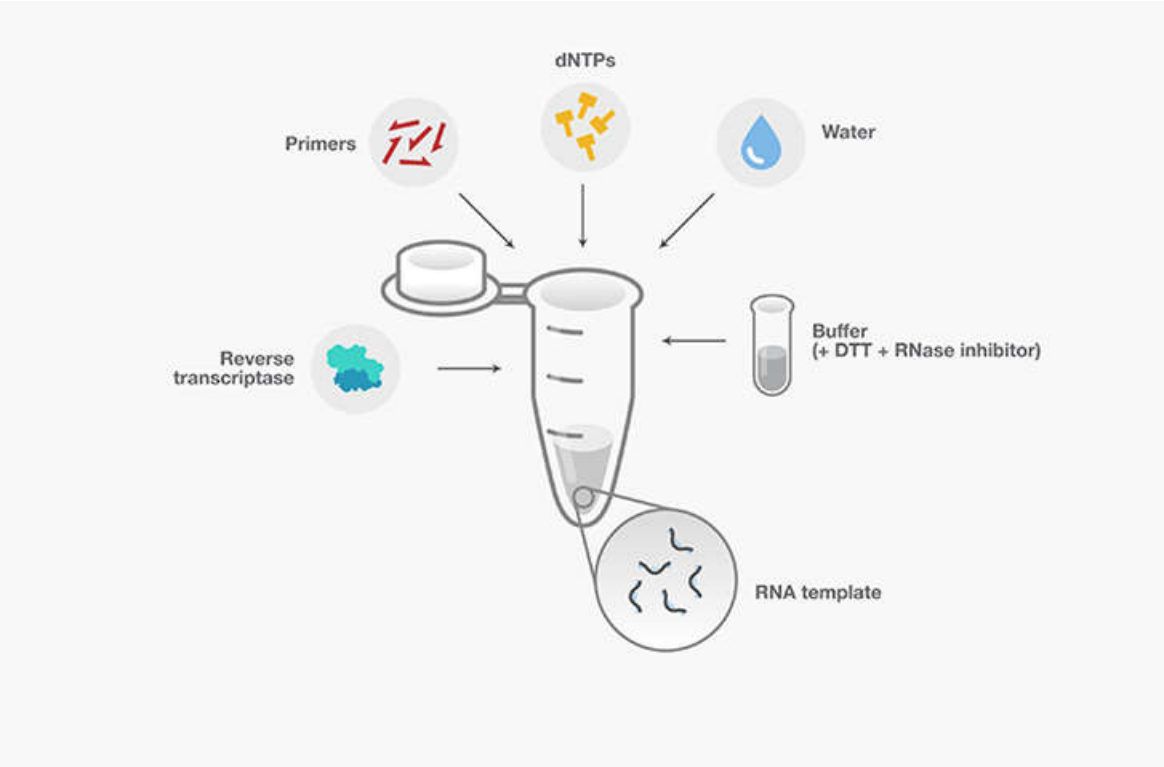
Principle of cDNA synthesis

Mature (fully spliced) mRNA is used as a template for preparing cDNA. In fact, cDNA can be produced from any RNA molecule. This conversion is brought about by reverse transcriptase. cDNA can be obtained both from prokaryotes and eukaryotes.

Reverse transcriptase is a RNA-dependent DNA polymerase. It acts on a single strand of mRNA. Using mRNA as a template, reverse transcriptase produces its complementary DNA based on the pairing of RNA base pairs. This enzyme executes reactions in the same way as DNA polymerase. It also requires a primer with a free 3′-hydroxyl group. For transcribing RNA having secondary structures, a reverse transcriptase with high temperature performance is recommended.

Main reaction components

In addition to enzyme and primers, the main reaction components for reverse transcription include RNA template (pre-treated to remove genomic DNA), buffer, dNTPs, DTT, RNase inhibitor, and RNase-free water.



Procedure of cDNA synthesis

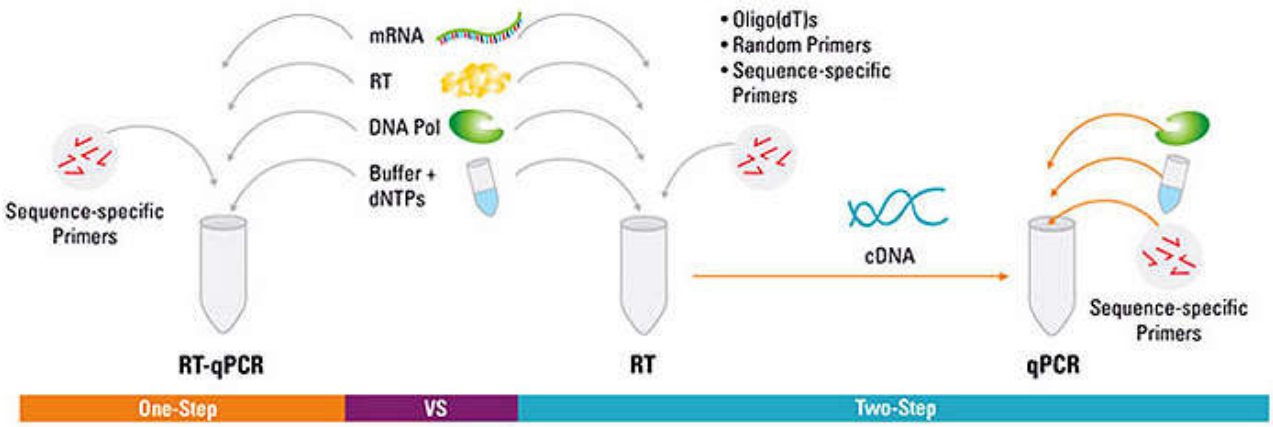
First of all, good quality intact mRNA or total RNA is isolated. Then, you need a few more reagents to prepare cDNA: dNTPs (dATP, dTTP, dCTP, dGTP), primers and reverse transcriptase.

In case of eukaryotic mRNAs, a poly-A tail is present at their 3′-ends. Therefore, a poly-T oligonucleotide is used as a primer. But certain modifications are needed when you use other RNAs which lack poly-A tail, e.g. prokaryotic mRNA, rRNA, RNA virus genomes, etc. In such cases, a poly-A tail is added to the 3′-end of the RNA. This makes it analogous to the eukaryotic mRNA.

The primer gets annealed to the 3′-end of the mRNA. Now, the 3′-end of the primer is extended with the help of the reverse transcriptase using mRNA strand as a template. This is known as “first strand reaction”. As a result of this, RNA-DNA hybrid molecule is produced. By the use of RNase H or alkaline hydrolysis, the RNA strand of this RNA-DNA hybrid molecule is digested. Now, the single stranded cDNA becomes free.

The reverse transcriptase used (most commonly used is Moloney Murine Leukemia Virus Reverse Transcriptase, MMLV RT) displays terminal transferase activity on reaching the end of

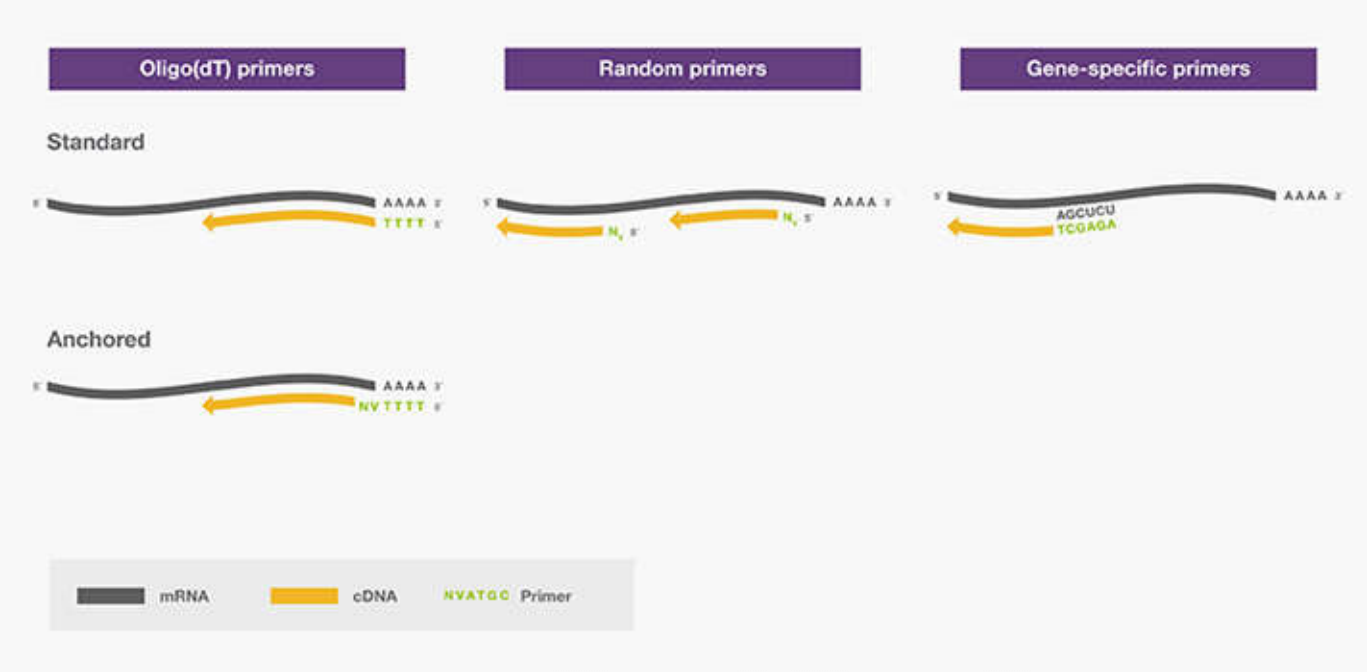
the RNA template. It adds 3-5 residues (usually dC) to the 3′-terminal of the first strand cDNA. An oligo containing a stretch of G residues is used. This oligo gets annealed to the dC rich cDNA tail and serves as an extended template for reverse transcriptase. Now, the synthesis of the complementary strand of the first strand cDNA begins. This is called "second strand reaction". Finally, a regular double stranded DNA is produced.



Types of primers used

Various types of primers can be used, in accordance to the requirements, to synthesize cDNA.

1) **Oligo-dT primer** –It is used when the mRNAs have poly-A tail, as in the case of eukaryotic mRNAs; or when a poly-A tail is attached to the existing RNA. Oligo-dT primer anneals to all the mRNAs simultaneously.
2) **Sequence-specific primer-**To generate cDNA from a particular population of mRNA among all the mRNAs, then sequence-specific primer is used. It will bind to a particular mRNA sequence only. This will give rise to a pure cDNA population generated from the desired mRNA. For designing sequence-specific primer, you must know the sequence of the mRNA of interest. Generally, the 3′-terminal sequence is preferred.
3) **Random primer**- A random primer cocktail is used to produce cDNA from all the mRNAs. The cDNAs produced are not full length. Random primer is extremely useful if production of the shorter cDNA fragments is desirable. Its use increases the probability of converting the entire 5′-end of the mRNA into the cDNA. In case of long mRNAs, reverse transcriptase is usually not able to reach the 5′-end. Therefore, random primer proves to be extremely advantageous in such cases.



Types of cDNA

cDNAs can be single stranded or double stranded. After the first strand reaction, cDNA obtained is single stranded. This single stranded cDNA can be converted to the double stranded form by second strand reaction. On the basis of the applications, single or double stranded form of the cDNA is used.

Applications of single stranded cDNA

1) Single stranded cDNA is most commonly used for RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction). RT-PCR is done for gene expression studies. It determines whether the gene of interest is expressed or not, and the level of its expression.

2) It is also used to amplify particular gene of interest. For this, sequence-specific primers are used.

3) Real-time PCR (also known as quantitative RT-PCR, qRT-PCR) also makes use of single stranded cDNA. It is done for performing gene expression analysis. As the amplification progresses, the amplicons can be visualized with the help of a fluorescent reporter molecular. It is highly sensitive and effective as compared to RT-PCR.

Applications of double stranded cDNA

1) Double stranded cDNAs are used to clone them into the appropriate vector to prepare libraries of cDNA (i.e. cDNA libraries). These libraries contain all the mRNA sequences in the form of cDNA, which are all expressed in a cell.

2) Double stranded form of a particular cDNA of interest can be cloned. Then, expression of the desired genes is allowed at the RNA and protein level for further study.

3) Sequencing of the double stranded cDNA is carried out to obtain the expressed sequence tags (ESTs).

4) They are also used for doing microarray for analysing global gene expression.

5) Suppression subtractive hybridization (SSH) is also performed with double stranded cDNA. It is done to find out differential gene expression.

Screening Representative cDNA Libraries

Library screening relies on information available about vector and genes of interest. In this, clones of the library are subjected to analysis to identify desired sequences. Differentmethodsareusedto identify clones harboring fragments of genes of interest in the library. DNA sequences can be screenedfor the gene sequence of interest among the clones of library with oligonucleotide probes that match to the sequence of the clone of interest.Functional screening for the encoded protein products by the expression of the gene of interest can also be performed to identify it.

Colony Hybridization

The method employing labelled oligonucleotideDNA probe to screen a library is known as colony hybridization.It was applied for the first time by M.Grunstein and D. S.Hogness in 1975. The basis of hybridization is complementarity between single stranded DNA or RNA molecules to associate with each other. In this method single stranded fragment of labelled DNA or RNA known as oligonucleotide probeis used to detect the gene of interest on the basis of homology. A library contains large numbers of clones, each containing a common vector backbone with unique insert. The oligonucleotide probe is used to label the colonies of cells containing the region of gene whose sequence matches with the probe. Firstly, recombinant vectors are transformed into suitable bacterial host cells. The cells are spread on nutrient agar plates containing solid growth medium. Each cell multiplies and is converted to a colony of cells, with

each colony containing same cells with similar vector and insert from the library. Pieces of nitrocellulose membrane are pressed on top of petri plates containing colonies of cells to produce a replica of the plate. This ensures that nitrocellulose filter retains some cells from each colony in the same position as they were on the petri plate. This is important because if the desired clone is discovered, the colony of cells containing that specific clone can be identified and insert DNA can be recovered. The petri plate acts as a reference for the nitrocellulose membrane.

The cells on the nitrocellulose membrane are treated with chemicals which open up cells and release the DNA on the filter. DNA is then denatured to make it single-stranded. Nitrocellulose filters are incubated with labelled oligonucleotide probe, whose sequence is complementary to the gene of interest, under conditions favoring hybridization. The hybridization step is performed at non-stringent conditions ensuring that the probe will bind to any clone harboring complementary sequence. These non-stringent hybridization conditions allow non-specific binding of probe to clones which have even little complementary to the probe.

Hybridization is followed by washing step, which is performed under stringent condition. This ensures that any excess probe that has not hybridized or bound non-specifically is washed away.

Membrane is then assayed and bound probes can be detected. If the probe has been labelled using radioactive isotopes, X-Ray film is overlaid on the membrane. Radioactive probe in DNA-DNA hybrid molecule bound to the membrane will expose the X-Ray film, producing black spots on the film. Membrane maintains the same pattern of colonies as it was there on the petri-plate, therefore black spots on the film can provide details about corresponding colonies on the petri-plate. This technique of colony hybridization can be employed to screen both genomic and cDNA libraries without the expression of inserted sequence. Large number of recombinant clones can be screened by this method to determine the recombinant plasmid containing the sequence of interest. At the same time, this method is time consuming and can be followed for microbes only, which can withstand plating process.

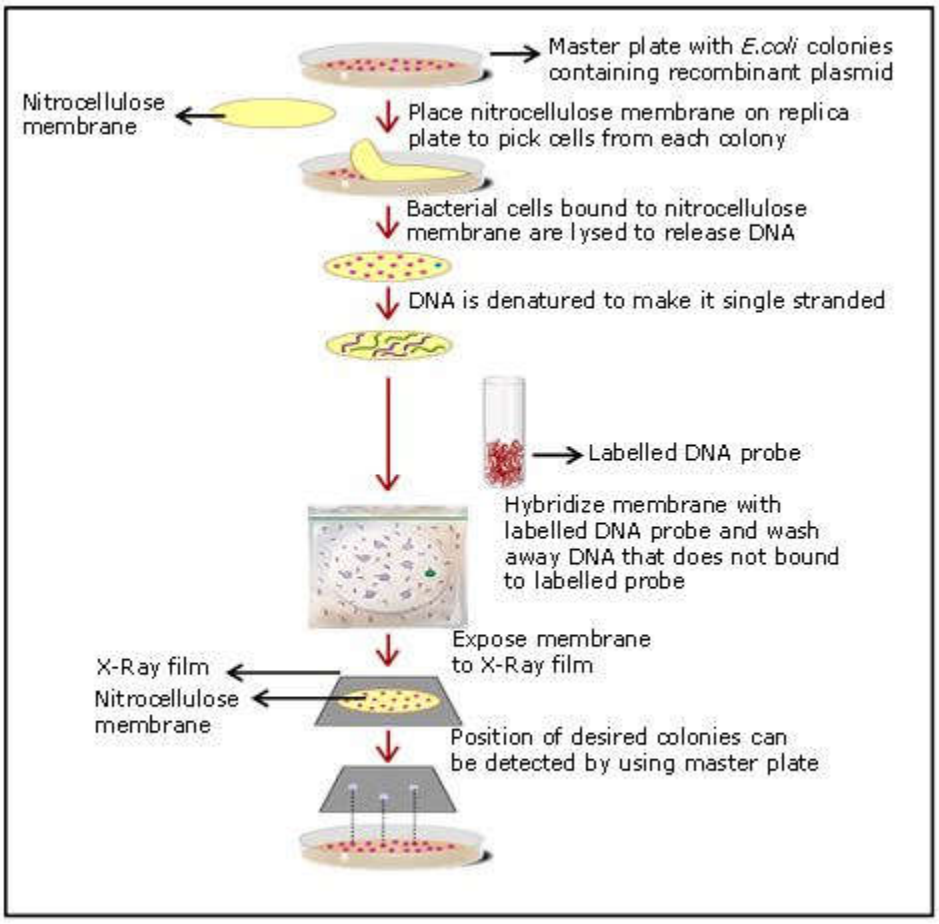


Figure: DNA libraries can be screened by colony hybridization with a radiolabeled probe to detect a clone of interest

Nowadays, with the concern over the usage of some non-radioactive materials which undergo chemical or color reactions are used to label the probe. For example, digoxigenin is a non – radioactive antibody based probe and labelling withd eoxyuridine triphosphate (dUTP) nucleotides and labelling with the enzyme horseradish peroxidase are becoming increasingly popular.

Polymerase Chain Reaction

Colony hybridization is a laborious and time-consuming process and requires several rounds of plating and filter hybridization. It is also prone to false positives results. Polymerase chain reaction(PCR) has emerged as a robust technique in the area of molecular biology and is also efficient method for library screening. PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. Although it is tedious to screen

thousands of clones by but becauseof the specificity and sensitivity of PCR (if primer pair for gene of interest is availablei.e. the sequence of the desirable fragment is known), it can be a useful way to identify clones of interest. Individual clones of the library can be subjected to PCR analysis.

Typically the PCR procedure consists of a three-step cycle at discrete temperatures to bring about achain reaction that produces an amplified product of identical DNA molecules. First regular cycling step is denaturation where the reaction mixture is heated at 94–98 °C for 20–30 seconds to separatethetwoDNA strands. Second step is annealing wherein the reaction temperature is lowered to 50–65 °C for 20–40 seconds so that short single-stranded DNA primers complementary to sequences on opposite strands anneals at each end of the target sequence. Lastly, a heat-stable DNA polymerase extends the primers in the 5' ---> 3' direction and synthesizes a new DNA strand complementary to the DNA of the template strand. The PCR technique is explained elaborately in the chapter Polymerase chain reaction.

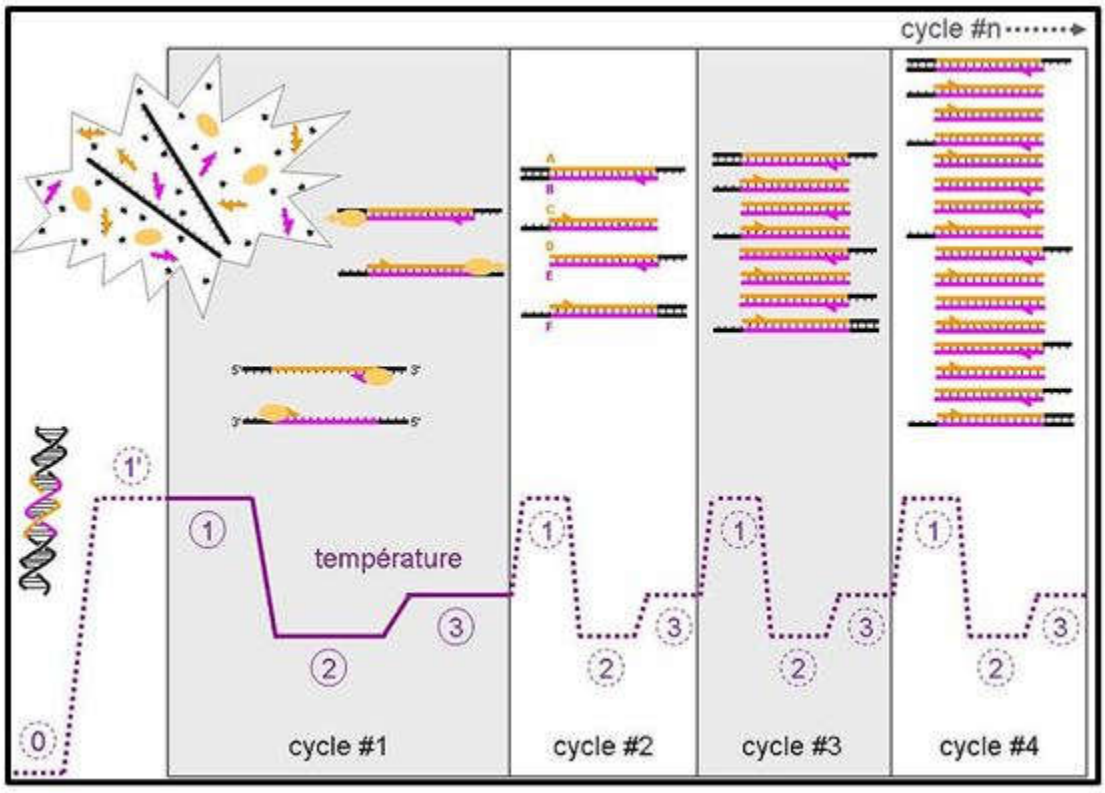


Figure: Schematic representation of the basic principle of PCR

For library screening, multiple clonescanbe pooled and a PCR reaction can beset on the pooled samples. This is known as combinatorial screening. The high sensitivity and specificity of PCR allows the detection of target sequences from pooled samples as well. Samples from clones in the rows and columns of the plate are pooled respectively and a PCR is performed on the mixed samples. If a clone is present in a sample from row pool, it must be in one of the wells in that row. Likewise, if a clone is present in a sample from column pool, it must be the clone in that column. If a positive clone is obtained, the clone is identified by PCR of independent samples of the row and column.

Immunological Screening

The desired gene can also be identified by the activities of the protein product of the encoded gene. This type of strategy is used for cDNA expression libraries. The fragments are cloned into special cloning vectors which allow the functional expression of cloned DNA fragments. The protocol of immunological screening is similar to hybridization screening except the fact that antibodies are used as probe instead of nucleic acid. Clones of the library are spread on replica plates.Transformed bacterial colonies on petri plates are lysed to release protein (antigen) from the positive colonies. Protein samples are transferred to similar position on the nitrocellulose membrane so thatthe pattern of clones remains same on the membrane and the replica plates. Released bacterial and expressed proteins are bound to the membrane. Membrane is overlaid with primary antibody which binds to protein of interest. A secondary antibody with a detection system is then overlaid on the membrane to detect protein-primary antibody complex. Unbound antibodies are then washed out. Bound antibodies are detected as it produces a colored compound. The colonies with positive results are isolated to identify the vector and correct gene of interest. The limitation of this technique is that it can be used only when specific antibody is available.

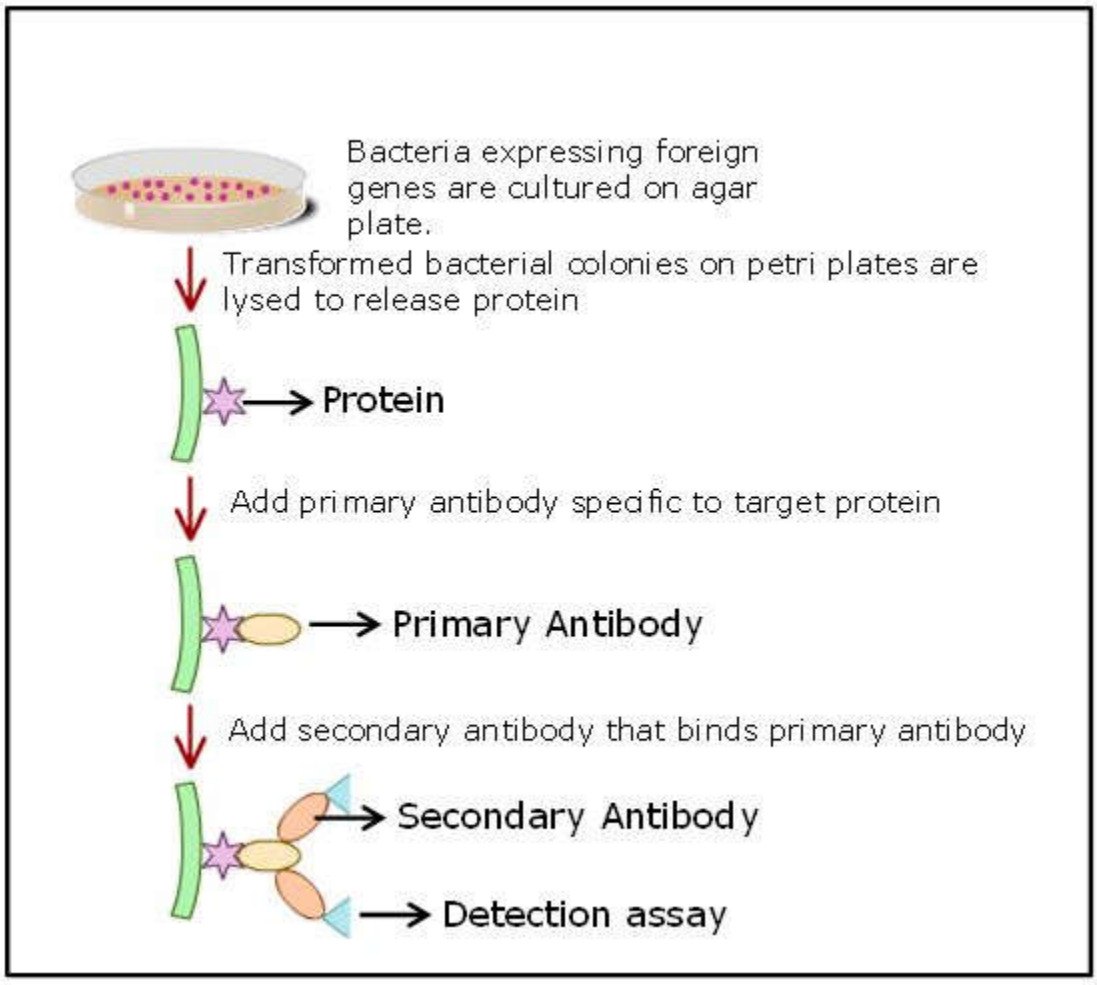


Figure: Immunological screening of a functional protein from DNA library. The figure shows one reference protein.

Complementation

Genomic libraries can also be screened for the ability of the cloned gene to expressa functional protein. This approach is effective if the functional protein complements a recessive mutation and is known as functional complementation. Yeast is a model eukaryotic organism, which is easy to grow and perform genetic manipulations. In this method, a yeast mutant defective in the gene of interest is chosen and a yeast genomic library is transformed in bacterial cells. Yeast mutant is complemented when expression of transgene from library is induced.

Yeast genes do not contain multiple introns and therefore entire gene sequences can be inserted into a plasmid vector. Functional complementation is tested for yeast genescloned in plasmid vector and therefore shuttle vectors (which are capable of propagation in two different hosts,

yeast and E. coli in the present case) are used for screening of recombinant plasmids. This shuttle vector contains essential elements to allow cloning of yeast DNA fragments in E. coli. Additionally it contains origin for DNA replication in yeast (ARS, Autonomously replicating sequence), yeast centromere (CEN) to allow faithful segregation of plasmid during yeast cell division and a yeast gene encoding a selectable marker URA3 fororotidine-5'phosphate decarboxylase, an enzyme which is required for the synthesis of uracil. A particular yeast DNA sequence is digested to produce overlapping restriction fragments.Shuttle vector is cleaved with the same restriction enzyme to produce sticky ends complementary to the DNA sequence. Vector is transformed to E. coli cells, and cells that grow after selection for ampicillin resistance contain single type of yeast cDNA fragment.

A yeast genomic library can be cloned in shuttle vector and screened to identify the wild type gene corresponding to recessive and temperature sensitive cdc28 mutations. These cell cycle mutants can grow easily at 23°C but are unable to make colonies at 36°C. The assay utilizes double mutants, which requires uracil for growth due to ura3 mutation and is temperature sensitive due to cdc28 mutation. A genomic DNA clone that complements this mutation can be identified by transforming recombinant plasmids isolated from yeast genomic library with yeast double mutants. Two steps are important to prove that insert present on the plasmid contains wild-type CDC28 gene. Firstly, transformed yeast cells will carry a plasmid borne selectable marker, a gene for synthesis of uracil, and can be selected by their ability to grow in absence of uracil. Transformed colonies are then allowed to grow under permissive conditions ie at 23°C. Replica plates of transformants are then transferred to non- permissive conditions ie at 36°C. Only the yeast colonies that carry dominant form of CDC28 gene will be grow in non-permissive conditions. Plasmid DNA can be extracted from yeast cells and further analyzed by sequencing.

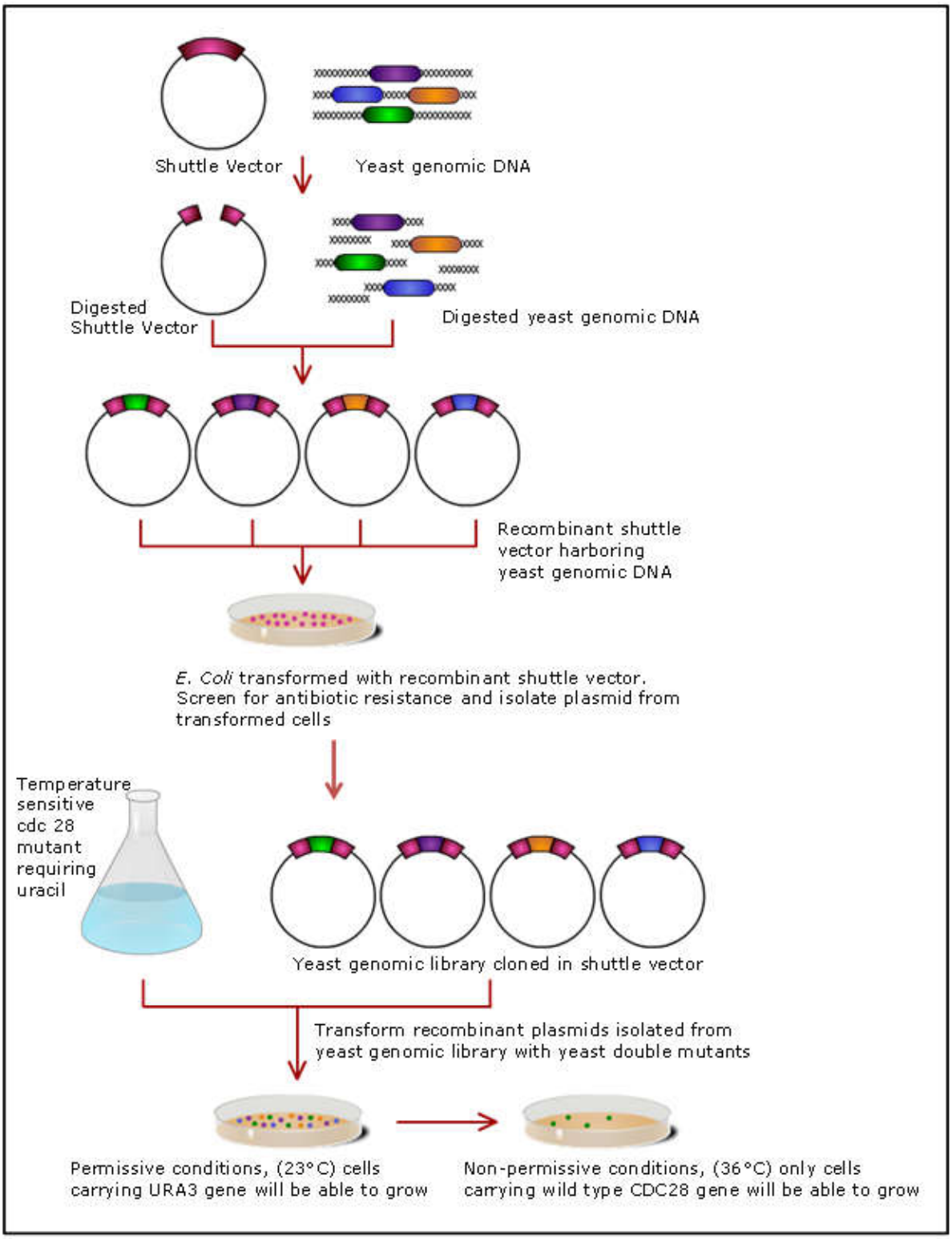
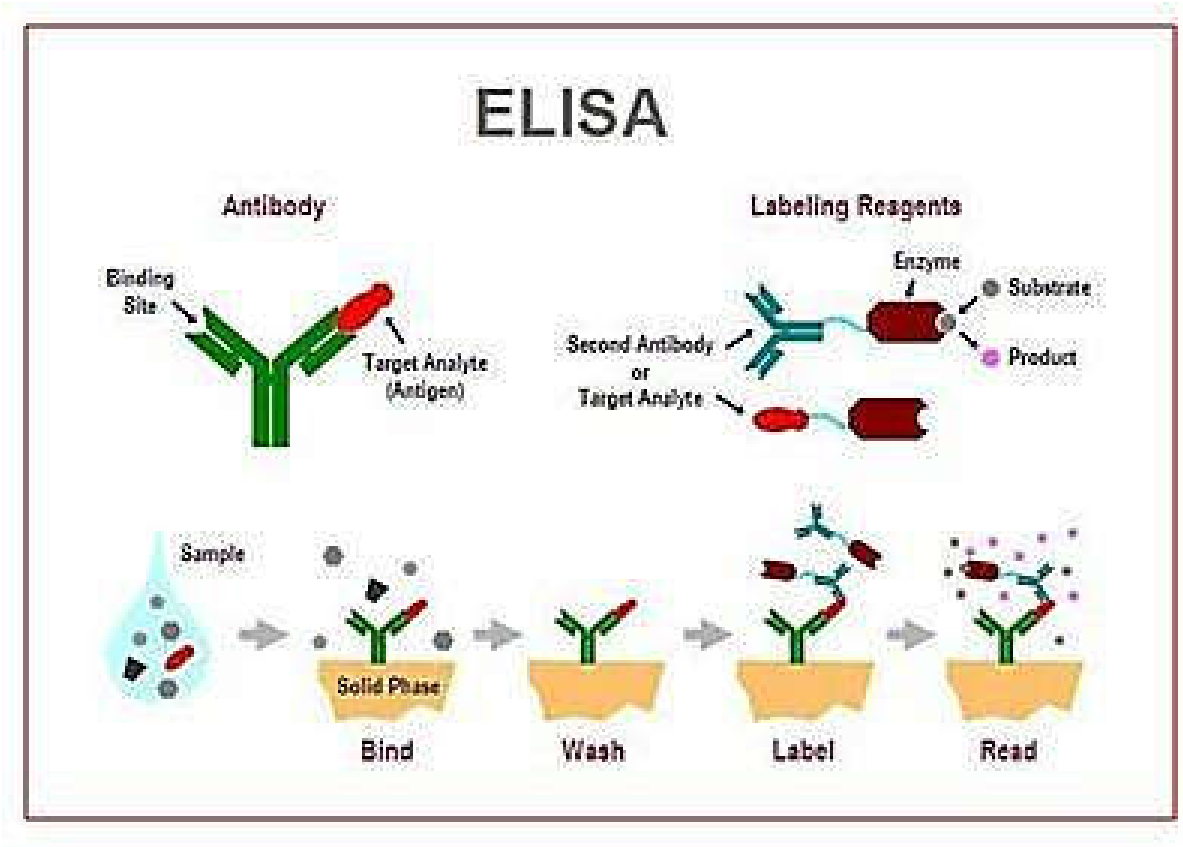


Figure: Construction of yeast genomic libraries in shuttle vector and screening by functional complementation to identify clones carrying the normal form of a mutant yeast gene.

An Enzyme Linked Immunosorbent Assay (ELISA)

An Enzyme Linked Immunosorbent Assay (ELISA) is a common quick and simple biochemical technique and involves specific and non-specific interactions by sequential binding to a solid surface. It detects the presence of an antibody or an antigen in the sample. The procedure of the ELISA results in a colored end product which correlates to the amount of specific substance present in the original sample. Steps of ELISAs begin with a coating step, where an antigen containing solution is adsorbed to a polystyrene 96 well plate. A small proportion of protein coats the surface of tubes or wells. Large number of samples can be run together.Fisrt step of coating is followed by washing and detection steps as shown in the schematic diagram below.The procedure uses surface binding for separation; several washing steps are performed between each ELISA step to remove any unbound substance. After the unbound antigens are washed away, the samples of antibody (known or unknown) are incubated in the antigen coated wells. Antibody which remains bound to immobilized antigen, after washing can be detected using labeled anti-immunoglobulin or immunoglobulin binding protein. These assays employ enzyme labelled detecting agent are therefore known as enzyme linked immunosorbent assays. These assays can also be quantified by using a standard solution of known antibody content.



Functional Sequencing of cDNA Expression Libraries

To understand the complexities of gene structure, its expression, its regulation, protein interactions, and molecular mechanisms of genetic diseases—the detailed and exact sequences of the bases in DNA is very essential. During the late 1970s two different sequencing techniques were developed. They are:

1. The **chemical cleavage method** developed by Allan Maxam and Walter Gilbert (the Maxam-Gilbert Method). This method was very important in the beginning but is no longer practiced today.

2. The enzyme-mediated chain termination method developed by Frederick Sanger along with Andrew Coulson is popularly known as the **Sanger sequencing method** or the **enzymatic method of DNA sequencing**. Both methods involve the labeling of the terminal nucleotide followed by separation and detection of the generated oligonucleotides.

1.Sanger Method or Dideoxynucleotide Chain Termination DNA Sequencing

DNA sequencing reactions are just like the PCR reactions for replicating DNA or synthesizing a new strand of DNA. The reaction mix includes the template DNA, free 5′deoxyribonucleotide triphosphates, an enzyme, the DNA polymerase (usually a variant of Taq polymerase) and a 'primer'—a small piece of single-stranded DNA about 20 to 30 nucleotides along with a free 3′-hydroxyl group that can hybridize to one strand of the template DNA. The primer with a free 3′OH group can initiate the synthesis of DNA strand with the addition of the free nucleotides to the primer by the polymerase enzyme. **Sanger's chain termination method** utilizes 2′3′-dideoxyribonucleoside triphosphates (ddNTPs). This molecule differs from the normal deoxyribonucleoside triphosphates (dNTPs) by having a hydrogen atom at the 3′ carbon instead of a hydroxyl group (Figure).

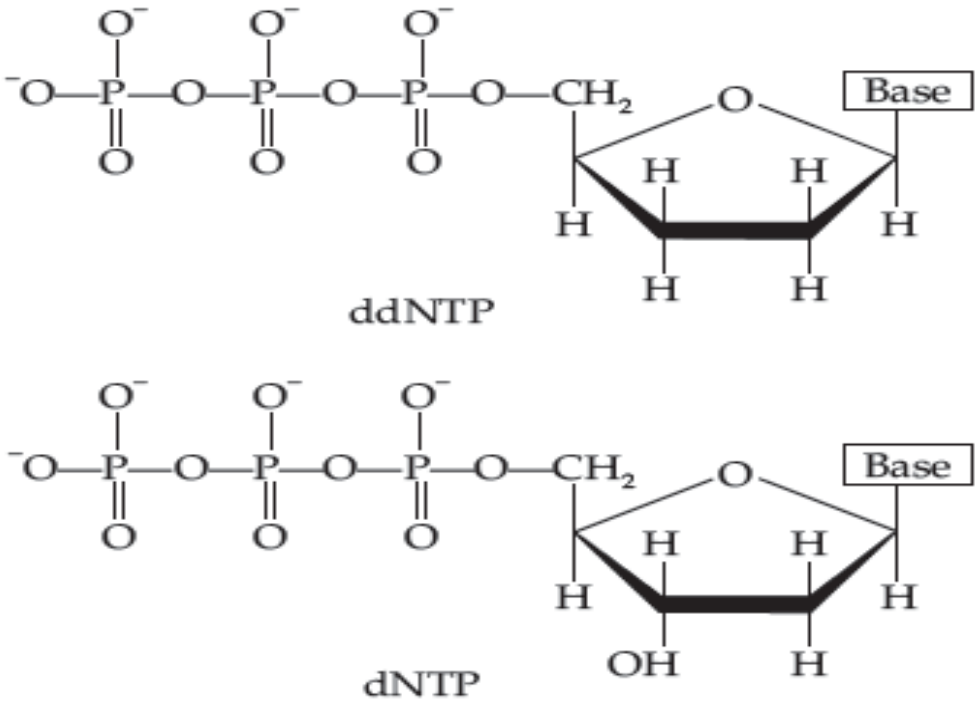


FIGURE: Chemical structure of ddNTP and dNTP.

The reaction is initiated with heat until the two strands of DNA (template) separate, then the primer binds to its intended location and the DNA polymerase starts elongating the primer by adding the appropriate dNTPs as indicated in Figure 15.20. If allowed to go to completion, a new strand of DNA would be the result. If we start with a billion identical pieces of template DNA, we will get a billion new copies of one of its strands. However, we run the sequencing reactions in the presence of a dideoxyribonucleotide. Once this nucleotide, which doesn't have the 3′-

hydroxyl group, is added to the end of a growing DNA strand, there's no way to continue its elongation. The key point here is that in the reaction mixture that we use for DNA sequencing most of the nucleotides are regular ones (dNTPs),and just a fraction of them are dideoxy nucleotides (ddNTPs). The double-stranded DNA (the template) can be converted into single strands by NaOH or by heating to 94°C as in the case of PCR reaction. A DNA-sequencing reaction following Sanger's method consists of the following components:

1. **DNA sample to be sequenced or the template**
2. **DNA primers**
3. **A mixture of all dNTPs—dATP, dGTP, dCTP, and dTTP**
   All this as mixture is distributed among four different reaction tubes and labeled A, T, G, and C.
4. **ddNTP: ddATP, ddTTP, ddGTP, and ddCTP (separately in four tubes)**
5. **Taq DNA polymerase**

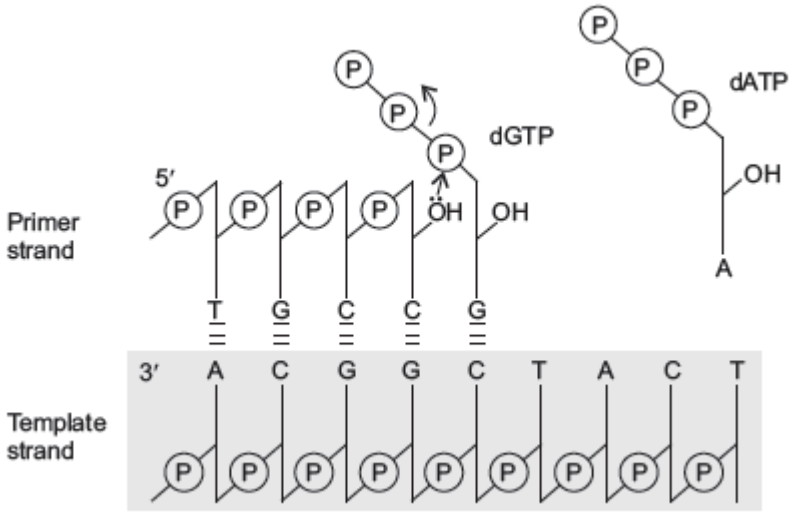


FIGURE: Binding of primer to the template and the elongation of the primer with the addition of dNTPs.

The DNA sample to be sequenced is the template. The template has to be converted into a single strand by denaturing with NaOH. But if you are carrying out the sequencing reaction using a PCR machine, denaturation of the template occurs as a part of the reaction cycle.

DNA primers.5′ end radio-labeled DNA primers, which are short fragments

of DNA complementary to the template DNA. Primers are labeled with radioactive phosphate at the 5′ end.

A mixture of all dNTPs—dATP, dGTP, dCTP, and dTTP. All this as a mixture is distributed among four different reaction tubes in appropriate quantities and labeled**'A', 'T', 'G', and 'C'.**

ddNTPs. In each tube a small quantity of the corresponding ddNTP is added. In a tube labeled 'A' a small amount of ddATP is added. In tube 'T' ddTTP is added, in tube 'G' ddGTP is added, and in tube 'C' a small amount of ddCTP is added. The concentration of ddNTP is approximately 1% of the concentration of the dNTPs.

Taq DNA polymerase. When all the components are ready, Taq DNA polymerase is added to all the four tubes and the reaction, the synthesis of DNA or the elongation of the primer, starts.

The elongation of the primer with the addition of dNTPs directed by the template continues until a dideoxynucleotide is incorporated instead of a dNTP.

For example, consider the DNA synthesis in the presence of a small amount of ddTTP (the reaction in the tube labeled 'T'). Most of the time when a 'T' is required to make the new strand, the enzyme will get a dTTP and there's no problem. After adding a T, the enzyme will go ahead and add more nucleotides and thus the elongation continues. However, 5% of the time, the enzyme will get a dTTP, but instead it will get a dideoxyT(ddTTP) and that strand can never again be elongated. It eventually breaks away from the enzyme, resulting in a dead-end product. Sooner or later all of the copies will get terminated by a 'T.' But each time the enzymemakes a new strand and the places at which it stops will be random. In millions of starts, there will be strands stopping at every possible T along the way. This is illustrated in Figure

5′— TACGCGGTAACGGTATGTTCGACCGTTTAGCTACCGAT

3′— ATGCGCCATTGCCATACANGCTGGCAAATCGATGGCTAGAGATCCAA — 5

Normal synthesis of DNA strand, when the reaction Mixture contains only dTTPs

5′— TACGCGGTAACGGTATGTTCGACCGTTTAGCTACCGAT—

5′— TACGCGGTAACGGTATGTTCGACCGTTTAGCT—

5′— TACGCGGTAACGGTATGTTCGACCGTTT—

5′— TACGCGGTAACGGTATGTTCGACCGTT—

5′— TACGCGGTAACGGTATGTTCGACCGT—

5′□— TACGCGGTAACGGTATGTT—

5′□— TACGCGGTAACGGTATGT—

5′□— TACGCGGTAACGGTAT—

5′□— TACGCGGTAACGGT—

5′□— TACGCGGT—

If 1% of the T nucleotides are ddTTPs then each strand will terminate when it gets a ddTTP on its growing end.

FIGURE: Diagram illustrating DNA chain termination due to the incorporation of ddNTPs and the generation of fragments of various types with terminal ddNTP.

The length of the strands thus produced will depend on the position at which the ddNTP is incorporated and can be determined by electrophoresis. When the reaction is completed, the products from each tube labeled 'A', 'T', 'G', and 'C' are taken and electrophoresed under denaturing conditions in four different lanes in a polyacrylamide gel. The gel is dried and exposed to an x-ray film by autoradiography. Only those fragments, which are labeled with the primer, will appear on the autoradiogram. Each fragment or the bands that are present in a lane end with the corresponding ddNTPs. For example, in the lane meant for the base 'T' all the bands or the fragments will have the ddTTP at the terminals. Thus, by reading the four lanes 'A', 'T', 'G' and 'C' from the bottom of the gel to the top, the sequence of the DNA can be elucidated. But it should be noted that whatever sequence you are reading from the gel is the complementary of the template DNA.

It is well-illustrated in the following Figures

DNA sequence can be directly read from the autoradiogram. If the labeling is by fluorescent dyes, instead of using radiolabeled primers, fluorescently labeledddNTPs are used in the reaction mixture. Each type of ddNTPs is labeled with a specific color and can be detected directly from the gel when illuminated with a laser or UV light

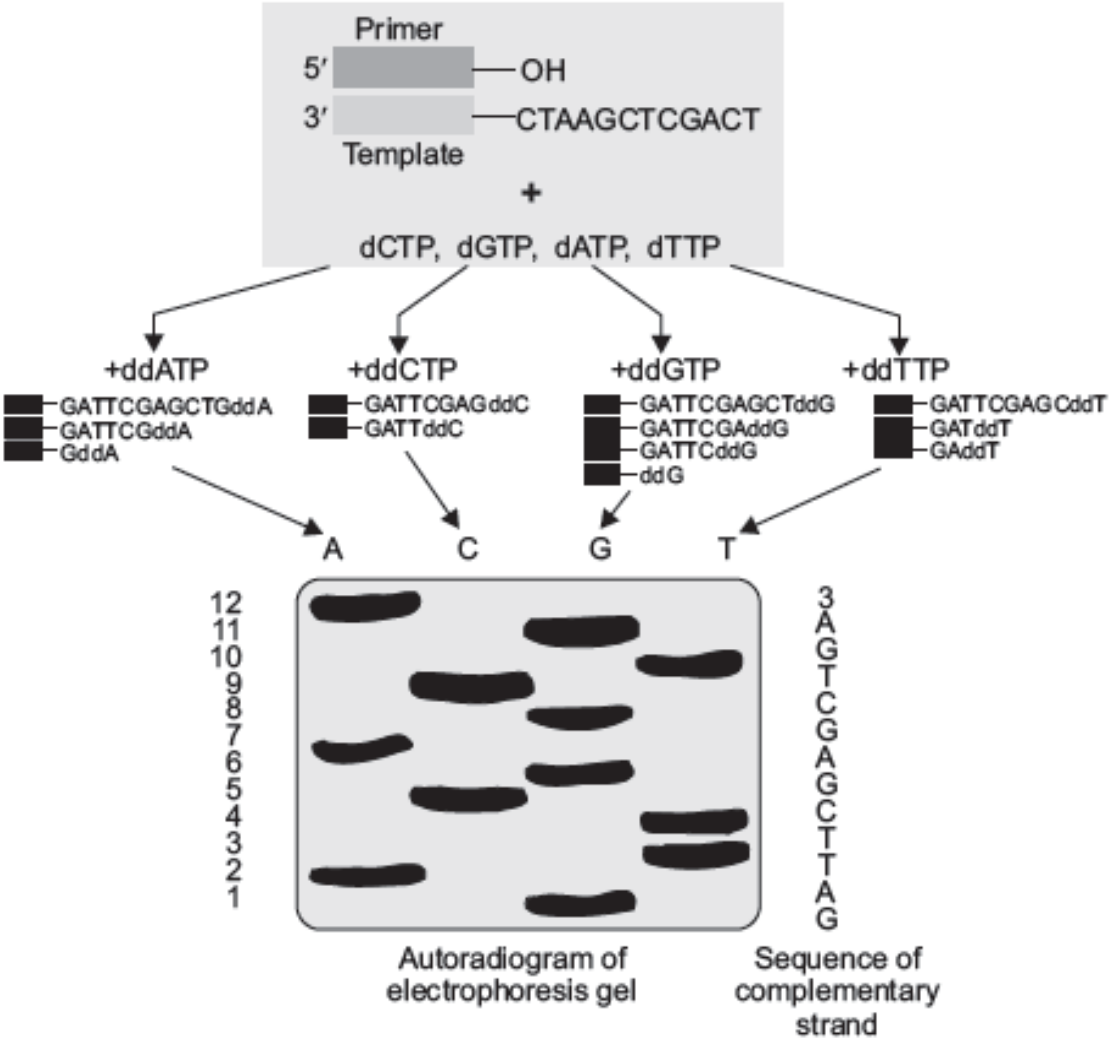


FIGURE: Chain termination after incorporating the ddNTPs and its interpretation from an autoradiography of a DNA sequencing gel.

Expressed cDNAsCompared with Computer Databases

Genomics encompasses the study of all features of genomes and individual genes at the DNA level, including mutations, polymorphisms, and phylo-genetic relationships that are based on sequence differences. Another aspect of genomics that is often called functional genomics (or transcriptomics) is concerned with the patterns of transcription, either qualitatively to determine which genes are expressed or quantitatively to measure changes in the levels of transcription of genes. Transcription at the whole-genome level is assessed as a function of clinical conditions, as a consequence of mutations, in response to natural or toxic agents, in different cells or tissues, or

at different times during biological processes, such as cell division or the development of an organism. One of the aims of gene expression studies is to discover the genes that are up- and downregulated under specific conditions (the transcriptome). In the past, the transcription of only one or a few genes could be followed at a time. Currently, functional genomics methodology can track the simultaneous transcription of thousands of genes (gene expression profiling) of either a cell or a tissue sample. The main experimental approaches for determining gene expression profiles are DNA microarrays and serial analysis of gene expression (SAGE). Because of the large amount of data that is generated from these experiments, special computational tools are required for obtaining, storing, and analyzing the results.

DNA Microarray Technology

A DNA microarray (DNA chip, or gene chip) experiment consists of hybridizing a nucleic acid sample (target) derived from the messenger RNAs (mRNAs) of a cell or tissue to single-stranded DNA sequences (probes) that are bound in an ordered arrangement to a solid platform. One type of DNA microarray is constructed by spotting polymerase chain reaction (PCR)-amplified cDNA sequences from the mRNAs of a single cell or all or specific sets of the coding sequences of an organism onto a glass slide or nylon membrane. In this case, about 10,000 different probes can be arrayed in a 1-cm2 area.

An alternative microarray system utilizes sets of oligonucleotides as probes, usually representing thousands of genes. For one commonly used platform, the probes are synthesized directly (in situ) on a solid surface (quartz wafer) by a light-directed process known as photolithography. Thousands of copies of an oligonucleotide with the same specific nucleotide sequence are synthesized in a predefined position (probe cell or feature) on the array surface. For this type of microarray, the probes are typically 10 to 40 nucleotides, and several probes with different sequences for each gene will be synthesized on the microarray. Longer oligonucleotides up to 100 nucleotides can also be used. A complete whole-genome oligonucleotide array may contain more than 500,000 probes representing as many as 30,000 genes.

Generally, the design of the probes (probe set) for a microarray depends on the objective of the experiment and the degree of resolution that is required. Computer programs determine probe sequences that are specific for their target sequences, are least likely to hybridize with non-target sequences (cross-hybridize), have no secondary structure (foldback) that would prevent

hybridization with the target sequence, and have similar melting (annealing) temperatures, so that all target sequences can bind to their complementary probe sequences under the same conditions. Oligonucleotide microarrays may consist of probes that represent an entire genome, a single chromosome, selected genomic regions, or selected coding regions from one or several different organisms. Repetitive sequences are not included in genomic DNA microarrays.

Typically, for most gene expression profiling experiments that utilize microarrays, mRNA is extracted from cells or tissues and purified, and cDNA is synthesized using reverse transcriptase and the extracted mRNA as a template. Usually, mRNA is extracted from two or more sources whose expression profiles are compared; for example, from diseased versus normal tissue or from cells grown under different conditions. The cDNA from each source is labeled with a different fluorophore by incorporating fluorescently labelednucleotides during cDNA synthesis. For example, a green-emitting fluorescent dye (Cy3) is used for the normal (reference) sample and a red-emitting fluorescent dye (Cy5) for the test sample. After being labeled, the cDNA samples are mixed and hybridized tothe same microarray. A laser scanner determines the intensities of Cy5 and Cy3 for each probe cell. The probe cells have different colors depending on the amounts of Cy3 and Cy5 that are present, and the ratio of red (Cy5) to green (Cy3) fluorescence intensity of a probe cell indicates the relative expression levels of the represented gene in the two samples (Fig. 5.5). To avoid variation due to inherent and sequence-specific differences in labeling efficiencies between Cy3 and Cy5, reference and test samples are often reverselabeled and hybridized to another microarray. In the above example, reverse labeling (dye swapping) would entail labeling the reference sample with Cy5 and the test sample with Cy3. Alternatively, for some microarray platforms, the target sequences from each source are labeled with the same fluorescent dye, and reference and test samples are hybridized to different microarrays.

Serial Analysis of Gene Expression

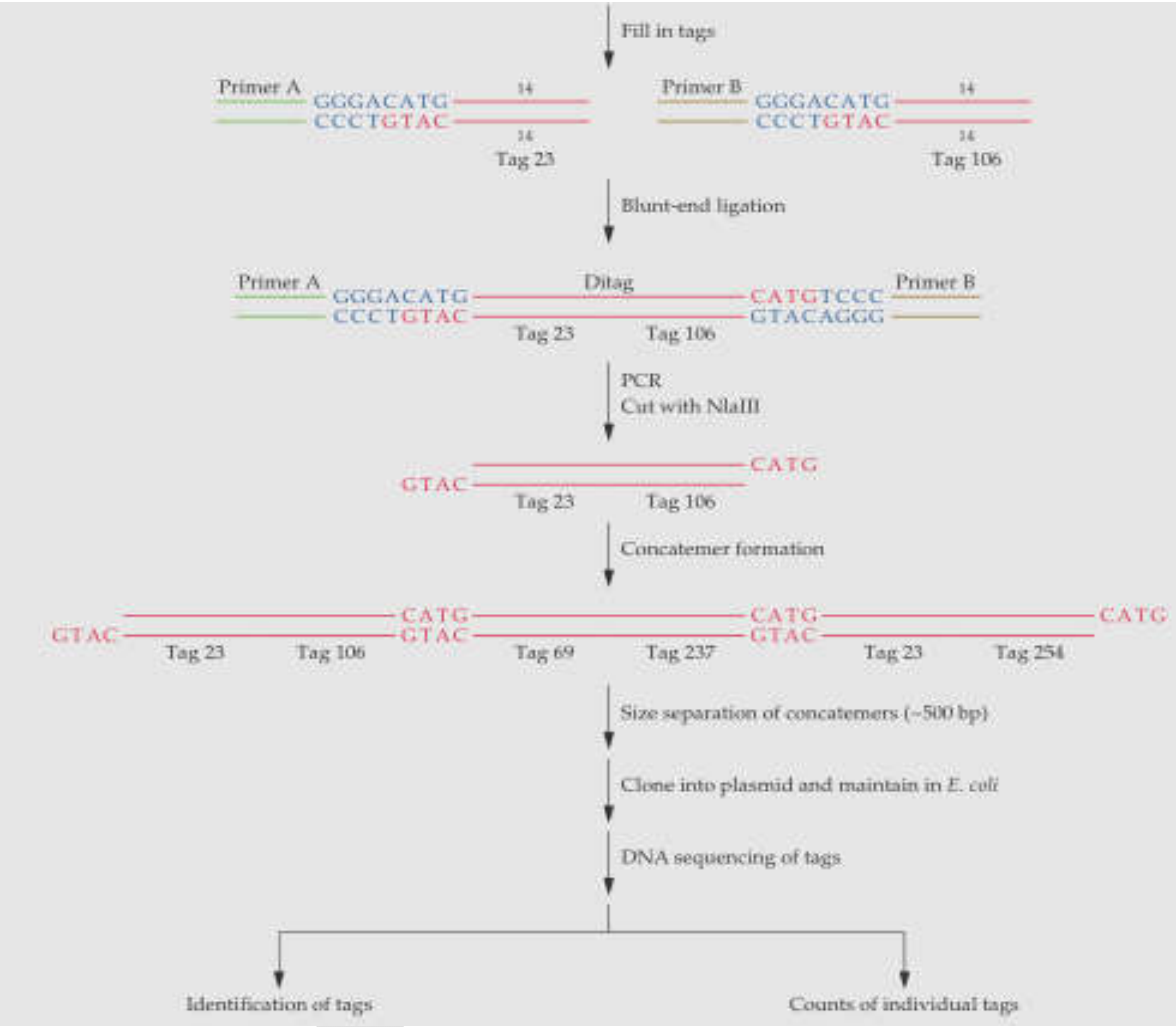
Unlike DNA microarrays that rely on hybridization and signal detection, SAGE uses recombinant DNA techniques to clone randomly linked short sequences of cDNA prepared from extracted cellular mRNA that can be efficiently sequenced to identify expressed genes (Fig. 5.9). Polyadenylated mRNA is captured by an oligo(dT) sequence that is labeled with biotin and attached to a streptavidin-coated magnetic bead. Double-stranded cDNA is synthesized from the

purified mRNA using reverse transcriptase to synthesize the first strand of cDNA from the oligo(dT) primer and mRNA template and then DNA polymerase to synthesize the second, complementary, strand. A strong magnet is used to retain the magnetic beads with attached cDNAs during successive treatments and washings. The cDNAs are cut with the restriction endonuclease NlaIII, which recognizes thesequence CATGGTAC and cuts outside the G:C base pairs, leaving a 3′ GTAC extension. Since NlaIII cuts, on average, 1 in 256 base pairs (bp), there is a high probability that each cDNA will have at least one NlaIII recognition site. Because the cDNAs are bound to beads, each NlaIII cut site that is closest to the 3′ end of a cDNA is retained, and all unbound fragments are washed away. In the nomenclature developed for SAGE, NlaIII is called an anchoring enzyme. Next, the NlaIII-digested cDNA sample is divided in two. One aliquot is ligated with adaptor A and the other with adaptor B.

Both adaptors have a CATG extension that is complementary to the extension produced by NlaIII digestion, a 5-bp recognition site for the restriction endonuclease BsmFI, and a sequence for priming a PCR. The adaptors have different primer sequences to prevent intrastrand base pairing (snap back) during subsequent PCR steps. After the adaptors are ligated to NlaIII-digested cDNA, the products are treated with BsmFI. Unlike NlaIII and other type II restriction endonucleases, this type IIs restriction endonuclease cuts 10 nucleotides downstream from its recognition site in one DNA strand and 14 nucleotides in the other strand regardless of the intervening nucleotide sequence. In SAGE, BsmFI is known as a tagging enzyme, and the segment of cDNAproduced by the BsmFI treatment is called a tag. BsmFI digestion releases the adaptor–tag molecules from the beads into solution, from which they can be recovered. The 4-nucleotide extension of the BsmFI-cut DNA is filled in to form a blunt-end molecule, the pools of adaptor A– and adaptor B–tags are mixed, and T4 DNA ligase is added to the mixture. Under these conditions, the blunt ends of two tags are joined to form a two-tag molecule (a ditag) that is flanked by primer sequences. Since ditag formation is completely random, tags from different cDNAs are joined during ligation, and the ditags are readily amplified during PCR using primer sequences present in the adaptors.

The amplified ditags are treated with NlaIII to release the adaptor sequences and produce ditags with an NlaIII extension at each end. The NlaIII-digested ditags are ligated to form multiple, randomly joined combinations (concatemers) of ditags. Concatemers that are about 500

bp in length are isolated and cloned in an E. coli plasmid. The concatemers are sequenced, the sequence of each tag is recorded, and a specialized "tag to gene" database is searched to identify the corresponding gene. The sequenced tag is derived from the 3′ end of the mRNA and therefore corresponds to the 3′ end of the gene. The number of times each tag is sequenced, which represents its abundance in the initial sample, is determined.



Up- and downregulated mRNAs can be identified by comparing the frequencies of tags in different samples. Generally, more than 10,000 unique tags are collected from a single experiment. Over 30 million SAGE tags have been assembled from humans and another 35 million from various organisms. In principle, SAGE can detect all the transcripts in a sample, whereas with a DNA microarray, only the sequences that correspond to probes on the array are identified.Additional SAGE protocols, such as LongSAGE and SuperSAGE, have been

developed to produce longer tags. Also, other anchoring enzymes, e.g., Sau3AI and other restriction endonucleases that recognize and cleave at specific 4-bp sequences (4-base cutters), have been used to identify transcribed genes that do not have a convenient NlaIII site. An online resource called SAGE Genie (http://cgap.nci.nih.gov/SAGE) is available for matching tags to likely genes, determining the frequency of a tag among various SAGE libraries, and providing other pertinent information.

Recombinant DNA Technology in the Synthesis of Human Insulin

The nature and purpose of synthesising human insulin.

Since Banting and Best discovered the hormone, insulin in 1921. diabetic patients, whose elevated sugar levels (see fig. 1) are due to impaired insulin production, have been treated with insulin derived from the pancreas glands of abattoir animals. The hormone, produced and secreted by the beta cells of the pancreas' islets of Langerhans, regulates the use and storage of food, particularly carbohydrates.

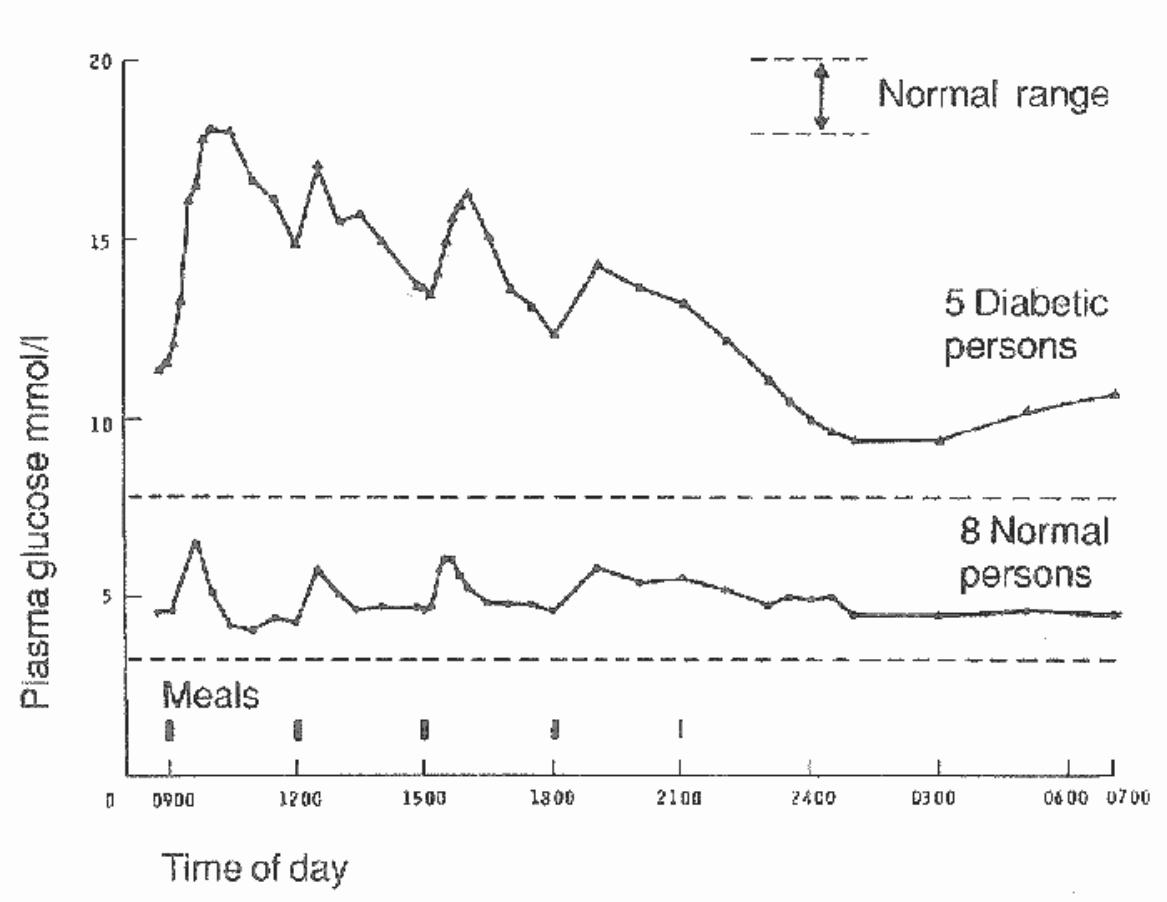


Fig. 1

Although bovine and porcine insulin are similar to human insulin, their composition is slightly different. Consequently, a number of patients' immune systems produce antibodies against it, neutralising its actions and resulting in inflammatory responses at injection sites. Added to these adverse effects of bovine and porcine insulin, were fears of long term complications ensuing from the regular injection of a foreign substance, as well as a projected decline in the production of animal derived insulin. These factors led researchers to consider synthesising *Humulin* by inserting the insulin gene into a suitable vector, the E. coli bacterial cell, to produce an insulin that is chemically identical to its naturally produced counterpart. This has been achieved using Recombinant DNA technology. This method (see fig. 2) is a more reliable and sustainable method than extracting and purifying the abattoir by-product.

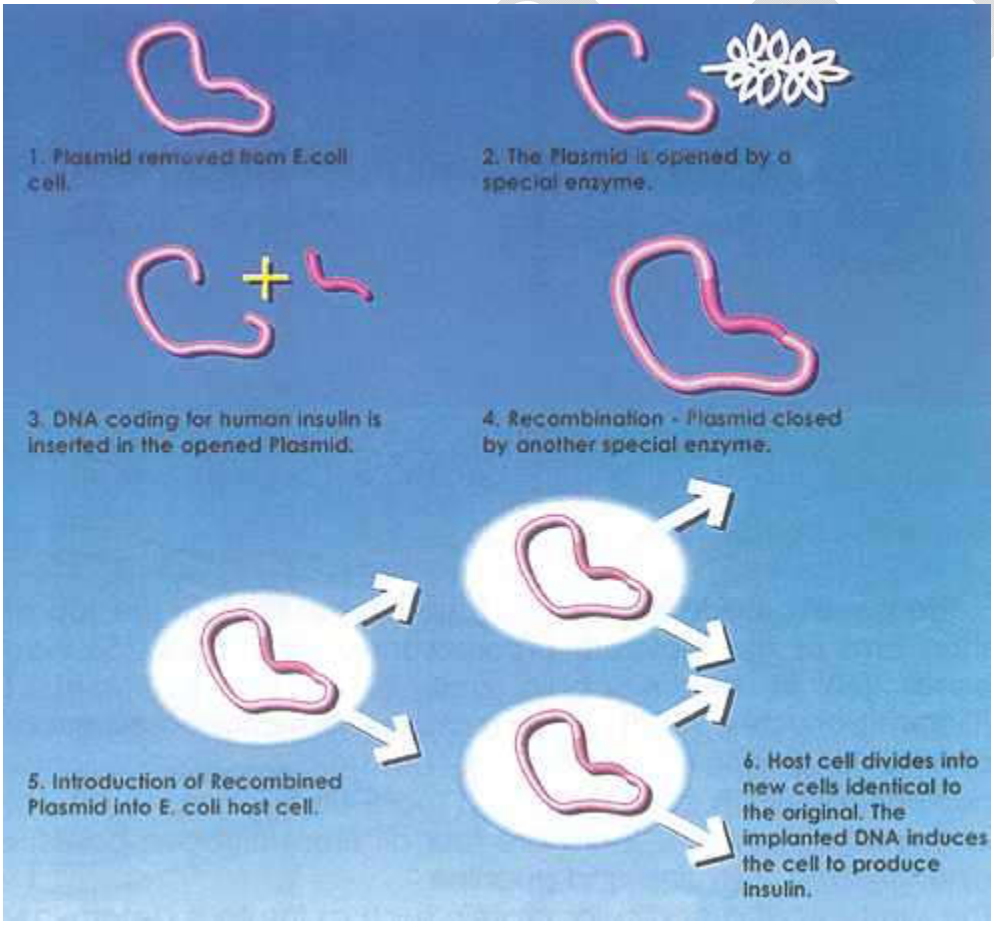


Fig. 2

Understanding the genetics involved.

The structure of insulin.

Chemically, insulin is a small, simple protein. It consists of 51 amino acid, 30 of which constitute one polypeptide chain, and 21 of which comprise a second chain. The two chains (see fig. 3) are linked by a disulfide bond.

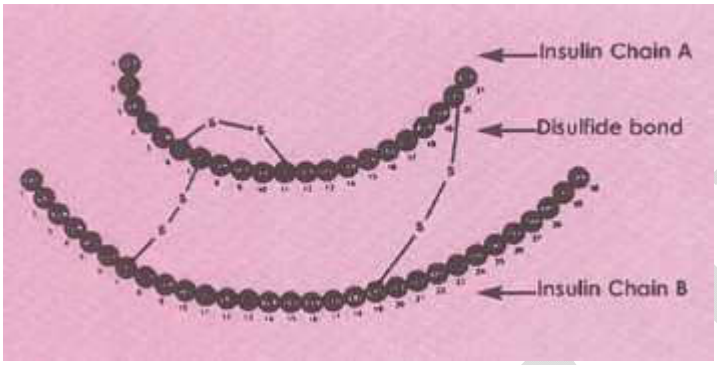


Fig. 3

Inside the Double Helix.

The genetic code for insulin is found in the DNA at the top of the short arm of the eleventh chromosome. It contains 153 nitrogen bases (63 in the A chain and 90 in the B chain).DNA Deoxyribolnucleic Acid), which makes up the chromosome, consists of two long intertwined helices, constructed from a chain of nucleotides, each composed of a sugar deoxyribose, a phosphate and nitrogen base. There are four different nitrogen bases, adenine, thymine, cytosine and guanine. The synthesis of a particular protein such as insulin is determined by the sequence in which these bases are repeated (see fig. 4).

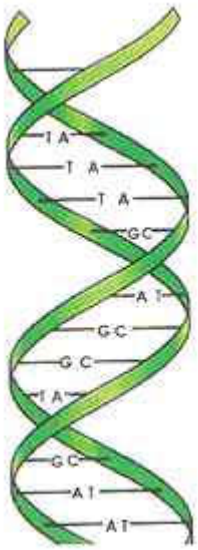


Fig. 4

Insulin synthesis from the genetic code.

The double strand of the eleventh chromosome of DNA divides in two, exposing unpaired nitrogen bases which are specific to insulin production (see fig. 5).

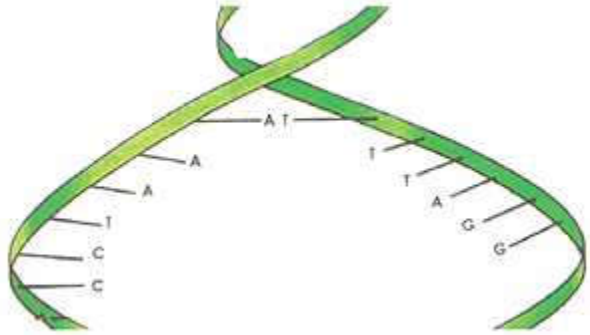


Fig. 5

Using one of the exposed DNA strands (see fig.6) as a template, messenger RNA forms in the process of transcription (see fig. 7).

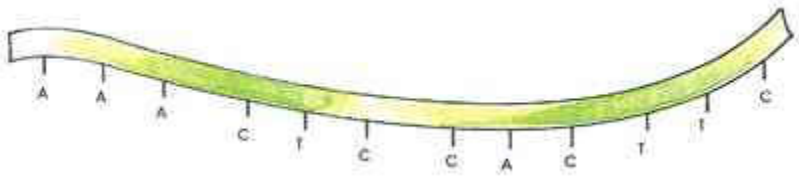


Fig 6

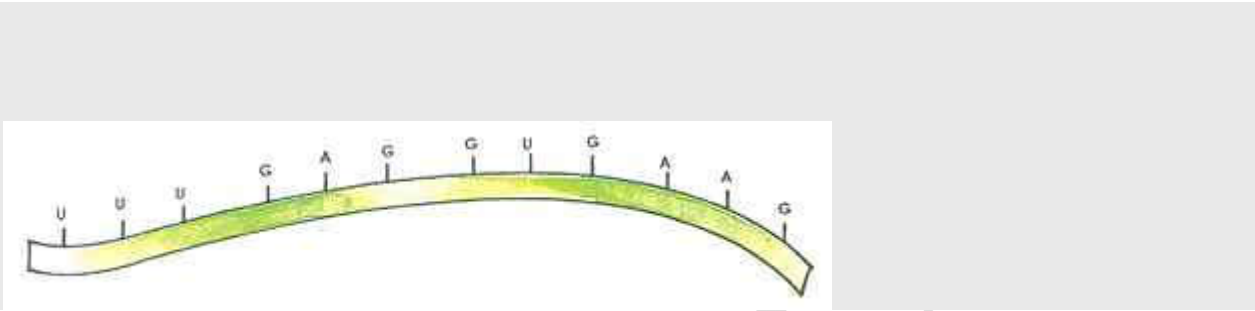


Fig. 7

The role of the mRNA strand, on which the nitrogen base thymine is replaced by uracil, is to carry genetic information, such as that pertaining to insulin,from the nucleus into the cytoplasm, where it attaches to a ribosome (see fig. 8).

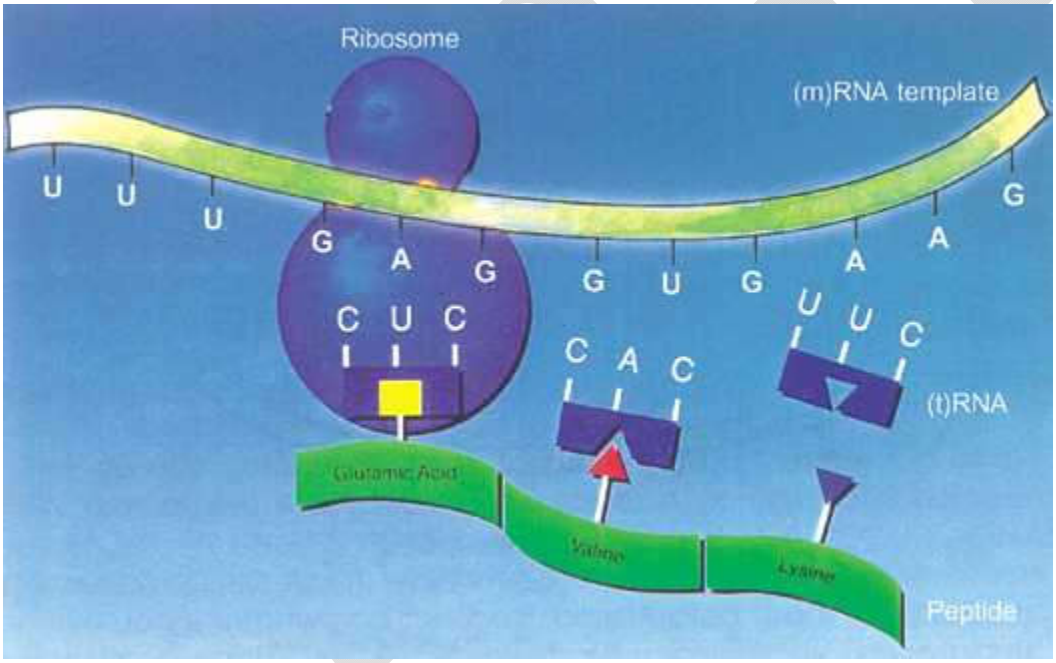


Fig. 8

The nitrogen bases on the mRNA are grouped into threes, known as codons. Transfer RNA (tRNA) molecules, three unpaired nitrogen bases bound to a specific amino acid, collectively known as an anti-codon (see fig.9) pair with complementary bases (the codons) on the mRNA.

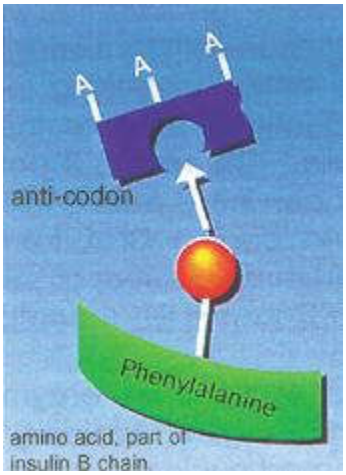


Fig. 9

he reading of the mRNA by the tRNA at the ribosome is known as translation. A specific chain of amino acids is formed by the tRNA following the code determined by the mRNA. The base sequence of the mRNA has been translated into an amino acid sequence which link together to form specific proteins such as insulin.

The Vector (Gram negative E. coli).

A weakened strain of the common bacterium, Escherrichia coli (E. coli) (see fig. 10), an inhabitant of the human digestive tract, is the 'factory' used in the genetic engineering of insulin.

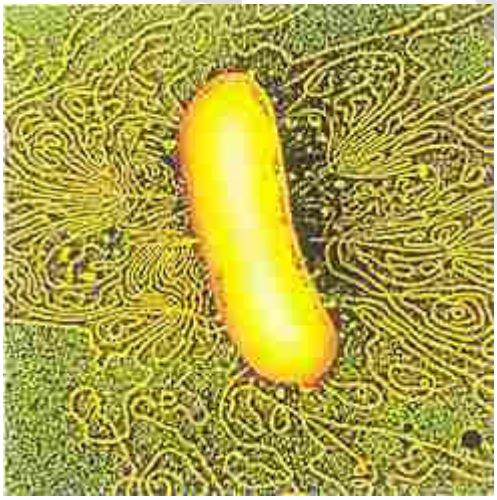


Fig. 10

When the bacterium reproduces, the insulin gene is replicated along with the plasmid, a circular section of DNA (see fig. 11). E. coli produces enzymes that rapidly degrade foreign proteins such as insulin. By using mutant strains that lack these enzymes, the problem is avoided.

Fig. 11

n E. coli, B-galactosidase is the enzyme that controls the transcription of the genes. To make the bacteria produce insulin, the insulin gene needs to be tied to this enzyme.

Inside the genetic engineer's toolbox.

Restriction enzymes, naturally produced by bacteria, act like biological scalpels (see fig.12), only recognising particular stretches of nucleotides, such as the one that codes for insulin.

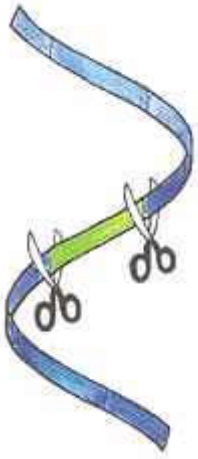


Fig 12

This makes it possible to sever certain nitrogen base pairs and remove the section of insulin coding DNA from one organism's chromosome so that it can manufacture insulin (See fig. 13). DNA ligase is an enzyme which serves as a genetic glue, welding the sticky ends of exposed nucleotides together.

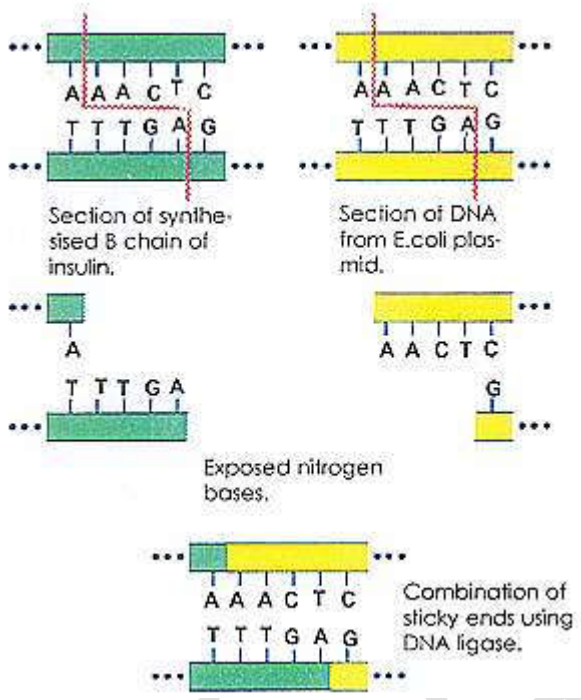


Fig. 13

Manufacturing Humulin.

The first step is to chemically synthesise the DNA chains that carry the specific nucleotide sequences characterising the A and B polypeptide chains of insulin (see fig. 14).

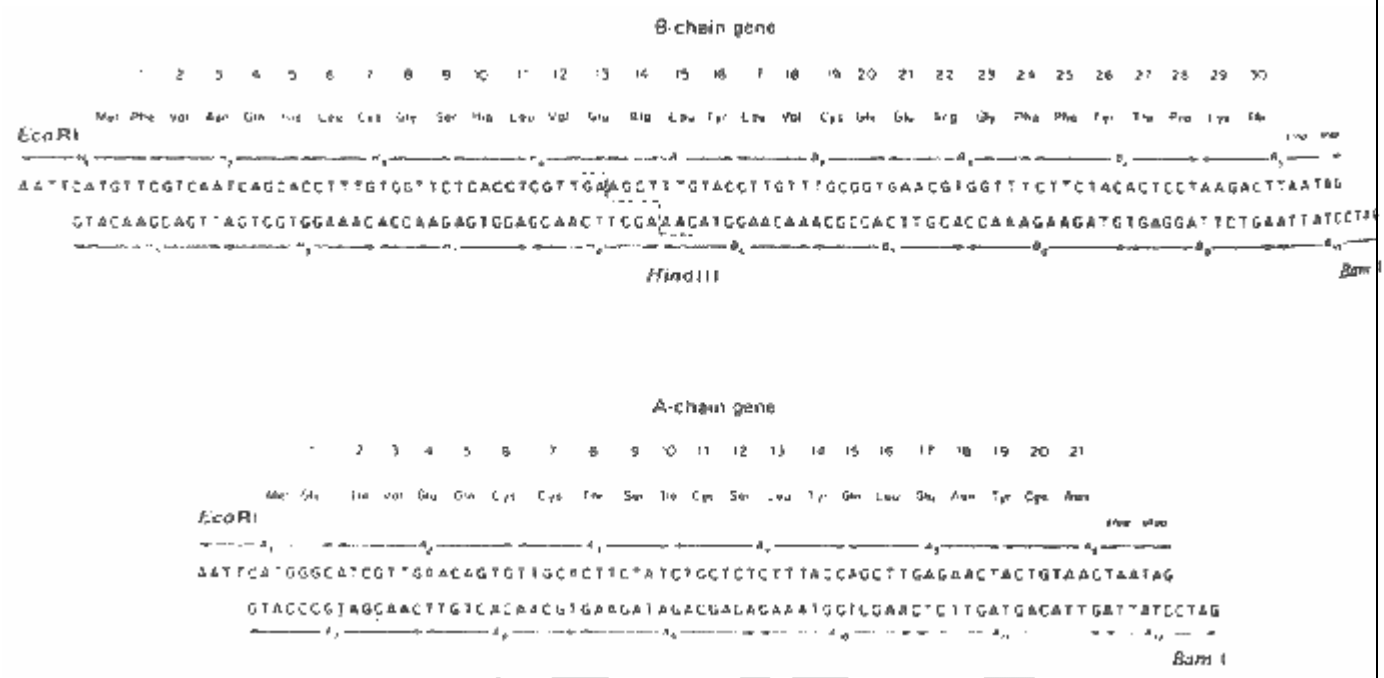


Fig. 14

he required DNA sequence can be determined because the amino acid compositions of both chains have been charted. Sixty three nucleotides are required for synthesising the A chain and ninety for the B chain, plus a codon at the end of each chain,signalling the termination of protein synthesis. An anti-codon, incorporating the amino acid, methionine, is then placed at the beginning of each chain which allows the removal of the insulin protein from the bacterial cell's amino acids. The synthetic A and B chain 'genes' (see fig. 15) are then separately inserted into the gene for a bacterial enzyme, B-galactosidase, which is carried in the vector's plasmid. At this stage, it is crucial to ensure that the codons of the synthetic gene are compatible with those of the B-galactosidase.

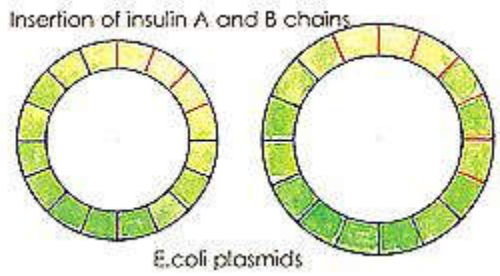


Fig. 15

The recombinant plasmids are then introduced into E. coli cells. Practical use of Recombinant DNA technology in the synthesis of human insulin requires millions of copies of the bacteria whose plasmid has been combined with the insulin gene in order to yield insulin. The insulin gene is expressed as it replicates with the B-galactosidase in the cell undergoing mitosis (see fig. 16).

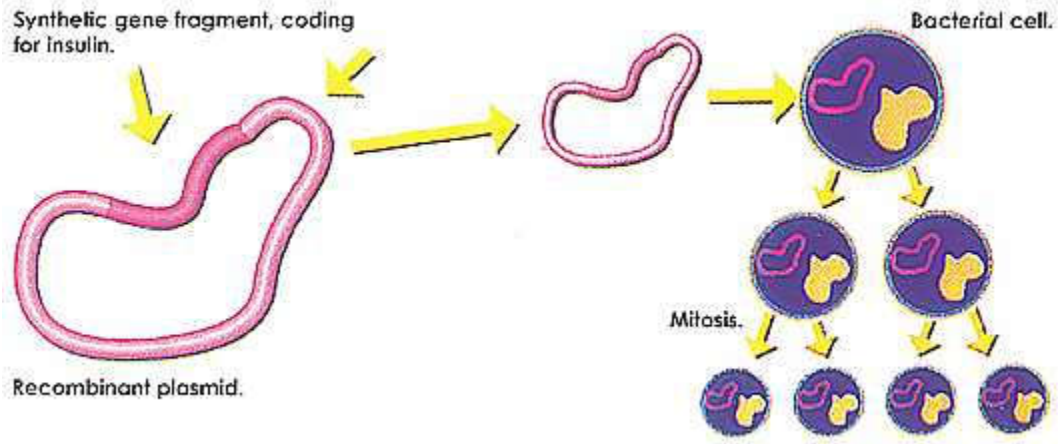


Fig. 16

The protein which is formed, consists partly of B-galactosidase, joined to either the A or B chain of insulin (see fig.17). The A and B chains are then extracted from the B-galactosidase fragment and purified.

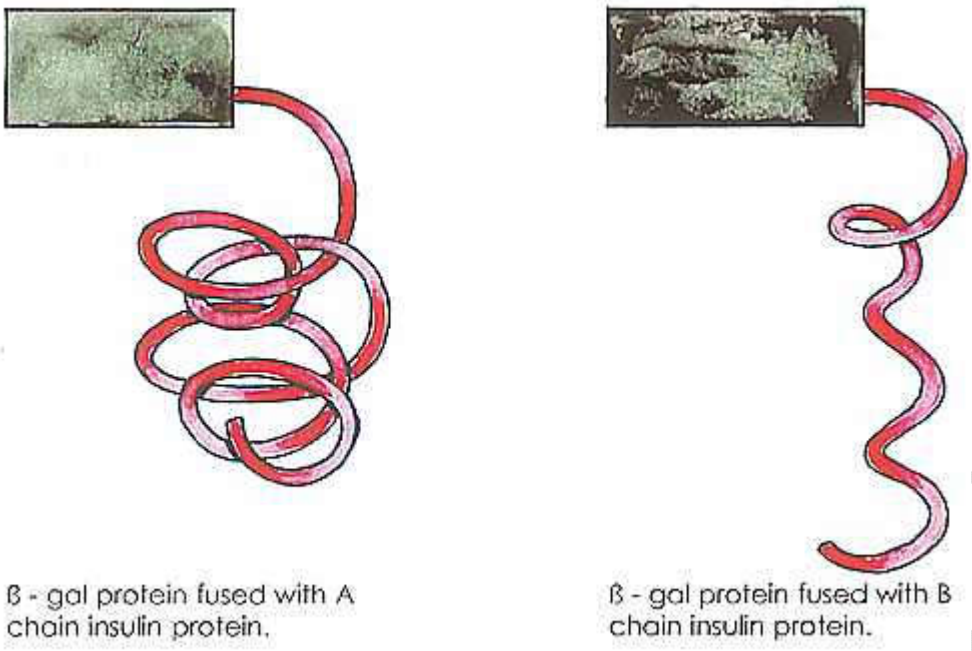


Fig. 17

The two chains are mixed and reconnected in a reaction that forms the disulfide cross bridges, resulting in pure *Humulin* - synthetic human insulin (see fig. 18).

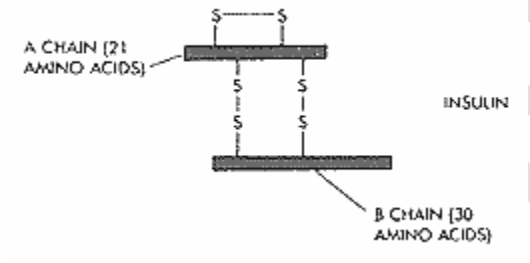


Fig. 18

Biological implications of genetically engineered Recombinant human insulin.

Human insulin is the only animal protein to have been made in bacteria in such a way that its structure is absolutely identical to that of the natural molecule. This reduces the possibility of complications resulting from antibody production. In chemical and pharmacological studies, commercially available Recombinant DNA human insulin has proven indistinguishable from pancreatic human insulin. Initially the major difficulty encountered was the contamination of the final product by the host cells, increasing the risk of contamination in the fermentation broth. This danger was eradicated by the introduction of purification processes. When the final insulin

product is subjected to a battery of tests, including the finest radio-immuno assay techniques, no impurities can be detected. The entire procedure is now performed using yeast cells as a growth medium, as they secrete an almost complete human insulin molecule with perfect three dimensional structure. This minimises the need for complex and costly purification procedures.

The issue of hypoglycaemic complications in the administration of human insulin.

Since porcine insulin was phased out, and the majority of insulin dependent patients are now treated with genetically engineered recombinant human insulin, doctors and patients have become concerned about the increase in the number of hypoglycaemic episodes experienced. Although hypoglycaemia can be expected occasionally with any type of insulin, some people with diabetes claim that they are less cognisant of attacks of hypoglycaemia since switching from animal derived insulin to Recombinant DNA human insulin. In a British study, published in the 'Lancet", hypoglycaemia was induced in patients using either pork or human insulin, The researchers found "no significant difference in the frequency of signs of hypoglycaemia between users of the two different types of insulin."

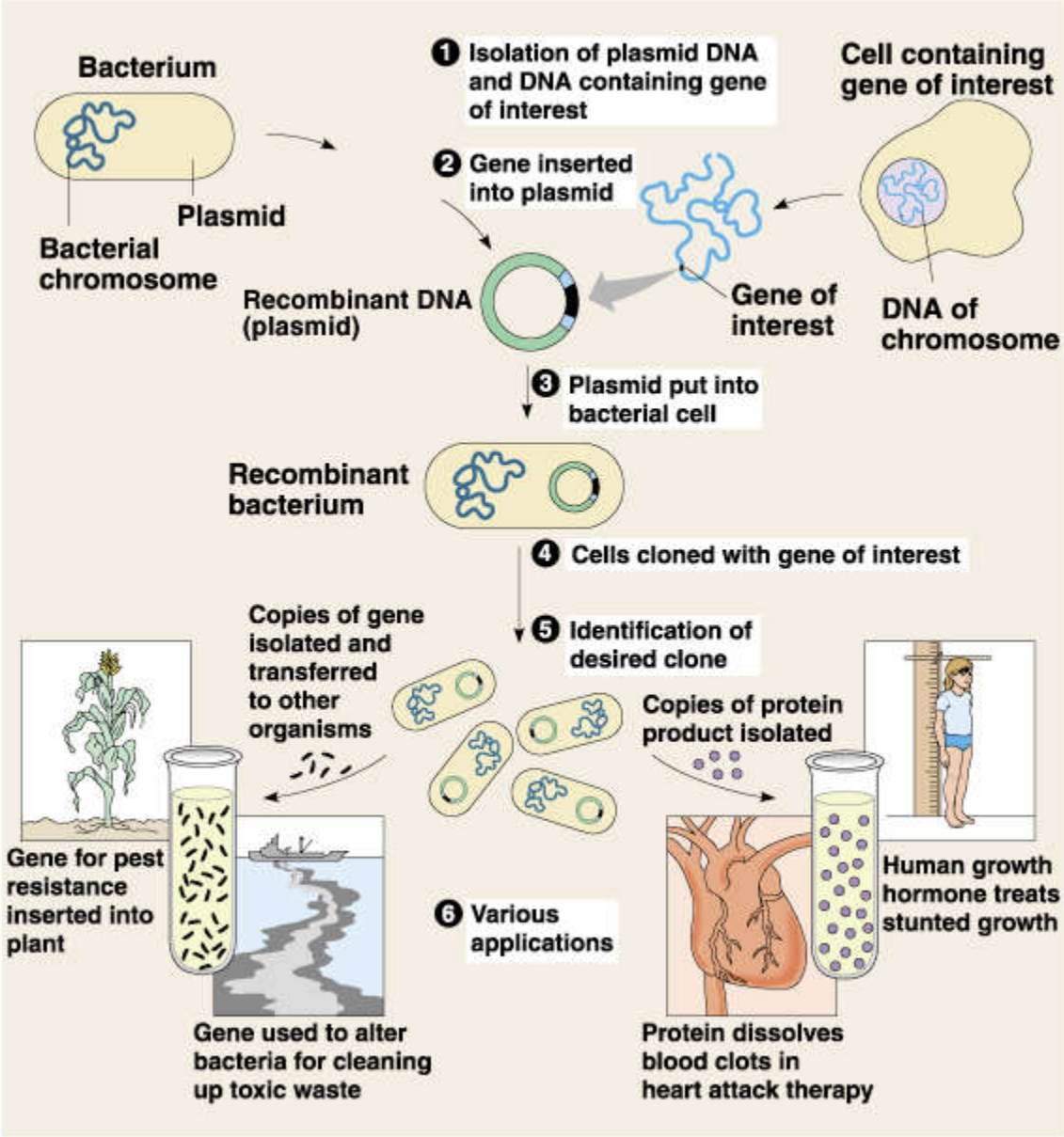
An anecdotal report from a British patient who had been insulin dependent for thirty years, stated that she began experiencing recurring, unheralded hypoglycaemia only after substituting Recombinant DNA human insulin for animal derived insulin. After switching back to pork insulin to ease her mind, she hadn't experienced any unannounced hypoglycaemia. Eli Lilly and Co., a manufacturer of human insulin, noted that a third of people with diabetes, who have been insulin dependent for over ten years, "lose their hypoglycaemic warning signals, regardless of the type of insulin they are taking."

Dr Simon P. Wolff of the University College of London said in an issue of *Nature* , "As far as I can make out, there's no fault (with the human insulin)." He concluded, "I do think we need to have a study to examine the possible risk."

Although the production of human insulin is unarguable welcomed by the majority of insulin dependent patients, the existence of a minority of diabetics who are unhappy with the product cannot be ignored. Although not a new drug, the insulin derived from this new method of production must continue to be studied and evaluated, to ensure that all its users have the opportunity to enjoy a complication free existence.

Human growth hormone (hGH)

Human growth hormone (hGH) is a single-chain polypeptide that participates in a wide range of biological functions such as metabolism of proteins, carbohydrates and lipids as well as in growth, development and immunity. Growth hormone deficiency in human occurs both in children and adults. The routine treatment for this condition is administration of recombinant human growth hormone (rhGH) made by prokaryotes. Since nonglycosylated human growth hormone is a biologically active protein, prokaryotic expression systems are preferred for its production. Different strains of *E.coli* were transformed by plasmid containing human growth hormone gene and cultured in different conditions. After induction by IPTG, recombinant human growth hormone production was assessed using ELISA, dot blotting and western blotting techniques.



tPA

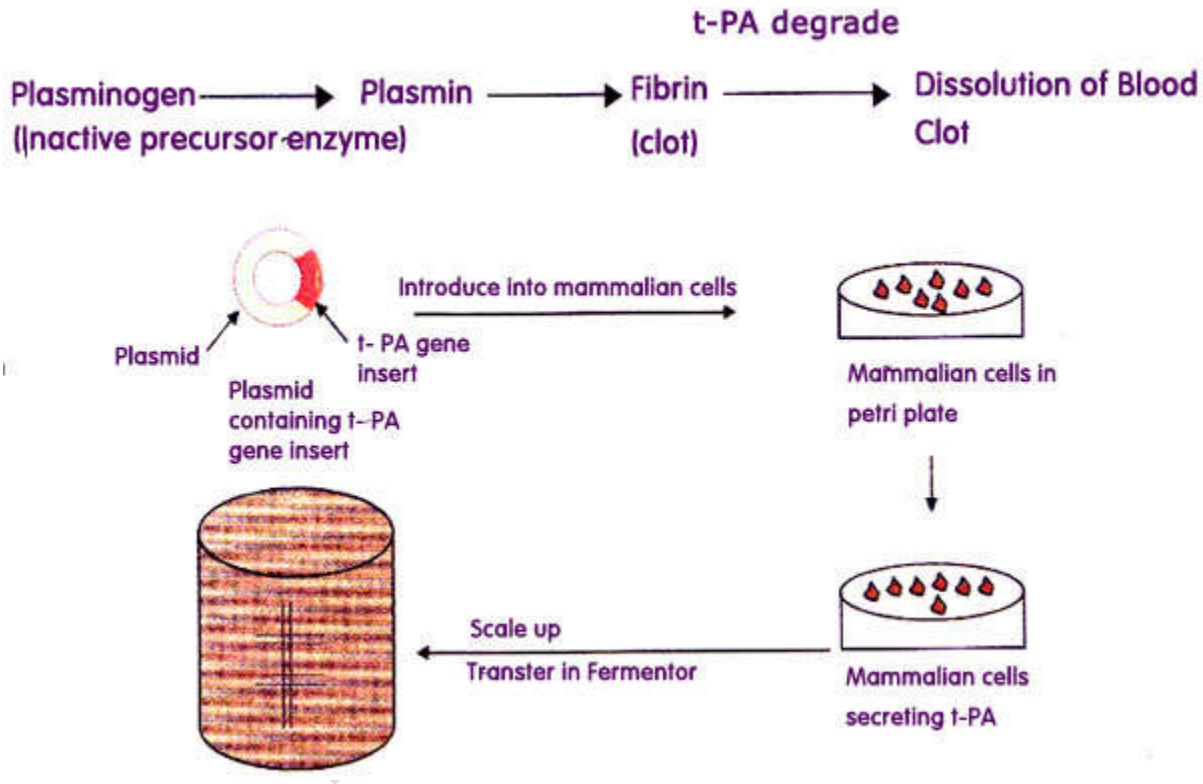
Tissue plasminogen activator (abbreviated tPA or PLAT) is a protein involved in the breakdown of blood clots. It is a serine protease (EC 3.4.21.68) found on endothelial cells, the cells that line the blood vessels. As an enzyme, it catalyzesthe conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown.

tPA can be manufactured using recombinant biotechnology techniques and is then referred to as recombinant tissue plasminogen activator (rtPA). Specific rtPAs include alteplase, reteplase, and tenecteplase. They are used in clinical medicine to

treat embolic or thrombotic stroke. Use is contraindicated in hemorrhagic stroke and head trauma. The antidote for tPA in case of toxicity is aminocaproic acid.

Medical Uses

tPA is used in some cases of diseases that feature blood clots, such as pulmonary embolism, myocardial infarction, and stroke, in a medical treatment called thrombolysis. The most common use is for ischemic stroke. It can either be administered systemically, in the case of acute myocardial infarction, acute ischemic stroke, and most cases of acute massive pulmonary embolism, or administered through an arterial catheter directly to the site of occlusion in the case of peripheral arterial thrombi and thrombi in the proximal deep veins of the leg.

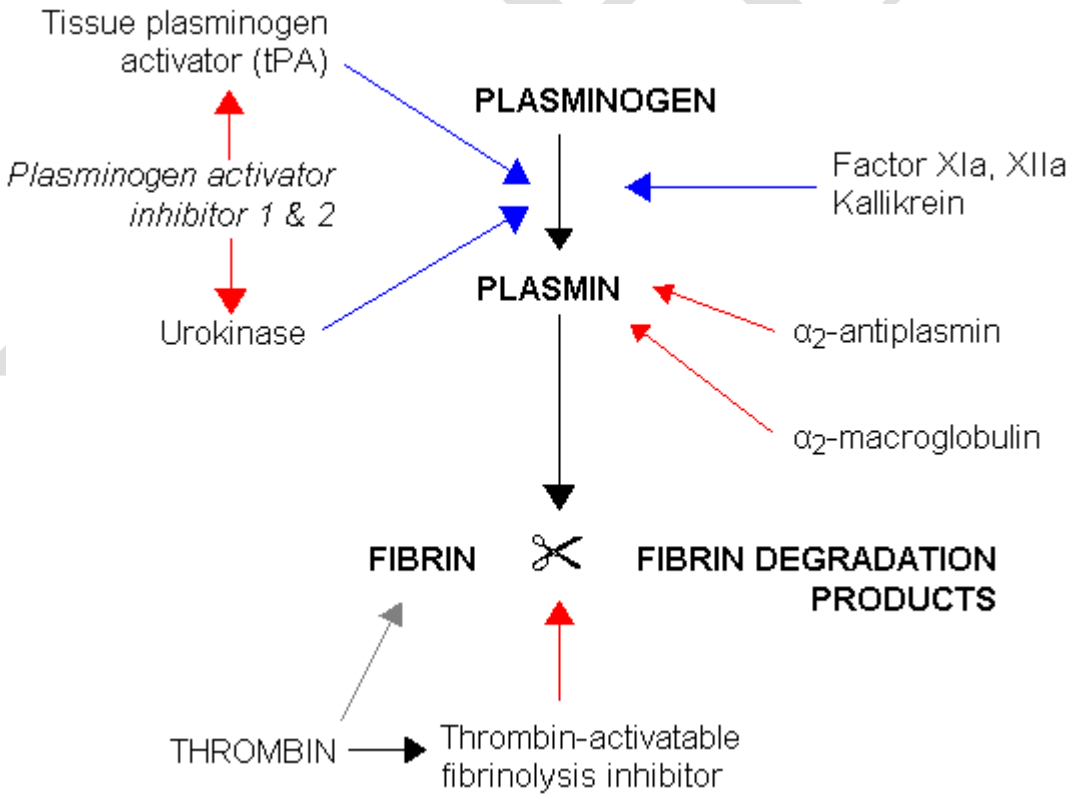


Recombinant tissue plasminogen activators (r-tPA)

tPA was first produced by recombinant DNA techniques at Genentech in 1982. Tissue-type plasminogen activators were initially identified and isolated from mammalian tissues after which a cDNA library was established with the use of reverse transcriptase and mRNA from human melanoma cells. The aforementioned mRNA was isolated using antibody based

immunoprecipitation. The resulting cDNA library was subsequently screened via sequence analysis and compared to a whole genome library for confirmation of specific protein isolation and accuracy. cDNA was cloned into a synthetic plasmid and initially expressed in E. coli cells, followed by yeast cells with successful results confirmed via sequencing before attempting in mammalian cells. The transformants were selected with the use of Methotrexate. Methotrexate strengthens selection by inhibiting DHFR activity which then compels the cells to express more DHFR (exogenous) and consequently more recombinant protein to survive. The highly active transformants were then subsequently placed in a industrial fermenter. The tPA which was then secreted into the culture medium was isolated and collected for therapeutic use. For pharmaceutical purposes, tPA was the first pharmaceutical drug produced synthetically with the use of mammalian cells, specifically Chinese hamster ovarian cells (CHO). Recombinant tPA is commonly referred to as r-tPA and sold under multiple brand names.

Functions



Applications of recombinant proteins

Protein is an important component of all organisms. Each protein molecule consists of one or more polypeptide chains made of amino acids. There are 20 naturally occurring amino acids. Different series of amino acids are linked together by peptide bonds to form protein chains. Each protein has a unique sequence of amino acids. All the functions of proteins are dependent on their structures. Although information necessary for life is encoded by DNA or RNA, proteins perform a wide range of biological functions within organisms, including enzyme catalysis, defense, transport, support, motion, and regulation. According to their functions in the body, proteins can be divided into different categories, such as antibody, enzyme, messager, structural component, and transport/ storage protein. Given the important functions of proteins, proteins have been studied intensively and applied widely.

In the past, the major way to obtain a specific protein was to isolate it from a natural source, which is generally inefficient and time-consuming. Recent advances in recombinant molecule biological techniques have made it possible to clone the DNA encoding a specific protein into an expression vector and express the protein in expression systems, such as bacteria, yeast, insect cells, and mammalian cells. Simply put, recombinant proteins are translated products of exogenous DNA in living cells. The production of recombinant proteins generally contains two major steps: molecule cloning and protein expression. Currently, recombinant protein production is one of the most powerful techniques used in life sciences. Recombinant proteins have wide applications in medicine, research, and biotechnology.

1. Medicine

Therapeutic proteins provide important therapies for a variety of diseases, such as diabetes, cancer, infectious diseases, hemophilia, and anemia. Common therapeutic proteins include

antibodies, FC fusion proteins, hormones, interleukins, enzymes, and anticoagulants. There is a growing demand for recombinant proteins for therapeutic applications. Human proteins obtained through genetic engineering play a key role in therapeutic medicines market. Currently, most of all recombinant therapeutic proteins are produced in mammalian cells because mammalian cells are capable of producing high-quality proteins similar to the naturally occurring ones. In addition, many approved recombinant therapeutic proteins are generated in Escherichia coli due to its well-characterized genetics, rapid growth, and high-yield production.

Basically, therapeutic proteins can be classified into four groups.

Group I: Therapeutic proteins with enzymatic or regulatory activity. These proteins replace a protein that is deficient or abnormal, up-regulate an existing pathway, or provide a new function or activity.

Group II: Therapeutic proteins with special targeting activity. These proteins interfere with a molecule or organism or deliver other molecules.

Group III: Therapeutic proteins as vaccines. These proteins help protect against foreign agents, autoimmune diseases, and cancer.

Group IV: Therapeutic proteins as diagnostics. These proteins are generally purified and recombinant proteins.

2. Research

Recombinant proteins help to elucidate the basic and fundamental principles of an organism. These molecules can be used to identify and locate the position of the protein encoded by a specific gene, and to uncover the function of other genes in various cellular activities such as cell signaling, metabolism, growth, replication and death, transcription, translation, and

protein modification. Thus, recombinant proteins are frequently used in molecular biology, cell biology, biochemistry, structural and biophysical studies, and many other research fields.

Recombinant proteins are useful tools in understanding protein-protein interactions. They have proven performance in several laboratory techniques, such as ELISA, Western Blot, and immunohistochemistry (IHC). Recombinant proteins can be used to develop enzymatic assays. When used in conjunction with a matched antibody pair, recombinant proteins can be used as standards such as ELISA standards. Moreover, recombinant proteins can be used as positive controls in Western blots.

3. Biotechnology

Recombinant proteins are also used in industry, food production, agriculture, and bioengineering. For example, in breeding industry, enzymes can be added to animal feed to increase the nutritional value of feed ingredients, reduce feed and waste management costs, support animal gut health, enhance animal performance and improve the environment. Besides, lactic acid bacteria (LAB) have been used for a long time for the production of fermented foods, and recently, LAB has been engineered for the expression of recombinant proteins, which have wide applications such as improving human/animal digestion and nutrition.

However, recombinant proteins also have limitations.

1. In some cases, the production of recombinant proteins is complex, expensive, and time-consuming.

2. The recombinant proteins produced in cells may not be the same as the natural forms. This difference may reduce the effectiveness of therapeutic recombinant proteins and even cause side effects. Additionally, this difference may affect the results of experiments.

3. A major concern for all recombinant drugs is immunogenicity. All biotechnologically produced therapeutics may exhibit some form of immunogenicity. It is difficult to predict the safety of novel therapeutic proteins.

Overall, advancements in the field of biotechnology have increased and facilitated the production of recombinant proteins for various applications. Although recombinant proteins still have some drawbacks, they are important in medicine, research, and biotechnology.

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POSSIBLE QUESTIONS

1. Explain the conversion of mRNA transcripts into cDNA.
2. Enumerate characterization of recombinant proteins.
3. Write about functional sequencing of cDNA.
4. Explain the following,
   i) hGH ii) tpA
5. Describe in detail about the principle and applications of PCR technique.
6. Explain about protein purification and refolding.
7. Describe in detail about Functional sequencing of cDNA expression libraries.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
I-M.Sc., BIOCHEMISTRY
RECOMBINANT DNA TECHNOLOGY (17BCP205A)
MULTIPLE CHOICE QUESTIONS

UNIT III

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	Which organism produces Taq DNA polymerase	Thermus aquaticus	E.coli	Bacillus spp	Pseudomonas spp	Thermus aquaticus
2	Recoginition sequence for Eco R1	AATTCCTTAA	AG/CT	TC/GA	GA/TC	GA/TC
3	What is the product produced by reverse transcriptase	single strand DNA	double strand DNA	single strand RNA	double strand RNA	single strand DNA
4	Restriction enzymes cleave DNA at----------	Specific nucleotide sequence	Interior part of nucleotide sequence	Ends of nucleotide sequence	Both A and B	Specific nucleotide sequence
5	Endonucleases are enzymes that cleaves DNA at	Defined sequence	3' end of nucleotide	Internal position in random manner	Both a and b	Internal position in random manner
6	Term endonucleases was coined by----------	Lederberg and Meselson	Lederberg and tatum	Lederberg and yaun	Smith and Nathans	Lederberg and Meselson
7	Restriction enzyme from Escherichia coli K 12 was first isolated from	Meselson and yaun	Lederberg and Meselson	yaun	Meselson	Meselson and yaun
8	Enzymes were classified by	Smith	Nathans	Both a and b	Boliver	Both a and b
9	Type I restriction enzymes cleave DNA at	The point of recognition	1000 to 5000 nucleotides away	25 base pairs away	30 base pairs away	1000 to 5000 nucleotides away
10	co factor needed for type I restriction enzyme were	ATP and Mg 2+	S- Adenosyl Methionine	Both a and b	DNP+	Both a and b
11	Type I restriction enzyme have	3 subunit	5 subunit	2 subunit	1 subunit	3 subunit
12	Type I restriction enzymes are isolated from the organisms	Escherichia coli B	Escherichia coli K12	Both a and b	Escherichia coli Dam	Both a and b
13	Type II restriction enzymes cleaves DNA at	Defined recognition site	Random sites	25 bases away	1000 basepairs away	Defined recognition site
14	Smith and Nathans received nobel prize during	1973	1978	1980	1981	1978
15	Large quantities of specific DNA can be obtained by	Chromosome walking	Chromosome jumping	Gene duplication	Polymerase chain reaction	Polymerase chain reaction
16	The order of three steps in PCR is	Denaturation, Renaturation and synthesis	Denaturation, Synthesis and renaturation	Renaturation, denaturation and synthesis	Synthesis, denaturation and renaturation	Denaturation, Renaturation and synthesis
17	Denaturation in PCr is achieved by	95°C for 2 min	95°C for 1 min	90°C for 2 min	90°C for 1 min	95°C for 1 min
18	Annealing temperature in PCR is	95°C	72°C	55°C	80°C	55°C
19	The other name for annealing is	Renaturation	Denaturation	Polymerisation	Extension	Renaturation
20	Enzyme used in PCR is	DNApolymerase	RNA polymerase	Taq polymerase	Reverse transcriptase	Taq polymerase
21	Enzyme used in PCR is tolerant to	Salt	Heat	Pressure	Ions	Heat
22	Optimum temperature of Taq polymerase is	95°C	75°C	37°C	60°C	75°C
23	Source of Taq polymerase is	Thermus asiaticus	Thermus aquaticus	Thermus asennensis	Thermus areius	Thermus aquaticus
24	Synthetic single or double strand DNA or RNA molecules used to detect complementary sequence is	Template	Primer	Probe	Antibody	Probe
25	Main enzyme used for cDNA synthesis is	DNApolymerase	RNA polymerase	Taq polymerase	Reverse transcriptase	Reverse transcriptase
26	DNA probe is usually labeled with _____	P32	S16	H1	C14	P32
27	cDNA can be obtained from	DNA	mRNA	tRNA	rRNA	mRNA
28	PCR was first proposed by	Kary Mullis	Hargobind khurrana	James Watson	Barbara mcclintock	Kary Mullis
29	PCR is a technique of	DNA degradation	DNA amplification	DNA sequencing	DNA ligation	DNA amplification
30	The basic requirements of PCR reaction includes	DNA	primers	heat stable DNA polymerase	All of these	All of these
31	The process of binding of primer to the denatured DNA strand is called as	Denaturation	Annealing	Renaturation	None of these	Annealing
32	The technique used to identify specific DNA sequence in bacterial colonies is	Colony hybridization	In situ hybridization	Dot blot	Western blotting	Colony hybridization
33	Asymmetric PCR is to generate	single stranded copies	double stranded copies	Both a and b	None of these	single stranded copies
34	Reverse transcriptase PCR uses	mRNA as template to form cDNA	protein as template to form DNA	DNA as tempelate to form protein	All of these	mRNA as template to form cDNA
35	Reverse transcriptase enzyme is	DNA dependent DNA polymerase	RNA dependent DNA polymerase	RNA dependent RNA polymerase	DNA dependent RNA polymerase	RNA dependent DNA polymerase
36	RNA can be generated from DNA by a process known as	Reverse transcription	Forward reversion	Mutation	Ligation	Reverse transcription
37	Conjugation is biological phenomena observed in the bacterial genetics directly by	Functional genes	Transfer genes and Mobilizing genes	Nick gene	Basis of mobilizing genes	Transfer genes and Mobilizing genes
38	Transfer genes have cluster of	12 different genes	11 different genes	10 different genes	20 different genes	12 different genes
39	Mob function defined by	3 regions of plasmid DNA	5 regions of plasmid DNA	7 regions of plasmid DNA	2 regions of plasmid DNA	2 regions of plasmid DNA
40	The bacteria associated with the discovery of R plasmids	Bacillus	Pseudomonas	Shigella	Salmonella	Shigella
41	Non-conjugative plasmids have	tra+/ mob+ genes	tra-/ mob- genes	tra-/ mob+ genes	Mob+/ mob+ genes	tra-/ mob+ genes
42	Plasmids carrying genes, which is responsible for antibiotic resistance known as	R plasmids	Cancer inducing plasmid	Col E 1 plasmid	F plasmid	R plasmids

43	Plasmid contain autonomous replication (rep) includes	Origin of replication	Selectable marker	Copy number	All the above	All the above
44	The plasmid that maintains low copy number in a cell	Stringent plasmid	Relaxed plasmid	Conjugative plasmids	None of the above	Stringent plasmid
45	In the Sanger method, what causes the termination of chain elongation	incorporation of DNA	dideoxy nucleotide	Denaturation	amplification	dideoxy nucleotide
46	Which of the following is a chemical nucleotide sequencing method	Sanger	Maxam-Gilbert's	Edmans	Automated sequencing	Maxam-Gilbert's
47	Satellite DNA contains	Highly repetitive sequences	Moderately repetitive sequences	Non-repetitive sequences	DNA-RNA hybrids	Highly repetitive sequences
48	Synthesis of RNA and a DNA template is known as	Replication	Translation	Transcription	Mutation	Transcription
49	DNA-dependent RNA polymerase is a	Monomer	Dimer	Trimer	Tetramer	Tetramer
50	The initiation site for transcription is recognized by	α-Subunit of DNA-dependent RNA polymerase	β-Subunit of DNA-dependent RNA polymerase	Sigma factor	Rho factor	Sigma factor
51	A mammalian DNA polymerase among the following is	DNA polymerase α	DNA polymerase I	DNA polymerase II	DNA polymerase IV	DNA polymerase α
52	Mammalian DNA polymerase γ is located in	Nucleus	Nucleolus	Mitochondria	Cytosol	Mitochondria
53	Novobicin inhibits the synthesis of	DNA	mRNA	tRNA	rRNA	DNA
54	Ciprofloxacin inhibits the synthesis of	DNA	mRNA	tRNA	rRNA	DNA
55	Ciprofloxacin inhibits	DNA topisomerase II	DNA polymerase I	DNA polymerase III	DNA gyrase	DNA gyrase
56	Actinomycin D binds to	Double stranded DNA	Single stranded DNA	Single stranded RNA	DNA-RNA hybrid	Double stranded DNA
57	Non-coding sequences in a gene are known as	Cistrons	Nonsense codons	Introns	Exons	Introns
58	The first amino acyl tRNA which initiates translation in eukaryotes is	Mehtionyl tRNA	Formylmethionyl tRNA	Tyrosinyl tRNA	Alanyl tRNA	Mehtionyl tRNA
59	The first amino acyl tRNA which initiates translation in prokaryotes is	Mehtionyl tRNA	Formylmethionyl tRNA	Tyrosinyl tRNA	Alanyl tRNA	Formylmethionyl tRNA
60	The codon which serves as translation start signal is	AUG	UAG	UGA	UAA	AUG

UNIT-IV

SYLLABUS

Mutagenesis: Site-directed mutagenesis, *In vitro* mutagenesis-Linkers, synthetic oligonucleotides and transposons, Role of Tagging in gene analysis, Identification and isolation of genes through T-DNA or transposons.

Gene therapy- Different strategies for gene therapy, therapeutics based on targeted exhibition of gene expression and mutation correction *in vivo*, Gene therapy for inherited diseases, ADA, FH, Cystic fibrosis.

Site-directed mutagenesis

Site-directed mutagenesis is a molecular biologymethod that is used to make specific and intentional changes to the DNA sequence of a gene and any gene products. Also called site-specific mutagenesis or oligonucleotide-directed mutagenesis, it is used for investigating the structure and biological activity of DNA, RNA, and protein molecules, and for protein engineering. Site-directed mutagenesis is one of the most important techniques in laboratory for introducing a mutation into a DNA sequence. There are numerous methods for achieving site-directed mutagenesis, but with decreasing costs of oligonucleotide synthesis, artificial gene synthesis is now occasionally used as an alternative to site-directed mutagenesis. Since 2013, the development of the CRISPR/Cas9 technology, based on a prokaryotic viral defense system, has also allowed for the editing of the genome, and mutagenesis may be performed in vivo with relative ease.

Basic mechanism

The basic procedure requires the synthesis of a short DNA primer. This synthetic primer contains the desired mutation and is complementary to the template DNA around the mutation site so it can hybridize with the DNA in the gene of interest. The mutation may be a single base change (a point mutation), multiple base changes, deletion, or insertion. The single-strand primer is then extended using a DNA polymerase, which copies the rest of the gene. The gene thus copied

contains the mutated site, and is then introduced into a host cell as a vector and cloned. Finally, mutants are selected by DNA sequencing to check that they contain the desired mutation. The original method using single-primer extension was inefficient due to a low yield of mutants.

This resulting mixture contains both the original unmutated template as well as the mutant strand, producing a mixed population of mutant and non-mutant progenies. Furthermore, the template used is methylated while the mutant strand is unmethylated, and the mutants may be counter-selected due to presence of mismatch repairsystem that favors the methylated template DNA, resulting in fewer mutants. Many approaches have since been developed to improve the eficiency of mutagenesis.

Kunkel's method

In 1985, Thomas Kunkel introduced a technique that reduces the need to select for the mutants. The DNA fragment to be mutated is inserted into a phagemid such as M13mp18/19 and is then transformed into an *E. coli* strain deficient in two enzymes, dUTPase (dut) and uracil deglycosidase (ung). Both enzymes are part of a DNA repair pathway that protects the bacterial chromosome from mutations by the spontaneous deamination of dCTP to dUTP. The dUTPase deficiency prevents the breakdown of dUTP, resulting in a high level of dUTP in the cell. The uracil deglycosidase deficiency prevents the removal of uracil from newly synthesized DNA. As the double-mutant E. coli replicates the phage DNA, its enzymatic machinery may, therefore, misincorporate dUTP instead of dTTP, resulting in single-strand DNA that contains some uracils (ssUDNA). The ssUDNA is extracted from the bacteriophage that is released into the medium, and then used as template for mutagenesis. An oligonucleotide containing the desired mutation is used for primer extension. The heteroduplex DNA, that forms, consists of one parental non-mutated strand containing dUTP and a mutated strand containing dTTP. The DNA is then transformed into an *E. coli* strain carrying the wildtype dut and ung genes. Here, the uracilcontaining parental DNA strand is degraded, so that nearly all of the resulting DNA consists of the mutated strand.

Cassette mutagenesis

Unlike other methods, cassette mutagenesis need not involve primer extension using DNA polymerase. In this method, a fragment of DNA is synthesized, and then inserted into a plasmid. It involves the cleavage by a restriction enzyme at a site in the plasmid and subsequent

ligation of a pair of complementary oligonucleotides containing the mutation in the gene of interest to the plasmid. Usually, the restriction enzymes that cut at the plasmid and the oligonucleotide are the same, permitting sticky ends of the plasmid and insert to ligate to one another. This method can generate mutants at close to 100% efficiency, but is limited by the availability of suitable restriction sites flanking the site that is to be mutated.\

PCR site-directed mutagenesis

The limitation of restriction sites in cassette mutagenesis may be overcome using polymerase chain reaction with oligonucleotide "primers", such that a larger fragment may be generated, covering two convenient restriction sites. The exponential amplification in PCR produces a fragment containing the desired mutation in sufficient quantity to be separated from the original, unmutated plasmid by gel electrophoresis, which may then be inserted in the original context using standard recombinant molecular biology techniques. There are many variations of the same technique. The simplest method places the mutation site toward one of the ends of the fragment whereby one of two oligonucleotides used for generating the fragment contains the mutation. This involves a single step of PCR, but still has the inherent problem of requiring a suitable restriction site near the mutation site unless a very long primer is used. Other variations, therefore, employ three or four oligonucleotides, two of which may be non-mutagenic oligonucleotides that cover two convenient restriction sites and generate a fragment that can be digested and ligated into a plasmid, whereas the mutagenic oligonucleotide may be complementary to a location within that fragment well away from any convenient restriction site. These methods require multiple steps of PCR so that the final fragment to be ligated can contain the desired mutation. The design process for generating a fragment with the desired mutation and relevant restriction sites can be cumbersome. Software tools like SDM Assist can simplify the process.

Whole plasmid mutagenesis

Site-directed mutagenesis is used to generate mutations that may produce a rationally designed protein that has improved or special properties (i.e.protein engineering). Investigative tools– specific mutations in DNA allow the function and properties of a DNA sequence or a protein to be investigated in a rational approach.

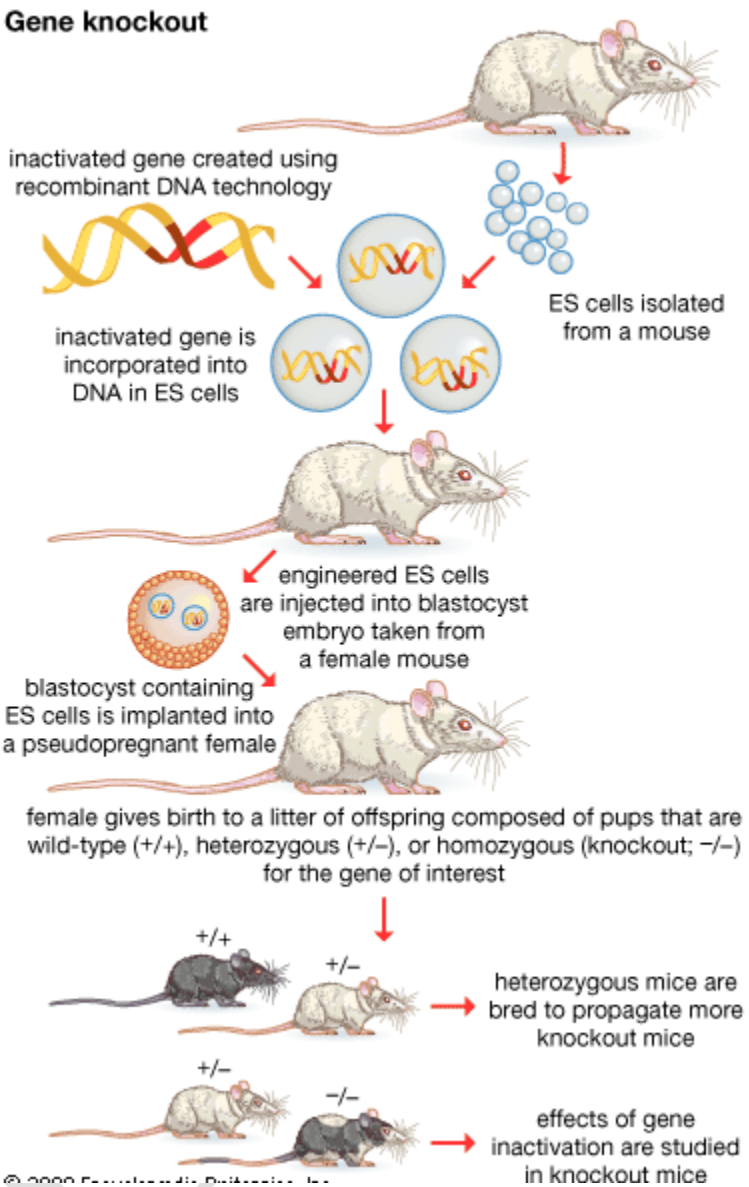
Commercial applications – Proteins may be engineered to produce mutant forms that are tailored for a specific application. For example, commonly used laundry detergents may contain subtilisin, whose wild-type form has a methionine that can be oxidized by bleach, significantly reducing the activity the protein in the process. This methionine may be replaced by alanine or other residues, making it resistant to oxidation thereby keeping the protein active in the presence of bleach.

In Vitro Mutagenesis

Another use of cloned DNA is in vitro mutagenesis in which a mutation is produced in a segment of cloned DNA. The DNA is then inserted into a cell or organism, and the effects of the mutation are studied. Mutations are useful to geneticists in enabling them to investigate the components of any biological process. However, traditional mutational analysis relied on the occurrence of random spontaneous mutations—a hit-or-miss method in which it was impossible to predict the precise type or position of the mutations obtained. In vitro mutagenesis, however, allows specific mutations to be tailored for type and for position within the gene. A cloned gene is treated in the test tube (in vitro) to obtain the specific mutation desired, and then this fragment is reintroduced into the living cell, where it replaces the resident gene.

One method of in vitro mutagenesis is oligonucleotide-directed mutagenesis. A specific point in a sequenced gene is pinpointed for mutation. An oligonucleotide, a short stretch of synthetic DNA of the desired sequence, is made chemically. For example, the oligonucleotide might have adenine in one specific location instead of guanine. This oligonucleotide is hybridized to the complementary strand of the cloned gene; it will hybridize despite the one base pair mismatch. Various enzymes are added to allow the oligonucleotide to prime the synthesis of a complete strand within the vector. When the vector is introduced into a bacterial cell and replicates, the mutated strand will act as a template for a complementary strand that will also be mutant, and thus a fully mutant molecule is obtained. This fully mutant cloned molecule is then reintroduced into the donor organism, and the mutant DNA replaces the resident gene.

Another version of in vitro mutagenesis is gene disruption, or gene knockout. Here, the resident functional gene is replaced by a completely nonfunctional copy. The advantage of this technique over random mutagenesis is that specific genes can be knocked out at will, leaving all other genes untouched by the mutagenic procedure.


Gene knockout
inactivated gene created using recombinant DNA technology
ES cells isolated from a mouse
inactivated gene is incorporated into DNA in ES cells
engineered ES cells are injected into blastocyst embryo taken from a female mouse
blastocyst containing ES cells is implanted into a pseudopregnant female
female gives birth to a litter of offspring composed of pups that are wild-type (+/+), heterozygous (+/–), or homozygous (knockout; –/–) for the gene of interest
+/+
+/–
heterozygous mice are bred to propagate more knockout mice
+/–
–/–
effects of gene inactivation are studied in knockout mice

MUTAGENESIS

SITE-DIRECTED MUTAGENESIS

Any heritable change in the genome is commonly called a **mutation**. Biochemically, it is a chemical change or alteration in a nitrogen base of a DNA sequence resulting in the production of a defective protein or a truncated protein, which is not functional. These altered proteins can cause serious problems in metabolism leading to changes in the morphology and physiology of the organism. Mutations, in most cases, are spontaneous and may not be dangerous. Even though the natural mutations are spontaneous and rare, biologists can induce mutations using different methods, which in most cases are not desirable and precise. But, now molecular biologists can alter any amino acid of a protein by changing the corresponding bases in its gene very precisely and accurately resulting in desirable mutations. It is possible to alter properties such as increased stability, temperature resistance, product inhibition, substrate specificity, etc. of any enzyme.

The accurate induction of one or more point mutations on selected regions of a gene resulting in amino acid substitutions or deletion or addition is known as **site directed mutagenesis**. It can be defined as the controlled alteration of selected regions of a DNA molecule.

The **principle of site-directed mutagenesis** is that a mismatched oligonucleotide primer is extended, incorporating the 'mutation' into a strand of DNA that can be cloned. This technique of creating desired molecular mutations in a gene has contributed greatly to the basic understanding of functions of genes, DNA-protein interactions, gene regulations, the role of amino acids in the structure and functions of proteins, role of active centres in the enzyme-substrate interactions, etc. A single base change in a gene permits the evaluation of the role of specific amino acids in the function and structure of a protein. This technique also allows one to create or destroy a restriction site at specific locations within a DNA sequence or gene.

Site-directed mutagenesis is actually one of the applications of PCR. The gene, which has to be mutated, should be made into a single-stranded DNA by cloning into a M13 plasmid vector. By following modern PCR methods, it is possible to carry out the site directed mutation without the participation of M13 vector. The designing and chemical synthesis of the primer is the key factor in this technique. The part of the DNA where the mutation has to be introduced should be synthesized as an oligonucleotide primer, which is complementary to the respective region of the

DNA except for the nucleotide that has to be changed. In short, the mutation is introduced to the gene in the form of a primer and the primer is extended with a polymerase reaction.

The site-directed mutagenesis is a multistep process that begins with the cloning of the gene in a bacteriophage like M13 to generate single stranded DNA. M13 is a filamentous bacteriophage that specifically infects e. coli that expresses sex pili encoded by a plasmid F factor. M13 bacteriophage contains DNA in a single-stranded or replicative form, which is replicated to double-stranded DNA within a bacterial cell. The primer is designed and synthesized, which is an oligonucleotide complementary to the region of the DNA to be mutated except for the nucleotide to be changed. This oligonucleotide with the mismatched base or bases hybridize to the single-stranded DNA and serve as the primer to start synthesizing the complementary DNA strand with the help of a suitable DNA polymerase such as T4 DNA polymerase or Taq polymerase. The resulting doublestranded DNA will be a hybrid of the wild type parent strand and the mutated newly synthesized DNA strand. This DNA molecule can be transformed into an E.coli cell, where the mutated DNA strand serves as a template to replicate new strands carrying the mutation along with the wild strands. The bacteriophage plaques containing the mutated DNA can be screened by hybridizing with the labeled probe of the original mutated oligonucleotide. By adjusting the hybridization time and wash temperature of the hybridized probe, only the perfectly matched hybrid will remain and all other mismatched hybrids will dissociate. The presence of the desired mutation in the gene can be checked and confirmed afterisolating the plasmid DNA from the single positive plaques and sequencing it.

A single base can be mutated in recombinant DNA plasmids with a process called **inverse PCR**. Two primers are synthesized with their antiparallel 5' ends complementary to the adjacent bases on the two strands of DNA. One of the two primers carries a specific mismatched base that is faithfully copied during the PCR amplification resulting in a recombinant plasmid with a single mutated base.

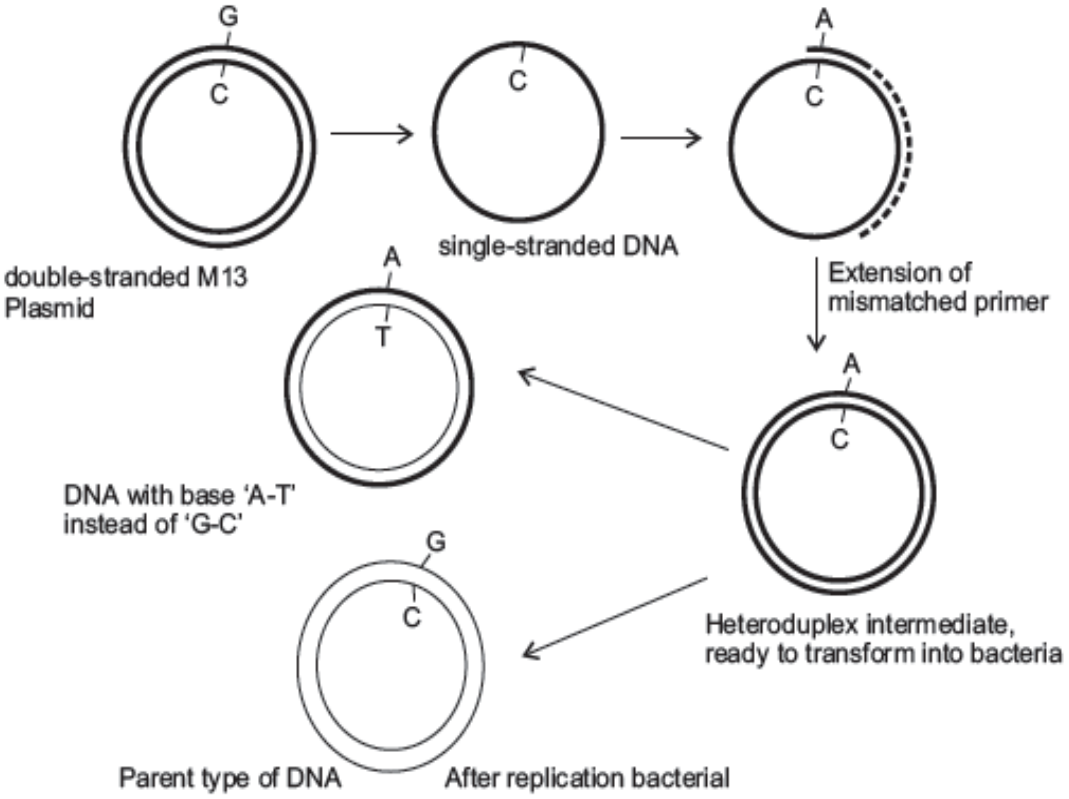


FIGURE: Site-directed mutagenesis of a gene mediated through M13 plasmid.

IN VITRO MUTAGENESIS

Linkers

The first of these methods involves the use of linkers. These are short pieces of double stranded DNA, of known nucleotide sequence, that are synthesized in the test tube. A typical linker is shown in Figure a. It is blunt-ended, but contains a restriction site, BamHI in the example shown. DNA ligase can attach linkers to the ends of larger blunt ended DNA molecules. Although a blunt end ligation, this particular reaction can be performed very efficiently because synthetic oligonucleotides, such as linkers, can be made in very large amounts and added into the ligation mixture at a high concentration. More than one linker will attach to each end of the DNA molecule, producing thechain structure shown in Figure. However, digestion with BamHI cleaves the chains at the recognition sequences, producing a large number of cleaved linkers and the original DNA fragment, now carrying BamHI sticky ends. This modified fragment is ready for ligation into a cloning vector restricted with BamHI.

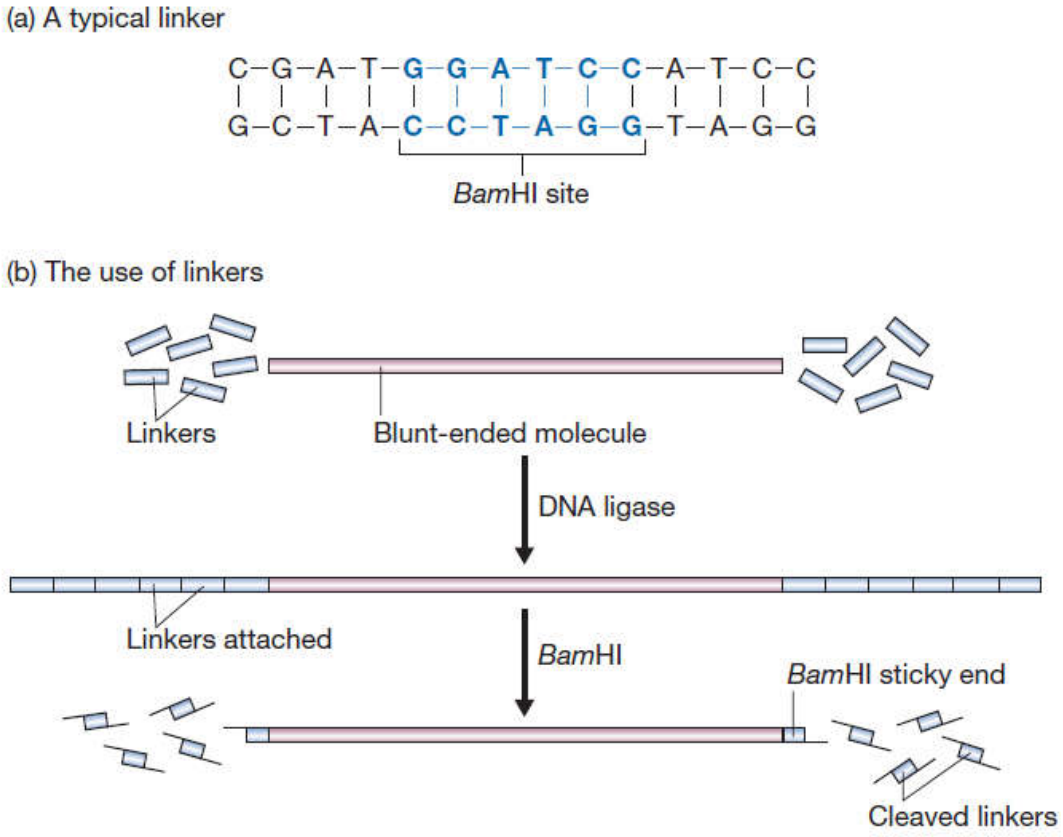


Fig: Linkers and their use: (a) the structure of a typical linker; (b) the attachment of linkers to a blunt-ended molecule

Synthesized Oligonucleotide

Chemically synthesized oligonucleotides (<100-mers) have a myriad of functions. Single-stranded hybridization oligonucleotide probes (20- to 40-mers) can be formulated by deducing the codons from the amino acid sequence of a protein and then used to screen a genomic library for the gene (Fig). Since the actual codons representing a conserved amino acid sequence are unknown because of codon redundancy, especially at the third position, a single arbitrary synthetic probe may not contain sufficient complementary bases (matches) to produce significant hybridization witha heterologous sequence. For this reason, a set of mixed probes is often used to screen a genomic library. The formation of a sample of mixed probes is straightforward. Briefly, during chemical DNA synthesis, instead of providing a specific phosphoramidite for a particular nucleotide site, a mixture of different bases is added to the reaction. For example, with the addition of equal concentrations of four different bases for one nucleotide position, four different

probes are produced. If two sites are treated this way, 16 (42) different probes will be synthesized (Fig. 4.8B); for n sites, there are 4n different oligonucleotides. Moreover, the frequencies of various probes in the mixture can be skewed by varying the proportions of bases in the reaction mixture for specific sites. As a consequence, in contrast to a single probe, a set of mixed probes is likely to contain sequences that are highly complementary to a heterologous gene. As discussed below, single-stranded oligonucleotides (~17- to 24-mers, or primers) are also required for DNA sequencing and PCR. In some cases, additional sequences are added to the PCR primers as part of the synthesis process to create molecules with restriction endonuclease sites for cloning or sequences that contain regulatory elements for transcription and translation of the amplified DNA after it is inserted into a vector.

Transposons

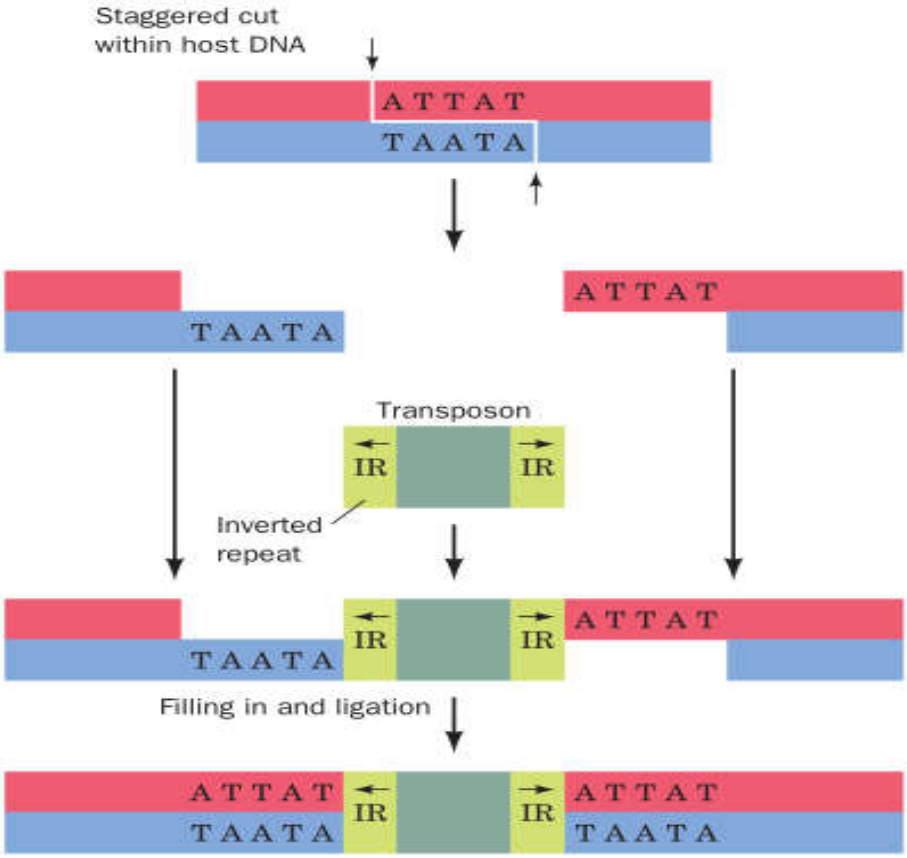
In the early 1950s, Barbara McClintock reported that the variegated pigmentation pattern of maize (Indian corn) kernels results from the actionof genetic elements that can move within the maize genome.This proposal was resoundingly ignored because it was contrary to the then-held orthodoxy that chromosomes consist ofgenes linked in fixed order.Another 20 years were to pass before evidence of mobile genetic elements was found in anotherorganism, E. coli.

It is now knownthat transposable elements, or transposons, are common in both prokaryotes and eukaryotes, where they influence the variation of phenotypic expression over the short term and evolutionary development over the longterm. Each transposon codes for the enzymes that insert it into the recipient DNA. This process differs from homologous recombination in that itrequires no homology between donor and recipient DNA and occurs at arate of only one event in every 104to 107cell divisions.

Prokaryotic transposons with three levels of complexity have been characterized:

1. The simplest transposons are named insertion sequences or IS elements. They are normal constituents of bacterial chromosomes and plasmids (autonomously replicating circular DNA molecules that usually consist of several thousand base pairs). For example, a common E. coli strain has eight copies of IS1 and five copies of IS2. IS elements generally consist of <2000 bp, comprising a so called transposase gene and, in some cases, a regulatory gene, flanked by short inverted (having opposite orientation) terminal repeats. On each side of an

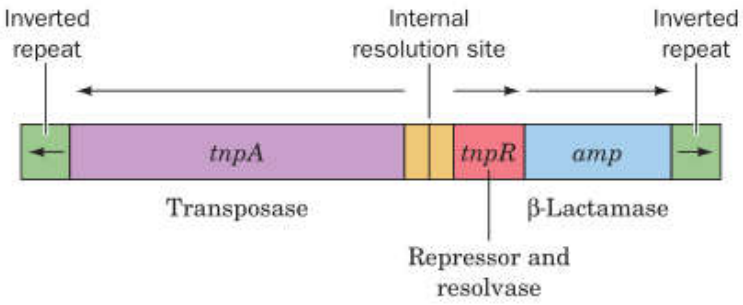
inserted IS element is a directly (having the same orientation) repeated segment of host DNA (Fig). This suggests that an IS element is inserted in the host DNA at a staggered cut that is later filled in (Fig).The length of the target sequence (most commonly 5–9 bp), but not its sequence, is characteristic of the IS element.



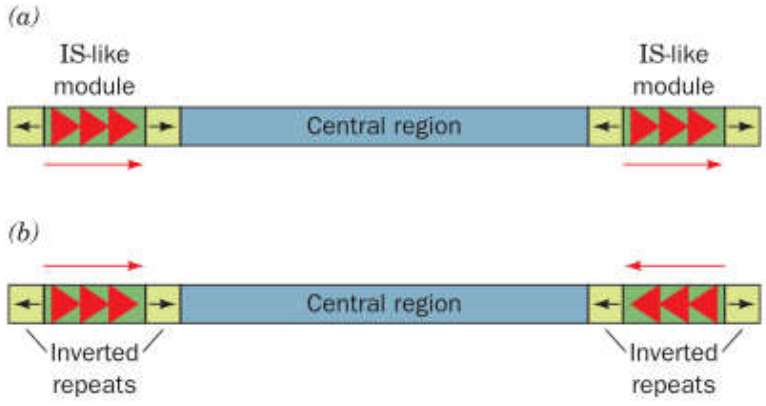
A model for transposon insertion. A staggered cut that is later filled ingenerates direct repeats of the target sequence.

2. More complex transposons carry genes not involved in the transposition process, such as antibiotic resistance genes. For example, Tn3 consists of 4957 bp and has inverted terminal repeats of 38 bp each. The central region of Tn3 codes for three proteins: (1) a 1015-residue transposase named TnpA; (2) a 185-residue protein known as TnpR, which represses the expression of both tnpA and tnpR and mediates the site-specific recombination reaction necessary for transposition and (3) a β-lactamase (encoded by amp) that inactivates the

antibiotic ampicillin. The site-specific recombination occurs in an AT-rich region, the internal resolution site, between tnpA and tnpR.



3. The so-called composite transposons consist of a gene- containing central region flanked by two identical or nearly identical IS-like modules that have either the same or an inverted relative orientation. Composite transposons apparently arose by the association of two originally independent IS elements. Experiments demonstrate that composite transposons can transpose any sequence of DNA in their central region.



A composite transposon.This element consists of two identical or nearlyidentical IS-like modules (green) flanking a centralregion carrying various genes. The IS-like modulesmay have either (a) direct or (b) inverted relative orientations

Role of Tagging in Gene Analysis

Mutation in genes oten produces alterations in phenotype. To identify a gene that has been mutated and where the phenotype has been changed consequently, it would be necessary to identify a gene on the basis that it carrie a mutation. This is the main idea behind gene tagging. A

number of molecular tags have been determined by many genes in most of the plants. These molecular tags include cloned restriction fragment length polymorphism (RFLP) probes, oligonucleotides RFLP probes, variable number of tandem repeats (VNTR), microsatellite, minisatellite, DNA fingerprint loci. These markers must satisfy the given criteria to be used as tags. Gene tagging and marker assisted selection is an essentail component of molecular breeding and is based on the saturation mapping of the genome. This has opened up the possibilities of identifying, mapping tagging and even isolating or transferring quantitative trait loci(QTL). Thus the most powerful application of DNA markers in plant breeding might help in cloning genes. Earlier, cloning such genes was difficult. With the invention of DNA markers and transposon tagging, important genes have now become accessible to molecular cloning. DNA markers provide the essential starting point for physical isolation of genomic regions containing the gene of interest. The efforts that are involved in tagging a gene can be used further as a part of marker assisted selection programme as economically important genes are tagged, they can even be transferred to unrelated species.

IDENTIFICATION AND ISOLATION OF GENES THROUGH T-DNA OR TRANSPOSONS

Introduction

This Chapter gives a guide to the use of Agrobacterium T-DNA as an insertion mutagen in the molecular genetic analysis of plant gene functions. T-DNA represents a segment of Ti and Ri plasmids that is transferred from Agrobacterium into the nuclei of infected plant cells where it is randomly integrated into potentially transcribed domains of the chromosomes by non-homologous (i.e. illegitimate) recombination. Insertion of T-DNA into plant genes causes gene mutations. Hence, T-DNA is an efficient mutagen. T-DNA tagging, which results in the labelling of gene mutations with known sequences and dominant selectable markers, provides a simple means for genetic llnkagemappingfYnchna1 analysis and nlolecular characterization of plant gene mutations.

Promoterless reporter genes. with or without a translation initiation codon, are inserted into plant genes with the aid of the T-DNA. Thus, gene mutations are identified by selection or screening for the expression of transcriptional or translational reporter gene fusions controlled by T-DNA tagged plant genes. Reporter genes bearing only the TATA-region of so-called mininlal

pronlotcrv are used as enhancer traps to identifjl cis-acting regulato~y sequences with the help of T-DNA tags in the plant genome.

Mutagenesis with T-DNAs, carrying multiple copies of enhancers derived, for example. from the well-known promoter of the Cauliflower Mosaic Virus (CaMV) 35s RNA, are employed to induce cis-activation of plant genes located in the vicinity of T-DNA tags (7). This activation T-DNA tagging technology is used for the isolation of dominant mutations that can also be identified in tetraploid and allotetraploid plants. Promoters orientated towards the termini of T-DNA tags provide a means for induction of either constitutive or regulated transcription of neighbouring plant DNA sequences. This may either activate or inactivate genes by transcriptional read-through or interference, as well as lead to alterations in chromatin stiucture implying long-range *cis*-effects.

Applications, aiming at either a removal of certain T-DNA tags from the genome or isolation of a particular type of gene nlutation apply conditional'suicide' genes for negative selection. A combination of these methods facilitates the isolation of regulatory mutations that affect the activity of single genes, including second site suppressors of known mutations. T-DNA insertions carrying target sites and genes for site-specific recombination also allow chromo-some engineering by generation of defined deletions, additions, inversions and translocations. Finally, T-DNA serves as a common vehicle to deliver autonomous or defective, but mobilizable, transposons and retrotransposons into diverse plant species.

Analysis of T-DNA mutants and cloning a tagged gene

The strategy is based on the assumption that the mutation is recessive, gives a clear phenotype, the homozygous mutant is fertile, and the T-DNA carries a dominant selection marker, such as antibiotic and/or herbicide resistance.

A problem encountered when screening a population of T-DNA transform- ants may be the occurrence of untagged mutations. I t is therefore essential to rigorously confirm genetic linkage between the mutation and the T-DNA insert before attempting the isolation of the tagged locus. Because the T-DNA carries a dominant marker, rather large F2/M2 and F3/& p ~ p ~ l a t i o n ~ have to be used to attempt the separation of untagged mutations from potentially closely linked T-DNA inserts. Finding no mutant without T-DNA among 1000 mutants means linkage within about 3.2 cM. Protocol 4 describes the screening of mutants for presence of T-DNA. When

working with a single T-DNA insert, alternatively the progeny of a wild-type plant (i.e. an M, family) carrying the T-DNA can be screened for mutants after selfing (i.e. this wild-type M2 family is expected to be hemizygous for the T-DNA tagged locus). If within the T-DNA transformed progeny a wild-type family is found, not segregating mutant phenotype, the mutation is not T-DNA tagged. When a different ecotype than the one used for transformation, is crossed with the mutant for making a segregating F2 (provided the mutant phenotype is expressed in a different genetic background), ARMS (70) or CAPS (71) markers (see Chapter 3) can provide help to map the mutant to a chromo- some arm.

Isolation of plant DNA fragments flanking the T-DNA by plasmid rescue (as described in Protocol 5) is only possible when the T-DNA contains an E. coli plasmid replication origin (ori). Otherwise the T-DNA insert junctions can be isolated by IPCR (see Protocols 2 and 6) using T-DNA-specific primers (69,72). When the complementation of the mutant by transformation is complicated, for example when fertility is affected, different EMS or radiation- induced alleles can be sequenced and compared with the T-DNA locus

GENE THERAPY

Introduction

There are many diseases which can be cured by using specific medicine synthesized biochemically. Now-a-days techniques have been developed to produce recombinant therapeutic biochemicals, for example, insulin, interferon, somatotropin, somatostatin, endorphin, human blood clotting factor VIII:C, immunogenic proteins, etc. Several companies viz., Eberstadt & Co. (New York), E. Lily (USA), National Pituitary Agency (USA), Kabi Vitrum AB (Sweden), Genetech Co (USA), Biogen (Switzerland), Hybritech (USA), Astra Research Center (India), etc. are producing or trying to produce on mass scale to make available at low cost.

However, after 1975, a remarkable advancement in recombinant DNA technology has occurred and accumulated such knowledge that has made possible to transfer genes for treatment of human diseases. Several protocols have been developed for the introduction and expression of genes in humans, but the clinical efficiency has to be demonstrated conclusively. Success of gene therapy depends on the development of better gene transfer vectors for sustained, long term expression of foreign gene as well as a better understanding of gene physiology of human diseases (Rangarajan and Padmanaban, 1996).

Genes are the ultimate molecular switches that control various cellular process. The abnormal gene expression can manifest in the form of specific genetic disorders. Until the last decade, delivering genes into humans to correct diseases has been accepted as scientifically viable and recognized as an independent discipline and christened 'gene therapy'.

The ultimate goal of gene therapy is the gene replacement therapy. Gene replacement therapy permits physiological regulation of the transgenes and elimination of the possibility of insertional activation of other cellular genes which occur at the time of random integration of the foreign gene. At present the current strategy for gene therapy largely centers around gene augmentation therapy, where the foreign gene replaces the detective or missing gene.

Overall, there are two gene transfer strategies: (i) the in vivo approach which involves introduction of genes directly into the target organs of an individual (it is done in patients therefore, also called patient therapy), (ii) ex vivo approach where cells are isolated for gene transfer in vitro followed by transplantation of genetically modified cells back into the patients (Verma. 1990).

Types of gene therapy

All the gene therapies that can be done in humans can be classified into the following four types :

(i) Somatic gene therapy. The genetic defects are corrected in somatic cells of the body. It was initially formulated for the treatment of monogenetic defects, but now holds promises for a wide range of disorders such as cancer, neurological disorders, heart diseases and infectious diseases (Table). Sufficient expertise in performing successful gene transfer in somatic cells is required before carrying out gene manipulation in humans (Anderson, 1992).

(ii) Germ-line gene therapy. The functional genes are introduced into the germ cells for correction of genetic defects in the offspring. This therapy is being carried out in laboratory and farm animals. However, it has not been attempted in humans due to technical and ethical problems. One of its types is the embryo therapy where embryos are diagnosed for genetic defects. If any such disease is present the patients are advised for embryo therapy or abortion. In young embryo a functional gene is transferred through microinjection technique (Mandal, 1988).

(iii) Enhancement genetic engineering. This type of gene transfer is done for the improvement of a specific trait in animals; for example introduction of growth hormone gene to increase height. It is being carried out in laboratory and farm animals.

(iv) Eugenic genetic engineering. Novel genes can be introduced in humans to alter or improve complex traits such as intelligence and personality. This type of therapy is not being attempted in humans because it is far beyond our technical capabilities, and ethical problems.

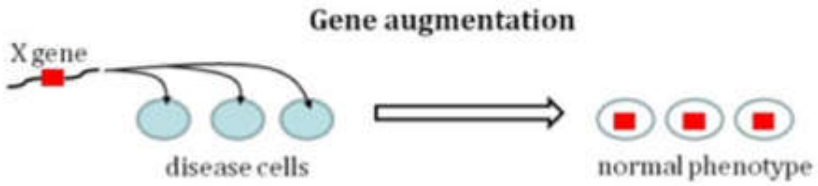
DIFFERENT STRATEGIES FOR GENE THERAPY

The term gene therapy is a broad one: it encompasses many different strategies, all of which are designed to overcome or alleviate disease by a procedure in which genes, gene segments or oligonucleotides are introduced into the cells of an affected individual.

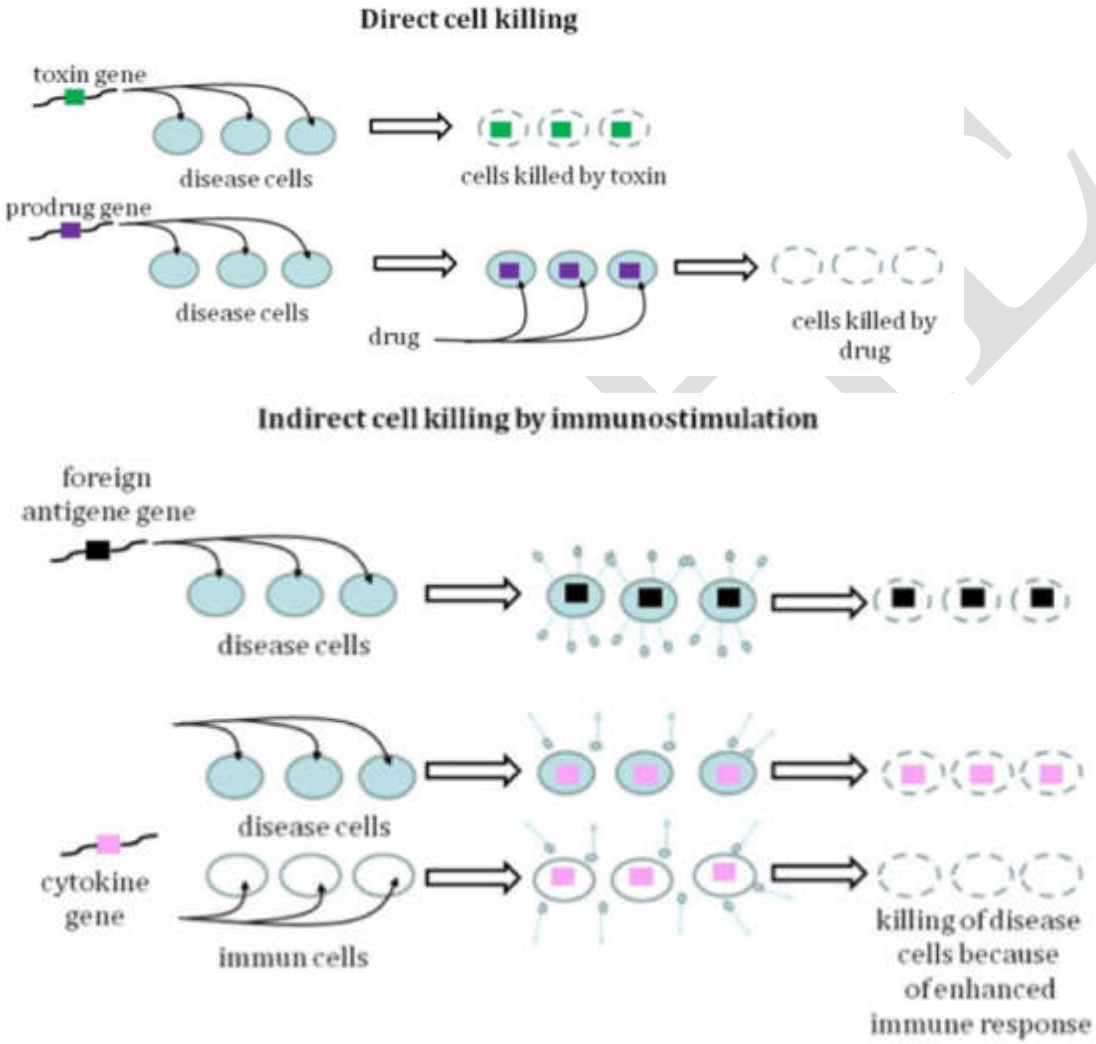
The genetic material may be transferred directly into cells within a patient (in vivo gene the-rapy), or cells may be removed from the patient and the genetic material inserted into them in vitro, prior to replacing the cells in the patient (ex vivo gene therapy). Because the molecular basis of diseases can vary widely, some gene therapy strategies are particularly suited to certain types of disorder, and some to others.

General gene therapy strategies

Gene augmentation therapy (GAT). For diseases caused by loss of function of a gene, introducing extra copies of the normal gene may increase the amount of normal gene product to a level where the normal phenotype is restored. As a result GAT is targeted at clinical disorders where the pathogenesis is reversible. It also helps to have no precise requirement for expression levels of the introduced gene and a clinical response at low expression levels. GAT has been particularly applied to autosomal recessive disorders where even modest expression levels of an introduced gene may make a substantial difference. Dominantly inherited disorders are much less amenable to treatment: gain-of-function mutations are not treatable by this approach and, even if there is a loss-of-function mutation, high expression efficiency of the introduced gene is required: individuals with 50% of normal gene product are normally affected, and so the challenge is to increase the amount of gene product towards normal levels.

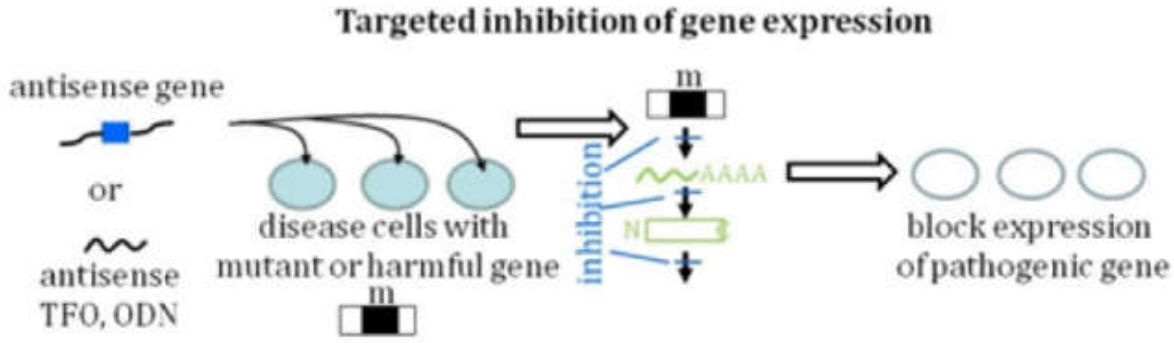


Targeted killing of specific cells. This general approach is popular in cancer gene therapies. Genes are directed to the target cells and then expressed so as to cause cell killing. Direct cell killing is possible if the inserted genes are expressed to produce a lethal toxin (suicide genes), or a gene encoding a prodrug is inserted, conferring susceptibility to killing by a subsequently administered drug. Alternatively, selectively lytic viruses can be used. Indirect cell killing uses immune-stimulatory genes to provoke or enhance an immune response against the target cell.

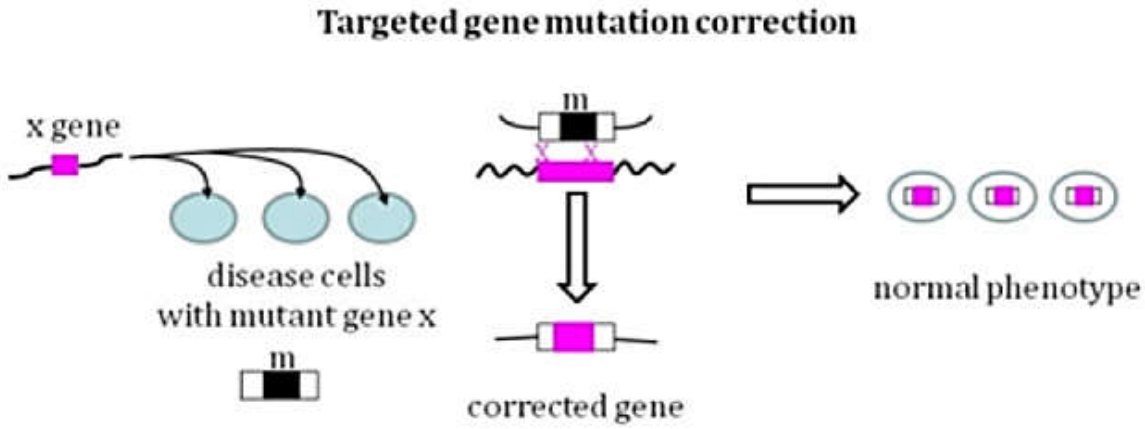


Targeted inhibition of gene expression. If disease cells display a novel gene product or inappropriate expression of a gene (as in the case of many cancers, infectious diseases, etc.), a variety of different systems can be used specifically to block the expression of a single gene at the DNA, RNA or protein levels. Allele-specific inhibition of expression may be possible in some cases, permitting therapies for some disorders resulting from dominant negative effects.

(The example shows correction of a mutation in a mutant gene by homologous recombination, but mutation correction may also be possible at the RNA level. ODN, oligodeoxynucleotide; TFO, triplex-forming oligonucleotide.)



Targeted mutation correction. If an inherited mutation produces a dominant-negative effect, gene augmentation is unlikely to help. Instead the resident mutation must be corrected. Because of practical difficulties, this approach has yet to be applied but, in principle, it can be done at different levels: at the gene level (e.g. by gene targeting methods based on homologous recombination); or at the RNA transcript level (e.g. by using particular types of therapeutic ribozymes or therapeutic RNA editing).



THERAPEUTICS BASED ON TARGETED EXHIBITION OF GENE EXPRESSION AND MUTATION CORRECTION IN VIVO

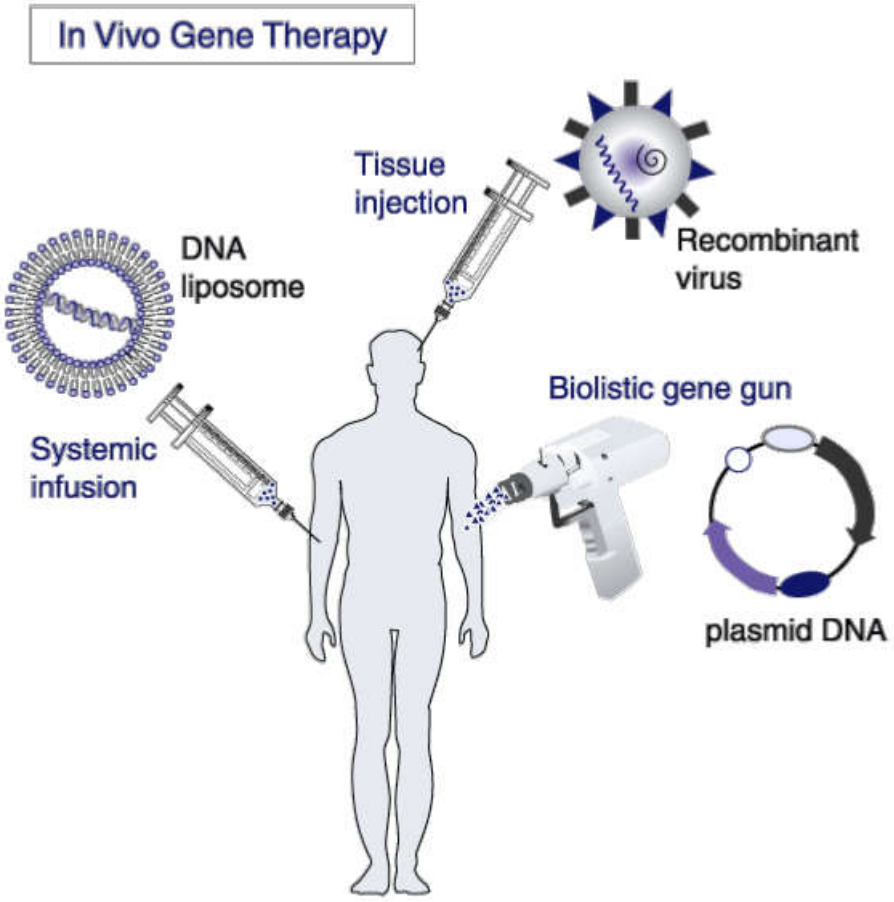
The direct delivery of the therapeutic gene (DNA) into the target cells of a particular tissue of a patient constitutes in vivo gene therapy. Many tissues are the potential candidates for this

approach. These include liver, muscle, skin, spleen, lung, brain and blood cells. Gene delivery can be carried out by viral or non- viral vector systems. The success of in vivo gene therapy mostly depends on the following parameters.

i. The efficiency of the uptake of the remedial (therapeutic) gene by the target cells.

ii. Intracellular degradation of the gene and its uptake by nucleus.

iii. The expression capability of the gene.



RECOMBINANT VIRUS

Retrovirus vector system:

Replication defective retrovirus vectors that are harmless are being used. A plasmid in association with a retrovirus, a therapeutic gene and a promoter is referred to as plasmovirus. The plasmovirus is capable of carrying a DNA (therapeutic gene) of size less than 3.4 kb. Replication defective virus particles can be produced from the plasmovirus.

As such, for the delivery of genes by retroviral vectors, the target cells must be in a dividing stage. But majority of the body cells are quiescent. In recent years, viral vectors have been engineered to infect non-dividing cells. Further, attempts are on to include a DNA in the retroviral vectors (by engineering env gene) that encodes for cell receptor protein. If this is successfully achieved, the retroviral vector will specifically infect the target tissues.

Adenoviral vector system:

Adenoviruses (with a DNA genome) are considered to be good vectors for gene delivery because they can infect most of the non-dividing human cells. A common cold adenovirus is a frequently used vector. As the target cells are infected with a recombinant adenovirus, the therapeutic gene (DNA) enters the nucleus and expresses itself.

However, this DNA does not integrate into the host genome. Consequently, adenoviral based gene therapy required periodic administration of recombinant viruses. The efficiency of gene delivery by adenoviruses can be enhanced by developing a virus that can specifically infect target cells. This is possible by incorporating a DNA encoding a cell receptor protein.

Adeno-associated virus vector system:

Adeno-associated virus is a human virus that can integrate into chromosome 19. It is a single-stranded, non-pathogenic small DNA virus (4.7 kb). As the adeno-associated virus enters the host cell, the DNA becomes double- stranded, gets integrated into chromosome and expresses.

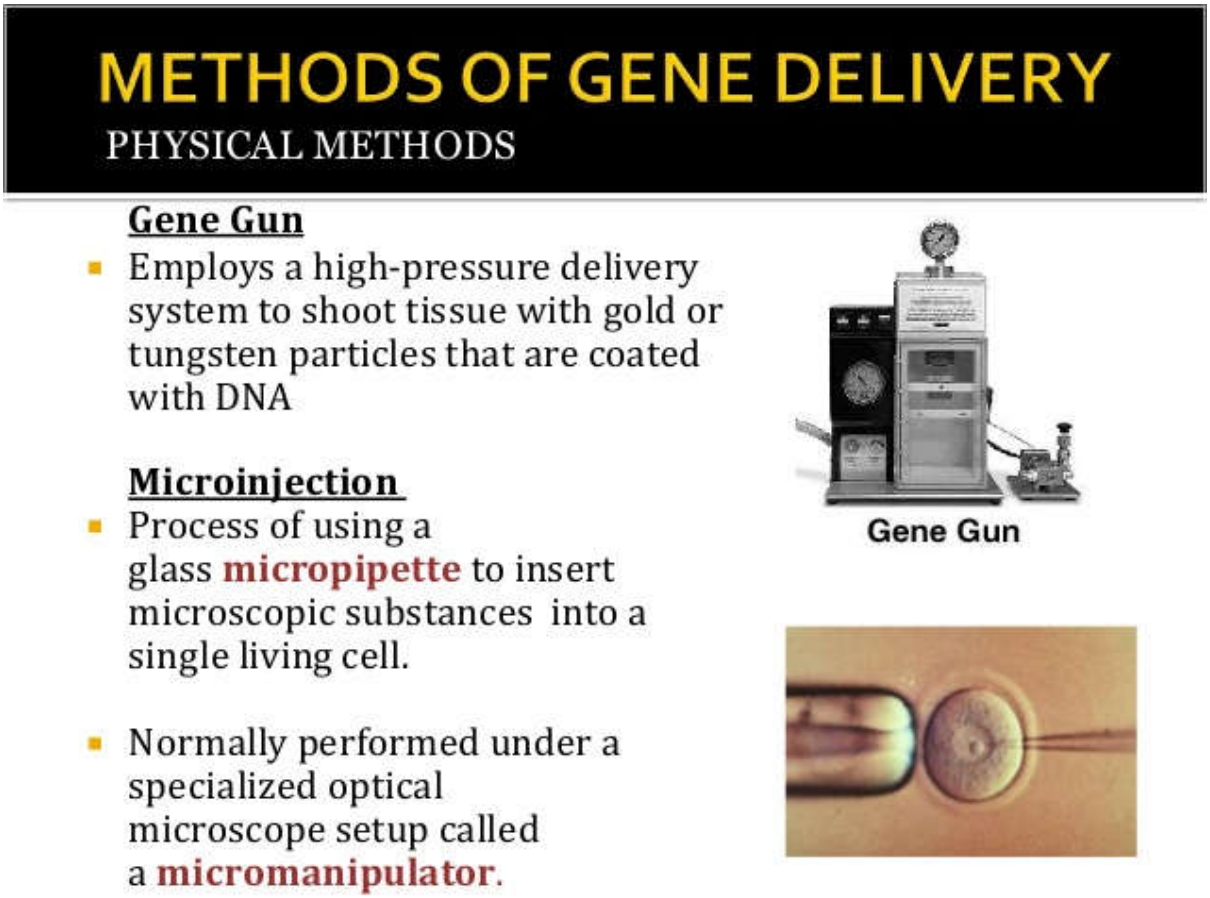
Adeno-associated viruses can serve as good vectors for the delivery of therapeutic genes. Recombinant viruses are created by using two plasmids and an adenovirus (i.e., helper virus) by a special technique. Some attempts were made to use therapeutic genes for the treatment of the human diseases-hemophilia (for production of blood clotting factor IX) and cystic fibrosis (for synthesis of cystic fibrosis trans membrane regulator protein) by employing adeno-associated viruses.

Lipoplexes:

The lipid-DNA complexes are referred to as lipoplexes or more commonly liposomes. They have a DNA construct surrounded by artificial lipid layers. A large number of lipoplexes have been prepared and used. They are non-toxic and non-immunogenic.

The major limitation with the use of lipoplexes is that as the DNA is taken up by the cells, most of it gets degraded by the lysosomes. Thus, the efficiency of gene delivery by lipoplex is very

low. Some clinical trials using liposome-CFTR gene complex showed that the gene expression was very short-lived.



GENE THERAPY FOR INHERITED DISEASES

Therapy for Adenosine Deaminase Deficiency:

The first and the most publicized human gene therapy was carried out to correct the deficiency of the enzyme adenosine deaminase (ADA). This was done on September 14, 1990 by a team of workers led by Blaese and Anderson at the National Institute of Health, USA (The girl's name is Ashanti, 4 years old then).

Severe combined immunodeficiency (SCID):

This is rare inherited immune disorder associated with T-lymphocytes, and (to a lesser extent) B-lymphocytes dysfunction. About 50% of SCID patients have a defect in the gene (located on chromosome 20, and has 32,000 base pairs and 12 exons) that encodes for adenosine deaminase.

In the deficiency of ADA, deoxyadenosine and its metabolites (primarily deoxyadenosine 5′-triphosphate) accumulate and destroy T-lymphocytes.

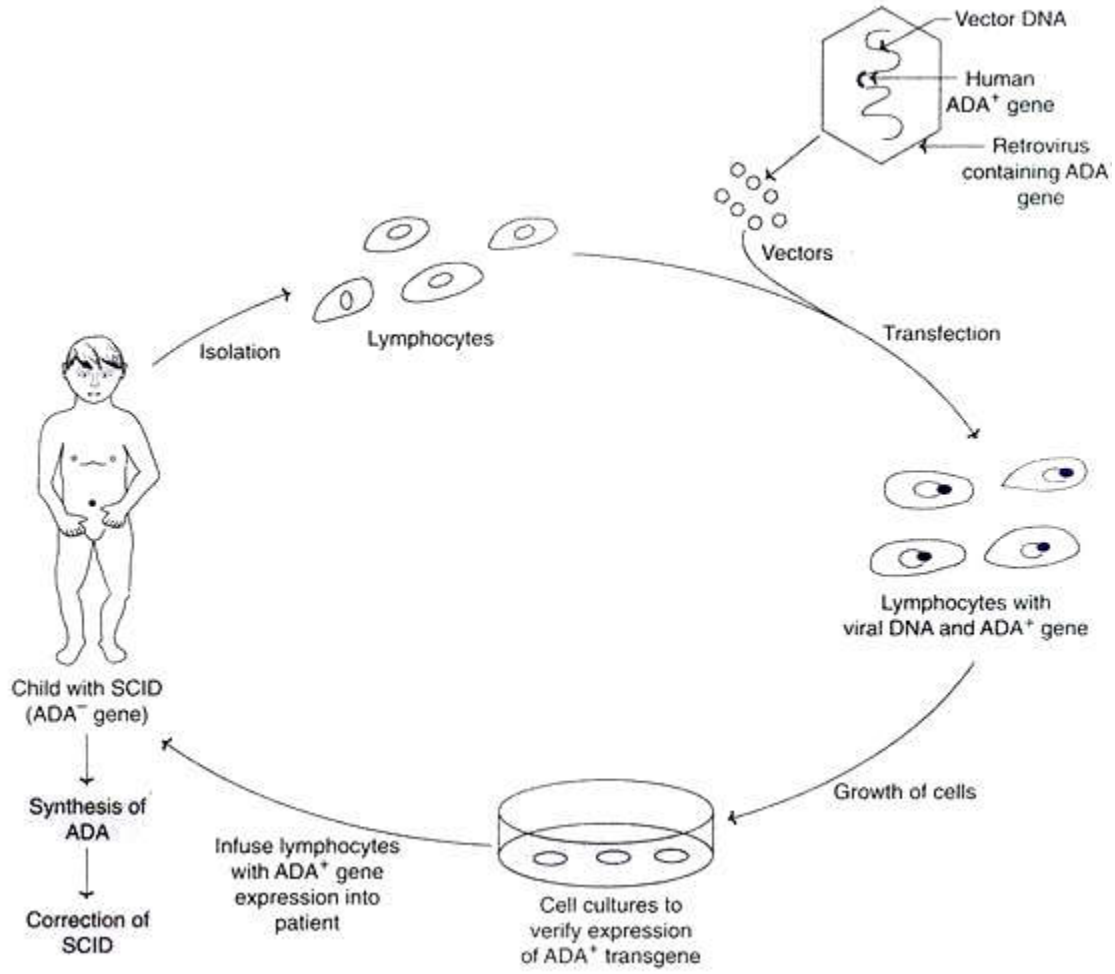
T-Lymphocytes are essential for body's immunity. Besides participating directly in body's defense, they promote the function of B-lymphocytes to produce antibodies. Thus, the patients of SCID (lacking ADA) suffer from infectious diseases and die at an young age. Previously, the children suffering from SCID were treated with conjugated bovine ADA, or by bone marrow transplantation.

Technique of therapy for ADA deficiency:

The general scheme of gene therapy adopted for introducing a defective gene in the patient has been depicted in Fig 13.2. The same procedure with suitable modifications can also be applied for other gene therapies.

A plasmid vector bearing a pro-viral DNA is selected. A part of the pro-viral DNA is replaced by the ADA gene and a gene (G 418) coding for antibiotic resistance, and then cloned. The antibiotic resistance gene will help to select the desired clones with ADA gene.

A diagrammatic representation of the treatment of ADP deficient patient is depicted in Fig. 13.5.



Circulating lymphocytes are removed from a patient suffering from ADA deficiency. These cells are transfected with ADA gene by exposing to billions of retroviruses carrying the said gene. The genetically-modified lymphocytes are grown in cultures to confirm the expression of ADA gene and returned to the patient. These lymphocytes persist in the circulation and synthesize ADA. Consequently, the ability of the patient to produce antibodies is increased. However, there is a limitation. The lymphocytes have a short life span (just live for a few months), hence the transfusions have to be carried out frequently.

Transfer of ADA gene into stem cells:

In 1995, ADA gene was transferred into the stem cells, obtained from the umbilical cord blood, at the time of baby's delivery. Four days after birth, the infant received the modified cells back. By this way, a permanent population of ADA gene producing cells was established.

Therapy for Familial Hypercholesterolemia:

The patients of familial hypercholesterolemia lack the low density lipoprotein (LDL) receptors on their liver cells. As a result, LDL cholesterol is not metabolised in liver. The accumulated LDL- cholesterol builds up in the circulation, leading to arterial blockage and heart diseases.

Attempts are being made by gene therapists to help the victims of familial hypercholesterolemia. In fact, there is some success also. In a woman, 15% of the liver was removed. The hepatocytes were transduced with retroviruses carrying genes for LDL receptors. These genetically modified hepatocytes were infused into the patient's liver.

The hepatocytes established themselves in the liver and produced functional LDL-receptors. A significant improvement in the patient's condition, as assessed by estimating the lipid parameters in blood, was observed. Further, there were no antibodies produced against the LDL-receptor molecules, clearly showing that the genetically modified liver cells were accepted.

Cystic Fibrosis

Therapy for cystic fibrosis:

Cystic fibrosis (CF) is one of the most common (frequency 1: 2,500) and fatal genetic diseases. It is characterized by the accumulation of sticky, dehydrated mucus in the respiratory tract and lungs. Patients of CF are highly susceptible to bacterial infections in their lungs and most of them die before reaching the age of thirty. Cystic fibrosis can be traced in European folklore, the following statement used to be said "Woe to that child which when kissed on the forehead tastes salty. He is be witched and soon must die".

Biochemical basis:

In the normal persons the chloride ions of the cells are pushed out through the participation of a protein called cystic fibrosis trans membrane regulator (CFTR). In the patients of cystic fibrosis, the CFTR protein is not produced due to a gene defect. Consequently, the chloride ions concentrate within the cells which draw water from the surroundings. As a result, the respiratory tract and the lungs become dehydrated with sicky mucus, an ideal environment for bacterial infections.

Gene therapy:

As the defective gene for cystic fibrosis was identified in 1989, researchers immediately started working on gene therapy for this disease. Adenoviral vector systems have been used, although

the success has been limited. The major drawback is that the benefits are short-lived, since the adenoviruses do not integrate themselves into host cells. Multiple administration of recombinant adenovirus caused immunological responses that destroyed the cells.

By using adeno-associated virus vector system, some encouraging results were reported in the gene therapy of CF. In the phase I clinical trials with CF patients, the vector persisted for about 70 days and some improvement was observed in the patients. Some researchers are trying to insert CF gene into the developing fetal cells (in experimental animals such as mice) to produce CFTR protein. But a major breakthrough is yet to come.

POSSIBLE QUESTIONS

1. Explain the following
   i) Artificial insemination ii) Gene therapy
2. Elaborate *in vitro* mutagenesis.
3. Discuss the role of tagging in gene analysis.
4. Describe about different strategies for gene therapy.
5. Give an account on synthetic oligonucleotides and transposons.
6. Explain about the inherited diseases ADA, FH and cystic fibrosis.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
I-M.Sc., BIOCHEMISTRY
RECOMBINANT DNA TECHNOLOGY (17BCP205A)
MULTIPLE CHOICE QUESTIONS

UNIT IV

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	Amino acid coding changes in DNA can be achieved by	PCR	Site directed mutagenesis	FISH	FACS	Site directed mutagenesis
2	All are applications of site directed mutagenesis except	Increase the substrate affinity of an enzyme	Making the enzyme thermo tolerant	Improving the stability of an enzyme	Decreasing the activity of an enzyme	Decreasing the activity of an enzyme
3	Design, develop and produce protein with improved character is called	Protein engineering	Codon bias	Codon optimization	Protein enhancing	Protein engineering
4	If the fragment of gene to be mutated lies between two restriction enzyme cleavage site_____ mutagenesis is possible	Oligo nucleotide	Casette	PCR	Direct	Casette
5	Kinetic properties of the enzymes can be improved by	Protein engineering	Codon enhancement	Codon optimization	Protein enhancing	Protein engineering
6	All are used in in vivo gene therapy as vector except _____	Herpes virus	Adeno virus	Cauli flower mosaic virus	Adeno associated virus	Cauli flower mosaic virus
7	Host for hepatitis B vaccine is	*E.coli*	*S.Cerevisiae*	*B.subtilis*	*P.vulgaris*	*S.Cerevisiae*
8	First synthetic vaccine is against	Human simplex virus	Hepatitis B	Tuberculosis	Measles	Hepatitis B
9	AIDS subunit vaccine contain	Nucleic acid	Carbohydrate	Lipid	Glycoprotein	Glycoprotein
10	_____ gene expression can be quantified	Scorable	Selectale	Reporter	Readable	Reporter
11	Which gene transfer technique involves the use of a fatty bubble to carry a gene into a somatic cell?	Electroporation	Micro injection	Particle bombardment	Lipofection	Lipofection
12	The process by which the recombinant DNA is introduced into bacterium through bacterio phage	Transfection	Transformation	Transduction	Transdution	Transfection
13	Uptake of naked DNA into the cell is called as _____	Transfection	Transformation	Transduction	Transdution	Transformation
14	The process of physical and chemical treatment that enhance the ability of cell to take up DNA is	Transfection	Transformation	Transduction	Competent	Competent
15	Trnasfer gene in cultured cell carried out in ____ gene therapy	*Ex vivo*	*In vivo*	*In situ*	*Ex situ*	*Ex vivo*
16	Transfer gene in to the targeted cell occur in _____ gene therapy	*Ex vivo*	*In vivo*	*In situ*	*Ex situ*	*In vivo*
17	All are vector for gene therapy except	Virus	Plasmid	Human artificial chromosome	Bone marrow cell	Bone marrow cell
18	The first human gene therapy was carried out to correct_____	SCID	Cystic fibosis	AIDS	Sickle cell anemia	SCID
19	The process somatic cell gene therapy involves	introduction of remedial gene into zygote	introduction of remedial gene into blood cells	introduction of remedial gene into sperm	introduction of remedial gene into egg	introduction of remedial gene into blood cells
20	An example of binary vector	pBR322	pUC8	pAL4404	pGV2260	pAL4404
21	An example of co integrative vector	pGV2260	PTi c58	pBR322	pUC8	pGV2260
22	Human embryo can be cryopreserved at_____ in liquid nitrogen	-180^{o}C	-196^{o}C	-160^{o}C	-220^{o}C	-196^{o}C
23	First mammalian clone was developed by	Maxim and Gilber, 1971	Wilmut and Camphel, 1997	Salk and Sabin, 1968	Jacob and Mond, 1973	Wilmut and Camphel, 1997
24	Among the following which one hasthe totipotency capacity	Muscle cell	Embryonic cell	Neuronal cell	Fibroblasts	Embryonic cell
25	First cloned animal	Dolly sheep	Dog	Mule	Cat	Dolly sheep
26	The chromosome used for embryo sexing is	X-chromosome	Y-chromosome	XY chromosome	Polytene chromosome	Y-chromosome
27	Antisence technology	Selectively block expression of gene	Combine genetic material from different species	Combine organelle in a cell	Transfer a cell	Selectively block expression of gene
28	Marker gene in transgenic mice production	Creatinine kinase	Thymidine kinase	Asparaginase	Alkaline phosphatase	Thymidine kinase
29	In knockout mice existing functions can be _____	Inctrase	Decreased	Altered	Blocked	Blocked
30	Developmental and physiological consequence of a gene in an organism can be studied using	Transgenic mice	Transgenic cattle	Knockout mice	Transgenic Fish	Knockout mice
31	Gene knockout is a genetically engineered organism that carries	One or more genes in its chromosomes	One or more genes in its cell	One gene of assortment	None of the above	One or more genes in its chromosomes
32	Knockout is a route to learning about a gene	That is active	That has been sequenced.	That are suppressed	That are ordered	That are suppressed
33	Knockout is accomplished through	a combination of techniques	a single technique	Bifunctional technique	None of the above	a combination of techniques
34	Knockout require	a plasmid	bacterial artificial chromosome	cell culture	All the above	All the above
35	Gene therapy is the insertion of genes into an individual's	Bones	cells and tissues	Ligaments	Skull	cells and tissues
36	---------- to treat a disease, and hereditary diseases	Gene therapy	Genetic instability	Genome stability	Transplantation	Gene therapy
37	A carrier called a ---------- must be used to deliver the therapeutic gene to the patient's	Fusion agent	Trancription initiater	Vector	Illucitor	Vector

38	The most common type of vector are ---------- that have been genetically altered to carry normal human DNA	PBR322	Cosmids	Viruses	Yeast	Viruses
39	All gene therapy to date on humans has been directed	somatic cells	Gene level	RNA level	Plasmid level	somatic cells
40	Somatic gene therapy can be broadly split in to	one category	Five category	Six category	Two categories	Two categories
41	ex vivo, which means	Cells are modified outside the body and then transplanted back in again	genes are changed in cells still in the body	recombination with a very low probability	Recombination approach	Cells are modified outside the body and then transplanted back in again
42	vivo, which means	Cells are modified outside the body and then transplanted back in again	genes are changed in cells still in the body	recombinatiorr with a very low probability	Recombination approach	genes are changed in cells still in the body
43	Gene therapy in humans was first practised by Blease and Andresco to cure	Cystic fibrosis	Hemophilia	Thalassemia	Severe combined immunodeficiency disease	Severe combined immunodeficiency disease
44	If the remedial gene does the function of defective gene. The approach is called as	Gene replacement and Gene augmentation therapy	Gene replacement therapy	Gene augmentation therapy	Corrective gene therapy	Gene replacement and Gene augmentation therapy
45	The introduction of remedial gene to bone marrow cells comes under	Germ line therapy	Somatic cell therapy	Germ line and somatic cell therapy	Corrective gene therapy	Somatic cell therapy
46	The possibility of introducing correct version of	Germ line therapy	Somatic cell therapy	Gene augmentation	Corrective gene	Germ line therapy
47	Introduction of healthy gene into cells, tissues or organs cultured in vitro and reimplanting back to the patient is referred as	Germ line therapy	Somatic cell therapy	*Ex vivo* therapy	*In vivo* therapy	*Ex vivo* therapy
48	Embryo therapy was devised by Handyside et al to cure	Cystic fibrosis	Hemophilia	Thalassemia	Severe combined immunodeficiency disease	Cystic fibrosis
49	Introduction of healthy gene at specific sites to displace the defective gene is referred as	Germ line therapy	Somatic cell therapy	Germ line and somatic cell therapy	Corrective gene therapy	Corrective gene therapy
50	The common gene delivery system for in vivo gene therapy is	Micro injection	Lipofection	Adeno viral vectors	Electroporation	Adeno viral vectors
51	The method of treating genetic diseases by introducing a remedial gene that prevents the expression of a specific defective gene is	Ex vivo therapy	In vivo therapy	Antisense therapy	All of these	Antisense therapy
52	Which of the statement is false regarding gene augmentation therapy	Suitable for single gene disorders	Random insertion of healthy gene	Suitable for multi gene disorders also	No recombination event is required	Suitable for multi gene disorders also
53	Which of the following is most controversial approach in gene therapy	Germ line therapy	Somatic therapy	Ex vivo therapy	Anti sense therapy	Germ line therapy
54	Bisulphite ions are used to deaminate _____ residues in _____ DNA.	C, double stranded	C, single stranded	U, double stranded	U, single stranded	C, single stranded
55	For mutagenesis without PCR, which of the following can be used as a template?	Single stranded DNA	Double stranded DNA	Circular DNA	Both single and double stranded DNA	Both single and double stranded DNA
56	How many sites can be mutated at a time?	1	2	3	Many	Many
57	If mixed oligonucleotides are used, it is regarded as:	Mixed mutagenesis	Multiple mutagenesis	Cassette mutagenesis	Polymutagenesis	cassette mutagenesis
58	It is easier to subclone a restriction fragment if it belongs to?	Small gene	Large gene	Prokaryotic organism	Eukaryotic organism	Large gene
59	Once the double stranded molecule with mutation is introduced into E. coli for replication, how many types of molecules are produced?	1	2	3	4	2
60	Which of the following statement is incorrect for synthesis of second strand?	The oligonucleotide is acting as a primer for the synthesis of second strand	DNA polymerase and dNTPs are added for synthesis	The polymerase should have 5'-3' exonuclease activity	A polymerase having 5'-3' exonuclease activity would degrade the primer that carries the mutant sequence	The polymerase should have 5'-3' exonuclease activity

UNIT-V

SYLLABUS

Transgenics: Gene transfer techniques- Microinjection, biolistic methods, vector based transfer.

Transgenic plants: Agrobacterium & Ti plasmids. Methods of engineering herbicide resistance plants, Stress resistance plants and modification of plant nutritional content (amino acids, β-carotene) Plants as bioreactors: edible vaccines.

Transgenic animals: Method of Engineering transgenic mice, transgenic cattle- applications.

Biosafety- regularities and concerns. Societal impact of genetically modified food.

Transgenics

Gene transfer techniques

Microinjection

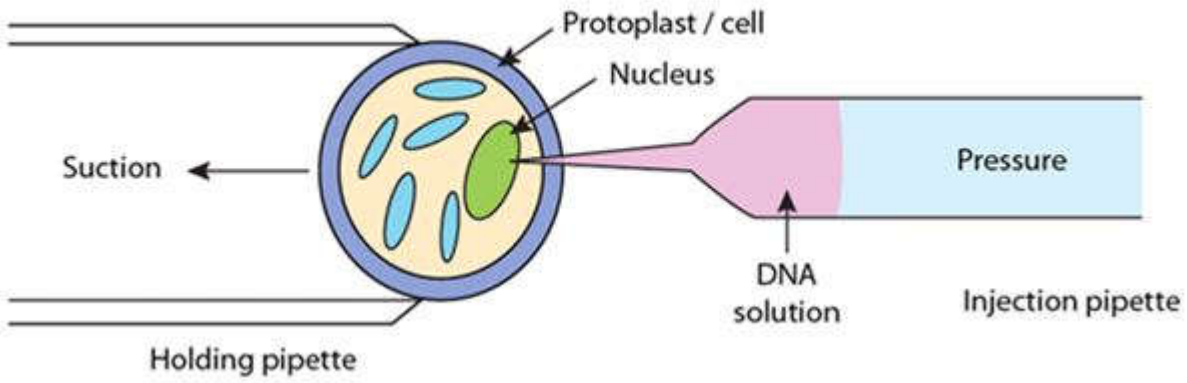
The process of using a fine glass micropipette to manually inject transgene at microscopic or borderline macroscopic level is known as microinjection.The transgene, in the form of plasmids, cosmids, phage, YACs, or PCR products, can be circular or linear and need not be physically linked for injection.

Microinjection involves direct mechanical introduction of DNA intothe nucleus or cytoplasm using a glass microcapillary injectionpipette. The protoplasts are immobilized in low melting agar, while working under a microscope, using a holding pipette and suction force. DNA is then directly injected into the cytoplasm or the nucleus. Theinjected cells are then cultured *invitro* and regenerated into plants. Successful examples of this process has been shown in rapeseed, tobacco and various other plants.

Stable transformants can be achieved through this method but it requires technical expertise and is atime consuming process. Also,microinjection has achieved only limited success in plant transformationdue to the thick cell walls of plants and a lack of availability of a single-cell-to-plant regenerationsystem in most plant species.

In this technique a traditional compound microscope (around 200X magnification) or aninverted microscope (around 200x magnification) or a dissecting stereomicroscope (around 40-50x)is used.Under the microscope target cell is positioned and cell membrane and nuclear envelope are

penetrated with the help of two micromanipulators.One micromanipulator holds the pipette and another holds the microcapillary needle.



There are two types of microinjection systems; constant flow system and pulsed flow system.

- In the constant flow system the amount of sample injected is determined by the duration for which needle remains in the cell. The constant flow system is relatively simple and inexpensive but outdated.
- The pulsed flow system has greater control over the volume of substance delivered, needle placement and movement and has better precision. This technique results in less damage to the receiving cell, however, the components of this system are quite expensive.

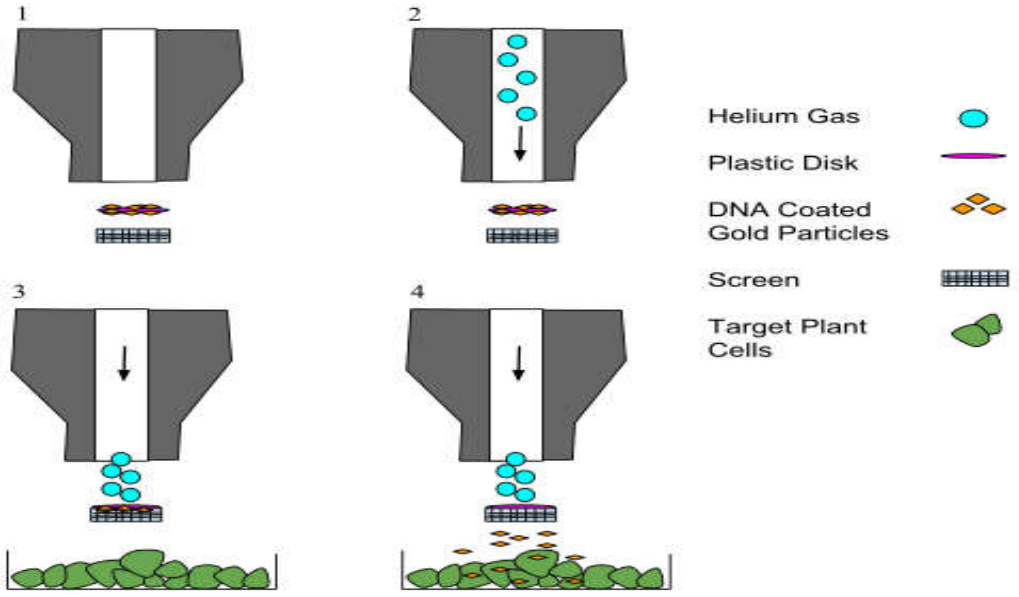
BIOLISTIC METHODS

A gene gun or a biolistic particle delivery system, originally designed for plant transformation, is a device for delivering exogenous DNA (transgenes) to cells. The payload is an elemental particle of a heavy metal coated with DNA (typically plasmid DNA). This technique is often simply referred to as biolistics.

This device is able to transform almost any type of cell, including plants, and is not limited to transformation of the nucleus; it can also transform organelles, including plastids.

A gene gun is used for delivery of exogenous DNA to cells. This method is known as 'biolistics'. Gene guns can be used effectively on most cells but are mainly used on plant cells. Step 1 The gene gun apparatus is ready to fire. Step 2 Helium fills the chamber and pressure builds against the rupture disk. Step 3 The pressure eventually reaches the point where the rupture disk breaks, and the resulting burst of helium propels the DNA/gold-coated macrocarrier ('Plastic Disk') into

the stopping screen. Step 4 When the macrocarrier hits the stopping screen, the DNA-coated gold particles are propelled through the screen and into the target cells.



Advantages

Biolistics has proven to be a versatile method of genetic modification and it is generally preferred to engineer transformation-resistant crops, such as cereals. Notably, Bt maize is a product of biolistics. Plastid transformation has also seen great success with particle bombardment when compared to other current techniques, such as Agrobacterium mediated transformation, which have difficulty targeting the vector to and stably expressing in the chloroplast. In addition, there are no reports of a chloroplast silencing a transgene inserted with a gene gun. Additionally, with only one firing of a gene gun, a skilled technician can generate two transformed organisms. This technology has even allowed for modification of specific tissues in situ, although this is likely to damage large numbers of cells and transform only some, rather than all, cells of the tissue.

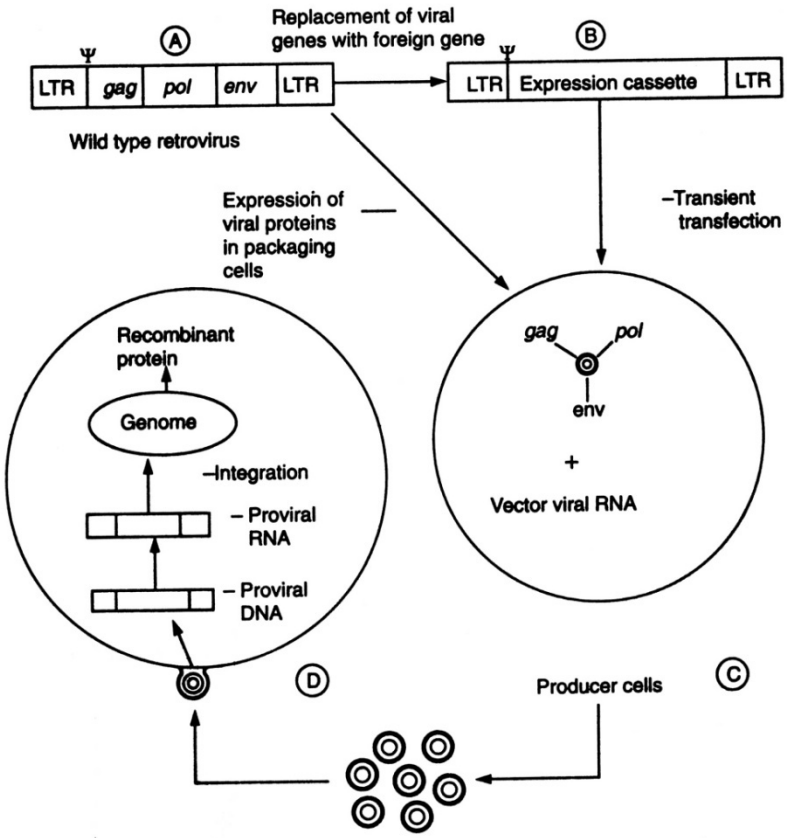
VECTOR BASED TRANSFER

The salient features of the commonly used gene (DNA) transfer methods are given in Table.

Method	Salient features
I. Vector–mediated gene transfer	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
II. Direct or vectorless DNA transfer	
(A) Physical methods	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/ tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
(B) Chemical methods	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

A variety of gene transfer strategies have been developed during the last decade for the treatment of human diseases which can be grouped into the two major categories: the viral and non-viral methods.

Virus vectors: After 1980, much work has been done on retroviruses as gene transfer vectors, more specifically on murine-leukemia virus (MLV) for gene therapy. Efforts are being made to develop HIV-based vectors so that even non-dividing cells can be injected. The steps of developing a replication-defective recombinant retroviral vectors are : *(i)* the replacement of viral structural genes *e.g. gag, pol* and *env* by the therapeutic foreign genes of interest, *(ii)* transfection of this vector into packaging cell line *(i.e.* producer cells) that provide the viral structural proteins *in trans* so that the recombinant retroviral genome is packed and replication defective retroviruses are generated, *(iii)* transfection of host cells by such viruses, and reverse transcription of recombinant retroviral RNA and random integration into the host genome. In the absence of viral genes, the foreign genes (therapeutic in nature is transcribed from the viral LTRs, the long terminal repeats) and desired protein is synthesized. The retroviral vectors are used in *ex vivo* gene transfer experiment, although it has been shown that they can infect a regenerating liver when administered intravenously into hepatectomized animal.



Transgenic plants

GENETIC ENGINEERING OF PLANTS

Genetically modified crops (GMCs, GM crops, or biotech crops) are plants used in agriculture, the DNA of which has been modified using genetic engineering techniques. In most cases, the aim is to introduce a new trait to the plant which does not occur naturally in the species.

Organization of Ti plasmid:

The Ti plasmids (approximate size 200 kb each) exist as independent replicating circular DNA molecules within the Agrobacterium cells. The T-DNA (transferred DNA) is variable in length in the range of 12 to 24 kb, which depends on the bacterial strain from which Ti plasmids come. Nopaline strains of Ti plasmid have one T-DNA with length of 20 kb while octopine strains have two T-DNA regions referred to as T_L and T_R that are respectively 14 kb and 7 kb in length.

A diagrammatic representation of a Ti plasmid is depicted in Fig. The Ti plasmid has three important regions.

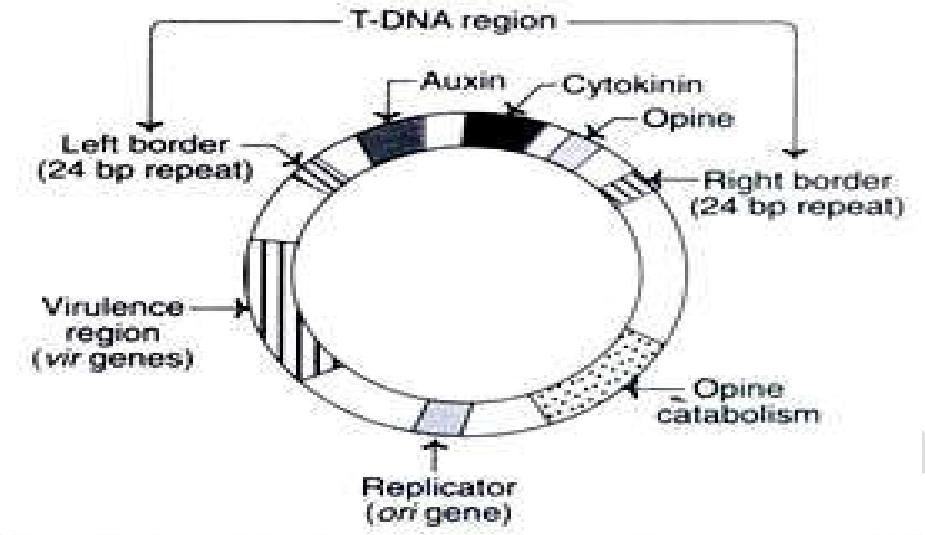


Fig. 49.3 : A dagrammatic representation of a Ti plasmid.

1. T-DNA region:

This region has the genes for the biosynthesis of auxin (aux), cytokinin (cyt) and opine (ocs), and is flanked by left and right borders. These three genes-aux, cyto and ocs are referred to as oncogenes, as they are the determinants of the tumor phenotype.

T-DNA borders — A set of 24 kb sequences present on either side (right and left) of T-DNA are also transferred to the plant cells. It is now clearly established that the right border is more critical for T-DNA transfer and tumori-genesis.

2. Virulence region:

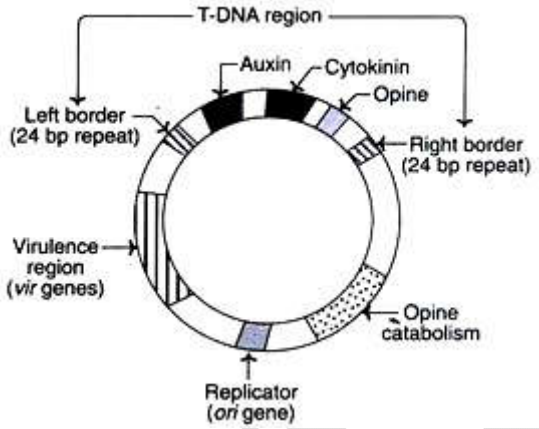
The genes responsible for the transfer of T-DNA into the host plant are located outside T-DNA and the region is referred to as vir or virulence region. Vir region codes for proteins involved in T-DNA transfer. At least nine vir-gene operons have been identified. These include vir A, vir G, vir B_1, vir C_1, vir D_1, D_2 and D_4, and vir E_1, and E_2.

3. Opine catabolism region:

This region codes for proteins involved in the uptake and metabolisms of opines. Besides the above three, there is ori region that is responsible for the origin of DNA replication which permits the Ti plasmid to be stably maintained in A. tumefaciens.

T-DNA transfer and integration:

The process of T-DNA transfer and it integration into the host plant genome is depicted in Fig, and is briefly described.



1. Signal induction to Agrobacterium:

The wounded plant cells release certain chemicals- phenolic compounds and sugars which are recognized as signals by Agrobacterium. The signals induced result in a sequence of biochemical events in Agrobacterium that ultimately helps in the transfer of T-DNA of T-plasmid.

2. Attachment of Agrobacterium to plant cells:

The Agrobacterium attaches to plant cells through polysaccharides, particularly cellulose fibres produced by the bacterium. Several chromosomal virulence (chv) genes responsible for the attachment of bacterial cells to plant cells have been identified.

3. Production of virulence proteins:

As the signal induction occurs in the Agrobacterium cells attached to plant cells, a series of events take place that result in the production of virulence proteins. To start with, signal induction by phenolics stimulates vir A which in turn activates (by phosphorylation) vir C. This induces expression of virulence genes of Ti plasmid to produce the corresponding virulence proteins (D1, D2, E_2, B etc.). Certain sugars (e.g. glucose, galactose, xylose) that induce virulence genes have been identified.

4. Production of T-DNA strand:

The right and left borders of T-DNA are recognized by vir D_1/vir D_2proteins. These proteins are involved in the production single-stranded T-DNA (ss DNA), its protection and export to plant cells. The ss T-DNA gets attached to vir D_2.

5. Transfer of T-DNA out of Agrobacterium:

The ss T-DNA — vir D_2 complex in association with vir G is exported from the bacterial cell. Vir B products form the transport apparatus.

6. Transfer of T-DNA into plant cells and integration:

The T-DNA-vir D_2 complex crosses the plant plasma membrane. In the plant cells, T-DNA gets covered with vir E_2. This covering protects the T-DNA from degradation by nucleases; vir D_2 and vir E_2 interact with a variety of plant proteins which influences T-DNA transport and integration.

The T-DNA-vir D_2-vir E_2 — plant protein complex enters the nucleus through nuclear pore complex. Within the nucleus, the T-DNA gets integrated into the plant chromosome through a process referred to illegitimate recombination. This is different from the homologous recombination, as it does not depend on the sequence similarity.

Hairy Root Disease of A. Rhizogenes — R_1 Plasmids:

Agrobacterium rhizogenes can also infect plants. But this results in hairy roots and not crown galls as is the case with A. tumefaciens. The plasmids, of A. rhizogenes have been isolated and characterized. These plasmids, referred to as Ri plasmids, (Ri stands for Root inducing) are of different types. Some of the Ri plasmid strains possess genes that are homologous to Ti plasmid e.g. auxin biosynthetic genes.

Instead of virulence genes, Ri plasmids contain a series of open reading frames on the T-DNA. The products of these genes are involved in the metabolism of plant growth regulators which gets sensitized to auxin and leads to root formation.

Vectors of A. rhizogenes:

As it is done with A tumefaciens, vectors can be constructed by using A. rhizogenes. These vectors are alternate strategies for gene transfer. However, employment of A. rhizogene-based vectors for plant transformation is not common since more efficient systems of A. tumefaciens have been developed.

Importance of hairy roots:

Hairy roots can be cultured in vitro, and thus are important in plant biotechnology. Hairy root systems are useful for the production of secondary metabolites, particularly pharmaceutical proteins.

Ti Plasmid-Derived Vector Systems:

The ability of Ti plasmid of Agrobacterium to genetically transform plants has been described. It is possible to insert a desired DNA sequence (gene) into the T-DNA region (of Ti plasmid), and then use A. tumefaciens to deliver this gene(s) into the genome of plant cell.

In this process, Ti plasmids serve as natural vectors. However, there are several limitations to use Ti plasmids directly as cloning vectors:

i. Ti plasmids are large in size (200-800 kb). Smaller vectors are preferred for recombinant experiments. For this reason, large segments of DNA of Ti plasmid, not essential for cloning, must be removed.

ii. Absence of unique restriction enzyme sites on Ti plasmids.

iii. The phytohormones (auxin, cytokinin) produced prevent the plant cells being regenerated into plants. Therefore auxin and cytokinin genes must be removed.

iv. Opine production in transformed plant cells lowers the plant yield. Therefore opine synthesizing genes which are of no use to plants should be removed.

v. Ti plasmids cannot replicate in E. coli. This limits their utility as E. coli is widely used in recombinant experiments. An alternate arrangement is to add an origin of replication to Ti plasmid that allows the plasmid to replicate in E. coli.

Considering the above limitations, Ti plasmid- based vectors with suitable modifications have been constructed.

These vectors are mainly composed of the following components:

1. The right border sequence of T-DNA which is absolutely required for T-DNA integration into plant cell DNA.

2. A multiple cloning site (poly-linker DNA) that promotes the insertion of cloned gene into the region between T-DNA borders.

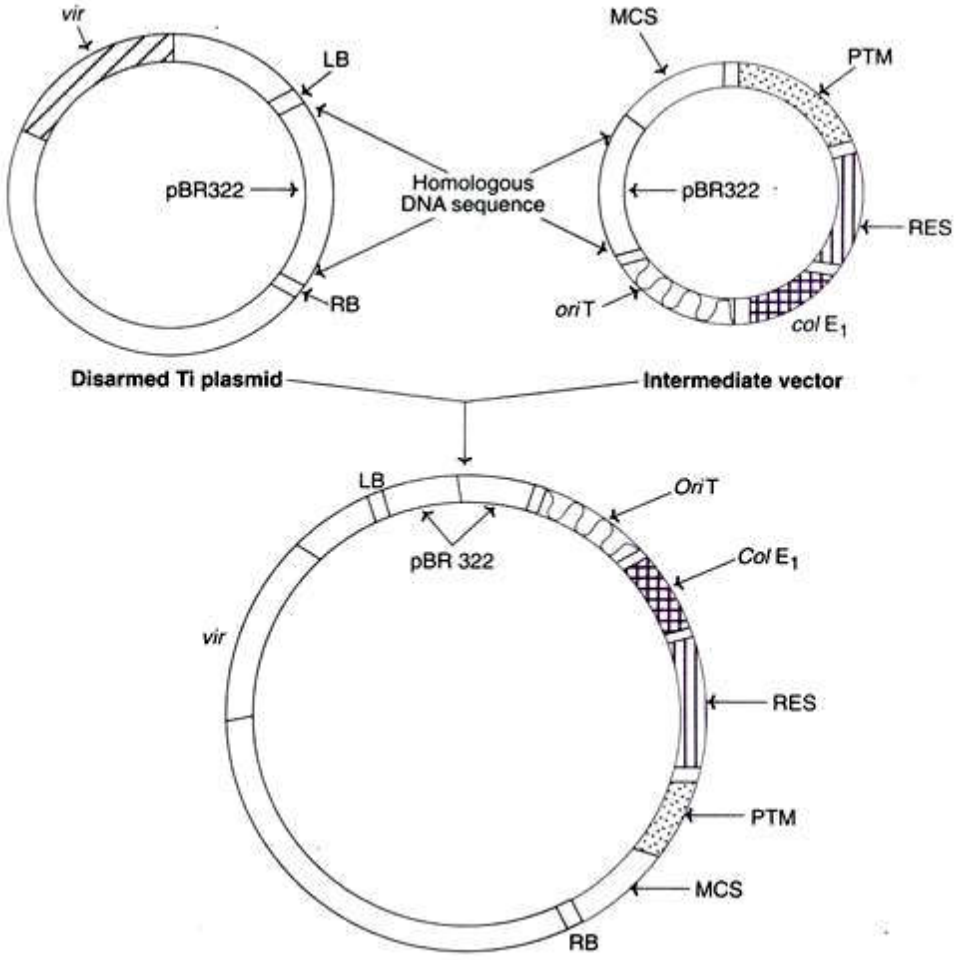
3. An origin of DNA replication that allows the plasmids to multiply in E. coli.

4. A selectable marker gene (e.g. neomycin phosphotransferase) for appropriate selection of the transformed cells.

Two types of Ti plasmid-derived vectors are used for genetic transformation of plants— co-integrate vectors and binary vectors.

Co-integrate vector:

In the co-integrate vector system, the disarmed and modified Ti plasmid combines with an intermediate cloning vector to produce a recombinant Ti plasmid (Fig. 49.5).



Production of disarmed Ti plasmid

The T-DNA genes for hormone biosynthesis are removed (disarmed). In place of the deleted DNA, a bacterial plasmid (pBR322) DNA sequence is incorporated. This disarmed plasmid, also referred to as receptor plasmid, has the basic structure of T-DNA (right and left borders, virulence genes etc.) necessary to transfer the plant cells.

Construction of intermediate vector:

The intermediate vector is constructed with the following components:

i. A pBR322 sequence DNA homologous to that found in the receptor Ti plasmid.

ii. A plant transformation marker (PTM) e.g. a gene coding for neomycin phosphotransferase II (npt II). This gene confers resistance to kanamycin in the plant cells and thus permits their isolation.

iii. A bacterial resistance marker e.g. a gene coding for spectinomycin resistance. This gene confers spectinomycin resistance to recipient bacterial cells and thus permits their selective isolation.

iv. A multiple cloning site (MCS) where foreign genes can be inserted.

v. A Co/E_1 origin of replication which allows the replication of plasmid in E. coli but not in Agrobacterium.

vi. An oriT sequence with basis of mobilization (bom) site for the transfer of intermediate vector from E. coli to Agrobacterium.

Production and use of co-integrate vectors:

The desired foreign gene (target-gene) is first cloned in the multiple cloning site of the intermediate vector. The cloning process is carried out in E. coli, the bacterium where the cloning is most efficient. The intermediate vector is mated with Agrobacterium so that the foreign gene is mobilised into the latter.

The transformed Agrobacterium cells with receptor Ti plasmid and intermediate vector are selectively isolated when grown on a minimal medium containing spectinomycin. The selection process becomes easy since E. coli does not grow on a minimal medium in which Agrobacterium grows.

Within the Agrobacterium cells, intermediate plasmid gets integrated into the receptor Ti plasmid to produce co-integrate plasmid. This plasmid containing plant transformation marker (e.g. npt II) gene and cloned target gene between T-DNA borders is transferred to plant cells. The transformed plant cells can be selected on a medium containing kanamycin when the plant and Agrobacterium cells are incubated together.

Advantages of co-integrate vector:

i. Target genes can be easily cloned

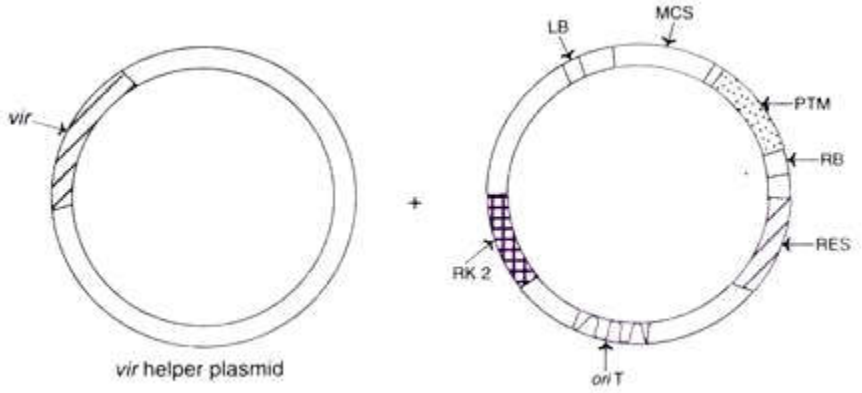
ii. The plasmid is relatively small with a number of restriction sites.

iii. Intermediate plasmid is conveniently cloned in E. coli and transferred to Agrobacterium.

Binary vector:

The binary vector system consists of an Agrobacterium strain along with a disarmed Ti plasmid called vir helper plasmid (the entire T-DNA region including borders deleted while vir gene is retained). It may be noted that both of them are not physically linked (or integrated). A binary vector with T-DNA can replicate in E. coli and Agrobacterium.

A diagrammatic representation of a typical binary vector system is depicted in Fig. 49.6. The binary vector has the following components.



1. Left and right borders that delimit the T-DNA region.
2. A plant transformation marker (PTM) e.g. npt II that confers kanamycin resistance in plant transformed cells.
3. A multiple cloning site (MCS) for introducing target/foreign genes.
4. A bacterial resistance marker e.g. tetracycline resistance gene for selecting binary vector colonies in E. coli and Agrobacterium.
5. oriT sequence for conjugal mobilization of the binary vector from E. coli to Agrobacterium.
6. A broad host-range origin of replication such as RK_2 that allows the replication of binary vector in Agrobacterium.

Production and use of binary vector:

The target (foreign) gene of interest is inserted into the multiple cloning site of the binary vector. In this way, the- target gene is placed between the right and left border repeats and cloned in E. coli. By a mating process, the binary vector is mobilised from E. coli to Agrobacterium. Now, the virulence gene proteins of T-DNA facilitate the transfer of T-DNA of the vector into plant cells.

Advantages of binary vectors:

i. The binary vector system involves only the transfer of a binary plasmid to Agrobacterium without any integration. This is in contrast to co-integrate vector system wherein the intermediate vector is transferred and integrated with disarmed Ti plasmid.

ii. Due to convenience, binary vectors are more frequently used than co-integrate vectors.

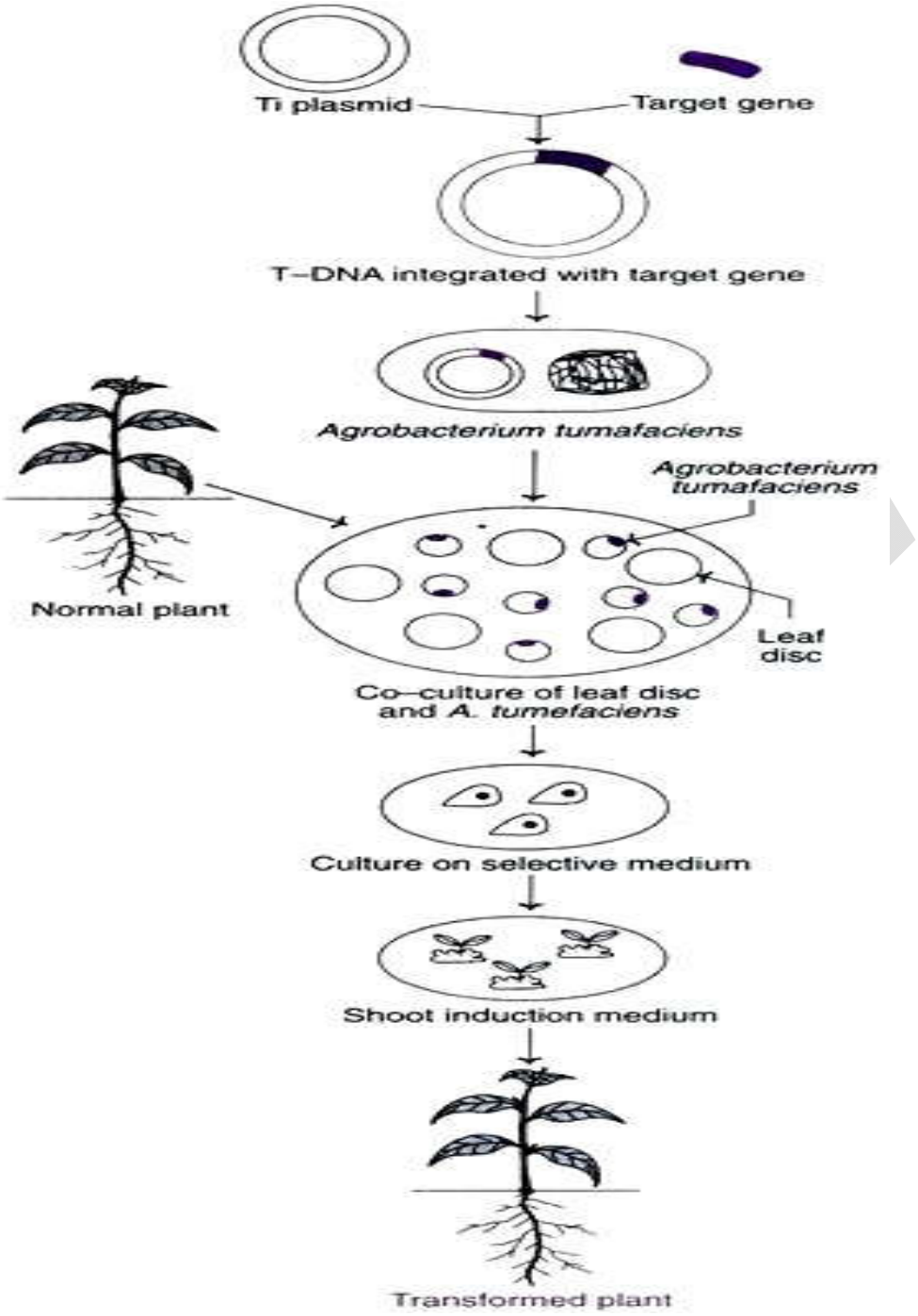
Plant Transformation Technique Using Agrobacterium:

Agrobacterium-mediated technique is the most widely used for the transformation of plants and generation of transgenic plants. The important requirements for gene transfer in higher plants through Agrobacterium mediation are listed.

i. The explants of the plant must produce phenolic compounds (e.g. autosyringone) for activation of virulence genes.

ii. Transformed cells/tissues should be capable to regenerate into whole plants.

In general, most of the Agrobacterium-mediated plant transformations have the following basic protocol (Fig.)



1. Development of Agrobacterium carrying the co-integrate or binary vector with the desired gene.

2. Identification of a suitable explant e.g. cells, protoplasts, tissues, calluses, organs.

3. Co-culture of explants with Agrobacterium.

4. Killing of Agrobacterium with a suitable antibiotic without harming the plant tissue.

5. Selection of transformed plant cells.

6. Regeneration of whole plants.

Advantages of Agrobacterium- mediated transformation:

i. This is a natural method of gene transfer.

ii. Agrobacterium can conveniently infect any explant (cells/tissues/organs).

iii. Even large fragments of DNA can be efficiently transferred.

iv. Stability of transferred DNA is reasonably good.

v. Transformed plants can be regenerated effectively.

Limitations of Agrobacterium- mediated transformation:

i. There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g. cereals) are not infected by it. In recent years, virulent strains of Agrobacterium that can infect a wide range of plants have been developed.

ii. The cells that regenerate more efficiently are often difficult to transform, e.g. embryonic cells lie in deep layers which are not easy targets for Agrobacterium.

Herbicide Tolerance Technology

Weeds are constant problem in farmers' fields. Weeds not only compete with crops for water, nutrients, sunlight, and space but also harbor insect and disease pests; clog irrigation and drainage systems; undermine crop quality; and deposit weed seeds into crop harvests. If left uncontrolled, weeds can reduce crop yields significantly.

Farmers can fight weeds with tillage, hand weeding, herbicides, or typically a combination of all techniques. Unfortunately, tillage leaves valuable topsoil exposed to wind and water erosion, a serious long-term consequence for the environment. For this reason, more and more farmers prefer reduced or no-till methods of farming.

Similarly, many have argued that the heavy use of herbicides has led to groundwater contaminations, the death of several wildlife species and has also been attributed to various human and animal illnesses.

Advantages of Herbicide Tolerant Crops

- Excellent weed control and hence higher crop yields;
- Flexibility – possible to control weeds later in the plant's growth;
- Reduced numbers of sprays in a season;

- Reduced fuel use (because of less spraying);
- Reduced soil compaction (because of less need to go on the land to spray);
- Use of low toxicity compounds which do not remain active in the soil; and
- The ability to use no-till or conservation-till systems, with consequent benefits to soil structure and organisms

Applications of Transgenic Plants

The genetic manipulations carried out in plants for the production of transgenic plants have been described .The ultimate goal of transgenic (involving introduction, integration, and expression of foreign genes) is to improve the crops, with the desired traits.

Some of the important ones are listed:

i. Resistance to biotic stresses i.e. resistance to diseases caused by insects, viruses, fungi and bacteria.

ii. Resistance to abiotic stresses-herbicides, temperature (heat, chilling, freezing), drought, salinity, ozone, intense light.

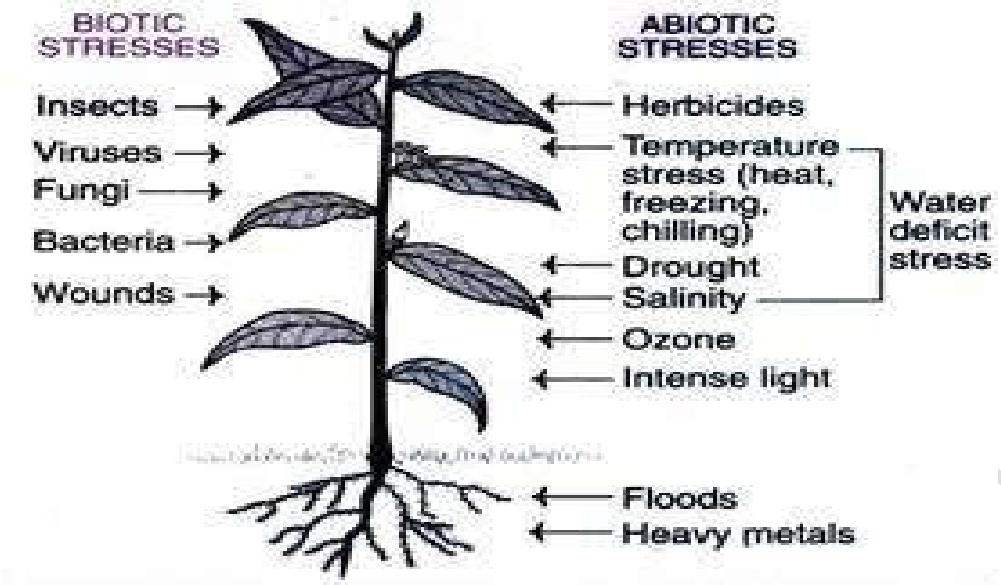
iii. Improvement of crop yield, and quality e.g. storage, longer shelf life of fruits and flowers.

iv. Transgenic plants with improved nutrition.

v. Transgenic plants as bioreactors for the manufacture of commercial products e.g. proteins, vaccines, and biodegradable plastics.

Environmental stresses to plants:

The different types of external stresses that influence the plant growth and development are depicted in Fig, These stresses are grouped based on their characters—biotic and abiotic stresses. The biotic stresses are caused by insects, pathogens (viruses, fungi, bacteria), and wounds. The abiotic stresses are due to herbicides, water deficiency (caused by drought, temperature, and salinity), ozone and intense light.



Almost all the stresses, either directly or indirectly, lead to the production of reactive oxygen species (ROS) that create oxidative stress to plants. This damages the cellular constituents of plants which is associated with a reduction in plant yield.

HERBICIDE RESISTANCE PLANT

Weeds (wild herbs) are unwanted and useless plants that grow along with the crop plants. Weeds compete with traps for light and nutrients, besides harbouring various pathogens. It is estimated that the world's crop yield is reduced by 10-15% due to the presence of weeds.

To tackle the problem of weeds, modern agriculture has developed a wide range of weed killers which are collectively referred to as herbicides. In general, majority of the herbicides are broad-spectrum as they can kill a wide range of weeds.

A good or an ideal herbicide is expected to possess the following characteristics:

i. Capable of killing weeds without affecting crop plants.

ii. Not toxic to animals and microorganisms.

iii. Rapidly trans-located within the target plant.

iv. Rapidly degraded in the soil.

None of the commercially available herbicides fulfills all the above criteria. The major limitation of the herbicides is that they cannot discriminate weeds from crop plants. For this reason, the crops are also affected by herbicides, hence the need to develop herbicide-resistant plants. Thus,

these plants provide an opportunity to effectively kill the weeds (by herbicides) without damaging the crop plants.

Strategies for engineering herbicide resistance:

A number of biological manipulations particularly involving genetic engineering are in use to develop herbicide-resistant plants.

1. Overexpression of the target protein:

The target protein, being acted by the herbicide can be produced in large quantities so that the affect of the herbicide becomes insignificant. Overexpression can be achieved by integrating multiple copies of the genes and/or by using a strong promoter.

2. Improved plant detoxification:

The plants do possess natural defense systems against toxic compounds (herbicides). Detoxification involves the conversion of toxic herbicide to non-toxic or less toxic compound. By enhancing the plant detoxification system, the impact of the herbicide can be reduced.

3. Detoxification of herbicide by using a foreign gene:

By introducing a foreign gene into the crop plant, the herbicide can be effectively detoxified.

4. Mutation of the target protein:

The target protein which is being affected by the herbicide can be suitably modified. The changed protein should be capable of discharging the functions of the native protein but is resistant to inhibition by the herbicide.

Once the resistant target protein gene is identified, it can be introduced into the plant genomes, and thus herbicide-resistant plants can be developed. For success in the development of herbicide resistant plants, good knowledge of the target protein and the action of herbicides is required.

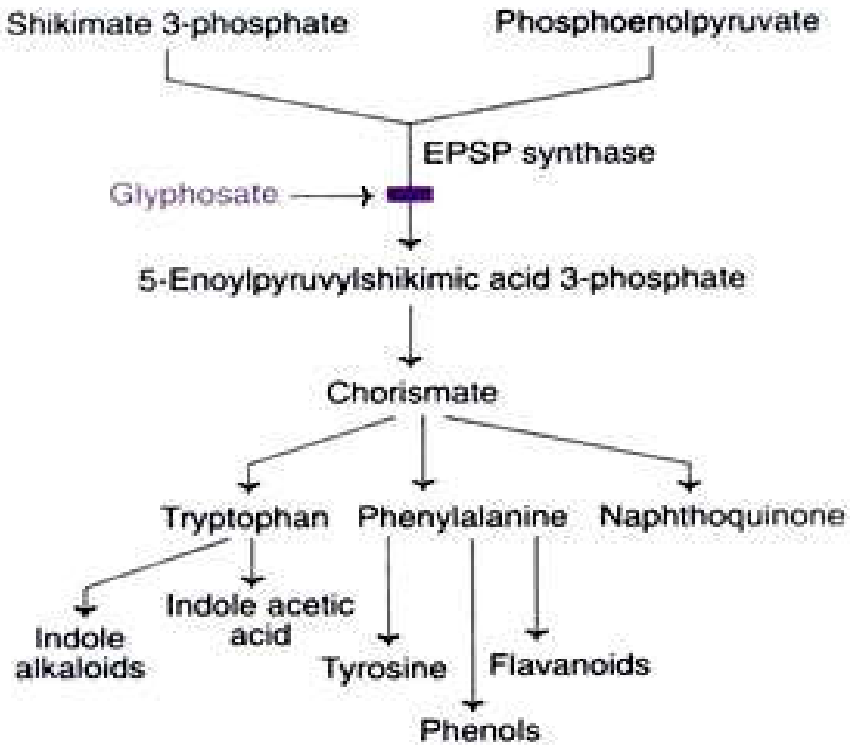
Some of the developments made in the herbicide resistance of plant are briefly described:

Glyphosate Resistance:

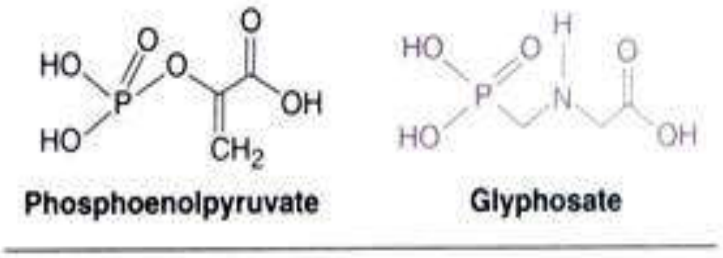
Glyphosate, is a glycine derivative. It acts as a broad-spectrum herbicide and is effective against 76 of the world's worst 78 weeds. Glyphosate is less toxic to animals and is rapidly degraded by microorganisms. In addition, it has a short half-life. The American chemical company Monsanto markets glyphosate as Round up.

Mechanism of action of glyphosate:

Glyphosate is rapidly transported to the growing points of plants. It is capable of killing the plants even at a low concentration. Glyphosate acts as a competitive inhibitor of the enzyme 5-enoylpyruvylshikimate 3-phosphate synthase (EPSPS). This is a key enzyme in shikimic acid pathway that results in the formation of aromatic amino acids (tryptophan, phenylalanine and tyrosine), phenols and certain secondary metabolites (Fig.).



The enzyme EPSPS catalyses the synthesis of 5-enoylpyruvylshikimate 3-phosphate from shikimate 3-phosphate and phosphoenoylpyruvate. Glyphosate has some structural similarly with the substrate phosphoenol pyruvate. Consequently, glyphosate binds more tightly with EPSPS and blocks the normal shikimic acid pathway. Thus, the herbicide glyphosate inhibits the biosynthesis of aromatic amino acids and other important products.



This results in inhibition of protein biosynthesis (due to lack of aromatic amino acids). As a consequence, cell division and plant growth are blocked. Further, the plant growth regulator indole acetic acid (an auxin) is also produced from tryptophan. The net result of glyphosate is the death of the plants. Glyphosate is toxic to microorganisms as they also possess shikimate pathway.

Glyphosate is non-toxic to animals (including humans), since they do not possess shikimate pathway. Of the three aromatic amino acids (synthesized in this pathway), tryptophan and phenylalanine are essential and they have to be supplied in the diet, while tyrosine can be formed from phenylalanine.

Strategies for glyphosate resistance:

There are three distinct strategies to provide glyphosphate resistance to plants:

1. Overexpression of crop plant EPSPS gene:

An overexpressing gene of EPSPS was detected in Petunia. This expression was found to be due to gene amplification rather than an increased expression of the gene. EPSPS gene from Petunia was isolated and introduced into other plants. The increased synthesis of EPSPS (by about 40 fold) in transgenic plants provides resistance to glyphosate. These plants can tolerate glyphosate at a dose of 2-4 times higher than that required to kill wild-type plants.

2. Use of mutant EPSPS genes:

An EPSPS mutant gene that conferred resistance to glyphosate was first detected in the bacterium Salmonella typhimurium. It was found that a single base substitution (C to 7) resulted in the change of an amino acid from proline to serine in EPSPS. This modified enzyme cannot bind to glyphosate, and thus provides resistance.

The mutant EPSPS gene was introduced into tobacco plants using Agrobacterium Ti plasmid vectors. The transgene produced high quantities of the enzyme EPSPS. However, the transformed tobacco plants provided only marginal resistance to glyphosate. The reason for this was not immediately identified.

It was later known that the shikimate pathway occurs in the chloroplasts while the glyphosate resistant EPSPS was produced only in the cytoplasm. This enzyme was not transported to the chloroplasts, hence the problem to provide resistance. This episode made scientists to realize the importance of chloroplasts in genetic engineering.

In later years, the mutant EPSPS gene was tagged with a chloroplast-specific transit peptide sequence. By this approach, the glyphosate-resistant EPSPS enzyme was directed to freely enter chloroplast and confer resistance against the herbicide.

3. Detoxification of glyphosate:

The soil microorganisms possess the enzyme glyphosate oxidase that converts glyphosate to glyoxylate and aminomethylphosponic acid. The gene encoding glyphosate oxidase has been isolated from a soil organism Ochrobactrum anthropi. With suitable modifications, this gene was introduced into crop plants e.g. oilseed rape. The transgenic plants were found to exhibit very good glyphosate resistance in the field.

Use of a combined strategy:

More efficient resistance of plants against glyphosate can be provided by employing a combined strategy. Thus, resistant (i.e. mutant) EPSPS gene in combination with glyphosate oxidase gene are used. By this approach, there occurs glyphosate resistance (due to mutant EPSPS gene) as well as its detoxification (due to glyphosate oxidase gene).

Resistance to other herbicides:

Besides the above, some other herbicide resistant plants have also been developed e.g. bromoxynil, atrazine, phenocarboxylic acids, cyanamide. A list of selected examples of gene transferred herbicide resistant plants is given in Table.

Herbicide	*Gene transfer/mechanism of resistance*	*Transgenic crop(s)*
Glyphosate	Inhibition of EPSPS	Soybean, tomato
Glyphosate	Detoxification by glyphosate oxidase	Maize, soybean
Phosphinothricin	*bar* gene coding phosphinothricin acetyltransferase	Maize, rice, wheat, cotton, potato, tomato, sugarbeet
Sulfonylureas/imidazolinones	Mutant plant with acetolactate synthase	Rice, tomato, maize, sugarbeet
Bromoxynil	Nitrilase detoxification	Cotton, potato, tomato
Atrazine	Mutant plant with chloroplast *psb A* gene	Soybean
Phenocarboxylic acids	Monooxygenase detoxification (e.g. 2,4-D and 2,4,5-T)	Maize cotton
Cyanamide	Cyanamide hydratase gene	Tobacco

It may however, be noted that some of the herbicide-resistant transgenic plants are at field-trial stage. Due to environmental concern, a few of these plants are withdrawn e.g. atrazine- resistant crops.

Environmental Impact of Herbicide-Resistant Crops:

The development genetically modified (GM) herbicide-resistant crops has undoubtedly contributed to increase in the yield of crops. For this reason, farmers particularly in the developed countries (e.g. USA) have started using these GM crops. Thus, the proportion of herbicide resistant soybean plants grown in USA increased from 17% in 1997 to 68% in 2001.

The farmer is immensely benefited as there is a reduction in the cost of herbicide usage. It is believed that the impact of herbicide-resistant plants on the environment is much lower than the direct use of the herbicides in huge quantities.

There are however, other environmental concerns:

i. Disturbance in biodiversity due to elimination of weeds.

ii. Rapid development of herbicide-resistance weeds that may finally lead to the production of super weeds.

Goals of biotechnological improvements in crops:

There are about 30-40 crops that have been genetically modified, and many more are being added. However, very few of them have got the clearance for commercial use. A selected list is already given in Table.

The ultimate goals of genetically modified (CM) crop plants are listed below:

i. Resistance to diseases (insect, microorganisms).

ii. Improved nitrogen fixing ability.

iii. Higher yielding capacity.

iv. Resistance to drought and soil salinity.

v. Better nutritional properties.

vi. Improved storage qualities.

vii. Production of pharmaceutically important compounds.

viii. Absence of allergens.

ix. Modified sensory attributes e.g. increased sweetness as in thaumatin.

Concerns about transgenic plants:

The fears about the harmful environmental and hazardous health effects of transgenic plants still exist, despite the fact that there have been no reports so far in this regard. The transfer of almost all the transgenic plants from the laboratory to the crop fields is invariably associated with legal and regulatory hurdles, besides the social and economic concerns.

The major concern expressed by public (also acknowledged by biotechnologists) is the development of resistance genes in insects, generation of super weeds etc. Several remedial measures are advocated to overcome these problems.

The farmers in developing countries are much worried about the seed terminator technology which forces them to buy seeds for every new crop. These farmers are traditionally habituated to use the seeds from the previous crop which is now not possible due to seed terminator technology.

Preparation of edible vaccines

Selection of the desired gene and plant

As the first important step, developing edible vaccines involves introduction of selected desired genes into plants and then inducing these altered plants to manufacture the encoded proteins. This process is known as transformation, and the altered plants are called transgenic plants. Toward development of edible vaccine subunit proteins, selection of important epitope region(s) from the pathogen of interest is the one of the key factors that determines the success of potential edible vaccines. A successful edible vaccine should ultimately be safe, non-pathogenic, and able to induce both mucosal and systemic immunity upon entry into the digestive tract. Efficacious edible vaccines should be able to resist the rigid acidic environment of the stomach, and reach the target cells in bioactive form. Selected antigen genes and their required expression machinery should be compatible with the selected plant type. Antigens in transgenic plants are delivered through bioencapsulation within the tough outer wall of plant cells. Bioencapsulation of recombinant antigens with transgenic plant cell vesicles protects the integrity of the antigens from gastric secretions until the plant cell walls degrade in the intestines. Upon degradation, antigens are released, taken up by M cells in the intestinal lining that overlay Peyer's patches and gutassociated lymphoid tissue (GALT). Subsequent antigen processing includes passage to macrophages, other antigen-presenting cells, and local lymphocyte populations.

Following vaccination and subsequent exposure to the native pathogen, serum IgG, IgE and local IgA responses, and memory cells are triggered, which would promptly neutralize the attack by the real infectious agent. Like conventional subunit vaccines, edible vaccines are composed of antigenic proteins and are devoid of pathogenic genes. As such, edible vaccines cannot establish infection, which better assures safety, a particularly important consideration for

vaccine regimens involving susceptible populations such as immunocompromised patients, children and the elderly. Conventional subunit vaccines can be expensive and technology-intensive, require complex purification, refrigeration, and produce poor mucosal responses. Oral administration protocols greatly reduce the need for trained medical personnel. Production of potential edible vaccine-quality proteins in transgenic plants is highly efficient and can be readily scaled up for commercial production. Transgenic plants can be engineered to produce immunoprotective proteins against infectious diseases, as well as some autoimmune diseases and human tumors. Transgenic potatoes, tomatoes, maize, rice, and soybeans have been developed and used in various plant bioreactor studies. The results of human trials that have tested several transgenic plant-produced recombinant therapeutic proteins have shown positive responses and no major safety concerns.

Plant/fruit	Advantages	Disadvantages
Tobacco	Good model for evaluating recombinant proteins Low-cost preserving system (numerous seeds, stored for long time) Easy purification of antibodies stored in the seeds, at any location Large harvests, number of times/year	Produces toxic compounds*
Potato	Dominated clinical trials Easily manipulated/transformed Easily propagated from its "eyes" Stored for long periods without refrigeration	Needs cooking, which can denature the antigens and decrease immunogenicity**
Banana	Do not need cooking Proteins not destroyed even if cooked Inexpensive Grown widely in developing countries	Trees take 2-3 years to mature Transformed trees take about 12 months to bear fruit Spoils rapidly after ripening Contains very little protein, so unlikely to produce large amounts of recombinant proteins
Tomato	Grow quickly Cultivated broadly High content of vitamin A may boost immune response Overcome the spoilage problem by freeze-drying technology Heat-stable, antigen-containing powders***, made into capsules Different batches blended to give uniform doses of antigen	Spoils readily
Rice	Commonly used in baby food because of low allergenic potential High expression of proteins/ antigens Easy storage/transportation Expressed protein is heat-stable	Grows slowly Requires specialized glasshouse conditions
Lettuce	Fast-growing Direct consumption	Spoils readily
Soybean and Alfalfa	Large harvests, number of times/year	
Musk melon (cantaloupe)	Fast growing Easily propagated by seed Easily transformed	
Others	Carrots, peanuts, wheat, corn	

Methods of creation of transgenic animals

For practical reasons, i.e., their small size and low cost of housing in comparison to that for larger vertebrates, their short generation time, and their fairly well defined genetics, mice have become the main species used in the field of transgenics.

The three principal methods used for the creation of transgenic animals are DNA microinjection, embryonic stem cell-mediated gene transfer and retrovirus-mediated gene transfer.

a) DNA microinjection.

This method involves the direct microinjection of a chosen gene construct (a single gene or a combination of genes) from another member of the same species or from a different species, into the pronucleus of a fertilized ovum. It is one of the first methods that proved to be effective in

mammals (Gordon and Ruddle, 1981). The introduced DNA may lead to the over- or under-expression of certain genes or to the expression of genes entirely new to the animal species. The insertion of DNA is, however, a random process, and there is a high probability that the introduced gene will not insert itself into a site on the host DNA that will permit its expression. The manipulated fertilized ovum is transferred into the oviduct of a recipient female, or foster mother that has been induced to act as a recipient by mating with a vasectomized male.

A major advantage of this method is its applicability to a wide variety of species.

b) Embryonic stem cell-mediated gene transfer.

This method involves prior insertion of the desired DNA sequence by homologous recombination into an in vitro culture of embryonic stem (ES) cells. Stem cells are undifferentiated cells that have the potential to differentiate into any type of cell (somatic and germ cells) and therefore to give rise to a complete organism. These cells are then incorporated into an embryo at the blastocyst stage of development. The result is a chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knock-out method.

This technique is of particular importance for the study of the genetic control of developmental processes. This technique works particularly well in mice. It has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination.

c) Retrovirus-mediated gene transfer.

To increase the probability of expression, gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

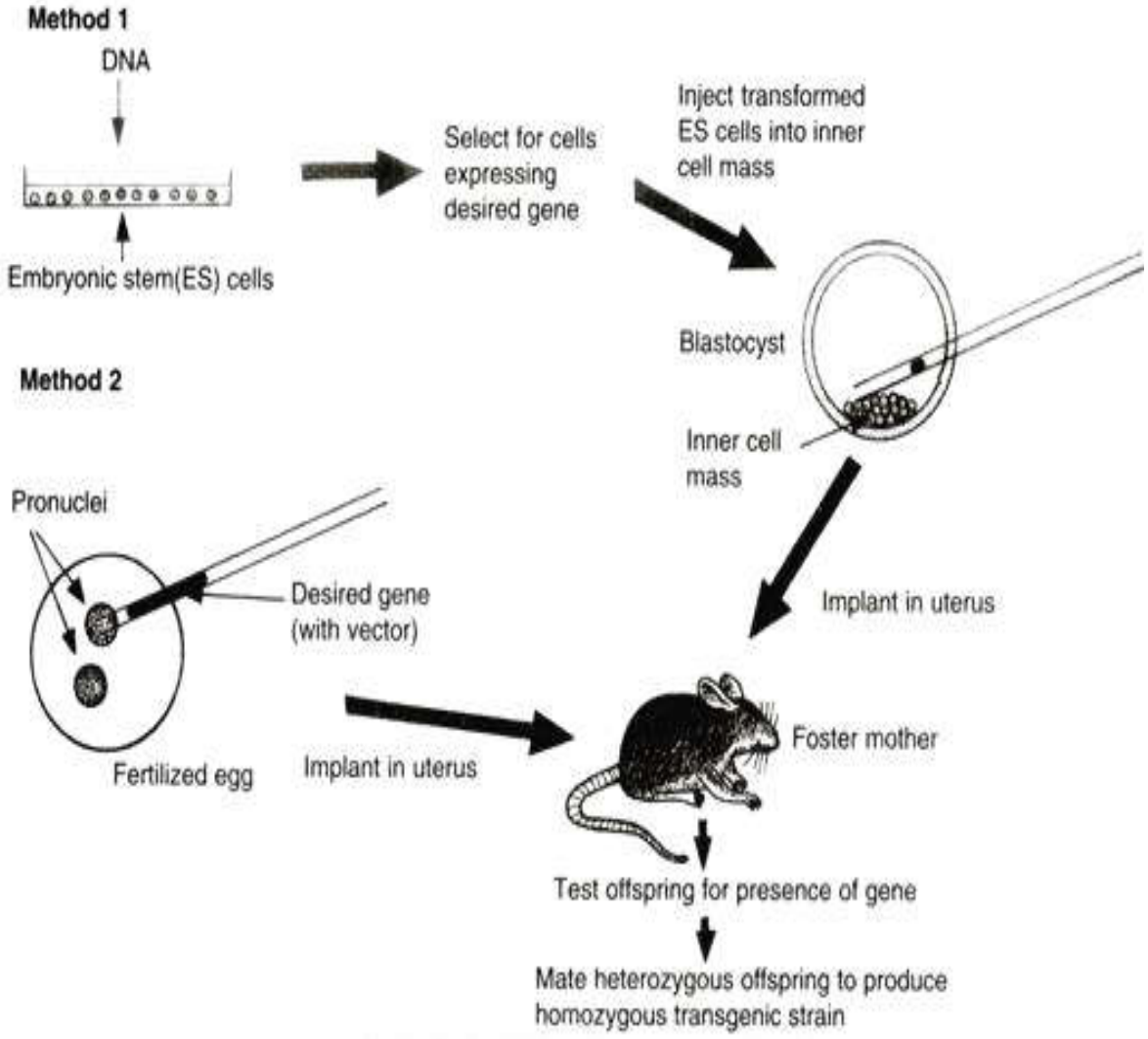
For any of these techniques the success rate in terms of live birth of animals containing the transgene is extremely low. Providing that the genetic manipulation does not lead to abortion, the result is a first generation (F1) of animals that need to be tested for the expression of the transgene. Depending on the technique used, the F1 generation may result in chimeras. When the transgene has integrated into the germ cells, the so-called germ line chimeras are then inbred for

10 to 20 generations until homozygous transgenic animals are obtained and the transgene is present in every cell. At this stage embryos carrying the transgene can be frozen and stored for subsequent implantation.

The Transgenic Mouse:

It is designed to support investigators doing biology of aging research by creating mice that have been genetically altered by either inserting a new gene or removing a normal gene.

This method has become one of the most exciting approaches of discovering the functions and interactions of genes in mammals. At the University Of Washington, Nathan Shock Center, this transgenic technology is used to develop new animal models for studying genetic mechanisms of the aging process.



Method of producing transgenic mice

During the previous year, transgenic mouse production has focused on constructs with enhanced defense against free radical injury in aging (e.g., catalase, superoxide dismutase, glutathione S-transferase), Werner Syndrome, adult onset diabetes, Alzheimer's disease, thrombospondin, and rheumatoid arthritis in aging. Almost 4000 embryos, mainly of the C57BL/6 ingred strain, have been transferred, 498 pups analyzed and at least 40 contained the integrated construct.

The creation of "transgenic" animals that make a specified gene product presents a spectrum of opportunities for basic studies in molecular pathogenesis and pre-clinical investigations applicable to a wide variety of medical problems of aging. An additional gene transfer technology developed in the 1980's involved the use of stem cells from the early embryo, so-called embryonic stem (ES) cells. The capacity of ES cells to undergo differentiation makes them useful for investigating the effects of genetic modifications of either the gain of function or loss of function.

Recombinant insulin

Insulin, synthesized by the b-cells of the islets of Langerhans in the pancreas, controls the level of glucose in the blood. An insulin deficiency manifests itself as diabetes mellitus, a complex of symptoms which may lead to death if untreated. Fortunately, many forms of diabetes can be alleviated by a continuing program of insulin injections, thereby supplementing the limited amount of hormone synthesized by the patient's pancreas. The insulin used in this treatment was originally obtained from the pancreas of pigs and cows slaughtered for meat production. Although animal insulin is generally satisfactory, problems may arise in its use to treat human diabetes. One problem is that the slight differences between the animal and the human proteins can lead to side effects in some patients. Another is that the purification procedures are difficult, and potentially dangerous contaminants cannot always be completely removed. Insulin displays two features that facilitate its production by recombinant DNA techniques.

The first is that the human protein is not modified after translation by the addition of sugar molecules (p. 236): recombinant insulin synthesized by a bacterium should therefore be active. The second advantage concerns the size of the molecule. Insulin is a relatively small protein, comprising two polypeptides, one of 21 amino acids (the A

chain) and one of 30 amino acids (the B chain). In humans these chains are synthesized as a precursor called preproinsulin, which contains the A and B segments linked by a third chain (C) and preceded by a leader sequence. The leader sequence is removed after translation and the C chain excised, leaving the A and B polypeptides linked to each other by two disulphide bonds.

Several strategies have been used to obtain recombinant insulin. One of the first projects, involving synthesis of artificial genes for the A and B chains followed by production of fusion proteins in *E. coli*, illustrates a number of the general techniques used in recombinant protein production.

Synthesis and expression of artificial insulin genes

In the late 1970s, the idea of making an artificial gene was extremely innovative. Oligonucleotide synthesis was in its infancy at that time, and the available methods for making artificial DNA molecules were much more cumbersome than the present-day automated techniques. Nevertheless, genes coding for the A and B chains of insulin were synthesized as early as 1978.

The procedure used was to synthesize trinucleotides representing all the possible codons and then join these together in the order dictated by the amino acid sequences of the A and B chains. The artificial genes would not necessarily have the same nucleotide sequences as the real gene segments coding for the A and B chains, but they would

still specify the correct polypeptides. Two recombinant plasmids were constructed, one carrying the artificial gene for the A chain, and one the gene for the B chain. In each case the artificial gene was ligated to a *lacZ′*reading frame present in a pBR322-type vector. The insulin genes were therefore under the control

of the strong *lac* promoter (p. 231), and were expressed as fusion proteins, consisting of the first few amino acids of b-galactosidase followed by the A or B polypeptides. Each gene was designed so that its b-galactosidase and insulin segments were separated by a methionine residue, so that the insulin polypeptides could be

cleaved from the b-galactosidase segments by treatment with cyanogen bromide (p. 233). The purified A and B chains were then attached to each other by disulphide bond formation in the test tube.

The final step, involving disulphide bond formation, is actually rather inefficient. A subsequent improvement was to synthesize not the individual A and B genes, but the entire proinsulin reading frame, specifying B chain–C chain–A chain. Although this is a more daunting proposition in terms of DNA synthesis, the prohormone

has the big advantage of folding spontaneously into the correct disulphide-bonded structure. The C chain segment can then be excised relatively easily by proteolytic cleavage.

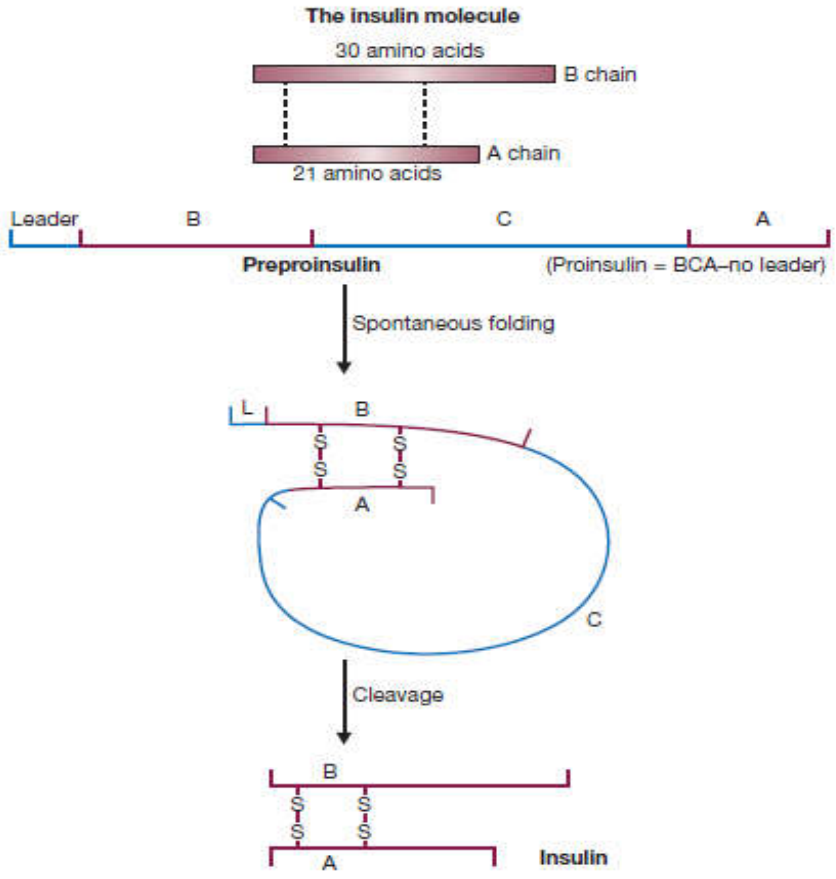


Fig: The structure of the insulin molecule and a summary of its synthesis by processing from preproinsulin

Knockout mice

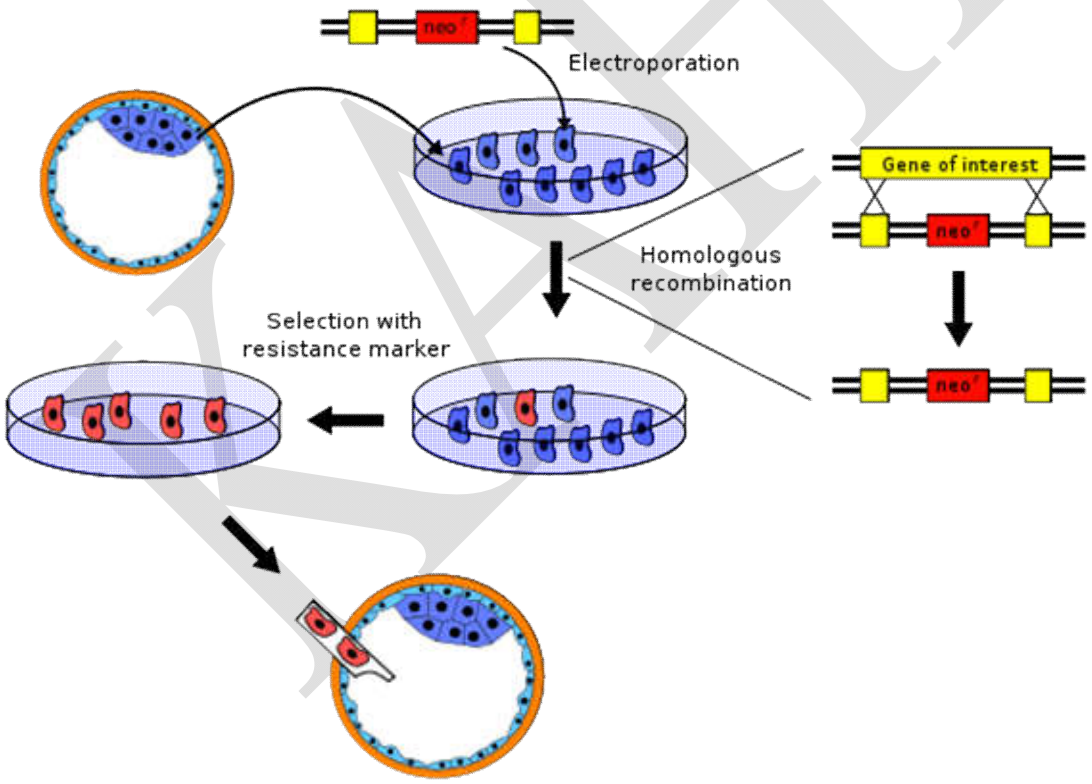
A knockout mouse or knock-out mouse is a genetically modified mouse (*Mus musculus*) in which researchers have inactivated, or "knocked out", an existing gene by replacing it or disrupting it with an artificial piece of DNA. They are important animal models for studying the role of genes which have been sequenced but whose functions have not been determined. By

causing a specific gene to be inactive in the mouse, and observing any differences from normal behaviour or physiology, researchers can infer its probable function.

Use

Knocking out the activity of a gene provides information about what that gene normally does. Humans share many genes with mice. Consequently, observing the characteristics of knockout mice gives researchers information that can be used to better understand how a similar gene may cause or contribute to disease in humans. Examples of research in which knockout mice have been useful include studying and modeling different kinds of cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson's disease. Knockout mice also offer a biological and scientific context in which drugs and other therapies can be developed and tested. Millions of knockout mice are used in experiments each year.

Procedure

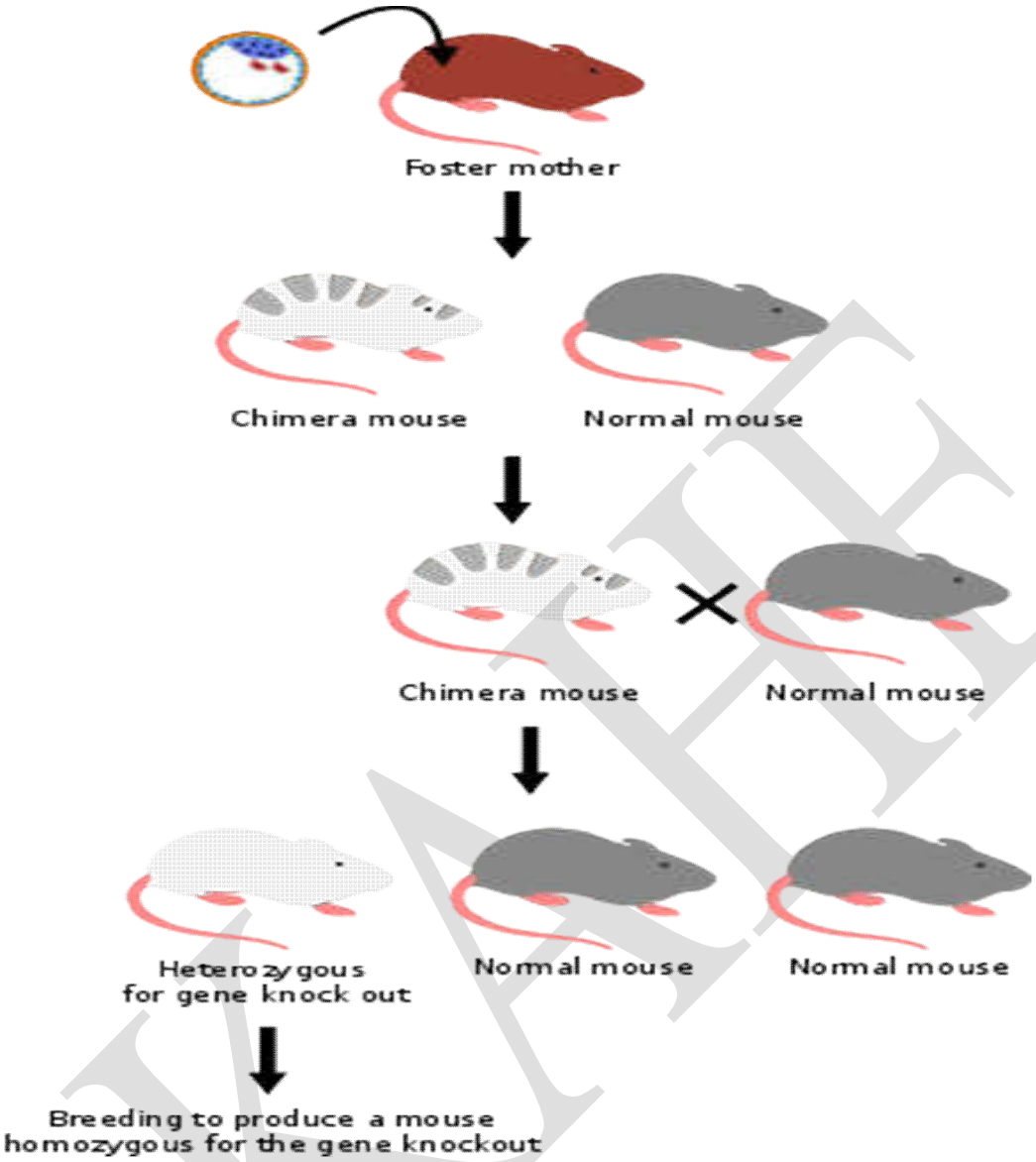


There are several variations to the procedure of producing knockout mice; the following is a typical example.

1. The gene to be knocked out is isolated from a mouse gene library. Then a new DNA sequence is engineered which is very similar to the original gene and its immediate neighbour sequence, except that it is changed sufficiently to make the gene inoperable. Usually, the new sequence is also given a marker gene, a gene that normal mice don't have and that confers resistance to a certain toxic agent (e.g., neomycin) or that produces an observable change (e.g. colour or fluorescence). In addition, a second gene, such as herpes tk+, is also included in the construct in order to accomplish a complete selection.
2. Embryonic stem cells are isolated from a mouse blastocyst (a very young embryo) and grown *in vitro*. For this example, we will take stem cells from a white mouse.
3. The new sequence from step 1 is introduced into the stem cells from step 2 by electroporation. By the natural process of homologous recombination some of the electroporated stem cells will incorporate the new sequence with the knocked-out gene into their chromosomes in place of the original gene. The chances of a successful recombination event are relatively low, so the majority of altered cells will have the new sequence in only one of the two relevant chromosomes – they are said to be heterozygous. Cells that were transformed with a vector containing the neomycin resistance gene and the herpes tk+ gene are grown in a solution containing neomycin and Ganciclovir in order to select for the transformations that occurred via homologous recombination. Any insertion of DNA that occurred via random insertion will die because they test positive for both the neomycin resistance gene and the herpes tk+ gene, whose gene product reacts with Ganciclovir to produce a deadly toxin. Moreover, cells that do not integrate any of the genetic material test negative for both genes and therefore die as a result of poisoning with neomycin.
4. The embryonic stem cells that incorporated the knocked-out gene are isolated from the unaltered cells using the marker gene from step 1. For example, the unaltered cells can be killed using a toxic agent to which the altered cells are resistant.
5. The knocked-out embryonic stem cells from step 4 are inserted into a mouse blastocyst. For this example, we use blastocysts from a grey mouse. The blastocysts now contain two types of stem cells: the original ones (from the grey mouse), and the knocked-out cells (from the white mouse). These blastocysts are then implanted into the uterus of female

mice, where they develop. The newborn mice will therefore be chimeras: some parts of their bodies result from the original stem cells, other parts from the knocked-out stem cells. Their fur will show patches of white and grey, with white patches derived from the knocked-out stem cells and grey patches from the recipient blastocyst.

6. Some of the newborn chimera mice will have gonads derived from knocked-out stem cells, and will therefore produce eggs or sperm containing the knocked-out gene. When these chimera mice are crossbred with others of the wild type, some of their offspring will have one copy of the knocked-out gene in all their cells. These mice will be entirely white and are not chimeras, however they are still heterozygous.
7. When these heterozygous offspring are interbred, some of their offspring will inherit the knocked-out gene from both parents; they carry no functional copy of the original unaltered gene (i.e. they are homozygous for that allele).



Applications of transgenic animals

The animal whose genetic material is taken from another organism is called as transgenic animal. The artificial genes are inserted in the germline cells of the organisms so they pass from one generation to the other. First transgenic animal was mouse which is the most important animal for doing experiments in the laboratory. But after that many other animals were also genetically modified through genetic engineering techniques for example rabbit, pig, sheep and other cattle. There are two reasons due to which the animals are produced through transgenic techniques:

1) There is one reason of producing transgenic animals that they can be beneficial economically. For example genetically modified cows give more milk then other cows and their milk contains human proteins which can be used to treat the disease of emphysema in humans.

2) Some transgenic animals are used as models for detecting, diagnosing and treating different diseases. For example most common laboratory animals are mice and rabbits. Scientists of Harvard produced a mouse carrying the genes which help in development of cancer. They named the mouse oncomouse or Harvard mouse.

Applications of Transgenic Animals in

Medicine:-

We can say that medicine is one field which has took benefit of transgenic animals most. Every year people die of kidney failure, heart failure and due to other organs failure. But now scientists have succeeded in producing transgenic animals like pigs which carry the organs needed for humans. Pig is responsible for providing the organs to the humans and these organs can be inserted into the human body through transplantation. There are chances that pig protein might hinder organ transplantation but scientists are working on this issue and are thinking of replacing this protein with the human protein. United Kingdom is in the need of organs for its patients and according to the survey, almost 5000 organs are needed to be transplanted in patients with different diseases.

Transgenic animals have already helped in developing certain growth hormones. For this purpose, special genes are inserted in the milk of the cow, which increase the production of the milk. Similarly various genetic and hereditary diseases like cystic fibrosis and phenylketonuria can be treated by manufacturing milk which is developed with transgenesis.

Human gene therapy has provided opportunities for medicine that in a certain genetic or acquired disease, damaged genes can be replaced with the healthy genes and their function is secured.

Agriculture:-

Agriculture is another field which has taken advantage of transgenic animals. In livestock, when transgenic animals are bred with the normal animals, the generation produced contains healthy organs, meat and milk. Transgenic techniques have made quick production of animals while traditional methods of animals breeding are time taking. Quality of the animals also increases and they are the source of providing good quality food to humans in the form of milk and meat.

Industry:-

Two scientists of Canada succeeded in inserting spider genes in the goats who gave milk. The goats produced silk with the milk in large amounts. It was a good source of economy for the industrialists. Polymer strands of the silk were extracted from the milk and light threads were made. This thread could be used in the making uniforms of soldiers and also the tennis rockets.

Animal biotechnology is a branch of **biotechnology** in which molecular biology techniques are used to genetically engineer (i.e. modify the genome of) **animals** in order to improve their suitability for pharmaceutical, agricultural or industrial applications.

Genetically Modified Foods and Social Concerns

Biotechnology is providing us with a wide range of options for how we can use agricultural and commercial forestry lands. The cultivation of genetically modified (GM) crops on millions of *hectares* of lands and their injection into our food chain is a huge global genetic experiment involving all living beings. Considering the fast pace of new advances in production of genetically modified crops, consumers, farmers and policymakers worldwide are challenged to reach a consensus on a clear vision for the future of world food supply.

The current food biotechnology debate illustrates the serious conflict between two groups: 1) Agri-biotech investors and their affiliated scientists who consider agricultural biotechnology as a solution to food shortage, the scarcity of environmental resources and weeds and pests infestations; and 2) independent scientists, environmentalists, farmers and consumers who warn that genetically modified food introduces new risks to food security, the environment and human health such as loss of biodiversity; the emergence of superweeds and superpests; the increase of antibiotic resistance, food allergies and other unintended effects. This article reviews major viewpoints which are currently debated in the food biotechnology sector in the world. It also lays the ground-work for deep debate on benefits and risks of Biotech-crops for human health, ecosystems and biodiversity. In this context, although some regulations exist, there is a need for continuous vigilance for all countries involved in producing genetically engineered food to follow the international scientific bio-safety testing guidelines containing reliable pre-release experiments and post-release track of transgenic plants to protect public health and avoid future environmental harm.

MAJOR ECOLOGICAL RISKS ASSOCIATED WITH GENETICALLY MODIFIED FOOD

Conventional breeding allows mixing and recombination of genetic material between species that share a recent evolutionary history. On the other hand, genetic engineering is an imprecise, haphazard technology and is completely different from traditional plant breeding. With alarming regularity, biotechnology companies have demonstrated that scientists cannot control where genes are inserted and cannot guarantee the resulting outcomes. Unexpected field results highlight the unpredictability of the science, yet combinations previously unimaginable are being field tested and used commercially. The nature of the process of genetic engineering produces unpredictable effects at the genetic and cellular level, which will inevitably have impacts at the ecological level. The followings are some major ecological concerns related to the introduction of GM crop in field conditions.

Potential health hazards: Several studies on Bt crops in particular and GM crops in general show that there are many potential health hazards in foods bio-engineered in this manner. GM-fed animals in various studies have shown that there are problems with growth, organ development and damage, immune responsiveness and so on. It has also been shown from studies elsewhere that genes inserted into GM food survive digestive processes and are transferred into the human body.They are known to have transferred themselves into intestinal bacteria too. Bt toxin had caused powerful immune responses and abnormal cell growth in mice.It has also been shown that all the Cry proteins in Bt crops have amino acid sequence similar to known allergens and are hence potential allergens.

Origin of super weeds: Research suggests that bees may be important pollen vectors over a range of distances and farm-to-farm spread of oilseed rape transgenes will be widespread. Pollen can also travel for miles in the wind and integrate its DNA into the genome of conventional plants. Genes from GE crops can spread to wild plants and native species, resulting in herbicide resistant superweeds. The traditional weed then becomes a stronger "superweed." This outcrossing has started to produce superweeds that are resistant to a wide range of herbicides.

Loss of biodiversity: With development of transgenic crops, traditional varieties may be eliminated as farmers will grow only GM crops to obtain the highest yields for commercial production. Bt (Bacillus thuringiensis) toxins are becoming ubiquitous, highly bioactive

substances in agroecosystems. Bt crops are pumping out huge amounts of toxin from all tissues throughout the growing season, from germination to senescence. Most non-target herbivore insects, although not lethally affected, ingest plant tissue containing Bt protein which they can pass on to their natural enemies. The spread of transgenes into the wild and the effect this will have on biodiversity may be especially severe in less developed countries where native varieties of agricultural crops exist.

Loss of soil fertility: Many crops are engineered with the Bt toxin in order to resist infestation from insects. Yet root exudates from these plants release the toxin into the soil. This stimulates major changes in soil biota that could affect nutrient cycling processes and reduce soil fertility. Scientific studies have shown that Roundup Ready soybeans are toxic to earthworms, beneficial insects, birds and mammals (in addition to destroying the vegetation on which they depend for food and shelter). A study of University of Missouri revealed that Roundup Ready soybeans receiving glyphosate at recommended rates had significantly higher incidence of Fusarium on roots compared with soybeans that did not receive glyphosate.

Effects on non-target insects: The Bt toxin has been shown to be lethal to non-target organisms such as Monarch butterflies, lacewings and ladybird beetles. The issue is broader than whether Bt toxin produced by genetically modified crops imperils beneficial insects. The real issue is that a strategy to establish expression of an insecticidal compound in largescale crop monocultures and thus expose a homogeneous sub-ecosystem continuously to the toxin can cause irreparable damage to natural habitats forever.

Sustainable agriculture and organic farming threatened: The entire future of organic farming is being threatened because pollen transfers by insects and the wind from GE crops to organic farms. Cross pollination can move transgenes into the crops so that, against their intentions, farmers are growing GE crops. Bt microbes are applied by organic farmers as a surface agent (when one is absolutely necessary) and will become ineffective as an important biological insect control tool. Transgenes may cause significant damage to that genetic diversity, and commercialization of a few varieties of patented seeds will also erode this vital heritage. “Terminator” systems designed to protect seed companies’ profits by ensuring that farmers can’t save seed (the succeeding crop will be sterile) are a further step away from sustainable agricultural practices and respect for the diversity of our agricultural heritage.

GM crops provoke immune reactions: Research showed significant immune system changes in mammals fed Bt corn. GM soy and corn each contain two new proteins with allergenic properties (Irina, 2006). GM soy has up to seven times more trypsin inhibitor-a known soy allergen and skin prick tests show some people react to GM, but not to non-GM soy. Perhaps the US epidemic of food allergies and asthma is a casualty of genetic manipulation.

Safety

Potential human health impacts, including allergens, transfer of antibiotic resistance markers, unknown effects.

Potential environmental impacts, including: unintended transfer of transgenes through cross-pollination, unknown effects on other organisms (e.g., soil microbes) and loss of flora and fauna biodiversity.

Access and Intellectual Property

Domination of world food production by a few companies.

Increasing dependence on industrialized nations by developing countries.

Biopiracy, or foreign exploitation of natural resources.

Ethics

Violation of natural organisms' intrinsic values

Tampering with nature by mixing genes among species.

Objections to consuming animal genes in plants and vice versa.

Stress for animal.

Labeling

Not mandatory in some countries (e.g., United States).

Mixing GM crops with non-GM products confounds labeling attempts.

Society

New advances may be skewed to interests of rich countries.

POSSIBLE QUESTIONS

1. Give an account on gene transfer techniques.
2. Discuss in detail about the method of engineering transgenic mice
3. Detailed note on herbicide resistant plants and their importance.
4. Describe the societal impact of genetically modified food.
5. Detailed note on Agro bacterium mediated gene transfer in plants.
6. Explain about stress resistance plants.
7. Discuss in detail about edible vaccines.
8. Explain in detail about the method of engineering transgenic cattle.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
I-M.Sc., BIOCHEMISTRY
RECOMBINANT DNA TECHNOLOGY (17BCP205A)
MULTIPLE CHOICE QUESTIONS

UNIT V

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	The recombinant DNA is introduced into bacterium through _____process	Transfection	Transformation	Transduction	Transdution	Transformation
2	Which gene transfer technique involves a tiny needle which is used to inject DNA into a cell lacking that DNA sequence?	Electroporation	Micro injection	Particle bombardment	Lipofection	Micro injection
3	Agrobacterium tumefaciens infects	Monocotyledon Palnts	Dicotyledon Plants	Both a and b	None	Dicotyledon Plants
4	Ti Plasmids are	Transgenic plasmids	Tumour inducing plasmids	Inhibiting plasmids	Haemorhageic plasmids	Tumour inducing plasmids
5	Which of the following methods for introducing DNA into cells is used only for plants?	A gene 'gun'	Electroporation	Microinjection	Transformation of competent cells	A gene 'gun'
6	Except _____ all are gene transfer technique in plants	Adeno virus	Electroporation	Microinjection	Shot gun	Adeno virus
7	All are vectors in plant except_____	Gemini virus	CaMV	Adeno virus	Monopartite virus	Adeno virus
8	_____ issues are those that involves the impact o	Ethical	Legal	social	scientific	social
9	Use of high electric impulse used to alter cell membrane permeability in	Lipofection	Electroporation	Microinjection	Shot gun	Electroporation
10	Except _____ all are chemical mediated gene transfer	DEAE	PEG	$CaPO_4$	Liposome	Liposome
11	Inert materials used in microcarriers are	Gold, Copper , tungsten	Gold, Platinum , tungsten	Gold, platinum, Copper	Platinum, copper, tunsten	Gold, Platinum , tungsten
12	The genetic material in retroviruses is in the form	DNA molecule	RNA molecules	Proteims	Lipids	RNA molecules
13	Antisense refers to short	DNA sequences	RNA sequences	Both DNA and RNA sequences	Any chemical substances	Both DNA and RNA sequences
14	--------- are designed to be complementary to a specific gene sequence to inhibit activity	Oligonucleotides	Gene fragments	Similar sequences	Antisense oligonucleotide	Antisense oligonucleotide
15	In principle, antisense technology is supposed to prevent	Protein production from a targeted gene	Amino acid synthesis	Mutate cells	Elongate the cell cycle	Protein production from a targeted gene
16	RNA interference is a mechanism	DNA -guide regulation of gene expression	RNA-guided regulation of gene expression	Reverse trancriptase - guided regulation mechanism	Conserved pathway modifiction	RNA-guided regulation of gene expression
17	In RNAi_____ inhibits the expression of genes	Viruses	Double stranded DNA	double-stranded ribonucleic acid	Bacterial DNA	double-stranded ribonucleic acid
18	RNAi pathway is thought to have evolved as a form of	innate immunityagainst viruses	Innate immunity against protozao	Innate immunity against bacteria	Innate immunity against fungi	innate immunityagainst
19	The RNA interference pathway is often exploited in experimental biology	To study the activation of genes	To study the biology of gene	to study the function of genes in cell culture and in vivo in model organisms.	To study the history of genes	to study the function of genes in cell culture and in vivo in model organisms.
20	Vaccines that use components of a pathogenic organism rather than whole organism is called	Peptide vaccines	Triplet vaccines	MAB	Subunit vaccines	Subunit vaccines
21	Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as	knock out	knock Down	knock up	Knock in	knock Down
22	In subunit vaccine for Herpes simplex virus are cloned into	Chines hamster ovary	Lung cell lines	Mamalian cell lines	Hela cell lines	Chines hamster ovary
23	The genome of FMDV consist of	Single stranded DNA	Single stranded RNA	Double stranded DNA	Double stranded RNA	Single stranded RNA
24	Hexadimeric enterotoxin of Vibryo cholerae made of	Single A and 5 identical B subunit	Single B and identical a subunit	Single alpha subunit	Sinale beta subunit	Single A and 5 identical B subunit
25	There are ---------- of interferons	Two major classes	Three major classes	Five major classes	Six major classes	Three major classes
26	Golden rice is a variety of	*Oryza glaberrima*	Oryza sativa	*Oryza triticum*	*Oryza annum*	*Oryza sativa*
27	Which vector is mostly used in crop improvement?	Plasmid	Cosmid	Phagemid	Agrobacterium	Agrobacterium
28	Substances released by plants in response to wounding and can induce vir genes of Ti plasmid is	Opines	Acetosyringone	Nopaline	Auxin	Acetosyringone
29	The genes which help in transfer of T-DNA	Tra genes	vir genes	Occ genes	Noc gene	vir genes
30	Genes of Ti plasmid that help in auxin biosynthesis is	vir D	vir E	vir B	vir G	vir D
31	Genes of Ti plasmid that help in auxin biosynthesis is	iaaH	iaaM	ipt	iaaH and iaaM	iaaH and iaaM
32	Vir E codes for	Single strand binding proteins	Topoisimerase	Polymerase	Helicase	Single strand binding proteins
33	The genes which produces toxic crystals in Bacillus thuringenesis is	Vir genes	Cry genes	Nif genes	Tra genes	Cry genes
34	The tonic crystals in B.thuringenesis kills the insect by	Causing pores in epithelial cells	By blocking respiration c	By inhibition of metabolic enzymes	By inhibiting production of eggs	Causing pores in epithelial cells

35	Glyophosate acts as herbicide by inhibiting	5-end pyruvyl shikimate 3 phosphate synthetase	Acetolactate synthetase	Photosynthesis	Photothricin acetyl transferase	5-end pyruvyl shikimate 3 phosphate synthetase
36	Bromoxynil, a herbicide acts by inhibiting	Acetolactase synthetase	Photosynthesis	EPSP synthetase	Auxin production	Photosynthesis
37	The herbicide bromoxynil is inactivated by the enzyme	ALA synthetase	Nitrilase	EPSP synthetase	PA transferase	Nitrilase
38	Genes coding for …… are integrated into plants for preventing fruit ripening and softening during transport	Poly galacturonase	Pectinase	Ethylene	Auxin	Poly galacturonase
39	Genes which code for nitrilase confers bromoxynil resistance is	Bar genes	Bxn genes	Cry genes	Trp genes	Bxn genes
40	The gene coding for…… is used to produce phosphinothricin tolerant plants	Phosphinothricin	Phosphinothricin acetyl transferase	Phosphinothricin butyl transferase	Phosphino acetyl transferase	Phosphinothricin acetyl transferase
41	The first transgenic plant was	Tobacco	Pea	Flax	Cotton	Tobacco
42	Which of the following gene can be used for making resistance against herbicide broxinil?	Nitrilase	Glutathione S Transferase	Phosphinothricin acetyl transferase	Nitratase	Nitrilase
43	Which cell based plant technology involve the combining of two cells without cell wall from different species?	Clonal propagation	Cybradisation	Protoplast fusion	Mutant selection	Protoplast fusion
44	Which of the following gene cannot be used for making resistance against viral infection?	Gene for capsid protein	Gene for nucleocapsid protein	Satellite DNA	Gene for revervse transcriptase	Gene for revervse transcriptase
45	Which of the following dies from Ti plasmid infection	Rice	Corn	Sorghum	Rice, corn and sorghum	Rice, corn and sorghum
46	Which tropical fruit crop has been successfully engineered to be protected against lethal virus?	Papaya	Passion fruit	Mango	Lemon	Papaya
47	_____ can be grown in suspension culture	MCF-7	3T3-Adipocyte	Hela -cell line	Cells of leukemia	Cells of leukemia
48	Embryonic cells can be used in the treatment of all except	Diabetes	Parkinsons disease	Kidney stone	Aging	Kidney stone
49	Dye used in cell viability assay is	Methylene blue	Tryphan blue	Crystal violet	Phenol red	Tryphan blue
50	Which is not a cell viability assay	Dye exclusion	Dye inclusion	TUNEL assay	Chromium uptake assay	TUNEL assay
51	Successful maintenance of stem cells in culture requires	Trypsin	Feeder layer	Stirrer	CO_2	Feeder layer
52	The gene introduced in to animal is called as	Trnas marker	Transgene	Foreign DNA	Recombinant DNA	Transgene
53	GMO refers to	Gene Modifying option	Genetically modifying organism	Genetic markers organisation	Gen making option	Genetically modifying organism
54	Important animal for most transgenic experiment is	Pig	Cow	Mice	Fish	Mice
55	All techniques except_____ is used in transgenic mice production	Retro viral method	Micro injection method	Embryonic stem cell method	Lipofection	Lipofection
56	The size of DNA transfer in retro viral method in transgenic mice production is	10kb	8 kb	12 kb	14 kb	8 kb
57	In transgenic mice production the gene is introduced into _____	Sperm	Egg	Fertilised egg	Ovum	Fertilised egg
58	In ES method of transgenic mice production the gene in introduced through	Microinjection	Electroporation	a&b	lipofection	a&b
59	Selection of tranformed cell in transgenic mice production takes place in _____medium	EMEM	DMEM	HAT	RPI	HAT
60	Which promoter is used for increasing the expression of transgene in mice	trp	tac	lac	MMT	MMT