Semester – IV

17MBU413 RECOMBINANT DNA TECHNOLOGY – PRACTICAL (4H – 2C)

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 9 Hours

SCOPE

This paper imparts the concepts of rDNA technology and their applications and Acquire knowledge on the applications of genetic engineering.

OBJECTIVES

- To learn the basic tools in recombinant technology
- To understand the various concepts of cloning vectors and cloning strategies
- To emphasize the knowledge in biotechnology and techniques.

EXPERIMENTS

1. Preparation of competent cells for transformation.
2. Demonstration of Bacterial Transformation and calculation of transformation efficiency.
3. Digestion of DNA using restriction enzymes and analysis by agarose gel electrophoresis
4. Ligation of DNA fragments.
5. Cloning of DNA insert and Blue white screening of recombinants.
6. Interpretation of sequencing gel electropherograms.
7. Designing of primers for DNA amplification.
8. Amplification of DNA by PCR.
9. Demonstration of Southern blotting.

Suggested reading

1. Brown TA. (2010). Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
2. Clark DP and Pazdernik NJ. (2009). Biotechnology: Applying the Genetic Revolution. Elsevier Academic Press, USA.
3. Primrose SB and Twyman RM. (2006). Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
4. Sambrook J and Russell D. (2001). Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Press
5. Wiley JM, Sherwood LM and Woolverton CJ. (2008). Prescott, Harley and Klein's Microbiology. McGraw Hill Higher Education
6. Brown TA. (2007). Genomes-3. Garland Science Publishers
7. Primrose SB and Twyman RM. (2008). Genomics: Applications in human biology. Blackwell Publishing, Oxford, U.K.

UNIT I

Duration	Topic	Reference
01	Milestones in genetic engineering and biotechnology cloning tools	T1 : 1-20
01	Cloning requires specialized tools	T1 -3-13
01	Restriction modification systems: Types I, II and III.	T1 : 52-56
01	Restriction Enzymes Types I, II and III.	T1 -52-60
01	Mode of action, nomenclature, applications of Type II restriction enzymes in genetic engineering.	T1 – 52 -56
01	DNA modifying enzymes and their applications: DNA polymerases.	T1: 57- 58
01	Terminal deoxynucleotidyl transferase	T1 59-60
01	kinases and phosphatases	T1: 1,9-50
01	DNA ligases	T1: 56-57
01	Revision	
	Total hours: 10 h	

UNIT II

Duration	Topic	Reference
01	Cloning Vectors: Definition and Properties	T1: 89-90
01	Plasmid vectors: pBR	T1: 92-93
01	pUC series	T1: 92-93
01	BacteriophageVectorM13 based vectors Cosmids	T1: 94-100 102-103
01	cosmids	T2 -18-25
01	BACs, YACs	W1
01	Use of linkers and adaptors. Expression vectors: *E.coli* lac based vectors	T1: 64-67 T2: 372-374
01	T7 promoter-based vectors, yeast YIP, YEP vectors	J1
01	YCP vectors, Baculovirus based vectors	W1
01	mammalian SV40-based vectors	J2
01	Revision	
	Total hours: 9 h	

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UNIT III

Duration	Topic	Reference
01	Transformation of DNA: Chemical method, Electroporation method	
01	Gene delivery: Microinjection, electroporation	T1: 124-125
01	gene gun and liposome	T1: 85-87
01	viral- mediated delivery	T1: 119-120
01	*Agrobacterium* - mediated gene (DNA) delivery	T1: 112-114
01	Agarose gel electrophoresis	T1: 58-59
01	Blotting techniques	W2
01	Dot blot, DNA microarray analysis	W2
01	SDS-PAGE	W2
01	Western blotting	T3 -120-125
01	Revision	
	Total hours: 10 h	

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UNIT IV

Duration	Topic	Reference
01	PCRasics, RT-PCR	T1: 6-9
01	Real-Time PCR, Sanger's method of DNA Sequencing introduction	T1: 173-176
02	Traditional and automated sequencing	T1: 64-67
01	Primer walking	T1: 384-388
01	shotgun sequencing	T1: 177-179
01	Revision	
	Total hours: 7 h	

UNIT V

Duration	Topic	Reference
01	Genomic libraries	T4: 51-53
01	cDNA libraries	T4: 51-53
01	screening of libraries	W1
01	Colony hybridization	-217-225
01	Chromosome walking and jumping	T1: 177-179
01	Products of r DNA technology , site directed mutagenesis	T4- 371-373
01	Insulin and hGH	T4: 297-299 T4: 303-305
01	Bt cotton, brinjal, gene therapy recombinant vaccines	T5: 51-54
01	Protein engineering and site directed mutagenesis	T4: 371-373
01	Revision	
	Total hours: 10 h	

References

T1: Brown TA (2010) Gene cloning – An introduction DNA analysis 6th edition, Blackwell publishing, oxford, UK.

T2: Wiley, Sherwood and Woolverton (2008). Prescott, Harley and Klein's Microbiology, Mc graw Hill Higher Education.

T3: S.N.Jogdand. Gene biotechnology. Himalaya publishing house.2003.

T4: Gary Wash (2007). Pharmaceutical Biotechnology, concepts and applications, Wiley.

T5: Agnes, Swrinder, Shelby (2014) Plant biotechnology, Springer.

J1: Victoria Lundblad (2014) yeast cloning vector and genes, current protocols in molecular biology. 21(1):1-10.

J2: Khan kH (2013) Gene expression in mammalian cells and its application; DOI-10.5681/apb.2013.042.

W1: WWW. Nptel.ac.in.

W2: WWW.ncbi.nlm.nih.gov

Unit –I

Syllabus

Milestones in genetic engineering and Biotechnology cloning Tools; Restriction modification systems: Types I, II and III. Mode of action, nomenclature, applications of Type II restriction enzymes in genetic engineering. DNA modifying enzymes and their applications: DNA polymerases. Terminal deoxynucleotidyl transferase, kinases and phosphatases, and DNA ligases

GENETIC ENGINEERING

Biological engineering seems to fall naturally into three primary categories of means to modify organisms. These are:

1. The recombination of existing genes, or eugenics.
2. The production of new genes by a process of directed mutation (manipulation of genetic material in a organism), or genetic engineering.
3. Modification or control of gene expression, or to adopt Lederberg's suggested terminology, euphenic engineering.

Genetic engineering deals with the development and application of scientific methods, procedures, and technologies that permit direct manipulation of genetic material in order to alter the hereditary traits of a cell, organism, or population.

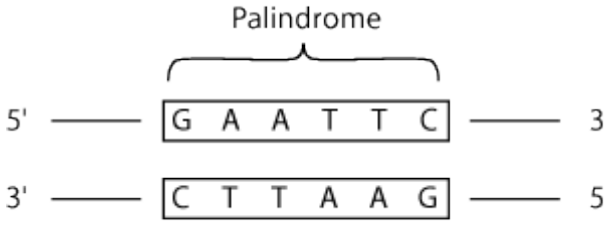
Milestones in genetic engineering:

- 1869: Friedrich Miescher discovered nuclein (a compound that consisted of nucleic acid that he extracted from white blood cells).
- In 1888, Heinrich Wilhelm Gottfried Von Waldeyer-Hartz, a German scientist, coined the term 'Chromosome.'
- In 1909, the term 'Gene' had already been coined by Wilhelm Johannsen (1857-1927), who described 'gene' as carrier of heredity. Johannsen also coined the terms 'genotype' and 'phenotype'.
- 1909: Genes are linked with hereditary disorders.
- The principle of genetics in inheritance was redefined by T.H. Morgan, who showed inheritance and the role of chromosomes in inheritance by using fruit flies. This landmark work was named, 'The theory of the Gene in 1926.'
- 1931: Barbara McClintock and Harriet Creighton demonstrate the linking of DNA from different chromosomes.
- 1941: The term 'genetic engineering' is first used by a Danish microbiologist.
- 1953: James Watson and Francis Crick proposed the double helix structure of DNA.
- 1955: An enzyme, DNA polymerase, involved in the synthesis of a nucleic acid, is isolated for the first time.
- 1960: French scientists discover messenger RNA (mRNA).
- 1961: Scientists understand genetic code for the first time.
- 1964: The existence of reverse transcriptase is predicted.

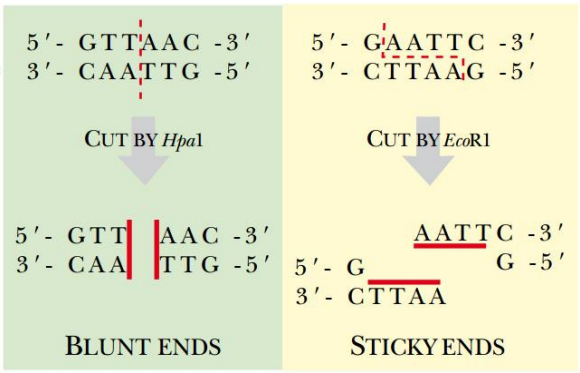
- 1966- Marshall Nirenberg & Har Gobind Khorana finished unravelling the genetic code.
- 1973- Stanley Cohen and Herbert Boyer invented the technique of DNA cloning, which allowed genes to be transplanted between different biological species (Cohen and Boyer perform the first successful recombinant DNA experiment, using bacterial genes).
- 1974- Stanley Cohen, Annie Chang and Herbert Boyer create the first genetically modified DNA organism (developed a technique for splicing together strands of DNA from more than one organism. The product of this transformation is called recombinant DNA (rDNA).
- Kohler and Milestein in 1975 came up with the concept of cytoplasmic hybridization and produced the first ever monoclonal antibodies, which has revolutionized diagnostics.
- 1978: Recombinant human insulin is produced for the first time.
- 1979: Human growth hormone is synthesized for the first time.
- 1980- First transgenic (genetically modified) mouse.
- In 1983, Kary Mullis developed polymerase chain reaction (PCR), which allows a piece of DNA to be replicated over and over again. PCR, which uses heat and enzymes to make unlimited copies of genes and gene fragments, later becomes a major tool in biotech research and product development worldwide.
- 1983: The first genetic transformation of plant cells by TI plasmids is performed.
- 1984- Development of genetic fingerprinting, a technique that has greatly helped the police force in finding and identifying criminals.
- 1985- First transgenic domestic animal, a pig
- 1986: The first recombinant vaccine for humans, a vaccine for hepatitis B, is approved.
- 1987- Only 7 years after the first transgenic mouse, a series of mouse are produced carrying human genes. - Also in this year, the first transgenic plant was created to resist a certain type of herbicide.
- 1988: The first pest-resistant corn, Bt corn, is produced.
- 1991- First gene therapy trials on humans.
- 1995 1996 Roundup Ready Soybeans (soy beans resistant to glyphosate herbicide (Roundup)) introduced in the USA.
- 1996 The birth of the first cloned animal, Dolly the sheep, was announced.
- 1999- September, first publicly reported patient death in a gene therapy trial caused by the gene therapy itself.
- 2003: The Human Genome Project completes sequencing of the human genome.
- 2007: Scientists discover how to use human skin cells to create embryonic stem cells
- 2009: FDA approves the first genetically engineered animal for production of a recombinant form of human antithrombin.
- 2013: Researchers in Japan developed functional human liver tissue from reprogrammed skin cells.

Note:

Palindromic sequence: A palindromic sequence is a sequence made up of nucleotides within double helix of DNA and/or RNA that is the same when read from 5' to 3' on one strand and 5' to 3' on the other, complementary, strand.



Blunt ends and sticky ends: At the cleavage site, different restriction enzymes cut DNA in one of two ways. Some enzymes make incisions in each strand at a point immediately opposite to another, producing "blunt end" DNA fragments. Most enzymes cut the two strands at a point not directly opposite each other, producing an overhang in each strand. These are called "sticky ends".



Cleavage site / restriction site / restriction recognition site are locations on a DNA molecule containing specific (4-8 base pairs in length) sequences of nucleotides, which are recognized by restriction enzymes.

Biotechnology cloning Tools:

Restriction modification systems

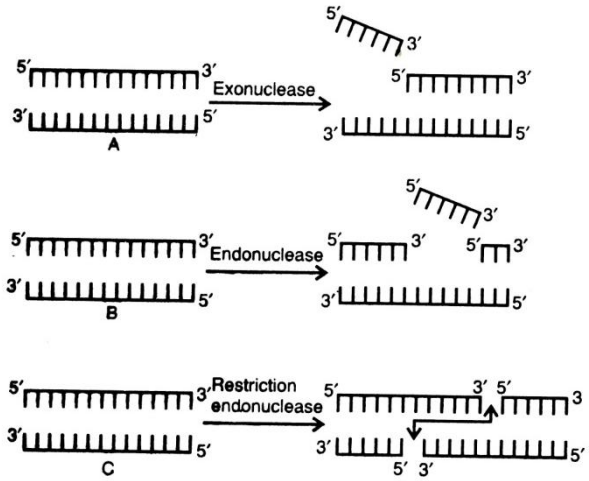
Restriction–modification systems allow bacterial cells to distinguish between their own DNA and any foreign DNA entering the cell, and to destroy the latter. They operate through two enzyme activities: a restriction nuclease that cleaves the foreign DNA, and a modification methyltransferase that protects the host DNA. The majority of R-M systems can be classified as type I, II, and III on the basis of enzyme structure, cofactor requirements, structure of the DNA recognition site, and location of DNA cleavage relative to the recognition site.

Types of nucleases:

1. Structure specific nuclease
2. Sequence specific nuclease

2.1 Exonuclease: A nuclease that functions by removing nucleotides from the ends of the DNA molecule is called an exonuclease

2.2 Endonuclease: Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain.



Types of Restriction nucleases

There are three distinct types of restriction nucleases.

I. Type I restriction nucleases are complex nucleases, and have recognition sequences of about 15 bp. They cleave the DNA about 1000 bp away from the 5'-end of the sequence "TCA" located within the recognition site. The cofactors S-Adenosyl methionine (AdoMet), hydrolyzed adenosine triphosphate (ATP), and magnesium (Mg^{2+}) ions, are required for their full activity (e.g. EcoRI, EcoRII).

II. Type II restriction nucleases are remarkably stable with recognition sites that are usually undivided and palindromic and 4–8 nucleotides in length and induce cleavage within their recognition sequences. It can either cleave at the center of both strands to yield a blunt end, or sticky ends. More than 350 different type II nucleases with over 100 different recognition sequences are known. They require Mg^{2+} ions for cleavage (e.g. HindIII).

III. Type III restriction nucleases are intermediate between the type I and type II enzymes. They recognize two separate non-palindromic sequences that are inversely oriented. They cut DNA about 20–30 base pairs after the recognition site (e.g. EcoP15).

Mechanism Type II restriction nucleases

Phosphodiester bond hydrolysis by Type II restriction endonucleases follows an SN2-type mechanism, which is characterized by inversion of configuration at phosphorous. The general mechanism of phosphodiester hydrolysis comprises three steps: (i) the preparation of the attacking nucleophile by deprotonation,

$H_2O + B + R'\text{-}O5'\text{-}(PO_2)\text{-}O3'\text{-}R'' \longrightarrow OH^- + BH^+ + R'\text{-}O5'\text{-}(PO_2)^-\text{-}O3'\text{-}R''$

(ii) the nucleophilic attack of the hydroxide ion on the phosphorous leading to the formation of the pentavalent transition state,

$OH^- + R'\text{-}O5'\text{-}(PO_2)\text{–}O3'\text{-}R'' \longrightarrow R'\text{-}O5'\text{-}(PO_2OH)^2\text{–}O3'\text{-}R''$

(iii) the departure of the 3' hydroxyl leaving group:

$-O5'\text{-}(PO_3)^2\text{–}O3'\text{-} + H_2O \longrightarrow R'\text{-}O5'\text{-}(PO_2)\text{-}O^- + HO_3'\text{-}R'' + OH^-$

To achieve efficient catalysis, all three steps require an assisting group: (i) a base to deprotonate the water molecule; (ii) a Lewis acid that stabilizes the pentavalent transition state with two negative charges; and (iii) an acid that protonates the leaving 3¢-oxyanion. The mechanism of restriction endonuclease catalysis can only be described if these groups are identified. The catalytic centres of Type II restriction endonucleases generally contain a PD…D/ExK motif. The negatively charged side chains serve to ligate a divalent metal ion cofactor, usually Mg2+ that is obligatory for catalysis. Lysine that is often considered as a general base candidate, however, is not strictly conserved; for example, in BamHI it is replaced by glutamate, and in BglII by glutamine. The major controversy regarding the mechanism of DNA cleavage by restriction endonucleases is about the number of divalent metal ions involved in the catalytic process. This is attributable to some crystal structures having one divalent cation, while others have two divalent cations associated with the catalytic centre, or that the divalent metal ions are located in different positions, or that individual subunits in a crystal differ in divalent metal ion occupancy. The mechanistic models for DNA cleavage by restriction endonuclease are based on the number of metal ions involved in the reaction.

Nomenclature of Restriction nucleases

The nomenclature of restriction endonucleases follows a general pattern.

(1) The first letter of the name of genus in which a given enzyme is first discovered is written in capital.

(2) This is followed by the first two letters of species name of the organism. These three letters are generally written in italics, e.g., Eco from Escherichia coli, Hin from Haemophilus influenzae, etc.

(3) Strain or type identification is depicted next in Roman. e.g., Ecok;

(4) When an organism produces more than one enzyme, they are identified by sequential Roman numerals, e.g., the different enzymes produced by H. influenzae strain Rd are named Hind II, Hind III, etc.

Restriction endonuclease	Source (organism and strain)	Recognition sequence
AluI	*Arthrobacter luteus*	AG/CT TC/GA
HindIII	*H. influenzae* Rd	A/AGCTTTTCGA/A
Sau3A	*Staphylococcus aureus* 3A	/GATCCTAG/
TaqI	*Thermus aquaticus* YTI	T/CGAAGC/T
EcoP15	*Escherichia coli*	$CAGCAGN_{20...}$/NN $GTCGTCN_{20}$NN

Apa I	*Acetobacter pasteurianus*	GGGCC|C
Bam HI	*Bacillus amiloliquifaciens*	G|GATCC
Bgl II	*Bacillus globigii*	A|GATCT
Cla I	*Caryophanon latum L*	AT|CGAT
Dde I	*Desulfovibrio desulfuricans*	C|TNAG
Dra I	*Deinococcus radiophilus*	TTT|AAA
Eco RI	*Escherichia coli RY13*	G|AATTC
Eco RV	*Escherichia coli J62*	GAT|ATC
Fnu4H I	*Fusobacterium nucleatum 4H*	GC|NGC
Hae III	*Haemophilus aegyptius*	GG|CC
Hind II	*Haemophilus influenzae Rd*	A|AGCTT
Hinf I	*Haemophilus influenzae Rf*	G|ANTC
Kpn I	*Klebsiella pnumoniae OK8*	GGTAC|C
Mbo I	*Moraxella bovis*	|GATC
Msp I	*Morazella sp.*	C|CGG
Nde I	*Neisseria dentrificans*	CA|TATG
Not I	*Nocardia otitidis-caviarum*	GC|GGCCGC
Pst I	*Providencia stuartii 164*	CTGCA|G
Pvu II	*Proteus vulgaris*	CAG|CTG
Rsa I	*Rhodopseudomonas sphaeroides*	GT|AC
Sma I	*Serratio marcescens S*	CCC|GGG
Taq I	*Thermus aquaticus YT1*	T|CGA
Xba I	*Xanthomonas badrii*	T|CTAGA

Applications:

- Restriction enzymes isolated from bacterial cells are used in the laboratory to manipulate fragments of DNA; for this reason they are indispensible tools of recombinant DNA technology
- Type II restriction nucleases are used for restriction mapping and gene cloning.

DNA modifying enzymes and their applications

Based on the type of reaction that they catalyse, five classes of DNA modifying enzymes are

I. DNA polymerases:

DNA polymerases are a group of polymerases that catalyze the synthesis of polydeoxyribonucleotides from mono-deoxyribonucleoside triphosphates (dNTPs). DNA polymerase adds nucleotides to the three prime (3')-end of a DNA strand, one nucleotide at a time.

In 1956, Arthur Kornberg and his colleagues discovered DNA polymerase I (Pol I), in *Escherichia coli*. DNA polymerase II was also discovered by Thomas Kornberg (the son of Arthur Kornberg) and Malcolm E. Gefter in 1970 while further elucidating the role of Pol I in *E. coli* DNA replication.

Types: (a) Prokaryotic polymerase:

Pol –I: The primary function of Pol I is polymerization (synthesis of DNA) and it has subsidiary role as an exo-nuclease (removal or degradation of nucleotides). It is mainly involved in short fragment synthesis and repair and requires magnesium as a cofactor. This enzyme is cleaved by trypsin into two fragments, a large fragment of 75 KD and a small fragment of 36 KD.

The large fragment shows $3' \rightarrow 5'$ exonuclease activity and the small fragment shows $5' \rightarrow 3'$ exonuclease activity. Due to $5' \rightarrow 3'$ exonuclease activity it involves in removal of RNA primer and filling the gap between Okazaki fragments by synthesis of DNA fragments.

Pol-II: This is a 90 KD polypeptide mainly involved in $5' \rightarrow 3'$ repair synthesis. It has $3' \rightarrow 5'$ exonuclease activity which shows its involvement in repair. DNA Pol II can synthesize DNA new base pairs at an average rate of between 40 and 50 nucleotides/second.

Pol-III: is the primary enzyme complex involved in prokaryotic DNA replication. It is heterodimer of two polypeptide chains having molecular weight of 900 KD. It is the main and most active enzyme which can add about 150,000 nucleotides per minute during DNA polymerization. However, it requires auxiliary proteins (co-polymerase II) for the synthesis of long DNA strand. It has only $3' \rightarrow 5'$ exonuclease activity. The complex has high processivity (i.e. the number of nucleotides added per binding event) and, specifically

referring to the replication of the *E.coli* genome, works in conjunction with four other DNA polymerases (Pol I, Pol II, Pol IV, and Pol V).

(b) Eukaryotic polymerase: There are four DNA polymerases in eukaryotes called α, β, γ, and δ.

Each polymerase has specific role(s) in DNA replication and repair, shown in the table below.

Polymerase	Function	Exonuclease Actvity
α	Synthesizes the RNA primer, initiations DNA synthesis and the lagging strand	None
β	Repair DNA	None
γ	Replicate mitochondrial DNA	3′ to 5′
δ	Synthesizes the leading strand, filling DNA gaps after removal of primer	3′ to 5′
ε	Repair DNA	3′ to 5′

Applications:

- DNA polymerase I obtained from *E. coli* is used extensively for molecular biology research.
- The Klenow fragments are generated by polymerases that are widely used in molecular biology.

II. Terminal deoxynucleotidyl transferase:

Terminal deoxynucleotidyl transferase (TDT), also known as DNA nucleotidylexotransferase (DNTT) or terminal transferase is a specialized DNA polymerase expressed in immature, pre-B, pre-T lymphoid cells, and acute lymphoblastic leukemia/lymphoma cells. The enzyme terminal deoxynucleotidyl transferase adds mononucleotides onto 3′ hydroxyl termini of either single- or double-stranded DNA. The mononucleotides are donated by a dNTP. Addition of each mononucleotide releases inorganic phosphate. Unlike most DNA polymerases it does not require a template.

The preferred substrate of this enzyme is a 3'-overhang, but it can also add nucleotides to blunt or recessed 3' ends. Cobalt is a necessary co-factor; however the enzyme catalyzes reaction upon Mg and Mn administration *in vitro*.

Applications:

- Terminal transferase has applications in molecular biology.
- It can be used in RACE to add nucleotides which can then be used as a template for a primer in subsequent PCR
- It can also be used to add nucleotides labeled with radioactive isotopes, for example in the TUNEL assay (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) for the demonstration of apoptosis (which is marked, in part, by fragmented DNA).

- Also used in the immunofluorescence assay for the diagnosis of acute lymphoblastic leukemia

III. Kinases:

Polynucleotide kinase (Pnk) was discovered by the Richardson and Hurwitz laboratories in T4 and T2 bacteriophage-infected *Escherichia coli*. T4 Polynucleotide Kinase is also called as 3'-phosphatase and a homotetramer. It consists of four identical subunits of 28.9 kDa. It catalyzes the transfer of the γ-phosphate from ATP of single- and double-stranded DNAs and RNAs as well as oligonucleotides to the 5´-terminus of polynucleotides or to mononucleotides bearing a 5´-hydroxyl group.

Applications:

The T4 polynucleotide kinase is used for labeling the 5'-termini of nucleic acids, and the labeled products can be used int he following ways: - markers for gel-electrophoresis - primers for DNA sequencing - primers for PCR - probes for hybridization - substrates for DNA and RNA ligases - probes for transcrip mapping 5'-phosphorlation of oligonucleotide linkers and DNA or RNA is required before ligation can be performed using the ^{32}P-postlabeling assay, DNA modification can be detected.

IV. Phosphatases:

Alkaline phosphatase is a homodimeric metalloenzyme which hydrolases the phosphomonoester into inorganic phosphate and corresponding alcohol or remove terminal mono-esterified phosphate from ribo-oligonucleotide, doxyribo-oligonucleotide, alkaloids and proteins i.e, enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called *dephosphorylation.*

The two immense uses of alkaline phosphates in DNA modification are

1. Removal of 5’ phosphates from plasmid and bacteriophage vectors by restriction enzyme. In subsequent ligation reactions, this incision prevents self ligation to facilitate the ligation of other fragments into the vector
2. Removal of 5’ phosphates from fragment of DNA prior to labelling with radioactive phosphate

Applications:

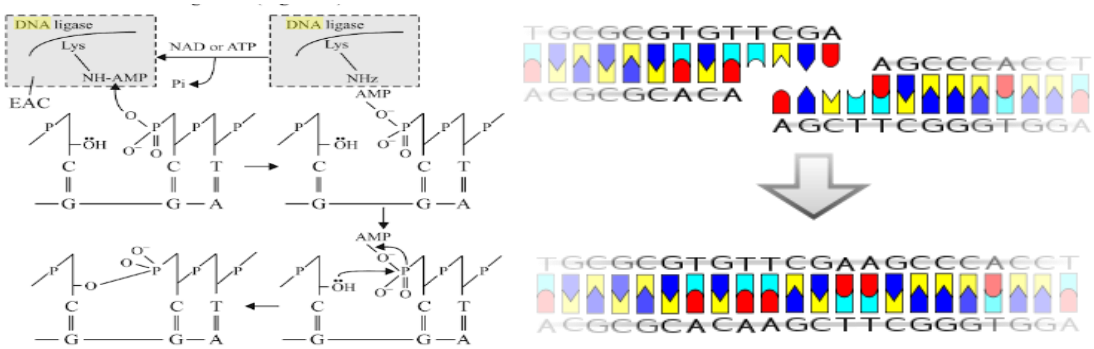
- Alkaline phosphatase has become a useful tool in molecular biology laboratories, since DNA normally possesses phosphate groups on the 5' end.
- Removing these phosphates prevents the DNA from ligating (the 5' end attaching to the 3' end), thereby keeping DNA molecules linear until the next step of the process for which they are being prepared; also, removal of the phosphate groups allows radiolabeling (replacement by radioactive phosphate groups) in order to measure the presence of the labeled DNA through further steps in the process or experiment.

- Another important use of alkaline phosphatase is as a label for enzyme immunoassays.

V. DNA ligase:

DNA ligase is a specific type of enzyme that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond. The first DNA ligase was purified and characterized in 1967.

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



1. Reorganization of activity site such as nicks in DNA segments or Okazaki fragments etc.
2. Adenylation (addition of AMP) of a lysine residue in the active centre of the enzyme, pyrophosphate is released;
3. Transfer of the AMP to the 5' phosphate of the so-called donor, formation of a pyrophosphate bond;
4. Formation of a phosphodiester bond between the 5' phosphate of the donor and the 3' hydroxyl of the acceptor.

Types: The commercially available DNA ligases were originally discovered in bacteriophage T4, *E. coli* and other bacteria.

I. T4 ligase: It can ligate either cohesive or blunt ends of DNA, oligonucleotides, as well as RNA and RNA-DNA hybrids, but not single-stranded nucleic acids. It can also ligate blunt-ended DNA with much greater efficiency than *E. coli* DNA ligase. Unlike *E. coli* DNA ligase, T4 DNA ligase cannot utilize NAD and it has an absolute requirement for ATP as a cofactor.

II. *E. coli* DNA ligase: DNA ligase in *E. coli*, as well as most prokaryotes, uses energy gained by cleaving nicotinamide adenine dinucleotide (NAD) to create the phosphodiester bond. It does not ligate blunt-ended DNA except under conditions.

III. In mammals, there are four specific types of ligase.

i. DNA ligase I: ligates the nascent DNA of the lagging strand after the Ribonuclease H has removed the RNA primer from the Okazaki fragments.

ii. DNA ligase III: complexes with DNA repair protein XRCC1 to aid in sealing DNA during the process of nucleotide excision repair and recombinant fragments. Of the all known mammalian DNA ligases, only Lig III has been found to be present in mitochondria.

iii. DNA ligase IV: complexes with XRCC4. It catalyzes the final step in the non-homologous end joining DNA double-strand break repair pathway.

iv. DNA ligase II: appears to be used in repair. It is formed by alternative splicing of a proteolytic fragment of DNA ligase III and does not have its own gene, therefore it is often considered to be virtually identical to DNA ligase III.

DNA ligase from eukaryotes and some microbes uses adenosine triphosphate (ATP) rather than NAD

Applications:

- DNA ligase is used in both DNA repair and DNA replication
- DNA ligase has extensive use in molecular biology laboratories for recombinant DNA experiments
- Purified DNA ligase is used in gene cloning to join DNA molecules together to form recombinant DNA

Possible questions

Part-A (1 mark)

Part-B (2 Marks)

1. What is Genetic engineering?
2. What is Palindromic sequence?
3. What are blunt ends and sticky ends?
4. What are exonuclease and endonuclease?
5. Mention the cofactor of restriction nucleases
6. Mention the applications of type-II restriction nuclease
7. Mention the types of prokaryotic and eukaryotic polymerase
8. What is terminal deoxynucleotidyl transferase
9. What is dephosphorylation?
10. What is the function of DNA ligase?
11. Mention the types of DNA ligase

12. Mention any to applications of DNA ligase
13. What is the function of terminal deoxynucleotidyl transferase?
14. What is the function of kinase?

Part-C (8 Marks)

1. Write a detailed note on milestones in genetic engineering.
2. Write a detailed note on type-II restriction nuclease.
3. Write a detailed note on DNA polymerases.
4. Write a detailed note on (a) Terminal deoxynucleotidyl transferase and (b) Kinases.
5. Write a detailed note on Phosphatases.
6. Write a detailed note on DNA ligase.

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: IIB.Sc MB COURSE NAME: RECOMBINANT DNA TECHNOLOGY
COURSE CODE:18MBU404B UNIT: I BATCH-2018-2021

Sl.No	Questions	opt1	opt2	opt3	opt4	Answer
1	Enzymes used to degrade nucleic acid are called as_____.	Nucleases	Ligases	Polymerases	Modifying enzymes	Nucleases
2	The enzyme used to add a methyl group to the DNA is called_____.	Amylase	Protease	Methylase	Ligases	Methylase
3	The enzyme used to joining nucleic acid molecules is_____.	Topoisomerases	Exonuclease	Endonuclease	Ligases	Ligases
4	Which enzyme used to make copies of nucleic acid molecules .	Nucleases	Ligases	Polymerases	Modifying enzymes	Polymerases
5	How many types of nucleases are present?	One	Two	Three	Four	Two
6	Which enzyme used to remove or add chemical groups?	Nucleases	Ligases	Polymerases	Modifying enzymes	Modifying enzymes
7	Which enzyme used to remove nucleotides from the ends of the DNA molecule?	Endonuclease	Exonuclease	Topoisomerases	Ligases	Exonuclease
8	The enzyme that introduce or remove supercoils from covalently closed circular DNA is _____.	Topoisomerases	Exonuclease	Endonuclease	Ligases	Topoisomerases
9	The functions of Endonuclease are_____.	Cleaving the DNA molecule	Add a methyl group to the DNA	Remove or add chemical groups to DNA	Introduce or remove supercoils from	Cleaving the DNA molecule
10	How many types of Restriction endonucleases are present?	One	Two	Three	Four	Three
11	Which types of Restriction endonucleases require Mg^{2+} ions for cleavage?	Type I	Type II	Type III	Type IV	Type II
12	How many types of DNA ligases are available?	One	Two	Three	Four	Four
13	Which enzyme helps to catalyse the polymerization of deoxyribonucleotides into a DNA strand?	Restriction endonucleases	DNA polymerase	RNA polymerase	Exonuclease	DNA polymerase
14	DNA polymerase III enzyme synthesizes at a rate of_____nucleotides per second.	1000	2000	3000	5000	1000
15	The enzyme that produces RNA is called_____.	Restriction endonucleases	DNA polymerase	RNA polymerase	Exonuclease	RNA polymerase
16	How many types in DNA polymerases are present in	One	Two	Three	Four	Three

	prokaryote?					
17	How many types in DNA polymerases are present in eukaryote?	2	3	4	5	5
`18	Which organism produces Taq DNA polymerase?	*Thermus aquaticus*	*E.coli*	*Bacillus* spp	*Pseudomonas* spp	*Thermus aquaticus*
19	Recognition sequence for *Eco* R1	GAA/GTTC	GAAG//TTC	GAAT/TTC	G/AATTC	G/AATTC
20	What is the product produced by reverse transcriptase?	Single strand DNA	Double strand DNA	Single strand RNA	Double strand RNA	Single strand DNA
21	Restriction enzymes cleave DNA at _____.	Specific nucleotide	Interior part of nucleotide sequence	Ends of nucleotide sequence	Middle part of Sequence	Specific nucleotide Sequence
22	Endonucleases are enzymes that cleave DNA at_____.	Defined sequence	3' end of nucleotide	Internal position in random manner	External position	Internal position in random manner
23	The term endonuclease was coined by _____.	Lederberg and Meselson	Lederberg and Tatum	Lederberg and Yaun	Smith and Nathans	Lederberg and Meselson
24	Restriction enzyme from *Escherichia coli* K 12 was first isolated from_____.	Meselson and Yaun	Lederberg	Yaun	Meselson	Meselson and Yaun
25	Enzymes were classified by_____.	Smith and Nathans	Arber	Rodriguez	Boliver	Smith and Nathans
26	Type I restriction enzymes cleave DNA at _____.	The point of recognition	1000 nucleotides away	25 base pairs away	30 base pairs away	1000 nucleotides away
27	Co-factor needed for type II restriction enzyme were _____.	Mg^{2+}	S- Adenosyl	Fe	DNP+	Mg^{2+}
28	Type I restriction enzyme have_____.	3 subunit	5 subunit	2 subunit	1 subunit	1 subunit
29	Type I restriction enzymes are isolated from the organisms _____.	*Escherichia coli*	*Virus*	Animals	Fungi	*Escherichia coli*
30	Type II restriction enzymes cleaves DNA at _____.	Defined recognition site	Random sites	25 base pairs away	1000 base pairs away	Defined recognition site

31	Smith and Nathans received Nobel prize during_____.	1973	1978	1980	1981	1978
32	Type II restriction enzymes cleaves DNA at _____.	Tetra nucleotide Sequence	Penta nucleotide Sequence	Hexa nucleotide sequence	Tri nucleotide Sequence	Penta nucleotide Sequence
33	Ligases are called_____.	Molecular	Molecular glue	Molecular tool	None of the above	Molecular glue
34	Ligases form phosphodiester bond between_____.	3" hydroxyl and 5" phosphate	3" hydroxyl	5" hydroxyl	3" hydroxyl & 5" triphosphate	3" hydroxyl and 5" phosphate
35	Commercial sources of Ligases are_____.	*Bacillus*	Bacteriophage	Bacteriophage infected bacterium	Retrovirus	Bacteriophage
36	*Escherichia coli* DNA ligase has the molecular weight of .	73 kDa	72 kDa	50 kDa	35 kDa	73 kDa
37	Bacteriophage ligases have molecular weight of_____.	68 kDa	70 kDa	60 kDa	95 kDa	68 kDa
38	Linkers used for producing sticky end have	Modified blunt end	A short nucleotide sequence with restriction site	Modified sticky end	Both a and c	A short nucleotide sequence with restriction site
39	*Escherichia coli* ligases seal nick at_____.	3 °C	4 °C	14 °C	40 °C	4 °C
40	Adaptors have a .	3' OH and 5' P end	a short nucleotide sequence with a restriction site	Linker end	Homopolymer tails	3' OH and 5' P end
41	T4 bacteriophage ligase need	Mg2+ as cofactor	ATP as cofactor	SAM as cofactor	NAD+ as cofactor	ATP as cofactor
42	Alkaline phosphatase catalyze	Removal of phosphate at 5'terminal	Removal of 3' terminal hydroxyl group	Removal of 3 'terminal phosphate	None of the above	Removal of phosphate at 5' terminal
43	Alkaline phosphatase is a_____.	Monomeric	Homo Dimeric protein	Polymeric protein	Single glycoprotein	Homo Dimeric protein

44	Alkaline phosphatase contains_____.	Zn^{2+}	Mg^{2+}	Fe	Energy containing enzyme	Zn^{2+}
45	The role of alkaline phosphatase is to _____.	Prevent recircularisation	Induce ligation	Cleavage	Induction of recircularisatio n	Prevent recircularisation
46	Bacterial Alkaline Phosphatase action is inhibited by_____.	Lysozyme	CTAB	EDTA	MgS04	EDTA
47	Methylases methylate nucleotides at_____.	C5 cytosine	Both C5 cytosine and N6 adenine	Two adenine at N6 position	Two cytosine at C5 position	Both C5 cytosine and N6 adenine
48	Dam methylase used in_____.	recombinational repair	Mismatch repair	SOS repair	Excision repair	Mismatch repair
49	Dam methylase are single nucleotide independent which under _____.	Type I modification/ Restriction system	Type II modification/ Restriction system	Type III modification/ Restriction system	None	Type II modification/ Restriction system
50	Dam methylase tranaferace methylate at _____.	.. .5" CCAGG 3"	. .. 5" CCTGG 3"	randam	.5 GATC 3	.. .5" CCAGG 3"
51	Source of methionine for methylation obtained from_____.	S- Adenosyl Methionine	Methionine	Protein synthesis	None	S- Adenosyl Methionine
52	PoIynucleotide kinase involves_____.	Transfer of hydroxyl group to the terminal end of DNA	Transfer of terminal phosphate group from ATP	Catalyst of biochemical reaction	Prevents the transfer	Transfer of hydroxyl group to the terminal end of DNA
53	DNA Polymerase I Holoenzyme are_____.	Single functional enzyme	Bi functional enzyme	Multifunctional enzyme	None	Bi functional enzyme
54	A Polymerase Klenow fragment	Posses Polymerase	Posses 5" exonuclease activity	Lack exonuclease activity	have no activity	Posses Polymerase activity
55	T4 DNA Polymerase optimum temperature is_____.	37 degree	45 degree	94 degree	50 degree	94 degree

56	Taq DNA Polymerase require_____cofactor for activity.	Mg^{2+}	Na^{2+}	Fe^{2+}	Cu^{2+}	Mg^{2+}
57	Reverse transcriptase isolated from_____.	Retrovirus	Adenovirus	Baculovirus	Gemini virus	Retrovirus
58	T4 bacteriophage ligase need_____.	Mg^{2+} as cofactor	ATP as cofactor	SAM as cofactor	NAD+ as cofactor	ATP as cofactor
59	Which organism produces Taq DNA polymerase?	*Thermus aquaticus*	*E.coli*	*Bacillus* spp	*Pseudomonas* spp	*Thermus aquaticus*
60	EcoRI is isolated from_____.	*Bacillus* sp	*Pseudomonas* sp	*Escherichia coli*	*Vibrio*	*Escherichia coli*

UNIT –II

Syllabus

Cloning Vectors: Definition and Properties Plasmid vectors: pBR and pUC series Bacteriophage lambda and M13 based vectors Cosmids, BACs, YACs. Use of linkers and adaptors. Expression vectors: *E.coli* lac and T7 promoter-based vectors, yeast YIp, YEp and YCp vectors, Baculovirus based vectors, mammalian SV40-based expression vectors

Cloning Vectors

- A cloning vector is a DNA molecule that has the ability to replicate in an appropriate host cell, and into which the DNA insert is integrated for cloning.
- Therefore, a vector must have an origin of DNA replication (denoted as ori) that functions in the host cell.
- Any extra chromosomal small genome, e.g., plasmid, phage and virus, may be used as a vector.

Properties of Good Vector

1. It should be able to replicate autonomously. When the objective of cloning is to obtain a large number of copies of the DNA insert, the vector replication must be under relaxed control so that it can generate multiple copies of itself in a single host cell
2. It should be easy to isolate and purify
3. It should be easily introduced into the host cells
4. The vector should have suitable marker genes that allow easy detection and/or selection of the transformed host cells.
5. When the objective is gene transfer, it should have the ability to integrate either itself or the DNA insert it carries into the genome of the host cell.
6. The cells transformed with the vector containing the DNA insert (recombinant DNA) should be identifiable be selectable from those transformed by the unaltered vector
7. A vector should contain unique target sites for as many restriction enzymes as possible into which the DNA inserts can be integrated when expression of the DNA insert is desired, the vector should contain at least suitable control elements, e.g., promoter, operator and ribosome binding sites.

It should be kept in mind that

- The DNA molecules used as vectors have coevolved with their specific natural host species, and hence are adapted to function well in them and in their closely related species. Therefore, the choice of vector depends largely on the host species into which the DNA insert of gene is to be cloned.
- Most naturally occurring vectors do not have all the required functions; therefore, useful vectors have been created by joining together segments performing specific functions (called modules) from two or more natural entities

Plasmid vector

- Plasmid was coined by Lederberg.
- Plasmids are extra chromosomal double-stranded circular DNA molecules that can be found in various bacteria and some eukaryotes.

- Plasmids are considered replicons, units of DNA capable of replicating autonomously within a suitable host.
- In their original form, the size of plasmids ranges between 1 and 200 kbp (kilo-base pairs).
- Plasmids often contain genes encoding enzymes that confer a certain selective advantage to the host cell. Such selective advantages include resistance to certain antibiotics.
- Plasmids are widely used as cloning vector
- If both strands of DNA are intact circles the molecules are described as covalently closed circles or CCC DNA.
- When isolated from cells, covalently closed circles often have a deficiency of turns in the double helix, such that they have a super coiled configuration.
- Cryptic Plasmids do not have genes that contribute to the phenotype of the host cell. They usually have genes to self replicate

Plasmids can be categorized into one of two major types –

1. Conjugative: Conjugative plasmids contain a set of transfer or *tra* genes which promote sexual conjugation between different cells
2. Non-conjugative: Non-conjugative plasmids do not contain *tra* genes.

Plasmids can also be categorized on the basis of copies per cell being maintained as

1. Relaxed plasmids - multiple copies per cell
2. Stringent plasmids - a limited number of copies per cell

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

- Fertility F-plasmids, which contain *tra* genes. They are capable of conjugation and result in the expression of sex pili.
- Resistance (R) plasmids, which contain genes that provide resistance against antibiotics or poisons. Historically known as R-factors, before the nature of plasmids was understood.
- Col plasmids, which contain genes that code for bacteriocins, proteins that can kill other bacteria. The best-studied Col plasmid is Col E plasmid.
- Degradative plasmids, which enable the digestion of unusual substances, e.g. toluene and salicylic acid.
- Virulence plasmids, which turn the bacterium into a pathogen.

Plasmids can belong to more than one of these functional groups

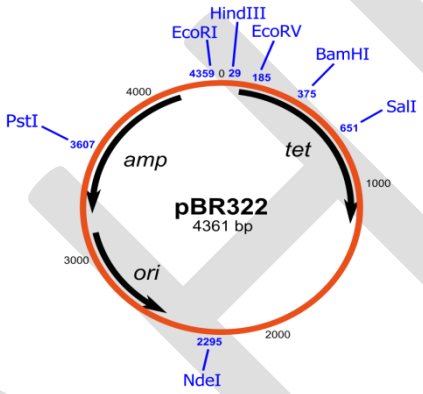
An ideal plasmid vector must have the following functions:

(1) Minimum amount of DNA,
(2) Relaxed replication control,
(3) At least two selectable markers,
(4) Only one (unique) recognition site for at least one restriction endonuclease, and
(5) For easy selection of the recombinant DNA, this unique restriction site must be located

(a) ***pBR322 plasmid*****:** is the most popular and most widely used plasmid of 4361 bp; The name pBR denotes the following: p signifies plasmid, B is from Boliver, and R is from Rodriguez, the two initials of the scientist who developed in 1977 in the laboratory.

- It has the replication module of *E. coli* plasmid Col El.

- This module has been incorporated in many other plasmid vectors since it permits plasmid replication even when chromosome replication and cell division are inhibited by amino acid starvation or chloramphenicol.
- Under such conditions, each cell accumulates several thousand copies of the plasmid so that one litre of bacterial culture easily yields a milligram of plasmid DNA.
- It has two selectable markers (tetracycline, tetr, and ampicillin, amp', resistance genes), and unique recognition sites for 12 different restriction enzymes (two unique sites, PstI and PvuI, are located within the amp' gene, and 4, e.g., BamHI, SalI, etc., are within tetr gene).
- The presence of restriction sites within the markers tetr and amp permits an easy selection for cells transformed with the recombinant pBR322.
- Insertion of the DNA fragment into the plasmid using restriction enzyme PstI or PvuI places the DNA insert within the gene amp'; this makes amp' nonfunctional.
- Bacterial cells containing such a recombinant pBR322 will be unable to grow in the presence of ampicillin, but will grow on tetracycline.

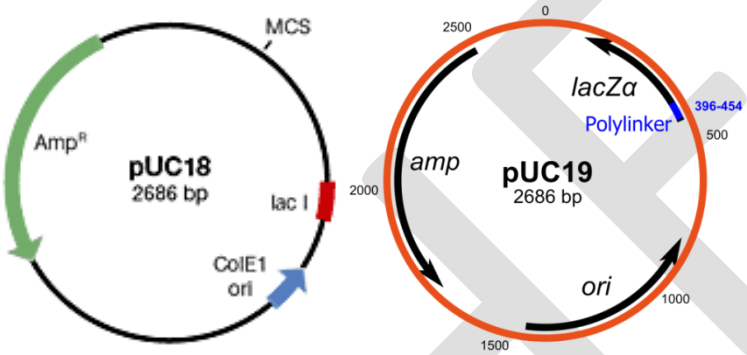


- Similarly, when restriction enzyme BamHI or SalI is used, the DNA insert is placed within the gene tetr making it nonfunctional.
- Bacterial cells possessing such a recombinant pBR322 will, therefore, grow on ampicillin but not on tetracycline.
- This feature allows an easy selection of a single bacterial cell having recombinant pBR322 from among 108 other types of cells.
- Transformed *E. coli* cells are first plated on an agar medium containing the antibiotic within the resistance gene for which the DNA fragment is not inserted, i.e., for which the bacterial cells having the recombinant DNA are expected to be resistant.
- This eliminates nontransformed bacterial cells; the resulting bacterial colonies will posses either recombinant or unaltered pBR322.
- The colonies so obtained are then replica plated on agar plates containing the other antibiotic (within the resistance gene for which the DNA insert is placed); all the colonies that develop on this plate will contain the unaltered pBR322.
- Therefore, the antibiotic sensitive colonies are identified and recovered from the master plate; these colonies will have the recombinant pBR322. This entire process may take up to 2 days.

(b) pUC18 and pUC19

pUC series of cloning vectors created by Joachim Messing and co-workers. The designation "pUC" is derived from the classical "p" prefix (denoting "plasmid") and the abbreviation for the University of California, where early work on the plasmid series had been conducted.

- It is a derivative of pBR322 and is much smaller (2.7 kb); it has all the essential parts of pBR322, e.g.,
  - ampicillin resistance gene and Col E1 origin.
  - The second marker is due to *E. coli* gene lacZα encoding the fragment of β-galactosidase, the enzyme that hydrolyses lactose.
  - The *E.coli* strains, e.g., JMIO3, JM1O9, used as hosts for the pUC series vectors have the lacZα deleted from their lacZ genes.
  - When pUC enters such an *E.coli* cell, the host genome and the plasmid encode for different parts of the β-galactosidase enzyme, which interact with each other to produce the active enzyme enabling these cells to hydrolyse lactose. β -galactosidase also hydrolyses X-gal (5-Bromo-4-chloro-3-indolyl-p-D-galactoside) to yield a blue dye.



- Therefore appropriate lacZ- *E. coli* cells transformed by the pUC vectors behave as lacZ+ and produce blue coloured colonies on a X-gal containing medium.
- A poly linker sequence located within the lacZα provides several (10 in case of pUC18/pUC19) unique restriction sites for DNA insertion.
- The polylinker sequence by itself does not interfere with lacZα expression. But when a DNA insert is placed within it, lacZα expression is prevented.
- Vectors pUC18 and pUC19 are identical, except for the orientation of the polylinker sequence, which is oriented in the opposite directions in the two vectors.
- The unique restriction sites used for integration of DNA inserts into pUC vectors interrupt the lacZα fragment so that appropriate *E. coli* cells possessing recombinant pUC DNA are β-galactosidase deficient and, as a result, produce white colonies on X-gal medium.
- Therefore, appropriate E.coli cells transformed with pUC recombinant DNA are grown on ampicillin, X-gal and IPG (isopropyl- β D-thiogalactoside; it serves as inducer of β - galactosidase, while X-gal itself cannot) containing medium to eliminate non transformed cells.
- The white colonies are selected as they contain the recombinant DNA (in contrast, blue colonies will contain the unaltered vector). The other vectors in pUC series are pUC 8, pUC 9, pUC 12, pUC 13, etc.

<u>The pUC series vectors offer the following advantages over pBR322:</u>

- Each *E. Coli* cell produces up to 700 copies without any treatment
- Cells containing recombinant DNA are selected in a single step
- The sites for DNA insert integration are confined to the poly linker, which permits the use of two restriction enzymes to open the vector, and they also allow sequencing of the DNA insert

Bacteriophage Vectors

- Bacteriophages are viruses that attack bacteria.

- Most phages lyse the bacterial cells they infect (lytic phages).
- But many others can choose to follow either a lytic or a lysogenic cycle; in the latter situation, the phage chromosome integrates into the bacterial chromosome and multiplies with the latter as prophage (temperate or lysogenic phages).
- The prophage may dissociate from the bacterial chromosome and follow the lytic cycle. Several bacteriophages are used as cloning vectors,
- The most commonly used *E. coli* phages being λ (lambda) and M13 phages. Plasmid vectors have to be introduced into bacterial cells, which are then cloned and selected for the recovery of recombinant DNA.
- In contrast, the phage vectors are directly tested on an appropriate bacterial lawn (a continuous bacterial growth on an agar plate) where each phage particle forms a plaque (a clear bacteria-free zone in the bacterial lawn).

Phage vectors present two advantages over plasmid vectors

(1) They are more efficient than plasmids for cloning of large DNA fragments; the largest cloned insert size in a λ vector is just over 24 kb, while that for plasmid vectors it is less than 15 kb. In addition, (2) it is easier to screen a large number of phage plaques than bacterial colonies for the identification of recombinant plaques/clones.

Two types of λ phage vectors

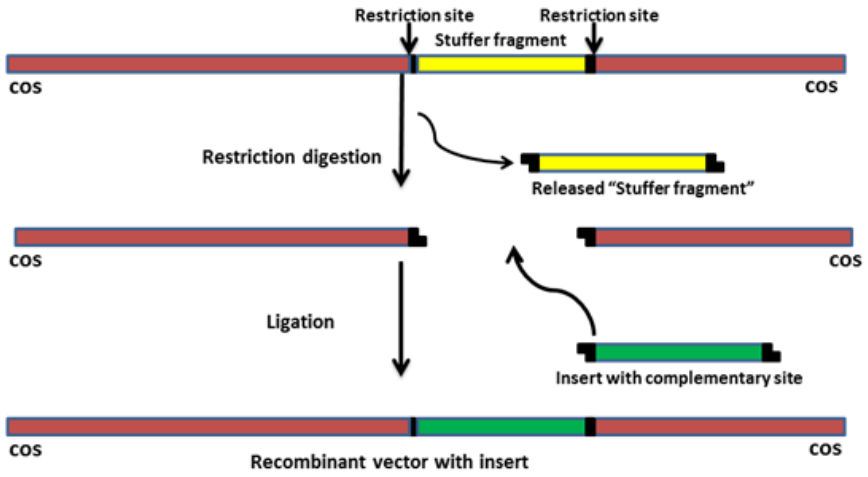
1. Insertional vectors
2. Replacment vectors

The λ insertional vectors – accept less DNA than the replacement type, the foreign DNA is simply inserted into a region of the phage genome with appropriate restriction sites.

Example – λ gt10, λ charon16A

In λ replacement vectors – a central region of DNA not essential for lytic growth is removed. This creates two DNA fragments, called right and left arms. The central stuffer fragment is replaced by inserting foreign DNA between the arms to form a functional recombinant λ phage.

Eg: λ EMBL 3, λ EMBL 4, λ DASH etc



Lambda (λ) Phage Vectors

- The λ genome (total 48,502 bp) contains an origin of replication, genes for head and tail proteins and enzymes for DNA replication, lysis and lysogeny, and single-stranded protruding cohesive ends of 12 bases (5' GGGCGGCGACCT; the other end is complementary to it, i.e., CCCGCCGCTGGA 3').

- The λ genome remains linear in the phage head, but within *E. coli* cells the two cohesive ends anneal to form a circular molecule necessary for replication.
- The sealed cohesive ends are called cos sites, which are the sites of cleavage during and are necessary for packaging of the mature phage DNA into phage heads.
- The λ DNA must be larger than 38 kb and smaller than 52 kb to be packaged into phage particles.
- The genes for lysogeny are located in the segment between 20 and 38 kb; the whole or a part of this segment is deleted to create λ vectors to accommodate larger DNA inserts and to ensure that the recombinant phage is always lytic.

Several vectors were produced from wild type λ genome by mutation and recombination *in-vivo* as well as by recombinant DNA techniques.

These vectors have the following basic features.

(1) The vector itself can be propagated as phage in *E. coli* cells enabling preparation of vector DNA.

(2) They contain restriction sites, which allow the removal of the lysogenic segment and also provide insertion site for the DNA fragment.

(3) During annealing and ligation of the DNA insert with the λ vector, two or more recombinant DNAs may join end-to-end producing a concatemer, which is the proper precursor for packaging of λ genome into phage heads.

Phage M13 Vectors

- The M13 family of vectors is derived from bacteriophage M13. This is lysogenic filamentous phage with a circular single-stranded DNA genome about 6,407 bp (6.4 kb) in length. Once inside the host-cell the single-stranded DNA of M13 phage acts as the template for synthesis of a complementary strand, resulting in normal double-stranded DNA
- M13 infects only F+ cells; it does not kill the cells, but forms turbid plaques due to growth retardation of infected cells.
- Ordinarily, the double stranded form is used to produce recombinant molecules since single-stranded DNAs are not cleaved by type II restriction endonucleases; this form is readily isolated from M13-infected E. coli cells.
- However, the single stranded form of M 13 is used to recover single-stranded copies of the DNA inserts; this form of vector is available from the phage particles abundant in the growth medium.

Attraction of M13 as a Cloning Vector:

- The genome is less than 10 kb in size, well within the range desirable for a potential vector. In addition, the double-stranded replicative form (RF) of the M13 genome behaves very much like a plasmid, and can be treated as such for experimental purposes.
- It is easily prepared from a culture of infected E. coli cells and can be reintroduced by transfection. Most importantly, genes cloned with an M13-based vector can be obtained in the form of single- stranded DNA. Single-stranded version of cloned genes is useful for several techniques, notably DNA sequencing and in vitro mutagenesis.

Properties of M13 Vectors:

M13 genome has been used to produce M13 series of vectors, e.g., M13mp 8, M13mp 9, etc.

The desirable features of M13 vectors are as follows:

(1) Very large inserts can be cloned since packaging does not depend on genome size (as is the case with λ vectors).

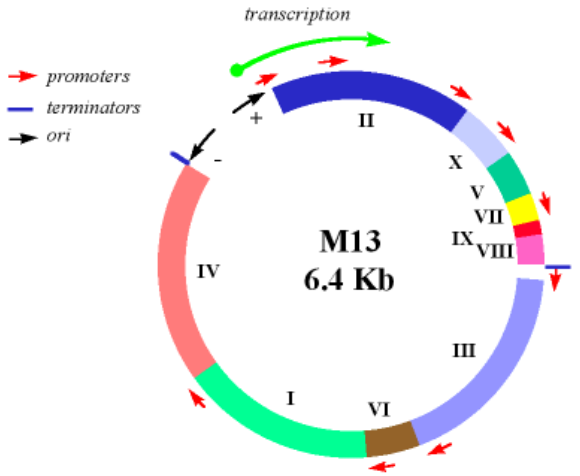
(2) Pure single-strand copies of double-strand DNA inserts are obtained in abundance.

(3) Since DNA inserts are accepted in either of the orientations (which is also the case for plasmid and λ vectors), some recombinant clones will produce single-strand copies of one strand of the DNA double-strand, while others would produce copies of the complementary strand of the DNA insert. The phage particles in a single plaque, as a rule, will yield copies of the same single-strand. This property is very useful for a precise DNA sequencing (using both the strands of a DNA molecule) and for the synthesis of specific radio-labelled DNA probes.

(4) Bacterial cells infected by these vectors remain viable as in the case of plasmid vectors; this allows easy maintenance of the vector.

(5) They form plaques like λ phage vectors making selection of the recombinant dnas rather easy,

(6) The recombinant DNA is obtained within stable bacteriophage particles.



Cosmid Vectors

A cosmid, first described by **Collins** and **Hohn** in 1978, is a type of hybrid plasmid with a bacterial "ori" sequence and a "*cos*" sequences derived from the lambda phage.

Cosmids contain a minimum of 250 bp of λ DNA, which includes the cos site (the sequence yielding cohesive ends) and sequences needed for binding of and cleavage by terminase so that under appropriate conditions they are packaged in vitro into empty λ phage particles.

A typical cosmid has

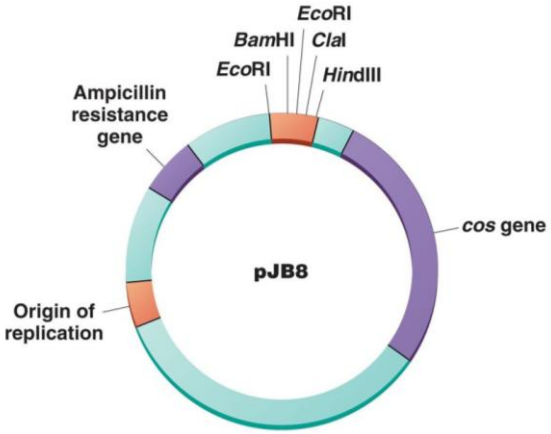
(1) replication origin,

(2) unique restriction sites and

(3) selectable markers from the plasmid; therefore, selection strategy for obtaining the recombinant DNA is based on that for the contributing plasmid.

- Cosmid vectors are constructed using recombinant DNA techniques.
- The cosmid vectors are opened by the appropriate restriction enzyme at a unique site, are then mixed with DNA inserts prepared by using the same enzyme and annealed.
- Among the several types of products, long cancatemers are present, which are the appropriate precursors for packaging in λ. particles.
- This procedure selects for long DNA inserts since for packaging the distance between two cos sites must be between 38 and 52 kb.

- Cosmids can accommodate upto 37-52 kb long DNA inserts.
- Packaged cosmids infect host cells like λ particles, but once inside the host they replicate and propagate like plasmids



The typical features of cosmids follow**:**

(1) These can be used to clone gene of interest up to 45 kb.

(2) As the lambda phage will insert the recombinant DNA into the host cell, an extra step of inserting the recombinant DNA into the host cell is not performed.

(3) Easy screening method is found.

(4) Cosmids are used for construction of genomic libraries of eukaryotes since these can be used for cloning large fragments of DNA.

Bacterial artificial chromosomes

Bacterial artificial chromosomes (BACs) are designed for the cloning of large DNA insert (typically 100 to 300 kb) in *E. coli* host. BAC vectors contain a single copy F-plasmid origin of replication (ori).

The F (fertility) plasmid is relatively large and vectors derived from it have a higher capacity than normal plasmid vectors. F-plasmid has F (fertility) factor which controls the replication and maintain low copy number. Also conjugation can take place between F+ bacteria (male) and F- bacteria (female) to transfer F-plasmid via pilus.

Common gene components of a bacterial artificial chromosome are:

1) oriS, repE – F for plasmid replication and regulation of copy number.
2) parA and parB for maintaining low copy number and avoiding two F plasmids in a single cell during cell division.
3) A selectable marker for antibiotic resistance; some BACs also have *lacZ* at the cloning site for blue/white selection.
4) T7 and Sp6 phage promoters for transcription of inserted genes.

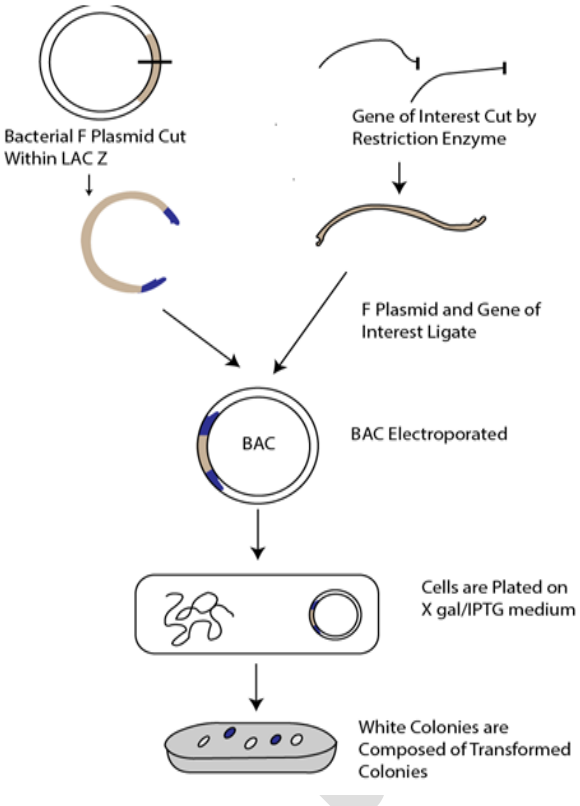
These vectors can hold DNA fragments of up to 300 kb. Since they are present in low copies, recombination between the highcopy plasmids.

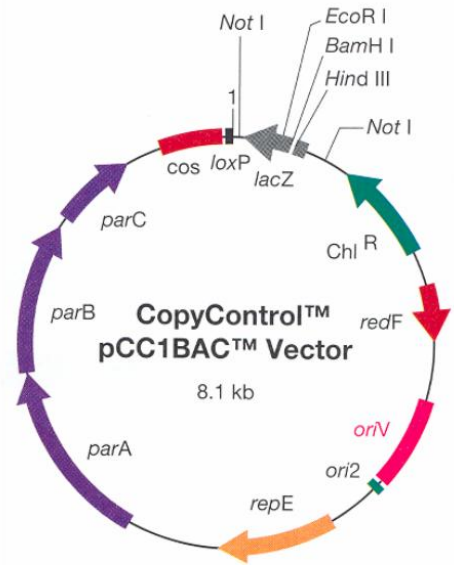
The *par* genes, derived from F plasmid assist in the even distribution of plasmids to daughter cells during cell division and increase the likelihood of each daughter cell carrying one copy of the plasmid, even when few copies are present. The low number of copies is useful in cloning large fragments of DNA because

it limits the opportunities for unwanted recombination reactions that can unpredictably alter large cloned DNA over time.

The first BAC vector, pBAC108L, did not contain a selectable marker for recombinants. Thus, positive recombinants had to be identified by colony hybridization. Two widely used BAC vectors, pBeloBAC11 and pECBAC1, are derivatives of pBAC108L in which the original cloning site is replaced with a *lac* Z gene carrying a multiple cloning site. PBeloBAC11 has two *Eco* RI sites, one in the *lac* Z gene and one in the CMR gene, whereas pECBAC1 has only one *Eco* RI site in the *lac* Z gene. Further improvements to BACs have been made by replacing the *lac* Z gene with the *sac* B gene which is a negative selection marker. The product of *sacB* gene is levansucrase which can convert sucrose present in the media into levan, a toxin for the bacteria. Hence the colonies without insert would have intact *sacB* gene and thus cells die before forming colonies.

The F plasmid is relatively large and vectors constructed on it have a higher capacity for accepting inserted DNA. A similar cloning vector called a P1-derived artificial chromosome or PAC has also been produced from the bacterial P1 bacteriophage DNA. Both BACs and PACs can be used to clone fragments of 300kb and longer. They are often used to sequence the genome of organisms in genome projects.





Yeast Artificial Chromosomes: YAC

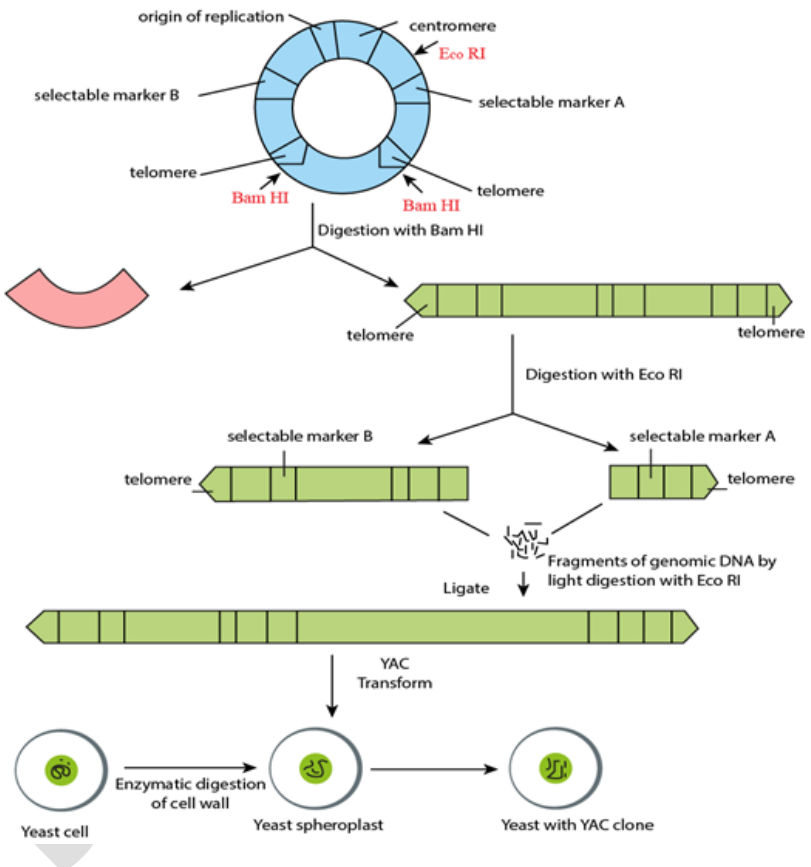
- First described in 1983 by **Murray** and **Szostak**, a yeast artificial chromosome has sequences to exist inside *E. coli* as a circular plasmid and contains sequences to maintain as linear nuclear chromosome in yeast.
- As YAC vectors can accommodate 100-500 kb of insert DNA. The number of clones in a genomic library can be greatly reduced.
- YAC vectors have following elements:
- *E. coli* origin of replication
- Yeast origin of replication

- Elements of eukaryotic yeast chromosome (centromere and telomere region)
- Selection markers for both the host.
- YAC vector is initially propagated as circular plasmid inside bacterial host utilizing bacterial *ori*sequence.

Circular plasmid is cut at specific site using restriction enzymes to generate a linear chromosome with two telomere sites at terminals. The linear chromosome is again digested at specific site with two arms with different selection marker. Genomic insert is then ligated into YAC vector using DNA ligase enzyme. The recombinant vectors are transformed into yeast cells and screened for the selection markers to obtain recombinant colonies.

- Yeast expression vectors, such as YACs, YIPs (yeast integrating plasmids), and YEPs (yeast episomal plasmids), have advantageous over bacterial artificial chromosomes (BACs). They can be used to express eukaryotic proteins that require post-translational modification. However, YACs have been found to be less stable than BACs.

Some recombinant plasmids have the ability to incorporate multiple replication origins and other elements that allow them to be used in more than one species (for example, yeast or *E. coli*).

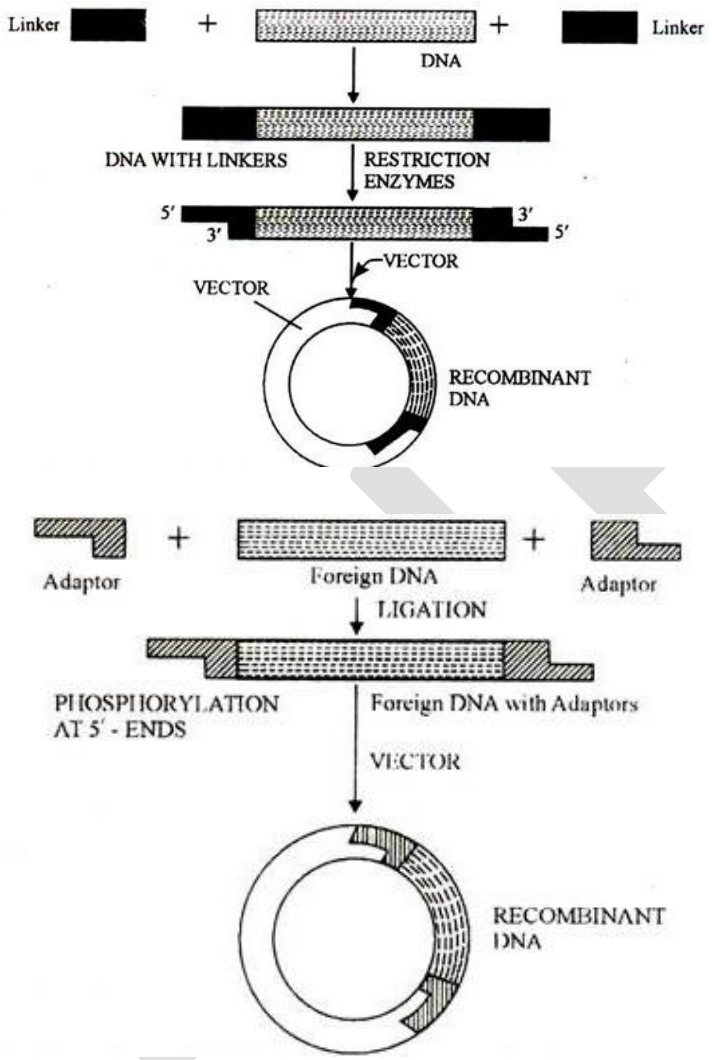


Linkers

Linkers are short stretches of double stranded DNA of length 8-14 bp of known nucleotide sequence that are synthesized in the test tube and have recognition site for restriction enzymes. Linkers are ligated to blunt end DNA by ligase enzyme.

- The linker ligation is more efficient as compared to blunt-end ligation of larger molecules because of the presence of high concentration of these small molecules in the reaction.
- The ligated DNA can be digested with appropriate restriction enzyme generating cohesive ends required for cloning in a vector.

- The restriction sites for the enzyme used to generate cohesive ends may be present within the target DNA fragment which may limit their use for cloning.



Adapters

These are short stretches of oligonucleotide with cohesive ends or a linker digested with restriction enzymes prior to ligation. Addition of adaptors to the ends of a DNA converts the blunt ends into sticky ends.

Expression vectors

A vector used for expression of a cloned DNA fragment in a host cell is called as an expression vector. These vectors are frequently engineered to contain regulatory sequences that act as promoter and/or enhancer regions and lead to efficient transcription of the insert gene. Commonly used expression vector series are: pET vectors, pBAD vetors etc.

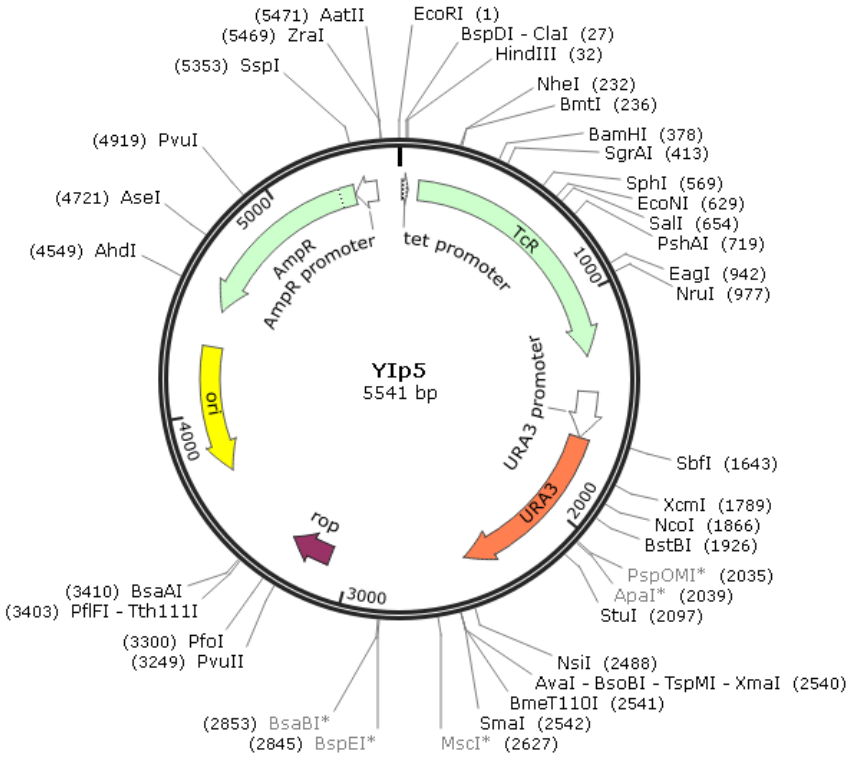
For an expression vector following features are essential:

- Promoter: Promoter is a sequence which is recognized by sigma subunit of RNA polymerase which is required for initiation of transcription of gene of interest.
- Terminator: It is a DNA element present at the end of a gene where transcription ends.
- Ribosome binding site: It is a short nucleotide sequence recognized by the ribosome as the point at which it should attach to the messenger molecule. The initiation codon of the gene is always a few nucleotides downstream of this.

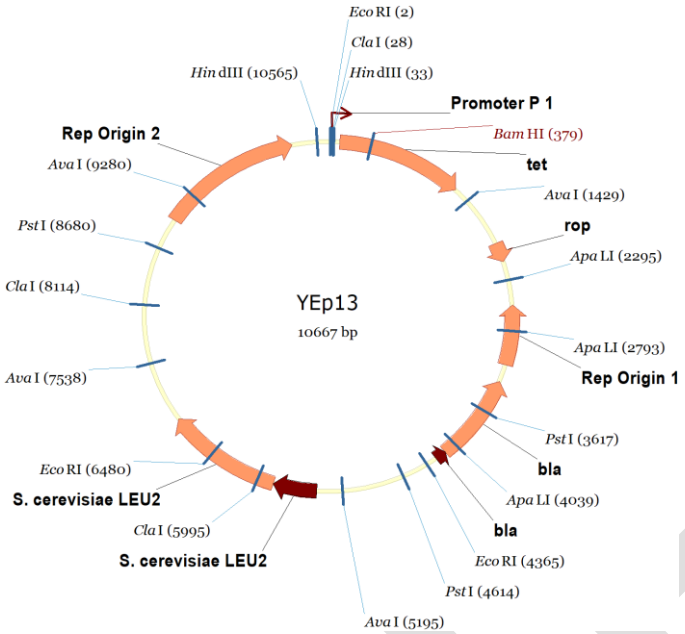
Yeast YIp, YEp and YCp vectors

Yeast expression plasmids used in the lab typically contain all the necessary components to allow shuttling between *E. coli* and yeast cells. Yeast vectors can be grouped into five general classes, based on their mode of replication in yeast: YIp, YRp, YCp, YEp, and YLp plasmids. With the exception of the YLp plasmids (yeast linear plasmids), all of these plasmids can be maintained in *E. coli* as well as in *S. cerevisiae* and thus are referred to as shuttle vectors.

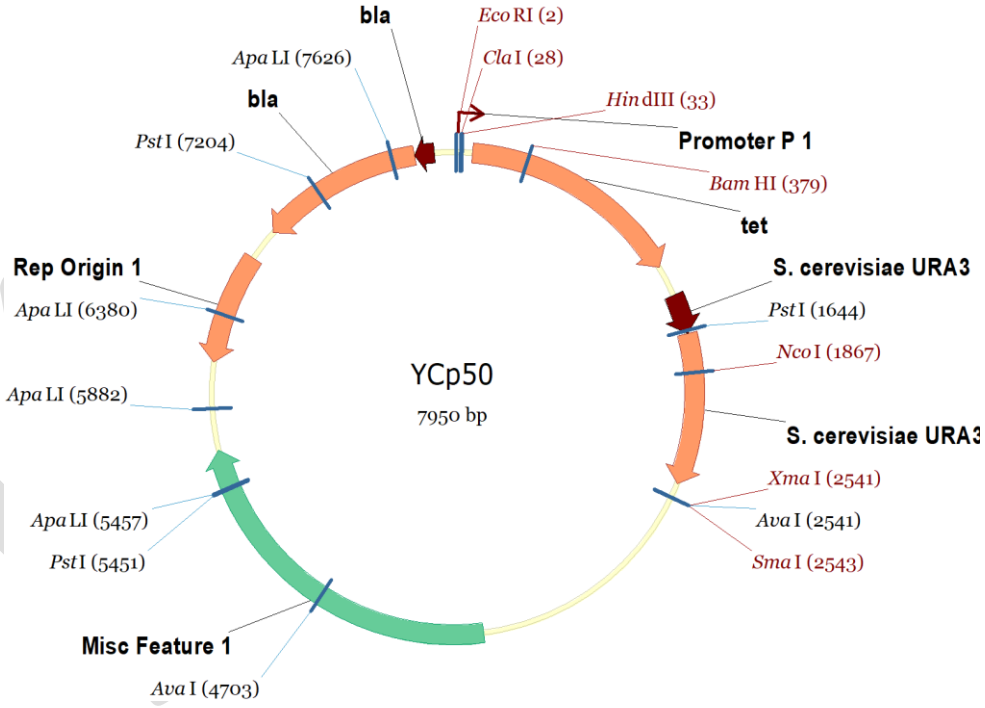
- **Yeast integrating plasmids (YIp)**: These plasmids lack an ORI and must be integrated directly into the host chromosome via homologous recombination.



- **Yeast Episomal plasmids (YEp)**: YEps were first constructed by Beggs (1978) by recombining an *E. coli* cloning vector with the naturally occurring yeast 2 µm plasmid.
  - The word "episomal" indicates that a YEp can replicate as an independent plasmid, but also implies that integration into one of the yeast chromosomes can occur.
  - Integration occurs because the gene carried on the vector as a selectable marker is very similar to the mutant version of the gene present in the yeast chromosomal DNA.
  - This plasmid is 6.3 kb in size, has a copy number of 50–100 per haploid cell and has no known function.
  - YEps have the highest transformation frequency providing between 10,000 and 100,000 transformed cells per µg.



- **Yeast Centromere plasmids (ycp)**: These are considered low copy vectors and incorporate part of an autonomously replicating sequence (ARS) along with part of a centromere sequence (CEN). These vectors replicate as though they are small independent chromosomes and are thus typically found as a single copy. Unlike the ARS vectors, CEN vectors are stable without integration.



Plasmid	Size	*E. coli* replicon	Yeast replicon	Phenotypes selectable in *E. coli*	Phenotypes selectable in yeast
YIp5	5541 bp	pMB1	none	Amp^r, Tet^r, $PyrF^+$	Ura^+
YRp7	5816 bp	pMB1	*ARS1*	Amp^r, Tet^r, $TrpC^+$	Trp^+
YEp13	10.7 kb	pMB1	2µm	Amp^r, Tet^r, $LeuB^+$	Leu^+
YEp24	7769 bp	pMB1	25µm	Amp^r; Tet^r in some constructions; $PyrF^+$	Ura+
YCp19	10.1 kb	pMB1	*ARS1*	Amp^r, $PyrF^+$, $TrpC^+$	Ura^+, Trp^+
YCp50	7.95 kb	pMB1	*ARS1*	Amp^r, Tet^r, $PyrF^+$	Ura^+

E.coli lac and T7 promoter-based vectors

Expression vectors produce proteins through the transcription of the vector's insert followed by translation of the mRNA produced, they therefore require more components than the simpler transcription-only vectors. Expression in different host organism would require different elements, although they share similar requirements, for example a promoter for initiation of transcription, a ribosomal binding site for translation initiation, and termination signals.

Prokaryotes expression vector

Promoter - commonly used inducible promoters are promoters derived from lac operon and the T7 promoter. A stronger promoter; Trp/Tryptophan Operon and Tac Promoter, a hybrid collection of both the Trp and Lac Operon promoters.

Ribosome Binding Site (RBS) Follows the promoter, and promotes efficient translation of the protein of interest.

Translation initiation site - Shine-Dalgarno sequence enclosed in the RBS, 8 base-pairs upstream of the AUG start codon.

The basic requirement for any expression system is a promoter cloning site(s) next to it and transcriptional terminator.

Origin suitable for replication initiation in a particular bacteria or any host, A selection marker gene such as antibiotic resistance gene, Regulator elements and Shine Delgarno sequence.

pLac-Z expression vectors

- Lac ‑Z promoter operator is in frame with lac-Z alpha fragment (the NH3 terminal part of Galactosidase gene. Multiple cloning sites are found in the border of NH3 end including ATG sequence.
- The presence of such restriction site sequences should not disturb the functional activity of the protein, which complements with the omega fragment of the Lac-Z produced by the bacterial cell as the complement.
- If any gene is placed in proper frame in the MCS the protein expressed will be is fused form.
- The expression of the gene can be regulated by IPTG (Isopropyl thio b-Galactoside).

pET Expression Vector:

- The size of the vector is 5700bp
- It has T7 promoter adjacent to lac operator
- Next to it is a sequence called Shine Delgarno sequence.
- Adjacent to S/D sequence there are few cloning sites such as Nde I, Nhe I and BamH I, at the end of which is T7 phage transcriptional terminator is found.
- For the expression of this gene the bacterial cell should provide T7 RNA polymerase, which is under the control of Lac-Z operator/promoter.
- The repressor produced by the bacterial lac-IQ can be regulated by IPTG.

Baculovirus based vectors

Baculovirus is a DNA virus with host range restricted to invertebrates, mostly insects. The baculovirus expression system has been used extensively for the expression of recombinant proteins in insect cells. Baculovirus is a group of insect virus and *Autographa californica* nucleopolyhedrovirus (AcMNPV) is the most extensively studied virus under this family.

The infection of AcMNPV is initiated by replication and transcription of of the DNA genome inside the nucleus and the assembly of the nucleocapsids. The nucleocapsids then bud off from the plasma membrane and initiate systemic infection.

Although, baculoviral vectors can transfect only insect cells, recombinant baculoviral vectors have been constructed containing mammalian cell specific promoters which can be used to infect mammalian cells as well.

Advantages of baculoviral vectors:

• Since insects cells are high eukaryotes, desired port-translational modification of complex protein can be achieved.
• They have higher packaging capacity of insert.
• Lower biosafety issue.
• High level of protein expression.

There are few drawbacks of baculoviral vectors such as-

• Foreign protein expression using an insect system is more complex and time consuming than a bacterial system.
• Sometime protein post-translational processing may be sub-optimal to compensate the secretory pathway of the protein.

Mammalian SV40-based expression vectors

Simian virus 40 (SV40) was the first mammalian expression vector whose genome size is 5.2kb. It is a DNA virus which can infect human as well other mammalian cell lines. SV40 may integrate into the host genome, permitting stable transmission of insert DNA to daughter cells. Recombinant SV40 vectors (rSV40) display some unique features:

• SV40 is a well-known virus and nonreplicative vectors are easy-to-make
• SV40 can be produced in high titers (10^{12} IU/ml).
• They can infect both resting and dividing cells.
• Stable transgene expression can be achieved in a wide range of cell types.

The major disadvantage of SV40 vector is the low packaging capacity with insert size of <5kb.

Possible questions

Part-B (2 Marks)

1. What are cloning vectors?
2. Define plasmid?
3. Mention any four Properties of Good Vector.
4. Mention the marker genes on pUC plasmid.
5. Mention the marker genes on pBR plasmid.
6. What as λ phage vectors and mention the types of λ phage vectors.
7. Mention any four properties of M13 vectors.
8. What are expression vectors?
9. What are linkers and adopters?
10. Expand YAC and BAC.
11. What is YACs?
12. What is BACs?

Part-C (8 Marks)

1. Write a detailed note on types of plasmids.
2. Write a detailed note on pUC and pBR plasmids.
3. Write a detailed note on bacteriophage vectors.
4. Write a detailed note on (a) cosmids and (b) linkers and adopters.
5. Write a detailed note on BACs.
6. Write a detailed note on YACs.
7. Write a detailed note on yeast YIp, YEp and YCp vectors.
8. Write a detailed note on *E.coli* lac and T7 promoter-based vectors.
9. Write a detailed note on (a) Baculovirus based vectors and (b) Mammalian SV40-based expression vectors.

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: II B.Sc MB COURSE NAME: RECOMBINANT DNA TECHNOLOGY
COURSE CODE: 18MBU404B UNIT: II BATCH-2018-2021

Sl.No	Questions	opt1	opt2	opt3	opt4	Answer
1	Plasmids are extra chromosomal DNA that vary in size from ____ to _____.	1 kb to more than 200 kb	2 kb to more than 100 kb	5 kb to more than 200 kb	More than 300 kb	1 kb to more than 200 kb
2	Plasmid was coined by _____.	Maccarthy	Avery	Lederberg	Smith	Lederberg
3	Plasmid carries _____.	PMBI replicon	PI5A replicon	ColEi replicon	PkN402	PMBI replicon
4	The copy number of plasmid was controlled by _____.	Host chromosome	Regulatory genes	Motar genes	Replicons	Replicons
5	Plasmids are commonly found in _____.	Algae	Virus	Protozoa	Prokaryotes	Prokaryotes
6	The term episome was coined by _____.	A.Jacob and Wollman	Lederberg	Watson	Collins	A.Jacob and Wollman
7	PBR322 plasmid vectors have _____.	Amp resistance gene	kan resistance gene	Lac z gene	X-gal	Amp resistance gene
8	Runaway vectors replicate upto _____.	34 °C	40 ^{o}C	94 °C	60 °C	34°C
9	Eukaryotes that habour a plasmid _____.	*Fusarium*	*Saccharromyces cerevisiae*	*Pencillium*	*Aspergillus*	*Saccharromyces cerevisiae*
10	There are two types of naturally occurring plasmids namely, _____ and _____.	Col plasmid and F plasmid	E plasmid and F plasmid	Both A and B	PBR322 and F plasmid	Col plasmid and F plasmid
11	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
12	Transfer genes have cluster of _____.	12 different genes	11 different genes	10 different genes	20 different genes	12 different genes
13	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

14	The bacteria associated with the discovery of R plasmids _____.	*Bacillus*	*Pseudomonas*	*Shigella*	*Salmonella*	*Shigella*
15	Non-conjugative plasmids have _____.	tra+/ mob+ genes	tra-/ mob- genes	tra-/ mob+ genes	Mob+/ mob+ genes	tra-/ mob+ genes
16	Plasmids carrying genes, which is responsible for antibiotic resistance known as _____.	R plasmids	Cancer inducing	Col E 1 plasmid	F plasmid	R plasmids
17	Plasmid contain autonomous replication (rep) includes _____.	Origin of replication	chemicals	Copy number	genes	Origin of replication
`18	The plasmid that maintains low copy number in a cell _____.	Stringent plasmid	Relaxed plasmid	Conjugative plasmids	None of the above	Stringent plasmid
19	The genes for drug resistance are initially coded on _____.	PBR322	Transposons	Yeast plasmid	None of the ooove	PBR322
20	PBR322 composed of _____.	3 section	2 section	1 section	5 section	3 section
21	Tn3 from transposon for PBR322 have _____.	Tetracyclin resistant	Streptomycin resistant	Ampicillin resistant	Chloramphenicol resistant	Ampicillin resistant
22	The best-studied Col plasmid is _____.	ColA	ColB	ColE	ColD	ColE
23	The replication of DNA is initiated at _____.	2534 position of PBR322	2000 position ofPBR322	2435 position of PBR322 .	1000 position ofPBR322	2534 position of PBR322
24	One gene important for ampicillin resistant in PBR322 is _____.	D galactose gene	P -galactose gene	B lactamase gene	B- Lactase gene	B- lactamase gene
25	PBR 322 vectors are restricted to _____.	Gram positive bacteria	Gram negative bacteria	Archae bacteria	Eubacteria.	Gram negative bacteria
26	Lambda DNA has a genetic material as _____.	ds DNA	Ss DNA	ds RNA	ssRNA	Ss DNA
27	Lambda genome has a length of _____.	48502bp	33402bp	40000bp	50,435bp	48502bp
28	The non-essential region of lambda phage is _____.	int,xis,mob	int,xis,att	tra,mob,xis	B2 region	B2 region
29	DNA replication during lytic growth required for the lysis	Sand R	Rand T	Sand Q	Q and U	Sand R

	of cellular membrane _____.					
30	Vectors, which contain a unique site (x) for the insertion of foreign DNA have been designated _____.	Expression vector	Replacement vector	Insertional vector	Shuttle vector	Insertional vector
31	λgt10 carry up to _____.	8 Kb of new DNA	10kb new DNA	15kb new DNA	20 kb new DNA	10kb new DNA
32	λgt10 E CORl site is located in _____.	R genes	C 1 genes	b 2 genes	D genes	C 1 genes
33	λEMBL4 carries up to _____.	10 Kb	20kb	l5kb	25 kb	10 Kb
34	λEMBL4 selected on the basis of _____.	Size /	Size / X gal Phenotype	Size / Non recombinants	None .	Size / Spiphenotype
35	The expression vector for somatostatin is _____.	Psom II 3	P som II	P BH 20	P som I	Psom II 3
36	The viral genome of SV40 is _____.	5243 bp	5842bp	3483bp	2800bp	5243 bp
37	Sv40 DNA is isolated from virus particles in the form of _____.	Super helical DNA	Double Stranded Super helix DNA	RNA	Inner DNA	Super helical DNA
38	Early region codes for two partially overlapping genes, which direct the synthesis of _____.	T -Antigen	t-antigen	R -antigen	r -antigen	T -Antigen
39	The function of T antigen is to _____.	Initiate DNA replication	Control of its own transcription	Translation control	Control of its own translation	Initiate DNA replication
40	Which organism is called as "Super Bug"?	*Escherichia coli*	Bacillus spp	*Pseudomonas putida*	*Streptococcus*	*Pseudomonas putida*
41	A Vir E code for which protein that binds to T-DNA during transfer _____.	Competence	SSB proteins	DSB proteins	Tyrosine	SSB proteins
42	PH V 33 Multiply both in _____ and _____.	*E.Coli* and Bacillus	Bacillus and Streptomyces	Bacillus and Yeast	Bacillus and *Pseudomonas*	*E.Coli* and Bacillus
43	*Agrobacterium tumefaciens* infects _____	Monocotyledon	Dicotyledon	Trees	Creepers	Dicotyledon

		Palnts	Plants			Plants
44	Ti Plasmids are _____.	Transgenic plasmids	Tumour inducing plasmids	Inhibiting plasmids	Haemorhageic plasmids	Tumour inducing plasmids
45	In retrovirus gag codes for _____.	Reverse transcriptase	Polymerase	Synthase	Envelop proteins	Reverse transcriptase
46	In retrovirus pol codes for _____.	Reverse transcriptase	Polymerase	Synthase	Envelop proteins	Polymerase
47	In retrovirus env codes for _____.	Reverse transcriptase	Polymerase	Synthase	Envelope proteins	Envelope proteins
48	Vectors, which contain two restriction sites for the insertion of foreign DNA have been called as _____.	Expression vector	Replacement vector	Insertional vector	Shuttle vector	Replacement vector
49	The first step I to create recombinant AcMNPV is to generate _____.	Transfer vector	Helper vector	Modified vector	Initiator sequence	Transfer vector
50	Copy number are either strictly controlled by correlating with the number of _____.	Plasmid DNA	Chromosomal DNA	Insertional DNA	None of the above	Chromosomal DNA
51	During infection _____ forms of virons are produced.	One form	Two form	Three form	Fiveform	Two form
52	In second form the protein of matrix is called as _____.	Monohedrin	Polyhedrin	Dihedrin	Trihedrin	Polyhedrin
53	λgt10 E CORl site is located in _____.	R genes	C 1 genes	b 2 genes	D genes	C 1 genes
54	pUC18 vector is _____ selection vector.	Amp selection vector	Kan selection vector	Lac selection vector	Spi selection vector	Lac selection vector
55	PBR322 the word BR stands for _____.	Berger	Bunsen rodergh	Beep run	Boliver and	Boliver and Rodriguez
56	pBR322 was developed in _____.	1960	1980	1970	1990	1970

57	EM vector have _____.	SP6 promoter	T7 Promoter	Both SP6 and T7	T8 Promoter	Both SP6 and T7
58	_____is produced from *E.coli*.	F plasmid and COL E plasmid	Ti plasmid	degradative plasmid	None of the above	F plasmid and COL E plasmid
59	T4 DNA polymerase optimum temperature is _____.	37	45	55	94	94
60	Reverese transcriptase is isolated from _____.	Retrovirus	Bacteria	Fungus	Aedinovirus	Retrovirus

UNIT –III

Syllabus

Transformation of DNA: Chemical method, Electroporation. Gene delivery: Microinjection, electroporation, biolistic method (gene gun), liposome and viral- mediated delivery, *Agrobacterium* - mediated delivery DNA, RNA and Protein analysis: Agarose gel electrophoresis, Southern - and Northern - blotting techniques, dot blot, DNA microarray analysis, SDS-PAGE and Western blotting.

Transformation of DNA

Transformation is the process by which genetic makeup of an organism is altered by the insertion of new gene (or exogenous DNA) into its genome.

- Transformation was first demonstrated in 1928 by British bacteriologist Frederick Griffith in S *pneumonia*.
- In 1944 this "transforming principle" was identified as being genetic by Oswald Avery, Colin MacLeod, and Maclyn McCarty.
- Transformation can occur in two ways: natural transformation and artificial transformation.
- Natural transformation describes the uptake and incorporation of naked DNA from the cell's natural environment.
- Artificial transformation encompasses a wide array of methods for inducing uptake of exogenous DNA.

Chemical method

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

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

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

The commonly used methods of chemical transfection use the following,

(i) Calcium phosphate

(ii) DEAE dextran

(iii) Cationic Lipid

(iv) Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

(i) Calcium phosphate transfection:

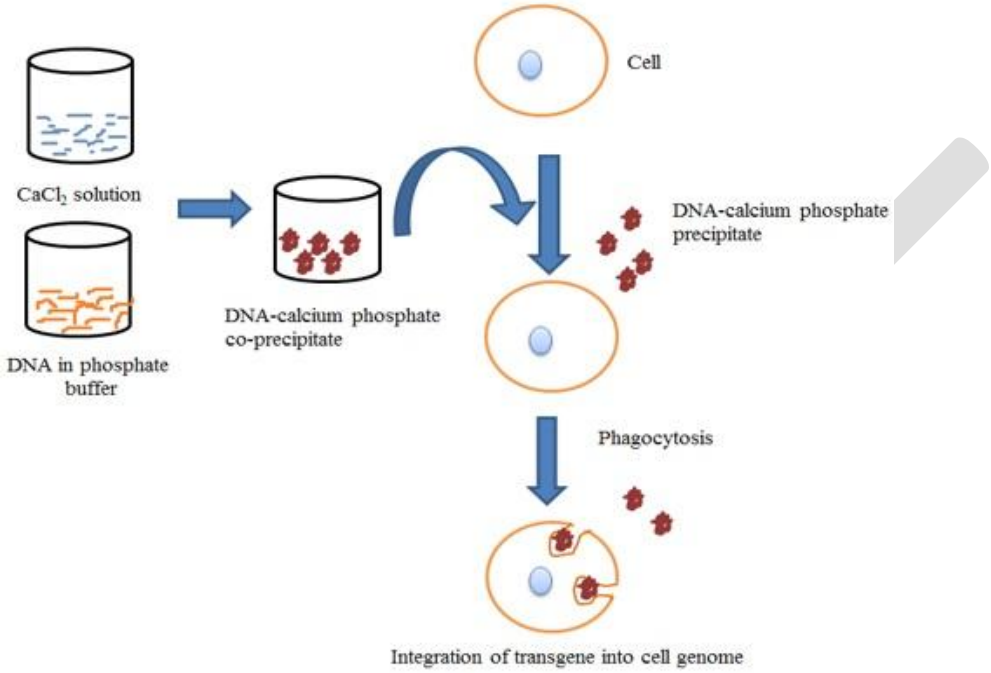
This method is based on the precipitation of plasmid DNA and calcium ions by their interaction. In this method, the precipitates of calcium phosphate and DNA being small and insoluble can be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis and the DNA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

Applications:

- Production of recombinant viral vectors; It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines; Simple and inexpensive; Applicability to generate stably transfected cell lines; Highly efficient (cell type dependent) and can be applied to a wide range of cell types; Can be used for stable or transient transfection

Disadvantages

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



This technique is simple, expensive and has minimal cytotoxic effect but the low level of transgene expression provoked development of several other methods of transfection.

(ii) DEAE-Dextran (Diethylaminoethyl Dextran)mediated DNA transfer:

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

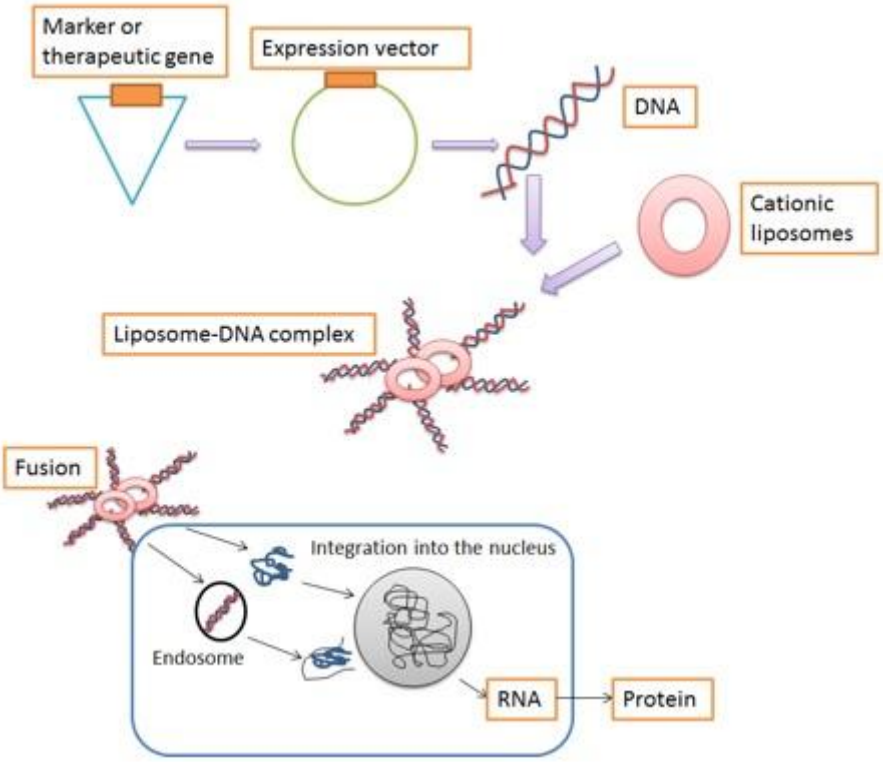
Advantages: Simple and inexpensive; More sensitive; Can be applied to a wide range of cell types; Can be used for transient transfection.

Disadvantages:

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

(iii) Lipofection:

- Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.
- Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of 0.1 to 10 micrometer or 20-25 nanometers respectively.
- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.
- Liposomes can be classified as either cationic liposome or pH-sensitive.
- Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) is some commonly used neutral co-lipids.



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

Advantages: Economic; Efficient delivery of nucleic acids to cells in a culture dish; Delivery of the nucleic acids with minimal toxicity; Protection of nucleic acids from degradation; Measurable changes due to

transfected nucleic acids in sequential processes; Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

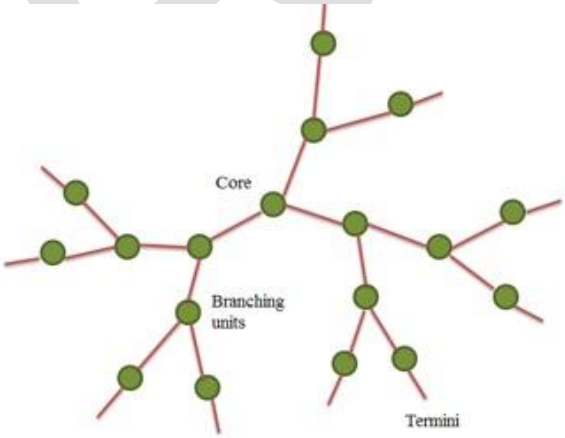
Disadvantages:
It is not applicable to all cell types; It fails for the transfection of some cell lines with lipids.

Other Methods
Other methods of chemical transfection involve the use of chemicals such as polyethylenimine, chitosan, polyphosphoester, dendrimers.

- Polyethylenimine (PEI) is a non-degradable, high molecular weight polymer which may accumulate in the body. PEI, due to its polycationic nature, condenses with the DNA molecule resulting in the formation of PEI-DNA complex which enters the cell by endocytosis, thus mediating gene transfer.

- Chitosan, a biodegradable polysaccharide is composed of D-glucosamine repeating units and can be used as a non-viral gene carrier. It can efficiently bind and protect DNA from nuclease degradation.
- Polyphosphoesters (PPE) are biocompatible and biodegradable, particularly those having a backbone analogous to nucleic acids and teichoic acids and used in several biomedical applications. They may result in extracellular persistent release of the DNA molecules thus enhancing the expression of transgene in the muscle as compared to naked DNA intake. Several polyphosphoesters with positive charges both in the backbone and in the side chain can be used as non-viral gene carriers.

- Dendrimers are a new class of polymeric materials that are highly branched and monodisperse macromolecules. Due to their unique behaviour, they are suitable for a wide range of biomedical applications. They have positively charged amino groups (termini) on their surface which interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.

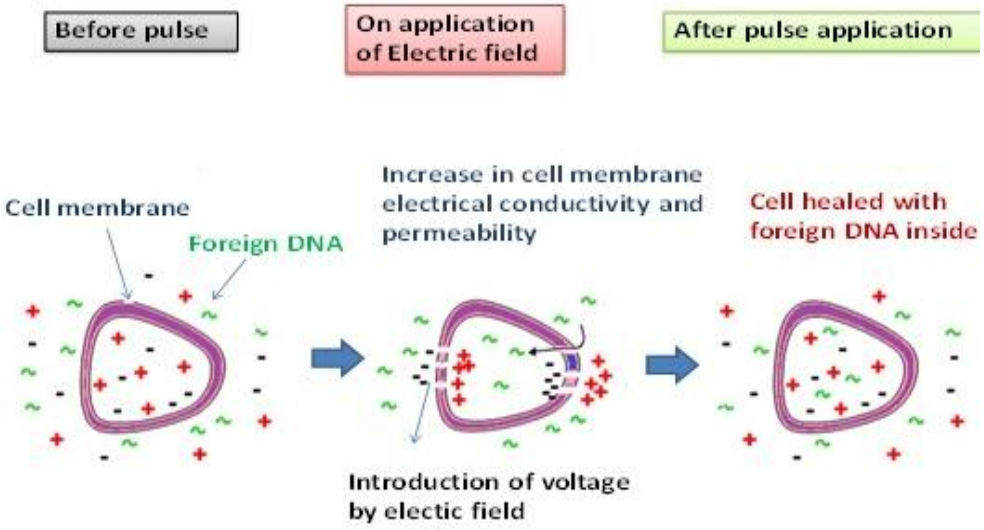


Electroporation

A mechanical method used to apply an electric current across a cell membrane resulting in temporary "pore" formation enabling the uptake of exogenous molecules found in the medium to either the cytoplasm or into the nucleus, thereby transfecting or transforming the cell.

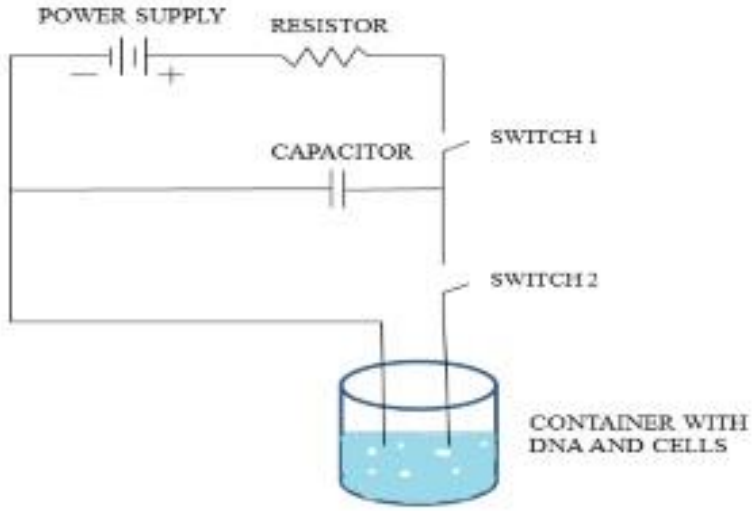
- This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer in mouse cells.

- It is now a widely used method for the introduction of transgene either stably or transiently into bacterial, fungal, plant and animal cells.



Procedure:

- The host cells and the DNA molecules to be transported into the cells are suspended in a solution. The basic process inside an electroporation apparatus is represented in a schematic diagram.
- When the first switch is closed, the capacitor charges up and stores a high voltage which gets discharged on closing the second switch.
- Typically, 10,000-100,000 V/cm in a pulse lasting a few microseconds to a millisecond is essential for electroporation which varies with the cell size.
- This electric pulse disrupts the phospholipid bilayer of the membrane causing the formation of temporary aqueous pores.
- When the electric potential across the cell membrane is increased by about 0.5-1.0 V, the charged molecules e.g. DNA migrate across the membrane through the pores in a similar manner to electrophoresis.
- The initiation of electroporation generally occurs when the transmembrane voltage reaches at 0.5-1.5 V.
- The cell membrane discharges with the subsequent flow of the charged ions and molecules and the pores of the membrane quickly close reassembling the phospholipid bilayer.



Advantages:

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

Disadvantages:

- Cell Damage: If the pulses are of the wrong length or intensity, some pores may become too large or fail to close after membrane discharge causing cell damage or rupture
- Nonspecific Transport: The transport of material into and out of the cell during the time of electro-permeability is relatively nonspecific. This may result in an ion imbalance that could later lead to improper cell function and cell death.

Applications:

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

- DNA transfection or transformation
- Direct transfer of plasmids between cells
- Gene transfer to a wide range of tissues
- Chemotherapy, Gene Therapy, Transdermal Drug Delivery

Gene delivery

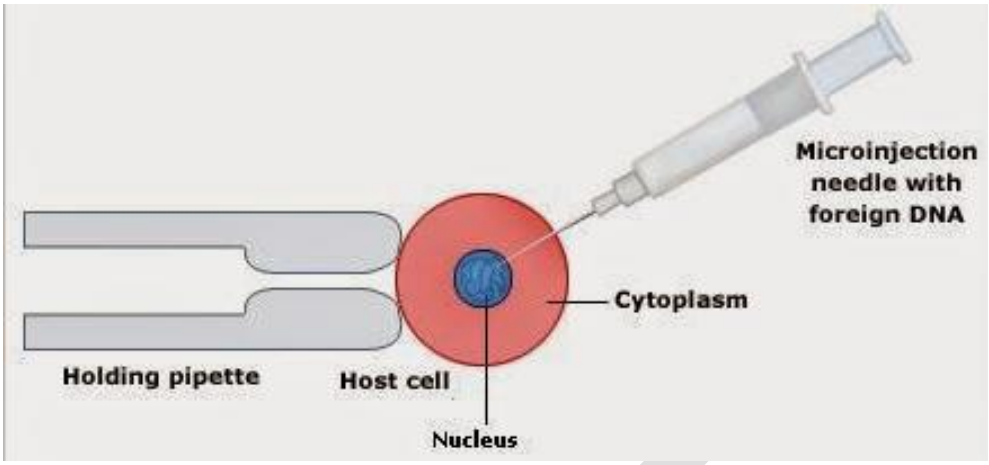
Gene delivery is the process of introducing foreign genetic material, such as DNA or RNA, into host cells. Genetic material must reach the nucleus of the host cell to induce gene expression.

Microinjection:

- Microinjection is a technique of delivering foreign DNA into a living cell (a cell, egg, oocyte, embryos of animals) through a glass micropipette.
- Rubin and Spradling (1982) for the first time introduced *Drosophila* gene for xanthine dehydrogenase into a P-element (parental element) which was microinjected with an intact helper P-element into embryo deficient for this gene.

Procedure:

- One end of a glass micropipette is heated until the glass becomes somewhat liquified.
- It is quickly stretched which forms a very fine tip at the heated end.
- The tip of the pipette attains to about 0.5 mm diameter which resembles an injection needle.
- The process of delivering foreign DNA is done under a powerful microscope.
- Cells to be microinjected are placed in a container.
- A holding pipette is placed in the field of view of the microscope.
- The holding pipette holds a target cell at the tip when gently sucked.
- The tip of the micropipette is injected through the membrane of the cell.
- Contents of the needle are delivered into the cytoplasm and the empty needle is taken out.



Advantages:

- High precision on the injection of thermoplastic, ceramic and metallic pieces.
- Three steps injection. Reduction of resident time of materials at high temperature. The degraded material usually generated by conventional injection is eliminated.
- Reproducibility and repeatability.
- Automatic high production system based on the double mould configuration. Processing and injection process can be simultaneously performed.
- Reduction of wastes, material injection optimized. There is a reduction of materials costs per injected unit.

Limitations: Costly; Skilled personal required; More useful for animal cells

Applications:

Process is applicable for plant cell as well as animal cell but more common for animal cells; Technique is ideally useful for producing transgenic animal quickly; Procedure is important for gene transfer to embryonic cells; Applied to inject DNA into plant nuclei.

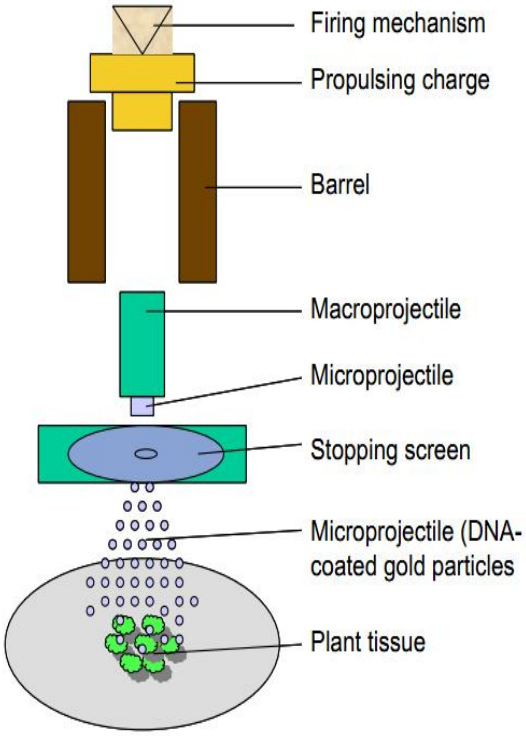
Biolistic method (gene gun):

- Prof Sanford and colleagues at Cornell University (USA) developed the original bombardment concept in 1987 and coined the term “biolistics” (short for “biological ballistics”) for both the process and the device.
- Also termed as particle bombardment, particle gun, micro projectile bombardment and particle acceleration.
- It employs high-velocity micro projectiles to deliver substances into cells and tissues.

The biolistic gun employs the principle of conservation of momentum and uses the passage of helium gas through the cylinder with arrange of velocities required for optimal transformation of various cell types. It consists of a bombardment chamber which is connected to an outlet for vacuum creation. The bombardment chamber consists of a plastic rupture disk below which macro carrier is loaded with micro carriers. These micro carriers consist of gold or tungsten micro pellets coated with DNA for transformation.

The apparatus is placed in Laminar flow while working to maintain sterile conditions. The target cells/tissue is placed in the apparatus and a stopping screen is placed between the target cells and micro

carrier assembly. The passage of high pressure helium ruptures the plastic rupture disk propelling the macro carrier and micro carriers.



The stopping screen prevents the passage of macro projectiles but allows the DNA coated micro pellets to pass through it thereby, delivering DNA into the target cells.

Advantages:

- Simple and convenient method involving coating DNA or RNA on to gold microcarrier, loading sample cartridges, pointing the nozzle and firing the device.
- No need to obtain protoplast as the intact cell wall can be penetrated.
- Manipulation of genome of sub-cellular organelles can be done.
- Eliminates the use of potentially harmful viruses or toxic chemical treatment as gene delivery vehicle.
- This device offers to place DNA or RNA exactly where it is needed into any organism.

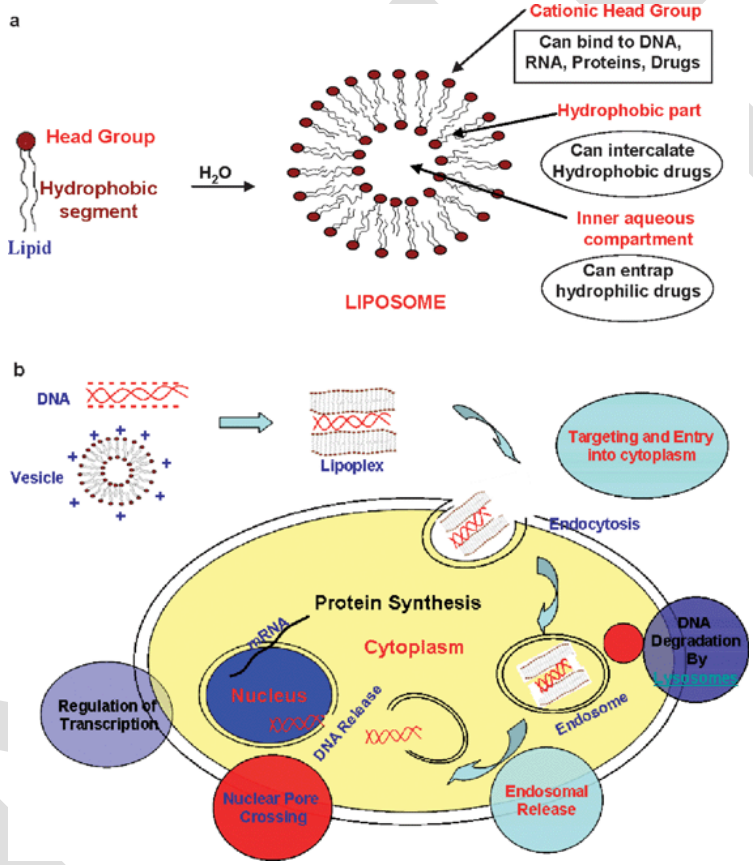
Disadvantages:

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

Applications: This method is commonly employed for genetic transformation of plants and many organisms; This method is applicable for the plants having less regeneration capacity and those which fail to show sufficient response to *Agrobacterium*- mediated gene transfer in rice, corn, wheat, chickpea, sorghum and pigeon-pea.

Liposomes:

- Liposomes are the small bladder having spherical shapes which are produced from glycolipids, cholesterols, non toxic surfactants and membranous proteins.
- These are generally a carrier loaded with different variety of molecules like minute drug molecule, proteins and (cationic liposomes) nucleotides.
- The liposomes were discovered in the year 1960 by British hematologist Dr. Alec D. Bangham. Liposomes have been manufactured and classified on the basis of size, composition, charge and speciality.
- The mechanism of action of liposomes have been attributed to their binding and dispersing with cellular membrane releasing the drug into the cell; engulfment by the cell and transfer of phospholipids into the cell membrane by which the drug is released; and the entrapment by the lipid bilayer acting upon by lysosomes and releasing the active ingredient of formulation.
- Moreover, the preparation of liposomes can be done by mechanical methods involving replacement of organic solvent; fusion of preformed vesicles; and by transformation of size.



Viral- mediated delivery:

There are several types of virus that can be adapted for use as viral vectors, including those based on herpes simplex virus (HSV-1), adenovirus (AV), adeno-associated virus (AAV), and lentivirus.

Herpes Simplex Virus:

- The herpes viruses are linear ds-DNA viruses of approximately 150 kb size. Most HSVs are transmitted without symptoms (varicella zoster virus is exceptional) and cause prolonged infections
- With the help of two viral glycoproteins, gB and gD, the virus binds to cells through an interaction with heparan sulphate moieties on the cell surface

- Most promised use of HSV vectors involves gene transfer to neural cells where it can cause a latent infection (e.g. spinal cord, brain, and peripheral nervous system)

Advantages:
Infects a wide range of cell types; Insert size up to 50 kb due to large viral genome size; Natural tropism to neuronal cells; Stable viral particles allow generation of high virus titres (1012pfu/ml)

Disadvantages:
No viral integration into host genome and transient transgene expression; High level of pre-existing immunity; Cytotoxicity effects; Risk of recombination with latently HSV-infected cells

Adenoviruses:

- Adenoviruses are medium-sized (90-100 nm), non-enveloped, icosahedral viruses containing linear, double-stranded DNA of approximately 36 kb
- First generation adenoviral vectors were replication deficient, lacking the essential E1A and E1B genes and often the non-essential gene E3 and were called 'E1 replacement vectors'
- They had a maximum capacity of about 7 kb and were propagated in the cell lines transfected with DNA containing E1 genes e.g. human embryonic kidney line 293
- To overcome the above limitations, an alternative strategy employs insertion of _stuffer DNA' into the nonessential E3 gene as part of the vector backbone so to maintain optimum vector size

Advantages:
High transduction efficiency; Insert size up to 7-8 kilobases; Generation of high virus titres; High level of expression in a wide variety of cell types; No mutagenic effects due to lack of random integration into the host genome

Disadvantages:
Transient expression due to lack of integration into the host; Pathogenic to humans

Adeno-associated virus:

- It was first discovered as a contaminant in an adenoviral isolate in 1965.
- It is a small, non-enveloped virus packaging a linear single stranded DNA belonging to Parvovirus family. It is naturally replication defective thus requiring a helper virus (usually adenovirus or herpes virus) for productive infection
- The AAV genome is small (about 5 kb) and comprises a central region containing rep (replicase) and cap(capsid) genes flanked by 145 base inverted terminal repeats (ITRs).
- In vitro manipulation of AAV is facilitated by cloning the inverted terminal repeats in a plasmid vector and inserting the transgene between them. Transfection of this construct into cells along with a helper plasmid produced recombinant viral particles.

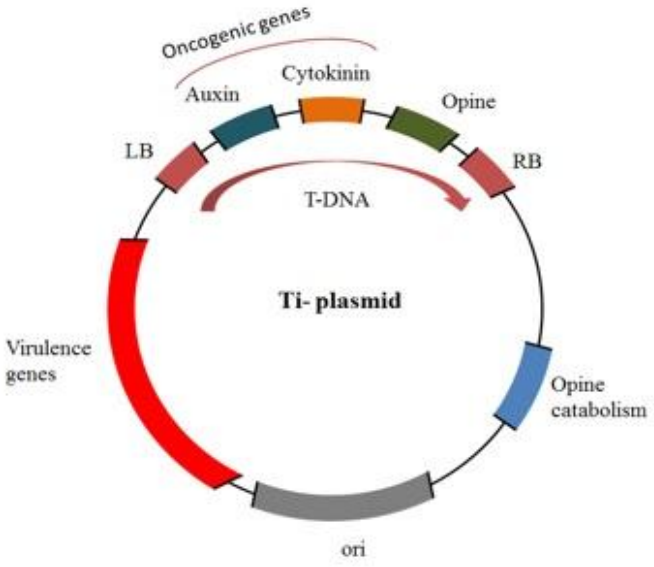
Advantages: Stable and have a wide host range; Lack of initiating an immune response; The dependence of AAV on a heterologous helper virus provides higher control over; vector replication, making AAV vectors safer for use in gene therapy; Potential of targeted/site-specific integration; Non-pathogenic

Disadvantages:

Uses concatemeric replication intermediates; they must be closely screened as they are often contaminated with adenovirus or Herpes Virus; Insert size is limited (4Kb); Difficult generation of high virus titres

***Agrobacterium* - mediated delivery:**

- *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are common gram-negative soil borne bacteria causing induction of 'crown gall' and 'hairy root' diseases. These bacteria naturally insert their genes into the genome of higher plants. The crown gall formation is due to the transfer of a segment of oncogenic (cancer causing) DNA into the plant cell at wounded sites.
- This DNA segment (transfer DNA or T-DNA) is present on large plasmid called Tumor- inducing (Ti) plasmids (140–235 kbp) in the bacterium.
- The T-DNA contains two groups of genes, which possess the ability to express in plants as follows-
  (a) Oncogenes for synthesis of auxins and cytokinins (phytohormones). The over-production of phytohormones leads to proliferation of callus or tumour formation.
  (b) Opine synthesizing genes for the synthesis of opines (a product from amino acids and sugars secreted by the crown gall infected cells and utilized by *A. tumefaciens* as carbon and nitrogen sources). Thus opines act as source of nutrient for bacterial growth, e.g. Octopine, Nopaline.
- Genes in the virulence region are grouped into the operons *virABCDEG*, which code for the enzymes responsible for mediating conjugative transfer of T-DNA to plant cells.
  - *virA* codes for a receptor which reacts to the presence of phenolic compounds such as acetosyringone, syringealdehyde or acetovanillone which leak out of damaged plant tissues.
  - *virB* encodes proteins which produce a pore/pilus-like structure.
  - *virC* binds the overdrive sequence.
  - *virD1* and *virD2* produce endonucleases which target the direct repeat borders of the T-DNA segment; virD4 is the coupling protein.
  - *virE* binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes, beginning with the right border.
  - *virG* activates vir-gene expression after binding to a consensus sequence, once it has been phosphorylated by *virA*



- When a plant root or a stem is wounded it gives off certain response. In response to those signals, the vir genes of *A. tumefaciens* become activated, and direct a series of events required for the transfer of the T-DNA from the Ti plasmid to the plant's chromosome. The function of different vir genes include a copy of T-DNA, followed by attachment of a product to the copied T-DNA strand to act as a leader, subsequently add proteins along with the length of the T-DNA, possibly as a protective mechanism.
- These eventually open a channel in the bacterial cell membrane, through which the T-DNA passes. The T-DNA then enters the plant through the wound.
- To use these bacteria as a vector its T-DNA region is removed excepting the border regions and the vir genes. The transgene is then inserted between the T-DNA regions, where it is transferred to plant cell and becomes integrated into the plant's chromosome. The T-DNA is cloned in Ti-plasmids, which are cut to size and replicated in *E.coli* to facilitate further manipulation. These vectors are mobilized into *Agrobacterium* host strains and used to infect the plant tissues.

Advantages:

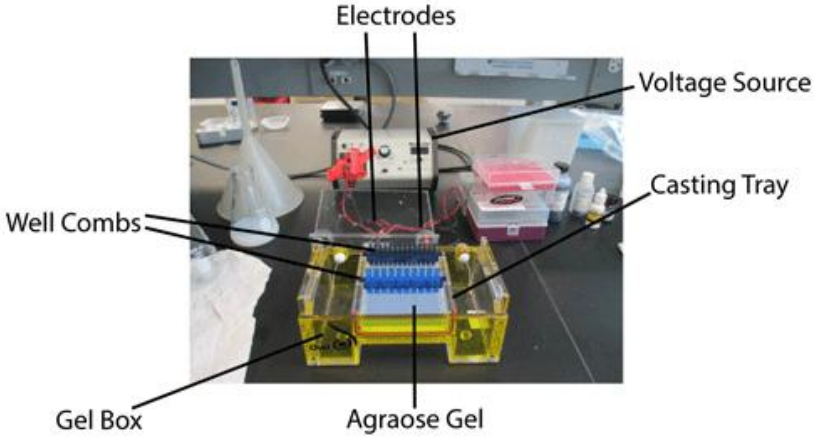
- This is a natural method of gene transfer.
- *Agrobacterium* can conveniently infect any explant (cells/tissues/organs).
- Even large fragments of DNA can be efficiently transferred.
- Stability of transferred DNA is reasonably good.
- Transformed plants can be regenerated effectively.

Limitations:

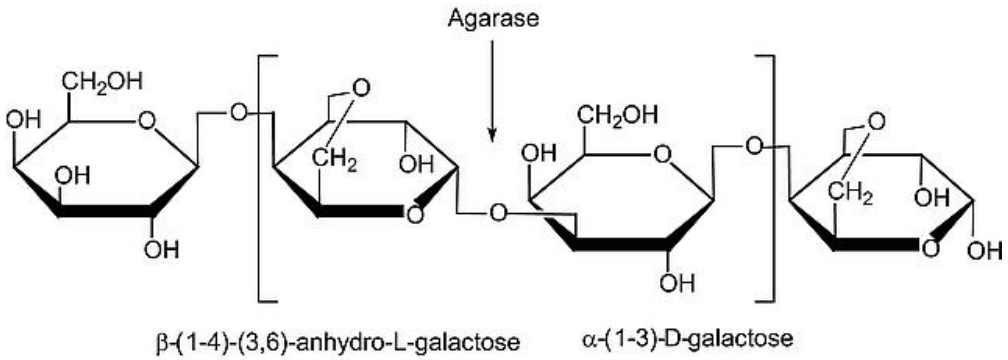
- There is a limitation of host plants for *Agrobacterium*, since many crop plants (monocotyledons e.g. cereals) are not infected by it. In recent years, virulent strains of *Agrobacterium* that can infect a wide range of plants have been developed.
- ii. The cells that regenerate more efficiently are often difficult to transform, e.g. embryonic cells lie in deep layers which are not easy targets for *Agrobacterium*.

Agarose gel electrophoresis:

Agarose gel electrophoresis is the easiest and most popular way of separating (100 bp to 25 kb DNA fragments) and analyzing nucleic acids. 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.



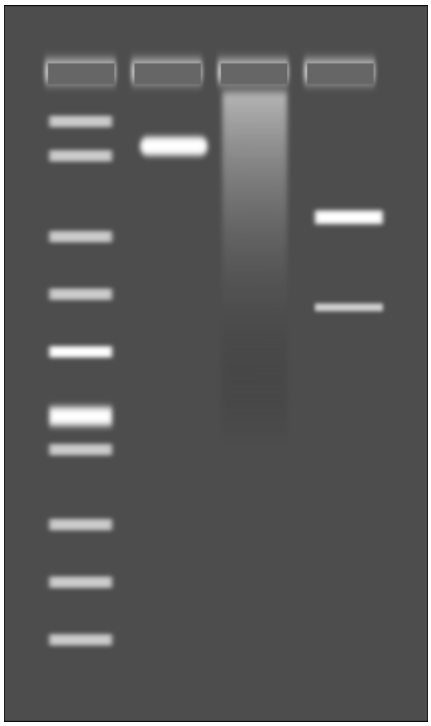
Agarose is isolated from the seaweed genera *Gelidium* and *Gracilaria*, and consists of repeated agarobiose (L- and D-galactose) subunits. Agarose makes an inert matrix. A 0.7 % gel will show good separation for large DNA fragments (5-10 kb) and a 2 % gel will show good resolution for small fragments with size range of 0.2-1.0 kb. Low percentage gels are very weak but high percentage gels are usually brittle and do not set evenly.



Procedure

- Agarose gels are commonly used in concentrations of 0.7 % to 2 % depending on the size of bands needed to be separated
- Mix agarose powder with 100 mL 1xTAE (Tris base, Acetic acid, EDTA and distilled water) in a microwavable flask.
- Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel).
- Let agarose solution cool down to about 50 °C, about 5 min.
- Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows to visualize the DNA under ultraviolet (UV) light.
- Pour the agarose into a gel tray with the well comb in place.
- Place newly poured gel at 4 °C for 10-15 min OR let sit at room temperature for 20-30 mins, until it has completely solidified.
- Add loading buffer (30 % Glycerol + 0.25 % Bomophenol blue dye) to each of your DNA samples.

  Note: Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and allows to gauge how far the DNA has migrated; 2) it contains a high percentage of glycerol that increases the density of your DNA sample causing it settle to the bottom of the gel well, instead of diffusing in the buffer.
- Once solidified, place the agarose gel into the gel box (electrophoresis unit).
- Fill gel box with 1xTAE (or TBE) until the gel is covered.
- Carefully load a molecular weight ladder into the first lane of the gel.
- Carefully load your samples into the additional wells of the gel.
- Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.



- Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
- If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5 µL of EtBr, place on a rocker for 20-30 min, replace EtBr solution with water and destain for 5 mins.
- Using UV transilluminator, visualize DNA fragments. The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel.

  Note: If purifying the DNA for later use, use long-wavelength UV and expose for as little time as possible to minimize damage to the DNA.

Factors Affecting the Movement of DNA:

(i) 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.

(ii) Ethidium Bromide (EtBr): After the running of DNA through an EtBr-treated gel, any band containing more than ~20 ng DNA becomes distinctly visible under UV light. Binding of Ethidium bromide to DNA alters its mass and rigidity, and thereby its mobility.

(iii) 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.

(iv) Conformation of DNA: Nicked or open circular DNA will move slowly than linear and super coiled DNA. Super helical circular, nicked circular and linear DNAs migrate gels at different rates through agarose gel.

Southern blotting technique:

- It was developed by Edward M. Southern (1975).
- Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.
- Procedure/ Steps
  - Restriction digest: by RE enzyme and amplification by PCR
  - Gel electrophoresis: SDS gel electrophoresis
  - Denaturation: Treating with HCl and NaOH
  - Blotting
  - Baking and Blocking with casein in BSA
  - Hybridization using labelled probes
  - Visualization by autoradiogram

Step I: Restriction digests

- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments
- The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

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

Step IV: Blotting

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

Step V: Baking and blocking

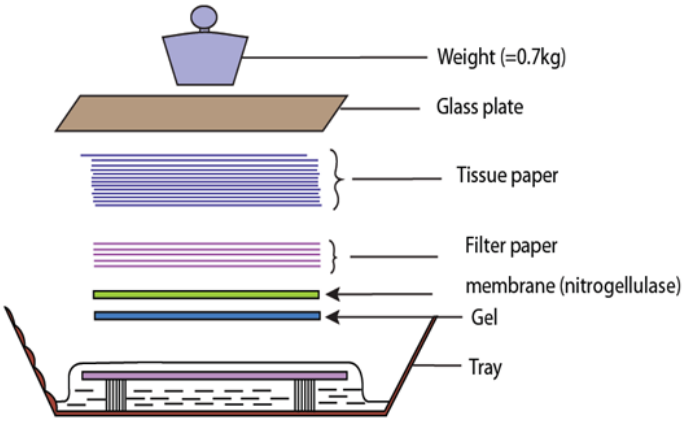
- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

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

Step VII: Visualization by Autoradiogram

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



Application:

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

Northern blotting technique:

- The northern Blot was developed at Stanford University in 1977 by James Alwine, David Kemp, and George Stark.
- The Northern blot, also known as the RNA blot, is one of the blotting techniques used to transfer RNA onto a carrier for sorting and identification.
- The formaldehyde was use in electrophoresis gel as a denaturant because the sodium hydroxide treatment used in the Southern blot procedure would degrade the RNA.

Procedure:

1) RNA isolation: mRNA is extracted from the cells and purified.

2) Probe generation: The mRNA is loaded onto a gel for electrophoresis. Lane 1 has size standards (a mix of known RNA fragments) Lane 2 has the RNA.

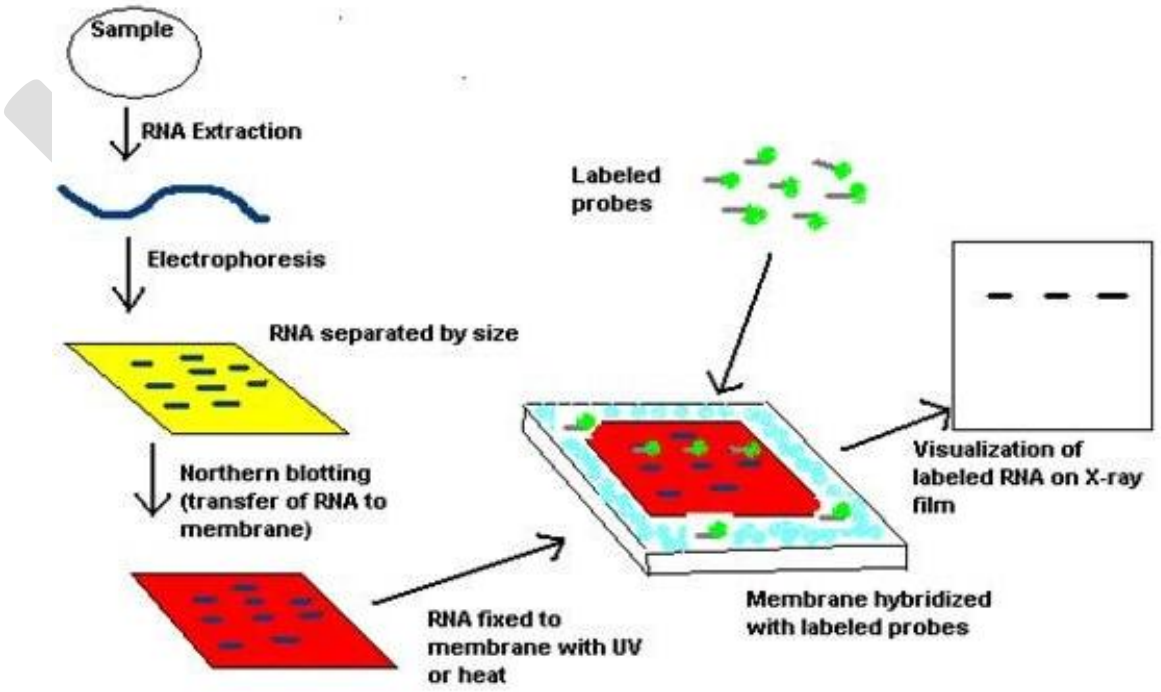
3) Agarose gel electrophoresis: An electric current is passed through the gel and the RNA moves away from the negative electrode. The distance moved depends on the size of the RNA fragment. Since genes are different sizes the size of the mRNAs varies also. This results in a smear on a gel. Standards are used to quantitate the size. The RNA can be visualized by staining first with a fluorescent dye and then lighting with UV.

4) Transfer to solid support and immobilization: RNA (After denatured using formaldehyde) is single-stranded, so it can be transferred out of the gel and onto a membrane without any further treatment. The transfer can be done electrically or by capillary action with a high salt solution.

5) Prehybridization and hybridization with probe: A labelled probe specific for the RNA fragment in question is incubated with the blot. The blot is washed to remove non-specifically bound probe and then a development step allows visualization of the probe that is bound.

6) Washing: The probe is bound specifically to the target mRNA and that there is negligible non-specific binding to other mRNA or the nylon membrane itself.

7) Detection: Hybridization signals are then detected.



Applications:

- Detecting a specific mRNA in a sample
- Used in the screening of recombinants by detecting the mRNA produced by the transgene
- In disease diagnosis
- In gene expression studies

SDS-PAGE:

- Sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis (PAGE) is one of the most widely used laboratory methods to separate proteins.
- SDS is a detergent with a strong protein-denaturing effect and binds to the protein backbone at a constant molar ratio.
- Polymerized acrylamide (polyacrylamide) forms a mesh-like matrix suitable for the separation of proteins of typical size.
- Polyacrylamide (both acrylamide and bisacrylamide copolymerize and makes a 3D network of straight chain of acrylamide with interconnection of bisacryamide) provides a matrix for the polypeptides to run. Polypeptides run towards the positive electrode (anode) through the gel when an electric field is applied.
  - Use 4-8% gels to separate proteins 100 to 500 kDa in size.
  - Use 4-20% gels to separate proteins 10 to 200 kDa in size.
- Electrophoretic mobility of the proteins depends upon 3 factors:
  - Shape – All the proteins are in the primary structure after the treatment with a reducing agent. So, shape doesn't affect the protein separation.
  - Charge – All the proteins are negatively charged proportional to their molecular weight after treatment with SDS. So charge doesn't affect the separation.
  - Size– proteins get separated solely on the basis of their molecular weight.
- Smaller polypeptides move faster because they have to face less hindrance, larger ones move slower because of a greater hindrance. Hence proteins get separated ONLY on the basis of their mass
- Ammonium persulfate provides the free radical necessary for the catalysis of the Polymerization of Acrylamide and Bis-acrylamide
- TEMED (N, N, N, N – tetramethylethylenediamine)**:** by catalyzing ammounium persulfate to form free radicals, TEMED accelerated the polymerization of acrylamide and bis-acrylamide.
- A reducing agent such as mercaptoethanol or dithiothreitol (DTT) (in the presence of a detergent i.e. SDS) breaks down the disulfide bridges that are responsible for protein folding, and a detergent such as SDS imparts a negative charge to the proteins thereby linearizing them into polypeptides.
- Bromophenol blue works as a tracking dye in electrophoresis and is used to monitor the progress of the molecules moving through the gel.
- Gel loading buffer: 1 M Tris-HCl pH 6.8, SDS, glycerol, 14.7 M β-mercaptoethanol, 0.5 M EDTA, bromophenol Blue.
- Staining solution: 0.25g of Coomassie Brilliant Blue R250 + 90 ml methanol:water (1:1 v/v) and 10 ml of Glacial acetic acid.
- Destaining solution: 90 ml methanol:water (1:1 v/v) + 10ml of Glacial acetic acid.

	Stacking Gel	**Resolving gel**
Polyacrylamide concentration	Low	High

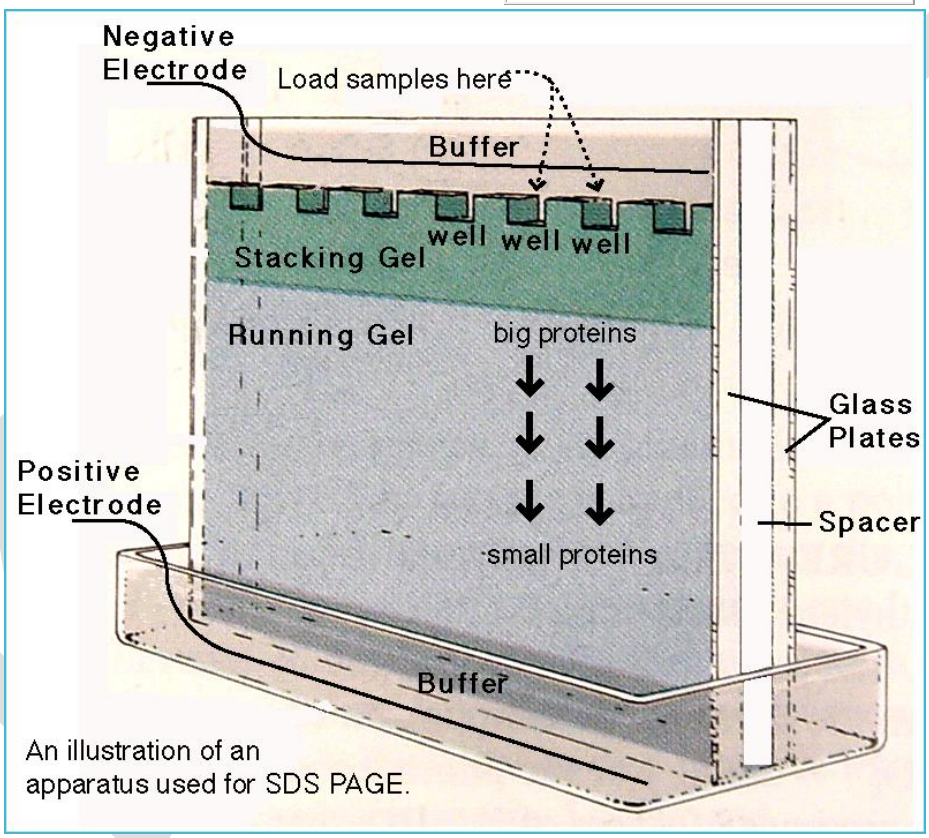
Pore size	Larger	Smaller
pH of Tris-Cl used	6.8	8.8
Purpose	To stack the polypeptides on the interface of stacking gel and resolving gel.	To separate the polypeptides solely on the basis of size.
Electrophoretic mobility	glycine <protein mixture <BPB <Cl.	Protein mixture <glycine <BPB <Cl.

Resolving gel (10%)

dH_20
30 % acrylamide mix
1.5 M Tris pH8.8
10 % SDS
10 % ammonium persulfate
TEMED

Stacking gel (5%)

dH_20
30 % acrylamide mix
1.0 M Tris pH 6.8
10 % SDS
10 % ammonium persulfate
TEMED



An illustration of an apparatus used for SDS PAGE.

Major steps of SDS-PAGE

- Pouring of the resolving gel: Resolving gel is poured between two glass plates (one is called short plate and the other one is tall plate), clipped together on a **casting frame.** Bubbles are removed by adding a layer of isopropanol on the top of the gel. The gel is then allowed to solidify. When the gel is solidified, remove the isopropanol by using a filter paper
- Pouring of the stacking gel: Stacking gel is loaded all the way to the top of the glass plates. Comb is placed after loading. The gel is, then, allowed to solidify. When stacking gel is solidified, comb is removed very carefully not damaging the well's shape

- Load the ladder into wells using a micropipette
- Load the samples into wells using a micropipette. At this stage, sample of the proteins appears to be blue because of a dye (bromophenol)
- Run the gel by applying voltage until the dye line is approximately 75-80 % of the way down the gel.
- The gel is rinsed with deionized water 3-5 times to remove SDS and buffer.
- Subsequent analysis: The gel is then dipped in Coomassie Blue stain (staining buffer) on a shaking incubator at room temperature. The invisible bands of the proteins to appear within minutes but it take approximately 1h for complete staining followed by destaining.

Applications:

SDS-PAGE is used mainly for the following purposes:

- Measuring molecular weight of proteins.
- Peptide mapping.
- Estimation of protein size.
- Determination of protein subunits or aggregation structures.
- Estimation of protein purity.
- Protein quantitation.
- Monitoring protein integrity.
- Comparison of the polypeptide composition of different samples.
- Analysis of the number and size of polypeptide subunits.
- Post-electrophoresis applications, such as Western blotting.
- Staining of Proteins in Gels with Coomassie G-250 without Organic Solvent and Acetic Acid.
- Pouring and Running a Protein Gel by reusing Commercial Cassettes.
- Selective Labelling of Cell-surface Proteins using CyDye DIGE Fluor Minimal Dyes.
- Detection of Protein Ubiquitination.

Western blotting technique:

- The procedure was first described by H. Towbin in 1979 and two years later given its name by W. Neal Burnette in 1981.
- Western blotting (protein blotting or immunoblotting) is a rapid and sensitive assay for detection and characterization of proteins. It is based on the principle of immunochromatography where proteins are separated into polyacrylamide gel according to their molecular weight.

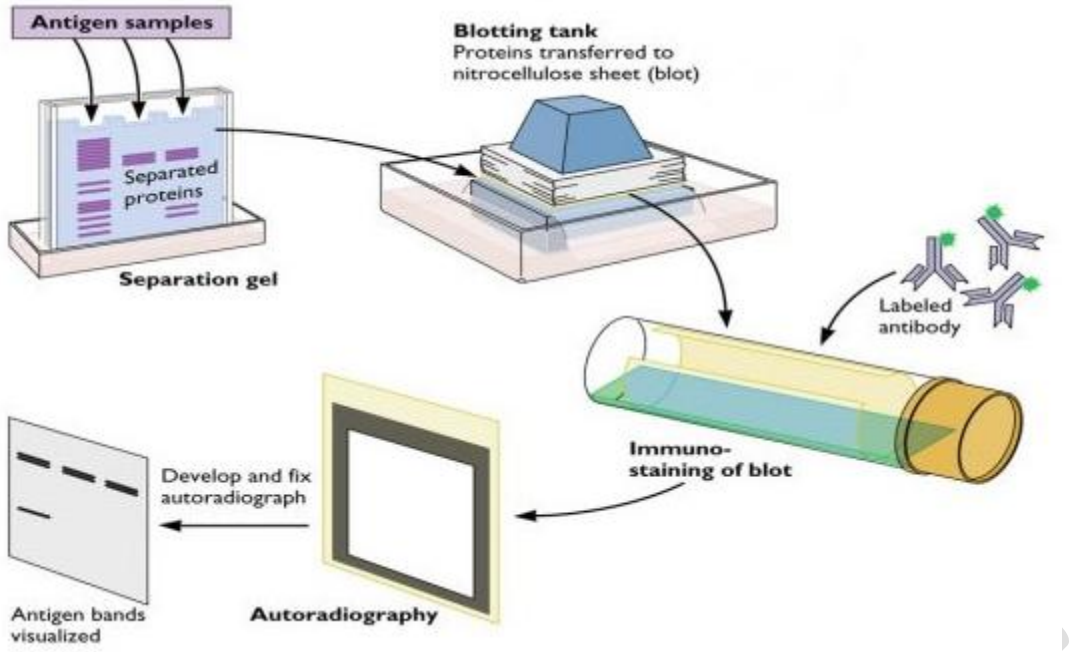
Procedure:

a) Proteins are separated by gel electrophoresis, usually with SDS-PAGE to have all the proteins carries negative charge(s).

b) The proteins then transferred to a nitrocellulose sheet although other types of paper or membranes can be used. The proteins retain in the same pattern of separation they had previous on the gel.

c) The blot is incubated with a generic protein (e.g. milk proteins) to bind to any remaining sticky places on the nitrocellulose. An antibody is then added to the solution which is able to bind to its specific protein. The antibody has an enzyme (e.g. alkaline phosphatase or horseradish peroxidase) or dye attached to it which cannot be seen at this time.

d) The location of the antibody is revealed by incubating it with a colorless substrate that the attached enzyme converts to a colored product, which can be seen and photographed.



Applications**:**

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

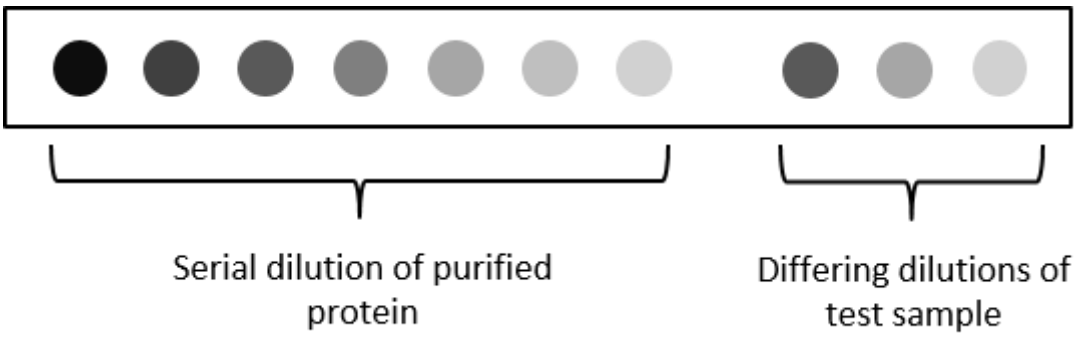
Dot blot:

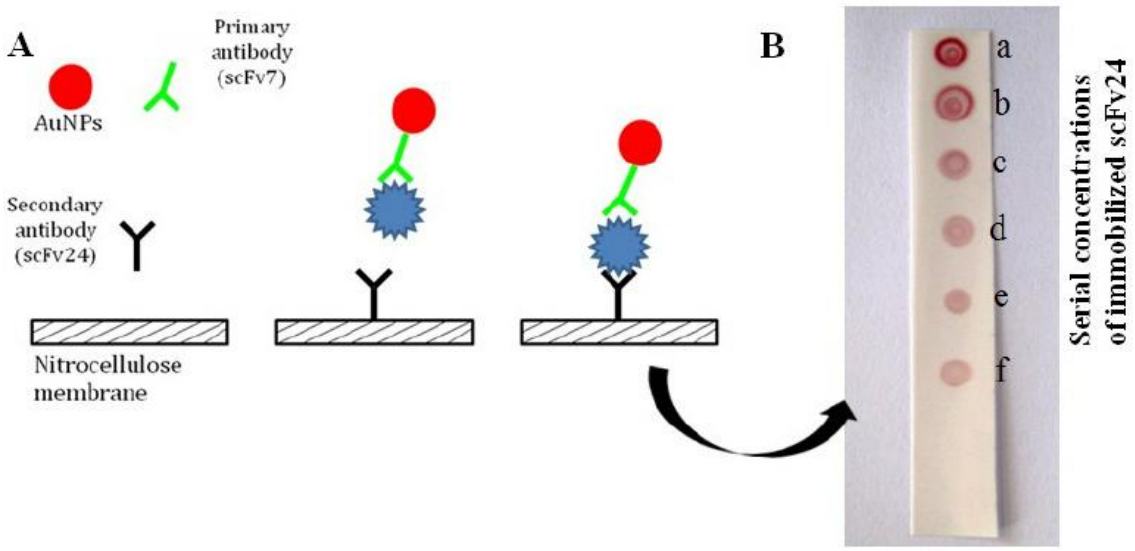
- A technique for detecting, analyzing, and identifying proteins, similar to the western blot technique but differing in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane or paper substrate.
- A general dot blot protocol involves spotting 1-2 microliters of samples onto a nitrocellulose or PVDF membrane and letting it air dry.
- Samples can be in the form of tissue culture supernatants, blood serum, cell extracts, or other preparations.
- The membrane is incubated in blocking buffer to prevent non-specific binding. It is then incubated with a primary antibody followed by detection antibody or a primary antibody conjugated to a detection molecule (commonly HRP or alkaline phosphatase).
- After antibody binding, the membrane is incubated with a chemiluminescent substrate and imaged

Applications:

- Performing a dot blot is similar in idea to performing a western blot, with the advantage of faster speed and lower cost.
- The technique offers significant savings in time, as chromatography or gel electrophoresis, and the complex blotting procedures for the gel are not required. However, it offers no information on the size of the target protein

- Dot blots are also performed to screen the binding capabilities of an antibody





DNA microarray analysis:

- An array is an orderly arrangement of samples where matching of known and unknown DNA samples is done based on base pairing rules.
- A DNA microarray also commonly known as DNA chip or biochip is a collection of microscopic DNA spots attached to a solid surface.
- Each DNA spot contains picomoles of a specific DNA sequence, known as probes.
- These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample under high-stringency conditions.
- The first DNA arrays were constructed by immobilizing cDNAs onto filter paper

A DNA micorarray allows scientists to perform an experiment on thousands of genes at the same time.

- Each spot on a microarray contains multiple identical strands of DNA.
- The DNA sequence on each spot is unique.
- Each spot represents one gene.
- Thousands of spots are arrayed in orderly rows and columns on a solid surface (usually glass).
- The precise location and sequence of each spot is recorded in a computer database.
- Microarrays can be the size of a microscope slide, or even smaller.

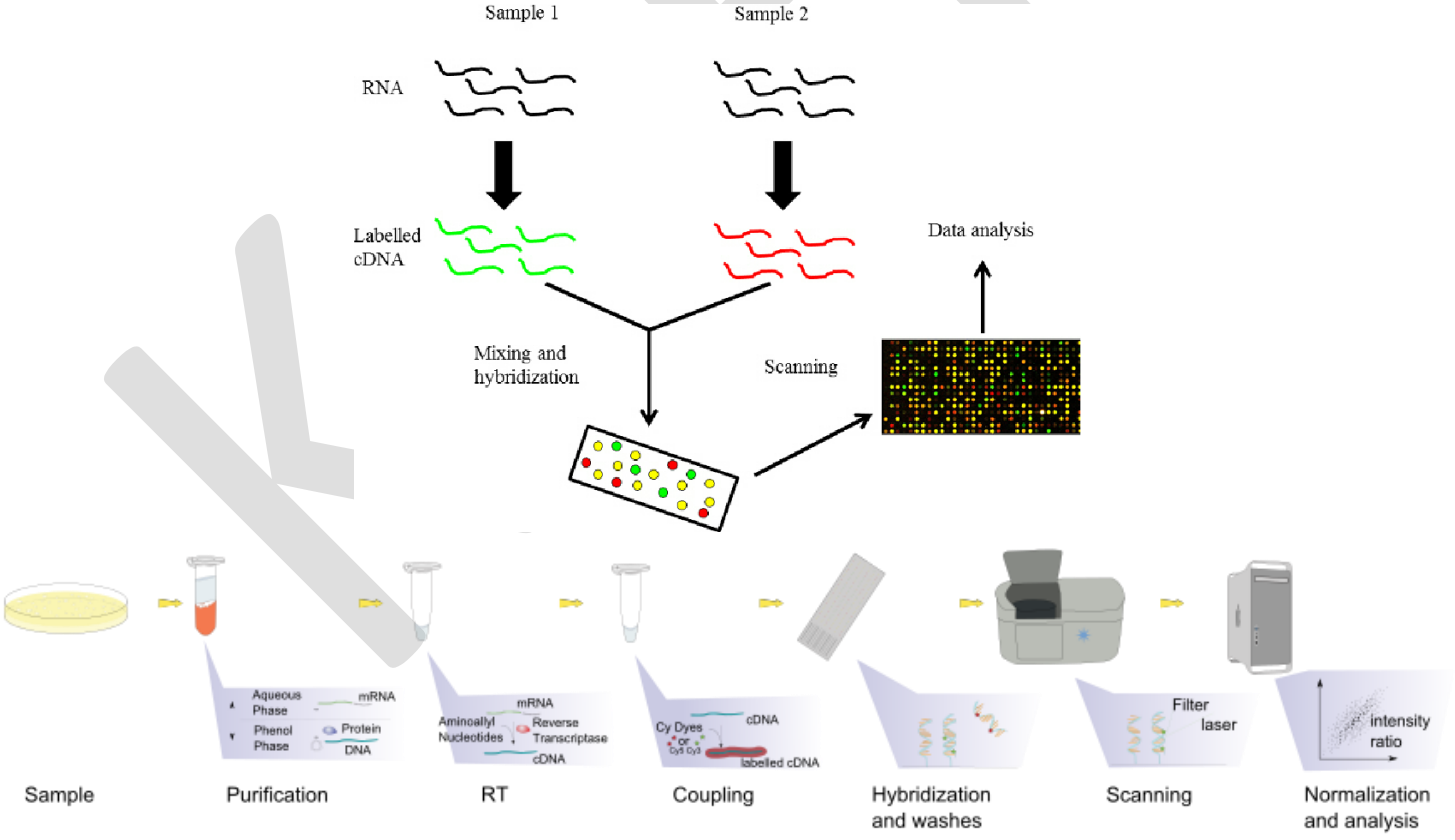
A basic protocol for a DNA microarray is as follows:

(i) Isolate and purify mRNA from samples of interest. Since we are interested in comparing gene expression, one sample usually serves as control, and another sample would be the experiment (healthy vs. disease, etc)

(ii)Reverse transcribe and label the mRNA. In order to detect the transcripts by hybridization, they need to be labeled, and because starting material maybe limited, an amplification step is also used. Labeling usually involves performing a reverse transcription (RT) reaction to produce a complementary DNA strand (cDNA) and incorporating a florescent dye that has been linked to a DNA nucleotide, producing a fluorescent cDNA strand. Disease and healthy samples can be labeled with different dyes and cohybridized onto the same microarray in the following step. Some protocols do not label the cDNA but use a second step of amplification, where the cDNA from RT step serves as a template to produce a labeled cRNA strand.

(iii) Hybridize the labeled target to the microarray. This step involves placing labeled cDNAs onto a DNA microarray where it will hybridize to their synthetic complementary DNA probes attached on the microarray. A series of washes are used to remove non-bound sequences.

(iv) Scan the microarray and quantitate the signal. The fluorescent tags on bound cDNA are excited by a laser and the fluorescently labeled target sequences that bind to a probe generate a signal. The total strength of the signal depends upon the amount of target sample binding to the probes present on that spot. Thus, the amount of target sequence bound to each probe correlates to the expression level of various genes expressed in the sample. The signals are detected, quantified, and used to create a digital image of the array.



Applications:

Gene Discovery; Disease Diagnosis; Drug Discovery; Toxicological Research

Possible questions

Part-A (1 Mark)

Part-B (2 Marks)

1. What is transformation?
2. Define transfection.
3. Mention any four chemical methods of transformation.
4. Mention the viruses used in the gene transfer.
5. Mention the denaturating agent used in southern blotting and northern blotting techniques.
6. What is electroporation?
7. Mention any four applications of DNA microarray analysis
8. What is DOT blot?
9. What is microinjection?
10. Expand SDS-PAGE
11. What is southern blotting technique?
12. What is western blotting technique?
13. What is northern blotting technique?
14. What are liposomes?

Part-C (8 Marks)

1. Write a detailed note on chemical methods of transformation.
2. Write a detailed note on electroporation.
3. Write a detailed note on DOT blot.
4. Write a detailed note on (a) Microinjection and (b) Gene gun.
5. Write a detailed note on *Agrobacterium* mediated gene transfer.
6. Write a detailed note on southern blotting technique.
7. Write a detailed note on western blotting technique.
8. Write a detailed note on northern blotting technique.
9. Write a detailed note on (a) SDS-PAGE and (b) Agarose gel electrophoresis
10. Write a detailed note on DNA microarray analysis.
11. Write a detailed note on liposomes.

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: II B.Sc MB COURSE NAME: RECOMBINANT DNA TECHNOLOGY
COURSE CODE: 18MBU404B UNIT: III BATCH-2018-2021

Sl.No	Questions	opt1	opt2	opt3	opt4	Answer
1	Microinjection in mice was performed at _____.	Male pronuclei	Female pronuclei	Blastocyst	Pronucleus	Male pronuclei
2	The protoplast fusion is done by _____.	PEG method	Electrofusion	Sodium nitrate method	Agrobacterium	PEG method
3	Microinjection technique proves direct delivery at _____.	Intranuclear	Intracytoplasmic region	Intro cellular region	Intra species region	Intranuclear region
4	Macroinjection technique involved injection of DNA in to _____.	Male pronuclei	Female pronuclei	Wound site within tissue-	Cytoplasm of cell	Wound site within tissue-
5	Liposomes are defined by _____.	Bongham	Winston	Hogness	Grunestein	Bongham
6	Liposome was first described for delivering _____.	Polio virus RNA	Adenovirus RNA	Baculovirus RNA	Adenovirus RNA	Polio virus RNA
7	The plasma membrane of pro top lasts is dissolved in the presence of _____.	Mg ions	Fe ions	Ca ions	None of the above	None of the above
8	The biolistic process currently in widest use employs gunpowder charge to drive _____.	Plastic cylindrical macro projectile	Plastic cylindrical microprojectile	DNA-tungsten suspension	Blank cartridge	Plastic cylindrical microprojectile
9	The microprojectile is commonly DNA coated with _____.	Gold or Tungsten	Silver or Molybdenum	Copper or zinc	Lithium or Molybdenum	Gold or Tungsten
10	The nick translation was performed by _____.	Making ss cuts in ds DNA molecule.	Making ds cuts in ds DNA molecule.	Making ss cuts in ss DNA molecule.	Making no cleavage in DNA molecule	Making ss cuts in ds DNA molecule.
11	The probes can be Non-radiolabelled by _____.	Biotin	Toluene	Epinephrin	Globulins	Biotin
12	Northern hybridization is used to measure _____.	The amount of RNA	Size of protein	Amount of DNA	Amount of micro	The amount of RNA

					satellites	
13	The first description of northern blotting was published by _____.	Alwine	EM Southern	Karry mullois	Hogness	Alwine
14	The most effective denaturating agent of RNA is _____.	Glyonol/	Formaldehyde	Dimethyl sulphide	Guanidine	Glyonol/ Formamide
15	The commonly used membrane in northern blotting is _____.	Polyethylene and nylon	Nylon and nitrocellulose	Terylene and nitrocellulose	Nylon and terylene	Nylon and nitrocellulose
16	The original name of nylon is _____.	Nylon	Polystyrene	Artificial wool	Filter 66	Polystyrene
17	Which of the following is tracking dye _____?	Methylene blue	Malachite green	Bromophenol blue	Xylene cyanol	Methylene blue
`18	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
19	Southern blotting technique was identified by _____.	Southern (1975)	Kary Mullis (1985)	Lederberg (1994)	Guray (1998)	Southern (1975)
20	DNA blotting technique is used to study about _____.	DNA	RNA	Proteins	Lipids	DNA
21	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
22	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
23	The polysaccharide contained in agarose is _____.	Sucrose	Galactose	Fructose	Maltose	Galactose
24	The glycosidic linkage in Agarose is _____.	a (1,2) ~ (1,4)	a (1,2) ~ (1,3)	a (1,3) ~ (1, 4)	a (1,4) f3 (1, 6)	a (1,4) f3 (1, 6)
25	The rate of the movement of DNA is _____ as the size of molecule increases.	Slow	Fast	No change	Scattering	Slow

26	The mostly preferred buffer in AGE _____.	TAE	TBE	TPE	TE	TAE
27	The DNA should move from _____.	Negative to	Positive to negative	Negative to neutral	Positive to neutral	Negative to Positive
28	Selection by complementation is the existence of _____.	Auxotroph	Prototroph	Heterotroph	Lethal genes	Auxotroph
29	Nonsense suppression is a special case of complementation operating only in the instance of	Frame Shift mutation	Transition mutation	Transversion	Nonsense mutation	Nonsense mutation
30	Electroporation can be other words called as _____.	Electro permeabilization	Induction	Diffusion	Localization	Electro permeabilization
31	Electro permeabilization, is a significant increase in _____.	electrical conductivity and permeability of the cell plasma membrane	Damage to membrane	Destroys cell structure	None of the above	electrical conductivity and permeability of the cell plasma membrane
32	Pores are formed when the voltage across a plasma membrane _____.	Exceeds its dielectric strength	Exceeds its trielectric value	Falls below the dielectric value	Maintains at the dielectric value	Exceeds its dielectric strength
33	Excessive exposure of live cells to electrical fields can cause _____.	Apoptosis and/or necrosis	Lineage	Pores	Condese mass	Apoptosis and/or necrosis
34	In molecular biology, the process of electroporation is often used for the _____.	transformation	recombination	congigation	transduction	transformation
35	Electroporation is done with _____.	Electroporators	Magnetic stirrers	Sonoicators	Hot plates	Electroporators
36	Biological ballistics is the method of transfecting cells by _____.	Bombarding them with Microprojectiles coated with	By mixing with DNA of interest	By encapsulation	By Precipitation	Bombarding them with Microprojectiles coated with

		DNA.				DNA.
37	RNA can be generated from DNA by a process known as _____.	Reverse transcription	Forward reversion	Mutation	Ligation	Reverse transcription
38	Biolistics has been a useful technique for _____.	Testing expression of genes particularly	Testing expression of genes particularly in earthworm	Testing expression of genes particularly in drosophila	Testing expression of genes particularly in plant	Testing expression of genes particularly in plant
39	_____ is the method used to burst of a gas.	Water flush	A capacitive electric discharge through a wire electrode	A burst of compressed carbon dioxide	A burst of compressed oxygen	A capacitive electric discharge through a wire electrode
40	Probes are short nucleotide of _____ in length.	15 - 20 base pair	20 - 40 base pair	80 base pair	100 base pair	15 - 20 base pair
41	Which one of the following is not used in nonradiolabelling?	Biotin	Horse radish peroxidase	Digitoxigenin	Cyanocobalamine	Cyanocobalamine
42	In _____ method the DNA should be pure.	Biolistic	Calcium phosphate	Lipofection	Electroporation	Biolistic
43	The marker gene present in PBR322 is _____.	Ampr	Kamr	Carnr	Metr	Ampr
44	The southern hybridization results were read by _____.	UV	Autoradiography	Stainiing	Visible light	Autoradiography
45	Biolistic operation cost _____.	351akhs	50lakhs	60lakhs	10lakhs	351akhs
46	The DNA should be mixed with _____ in biolistic process.	Calcium	Biotin	Digitoxigenin	Spermidine	Spermidine

47	_____ is the disadvantage in ETBR staining.	Limit to sensitivity less	Limit to sensitivity less than 25ng	Limit to sensitivity less than 35ng	Limit to sensitivity less than 45ng	Limit to sensitivity less than 25ng
48	Marker inactivation of antibiotics can be studied by _____.	Activity of antibiotics	Resistance of antibiotics	Both resistance and activity	None of the above	Resistance of antibiotics
49	_____ is the sample used for nucleic acid blotting.	DNA	RNA	Protein	Both DNA and RNA	Both DNA and RNA
50	For nucleic acid hybridization nucleic acid is digested with _____ restriction enzyme/s.	One	Two	More than two	Other than restriction enzymes	More than two
51	_____ refers to blotting of electrophoresed protein bands from SDS PAGE to membrane.	Southern blotting	Northern blotting	Western blotting	Dot blotting	Western blotting
52	Which technique purified nucleic acid sample directlyapplied to nitrocellulose filter?	Southern blotting	Northern blotting	Western blotting	Dot blotting	Dot blotting
53	Joining of donor DNA fragments and vector DNA fragments with the help of DNA ligase enzyme is known as _____.	Gene cloning	Splicing	Gene manipulation	Moleccular cloning	Gene cloning
54	Suitable host in genetic engineering to introduce DNA fragments of donor is _____.	Bacillus subtilis	Escherechia coli	Bacteriophages	virus	Bacillus subtilis
55	A southern blot is a technique used to detect _____.	proteins	DNA	RNA	None	DNA
56	Type of restriction endonucleases most useful in molecular cloning is _____.	Type I	Type II	Type III	None	Type II
57	Northern blots probes are _____.	DNA and RNA	DNA	RNA and protein	RNA	RNA
58	Which is the most specific recombinant library?	Genomic	protein	cDNA	cRNA	cDNA

59	Recombinant DNA research is dependent on _____.	Cloning	Hosts	Vectros	carrier	Vectros
60	The polymerase chain reaction is a technique that selectively replicates _____.	RNA	DNA	Protein	None	DNA

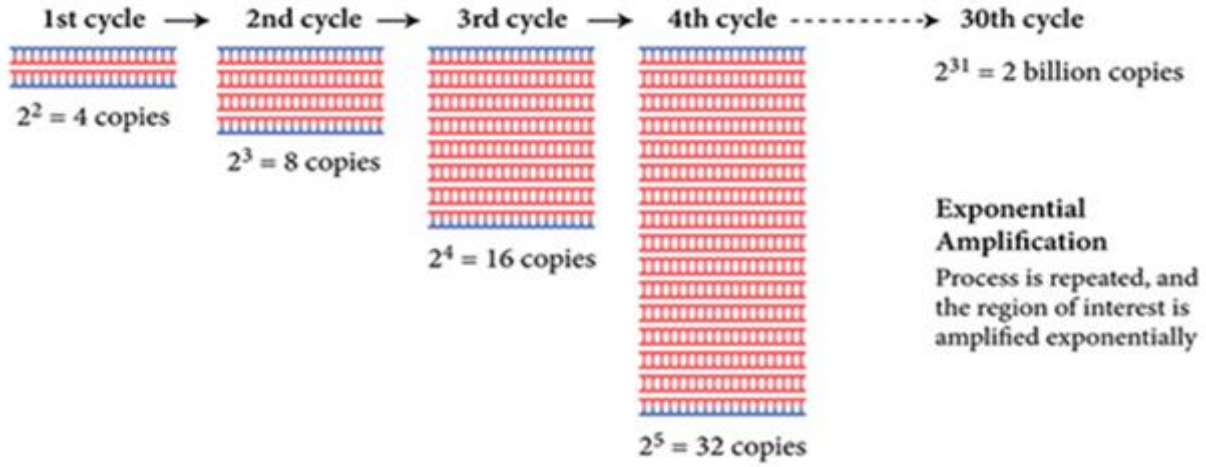
UNIT –IV

Syllabus

PCR: Basics of PCR, RT-PCR, Real-Time PCR (Quantitative). Sanger's method of DNA Sequencing: traditional and automated sequencing. Primer walking and shotgun sequencing

PCR:

- Polymerase chain reaction (PCR) is a widely employed technique in molecular biology to amplify single or a few copies of DNA, generating millions of copies of a particular DNA sequence.
- The polymerase chain reaction results in the selective amplification of a target region of a DNA or RNA molecule.
- In 1985, Kary Mullis invented a process Polymerase Chain Reaction (PCR) using the thermo-stable *Taq* polymerase for which he was awarded Nobel Prize in 1993.



Components and reagents:

A basic PCR set up requires the following essential components and reagents:

1. Template DNA containing the DNA region (target) to be amplified.
2. Primers that are complementary to the 5' ends of each of the sense (Forward primer) and anti-sense strand of the DNA target (Reverse primer).
3. *Taq* polymerase or other thermostable, high fidelity DNA polymerase (Pfu polymerase isolated from *Pyrococcus furiosus*).
4. Deoxyribonucleotide triphosphates (dNTPs), which are the building-blocks for a newly synthesized DNA strand.
5. Buffer solutions to provide a suitable chemical condition for optimum activity and stability of the DNA polymerases.
6. Divalent cations (eg. magnesium or manganese ions). They act as a co-factor for Taq polymerase which increases its polymerase activity. Generally Mg^{2+} is used, but Mn^{2+} can be applied to achieve PCR-mediated DNA mutagenesis. This is because higher Mn^{2+} concentration leads to higher error rate during DNA synthesis.

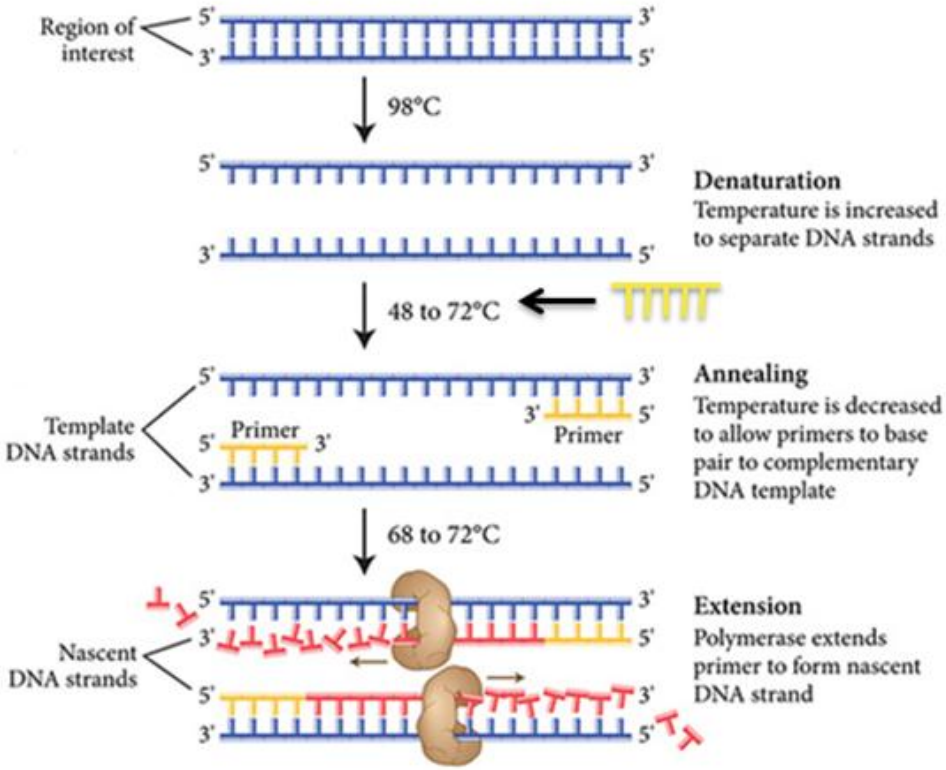
Procedure:

Typically, PCR is designed of 20-40 repeated thermal cycles. Various steps involved are:

a) Initial Denaturation

b) Denaturation
c) Annealing
d) Extension
e) Final extension

(i) Initial denaturation:
Initial denaturation involves heating of the reaction to a temperature of 94–96 °C for 7-10 min (or 98 °C if extremely thermostable polymerases are used).



(ii) Denaturation:
Denaturation requires heating the reaction mixture to 94–98 °C for 20–30 sec. It results in melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

(iii) Annealing:
Following the separation of the two strands of DNA during denaturation, the temperature of the reaction mix is lowered to 50–65 °C for 20–50 sec to allow annealing of the primers to the single-stranded DNA templates. Typically the annealing temperature should be about 3-5 °C below the T_m of the primers.

(iv) Extension / elongation:
Extension/elongation step includes addition of dNTPs to the 3' end of primer with the help of DNA polymerase enzyme. The type of DNA polymerase applied in the reaction determines the optimum extension temperature at this step. Conventionally, at its optimum temperature, DNA polymerase can add up to a thousand bases per minute. The drawback of Taq polymerase is its relatively low replication fidelity. It lacks a 3' to 5' exonuclease proofreading activity, and has an error rate measured at about 1 in 9,000 nucleotides.

(v) Final elongation & Hold:

Final elongation step is occasionally performed for 5–15 minutes at a temperature of 70–74°C after the last PCR cycle to ensure amplification of any remaining single-stranded DNA. Final hold step at 4°C may be done for short-term storage of the reaction mixture.

Applications:

1. PCR has been extensively exploited in cloning, target detection, sequencing etc.
2. Infectious disease diagnosis, progression and response to therapy

PCR-based diagnostics tests are available for detecting and/or quantifying a number of pathogens, including:

- HIV-1, which causes AIDS
- Hepatitis B and C viruses, might lead to liver cancer
- Human Papillomavirus, might cause cervical cancer
- *Chlamydia trachomatis*, might lead to infertility in women
- *Neisseria gonorrhoeae* , might lead to pelvic inflammatory disease in women
- Cytomegalovirus, might cause life threatening disease in transplant patients and other immunocompromised people, including HIV-1/AIDS patients
- *Mycobacterium tuberculosis*, which in its active state causes tuberculosis and can lead to tissue damage of infected organs.

3. Diagnosis of genetic diseases
4. Genetic counselling
5. Forensic sciences
6. Research in Molecular Biology

Real-Time Polymerase Chain Reaction (RT PCR)

A real-time polymerase chain reaction (Real-Time PCR), also known as quantitative polymerase chain reaction (qPCR), is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR.

Real Time PCR can perform detection, analysis and quantification of the sample.

- Detection: Find out the presence of targeted gene sequence which is assured by the presence of the amplification curve.
- Quantification: Quantification of targeted DNA in a sample can be done by using the cycle no. needed to obtain the threshold value of detector and PCR efficiency.
- Analysis: Analysis of the variants can be done by studying the melting curve or comparing the melting temperature with the sequences of the database.

Types: Real-time PCR technique can be classified by the chemistry used to detect the PCR product, specific or non-specific fluorochromes.

(i) Non-specific detection: Real-time PCR with double-stranded DNA-binding dyes as reporters

A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured at each cycle. However, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including

nonspecific PCR products (such as Primer dimer). This can potentially interfere with, or prevent, accurate monitoring of the intended target sequence.

(ii) Specific detection: fluorescent reporter probe method

Fluorescent reporter probes detect only the DNA containing the sequence complementary to the probe; therefore, use of the reporter probe significantly increases specificity, and enables performing the technique even in the presence of other dsDNA. Using different-coloured labels, fluorescent probes can be used in multiplex assays for monitoring several target sequences in the same tube.

Procedure:

Various steps involved are:

(i) Denaturation: 95 °C

(ii) Annealing: During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).

(iii) Extension / elongation: 70-72 °C

Real-Time PCR has been used for a wide number of applications. These include:

- Quantification of gene expression
- Diagnostic uses
- Microbiological uses
- Detection of phytopathogens
- Detection of genetically modified organisms
- Clinical quantification and genotyping
- microRNA and Non-Coding RNA analysis
- Genetic Variation
- Mutation Detection
- Genotyping/Allelic Discrimination

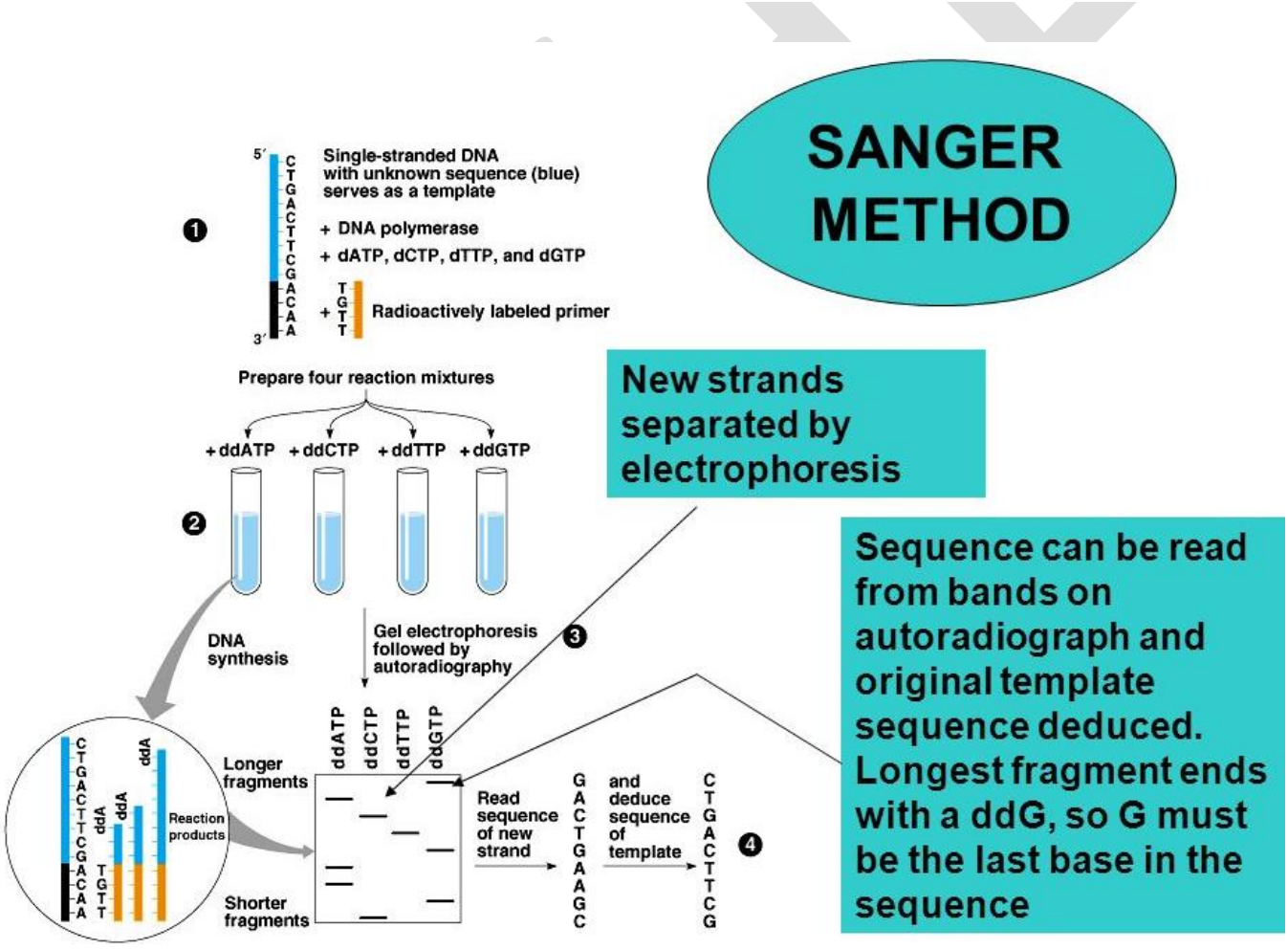
Sanger's method of DNA Sequencing: traditional sequencing

- Sanger's sequencing method was developed by Frederick Sanger and colleagues in 1977. This method is also known as chain termination method.
- Sanger's sequencing method requires single stranded DNA template, Primer, Taq DNA polymerase, dNTPs and modified nucleotides namely ddNTPs (dideoxy nucleotide triphosphate).
- These chain-terminating nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labelled for detection in automated sequencing machines.

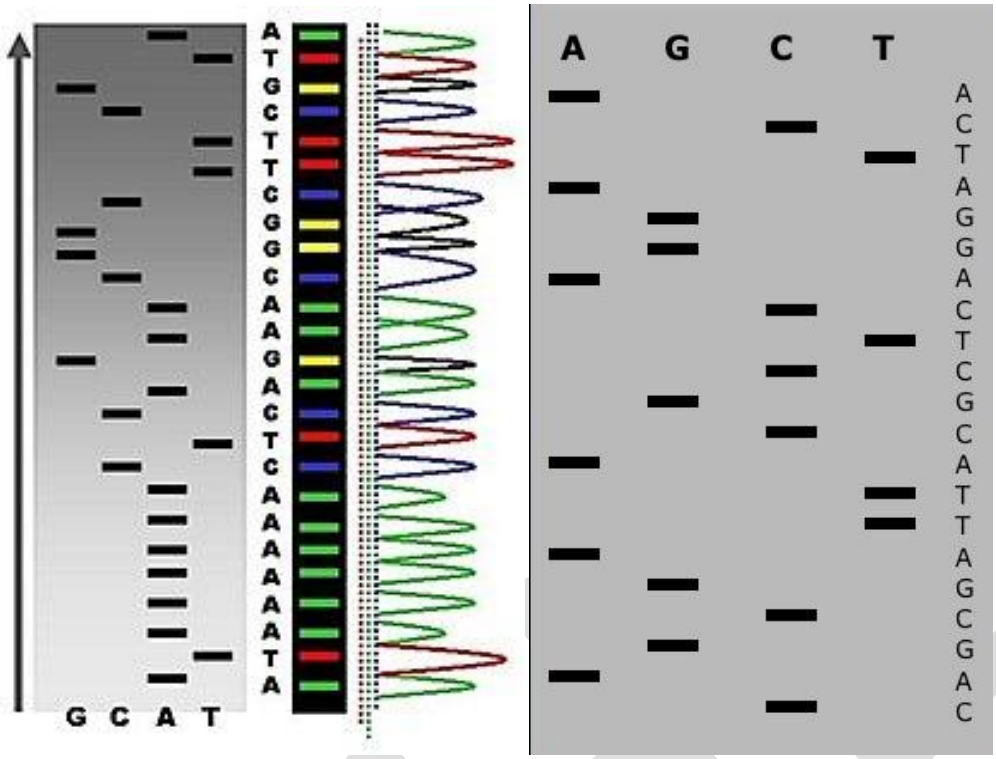
Procedure:

- The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase.
- To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP), while the other added nucleotides are ordinary ones.

- The dideoxynucleotide concentration should be approximately 100-fold lower than that of the corresponding deoxynucleotide (e.g. 0.005mM ddTTP : 0.5mM dTTP) to allow enough fragments to be produced while still transcribing the complete sequence.
- Putting it in a more sensible order, four separate reactions are needed in this process to test all four ddNTPs. Following rounds of template DNA extension from the bound primer, the resulting DNA fragments are heat denatured and separated by size using gel electrophoresis.
- In the original publication of 1977, the formation of base-paired loops of ssDNA was a cause of serious difficulty in resolving bands at some locations. This is frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C).
- The DNA bands may then be visualized by autoradiography or UV light and the DNA sequence can be directly read off the X-ray film or gel image.
- In the image on the right, X-ray film was exposed to the gel, and the dark bands correspond to DNA fragments of different lengths.
- A dark band in a lane indicates a DNA fragment that is the result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP).



- The relative positions of the different bands among the four lanes, from bottom to top, are then used to read the DNA sequence.



Sanger's method of DNA Sequencing: automated sequencing

- Automated DNA-sequencing instruments (DNA sequencers) can sequence up to 384 DNA samples in a single batch. Batch runs may occur up to 24 times a day.
- DNA sequencers separate strands by size (or length) using capillary electrophoresis, they detect and record dye fluorescence, and output data as fluorescent peak trace chromatograms.
- Sequencing reactions (thermocycling and labelling), cleanup and re-suspension of samples in a buffer solution are performed separately, before loading samples onto the sequencer.
- A number of commercial and non-commercial software packages can trim low-quality DNA traces automatically. These programs score the quality of each peak and remove low-quality base peaks (which are generally located at the ends of the sequence).
- The accuracy of such algorithms is inferior to visual examination by a human operator, but is adequate for automated processing of large sequence data sets.

Primer walking:

- Primer walking is a sequencing method of choice for sequencing DNA fragments between 1.3 and 7 kilobases. Such fragments are too long to be sequenced in a single sequence read using the chain termination method.
- This method works by dividing the long sequence into several consecutive short ones.
- The DNA of interest may be a plasmid insert, a PCRproduct or a fragment representing a gap when sequencing a genome.
- The term "primer walking" is used where the main aim is to sequence the genome.

The overall process is as follows:

I. A primer that matches the beginning of the DNA to sequence is used to synthesize a short DNA strand adjacent to the unknown sequence, starting with the primer (see PCR).

II. The new short DNA strand is sequenced using the chain termination method.

III. The end of the sequenced strand is used as a primer for the next part of the long DNA sequence, hence the term "walking".

The method can be used to sequence entire chromosomes (hence "chromosome walking"). Primer walking was also the basis for the development of shotgun sequencing, which uses random primers instead of specifically chosen ones.

Shotgun sequencing:

- The shotgun sequencing strategy is widely used in genome sequencing projects. Shotgun sequencing is a technique in which large pieces of DNA are sheared into smaller fragments that are then sequenced randomly.
- These random fragments must be realigned and ordered into larger contiguous pieces that are representative of the original large DNA unit.
- The random shotgun sequencing method is currently the most efficient and cost-effective strategy for completion of microbial genomes

STEPS IN SHOTGUN SEQUENCING-

1. RANDOM PHASE

Fragmentation: Fragmentation of the DNA can be done by using restriction enzymes, physically by breaking it into small pieces (usually 2, 10, 50, and 150 kb) by passing it through a narrow gauge syringe or sonicating it, which is the way of breaking the sample using sound waves, by mechanical method, shearing. It is a random process, so the sequences of the fragments will have some overlap between them. Fragments of about 150Mb are obtained.

Cloning: Next step is joining of DNA fragments with a vector which is a carrier DNA. This method is known as cloning. And a sequence library is created. Sequencing of entire genome will be created by using this library.

Sequencing: Sequencing of each clone in library is done. Individual fragments are sequenced individually using the chain termination method to obtain reads. Multiple overlapping reads are obtained for the target DNA by performing several rounds of this fragmentation and sequencing.

2. ASSEMBLY PHASE

Reassembling: Then based on overlapping regions, these fragments are reassembled into their original order. Assembling of the overlaps creates a “contig,”. It is a long continuous stretch of DNA sequence, or which is the de-coded version of the original source DNA. Larger and larger contigs will be produced, until a single ordered contig of the genome is achieved. These contigs are produced identifying the gaps (where there is no sequence available) and single stranded regions (where there is sequence for only one stand). These gaps and single stranded regions are then targeted to do additional sequencing to make a full sequenced molecule

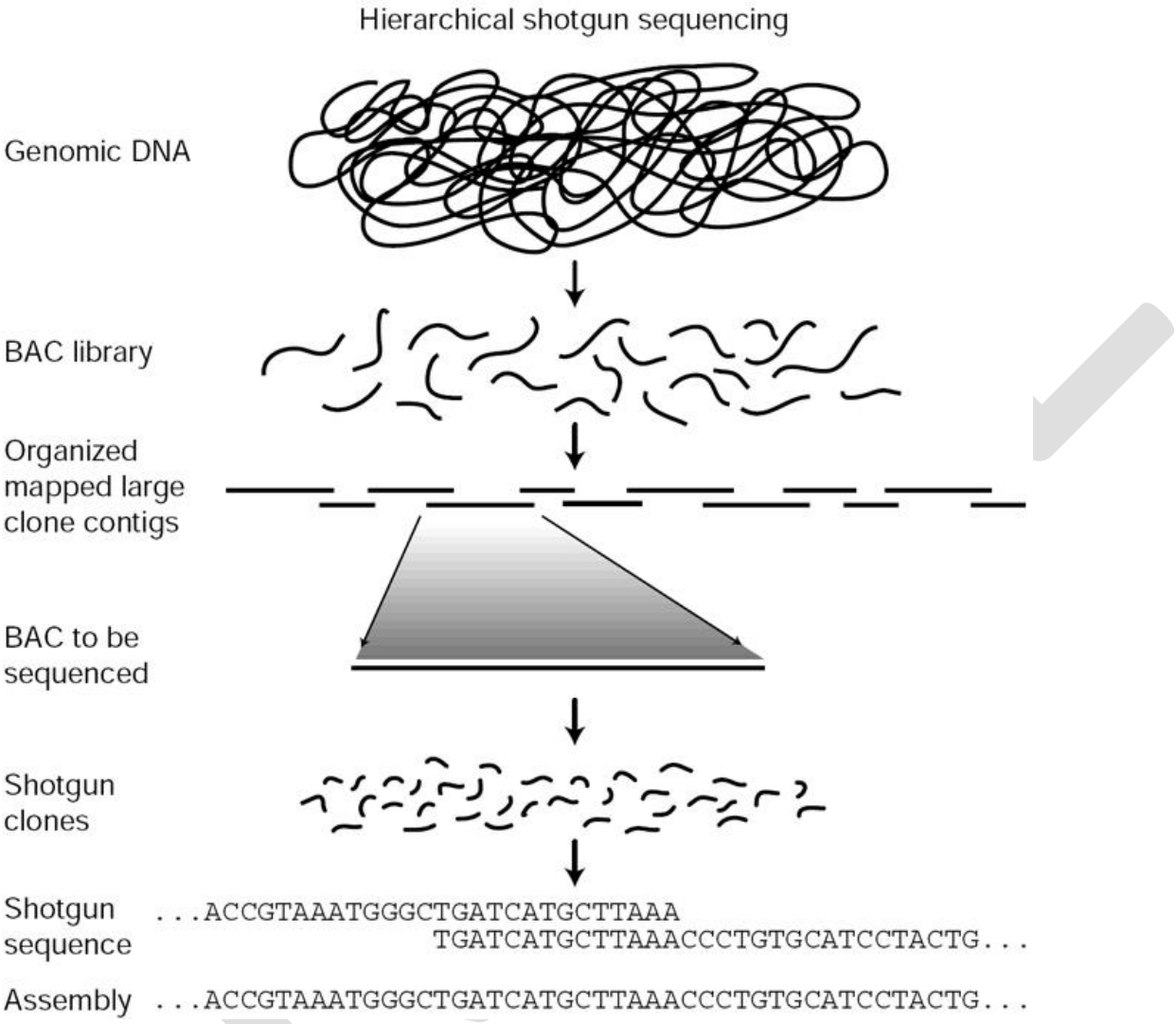
3. FINISHING PHASE

Alignment of the sequences of overlapping pieces is done by computer programs or sequence assembly softwares. In the case of the human genome project, a massive amount of data is involved, requiring supercomputer technology. Then it ultimately yields the complete sequence. It is a faster and a more complex technique.

Shotgun cloning usually results in some gaps between contigs because some sequences are missing from the library by chance. These gaps are filled by-

- Creating a new library or
- By using known sequences to extend outward from the contig.

Many fragments are sequenced more than once because shotgun sequencing sequences DNA fragments at random. Thus creates more certainty that the sequence is correct than if each fragment had only been sequenced once or twice



Advantages over previous methods:

- Faster because the mapping process was eliminated
- Uses less DNA than other methods
- Less expensive than approaches requiring a map

Disadvantages:

- Requires computer processing power beyond what an ordinary laboratory would possess
- Can introduce errors in the assembly process
- Requires a reference genome
- May not be able to assemble repetitive sequences

Possible questions

Part-A (1 Mark)

Part-B (2 Marks)

1. What is polymerase chain reaction?
2. Mention any four components of polymerase chain reaction.
3. Expand RT-PCR.
4. What is SYBR Green?
5. Mention any four applications of polymerase chain reaction.
6. What is chain termination method?
7. What is Primer walking?
8. What is shotgun sequencing?
9. Mention the steps involved in the RT-PCR.
10. Mention the types of RT-PCR.

Part-C (8 Marks)

1. Write a detailed note on polymerase chain reaction.
2. Write a detailed note on RT PCR.
3. Write a detailed note on (a) Primer walking and (b) shotgun sequencing.
4. Write a detailed note on Sanger's method of DNA Sequencing: traditional sequencing.
5. Write a detailed note on Sanger's method of DNA Sequencing: automated sequencing.

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: II B.Sc MB COURSE NAME: RECOMBINANT DNA TECHNOLOGY

COURSE CODE: 18MBU404B UNIT: IV BATCH-2018-2021

Sl.No	Questions	opt1	opt2	opt3	opt4	Answer
1	Gene knockout is a genetically engineered organism that carries _____.	One or more Genes in its	One or more genes in Its cell	One gene of assortment	None of the above	One or more genes in its Chromosomes
2	Knockout is a route to learning about a gene _____.	That is active	That has been	That are suppressed	That are ordered	That are suppressed
3	Knockout is accomplished through _____.	A combination of Techniques	A single technique	Bifunctional technique	None of the above	A combination of Techniques
4	Knockout requires _____.	A plasmid	Bacterial artificial Chromosome	Gene	Tissues	A plasmid
5	Gene therapy is the insertion of genes into an individual's _____.	Bones	Cells and tissues	Ligaments	Skull	Cells and tissues
6	_____ to treat a disease, and hereditary diseases.	Gene therapy	Genetic instability	Genome stability	Transplantation	Gene therapy
7	A carrier called a _____ must be used to deliver the therapeutic gene to the patient's.	Fusion agent	Trancription initiater	Vector	Illucitor	Vector
8	The most common type of vector are _____ that have been genetically altered to carry normal human DNA.	Pbr322	Cosmids	Viruses	Yeast	Viruses
9	All gene therapy to date on humans has been directed _____.	Somatic cells	Gene level	RNA level	Plasmid level	Somatic cells
10	Somatic gene therapy can be broadly split in to _____.	One category	Five category	Six category	Two categories	Two categories
11	*Ex vivo,* means _____.	Cells are modified outside the body and then transplanted back	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
12	*In vivo,* means _____.	Cells are modified outside the body and then	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

		transplanted back				
13	_____ are the methods to replace or repair the genes targeted in gene therapy.	A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene.	An abnormal gene could be swapped for a normal gene through homologous recombination	The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function	Insertion of artificial gene	The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function
14	The genetic material in retroviruses is _____.	DNA molecule	RNA molecules	Proteins	Lipids	RNA molecules
15	Antisense refers to short _____.	DNA sequences	RNA sequences	Both DNA and RNA sequences	Any chemical substances	Both DNA and RNA sequences
16	_____ are designed to be complementary to a specific gene sequence to inhibit activity.	Oligonucleotides	Gene fragments	Similar sequences	Antisense oligonucleotide	Antisense oligonucleotide
17	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
`18	RNA interference is a mechanism of_____.	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
19	In RNA ____ inhibits the expression of genes.	Viruses	Double stranded DNA	double-stranded ribonucleic acid	Bacterial DNA	double-stranded ribonucleic acid
20	RNA pathway is thought to have evolved as a form of _____.	innate immunity against viruses	Innate immunity against protozao	Innate immunity against bacteria	Innate immunity against fungi	innate immunity against viruses

21	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
22	Vaccines that use components of a pathogenic organism rather than whole organism is called _____.	Peptide vaccines	Triplet vaccines	MAB	Subunit vaccines	Subunit vaccines
23	Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred _____.	knock out	knock Down	knock up	Knock in	knock Down
24	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
25	The genome of FMDV consists of _____.	Single stranded DNA	Single stranded RNA	Double stranded DNA	Double stranded RNA	Single stranded RNA
26	Hexadimeric enterotoxin of *Vibrio cholerae* made of _____.	Single A and 5 identical B	Single B and identical a subunit	Single alpha subunit	Sinale beta subunit	Single A and 5 identical B subunit
27	There are _____ of interferons.	Two major classes	Three major classes	Five major classes	Six major classes	Three major classes
28	Golden rice is a variety _____.	*Oryza glaberrima*	*Oryza sativa*	*Oryza triticum*	*Oryza annum*	*Oryza sativa*
29	*Oryza sativa* produced through genetic engineering to biosynthesize the precursors of _____.	beta-carotene pro-vitamin A	Alpha - Pro-vitamin A	beta-carotene pro-Vitamin B	beta-carotene pro- vitamin c	beta-carotene pro-vitamin A
30	Beta carotene is present at _____ in the golden rice.	Capsule	Husk	Endospore	Stem	Endospore
31	Golden rice was created by transforming rice with _____.	Three beta-carotene	Two beta - carotene	Eive beta - carotene	Six beta carotene	Three beta-carotene'
32	*Psy* codes for _____ in golden rice.	Phosphoribosyl transferase	phytoene synthase	Phythose synthase	Phosphatase	phytoene synthase
33	In golden rice *crtl* gene is obtained from the soil bacterium, _____.	*Erwinia caratovora*	*Erwinia ungalculata*	*Erwinia uredova*	*Erwinia rhiza*	*Erwinia uredova*

34	_____causes second generation seeds to be sterile.	Antisense technology	Mutational technology	Terminator seed technology	Knock out technology	Terminator seed technology
35	The coagulation is an important part of _____.	Thrombosis	Emolbism	Hemostatis	Hemorrhage	Hemostatis
36	Eventually, all blood clots are reorganised and resorbed by a process termed _____.	Plastolysis	Fibrynolysis	Rectolysis	Clustering	Fibrynolysis
37	Fibrynogen forms _____.	Channels	Blood clots	Blood platelets	Permiations	Blood clots
38	Dicer is _____.	Chemical	Cofactor	Ribonulease enzyme	Ligase enzyme	Ribonulease enzyme
39	The RNAi pathway is initiated by the _____.	Protein P	REC·	Dicer enzyme	None ofthe above	Dicer enzyme
40	Galactose dehydrogenase is isolated from _____.	*Pseudomanas*	*E.Coli*	Bacillus	*Corynebacteriumi*	*Pseudomanas*
41	L- aspariginase have _____ property.	Antitumour	Antialleric property	Allergic property	Dehydration	Antitumour property
42	PCR was first proposed by _____.	Kary Mullis	Hargobind khurrana	James Watson	Barbara mcclintock	Kary Mullis
43	PCR is a technique of _____.	DNA degradation	DNA amplification	DNA sequencing	DNA ligation	DNA amplification
44	The basic requirements of PCR reaction exclude _____.	DNA	Primers	Heat stable DNA	Restriction enzymes	Restriction enzymes
45	The process of binding of primer to the denatured DNA strand is called as _____.	Denaturation	Annealing	Renaturation	None of these	Annealing
46	The technique used to identify specific DNA sequence in bacterial colonies is _____.	Colony	In situ hybridization	Dot blot	Western blotting	Colony hybridization
47	Asymmetric PCR is to generate _____.	Single stranded Copies	Double stranded copies	Multiple copies	Half copy	Single stranded copies
48	Reverse transcriptase PCR uses _____.	mRNA as template to form	Protein as template to form DNA	DNA as tempelate to form protein	Restriction enzymes	mRNA as template to form cDNA

49	Reverse transcriptase enzyme is _____.	DNA dependent DNA polymerase	RNA dependent DNA polymerase	RNA dependent RNA polymerase	DNA dependent RNA polymerase	RNA dependent DNA polymerase
50	Nucleic acid hybridization is the most commonly used method because _____.	Rapid	Screen cDNA libraries	Identifies clones	Restriction enzymes	Rapid
51	First step of PCR is _____.	Annealing	Renaturation	Denaturation	Ligation	Denaturation
52	Which of the following technique is used in DNA fingerprinting?	Western blotting	Southern blotting	Variable blotting	Eastern blotting	Southern blotting
53	Labeled antibody is used to detect _____.	DNA	RNA	Protein	Lipid	Protein
54	Which of the following technique doesn't involve electrophoresis for the separation of _____?	Dot blotting	Southern blotting	Northern blotting	Western blotting	Dot blotting
55	Probe is a ____.	Protein	Short piece of labeled DNA	Short piece of labeled protein	enzyme	Short piece of labeled DNA
56	Nitrocellulose membrane is used for _____.	Eastern blotting	Southern blotting	Northern blotting	Both B and C	Southern blotting
57	RT PCR is used for analysis of _____.	Forensic analysis of DNA	Genomic DNA	mRNA	Binding	mRNA
58	The molecular target for recognition in immuonological screening is generally	Paratope	Epitope	Antibody	Antigen	Paratope
59	In the Sanger method, what causes the termination of chain elongation?	Incorporation of DNA	Dideoxy nucleotide	Denaturation	Amplification	Dideoxy nucleotide
60	Which of the following is a chemical nucleotide sequencing method?	Sanger	Maxam-Gilbert's	Edmans	Automated	Maxam-Gilbert's

Unit 5

Syllabus

Construction of Genomic and cDNA libraries, screening of libraries: Colony hybridization and colony PCR, Chromosome walking and chromosome jumping. Products of recombinant DNA technology: Products of human therapeutic interest - insulin, hGH. Bt transgenic - cotton, brinjal, Gene therapy, recombinant vaccines, protein engineering and site directed mutagenesis

Construction of Genomic library

A genomic library is an organism specific collection of DNA covering the entire genome of an organism. It contains all DNA sequences such as expressed genes, non-expressed genes, exons and introns, promoter and terminator regions and intervening DNA sequences.

The first DNA-based genome ever fully sequenced was achieved by two-time Nobel Prize winner, Frederick Sanger, in 1977. Sanger and his team of scientists created a library of the bacteriophage, phi X 174, for use in DNA sequencing.

Steps in Genomic Library Construction:

Construction of genomic library involves following steps:

i. Isolation of genomic DNA and vector

Genomic libraries can be constructed by isolation of complete DNA from bacteria, virus, plants and animals. In eukaryotes, high molecular weight DNA is isolated by CTAB or SDS methods.

ii. Digestion genomic DNA and vector

Fragmentation can be done by mechanical shearing or using suitable restriction enzymes. Partial digestion is essential to procure proper size DNA fragments. Therefore, treatment times and concentration of enzyme is very important for desirable result.

iii. Ligation of fragmented DNA with the vector

Various vectors are available for cloning large DNA fragments. λ phage, yeast artificial chromosome, bacterial artificial chromosome etc. are considered as suitable vectors for larger DNA and λreplacement vectors like *λDASH* and *EMBL3* are preferred for construction of genomic DNA library.

The choice of vectors for the construction of genomic library depends upon three parameters:

1. The size of the DNA insert that these vectors can accommodate.
2. The size of the library that is necessary to obtain a reasonably complete representation of the entire genome.
3. The total size of the genome of the target organism.

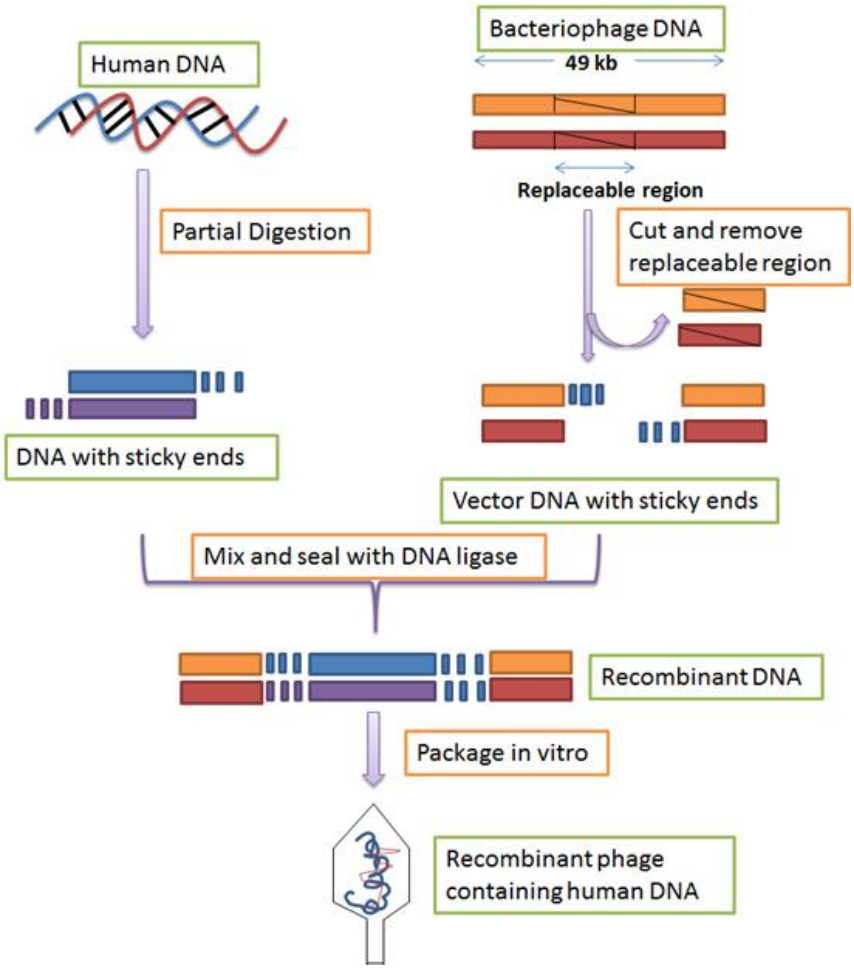
Vector	Insert size	Features
λ phages	Up to 20-30 kb	Genome size-47 kb, efficient packaging system, replacement vectors usually employed, used to study individual genes.
Cosmids	Up to 40 kb	Contains *cos* site of λ phage to allow packaging, propagate

		in *E. coli* as plasmids, useful for sub-cloning of DNA inserts from YAC, BAC, PAC etc.
Fosmids	35-45 kb	Contains F-plasmid origin of replication and λ *cos*site, low copy number, stable.
Bacterial artificial chromosomes (BAC)	Up to 300kb	Based on F-plasmid, relatively large and high capacity vectors.
P1 artificial chromosomes(PACs)	Up to 300 kb	Derived from DNA of P1 bacteriophage, combines the features of P1 and BACs, used to clone larger genes and in physical mapping, chromosome walking as well as shotgun sequencing of complex genomes.
Yeast artificial chromosomes (YAC)	Up to 2000kb	Allow identification of successful transformants. (BAC clones are highly stable and highly efficient)

The recombinant vectors and insert combinations are grown in *E. coli* such that a single bacterial colony or viral plaque arises from the ligation of a single genomic DNA fragment into the vector.

iv. Transformation of –DNA in the bacterial cells

v. Cloning the fragments in vector



Advantages:

- Identification of a clone encoding a particular gene of interest.
- It is useful for prokaryotic organisms having relatively small genomes.
- Genomic libraries from eukaryotic organisms are very important to study the genome sequence of a particular gene, including its regulatory sequences and its pattern of introns and exons.

Disadvantages:

- Genome libraries from eukaryotes having very large genomes contain a lot of DNA which does not code for proteins and also contain non-coding DNA such as repetitive DNA and regulatory regions which makes them less than ideal.
- Genomic library from a eukaryotic organism will not work if the screening method requires the expression of a gene.

Applications:

1. It helps in the determination of the complete genome sequence of a given organism.
2. It serves as a source of genomic sequence for generation of transgenic animals through genetic engineering.
3. It helps in the study of the function of regulatory sequences *in-vitro*.
4. It helps in the study of genetic mutations in cancer tissues.
5. Genomic library helps in identification of the novel pharmaceutical important genes.
6. It helps us in understanding the complexity of genomes.

Construction of cDNA library

Complementary DNA (cDNA) is the DNA produced on an RNA template by the action of reverse transcriptase (RNA-dependent DNA-polymerase). The sequence of the cDNA becomes complementary to the RNA sequence.

cDNA libraries are used to express eukaryotic genes in prokaryotes since it does not include introns, and therefore, can be expressed in prokaryotic cells.

cDNA libraries remove the large numbers of non-coding regions from the library, and it is also useful for subsequently isolating the gene that codes for that mRNA.

The construction of cDNA library involves following steps-

1. Isolation of mRNA

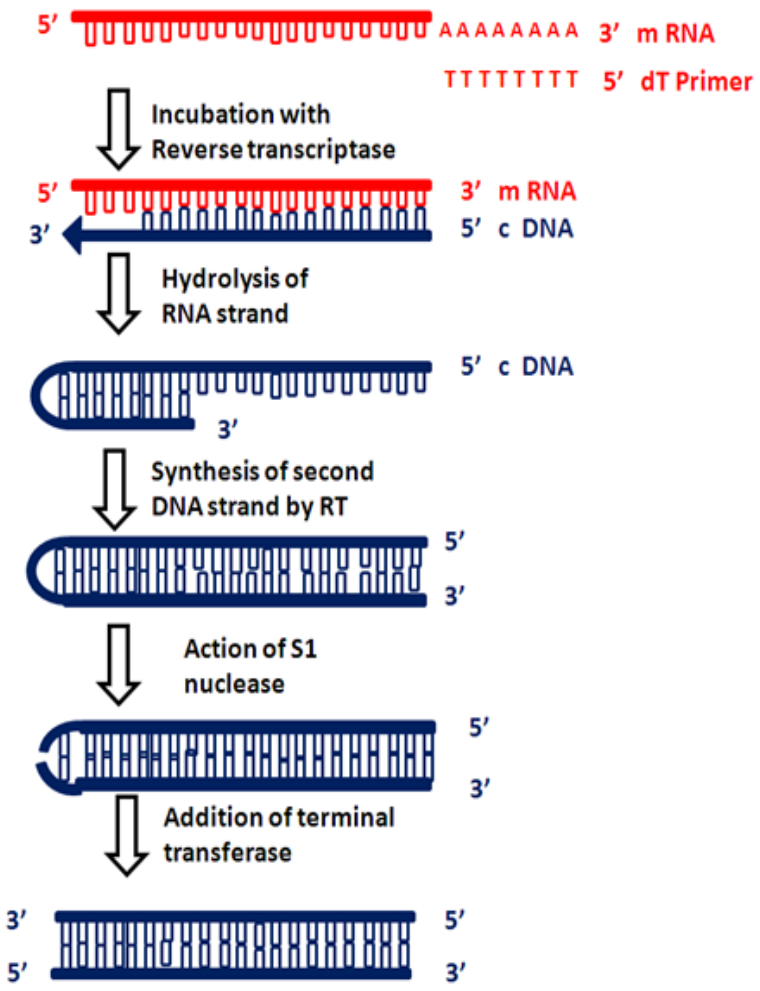
- It involves the isolation of total mRNA from a cell type or tissue of interest. The 3′ ends of eukaryotic mRNA consist of a string of 50 – 250 adenylate residues (poly A Tail) which makes the separation easy from the much more prevalent rRNAs and tRNAs in a cell extract using a column containing oligo-dTs tagged onto its matrix.
- When a cell extract is passed through an oligo-dT column, the mRNAs bind to the column due to the complementary base-pairing between poly (A) tail and oligo-dT. Other RNAs (ribosomal RNAs and

transfer RNAs) flow through as unbound fraction. The bound mRNAs can then be eluted using a low-salt buffer.

2. Synthesis of first and second strand of cDNA

mRNA being single-stranded cannot be cloned as such and is not a substrate for DNA ligase. It is first converted into DNA before insertion into a suitable vector which can be achieved using reverse transcriptase (RNA-dependent DNA polymerase or RTase) obtained from avian myeloblastosis virus (AMV).

- A short oligo (dT) primer is annealed to the Poly (A) tail on the mRNA.
- Reverse transcriptase extends the 3´-end of the primer using mRNA molecule as a template producing a cDNA: mRNA hybrid.
- The mRNA from the cDNA: mRNA hybrid can be removed by RNase H or Alkaline hydrolysis to give a ss-cDNA molecule.
- No primer is required as the 3´end of this ss-cDNA serves as its own primer generating a short hairpin loop at this end.This free 3´-OH is required for the synthesis of its complementary strand.
- The single stranded (ss) cDNA is then converted into double stranded (ds) cDNA by either RTase or *E. coli* DNA polymerase.
- The ds-cDNA can be trimmed with S1 nuclease to obtain blunt–ended ds-cDNA molecule followed by addition of terminal transferase to tail the cDNA with C's and ligation into a vector.

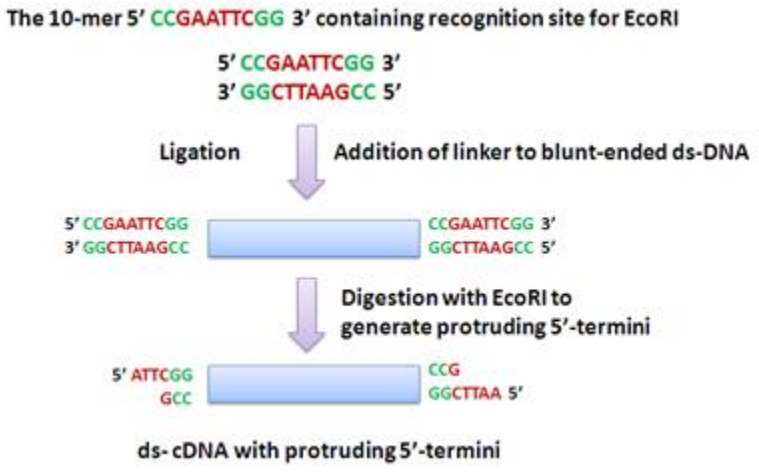


3. Incorporation of cDNA into a vector

The blunt-ended cDNA termini are modified in order to ligate into a vector to prepare ds-cDNA for cloning. Since blunt-end ligation is inefficient, short restriction-site linkers are first ligated to both ends.

The resulting double-stranded cDNAs with linkers at both ends are treated with a restriction enzyme specific for the linker generating cDNA molecules with sticky ends. Ligation of the digested ds-cDNA into a vector is the final step in the construction of a cDNA library. The vectors (e.g. plasmid or bacteriophage) should be restricted with the same restriction enzyme used for linkers. The *E. coli* cells are transformed with the recombinant vector, producing a library of plasmid or λ clones.

These clones contain cDNA corresponding to a particular mRNA



4. Cloning of cDNAs

cDNAs are usually cloned in phage insertion vectors. Bacteriophage vectors offer the following advantageous over plasmid vectors,

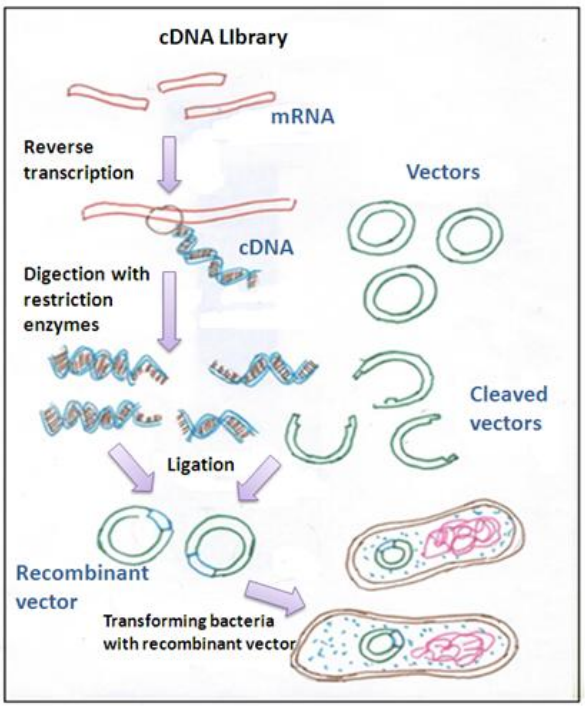
• are more suitable when a large number of recombinants are required for cloning low-abundant mRNAs as recombinant phages are produced by *in vitro* packaging.

• can easily store and handle large numbers of phage clones, as compared to the bacterial colonies carrying plasmids.

Plasmid vectors are used extensively for cDNA cloning, particularly in the isolation of the desired cDNA sequence involving the screening of a relatively small number of clones.

Disadvantages:

- cDNA libraries contain only the parts of genes found in mature mRNA. However, the sequences before and after the gene, for example, those involved in the regulation of gene expression, will not occur in a cDNA library.
- Construction of a cDNA library cannot be used for isolating the genes expressed at low levels as there will be very little mRNA for it in any cell type and may completely be out manoeuvred by the more abundant species.



Applications:

- Discovery of novel genes.
- *in vitro* study of gene function by cloning full-length cDNA.

- Determination of alternative splicing in various cell types/tissues.
- They are commonly used for the removal of various non-coding regions from the library.
- Expression of eukaryotic genes in prokaryotes as they lack introns in their DNA and therefore do not have any enzymes to cut it out in transcription process. Gene expression required either for the detection of the clone or the polypeptide product may be the primary objective of cloning.
- To study the expression of mRNA.

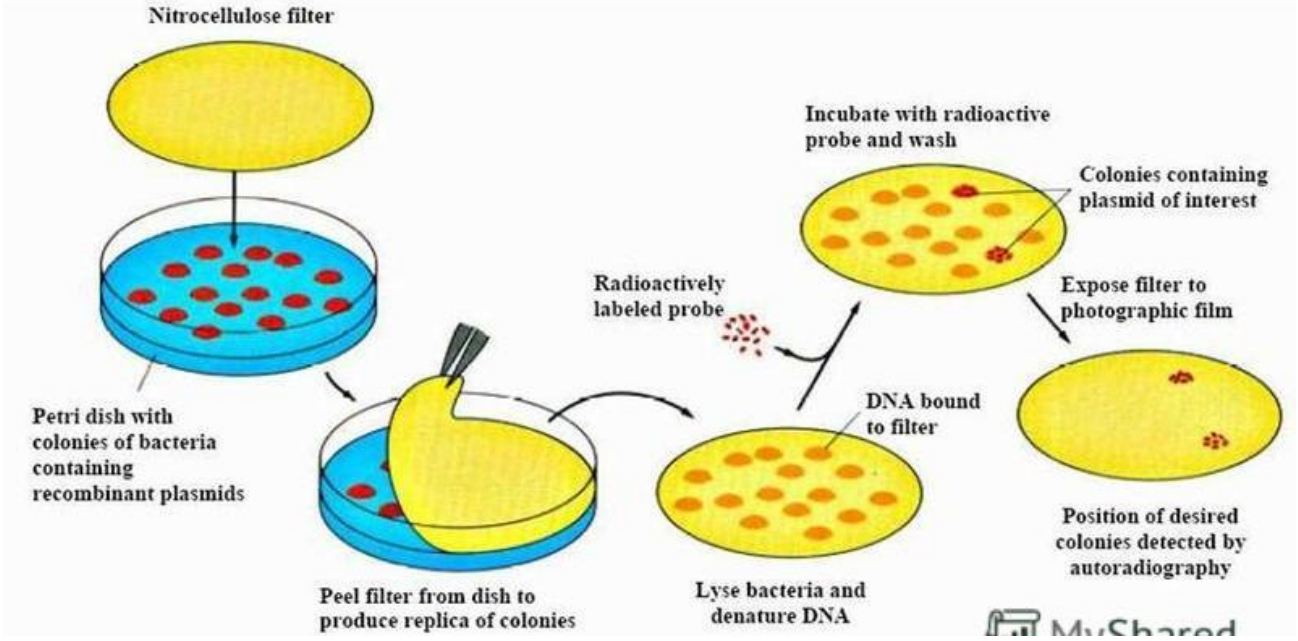
Screening of libraries

- Library screening is the process of identification of the clones carrying the gene of interest. Screening relies on a unique property of a clone in a library.
- The DNA libraries consist of a collection of probably many thousand clones in the form of either plaques or colonies on a plate.
- Screening of libraries can be done by following approaches based on detecting a particular DNA sequence and gene expression.

(a) Colony hybridization

Colony hybridization, also known as replica plating, allows the screening of colonies plated at high density using radioactive DNA probes. Colony hybridization can be used to screen plasmid or cosmid based libraries

Grunstein & Hogness (1975) developed a screening procedure to detect DNA sequences in transformed colonies by hybridization with radioactive RNA probes.



- The colonies to be screened are first replica-plated on to a nitrocellulose filter disc that has been placed on the surface of an agar plate prior to inoculation.
- Master plate is retained for reference set of colonies.
- The filter bearing the colonies is removed and treated with alkali so that the bacterial colonies are lysed and the DNA they contain is denatured.

- The filter is then treated with proteinase K to remove protein and leave denatured DNA bound to the nitrocellulose.
- The DNA is fixed firmly by baking the filter at 80°C.
- A labeled probe is hybridized to this DNA which is monitored by autoradiography.
- A colony whose DNA print gives a positive auto radiographic result on X-ray film can then be picked from the reference plate.

(b) Colony PCR

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

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

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

Chromosome walking and chromosome jumping

- Chromosome walking is a method of positional cloning used to find, isolate, and clone a particular allele in a gene library.
- Chromosome Walking was developed by Welcome Bender, Pierre Spierer, and David S. Hogness in the early 1980's.
- The technique of chromosome walking involves the following steps :

(i) From the genomic library select a clone of interest (identified by a probe) and subclone a small fragment from one end of the clone (there is a technique available to subclone a fragment from the end);

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

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

(iv) Third clone identified as above is also subcloned and hybridized with clones in the same manner and the procedure may be continued;

(v) Restriction map of each selected clo.ne may be prepared and compared to know the regions of overlapping as shown in Figure 40.11, so that identification of new overlapping restriction sites will amount to walking along the chromosome or along a long chromosome segment.

Application:

- This technique can be used for the analysis of genetically transmitted diseases, to look for mutations.
- Chromosome Walking is used in the discovery of single-nucleotide polymorphism of different organisms.

Disadvantages:

- There is a limitation to the speed of chromosome walking because of the small size of the fragments that are to be cloned.
- Another limitation is the difficulty of walking through the repeated sequence that are scattered through the gene.
- If the markers were too far away, it simply was not a viable option.
- Additionally, chromosome walking could easily be stopped by unclonable sections of DNA.
- A solution to this problem was achieved with the advent of chromosome jumping, which allows the skipping of unclonable sections of DNA.

- Chromosome jumping is used to bypass regions difficult to clone, such as those containing repetitive DNA that cannot be easily mapped by chromosome walking, and is useful in moving along a chromosome rapidly in search of a particular gene.
- Chromosome jumping allows more rapid movement through the genome compared to other techniques, such as chromosome walking, and can be used to generate genomic markers with known chromosomal locations.
- Chromosome jumping enables two ends of a DNA sequence to be cloned without the middle section. Genomic DNA may be partially digested using restriction endonucleases and with the aid of DNA ligase, the fragments are circularized.
- From a known sequence, a primer is designed to sequence across the circularised junction. This primer is used to jump 100 kb-300 kb intervals: a sequence 100 kb away would have come near the known sequence on circularisation. Thus, sequences not reachable by chromosome walking can be sequenced.
- Chromosome walking can be used from the new jump position (in either direction) to look for gene-like sequences or additional jumps can be used to progress further along the chromosome.

Products of recombinant DNA technology:

- Many practical applications of recombinant DNA are found in human and veterinary medicine, in agriculture, and in bioengineering. Biochemical products of recombinant DNA technology in medicine and research include: human recombinant insulin, growth hormone, blood clotting factors, hepatitis B vaccine, and diagnosis of HIV infection.
- Biochemical products of recombinant DNA technology in agriculture include: golden rice, herbicide-resistant crops, and insect-resistant crops.

Products of human therapeutic interest – Insulin:

- Insulin is a peptide hormone produced by β-cells of islets of Langerhans of pancreas. It was discovered by sir Edward Sharpey Schafer (1916)

- Chemically Human insulin is small, simple protein composed of 51 amino acids sequences and has a molecular weight of 5808 Da.
- Insulin hormone is a dimer of a A- chain and a B-chain which are linked together by a disulphide bond.
- Fredrick Sanger et al (1954) gave the first complete description of insulin. Insulin consists of two polypeptide chain,
  - o Chain A- 21 amino acids long
  - o Chain B-30 amino acids long
  - o Both chains are joined together by disulphide bond between two cysteine residue
- Some examples of human insulin:

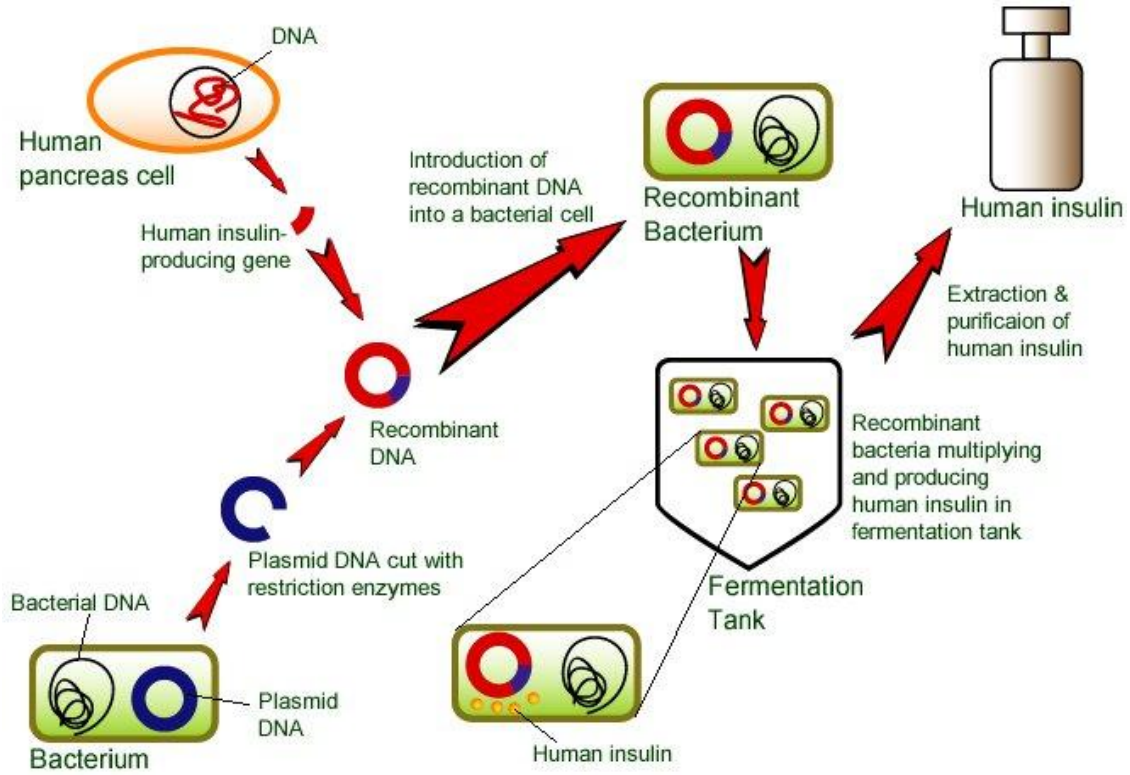
  Regular (short acting): Humulin S, Actrapid, Insuman Rapid

  NPH (intermediate acting):Humulin I, Insuman basal, Insulatard

  Premixed human insulins:Humulin M2, M3 and M5, Insuman Comb 15, 25 and 50

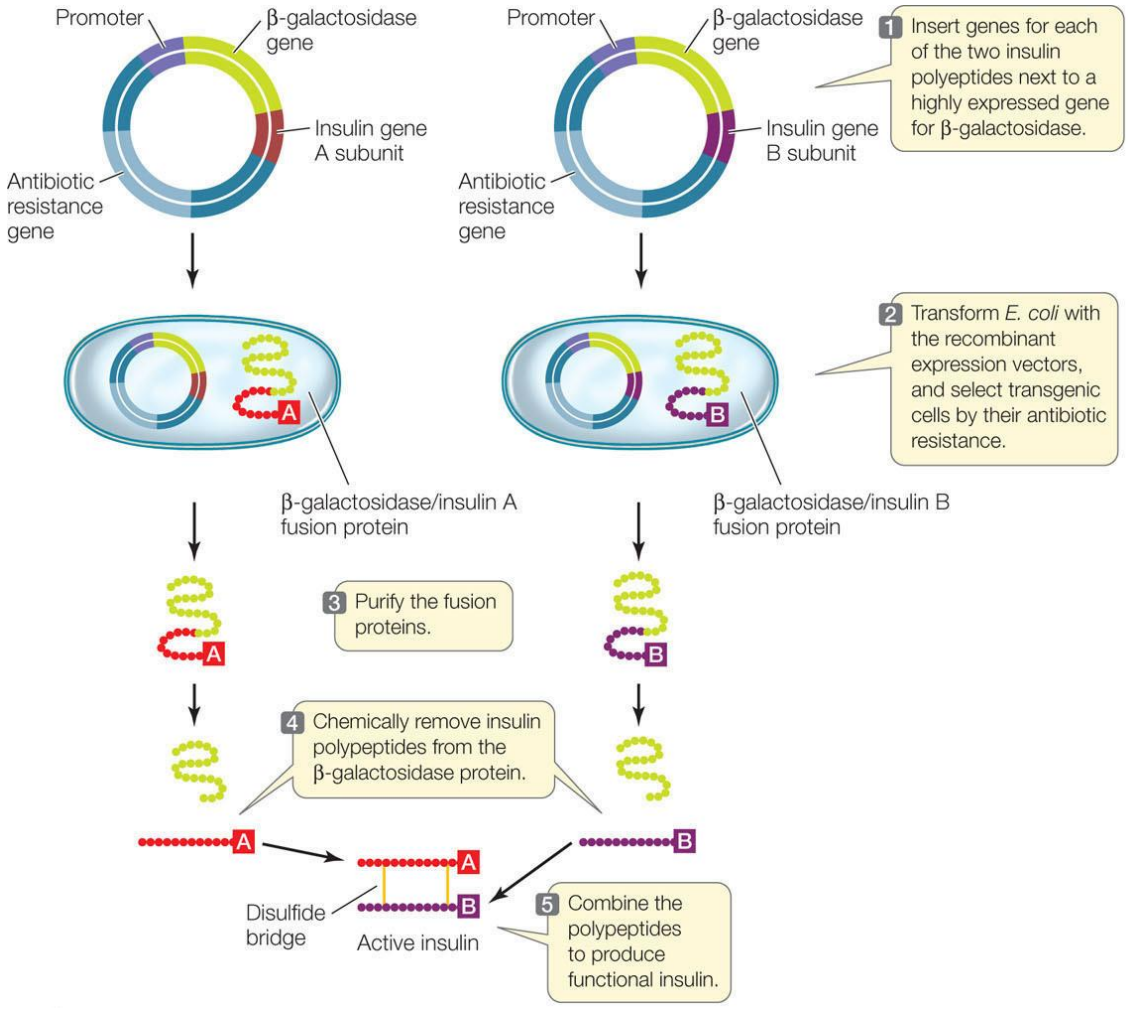
Manufacturing of insulin using microbes as a cell factory involves the following steps –

1. Isolation of gene: The gene for producing human insulin protein is isolated.
2. Preparation of target DNA: Circular piece of DNA called plasmid is obtained from bacteria.
3. Insertion of DNA into plasmid: The gene for insulin is inserted into the plasmid construct. The human insulin gene is now recombined with bacterial DNA.
4. Plasmid insertion: The bacterial DNA having insulin gene is inserted back into bacteria.
5. Plasmid multiplication: The bacterial cells having insulin gene are allowed to grow and multiply and during this process bacterial cells start to produce recombinant insulin. During division newly synthesized copy of cell are produced.
6. Human insulin produced by bacteria is purified.



Hakura et al (1977) chemically synthesize DNA sequence of insulin for two chains A and B and separately inserted into two PBR322 plasmid vector.

- These gene are inserted by the side of β-galactosidase gene of the plasmid.
- The recombinant plasmid were then separately transformed into *E. coli* host.
- The recombinant host produced pro-insulin chains ie. fused β-galactosidase-A chain and β-galactosidase-B-chain separately.
- These pro-insulin chains A and B were separated from β-galactosidase by treatment with cyanogen bromide. The detachment of pro-insulin chains from β-galactosidase is possible because an extra codon form methionine was added at N-terminal of each gene for A and B-chain.
- After detachment, A and B chains are joined invitro to reconstitute the naïve insulin by sulphonating the peptide chains with sodium disulphonate and sodium sulphite.



Human Growth hormone (hGH):

- Growth hormone is produced by the pituitary gland. It regulates the growth and development. Growth hormone stimulates overall body growth by increasing the cellular uptake of amino acids, and protein synthesis, and promoting the use of fat as body fuel. Insufficient human growth hormone (HGH) in young children results in retarded growth, clinically referred to as pituitary dwarfism.

- The gene for human growth hormone (hGH) is isolated from human pituitary gland.
- Insertion of whole hGH gene into plasmid vector and cloning into *E.coli* results into production of biologically inactive hormone because bacteria can translate the region of gene that are not translated in human thereby producing a prehormone containing an extra 26 aminoacids which might be difficult to remove.
- Hence the segment of gene that codes for the first 24 aminoacids of hormone is constructed chemically from blocks of nucleotide

Step I: Chemical synthesis of gene for first 24 aminoacids:

From the known aminoacids sequence of hGH, gene for first 24 aminoacids are constructed chemically. These genes are constructed in three small fragments and then they are joined by T4 DNA ligase to get whole gene for first 24 aminoacids.

Step II: Isolation of mRNA for hGH

In this step mRNA for hGH is isolated from human pituitary gland tissue.

Step III: Reverse transcription

Using reverse transcriptase enzyme complimentary DNA (cDNA) is synthesized from mRNA.

The cDNA obtained by reverse transcription process, is the gene for hGH.

The full gene is cut with restriction endonuclease enzyme to remove first 24 gene.

Step IV: Joining of synthetic gene and cDNA

In this step synthetic gene (gene for first 24 aminoacids) and cDNA are joined in order to obtain full gene with its own initiation codon (AUG). T4 DNA ligase joins these genes.

Step V: selection of suitable vector and recombination:

Expression vector phGH407 derived from plasmid vector PBR322 is used as carrier vector.

HGH gene is ligated into a restriction site just downstream of Lac; promotor/operator region of the expression vector.

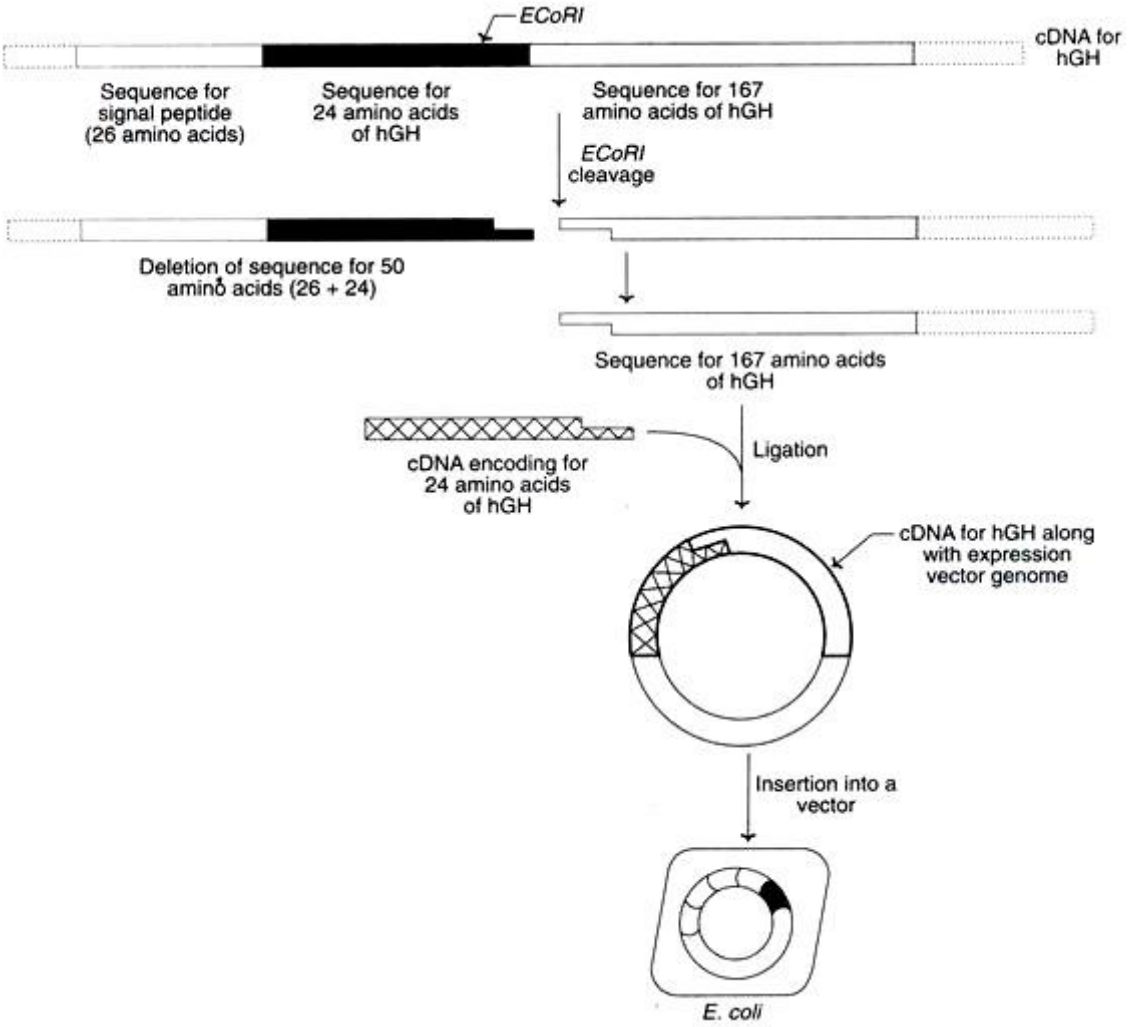
Step VI: selection and recombination into suitable host cell

E. coli is used as suitable host cell.

The recombinant expression vector is then transformed into *E.coli.*

The recombinant *E. coli* then starts producing hGH.

The recombinant *E. coli* are isolated from the culture and mass production by fermentation technology to obtain hGH.



Recombinant vaccines

A recombinant vaccine is a vaccine produced by inserting the DNA encoding an antigen (such as a bacterial surface protein) that stimulates an immune response into bacterial or mammalian cells, expressing the antigen in these cells and then purifying it from them**.**

The recombinant vaccines may be broadly categorized into three groups:

1. Subunit recombinant vaccines: These are the components of the pathogenic organisms. Subunit vaccines include proteins, peptides and DNA.

The advantages of these vaccines include their purity in preparation, stability and safe use.

The disadvantages are high cost factor and possible alteration in native conformation.

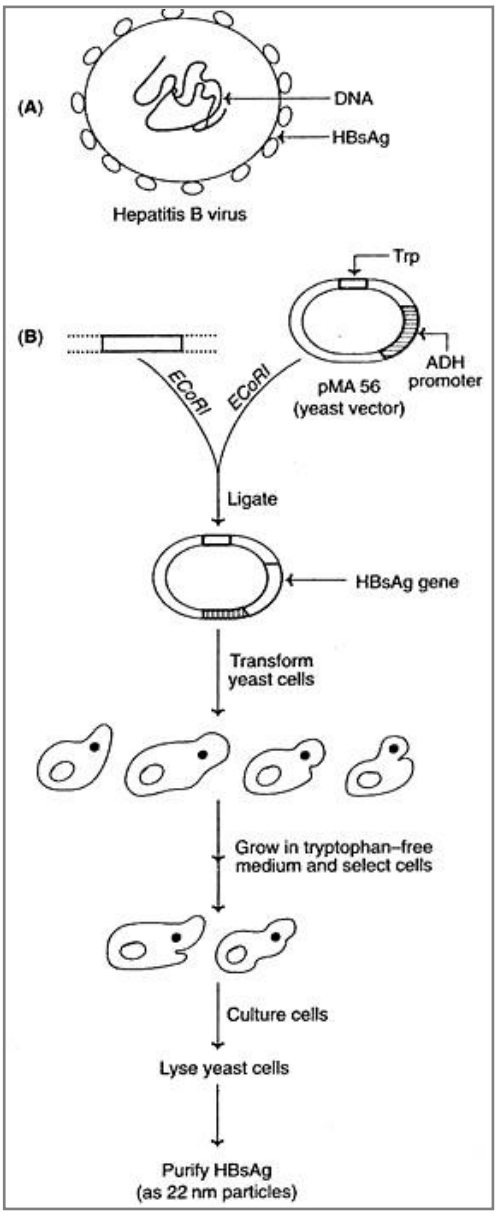
Hepatitis B primarily affects liver causing chronic hepatitis, cirrhosis and liver cancer. Hepatitis B virus consists of a core containing a viral genome (DNA) surrounded by a phospholipid envelope carrying surface antigens which are more immunogenic.

The gene encoding for hepatitis B surface antigen (HBsAg) has been identified. Recombinant hepatitis B vaccine as a subunit vaccine is produced by cloning HbsAg gene in yeast cells. *Saccharomyces*

cerevisiae. The gene for HBsAg is inserted (pMA 56) which is linked to the alcohol dehydrogenase promoter. These plasmids are then transferred and cultured.

The cells grown in tryptophan, free medium are selected and cloned. The HBsAg gene is expressed to produce 2nm sized particles similar to those found in patients infected with hepatitis B. (These particles are immunoreactive with anti-HBsAg antibodies). The subunit HBsAg as 22 nm particles can be isolated and used to immunize individuals against hepatitis B.

In 1987, the recombinant vaccine for hepatitis B (i.e. HBsAg) became the first synthetic vaccine for public use. It was marketed by trade names Recombivax and Engerix-B.



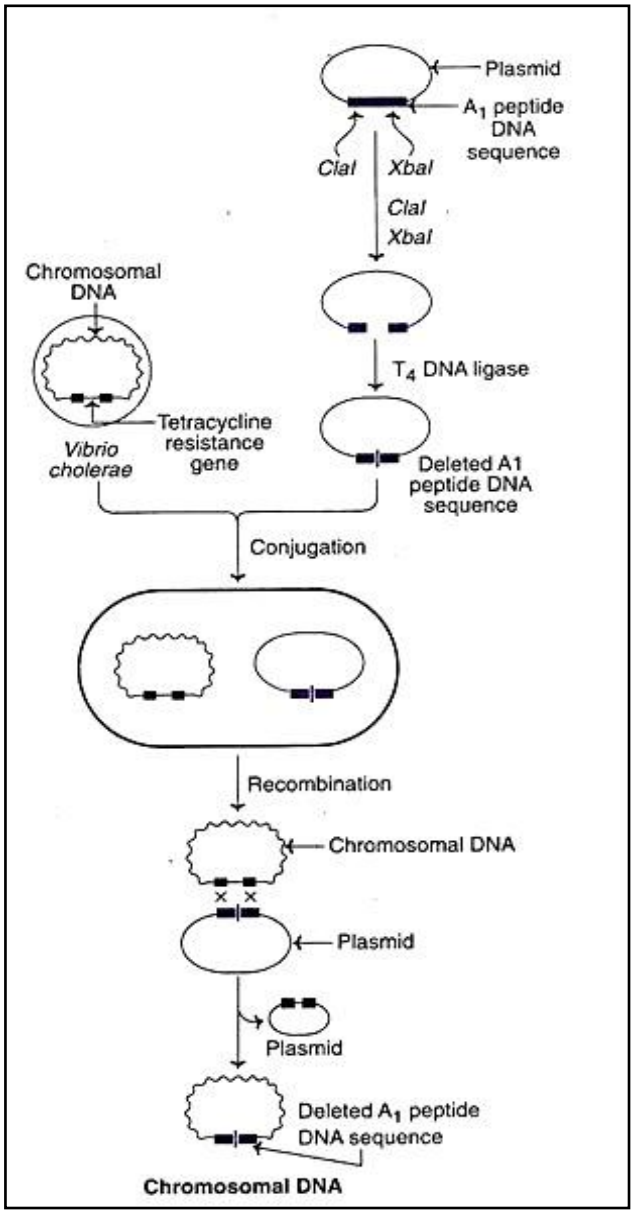
2. Attenuated recombinant vaccines: These are the genetically modified pathogenic organisms (bacteria or viruses) that are made non-pathogenic and used as vaccines.

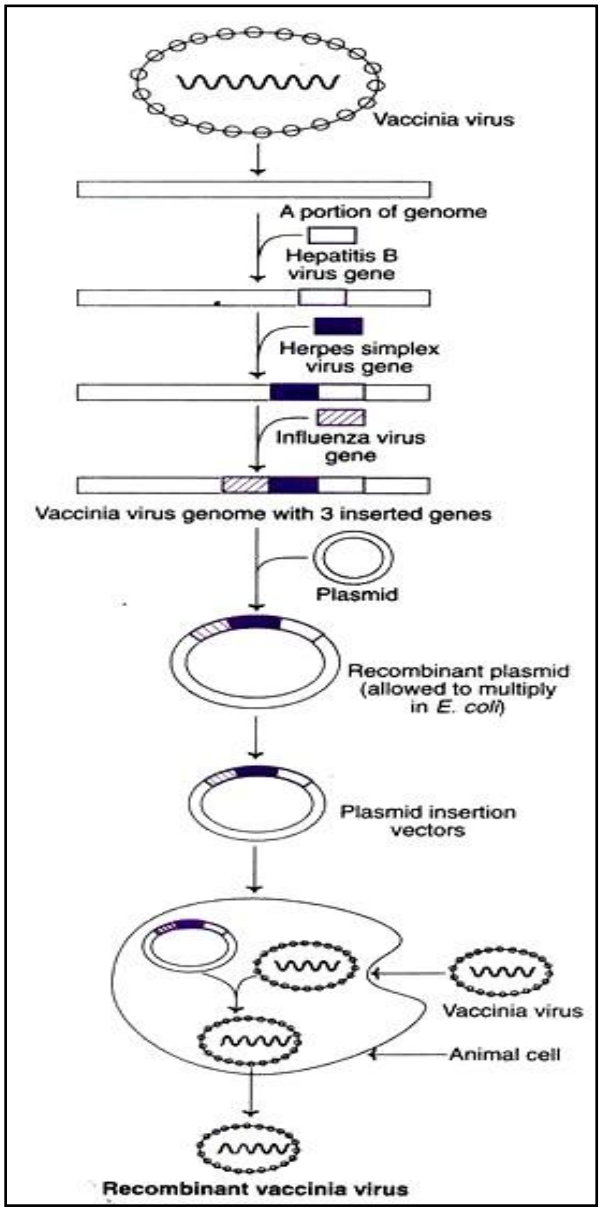
Cholera is caused by *Vibrio cholera*. This pathogenic organism is transmitted by drinking water contaminated with fecal matter. On entering the small intestine, *V. cholera* colonizes and starts producing large amounts of a toxic protein, Enterotoxin, an hexamer, consists of one A subunit and five identical B subunits. The A subunit has two functional domains-the A_1 peptide which possesses the toxic activity and A_2peptide that joins A subunit to B subunits.

By genetic engineering, it was possible to delete the DNA sequence encoding A_1 peptide and create a new strain of *V. cholera*. This strain is non-pathogenic, since it cannot produce enterotoxin. The genetically engineered *V. cholera* is a good candidate to serve as an attenuated vaccine.

- The DNA sequence of A_1 peptide is incorporated into a plasmid, cloned and digested with restriction enzymes (Clal and Xbal). In this manner, the A_1 peptide coding sequence is deleted (the DNA encoding for 183 of the 194 amino acids of the A_1 peptide is actually removed). By using T_4 DNA ligase, the plasmid is re-circularized. This plasmid contains a small portion of A_1 peptide coding sequence.
- The plasmid, containing the deleted A_1 peptide sequence is transferred by conjugation into the *V. cholera* strain carrying a tetracycline resistance gene.
- Recombination can occur between the plasmid (containing a small portion of peptide A_1 coding sequence) and the chromosome of *V. cholera* (carrying tetracycline resistance gene). The result of this double crossover is the formation of *V. cholera* containing a chromosomal DNA lacking A_1 peptide DNA sequence. As the bacterium, *V. cholera* multiplies, the plasmids are lost in the next few generations.

- The *V. cholera* cells defective in A_1 peptide are selected, based on tetracycline sensitivity.





3. Vector recombinant vaccines: These are the genetically modified viral vectors that can be used as vaccines against certain pathogens.

- Vaccinia viruses are basically the vaccine that was originally used by Jenner for the eradication of smallpox. Vaccinia virus contains a double-stranded DNA (187 kb) that encodes about 200 different proteins. The genome of this virus can accommodate stretches of foreign DNA which can be expressed along with the viral genes.
- The vaccinia viruses are generally harmless, relatively easy to cultivate and stable for years after lyophilization (freeze-drying). All these features make the vaccinia virus strong candidates for vector vaccine.

- The advantage with vector vaccine is that it stimulates B-lymphocytes (to produce antibodies) and T-lymphocytes (to kill virus infected cells). This is in contrast to a subunit vaccine which can stimulate only B-lymphocytes.

The development of recombinant vaccinia virus is carried out by a two-step procedure

1. Assembly of plasmid insertion vector:

Fresh vaccinia (cow pox) viruses are processed to release their DNAs. Now genes from hepatitis B virus, herpes simplex virus and influenza virus are added one after another and inserted into vaccinia virus genome. These DNA clusters are cloned in *E.coli* for increasing their number and to produce plasmid insertion vectors. The plasmid contains the foreign subunit genes, the natural vaccinia genes, including the promoter genes. The recombinant plasmids are isolated and purified and serve as plasmid insertion vectors.

2. Production of recombinant vaccinia viruses:

The animal cells are infected with plasmid insertion vectors and normal vaccinia viruses. As the viral replication occurs, the plasmids are taken up to produce recombinant vaccinia viruses. The plasmid insertion vector incorporates its genes into vaccinia virus genome at a place that encodes for the enzyme thymidine kinase (TK).

Thus the recombinant viruses have lost their ability to produce TK. There are two advantages of loss of TK gene. One is that it is easy to select recombined vaccinia viruses that lack TK gene and the second is that these viruses are less infectious than the normal viruses. The recombinant vaccinia viruses, released from the cultured animal cells, can be successfully used as vaccines. These live viral vaccines have some advantages over the killed or subunit vaccines.

Gene therapy

Gene therapy typically involves the insertion of a functioning gene into cells to correct a cellular dysfunction or to provide a new cellular function. For example, diseases such as cystic fibrosis, combined immunodeficiency syndromes, muscular dystrophy, hemophilia, and many cancers result from the presence of defective genes. Gene therapy has been especially successful in the treatment of combined immunodeficiency syndromes, showing lasting and remarkable therapeutic benefit.

There are two general approaches for introducing genes into a cell: viral and nonviral

- Viral vectors have been used in ~70% of the clinical trials to date. Viral vectors are extremely efficient at transferring genes but can create some safety risks. Gene transfer mediated by viral vectors is referred to as transduction.
- Nonviral vectors are considered to be much safer than viral vectors, but at present, they are fairly inefficient at transferring genes. Gene transfer mediated by nonviral vectors is referred to as transfection.
- The two most common viral vectors used in clinical trials have been those derived from a serotype 5 adenovirus (Ad5; ~26%) and Moloney murine leukemia virus (MoMLV; ~28%), a retrovirus.

Protein engineering

Protein engineering can be defined as the modification of protein structure with recombinant DNA technology or chemical treatment to get a desirable function for better use in medicine, industry and agriculture.

Site directed mutagenesis

Site-directed mutagenesis (SDM) is a method to create specific, targeted changes in double stranded plasmid DNA. There are many reasons to make specific DNA alterations (insertions, deletions and substitutions), including:

- To study changes in protein activity that occurs as a result of the DNA manipulation.
- To select or screen for mutations (at the DNA, RNA or protein level) that have a desired property
- To introduce or remove restriction endonuclease sites or tags

- 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.

Applications:

- Used to generate mutations that may produce rationally designed protein that has improved or special properties
- Proteins may be engineered to produce proteins that are tailored for a specific application.
- Site-directed mutagenesis has been widely used in the study of protein functions.

Bt transgenic – Cotton

This is a genetically modified version of cotton. 'Bt' stands for the microbe *Bacillus thuringiensis*. This microbe produces an insecticidal protein or toxin that kills other insects such as bollworm (*Helicoverpa armigera*) tobacco budworm, flies, mosquitoes, beetles etc.

Strains of the bacterium *Bacillus thuringiensis* produce over 200 different Bt toxins, each harmful to different insects. Most notably, Bt toxins are insecticidal to the larvae of moths and butterflies, beetles, cotton bollworms and ghtu flies but are harmless to other forms of life. The gene coding for Bt toxin has been inserted into cotton as a transgene, causing it to produce this natural insecticide in its tissues. Since most Bt toxins are insect-group specific, the choice of genes to be incorporated depends on the crop and the targeted pest. A gene named *cry* codes for the toxin protein and there a number of these genes. For example, the genes *cryIAc* and *cryIIAb* encode toxins that control cotton bollworms whereas the gene *cryIAb* controls the insect 'corn borer'.

Usually the toxin gets activated only once it comes in contact with the alkaline pH in the insect gut when the insect ingests it. The activated toxin then binds to the surface of epithelial cells and creates pores in it. This causes the cells to swell and lyse, eventually leading to the death of the insect.

Bt cotton has several advantages over non-Bt cotton. The important advantages of Bt cotton are briefly:

- Increases yield of cotton due to effective control of three types of bollworms, viz. American, Spotted and Pink bollworms.
- Reduction in insecticide use in the cultivation of Bt cotton in which bollworms are major pests.
- Potential reduction in the cost of cultivation (depending on seed cost versus insecticide costs).
- No health hazards due to rare use of insecticides (particularly who is engaged in spraying of insecticides).

Bt cotton has some limitations

- High cost of Bt cotton seeds as compared to non Bt cotton seeds.
- Effectiveness up to 120 days, after that the toxin producing efficiency of the Bt gene drastically reduces.
- Ineffective against sucking pests like jassids, aphids, whitefly etc.

Bt transgenic – Brinjal

The genetically modified brinjal is a suite of transgenic brinjals (also known as an eggplant or aubergine) created by inserting a crystal protein gene (Cry1Ac) from the soil bacterium Bacillus thuringiensis into the genome of various brinjal cultivars. The insertion of the gene, along with other genetic elements such as promoters, terminators and an antibiotic resistance marker gene into the brinjal plant is accomplished using Agrobacterium-mediated genetic transformation. The Bt brinjal has been developed to give resistance against lepidopteron insects, in particular the Brinjal Fruit and Shoot Borer (Leucinodes orbonalis)

When fruit and shoot borer larvae feed on Bt brinjal plants, they ingest the Bt protein Cry1Ac along with plant tissue. In the insect gut, which is alkaline with a pH >9.5, the protein is solubilized and activated by gut proteases. The Bt protein binds to specific receptor proteins present in the insect membrane, resulting in pore formation in the membranes. This leads to disruption of digestive processes, paralysis, and subsequent death of the fruit and shoot borer larvae

Advantages

1) non-infested, undamaged and good quality brinjal fruits. 2) Bt brinjal will significantly reduced level of pesticide residues, Farmers are expected to benefit at multiple levels. Some of these include: 3) Saving on cost of insecticides and lower labor cost as a result of reduced spraying. 4) Increase in yield per unit area by saving fruits from damage caused by FSB

Possible questions

Part A (1 Mark)

Part B (2 Marks)

1. What are genomic library and cDNA library?
2. Mention any two advantages and disadvantages of genomic library.
3. What is the function of reverse transcriptase?
4. What are colony hybridization and colony PCR
5. What is chromosome walking?
6. What is chromosome jumping?
7. Mention the steps involved in the synthesis of insulin
8. Mention the steps involved in the synthesis of hGH
9. What are recombinant vaccines? Give example
10. What is site directed mutagenesis?
11. Detail on Bt cotton and Bt brinjal
12. What is gene therapy?

Part C (8 Marks)

1. Write a detailed note on the steps involved in the construction of genomic library
2. Write a detailed note on the steps involved in the construction of cDNA library
3. Detail on the products of recombinant DNA technology human therapeutic interest – Insulin, hGH
4. Detail on the products of recombinant DNA technology Bt transgenic - cotton, brinjal
5. Write a detailed note on screening of libraries
6. Write a detailed note on recombinant vaccines and protein engineering
7. Write a detailed note on site directed mutagenesis and gene therapy
8. Write a detailed note on chromosome walking and jumping

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: II B.Sc MB COURSE NAME: RECOMBINANT DNA TECHNOLOGY

COURSE CODE: 18MBU404B UNIT: V BATCH-2018-2021

Sl.No	Questions	opt1	opt2	opt3	opt4	Answer
1	Which of the following statements are true regarding rDNA technology?	Obtain large number copies of specific DNA	Physics technology	Computer technology	Chemical technology	Obtain large number copies of specific DNA fragments
2	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
3	Which are responsible for over production of recombinant protein?	Strong promoter	Gene dosage	Week promoter	Small promoter	Strong promoter
4	Promoters used in bacterial expression vector are _____.	lac	BAX	TIC	LOCK	lac
5	Which of the following is used yeast as host?	*Saccharomyces cerivisiae*	*E. Coli*	*Bacillus subtilis*	*Aspergillus niger*	*Saccharomyces cerivisiae*
6	Which of the following would be useful selectable marker	Antibiotic	DNA	protein	RNA	DNA
7	Recombinant proteins expressed at high levels will sometimes form insoluble aggregates known _____.	Inclusion bodies	Exclusion bodies	partision bodies	microbodies	Inclusion bodies
8	The tac promoter is a fusion of _____.	Trp and Hfr	Trp and lac	T7 and lac	Lac and ypl	T7 and lac
9	Shuttle vectors contain _____.	Origin of replication of bacteria and	Origin of replication of eukaryote	marker	operon	Origin of replication of bacteria and eukaryotes
10	Recombinant insulin was expressed in _____.	Bacterial expression system	Plant expression system	Insect expression system	Mammalian expression system	Mammalian expression system
11	Molecular markers include _____.	RAPD	Satellite	SMP	GLP	RAPD
12	The variation in the restriction DNA fragment lengths between individuals of a species is called _____.	Restriction fragment length	Random amplified polymorphic DNA	Amplified fragment length polymorphisms	Simple sequence repeats	Restriction fragment length polymorphism

13	Specific biomolecules which shows easily detectable differences among different strains of a species is termed as _____.	DNA fingerprinting	Molecular marker	Molecular scissors	RFLP	Molecular marker
14	Locations of quantitative genes on chromosomes are called _____.	Qualitative trait	Quantitative trait loci	Allels	Recon	Qualitative trait loci
15	DNA of eukaryotic organisms has several repeating units of short sequences called _____.	Random repeats	Tandem repeats	Mini satellites	Mega satellite	Tandem repeats
16	____issues are those that ask to consider the potential moral outcomes.	Ethical	Legal	Social	Scientific	Ethical
17	____issues are those that help for policy making.	Ethical	Legal	Social	Scientific	Legal
`18	____issues are those that involves the impact of cloning technologies.	Ethical	Legal	Social	Scientific	social
19	The part of Ti plasmid which gets transferred to plant chromosome is ____.	A DNA	B DNA	Z DNA	T DNA	T DNA
20	Ti plasmids are isolated from _____.	*Staphylococcus aureus*	*Agrobacterium tumefaciens*	*Sacchromyces ceriviciae*	*Escherichia coli*	*Agrobacterium tumefaciens*
21	Treatment of genetic diseases by introducing proper genes into patients' cell is called _____.	Gene therapy	Enzyme therapy	Genetic engineering	Protein engineering	Gene therapy
22	Treatment of genetic diseases by introducing a remedial gene into sperm,egg or zygote is known as _____.	Somatic cell gene Therapy	Embryo therapy	Germ line gene therapy	Antisense therapy	Germ line gene therapy
23	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
24	Treatment of genetic diseases by introducing a remedial gene that prevents the expression of the specific defective gene is called _____.	Exvivo gene Therapy	Invivo gene therapy	Germline therapy	Antisense gene Therapy	Antisense gene therapy
25	The functional gene is introduced in addition to defective gene endogenous to the cells is known as ____.	Somatic cell gene Therapy	Augmentation gene	Targeted gene therapy	Germline gene Therapy	Augmentation gene

			Therapy			Therapy
26	Transfer of cloned genes into cells grown in culture and then introduction of these cells into _____.	*In vivo* gene	Embryo therapy	Somatic cell gene therapy	*Ex vivo* therapy	*Ex vivo* therapy
27	Synthetic vesicles composed of lipid bilayer used for transfer of genes are _____.	Lipoproteins	Liposomes	Lysosomes	Lysozymes	Liposomes
28	Binding of gene specific oligonucleotides to double stranded DNAto inhibit transcription of a gene is _____.	Triple helix Therapeutics	Antisense therapy	Ribozyme therapeutics	Antisense Oligonucleotide	Triple helix therapeutics
29	Self splicing RNA molecules are known as _____.	Exons	Introns	Ribozymes	Ribosomes	Ribozymes
30	*Agrobacterium tumefaciens* infect_____family.	Roseaceae	Solanaceae	Liliaceae	Malvaeceae	Roseaceae
31	The portion of Ti plasmid transferred to the chromosomal DNA of infected cells is called as _____.	vir gene	T-DNA	Ori c	Tra gene	T-DNA
32	Substance released by plants in response to wounding and can induce vir genes of Ti plasmid are _____.	Opines	Acetosyringone	Nopaline	Auxin	Acetosyringone
33	The genes which help in transfer of T-DNA ____.	Tra genes	vir genes	Occ genes	Noc gene	vir genes
34	Genes of Ti plasmid that helps in auxin biosynthesis is _____.	iaaH	iaaM	ipt	both a amd b	both a amd b
35	The vir gene responsible for topoisomerase activity ____.	vir D	vir E	vir B	vir G	vir D
36	The genes which produces toxic crystals in *Bacillus thuringenesis* is _____.	Vir genes	Cry genes	Nif genes	Tra genes	Cry genes
37	The toxic crystals in *B. thuringenesis* kills the insect by _____.	Causing pores in Epithelial cells	By blocking respiration	By inhibition of metabolic Enzymes	By inhibiting Production of eggs	Causing pores in epithelial Cells
38	Genes coding for _____ are integrated into plants for preventing fruit ripening and softening during transport.	Poly Galacturonase	Pectinase	Ethylene	Auxin	Poly galacturonase
39	The gene coding for _____is used to produce phosphinothricin tolerant plants.	Phosphinothricin	Phosphinothricin acetyl Transferase	Phosphinothricin butyl Transferase	Phosphino acetyl Transferase	Phosphinothricin acetyl Transferase

40	T7 promoter is derived from the _____ phage.	λ phage	T7 phage	M13 Phage	pUC phage	T7 phage
41	The insoluble recombinant protein aggregates are known as _____.	Inclusion bodies	Fusion proteins	Affinity proteins	Protein clot	Inclusion bodies
42	A strong promoter results _____ level of gene expression.	Low	High	Medium	very low	High
43	Which of the following is used yeast as host?	*Saccharomyces cerivisiae*	*E. Coli*	*Bacillus subtilis*	*Aspergillus niger*	*Saccharomyces cerivisiae*
44	Selectable marker in this is _____.	Antibiotic	DNA	Protein	RNA	DNA
45	During high levels of expression recombinant proteins will sometimes form insoluble aggregates _____.	Inclusion bodies	Perfusion bodies	Foreign bodies	Macro bodies	Inclusion bodies
46	The fusion combination of Tac promoter is _____.	Trp and Hfr	Trp and lac	T7 and lac	Lac and ypl	T7 and lac
47	The shuttle vectors contain _____.	Origin of replication of	Origin of replication of eukaryote	Origin of replication of bacteria and eukaryote	Promoter	Origin of replication of bacteria and eukaryote
48	Expression system for recombinant insulin is _____.	Bacterial expression system	Plant expression system	Insect expression system	Mammalian expression system	Bacterial expression system
49	Which of the following is molecular marker?	RFLP	RFPD	ALLP	SMP	RFLP
50	Restriction DNA fragment lengths variation between individuals of a species is called _____.	Restriction fragment length	Random amplified polymorphic DNA	Amplified fragment length polymorphisms	Simple sequence repeats	Restriction fragment length polymorphism
51	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
52	Quantitative genes location on chromosomes is called _____.	Qualitative trait	Quantitative trait loci	Quantum trait loci	Parallel gene	Qualitative trait loci
53	First step of PCR is _____.	Annealing	Renaturation	Denaturation	Ligation	Denaturation

54	Technique used in DNA fingerprinting is _____.	Western blotting	Southern blotting	Variable blotting	Eastern blotting	Southern blotting
55	In blotting, labeled antibody are used to detect _____.	DNA	RNA	Protein	Lipid	Protein
56	_____ technique doesn't involve electrophoresis for the separation of biomolecules.	Dot blotting	Southern blotting	Northern blotting	Western blotting	Dot blotting
57	Molecular Probe is a _____.	Protein	Short piece of labeled DNA	Short piece of labeled protein	Enzyme	Short piece of labeled DNA
58	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
59	Promoters used in bacterial expression vector are _____.	lac	BAX	TIC	LOCK	lac
60	The ____ gene responsible for topoisomerase activity.	vir D	vir E	vir B	vir G	vir D