Biyani's Think Tank

Concept based notes

Production of Recombinant Molecules

[B.Sc.(Biotechnology) Part-II]

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Preface

I am glad to present this book, especially designed to serve the needs of the students. The book has been written keeping in mind the general weakness in understanding the fundamental concepts of the topics. The book is self-explanatory and adopts the "Teach Yourself" style. It is based on question-answer pattern. The language of book is quite easy and understandable based on scientific approach.

Any further improvement in the contents of the book by making corrections, omission and inclusion is keen to be achieved based on suggestions from the readers for which the author shall be obliged.

I acknowledge special thanks to Mr. Rajeev Biyani, *Chairman* & Dr. Sanjay Biyani, *Director* (*Acad.*) Biyani Group of Colleges, who are the backbones and main concept provider and also have been constant source of motivation throughout this Endeavour. They played an active role in coordinating the various stages of this Endeavour and spearheaded the publishing work.

I look forward to receiving valuable suggestions from professors of various educational institutions, other faculty members and students for improvement of the quality of the book. The reader may feel free to send in their comments and suggestions to the under mentioned address.

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Tripti Agarwal

B.Sc. Part-II

PRODUCTION OF RECOMBINANT MOLECULES

Section -A

1. Introduction to gene cloning, tools and enzymes used in gene manipulation. Restriction enzymes, DNA ligases, DNA Polymerase Reverse Transcriptase, Polynucleotide kinease, End labeling and other processes used in rDNA technology
2. Major cloning vehicle and their applications-Plasmid vectors, cosmid, Phagemid, Phage vectors, Transposons.

Section -B

3. Making of genomic and DNA libraries, their screening and major application
4. Production of transgenic Microbes and their application in Biotechnology
5. Production of transgenic Animals and their application in Biotechnology
6. Production of transgenic Plant and their application in Biotechnology

Section -C

7. Requirement of recombinant molecules: in health, pharmaceutical, agriculture and industrial sectors in research laboratories, criteria of purity.
8. Rationale for the design of vectors for the over expression of recombinant proteins, selection of suitable promoter sequences, ribosome binding sites, transcription terminator, fusion protein tags, purification tags, protease cleavage sites and enzymes plasmid copy number, inducible expression system.

Section -D

9. Over expression conditions, production of inclusion bodies, solubilization insoluble proteins, Purification protocols and up scaling
10. Determination of purity and activity of over expressed protein.
11. Expeirments using model systems E.coli, Yeast, Baculovirus. Agro bacterium tumefaciens

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Section-A

Introduction to gene cloning

Q1 What is Recombinant DNA/protein "cloning"?

Ans "Recombinant DNA is an artificial DNA sequence resulting from the combining of two other DNA sequences in a plasmid."

"Recombinant proteins are proteins that are produced by different genetically modified organisms following insertion of the relevant DNA into their genome."

Cloning DNA/proteins different and much easier than "cloning" an organism

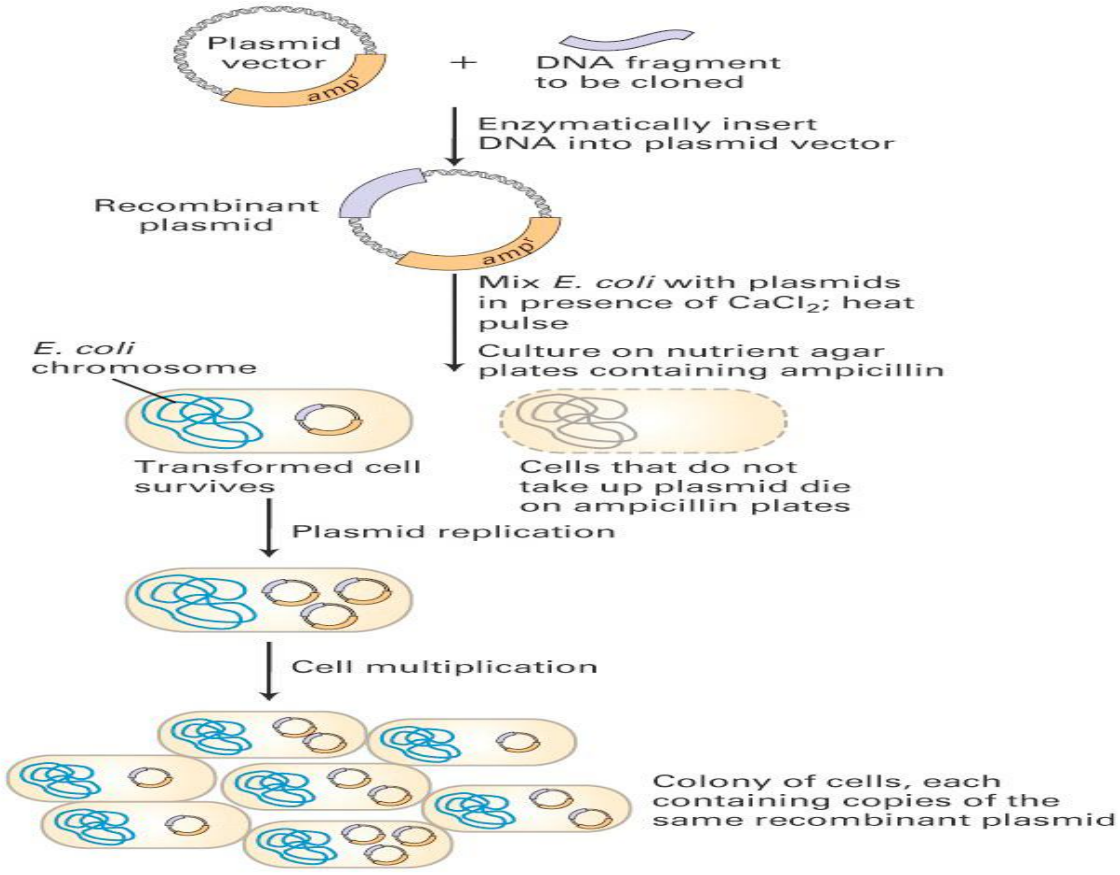


Figure 1 : Basic principle of DNA Cloning

Q.2 A restriction enzyme is a prototype, an isochizomer and a neoschizomer- what's the difference?

Ans A **prototype** is the first or a newly discovered restriction enzyme that possesses a unique recognition specificity for DNA.

Isoschizomers are restriction enzymes with the same specificity as prototypes but have been discovered subsequently.

Neoschizomers are restriction enzymes that recognize the same nucleotide sequence as their prototype but cleave at a different site. In some special applications this is a very helpful feature.

Q3 What is DNA ligase?

Ans A **ligase** (from the Latin verb *ligāre* — "to bind" or "to glue together") is an enzyme that can catalyse the joining of two large molecules by forming a new chemical bond, usually with accompanying hydrolysis of a small chemical group pendant to one of the larger molecules.

Q4) What is the function of Topoisomerase?

Ans Topoisomerase is not directly involved in replication at all; it temporarily "nicks" the sugar-phosphate backbone of one strand, allowing the ends to rotate with respect to one another to prevent excessive supercoiling of the DNA.

Q5 What is Reverse Transcription?

Ans The process by which DNA is synthesized from an RNA template by means of the enzyme reverse transcriptase.

Q6 What is a Cloning Vector? What are its general features?

Ans **"**Vector" is an agent that can carry a DNA fragment into a host cell. If it is used for reproducing the DNA fragment, it is called a "**cloning vector**". If it is used for expressing certain gene in the DNA fragment, it is called an "**expression vector**".

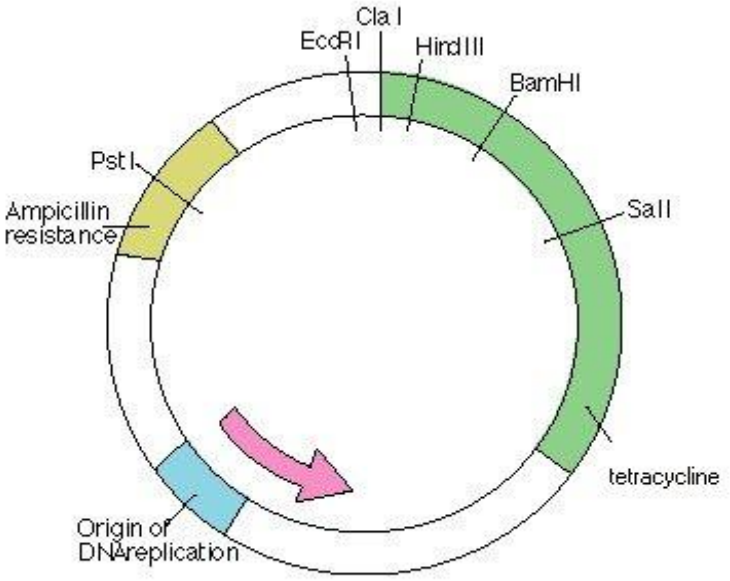
Commonly used vectors include **plasmid**, **Lambda phage**, **cosmid** and **yeast artificial chromosome (YAC)**.

General features:

- ✓ They are autonomously replicating DNA independent of host's genome.
- ✓ Easily to be isolated from the host cell
- ✓ Most are circular, some are linear
- ✓ Contains at least one selective marker, which allows host cells containing the vector to be selected amongst those which do not.
- ✓ Contains a multiple cloning site (MCS)

Q7 What is Plasmid?

Ans Plasmids are circular, double-stranded DNA molecules that exist in bacteria and in the nuclei of some eukaryotic cells. They can replicate independently of the host cell. The size of plasmids ranges from a few kb to near 100 kb.It have an origin of replication, antibiotic resistance genes as markers, and several unique restriction sites. After culture growth, the clone fragment can be recovered easily. The cells are lysed and the DNA is isolated and purified. A DNA fragment can be kept indefinitely if mixed with glycerol in a –70 degrees C freezer.



Q8 Explain how Cosmid is different from Plasmid ?

Ans The cosmid vector is a combination of the plasmid vector and the COS site which allows the target DNA to be inserted into the λ head. It has the following advantages:

- High transformation efficiency.
- The cosmid vector can carry up to 45 kb whereas plasmid and λ phage vectors are limited to 25 kb.

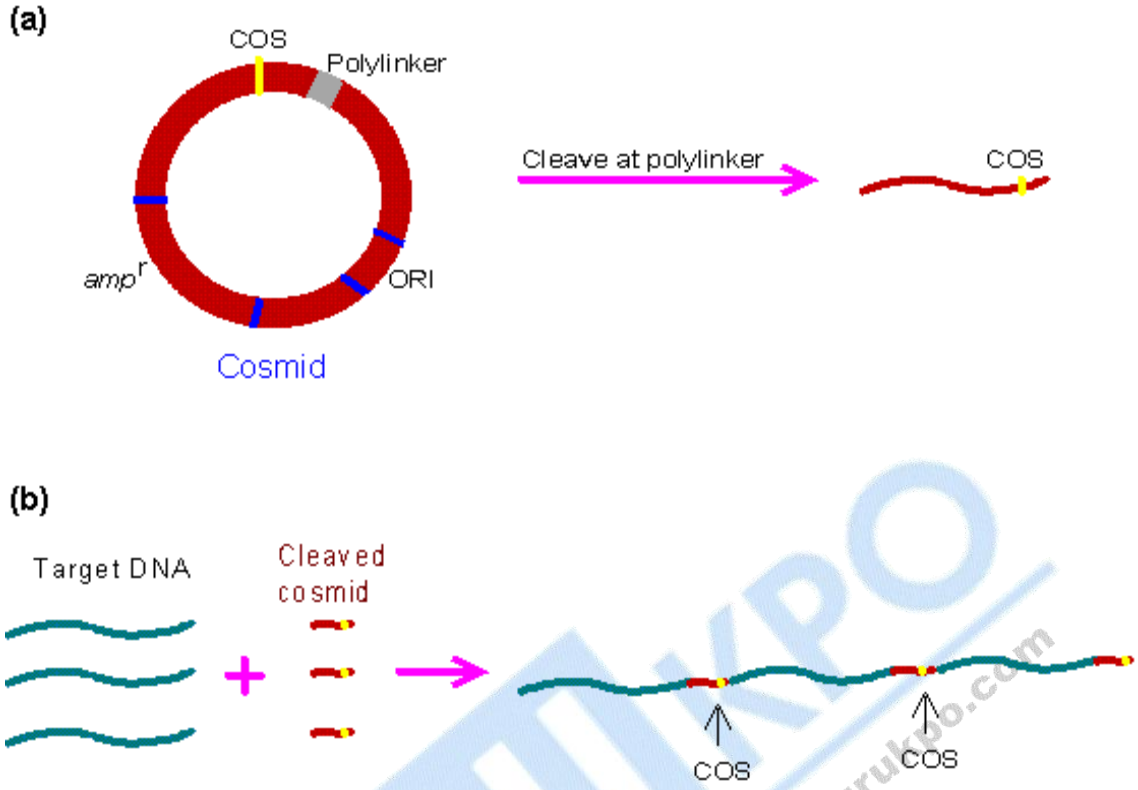


Figure 3. Cloning by using cosmid vectors.

(a) In addition to *amp*^{r}, ORI, and polylinker as in the plasmid vector, the cosmid vector also contains a COS site.

(b) After cosmid vectors are cleaved with restriction enzyme, they are ligated with DNA fragments. The subsequent assembly and transformation steps are the same as cloning with phages.

Q9 Explain diagrammatically DNA cloning using λ phages as vectors ?

Ans : λ phages are viruses that can infect bacteria. The major advantage of the phage vector is its high transformation efficiency, about 1000 times more efficient than the plasmid vector.

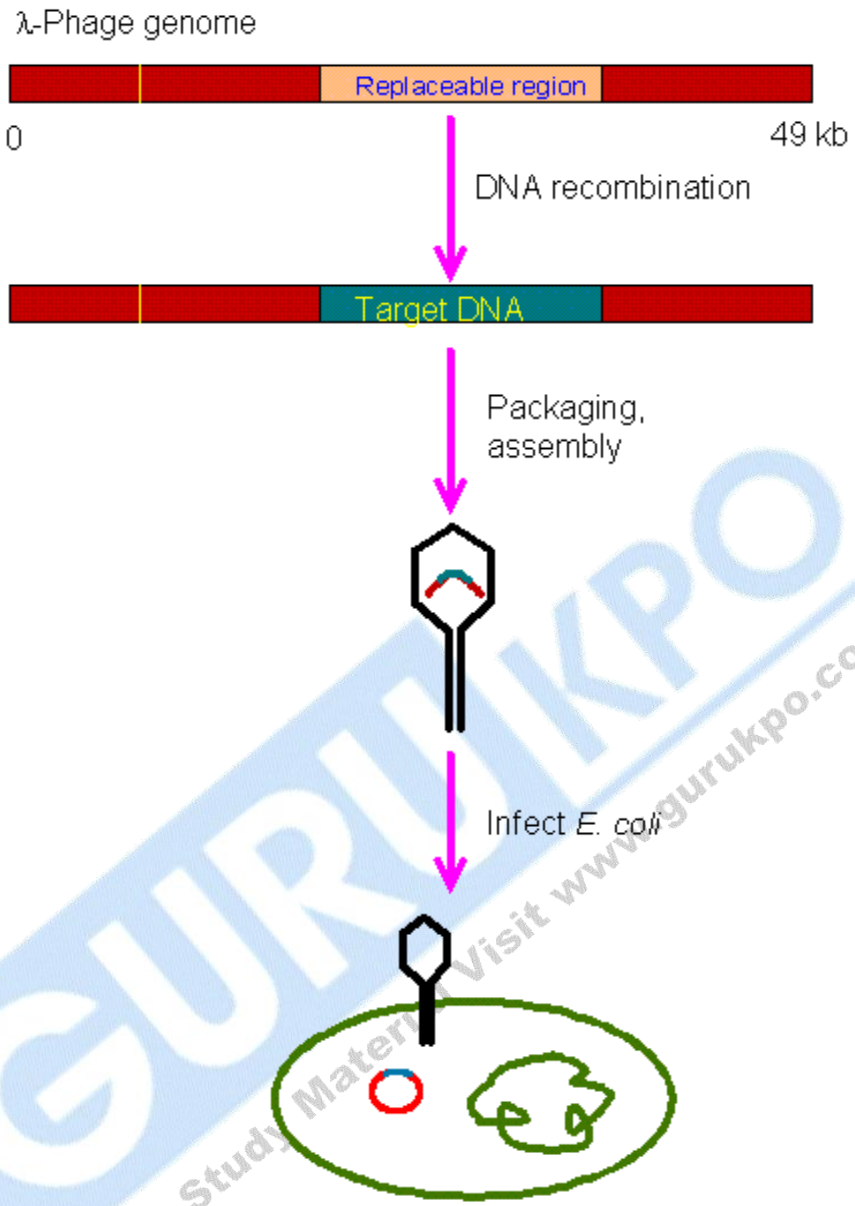


Figure 4 : Schematic drawing of the DNA cloning using λ phages as vectors.

Q 10 Give detailed account on Yeast Artificial Chromosome (YAC)?

Ans: The yeast artificial chromosome (YAC) vector is capable of carrying a large DNA fragment (up to 2 Mb), but its transformation efficiency is very low.

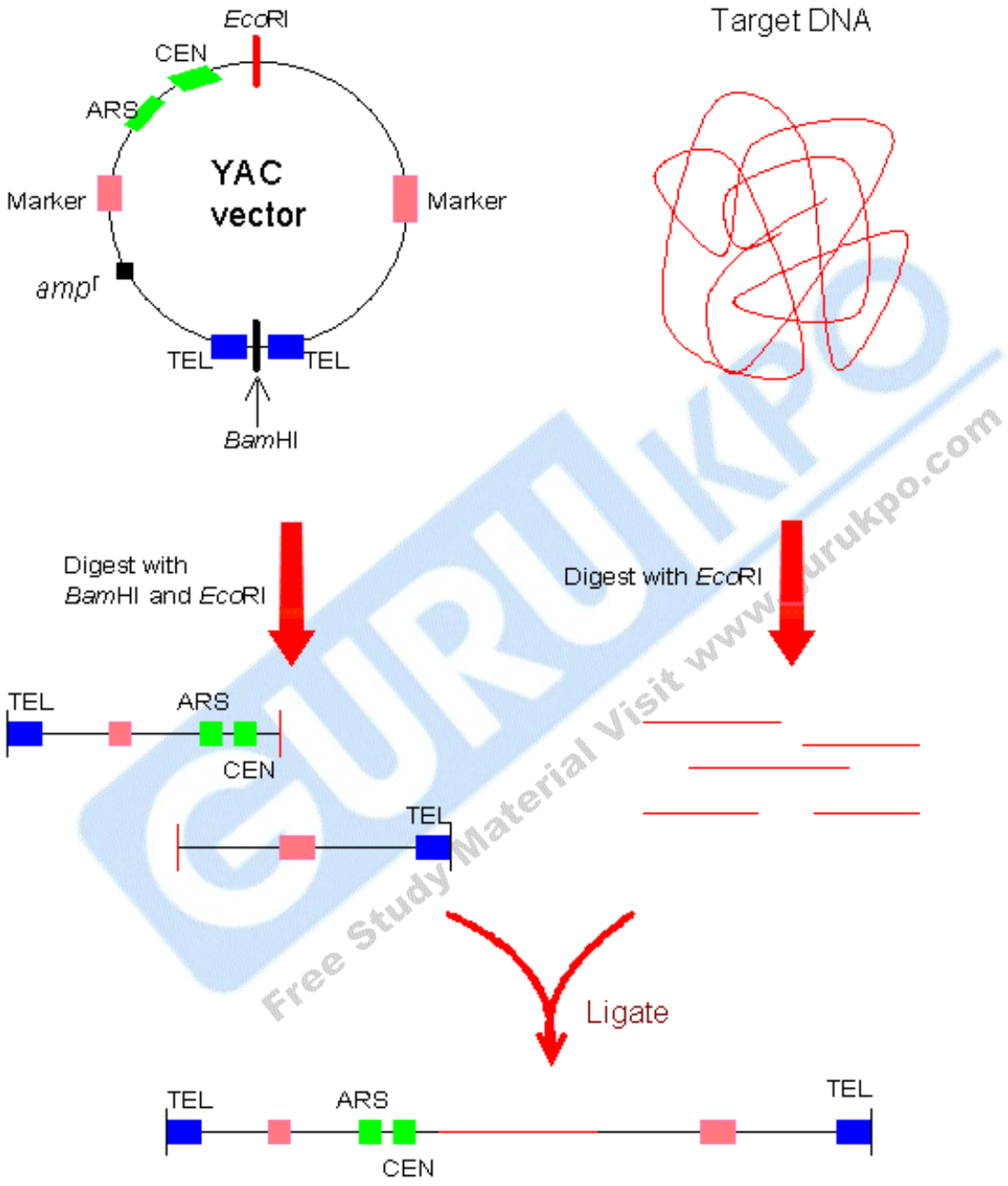


Figure 5. Cloning by the yeast artificial chromosome (YAC) vector.

Essential components of YAC vectors

- Centromers (CEN), telomeres (TEL) and autonomous replicating sequence (ARS) for proliferation in the host cell.
- *amp*^{r} for selective amplification and markers such as TRP1 and URA3 for identifying cells containing the YAC vector.
- Recognition sites of restriction enzymes (e.g., EcoRI and BamHI)

Procedure

1. The target DNA is partially digested by EcoRI and the YAC vector is cleaved by EcoRI and BamHI.
2. Ligate the cleaved vector segments with a digested DNA fragment to form an artificial chromosome.
3. Transform yeast cells to make a large number of copies

Q11) Give difference between Bacterial Artificial Chromosomes(BACs) and Yeast Artificial Chromosomes(YACs) ?

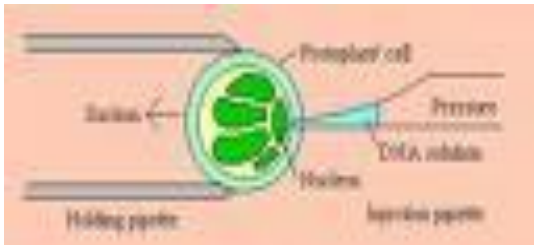
Ans:

• BACs can hold up to 300 kbs. • The F factor of E.coli is capable of handling large segments of DNA. • Recombinant BACs are introduced into E.coli by electroporation (a brief high-voltage current). Once in the cell, the rBAC replicates like an F factor. • Example: pBAC108L • Has a set of regulatory genes, OriS, and repE which control F-factor replication, and parA and parB which limit the number of copies to one or two. • A chloramphenicol resistance gene, and a cloning segment.	• YACs can hold up to 500 kbs. • YACs are designed to replicate as plasmids in bacteria when no foreign DNA is present. Once a fragment is inserted, YACs are transferred to cells, they then replicate as eukaryotic chromosomes. • YACs contain: a yeast centromere, two yeast telomeres, a bacterial origin of replication, and bacterial selectable markers. • YAC plasmid→Yeast chromosome • DNA is inserted to a unique restriction site, & cleaves the plasmid with another restriction endonuclease that removes a fragment of DNA and causes the YAC to become linear.Once in the cell,the rYAC replicates as a chromosome, also replicating the foreign DNA.

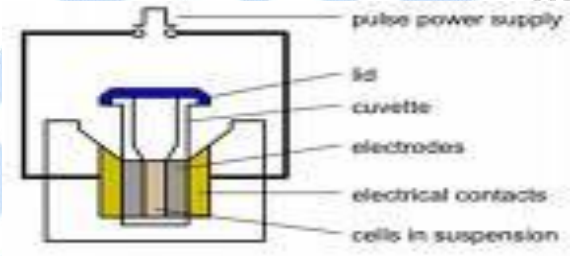
Q12 What are the methods of introduction of DNA into living cells?

Ans Several direct methods of gene transfer system have been designed :

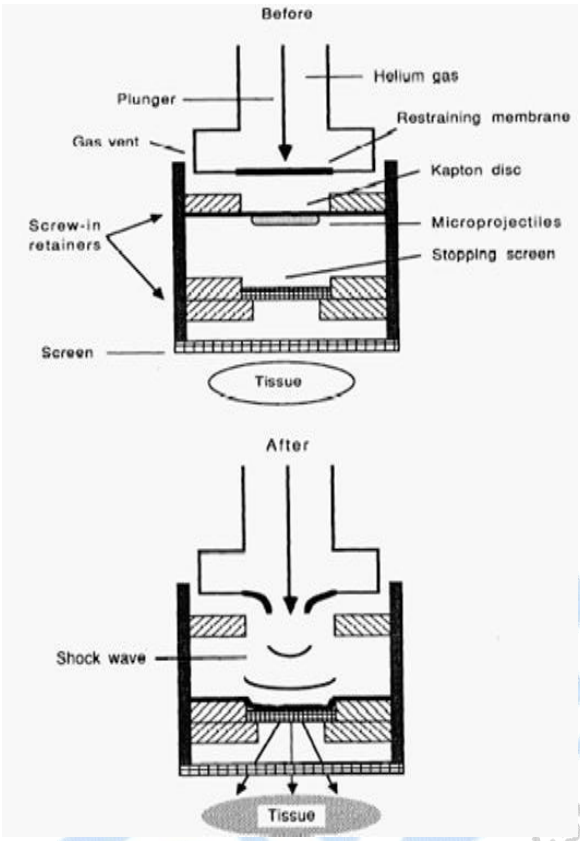
1) **Microinjection:** Microprojectile is a novel and efficient technique in which DNA is delivered directly inside the living cell. It uses microcappilaries and microscopic devices to deliver DNA into cells in such a way that the injected cell survives the treatment and is able to proliferate in culture.Two types of pipettes are used one is micropipette to deliver DNA and other is holding pipette which exerts suction pressure.



2) **Electroporation :** Electroporation facilitates cells to uptake DNA molecules by reversibly altering the permeability of cell membrane .The technique is more suitable for the transformation of plant protoplast and animal cells.A recombinant plasmid is mixed along protoplast in a container.A current of field strength 300 to 800 volts/cm is given to the protoplast following electroporation.

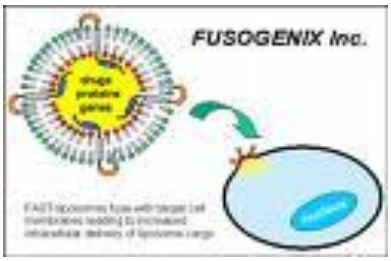


3) **Microprojectile Shot Gun Method :** This method is predominantly used for plant transformation. The technique is consist of basically two types of projectile systems,macro and microprojectile. The DNA sequence of interest is precipitated and coated on micro metals such as tungsten or gold particles of size 1-3 um.This is called as micro projectile .These DNA coated micro metals are then placed before a plastic or bullet known as macro projectile.Under high explosive discharge the high velocity micro projectile hits the tissues or cells placed next to it on the stage. Once micro projectile enters the cells,the DNA gets dissociated from the micro metals.



4) **Ultra Sonication :** DNA uptake is by directing ultrasound on the target cells .Small pieces of tissues or cells and known amount of exogenous DNA are taken in a vial, containing appropriate medium. The micro tip of sonicator is immersed in the suspension and pulses of ultrasound is delivered by sonicator.The vigorous vibration in the medium and violent collapse of bubbles generate high pressure and shock waves may result in sporadic localized rupture in the membrane and it can uptake exogenous DNA.

5) **Liposome Fusion:** Fusion of liposome have been applied to various tissues and cell cultures .In this technique it has been shown that liposome can carry small dye molecules in the cells within tissue and presuming that it can carry genes into target cells, "DNA or RNA encapsulated in liposome, can be transferred into plant protoplasm by direct fusion with plasma membrane."



6) **Micro laser :** The basic principle lying in this technique is localized rupture of cell membrane .A microlaser beam is focused into the light path of a microscope and it can be used to burn holes into cell walls and membranes .The localized rupture or burn hole region permits the entry of exogenous DNA .The extent of damage on the membrane is reversible.

Q13 Define Probe?

Ans In molecular biology, a **hybridization probe** is a fragment of DNA or RNA of variable length (usually 100-1000 bases long), which is used to detect in DNA or RNA samples the presence of nucleotide sequences (the DNA target) that are complementary to the sequence in the probe. To detect hybridization of the probe to its target sequence, the probe is tagged (or labeled) with a molecular marker; commonly used markers are ^{32}P (a radioactive isotope of phosphorus incorporated into the phosphodiester bond in the probe DNA) or Digoxigenin, which is non- radioactive antibody-based marker. DNA sequences or RNA transcripts that have moderate to high sequence similarity to the probe are then detected by visualizing the hybridized probe via autoradiography or other imaging techniques.

Q 14 Why is DNA cloning needed?

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

Q15 Explain Polymerase Chain Reaction in brief along with its advantages and disadvantages?

Ans PCR is based on DNA polymerase creating a second strand of DNA.

- Needs template DNA and two primers that flank the region to be amplified. Primers are short (generally 18-30 bases) DNA oligonucleotides complementary to the ends of the region being amplified.
- DNA polymerase adds new bases to the 3' ends of the primers to create the new second strand and go from 1 DNA to 2, then 4, 8, etc: exponential growth of DNA from this region
- A key element in PCR is a special form of DNA polymerase from *Thermus aquaticus*, a bacterium that lives in nearly boiling water in the Yellowstone National Park hot springs. This enzyme, Taq polymerase, can withstand the temperature cycle of PCR, which would otherwise kill DNA polymerase from E. coli.

Advantages:

- rapid, sensitive, lots of useful variations, robust (works even with partly degraded DNA)

Disadvantages:

- Only short regions (up to 2 kbp) can be amplified.
- limited amount of product made

PCR Cycle

- PCR is based on a cycle of 3 steps that occur at different temperatures. Each cycle doubles the number of DNA molecules :25-35 cycles produces enough DNA to see on an electrophoresis gel. Each step takes about 1 minute to complete.

1) **Denaturation:** make the DNA single stranded by heating to 94^{o}C

2) **Annealing**: hybridize the primers to the single strands. Temperature varies with primer, around 50^{o}C

3) **Extension**: build the second strands with DNA polymerase and dNTPs: 72^{o}C

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Section-B

Transgenic Microbes, Plants & Animals

Q1 What are Transgenic Animals?

Ans A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to a **structural gene**, the DNA usually includes other sequences to enable it

- to be incorporated into the DNA of the host and
- to be expressed correctly by the cells of the host.

Q2 Explain with example how transgenic mice have provided the tools for exploring many biological questions?

Ans Normal mice cannot be infected with polio virus. They lack the cell-surface molecule that, in humans, serves as the receptor for the virus. So normal mice cannot serve as an inexpensive, easily-manipulated model for studying the disease. However, transgenic mice expressing the human gene for the polio virus receptor

- can be infected by polio virus and even
- develop paralysis and other pathological changes characteristic of the disease in humans.

Q3 Explain method of milk production in sheep containing large amount of alpha1-antitrypsin?

Ans Sheep fibroblasts (connective tissue cells) growing in tissue culture were treated with a vector that contained these segments of DNA:

1. 2 regions homologous to the sheep *COL1A1* gene. This gene encodes Type 1 collagen. (Its absence in humans causes the inherited disease osteogenesis imperfecta.) This locus was chosen because fibroblasts secrete large amounts of collagen and thus one would expect the gene to be easily accessible in the chromatin.
2. A neomycin-resistance gene to aid in isolating those cells that successfully incorporated the vector.
3. The human gene encoding alpha1-antitrypsin.

Some people inherit two non- or poorly-functioning genes for this protein. Its resulting low level or absence produces the disease **Alpha1-Antitrypsin Deficiency** (**A1AD** or

Alpha1). The main symptoms are damage to the lungs (and sometimes to the liver).

4. Promoter sites from the **beta-lactoglobulin** gene. These promote hormone-driven gene expression in milk-producing cells.
5. Binding sites for ribosomes for efficient translation of the mRNAs.

Successfully-transformed cells were then

- fused with enucleated sheep eggs and
- implanted in the uterus of a ewe (female sheep).
- Several embryos survived until their birth, and two young lambs have now lived over a year.
- When treated with hormones, these two lambs secreted milk containing large amounts of alpha1-antitrypsin (650 µg/ml; 50 times higher than previous results using random insertion of the transgene).

Q4 Explain benefit of producing transgenic Chicken?

Ans Chickens:

- grow faster than sheep and goats and large numbers can be grown in close quarters;
- synthesize several grams of protein in the "white" of their eggs.

Two methods have succeeded in producing chickens carrying and expressing foreign genes.

- Infecting embryos with a viral vector carrying
  - the human gene for a therapeutic protein
  - promoter sequences that will respond to the signals for making proteins (e.g. lysozyme) in egg white.
- Transforming rooster sperm with a human gene and the appropriate promoters and checking for any transgenic offspring.

Preliminary results from both methods indicate that it may be possible for chickens to produce as much as 0.1 g of human protein in each egg that they lay.

Not only should this cost less than producing therapeutic proteins in culture vessels, but chickens will probably add the correct sugars to glycosylated proteins — something that *E. coli* cannot do.

Q5 Explain Bt Cotton in short?

Ans Bt Cotton has been genetically engineered to produce a natural insecticide that comes from a common soil bacterium, *Baccilus thuringiensis.* By using this type of cotton, that produces its own insecticide to kill insect pests, farmers spend much less on

pesticides and the environment is protected as well. The toxin made using the Bt gene is also environmentally friendly because it kills only Heliothis and closely related species.

Q 6 Explain transgenic plant as a source of bio pharmaceuticals?

Ans Plants are among the most efficient bioreactors which produce quantities of material with sunlight and soil based nutrients as inputs. Attempts are being made to replace the traditional fermentation procedure for the production of biopharmaceuticals to plant based production. The benefits of using plants are the ability to increase production at low cost by planting more acres, rather than building fermentation capacity, lower capital and operating cost, simplified downstream processing etc. Therapeutic drugs to treat cancer, infectious diseases, autoimmune diseases, cardiovascular diseases and other conditions and several vaccines can potentially be grown in plants. Plant transgenic technology is being used to produce a plant that will generate a seed that expresses a desired therapeutic protein. This seed can propagate under the right growing conditions to yield plants and seed stock for producing the desired protein. The desired protein can be extracted from the seed to make a biopharmaceutical. Plant based therapeutics are expected to be much more cost effective. For example, Dow Plant Pharmaceuticals is using corn to grow pharmaceuticals by designing and selecting the plant which will contain the active pharmaceutical within the endosperm seed compartment. Benefits of producing the pharmaceuticals in the corn include long term storage advantage, easier purification in view of limited number of soluble seed proteins in a corn seeds, low microbial load, low proteolytic activity and specialized promoters to enable expression of the protein in specific parts of the plants.

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Section-C

Requirement of recombinant molecules

Q1 Explain production of insulin using recombinant DNA technology?

Ans. Insulin is a protein hormone produced by beta cells of islets of Langerhans in the pancreas which regulates blood sugar by allowing uptake of glucose from bloodstream into body cells. Patients with diabetes have insufficient or impaired production of insulin.

It is composed of two polypeptide chains; one with 21 amino acids and the second with 30 amino acids which are linked via a disulfide bond.

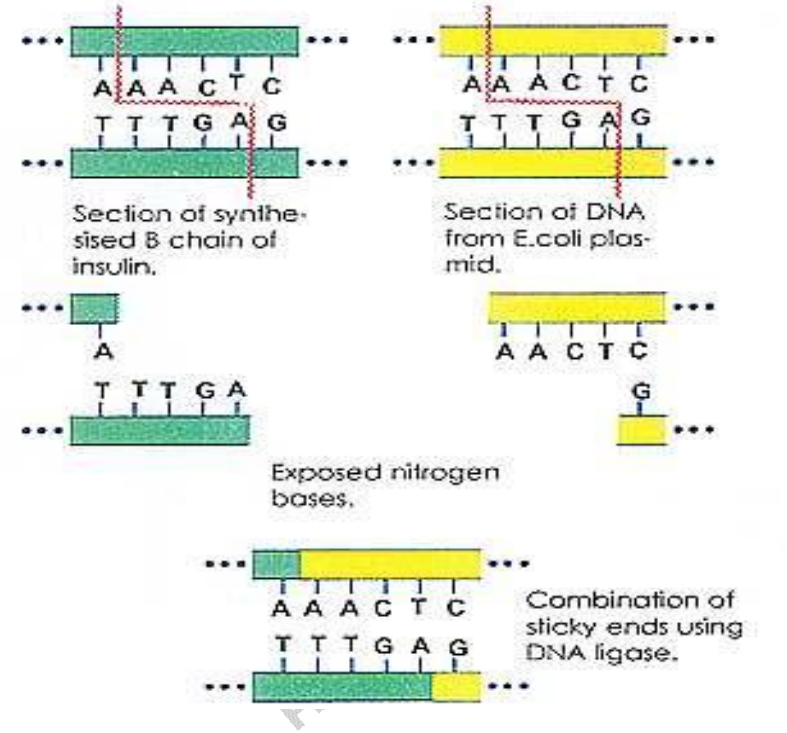

Figure 1: Restriction enzymes used to cut out insulin gene and to cut a bacterial (*E. coli*) plasmid at the same "sticky ends"

Recombinant DNA Technique :

- Insert insulin gene next to *E. coli* β -galactosidase gene which controls transcription
- Bacterial cells replicate and make copies of insulin gene
- Insulin protein is purified (β -galactosidase removed)
- Chains are mixed and disulfide bridges form
- Yeast cells provide a sterile growth medium
- Final product is Humulin - chemically identical to human insulin

Q2 Give some types of biomolecules produced through recombinant DNA technology?

Ans. Types of biomolecules produced through recombinant DNA technology are :

Recombinant Hormones
Insulin (and its analogs), growth hormone, follicle stimulating hormone, salmon calcitonin.

Blood products
Albumin, thrombolytics, fibrinolytics, and clotting factors (Factor VII, Factor IX, tissue plasminogen activator, recombinant hirudin)

Cytokines and growth factors
Interferons, interleukins and colony stimulating factors (Interferon, α, β and γ, erythropoietin, interlukin-2, GM-CSF, GCSF)

Monoclonal antibodies and related products
Mouse, chimeric or humanized; whole molecule or fragment; single chain or bispecific; and conjugated (rituximab, trastuzmab, infliximab, bevacizumab)

Recombinant Vaccines
Recombinant protein or peptides, DNA plasmid and anti-idiotype (HBsAg vaccine, HPV vaccine)

Recombinant Enzymes
Dornase– α (Pulomozyme), Acid glucosidase (Myozyme), α –L-iduronidase (Aldurazyme) and Urate Oxidase

Miscellaneous products
Bone morphogenic protein, conjugate antibody, pegylated recombinant proteins, antagonist

Q 3 Give application of Recombinant Technology in Agriculture?

Ans Application of recombinant technology is primarily for the production of transgenic plants with higher yield and nutritional values, increased resistance to stress and pests. Several commercially important transgenic crops such as maize, soybean, tomato, cotton, potato, mustard, rice etc. have been genetically modified. During the last couple of decades, considerable progress has been made to understand the function of genes, isolation of novel genes and promoters as well as the utilization of these genes for the development of transgenic crops with improved and new characters. Recombinant DNA technology has primarily helped in producing three major types of transgenic plant having improved performances. These are:

(1) Development of stress tolerant plant

(a) Plant resistant to environmental stress
(b) Herbicide Resistant plant
(c) Insect resistant plant
(d) Disease resistance plant

(2) Development of plant having improved yield

(3) Transgenic plant as a source of biopharmaceuticals

Q 4 What are edible vaccines?

Ans Crop plants offer cost-effective bioreactors to express antigens which can be used as edible vaccines. The genes encoding antigenic proteins can be isolated from the pathogens and expressed in plants and such transgenic plants or their tissues producing antigens can be eaten for vaccination/immunization (edible vaccines). The expression of such antigenic proteins in crops like banana and tomato are useful for immunization of humans since banana and tomato fruits can be eaten raw. The edible vaccines that are produced in transgenic plants have great advantages like the alleviation of storage problems, easy delivery system by feeding and low cost as compared to recombinant vaccines produced by bacterial fermentation. Vaccinating people against dreadful diseases like cholera and hepatitis B by feeding them banana/ tomato, and vaccinating animals against important diseases such as foot and mouth disease by feeding them sugar beets could be a reality in the near future.

Q 5 Give application of recombinant DNA technology in Environment?

Ans A vast majority of applications of environmental biotechnology use naturally occurring microorganisms (bacteria, fungi, etc.) to identify and filter manufacturing waste before it is introduced into the environment. Bioremediation program involving the use of microorganisms are currently in progress to clean up contaminated air, tracks of land, lakes and waterways. Recombinant technology helps in improving the efficacy of these processes so that their basic biological processes are more efficient and can degrade more complex chemicals and higher volumes of waste materials. Recombinant DNA technology also is being used in development of bioindicators where bacteria have been genetically modified as 'bioluminescors' that give off light in response to several chemical pollutants. These are being used to measure the presence of some hazardous chemicals in the environment. Other genetic sensors that can be used to detect various chemical contaminants are also undergoing trials and include sensors that can be used to track

how pollutants are naturally degrading in ground water. For example when gene such as the mercury resistance gene (mer) or the toluene degradation (tol) gene is linked to genes that code for bioluminescence within living bacterial cells, the biosensor cells can signal extremely low levels of inorganic mercury or toluene that are present in contaminated waters and soils by emitting visible light, which can be measured with fiber-optic fluro meters.

Q 6 What is genomic library?

Ans A genomic library contains clones of all the genes from a species genome. Restriction fragments of a genome can be packaged into phage using about 16 – 20 kb per fragment and its size will include the entirely of most eukaryotic genes. Once a library is established, it can be used to search for any gene of interest . A gene library is defined as "a collection of living bacterial colonies that have been transformed with different pieces of DNA from the organism that is the source of the gene of interest" The gene library then must be screened to find the colony with the gene in which the researchers are interested.

Q 7 Suppose we know the partial sequence of a gene (e.g., from the sequence of a homologous gene) and we want to determine its entire sequence, then what technique you may use?

Ans We may use genomic library that contains clones of all the genes from a species genome. Restriction fragments of a genome can be packaged into phage using about 16 – 20 kb per fragment and its size will include the entirely of most eukaryotic genes. Once a library is established, it can be used to search for any gene of interest . A gene library is defined as "a collection of living bacterial colonies that have been transformed with different pieces of DNA from the organism that is the source of the gene of interest" The gene library then must be screened to find the colony with the gene in which the researchers are interested.

Q 8 What is cDNA cloning?

Ans cDNA is the abbreviation for complementary DNA or copy DNA.A cDNA library is a set of clones representing as many as possible of the mRNAs in a given cell type at a given time. Such a library can contain tens of thousands of different clones.

It is made in following way:

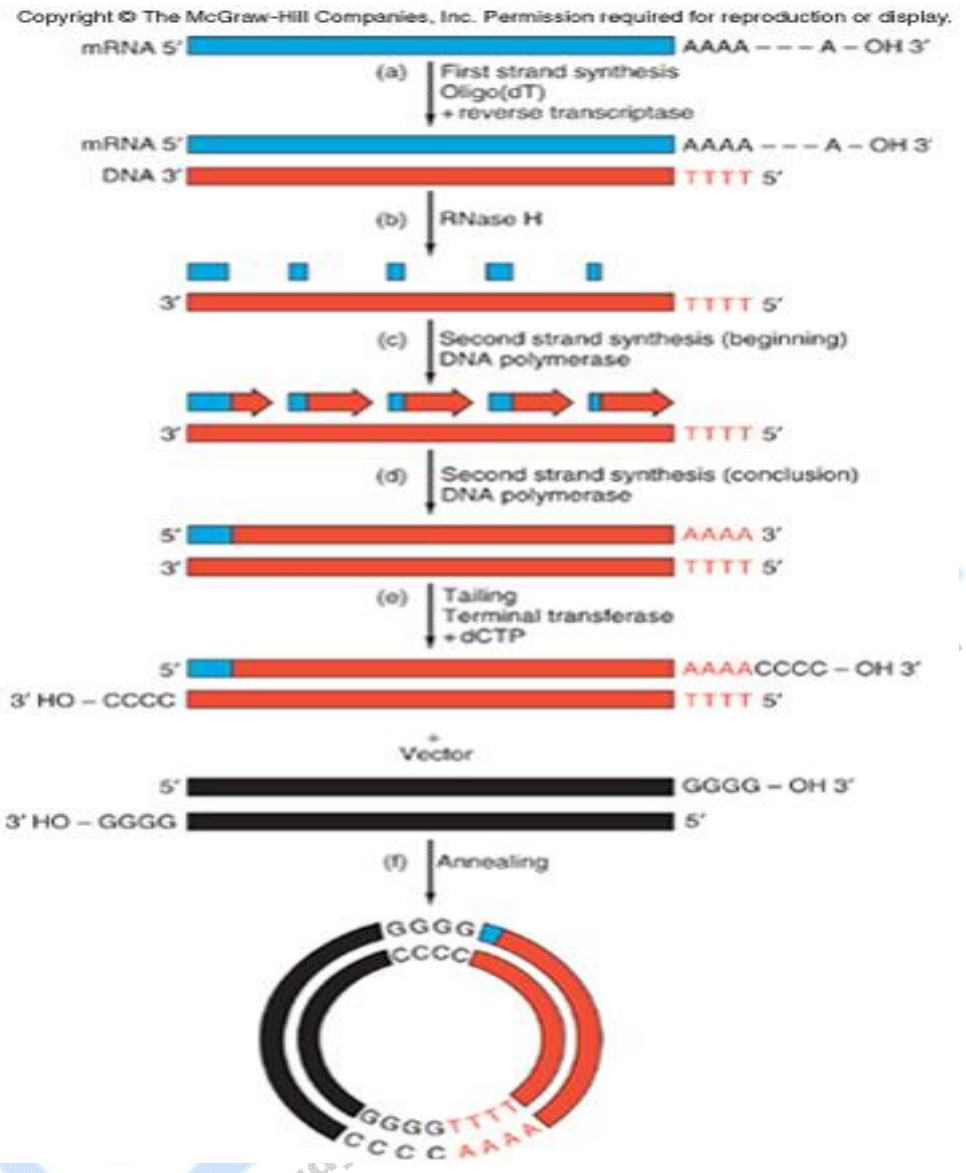

Figure 2 :Formation of cDNA library

Q 9 What are Fusion Proteins? Also give a brief account of phage display?

Ans Fusion proteins can simplify purification of recombinant proteins. Some cloning vectors, pUC and pBS, can work as expression vectors (those vectors that can yield protein products of the cloned genes) using *lac* promoter. If inserted DNA is in the same reading frame as interrupted gene, a fusion protein that results have a partial β-galactosidase sequence at amino end and inserted cDNA protein sequence at carboxyl end. Fusion protein are created because Foreign proteins in cells often degraded so by combining polypeptide with a host polypeptide can increase stability. It is created by ligating two open reading frames together in frame. Cleavage/release of host polypeptide after purification is generally necessary. Fused peptide commonly binds to a specific ligand during purification process.

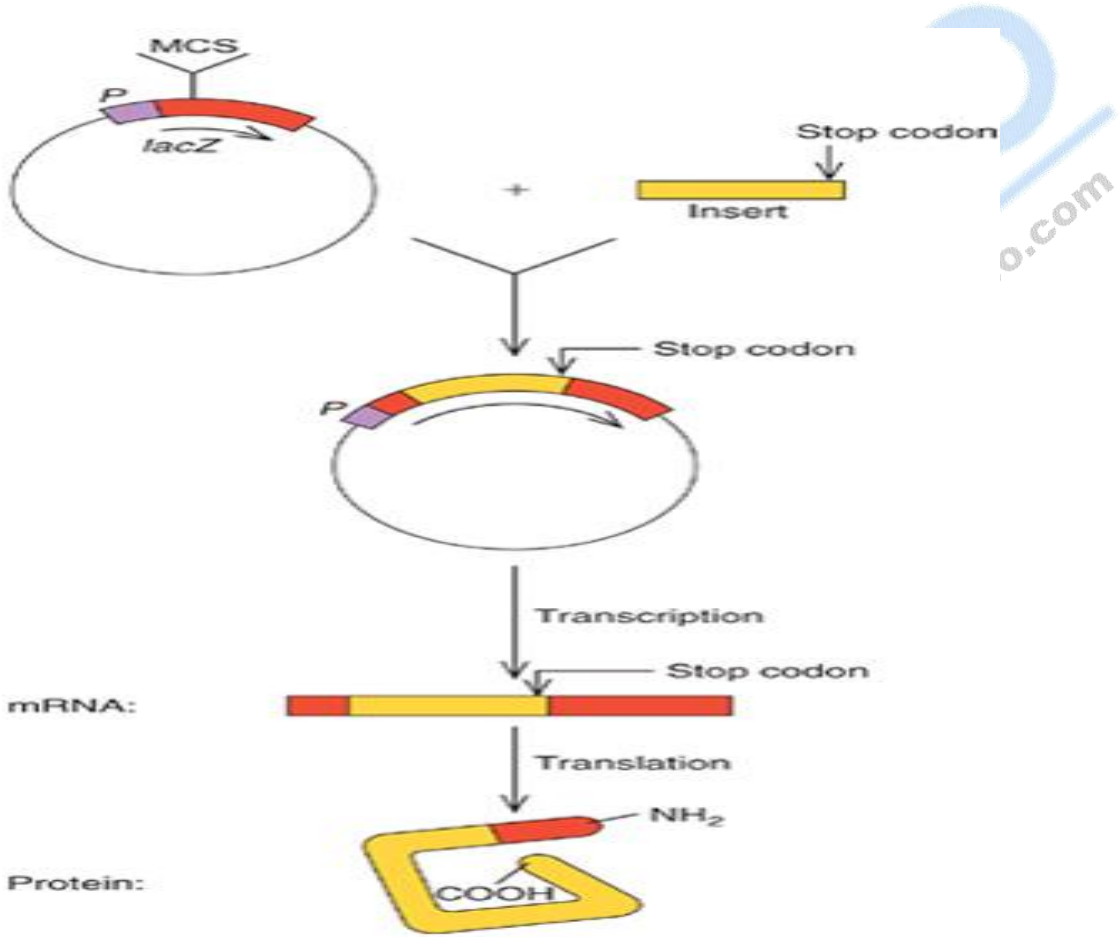

Figure 3 : Formation of Fusion Protein

Phage display is a method for the study of protein-protein, protein-peptide, and protein-DNA interactions that utilizes bacteriophage to connect proteins with the genetic information that encodes them.[1] This connection between genotype and phenotype

enables large libraries of proteins to be screened and amplified in a process called *in vitro* selection, which is analogous to natural selection. The most common bacteriophages used in phage display are M13 and fd filamentous phage,[2][3] though T4, T7, and λ phage have also been used.

Like the two-hybrid system, phage display is used for the high-throughput screening of protein interactions. In the case of M13 filamentous phage display, the DNA encoding the protein or peptide of interest is ligated into the pIII or pVIII gene. Multiple cloning sites are sometimes used to ensure that the fragments are inserted in all three possible frames so that the cDNA fragment is translated in the proper frame. The phage gene and insert DNA hybrid is then transformed into *E. coli* bacterial cells such as TG1 or XL1-Blue *E. coli*. The phage particles will not be released from the *E. coli* cells until they are infected with helper phage, which enables packaging of the phage DNA and assembly of the mature virions with the relevant protein fragment as part of their outer coat on either the minor (pIII) or major (pVIII) coat protein. The incorporation of many different DNA fragments into the pIII or pVIII genes generates a library from which members of interest can be isolated.

By immobilizing a relevant DNA or protein target(s) to the surface of a well, a phage that displays a protein that binds to one of those targets on its surface will remain while others are removed by washing. Those that remain can be eluted, used to produce more phage (by bacterial infection with helper phage) and so produce a phage mixture that is enriched with relevant (i.e. binding) phage. The repeated cycling of these steps is referred to as 'panning', in reference to the enrichment of a sample of gold by removing undesirable materials.

Phage eluted in the final step can be used to infect a suitable bacterial host, from which the phagemids can be collected and the relevant DNA sequence excised and sequenced to identify the relevant, interacting proteins or protein fragments.

Recent work published by Chasteen et al., shows that use of the helper phage can be eliminated by using a novel 'bacterial packaging cell line' technology.

General protocol

1. Target proteins or DNA sequences are immobilized to the wells of a microtiter plate.
2. Many genetic sequences are expressed in a bacteriophage library in the form of fusions with the bacteriophage coat protein, so that they are displayed on the surface of the viral particle. The protein displayed corresponds to the genetic sequence within the phage.
3. This phage-display library is added to the dish and after allowing the phage time to bind, the dish is washed.
4. Phage-displaying proteins that interact with the target molecules remain attached to the dish, while all others are washed away.

5. Attached phage may be eluted and used to create more phage by infection of suitable bacterial hosts. The new phage constitutes an enriched mixture, containing considerably less irrelevant (i.e. non-binding phage) than were present in the initial mixture.
6. The DNA within the interacting phage contains the sequences of interacting proteins, and following further bacterial-based amplification, can be sequenced to identify the relevant, interacting proteins or protein fragments.

Applications

The applications of this technology include determination of interaction partners of a protein (which would be used as the immobilized phage "bait" with a DNA library consisting of all coding sequences of a cell, tissue or organism) so that new functions or mechanisms of function of that protein may be inferred. The technique is also used to determine tumour antigens (for use in diagnosis and therapeutic targeting)[6] and in searching for protein-DNA interactions using specially-constructed DNA libraries with randomized segments.

Phage display is also a widely used method for *in vitro* protein evolution (also called protein engineering). As such, phage display is a useful tool in drug discovery. It is used for finding new ligands (enzyme inhibitors, receptor agonists and antagonists) to target proteins.. Competing methods for *in vitro* protein evolution are yeast display, bacterial display, ribosome display, and mRNA display.

Q 10 How can we produce recombinant vaccines?

Ans One of the highlights of modern medicine in the field of new generation drugs is in the production of recombinant vaccines .Vaccines produced through conventional methods are of two types inactivated vaccines (chemically or heat killed infectious organisms) and attenuated vaccines (live infectious viruses or bacteria that are not able to multiply in inoculated organism. Both these types could provide surface antigenic proteins to immune cells (lymphocytes).They respond immediately by producing antibodies and destroying infectious organisms. However these types of vaccines may continue to have contamination of organisms which are potentially dangerous .To overcome this problem recombinant vaccines can be used which are reliable and safe. Since recombinant vaccines are only antigenic proteins, devoid of genetic material is safe for therapeutic use.
Recombinant vaccines have been commercially produced for hepatitis B virus in yeast and in animal cells.

The general method for recombinant vaccine production in yeast cell is as follows :

Hepatitis B virus contains surface protein envelops enclosing small 3.2 kb DNA that has been sequenced completely .To prepare a vaccine against hepatitis B virus ,hepatitis B virus surface antigens (HBsAg) gene was cloned into high copy number vector for expression in yeast. The expression vector which contains a strong yeast promoter

of alcohol dehydrogenase was placed before HBsAg gene.A transcription terminator was placed downstream. The vector contains replication of origin and marker for both yeast and bacteria . Yeast transformed with the plasmid produced nearly 50-75 mg of the viral proteins in larger fermentor. The purification HBsAg protein was found to aggregate into particles .

The recombinant vaccines by trade name 'Shanvac' have been commercially produced by Hyderabad based company Shantha Biotech.

Sub Unit Vaccines for Foot and Mouth Disease:

Foot and mouth disease virus (FMDV) cattle are controlled to some extent by conventional vaccines that provide viral protein to immune cells for the production of antibodies.
In genetic engineering capsid viral protein cDNA was cloned in *E.coli* expression and vector expressed as fusion protein .The fusion protein containing containing viral protein fragment was able to synthesize neutralizing antibodies against FMDV.

Peptide Vaccines:

Peptide vaccines are small portion of protein (domain) which can effectively function as a subunit vaccine and are able to generate neutralizing antibody. These short peptides, that mimic may have been responsible for immunogenic responses and could be used as peptide vaccines. The classic example is the use of chemically synthesized domains of FMDV viral proteins. They were tested as potential peptide vaccine .The domain regions are located near the C and N terminal ends of viral protein. After a series of tests, a single of tests, a single inoculation with peptides of amino acids of 141 to 160 are sufficient to generate neutralizing antibody against foot and mouth disease virus. In USA the clinical trials are taken undertaken on peptide vaccine production based on epitope of plasmodium *Falciparum circumsporozite* protein.

Q 11 Name some Globally Approved Genetically Modified Plants?

Ans.

Product	**Genetically Altered Traits**
Tomato	Delayed ripening: Gene sequence for polygalaturonase production in tomato rearranged and reversed to minimise its expression by Antisense technology.
Cotton	Bt gene incorporated plants (ballworm & budworm resistant): CRY 1A c gene from Bt Kurstaki.
Soybean	Reisitant to glyphosate for control of weeds: Enolpyruvylashikimate-3-phosphate synthase gene from *Agrobacterium* sp.CP4

Potato	Bt gene incorporated (Colorado potato beetle resistant) : Cry III (A) gene from Bt. Tenebrionis.
Maize/Corn	Bt gene incorporated (resistant to comborer) : Cry 1A b gene from Bt. Kurstaki
Rapeseed / Canola	Altered oil composition (high lauric acid content): 12:0 acyl carrier protein thioesterase gene from Umbellularia californica. Resistant to glufosinate for Male sterility properties
Squash	Resistant to viruses: Coat protein genes of watermelon mosaic virus 2 and Zucchini yellow mosaic virus.
Papaya	Resistant to Papaya ring spot virus: Coat protein gene of p type of PRSV HA-5-1 from Hawai.
Chicory	Male starility resistant to glufosinate and fertility restores genes from bacteria.

Q 12 Give a short note on Recombinant Protein Production in bacterial species?

Ans: In 1973 Stanley Cohen and Herbert Boyer pioneered the use of recombinant DNA technology for cloning and expression of genes in foreign organisms. They cloned DNA from the *Salmonella typhimurium* streptomycin resistance plasmid into the *Escherichia coli* plasmid pSC101 and observed tolerance to streptomycin among the transformants .The first reported production of a human recombinant protein took place a few years later when the then newly started Biotec company Genentech announced that they had managed to express the gene encoding human somatostatin in *E. coli* .The value of the resulting bioactive substance was similar to that of somatostatin extracted from the brains of 500.000 sheep. In 1982 Genentech followed up this success with the product humulin, a recombinant insulin produced in *E. coli* and the first recombinant biotech drug to be accepted for market by the Food and Drug Administration. Today the production of recombinant proteins has become a global industry.

Recombinant protein production in gram-negative bacteria, with focus on *Escherichia coli*

Bacterial expression systems are the preferred choice for production of many prokaryotic and eukaryotic proteins. The reasons for this lie in the cost- effectiveness of bacteria, their well-characterized genetics, and the availability of many different bacterial expression systems. Among the hosts available for recombinant expression, *Escherichia coli* is an exceptional position. This stems from the many decades of intense research on its genetics as well as the broad scope of biotechnological tools available for genetic engineering of this organism. As a host for recombinant expression, *E. coli* is especially valued because of its rapid growth rate, capacity for continuous fermentation, low media costs and achievable high expression levels. One consequence of this popularity is that about 80% of all proteins used to solve three-dimensional structures submitted to the protein data bank (PDB) in 2003 were prepared in *E. coli* and during 2003 and 2006, nine out of 31 approved therapeutic proteins were produced in *E. coli*,among them important growth factors, insulins and interferons.

Q 13 Write a note on choosing and designing of suitable promoter for production of recombinant molecules?

Ans.

A promoter used for recombinant protein production should primarily give enough mRNA so that its level is not the limiting factor for the overall protein yield (i.e. it should be *strong*). Still, an increase in mRNA may not lead to more recombinant protein if other parts are limiting, e.g. if the translation machinery already works at maximum capacity. Secondly, the promoter should be controllable since constitutive promoters will not allow efficient production of toxic proteins and even some native proteins which are deleterious to the cell when over -expressed. One example is membrane proteins which when overproduced may cause cell death possibly by jamming the inner membrane .Thirdly, promoters used for recombinant expression should have a low basal transcription rate (i.e. they should have *low leakage*). The low leakage reduces pre-induction strain on the host from the metabolic burden of recombinant protein production and expression of host-toxic proteins. Some of the most commonly used promoters for recombinant protein production in *E. coli* are presented in Table 2. A few key promoters will be discussed with emphasis on how they have been optimized through rational engineering for maximized protein production.

One of the earliest operons to be studied in detail was the *E. coli* lactose utilization operon (the *lac* operon), and the classic model of gene regulation by Jacob and Monod was based on its properties (Jacob and Monod 1961). Hence, the *lac* operator with its repressor, LacI, were quickly adopted for use in recombinant expression (Polisky Bonehi et al. 1998).

Section D

Experiments using model systems

E.coli, Yeast, Baculovirus.

Agro bacterium tumefaciens

Q 1 What are the shortcomings of Bacterial Expression System?

Ans There are problems with expression of eukaryotic proteins in a bacterial system :

- Bacteria may recognize the proteins as foreign and destroy them.
- Posttranslational modifications are different in bacteria.
- Bacterial environment may not permit correct protein folding

> Very high levels of cloned eukaryotic proteins can be expressed in useless, insoluble form.

Q 2 Give use of Baculovirus As Expression Vector?

Ans Viruses in this class have a large circular DNA genome, 130 kb.

- Major viral structural protein is made in huge amounts in infected cells :
  - Promoter for this protein, polyhedrin, is very active
  - These vectors can produce up to 0.5 g of protein per liter of medium
  - Nonrecombinant viral DNA entering cells cannot result in infectious virus as it lacks an essential gene supplied by the vector.

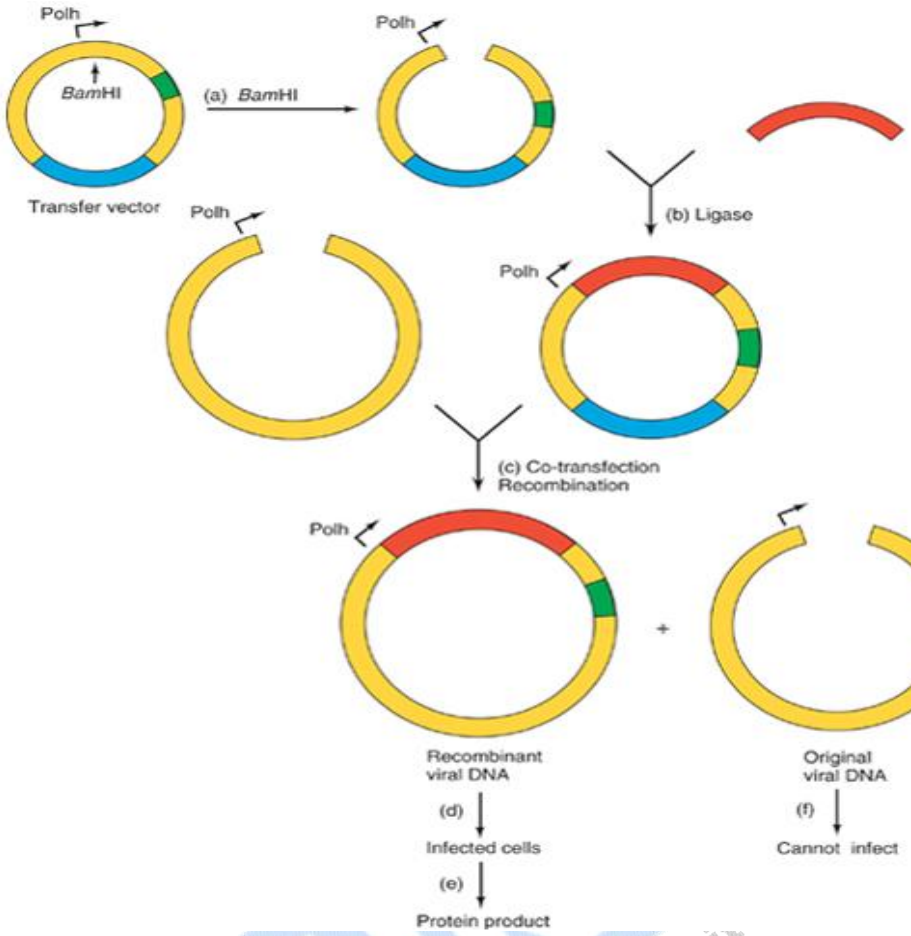

Figure 1

Q 3 Enumerate use of Ti Plasmid to Transfer Genes to Plants ?

Ans : Genes can be introduced into plants with vectors that can replicate in plant cells

- Common bacterial vector promoters and replication origins are not recognized by plant cells
- Plasmids are used containing T-DNA
  - T-DNA is derived from a plasmid known as tumor-inducing (Ti)
  - Ti plasmid comes from bacteria that cause plant tumors called crown galls.

Ti Plasmid Infection :

- Bacterium infects plant, transfers Ti plasmid to host cells.
- T-DNA integrates into the plant DNA causing abnormal proliferation of plant cells.
- T-DNA genes direct the synthesis of unusual organic acids, opines which can serve as an energy source to the infecting bacteria but are useless to the plant.

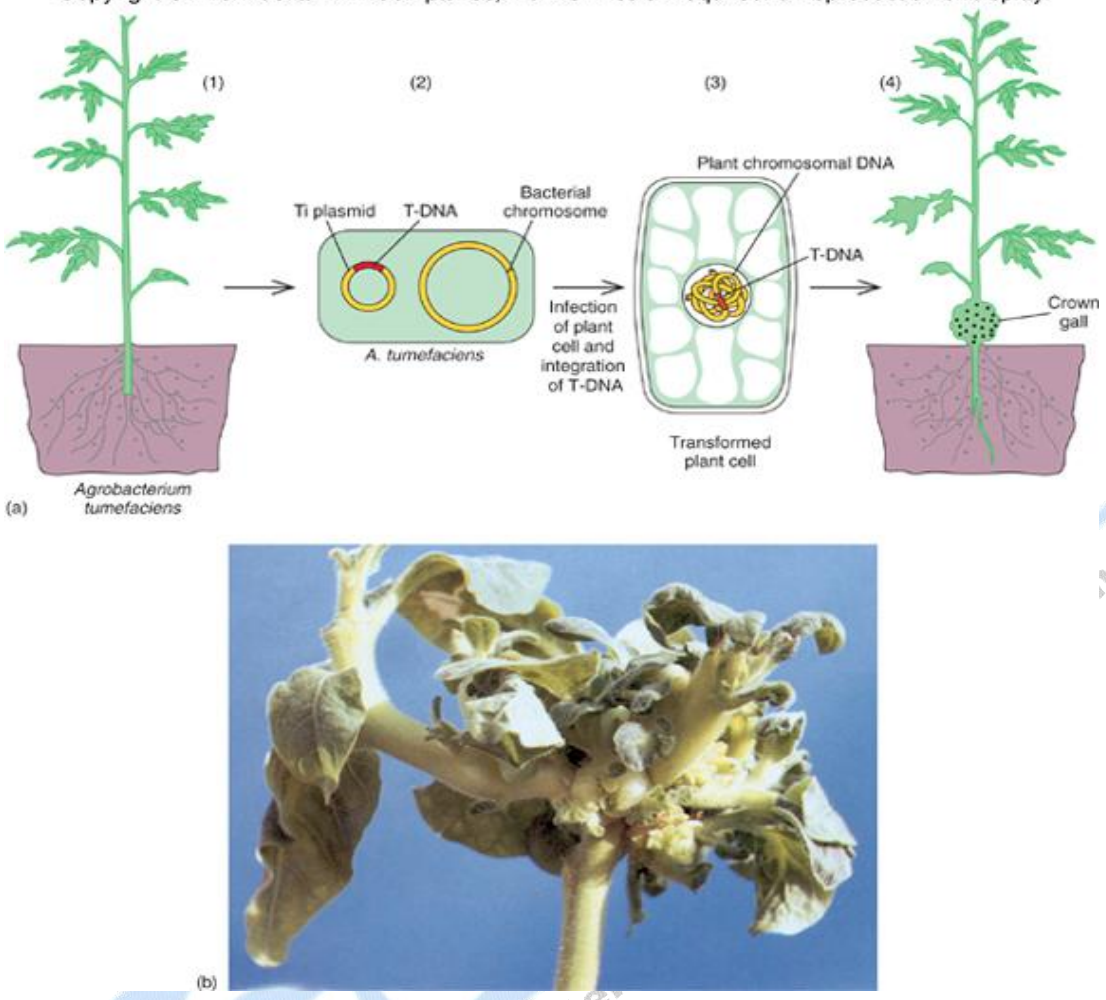

Figure 2 : **Ti Plasmid Infection**

Q 4 Why microbial cells are used for the production of human proteins?

Ans Microbial cells can be grown to large numbers at a relatively low cost and mammalian cells are expensive to culture

Level of expression of recombinant protein in microbial cells can be very high whereas expression of endogenous and foreign proteins in mammalian cells may be low.

Q5 Why it is needed to design a expression vector for the production of sufficient amount of foreign proteins?

Ans In gene cloning strategy if foreign gene is ligated into the vector and cloned in *E.coli* because it may not produce sufficient amount of foreign proteins. The signal system surrounded the gene can play a decisive role in optimizing gene expression. There are three important signals for *E.coli* genes.

a) The "promoter" which initiate transcription by facilitating binding of sigma subunit of RNA polymerase.
b) The "terminator" which makes the point at the end of the gene where transcription terminates .A terminator is usually a nucleotide sequence that can base pair with itself o form a stem loop structure.
c) The ribosome binding site, it is a short nucleotide sequence recognized by ribosome as the point at which it can attach to the mRNA molecule.

The genes of higher organisms are surrounded by several expression signals .Their nucleotide sequences are distinguished remarkably from prokaryotic sequence. If a foreign gene is inserted in *E.coli* .It is unlikely that bacterial RNA polymerase would recognize human promoter and initiate transcription. To overcome this problem foreign gene can be cloned in bacteria and placed under the control of bacterial promoter and termination signal. The cloning vehicle which provides three signals can be efficiently used in the production of recombinant protein are called expression vector.

In order to optimize gene expression for the production of sufficient amount of recombinant proteins .It is essential to place strong promoters before a foreign gene. Strong promoters are those that can sustain high rate of transcription and control genes whose proteins are required by large amounts by the cell .So expression vector should carry strong promoter to transcript at higher rate.

Another important factor that govern optimization of gene regulation is induction and repression .Any inducible gene is one whose gene is switched on by the addition of chemical to the growth media .In contrast a repressible gene is switched off by the addition of regulatory chemical.

Q 6 What is Site-directed Mutagenesis?

Ans Site-directed mutagenesis has been widely used in the study of protein functions. There are many approaches. This is an oligonucleotide-based method is illustrated diagrammatically below. This method was first developed by Michael Smith who was awarded a Nobel Prize in 1993 for this contribution. Site-directed mutagenesis can also be achieved by using PCR.

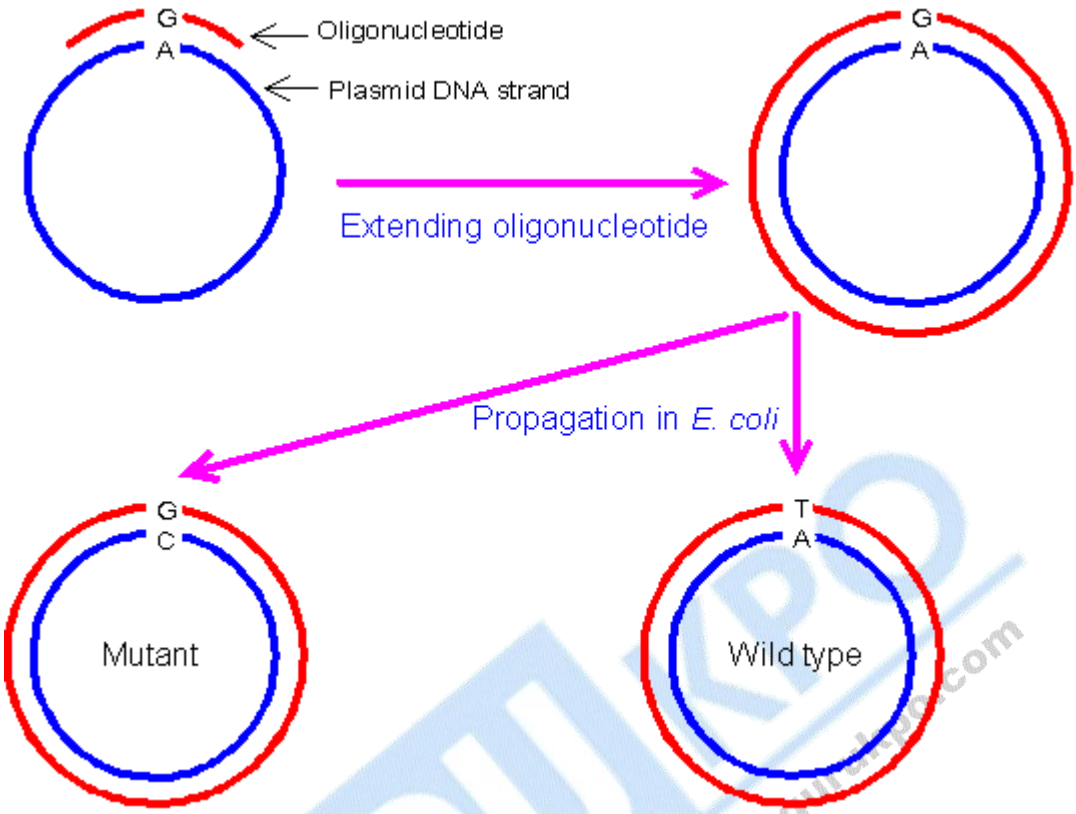

Figure 3. Illustration of the basic steps in a site-directed mutagenesis method.

(1) Cloning the DNA of interest into a plasmid vector.

(2) The plasmid DNA is denatured to produce single strands.

(3) A synthetic oligonucleotide with desired mutation (point mutation, deletion, or insertion) is annealed to the target region. In this figure, the T to G point mutation is used as an example.

(4) Extending the mutant oligonucleotide using a plasmid DNA strand as the template.
(5) The heteroduplex is propagated by transformation in *E. coli*.

After propagation, in theory, about 50% of the produced heteroduplexes will be mutants and the other 50% will be the "wild type" (no mutation). In commercial mutagenesis kits, some selection and enrichment methods have been used to favor the production of mutants.

Q.7 Write a short note on expression and purification of recombinant proteins?

Ans Recombinant proteins are expressed in two ways intracellular substances and extracellular substances. Intracellular substances include insoluble proteins and soluble proteins (cytoplasmic and periplasmic).

Primary techniques for extracellular protein purification are :

- Precipitation - pH and salt addition
- Ion exchange chromatography
- Hydrophobic interaction chromatography
- Gel filtration Chromatography
- Affinity Chromatography

Steps to purify Intracellular Insoluble Proteins (as Inclusion Bodies):
Dense aggregates of mainly the desired protein can be purified by cell lysis like enzymatic treatment, Sonication, Dynomill, Freeze thaw. Sometimes simple purification by centrifugation is also used. But when expressed protein is soluble it is extracted by simple lysis method (Lyse the cell then centrifuge the sample to collect the supernatant which can be further purified by Ion Exchange and Gel Filtration Chromatography.

□□□

Multiple Choice Questions

1. What enables scientists to take nucleotide fragments from other DNA and reassemble fragments into a new nucleotide sequence?
(a) Enzyme DNA technology
(b) Enzyme technology
(c) Recombinant DNA technology
(d) Recombinant enzyme technology

2. What is used to cut DNA double-helix strand DNA along the exterior of the strand?
(a) Overhang
(b) Restriction enzymes
(c) Restriction fragment
(d) Recognition sequence

3. What is the particular nucleotide sequence of a double-helical segment called?
(a) Overhang
(b) Restriction enzymes
(c) Restriction fragment
(d) Recognition sequence

4. What is another name for a restriction enzyme?
(a) Vector
(b) Plasmid
(c) Restriction endonucleases
(d) Agarose gel

5. Genetic engineering manipulates gene products at the level of:
A. The protein
B. Amino acids
C. DNA
D. RNA
E. Enzymes

6. A molecule that consists of a piece of DNA from one organism combined with the DNA from a member of another species is called:
A. Restricted DNA
B. Recombinant DNA
C. Transgenic DNA
D. Bioengineered DNA
E. Mutant DNA

7. In 1975, scientists convened in Asilomar, California and:
   A. Determined that restriction enzymes could cut DNA
   B. Created the first transgenic animals
   C. Reviewed the use of drugs produced by recombinant DNA technology
   D. Drew up guidelines to regulate recombinant DNA technology
   E. Developed PCR for amplifying DNA

8. Proteins isolated from bacteria that catalyze specific cleavage of DNA are:
A. Restriction enzymes
B. Bacteriophage enzymes
C. Plasmids
D. Methylating enzymes
E. Telomerases

8. Restriction enzymes cut DNA at:
   A. The sequence CTGGTC only
   B. A site specific for each enzyme
   C. Specific short methylated sequences
   D. Sites that are 10 bases apart

10. A small, circular DNA molecule used as a vector to transmit foreign DNA is a:
A. Plasmid
B. Prion
C. Liposome
D. Lipofectin
E. Ring chromosome

11. Bacteriophages can be used as vectors in recombinant DNA experiments because they:
A. Are small and made of double-stranded DNA
B. Are circular and easily imported into bacteria or yeast
C. Insert their genetic material into bacteria
D. Are resistant to protective restriction systems
E. Infect human and animal cells

12. A piece of foreign DNA was inserted into a plasmid with an antibiotic resistance gene and a lac Z gene. The plasmid DNA was cut with a restriction enzyme, which splits the lac Z gene and opens the circle. The foreign DNA was next inserted into the open restriction site of the plasmid. When the recombinant plasmid was introduced into bacterial cells and grown in the presence of antibiotic, some of the colonies turned blue in the presence of X-gal. The blue colonies contained:
A. Plasmid only
B. Foreign DNA only
C. Both foreign DNA and plasmid
D. Neither plasmid nor foreign DNA

13. In the above problem, colonies that were white in the presence of X-gal contained:
A. Plasmid DNA only
B. Foreign DNA only
C. Both foreign DNA and plasmid
D. Neither plasmid nor foreign DNA

14. The first drug produced using recombinant DNA technology was:
A. Insulin
B. Streptokinase
C. TPA
D. EPO
E. Telomerase

15. A drug produced using recombinant DNA technology that is used illegally by athletes is:
A. Insulin
B. Streptokinase
C. TPA
D. EPO
E. Telomerase

16. A multicellular organism that carries a specific genetic change in each cell because of an intervention at the fertilized egg stage is a:
A. Transversion
B. Transition
C. Transgenic
D. Transformant
E. Mosaic

17. Tiny fat bubbles used to deliver genes are:
A. Electropores
B. Phospholipids
C. Cholesterols
D. Liposomes
E. Plasmids

18. Which of these uses microscopic needles to inject DNA into cells (microinjection)?
A. Electrophoresis
B. Microinjection
C. Particle bombardment
D. Electroporation
E. Bacteriophage bombardment

19. To create a transgenic organism:
A. Introduce foreign DNA into a gamete or fertilized ovum
B. Inject a gene of interest into a somatic cell
C. Inject a gene of interest into several somatic cells
D. Introduce foreign DNA into somatic cells in culture and transplant them
E. Use site directed mutagenesis on the adult

20. Which of the following are used to introduce DNA into animal cells?
A. Liposomes
B. Electroporation
C. Microinjection
D. Particle bombardment
E. All of these

21. Which of these would not be used to introduce DNA in animal cells?
A. Liposomes
B. Electroporation
C. Microinjection
D. Particle bombardment
E. Ti plasmid

22. Transgenic organisms carry the transgene in:
A. Every cell
B. Gametes only
C. Somatic cells only
D. The cell that originally gets it
E. Viral cultures

23. DNA that is not associated with protein is called:
A. Recombinant DNA
B. Naked DNA
C. CDNA
D. Digested DNA
E. SiRNA
24. The Ti plasmid is used to genetically engineer:
A. Bacteria
B. Plants
C. Fungi
D. Animals
E. Pigs

25. Transgenic pharming involves using _____ to produce recombinant proteins in _____.
A. Cows; milk
B. Phage; bacteria
C. Bacteria; culture media
D. Fungi; culture media
E. Viruses; cell culture

26. Bt toxin producing plants are resistant to:
A. Fungal pathogens
B. Bacterial pathogens
C. Herbicides
D. Insect pests
E. Bacteriophage

27. Transcription-mediated amplification copies target RNA sequences into DNA which is then amplified using DNA polymerase and temperature shifts.
A. True
B. False

28. The polymerase chain reaction is used to:
A. Create millions of copies of an interesting piece of DNA
B. Speed the rate of DNA replication in cells
C. Make more copies of DNA polymerase
D. Copy protein into RNA
E. Make RNA in the cell nucleus

29. Automated PCR machines use a heat stable _____.
A. DNA ligase
B. Helicase
C. Primase
D. DNA polymerase
E. Amino acid synthetase

30. Which technique would be used to amplify viral RNA in a patient's blood specimen?
A. Antisense engineering
B. A knockout gene
C. A knockin gene
D. Transcription-mediated amplification
E. Traditional PCR

31. Which of the following are required for PCR?
A. Knowing part of the target DNA sequence that you want to amplify
B. Primers complimentary to opposite ends of the target DNA sequence
C. An excess of the four nucleotides A, T, G and C
D. Taq1 DNA polymerase
E. All of these are required for PCR

32. Which of the following would be used to study which genes are transcribed in a particular cell line?
A. Gene expression profiling
B. DNA variation screening
C. Microarray comparative genomic hybridization
D. Transcription-mediated amplification
E. Taq1 DNA polymerase

33. A genomic library is made from mRNAs and contains only the protein encoding genes.
A. True
B. False

34. A cDNA created from a cDNA library contains introns and promoters from the gene of interest.
A. True
B. False

35. Which of the following would be used in creating a transgenic plant?
A. Cross plants expressing a transgene to produce pure breeding lines
B. Insert a gene for a useful characteristic into a cloning vector
C. Deliver a recombinant vector into plant protoplasts
D. Grow whole plants from genetically altered cells
E. All of these

36. Which of the following is an example of a cloning vector?
A. Plasmid
B. Ribosomal RNA
C. Human growth hormone
D. Mosquito

37. Recombinant DNA technology is used for all of the following except:
A. hepatitis B vaccine production using yeast cells.
B. culturing unknown organisms.
C. amplification of DNA for microbe identification.
D. human insulin production by bacterial cells.
E. insertion of genes from humans or plants into bacteria or viruses

38. The process of making multiple copies of a DNA molecule is referred to as:
A. amplification.

B. hybridization.
C. DNA fingerprinting
D. transformation.
E. protoplast fusion

39. Which of the following is not an advantage of obtaining the protein product human growth hormone by recombinant DNA technology rather than extraction from cadavers?
A. Eliminates the need to extract the protein from tissues that might harbor pathogens.
B. Purity.
C. Cost-effectiveness
D. Production of endotoxins.
E. Speed

40. Which of the following is true for restriction enzymes?
A. A different restriction enzyme must be used to open the vector DNA than to excise the gene sequence to be cloned.
B. Each restriction enzyme known is able to make a staggered cut at its recognition site.
C. A given restriction enzyme will always recognize the same DNA sequence, but it will cut differently depending on the species of origin of the DNA.
D. Restriction enzymes are useful in genetic engineering when they make staggered cuts in DNA.
E. Any restriction enzyme can cut any piece of DNA.

41. When two DNA pieces cut with the same restriction enzyme are combined, sticky ends will:

A. associate due to DNA ligase.
B. not associate.
C. associate by complementary base pairing and hydrogen bonds.
D. associate by covalent bonds.
E. associate only if they are double stranded

42. If DNA ligase was not used in the creation of a recombinant plasmid:

A. the bacterium to receive the recombinant plasmid would not be competent and thus would be unable to take up the plasmid.
B. links between adenine and thymine would not occur.
C. links between guanine and cytosine would not occur.
D. base-pairing would occur but the sugar phosphate backbone would not be connected.
E. hydrogen bonds between complementary bases could not form

43. A good cloning vector:

A. should be large.
B. should not be capable of replication.
C. should have a gene or genes that allows for selection of transformed host cells.

D. should be readily degraded in the host.
E. should not be able to be cut by more than one restriction enzyme

43. All of the following are true of the polymerase chain reaction (PCR) except:

A. Large amounts of DNA must be isolated from the source organism
B. A heat-stable DNA polymerase is used in the reaction process
C. Short pieces of DNA called primers are added to the reaction mixtures
D. An automated thermocycler is used to heat and cool the reaction samples
E. Billions of copies of a DNA sequence are made in a few hours.

44. Which of the following is not a purpose of genetic modification?

A. To remove antibiotic resistant plasmids from bacteria.
B. To create hormones such as insulin or human growth hormone
C. To create multiple copies of a gene of interest.
D. To modify the characteristics of an organism
E. To create proteins used in vaccines (e.g. hepatitis B vaccine).

45. Recombinant DNA can be introduced into a host cell by any of the following methods except:

A. microinjection.
B. electroporation.
C. protoplast fusion
D. polymerase chain reaction.
E. transformation

46. For the introduction of a genetically modified plasmid into *E. coli*:

A. microinjection must be used
B. no treatment is used as the cells as naturally competent
C. protoplast fusion must be used
D. a gene gun must be used
E. calcium chloride and heat shock can be used.

47. A gene library:

A. involves plasmid but not phage clones
B. must be made with DNA isolated from a cell lysate
C. involves phage but not plasmid clones
D. is a collection of DNA fragments from a genome
E. is created without the aid of restriction enzymes

48. The advantage of a cDNA library of eukaryotic genes compared with a genomic library is that the cDNA library:

A. contains both introns and exons
B. lacks exons
C. consists of single-stranded DNA
D. consists of RNA and DNA
E. always has the entire gene sequence

49. An ampicillin-sensitive culture of *E. coli* is transformed with a plasmid that contains the gene of interest plus an ampicillin-resistance gene. If it is then plated on an ampicillin-containing growth medium:

A. no bacteria will grow.
B. only the lactose-positive bacteria will grow
C. only the bacteria with the plasmid will grow
D. all bacteria will grow.
E. only the ampicillin-sensitive bacteria will grow

50. Assume a cloning vector contains an antibiotic resistance gene and an appropriate restriction enzyme recognition site in the lacZ site. The gene of interest, if inserted, will:

A. inactivate the antibiotic resistance gene.
B. activate the beta-galactosidase gene.
C. **inactivate the beta-galactosidase gene**.
D. activate the antibiotic resistance gene
E. have no effect of either the beta-galactosidase gene or the ampicillin resistance gene

51. In the blue-white screening procedure, bacteria that are transformed with recombinant plasmid and cultured in media containing ampicillin and X-gal will:

A. not grow in this medium.
B. produce white colonies
C. produce blue colonies
D. grow more rapidly than cells without recombinant DNA
E. produce the enzyme beta-galactosidase.

52. Which of the following is not a step in Southern blotting?

A. Transfer DNA fragments to filters.
B. Addition of heat-stable DNA polymerase
C. Separation of DNA fragments by gel electrophoresis
D. Digestion of sample DNA with restriction enzyme
E. Addition of radioactive probe made from the gene of interest

53. Which of the following is not a method used to create plants with a human gene?

A. Growth of plants from genetically modified cells

B. Use of electroporation following creation of protoplasts
C. Use of gene gun
D. Crossing two plants
E. Use of *Agrobacterium tumefaciens* and the Ti plasmid

54. For *Agrobacterium tumefaciens* to be used to introduce foreign DNA into a plant cell, that DNA must first be:

A. inserted into the Ti plasmid of *A. tumefaciens* outside the T-DNA region
B. inserted into the T-DNA region of the Ti plasmid of *A. tumefaciens*
C. isolated from the crown gall using the appropriate restriction enzyme
D. inserted in an *A. tumefaciens* plasmid other than the Ti plasmid
E. inserted into the main chromosome of *A. tumefaciens*

Key Terms

Agrobacterium tumefaciens A bacterium that causes crown gall disease in some plants. The bacterium infects a wound, and injects a short stretch of DNA into some of the cells around the wound. The DNA comes from a large plasmid - the Ti (tumour induction) plasmid - a short region of which (called T-DNA, = **t**ransferred DNA) is transferred to the plant cell, where it causes the cell to grow into a tumour-like structure. The T-DNA contains genes which *inter alia* allows the infected plant cells to make two unusual compounds, nopaline and octopine, that are characteristic of transformed cells. The cells form a gall, which hosts the bacterium. This DNA-transfer mechanism is exploited in the genetic engineering of plants. The Ti plasmid is modified so that a foreign gene is transferred into the plant cell along with or instead of the nopaline synthesis genes. When the bacterium is cultured with isolated plant cells or with wounded plant tissues, the "new" gene is injected into the cells and ends up integrated into the chromosomes of the plant.

***Agrobacterium tumefaciens*-mediated transformation** A naturally occurring process of DNA transfer from the bacterium *A. tumefaciens* to plants.

Agrobacterium A genus of bacteria that includes several plant pathogenic species, causing tumour-like symptoms. *See Agrobacterium tumefaciens;* crown gall; hairy root culture; Ri plasmid; Ti plasmid.

agrobiodiversity; agricultural biological diversity That component of biodiversity that is relevant to food and agriculture production. The term agrobiodiversity encompasses within-species, species and ecosystem diversity.

Cosmid Artificially constructed cloning vector containing the cos gene of phage lambda. Cosmids can be packaged in lambda phage particles for infection into E. coli; this permits cloning of larger DNA fragments (up to 45 kb) than can be introduced into bacterial hosts in plasmid vectors.

E. coli Common bacterium that has been studied intensively by geneticists because of its small genome size, normal lack of pathogenicity, and ease of growth in the laboratory.
Electrophoresis A method of separating large molecules (such as DNA fragments or proteins) from a mixture of similar molecules. An electric current is passed through a medium containing the mixture, and each kind of molecule travels through the medium at a different rate, depending on its electrical charge and size. Separation is based on these differences. Agarose and acrylamide gels are the media commonly used for electrophoresis of proteins and nucleic acids.
Endonuclease An enzyme that cleaves its nucleic acid substrate at internal sites in the nucleotide sequence.
Enzyme A protein catalyst which is essential to the correct functioning of biochemical reactions.

Exogenous DNA DNA originating outside an organism.
Exons The protein-coding DNA sequences of a gene. Compare introns.
Exonuclease An enzyme that cleaves nucleotides sequentially from free ends of a linear nucleic acid substrate.

Gene therapy Insertion of normal DNA directly into cells to correct a genetic defect.
Genomic library A collection of clones made from a set of randomly generated overlapping DNA fragments representing the entire genome of an organism. Compare library, arrayed library.
Plasmid Autonomously replicating, extrachromosomal circular DNA molecules, distinct from the normal bacterial genome and nonessential for cell survival under nonselective conditions. Some plasmids are capable of integrating into the host genome. A number of artificially constructed plasmids are used as cloning vectors.
Polymerase chain reaction (PCR) A method for amplifying a DNA base sequence using a heat-stable polymerase and two 20-base primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other end. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce rapid and highly specific amplification of the desired sequence. PCR also can be used to detect the existence of the defined sequence in a DNA sample.

Polymerase, DNA or RNA Enzymes that catalyse the synthesis of nucleic acids on preexisting nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.

Probe Single-stranded DNA or RNA molecules of specific base sequence, labeled either radioactively or immunologically, that are used to detect the complementary base sequence by hybridisation.

Promoter A site on DNA to which RNA polymerase will bind and initiate transcription.

Protein A large molecule composed of one or more chains of amino acids in a specific order; the order is determined by the base sequence of nucleotides in the gene coding for the protein. Proteins are required for the structure, function, and regulation of the bodys cells, tissues, and organs, and each protein has unique functions. Examples are hormones, enzymes, and antibodies.

radioactive isotope; radioisotope An unstable isotope that emits ionizing radiation.

random amplified polymorphic DNA (RAPD; pronounced 'rapid') A technique using single, short (usually 10-mer) synthetic oligonucleotide primers for PCR. The primer, whose sequence has been chosen at random, initiates replication at its complementary sites on the DNA, producing fragments up to about 2 kb long, which can be separated by electrophoresis and stained with ethidium bromide. A primer can exhibit polymorphism between individuals, and polymorphic fragments can be used as markers.

random primer method A protocol for labelling DNA *in vitro*. A sample of random oligonucleotides (6 or 14 nucleotides long) containing all possible combinations of nucleotide

sequences is hybridized to a DNA probe. Then, in the presence of a DNA polymerase and the four deoxyribonucleotides - one of which is labelled - the 3´ hydroxy ends of the hybridized oligonucleotides provide initiation sites for DNA synthesis that uses the separated strands of the probe DNA as a template. This reaction produces labelled copies of portions of the probe DNA.

recognition site A nucleotide sequence - composed typically of 4, 6 or 8 nucleotides - that is recognized by and to which a restriction endonuclease (restriction enzyme) binds. For type II restriction enzymes (those used in gene-cloning experiments) it is also the sequence within which the enzyme specifically cuts (and their corresponding enzymes methylate) the DNA, i.e., for type II enzymes, the recognition site and the target site are the same sequence. Type I enzymes bind to their recognition site and then cleave the DNA at some more or less random position outside that recognition site. *cf* restriction site.

recombinant DNA technology A set of techniques which enable one to manipulate DNA. One of the main techniques is DNA cloning (because it produces an unlimited number of copies of a particular DNA segment), and the result is sometimes called a DNA clone or gene clone (if the segment is a gene), or simply a clone. An organism manipulated using recombinant DNA techniques is called a genetically modified organism (GMO).

recombinant DNA The result of combining DNA fragments from different sources.

recombinant protein A protein whose amino acid sequence is encoded by a cloned gene.

recombinant RNA A term used to describe RNA molecules joined *in vitro* by T4 RNA ligase.

recombinant toxin A single multifunctional toxic protein that has been created by combining the coding regions of various genes.

recombinant vaccine A vaccine produced from a cloned gene.

recombinant A term used in both classical and molecular genetics.

recombination fraction; recombination frequency The proportion of gametes that have arisen from recombination between two loci. It is estimated as the number of recombinant individuals among a set of offspring of a particular mating, divided by the total number of offspring from that mating. Represented by the Greek letter theta ( ). Linkage maps are created from estimates of recombination fraction between all pair-wise combinations of loci. *See* map distance.

recombination The process of crossing over, which occurs during meiosis I. It involves breakage in the same position of each of a pair of non-sister chromatids from homologous chromosomes, followed by joining of non-sister fragments, resulting in a reciprocal exchange of DNA between non-sister chromatids within an homologous pair of chromosomes.

reconstructed cell A viable transformed cell resulting from genetic engineering.

regulator Substance regulating growth and development of cells, organs, etc.

regulatory gene A gene whose protein controls the activity of other genes or metabolic pathways.

Regulatory regions or sequences A DNA base sequence that controls gene expression. **Resolution** Degree of molecular detail on a physical map of DNA, ranging from low to high. **Restriction enzyme cutting site** A specific nucleotide sequence of DNA at which a particular restriction enzyme cuts the DNA. Some sites occur frequently in DNA (e.g., every several hundred base pairs), others much less frequently (rare-cutter; e.g., every 10,000 base pairs). **Restriction enzyme, endonuclease** A protein that recognises specific, short nucleotide sequences and cuts DNA at those sites. Bacteria contain over 400 such enzymes that recognise and cut over 100 different DNA sequences. See restriction enzyme cutting site.

relaxed plasmid A plasmid that replicates independently of the main bacterial chromosome and is present in 10-500 copies per cell. *See* plasmid.

repetitive DNA DNA sequences that are present in a genome in multiple copies, sometimes a million times or more.

replacement therapy The administration of metabolites, co-factors or hormones that are deficient as the result of a genetic disease.

replacement; gene replacement A method of substituting a cloned gene, or part of a gene, which may have been mutated *in vitro*, for the wild-type copy of the gene within the host's chromosome. *See* homogenotization.

replica plating A procedure for duplicating the bacterial colonies growing on agar medium in one Petri plate to agar medium in another Petri plate.

replication The synthesis of duplex (double-stranded) DNA by copying from a single-stranded template.

replicative form (RF) The molecular configuration of viral nucleic acid that is the template for replication in the host cell.

replicon The portion of a DNA molecule which is replicable from a single origin. Plasmids and the chromosomes of bacteria, phages and other viruses usually have a single origin of replication and, in these cases, the entire DNA molecule constitutes a single replicon. Eukaryotic chromosomes have multiple internal origins and thus contain several replicons. The word is often used in the sense of a DNA molecule capable of independent replication, e.g., "The shuttle vector pJDB219 is a replicon in both yeast and *E. coli*."

resistance factor A plasmid that confers antibiotic resistance to a bacterium.

resistance Term commonly used to describe the ability of an organism to withstand a stress, a force or an effect of a disease, or its agent or a toxic substance.

restitution nucleus A nucleus with unreduced or doubled chromosome number that results from the failure of a meiotic or mitotic division.

restriction endonuclease [enzyme] A class of endonucleases that cleaves DNA after recognizing a specific sequence, e.g., *Bam*H1 (5´GGATCC3´), *Eco*RI (5´GAATTC3´), and *Hin*dIII (5´AAGCTT3´). There are three types of restriction endonuclease enzymes:

restriction exonuclease [enzyme] A class of nucleases that degrades DNA or RNA, starting from an end either 5´ or 3´.

restriction fragment length polymorphism (RFLP) The occurrence of variation in the length of DNA fragments that are produced after cleavage with a type II restriction endonuclease. The differences in DNA lengths are due to the presence or absence of recognition site(s) for that particular restriction enzyme. RFLPs were initially detected using hybridization with DNA probes after separation of digested genomic DNA by gel electrophoresis (Southern analysis). Now they are typically detected by electrophoresis of digested PCR product.

restriction fragment A fragment of DNA produced by cleaving (digesting, cutting) a DNA molecule with one or more restriction endonucleases.

restriction map The linear array of restriction endonuclease sites on a DNA molecule. *See* mapping.

restriction nuclease A bacterial enzyme that cuts DNA at a specific site.

restriction site The specific nucleotide sequence in DNA that is recognized by a type II restriction endonuclease and within which it makes a double-stranded cut. Restriction sites usually comprise four or six base pairs that typically are palindromic (q.v.), e.g., 5´GGCC3´

retro-poson; retro-transposon A transposable element that moves via reverse transcription (i.e., from DNA to RNA to DNA) but lacks the long terminal repeat sequences.

retroviral vectors Gene transfer systems based on viruses that have RNA as their genetic material.

retrovirus A class of eukaryotic RNA viruses that can form double-stranded DNA copies of their genomes by using reverse transcription; the double-stranded forms integrate into chromosomes of an infected cell. Many naturally occurring cancers of vertebrate animals are caused by retroviruses. Also, the AIDS virus is a retrovirus.

reversal transfer Transfer of a culture from a callus-supporting medium to a shoot-inducing medium.

reverse transcriptase; RNA-dependent DNA polymerase An enzyme that uses RNA molecule as a template for the synthesis of a complementary DNA strand.

reverse transcription The synthesis of DNA on a template of RNA, accomplished by reverse transcriptase.

reversion; reverse mutation Restitution of a mutant gene to the wild-type condition, or at least to a form that gives the wild phenotype; more generally, the appearance of a trait expressed by a remote ancestor.

rhizobacterium A micro-organism whose natural habitat is near, on or in plant roots.

Rhizobium (pl: rhizobia) Prokaryote able to establish symbiotic relationship with leguminous plants, as a result of which elemental nitrogen is fixed or converted to ammonia. *See* nitrogen fixation.

rhizosphere The soil region in the immediate vicinity of growing plant roots.

Ri plasmid A class of large conjugative plasmids found in the soil bacterium *Agrobacterium rhizogenes*. Ri plasmids are responsible for hairy root disease of certain plants. A segment of the Ri plasmid is found in the genome of tumour tissue from plants with hairy root disease.

ribonuclease Any enzyme that hydrolyses RNA.

ribozyme; gene shears RNA molecule that can catalyse chemical reactions, often cutting other RNAs.

RNA polymerase An enzyme that catalyses the synthesis of RNA from a DNA template. *See* polymerase; RNA.

RNA Ribonucleic acid. An organic acid composed of repeating nucleotide units of adenine, guanine, cytosine and uracil, whose ribose components are linked by phospho-diester bonds. The information-carrying material in some viruses. More generally, a molecule derived from DNA by transcription that may carry information (messenger RNA (mRNA)), provide sub-cellular structure (ribosomal RNA (rRNA)), transport amino acids (transfer RNA (tRNA)) or facilitate the biochemical modification of itself or other RNA molecules. *See* antigen RNA; gene splicing; heterogeneous nuclear RNA (hnRNA); mRNA; ribosomal RNA; RNA polymerase; small nuclear RNA; transfer RNA.

RNase Ribonuclease. A group of enzymes that catalyse the cleavage of nucleotides in RNA.

rRNA; ribosomal RNA The RNA molecules which are essential structural and functional components of ribosomes, the organelles responsible for protein synthesis. The different rRNA molecules are known by their sedimentation (Svedberg; symbol S) values. *E. coli* ribosomes contain one 16S rRNA molecule (1541 nucleotides long) in the same (small) sub-unit and a 23S rRNA (2904 nucleotides) and a 5S rRNA (120 nucleotides) in the large sub-unit. These three rRNA molecules are synthesized as part of a large precursor molecule which also contains the sequences of a number of tRNAs. Special processing enzymes cleave this large precursor to generate the functional moieties. *See* RNA.

The two strands may be cut either opposite to one another, to create blunt ends, or in a staggered manner, giving sticky ends, depending on the enzyme involved. *See* restriction endonuclease.

Type I: Cuts non-specifically a distance greater than 1000 bp from its recognition sequence and contains both restriction and methylation activities.

Type II: Cuts at or near a short, and often palindromic (q.v.) , recognition sequence. A separate enzyme methylates the same recognition sequence. They may make the cuts in the two DNA strands exactly opposite one another and generate blunt ends, or they may make staggered cuts to generate sticky ends. The type II restriction enzymes are the ones commonly exploited in recombinant DNA technology.

Type III: Cuts 24-26 bp downstream from a short, asymmetrical recognition sequence. Requires ATP and contains both restriction and methylation activities.

Yeast artificial chromosome (YAC) A vector used to clone DNA fragments (up to 400 kb); it is constructed from the telomeric, centromeric, and replication origin sequences needed for replication in yeast cells. Compare cloning vector, cosmid.

M.Sc./B.Sc. (Part II) Examination, 2011

(Faculty of Science)

(Common to Three and Five Year Integrated Course)

BIOTECHNOLOGY

Paper BT-602

Production of Recombinant Molecules

Year-2011

Time : 3 Hours ***Max. Marks : 50***

Attempt FIVE questions in all, including Questions No. 1 which is compulsory selecting ONE question from each Section. Each question carries equal 10 marks.

1. Answer the following questions (i to x) either by choosing the correct answer or filling in the blanks:

(i) Polymerase Chain Reaction was invented by:

(a) Hamilton Smith

(b) Karry Mullis

(c) Crick and Monad

(d) Hershey and Chase

(ii) Smith and Nathans are famous for the discovery of:

(a) Gene therapy

(b) Hybridoma technology

(c) Restriction enzymes

(d) Second Generation Vaccine

(iii) VNTRs are:

(a) Variable Number of Tandem Repeats

(b) Very Narrow Tandem Repeats

(c) Variable Noncistronic transposon Repeats

(d) Valuable Noncistronic Transposic Regions

(iv) Which of the following vectors is used in E.coli?

(a) PUC_8

(b) $_pGEM_{32}$

(c) $_PBR_{322}$

(d) All the above

(v) Humulin is:

(a) Antibiotic

(b) Human Insulin

(c) Digestive enzyme

(d) A form of chitin

(vi) Bal 31 is an example of…….

(vii) Genomic library stands for……..

(viii) YEps stands for……

(ix) RFLP stands for……

(x) Determination of the order of nucleotides in a DNA molecule is known as…….. 10 x 1 =10

Section-A

2. What is Recombinant DNA Technology? Write the different enzymes used in RDT. Write the method of preparation of plasmid DNA from Bacteria.

1 + 4 + 5 = 10

3. Write short notes on:

(a) Shuttle Vectors

(b) Ti Plasmid

(c) Phagemid 3 + 4 + 3 = 10

Section-B

4. Describe in detail about different methods of studying the transcript of a cloned gene. 10

5. Write short notes on:
   (a) PCR
   (b) Northern Blotting 5 + 5 = 10

Section-C

6. Write detailed account on production of any recombinant pharmaceutical. 10
7. Write short notes on any two of the following:
   (a) Transcriptome
   (b) Promoters used in Expression Vectors
   (c) Fusion protein tags and purification tags 5 + 5 = 10

Section-D

8. Write an account on determination of purity and activity of over expressed proteins. 10

9. Write short notes on:
   (a) E.coil as experimental model system for over expression of proteins.
   (b) Production of inclusion bodies and solubilising insoluble proteins. 5 + 5 = 10

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