Semester - VI

15MBU602 MICROBIAL TECHNOLOGY AND INTELLECTUAL PROPERTY RIGHTS

5H – 5C

Instruction Hours / week: L: 5 T: 0 P: 0 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

SCOPE

This paper helps the student to study theoretical concepts of rDNA technology and their applications and Acquire knowledge on the applications of genetic engineering. It creates awareness on the Intellectual property rights and patenting of biotechnological processes.

OBJECTIVES

To learn the basic tools in recombinant technology
To understand the various concepts of cloning vectors
To learn the cloning strategies
To familiarize the students, with the principles of bioethical concepts
To emphasize on IPR issues and need for knowledge in patents in biotechnology

UNIT – I

Restriction enzymes – types and nomenclature - classification, Target site of enzymes, Host controlled restriction and modification, Star activity and uses - Nucleases, methylases - ligases. Joining of DNA fragments with vector - linkers, adaptors and homopolymer tailing.

UNIT – II

Cloning vectors: Plasmid as Cloning Vectors, Binary vectors and co-integrate vectors. Plasmids types in Gram negative bacteria - *E. coli* - PBR322. Bacteriophage λ and M13, Cosmids - phagemid, Yeast vectors, Shuttle vectors, Ti – plasmid and Ri plasmid. Prokaryotic hosts: *E. coli*, Eukaryotic hosts - yeast cells.

UNIT – III

Gene cloning - basic steps in cloning, construction of cDNA and genomic libraries-selection and screening method of recombinants. Cloning strategies – transformation techniques and transfection. Blotting techniques: Southern, Northern and Western. PCR – methods and application. Finger printing.

UNIT – IV

Patenting – fundamental requirements – patenting multicellular organisms – patenting and fundamental research. Patenting the genes, patenting biological materials and biotechnology. Discrepencies in biotechnology / chemical patenting. Patenting Process.

UNIT – V

Intellectual Property Rights – historical perspective – recent developments, IPR in India, IPR and the rights of farmers in developing countries.

TEXT BOOKS

1. Dubey, R.C., 2006. Biotechnology. S. Chand and Company Ltd. New Delhi.
2. Ramadass, P. and S. Meerarani, 2000. Text book of Animal Biotechnology. 2nd Edition. Madras Veterinary College, Chennai.
3. Kumar, H.D. 2003. Biotechnology. Affiliated East West Press Private Limited, New Delhi.

REFERENCES

1. Brown, T.A., 1998. An Introduction to Gene Cloning. 3rd Edition. Stanley Thrones Publication, Cheltheham.
2. Glick, B.K. and J.J. Pasternak, 2003. Molecular Biotechnology: Principles and Applications of Recombinant DNA 3rd Edition. ASM Press, Washington.
3. Old, R.M. and S. B. Primrose, 2003. Principles of Gene Manipulation. 6th Edition. Blackwell Scientific Publication. London.
4. Smith, J., 2003. Biotechnology. Cambridge University Press. UK.
5. Winnacker, E.L., 1987. From Genes to Clones: Introduction to Gene Technology. VCH. Weinhein, Germany.
6. Kshitiji Kumar Singh, 2015. Biotechnology and Intellectual property rights: Legal and Social Implications. Springer.
7. Peppler and Pearlman, D and H.J 2014. Microbial Technology: Microbial Processes. 2nd Edition. Academic Press Inc. UK.

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LECTURE PLAN
DEPARTMENT OF MICROBIOLOGY

STAFF NAME: Dr. A. A. ARUNKUMAR
SUBJECT NAME: MTIPR SUB.CODE:15MBU602
SEMESTER: II CLASS: III B.Sc. (MB)

S. No	Lecture Duration Period	Topics to be Covered	Support Material/Page Nos
		UNIT-I	
1	1	Restriction Enzymes	T1: 45 – 52
2	1	Types and Nomenclature	T1: 52- 53
3	1	Classification	T1: 53
4	1	Target site of Enzymes	W1, W2
5	1	Host controlled restriction and modification	W2
6	1	Star activity and uses – Nucleases, Methylases- ligases	W2
7	1	Joining of DNA fragments with vectors – linkers	T1: 63 -65
8	1	Adaptors	T1: 65 -70
9	1	Homopolymer tailing	T1: 65 -70
10	1	Revision	
11	1	Possible Questions discussions	
12	1	One mark discussion	
13	1	Class test	
	Total No of Hours Planned for Unit 1=13		
		UNIT-II	
1	1	Cloning vectors	T1: 45 – 52
2	1	Plasmid as cloning vectores	T1: 52- 53
3	1	Binary vectors and Co – intergrate vectors	T1: 53
4	1	Plasmid Types in gram negative bacteria – Ecoli, PBR322	W1, W2
5	1	Bacteriophage λ and M13	W2
6	1	Cosmids and phagemids	W2
7	1	Yeast vectors and shuttle vectors	T1: 63 -65
8	1	Ti plasmid and Ri plasmid	T1: 65 -70
9	1	Prokaryotic and eukaryotic hosts	T1: 65 -70
10	1	Class test	
11	1	Revision	
12	1	Possible Questions discussions, One-mark discussion	
	Total No of Hours Planned For Unit II = 12		

		UNIT-III	
1	1	Gene cloning	T1: 45 – 52
2	1	Basic steps in cloning	T1: 52- 53
3	1	Construction of cDNA	T1: 53
4	1	Genomic libraries	W1, W2
5	1	Selection and screening method of recombinants	W2
6	1	Cloning strategies	W2
7	1	Transformation techniques	T1: 63 -65
8	1	Blotting techniques	T1: 65 -70
9	1	PCR – methods and applications	T1: 65 -70
10	1	Finger printing	
11	1	Revision	
12	1	Possible Questions discussions, One mark discussion	
13	1	Class test	
	Total No of Hours Planned For Unit III=13		
		UNIT-IV	
1	1	Patenting- fundamentals requirements	R2: 1 – 4
2	1	Patenting multicellular organisms	R2: 37, W1
3	1	Patenting and fundamental research	W1
4	1	Patenting genes	R2: 499
5	1	Patenting biological methods and materials	R2: 499
6	1	Discrepencies in biotechnology	R2 – 484
7	1	Chemical patenting, patenting process	R2: 484
8	1	Revision	
9	1	Possible Questions discussions	
10	1	One mark discussion	
11	1	Class test	
	Total No of Hours Planned For Unit IV=11		
		UNIT-V	R1 – 10, W1, W2
1	1	Intellectual property Rights	R1- 1 – 5, W1, W2
2	1	History and Recent development	W2, W3
3	1	IPR in India	R1: 17 -108, W2
4	1	IPR and the rights of farmers in developing countries	
5	1	Revision	
6	1	Possible Questions discussions	
7	1	One mark discussion	
8	1	Class test	
9	1	Discussion of Previous ESE Question Papers.	
	Total No of Hours Planned for unit V=9		
Total No of Hours Planned = 60			

TEXT BOOKS

1. Ananthanarayanan, R., and Panicker, C.K.J., (2005). *Text book of Microbiology*. (7th ed.). Orient Longman, New Delhi.
2. Carter, J., and Saunders, V., (2007). *Virology: Principles and Applications*. (1st ed). Wiley.
3. Chakraborty, P. (2003). *A Text book of Microbiology*. (2nd ed.). New Central Book Agency (P) Ltd, Calcutta.
4. Dubey, R.C., and Maheswari, D.K., (2004). *A Text book of Microbiology*. (1st ed.). S. Chand and Company Ltd, New Delhi.
5. Pelczar, Jr. M.J., Chan, E.C.S., and Kreig, K.R., (2003). *Microbiology*. (5th ed.). Tata McGraw-Hill Publishing Company, New Delhi.

REFERENCES

1. Acheson, N.H. (2006). *Fundamentals of Molecular Virology*. Wiley publication.
2. Cann, A.J. (2005). *Principles of Molecular Virology*, Academic Press.
3. Dimmock, N.J., Easton, A.J., and Leppard, K.N., (2007). *Introduction to Modern Virology*, (6th ed.). Blackwell Scientific Publications, Oxford, UK.
4. Flint, S.J., Racaniello, V.R., Enquist, L.W., Rancaniello, V. R., and Skalka, A. M., (2003). *Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses*. American Society Microbiology.
5. Jawetz, E., Melnic, J.L, and Adelberg, E.A., (2001). *Review of Medical Microbiology*. (22nd ed.). Lange Medical Publishers, NY.
6. Levy, J. A., Fraenkel-Conrat, H., and Owens, O. S., (1994). *Virology*. (3rd ed.). Benjamin Cummings.
7. Knipe D.M., Howley P.M., and Griffin D.E., (2006). *Fields Virology*. (5th ed). Vols - I, II. Lippincott, Williams & Wilkins.
8. Prescott, M., Harley, J.P., and Klein, D.A., (2007). *Microbiology*. (7th ed.). McGraw-Hill Inc. New York.
9. White, D. O., and Fenner, F.J., (1994). *Medical Virology*, (4th ed.). Academic Press, New York.

WEBSITES

W1: www.wikipedia.org

JOURNALS

J1: Lakshmi et al (2013) Detection of RNA Virus current technologies and future perspectives.

CLASS: III BSc MB COURSE NAME: MICROBIAL TECHNOLOGIES AND INTELLECTUAL PROPERTY RIGHTS

COURSE CODE: 15MBU602 UNIT: I

Restriction enzymes – types and nomenclature- classification, Target site of enzymes, Host controlled restriction and modification, Star activity and uses – Nucleases, Methylases – ligases. Joining of DNA fragments with vector – linkers, adaptors and hompolymer tailing.

A **restriction enzyme** is a nuclease enzyme that cleaves DNA sequence at a random or specific recognition sites known as restriction sites. In bacteria, restriction enzymes form a combined system (restriction + modification system) with modification enzymes that methylate the bacterial DNA. Methylation of bacterial DNA at the recognition sequence typically protects the own DNA of the bacteria from being cleaved by restriction enzyme.

There are two different kinds of restriction enzymes:

(1) Exonucleases catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5'to 3' direction or 3' to 5' direction. Example: exonuclease I, exonuclease II etc.

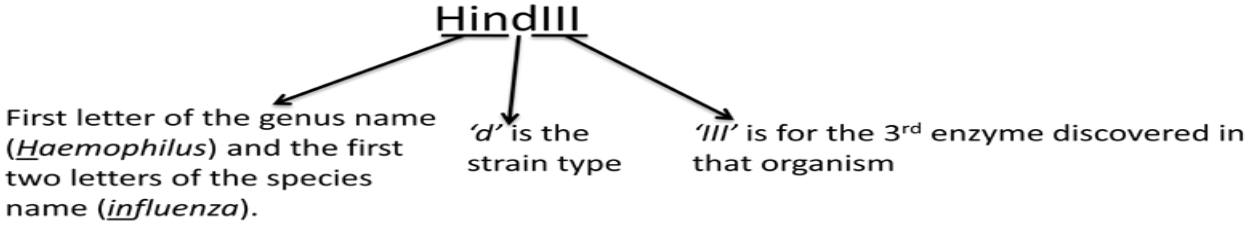
(2) Endonucleases can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. Example: EcoRI, Hind III, BamHI etc.

History:

In 1970 the first restriction endonuclease enzyme *Hind*II was isolated. For the subsequent discovery and characterization of numerous restriction endonucleases, in 1978 Daniel Nathans, Werner Arber, and Hamilton O. Smith awarded for Nobel Prize for Physiology or Medicine. Since then, restriction enzymes have been used as an essential tool in recombinant DNA technology.

Restriction Endonuclease Nomenclature:

Restriction endonucleases are named according to the organism in which they were discovered, using a system of letters and numbers. For example, *HindIII* (pronounced *"hindee-three"*) was discovered in *Haemophilus influenza* (strain d). The Roman numerals are used to identify specific enzymes from bacteria that contain multiple restriction enzymes indicating the order in which restriction enzymes were discovered in a particular strain.



Classification of Restriction Endonucleases:

There are three major classes of restriction endonucleases based on the types of sequences recognized, the nature of the cut made in the DNA, and the enzyme structure:

- Type I restriction enzymes
- Type II restriction enzymes
- Type III restriction enzymes

Type I restriction enzymes:

- These enzymes have both restriction and modification activities. Restriction depends upon the methylation status of the target DNA.
- Cleavage occurs approximately 1000 bp away from the recognition site.
- The recognition site is asymmetrical and is composed of two specific portions in which one portion contain 3–4 nucleotides while another portion contain 4–5

  nucleotides and both the parts are separated by a non-specific spacer of about 6–8 nucleotides.
- They require S-adenosylmethionine (SAM), ATP, and magnesium ions (Mg2+) for activity.
- These enzymes are composed of mainly three subunits, a specificity subunit that determines the DNA recognition site, a restriction subunit, and a modification subunit

Type II restriction enzymes:

- Restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification. Although the two enzymes recognize the same target sequence, they can be purified separately from each other.
- Cleavage of nucleotide sequence occurs at the restriction site.
- These enzymes are used to recognize rotationally symmetrical sequence which is often referred as palindromic sequence.
- These palindromic binding site may either be interrupted (e.g. BstEII recognizes the sequence 5´-GGTNACC-3´, where N can be any nucleotide) or continuous (e.g. KpnI recognizes the sequence 5´-GGTACC-3´).
- They require only Mg^{2+} as a cofactor and ATP is not needed for their activity.
- Type II endonucleases are widely used for mapping and reconstructing DNA *in vitro* because they recognize specific sites and cleave just at these sites.

The steps involved in DNA binding and cleavage by a type II restriction endonuclease:

- These enzymes have nonspecific contact with DNA and initially bind to DNA as dimmers.
- The target site is then located by a combination of linear diffusion or "sliding" of the enzyme along the DNA over short distances, and hopping/jumping over longer distances.
- Once the target restriction site is located, the recognition process (coupling) triggers large conformational changes of the enzyme and the DNA, which leads to activation of the catalytic center.
- Catalysis results in hydrolysis of phosphodiester bond and product release.

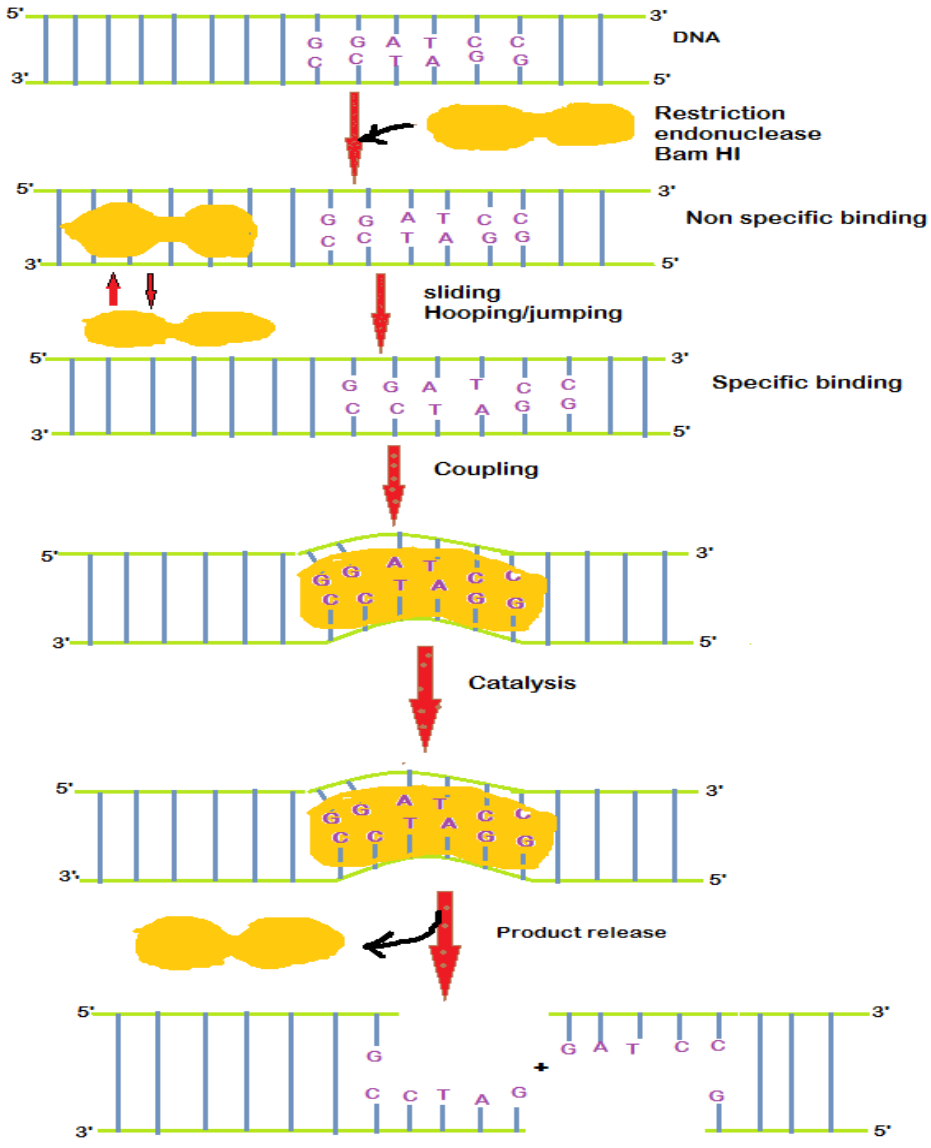


Fig 2-1.4.2: Structures of free, nonspecific, and specific DNA-bound forms of BamHI.

The two dimers are shown in brown, the DNA backbone is in green and the bases in gray. *BamHI* becomes progressively more closed around the DNA as it goes from the nonspecific to specific DNA binding mode.

Type III restriction enzymes:

- These enzymes recognize and methylate the same DNA sequence but cleave 24–26 bp away.
- They have two different subunits, in which one subunit (M) is responsible for recognition and modification of DNA sequence and other subunit (R) has nuclease action.
- Mg^{+2} ions, ATP are needed for DNA cleavage and process of cleavage is stimulated by SAM.
- Cleave only one strand. Two recognition sites in opposite orientation are necessary to break the DNA duplex.

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

Table 2-1.4: Comparative properties of restriction enzymes

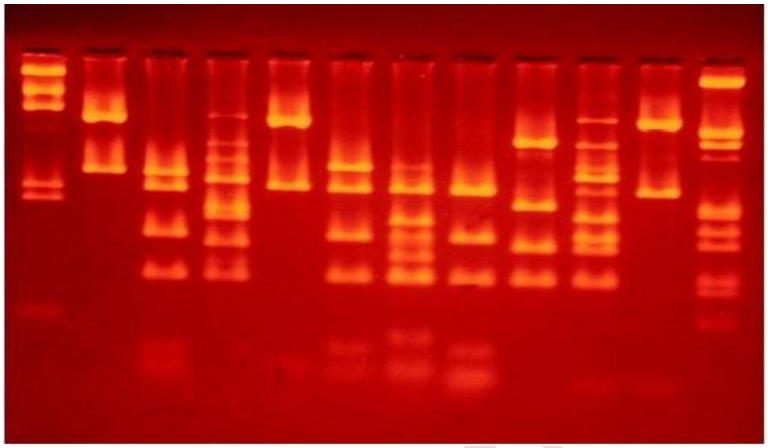


Fig 2-1.4.3: Cleaving a DNA sequence by a restriction enzyme creates a specific pattern.

Cleaving a single piece of DNA with multiple restriction enzymes creates a "DNA fingerprint." The pattern of fragments can be compared to similar DNA from another source treated with the same enzymes, to determine if the two are identical or different.

Cleavage Patterns of Some Common Restriction Endonucleases:

The recognition and cleavage sites and cleavage patterns of *HindIII, SmaI, EcoRI,* and *BamHI* are shown. Cleavage by an endonuclease creates DNA sequence with either a sticky end or blunt end. The blunt ended fragments can be joined to any other DNA fragment with blunt ends using linkers/adapters, making these enzymes useful for certain types of DNA cloning experiments.

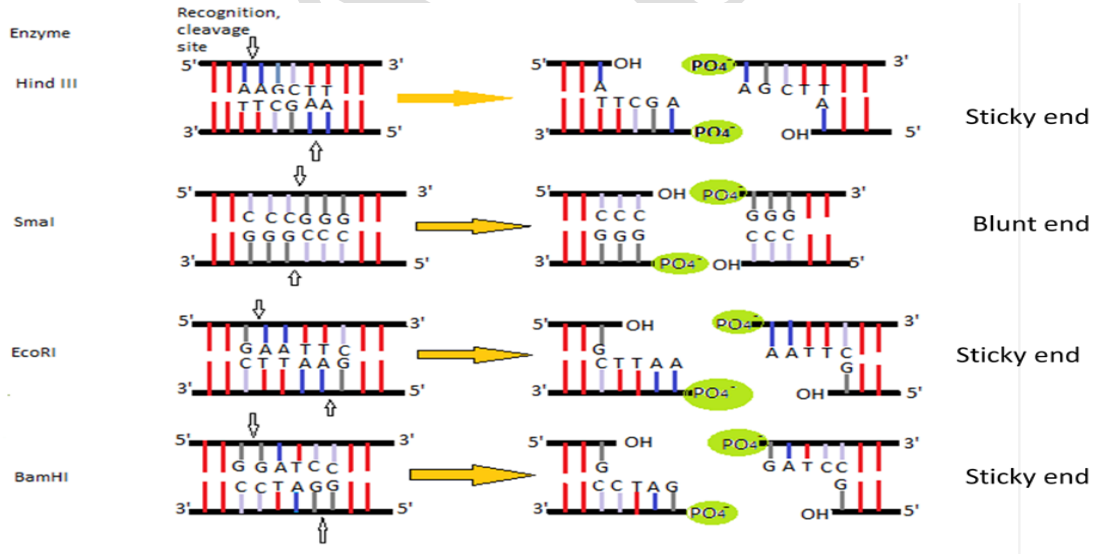


Fig 2-1.5: Cleavage patterns of *HindIII, SmaI, EcoRI* and *BamHI*

Applications:

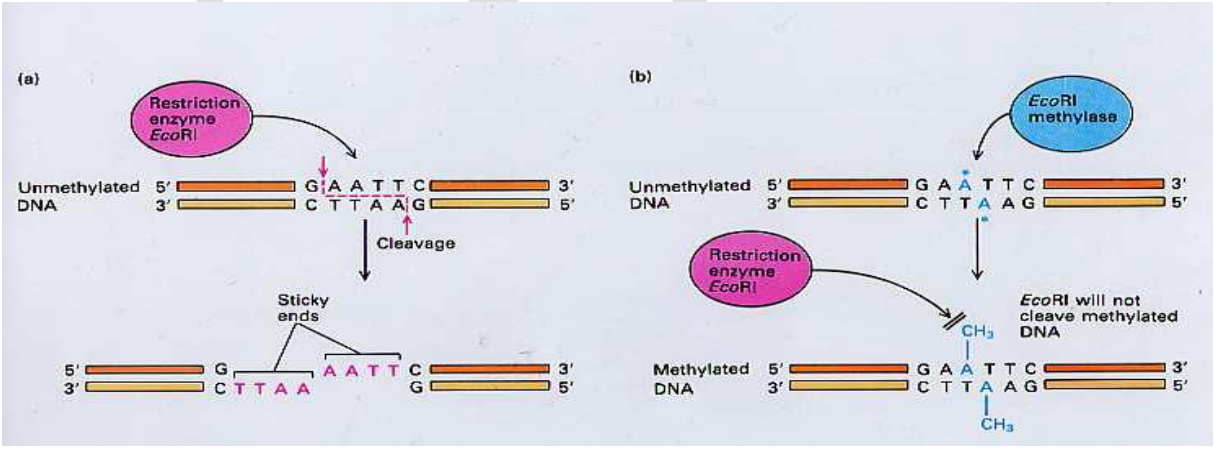
In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.

- They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.
- Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restriction site present in the allele.
- Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.

Host-controlled restriction and modification system

Restriction systems allow bacteria to monitor the origin of incoming DNA and to destroy it, if it is recognized as foreign. Restriction endonucleases recognize specific sequences in the incoming DNA (e.g. phages) and cleave the DNA into fragments, either at specific sites or more randomly, thus preventing it from successfully replicating and parasitizing the cell (immunity system). The restrictive host must, of course, protect its own DNA from the potentially lethal effects of the endonuclease and so its DNA must be appropriately modified. Modification involves methylation of certain bases at a very limited number of sequences within DNA.

EcoRI restriction endonuclease-methylase system (type II)



Together, a restriction endonuclease and its 'cognate' modification methyl-transferase form a restriction-modification system (R-M system)

Star Activity

It has been demonstrated that under extreme non-standard conditions, restriction endonucleases are capable of cleaving sequences which are similar but not identical to their defined recognition sequence. This altered or relaxed specificity has been termed "star" activity. It has been suggested that star activity may be a general

property of restriction endonucleases (1) and that any restriction endonuclease can be made to cleave noncanonical sites under certain extreme conditions. Testing at New England Biolabs has confirmed reports in the literature that the following restriction endonucleases can be made to exhibit star activity: Apo I (2), Ase I (2), BamH I (3), BssH II (2), EcoR I (4), EcoR V (5), Hind III (1), Hinf I (6,7), Pst I (8), Pvu II (9), Sal I (8), Sca I (2), Taq I (10), Xmn I (2). The manner in which an enzyme's specificity is altered depends on the enzyme and on the conditions employed to induce the star activity. The most common types of altered activity are single base substitutions, truncation of the outer bases in the recognition sequence, and single-strand nicking (10). Early studies with EcoR I by Polisky et al. (4) demonstrated that under conditions of elevated pH and low ionic strength, EcoR I cleaves the sequence N/AATTN, while more recent studies by Gardner et al. (11) showed that EcoR I* (EcoR I star activity) cleaves any site which differs from the canonical recognition sequence by a single base substitution, providing the substitution does not result in an (A) to (T) or a (T) to (A) change in the central (AATT) tetranucleotide sequence. SgrA I, which recognizes and cleaves the sequence CRCCGGYG, displays a new phenomenon of relaxation of sequence specificity. Under standard reaction conditions and in the presence of its cognate site, SgrA I is capable of cleaving non-cognate sites CRCCGGYN and CRCCGGGG (referred to as secondary sites). Studies performed with SgrA I reveal that DNA termini generated by cleaving the cognate site are an essential factor in the cleavage of secondary sites, as the secondary sites are not cleaved on DNA substrates that lack a cognate site (13). Star activity is completely controllable in the vast majority of cases and is generally not a concern when performing restriction endonuclease digests. Listed below are reaction conditions known to induce or inhibit star activity.

Conditions that Contribute to Star Activity

1. High glycerol concentration [>5% v/v]
2. High units to µg of DNA ratio [Varies with each enzyme, usually >100 units/µg]
3. Low ionic strength [<25 mM]
4. High pH [>pH 8.0]
5. Presence of organic solvents [DMSO, ethanol (9), ethylene glycol, dimethylacetamide, dimethylformamide, sulphalane
6. Substitution of Mg++ with other divalent cations [Mn++, Cu++, Co++, Zn++]

Inhibiting Star Activity

Recently, there has been much attention given to the fidelity of restriction endonucleases, particularly in forensic applications. If you are concerned about star activity, we recommend the following guidelines.

1. Use as few units as possible to get a complete digestion. This avoids overdigestion and reduces the final glycerol concentration in the reaction.

2. Make sure the reaction is free of any organic solvents such as alcohols which might be present in the DNA preparation.

3. Raise the ionic strength of the reaction buffer to 100-150 mM (provided the enzyme is not inhibited by high salt).

4. Lower the pH of the reaction buffer to pH 7.0.

5. Use Mg++ as the divalent cation.

Methylases:

- Methyltransferase or methylase catalyzes the transfer of methyl group (-CH_3) to its substrate. The process of transfer of methyl group to its substrate is called methylation.
- Methylation is a common phenomenon in DNA and protein structure.
- Methyltransferase uses a reactive methyl group that is bound to sulfur in S- adenosyl methionine (SAM) which acts as the methyl donor.
- Methylation normally occurs on cytosine (C) residue in DNA sequence. In protein, methylation occurs on nitrogen atom either on N-terminus or on the side chain of protein.
- DNA methylation regulates gene or silence gene without changing DNA sequences, as a part of epigenetic regulation.
- In bacterial system, methylation plays a major role in preventing their genome from degradation by restriction enzymes. It is a part of restriction – modification system in bacteria.

Methyltransferase can be classified in three groups:

a) m6A-generates N6 methyladenosine,

b) m4C-generates N4 methylcytosine,

c) m5C-generatesN5 methylcytosine.

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

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

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

Ligases:

- DNA ligase catalyses the formation of phosphodiester bond between two deoxynucleotide residues of two DNA strands.
- DNA ligase enzyme requires a free hydroxyl group at the 3´ -end of one DNA chain and a phosphate group at the 5´-end of the other and requires energy in the process.
- *E.coli* and other bacterial DNA ligase utilizes NAD^+ as energy donor, whereas in T4 bacteriophage, T4 DNA ligase uses ATP as cofactor.
- The role of DNA ligase is to seal nicks in the backbone of double-stranded DNA after lagging strand formation to join the okazaki fragments.
- This joining process is essential for the normal synthesis of DNA and for repairing damaged DNA. It has been exploited by genetic engineers to join DNA chains to form recombinant DNA molecules. Usually single stranded break are repaired using the complimentary strand as the template but sometimes double stranded breaks can also be repaired with the help of DNA ligase IV.
- The most widely used DNA ligase is isolated from T4 bacteriophage. T4 DNA ligase needs ATP as a cofactor. The enzyme from *E. coli* uses cofactor NAD. Except this, the catalysis mechanism is somewhat similar for both the ligases. The role of cofactor is splitting and forming an enzyme-AMP complex which further aids in formation of phosphodiester bonds between hydroxyl and phosphate groups by exposing them.

Mechanism of Action of DNA Ligases:

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

pathway.

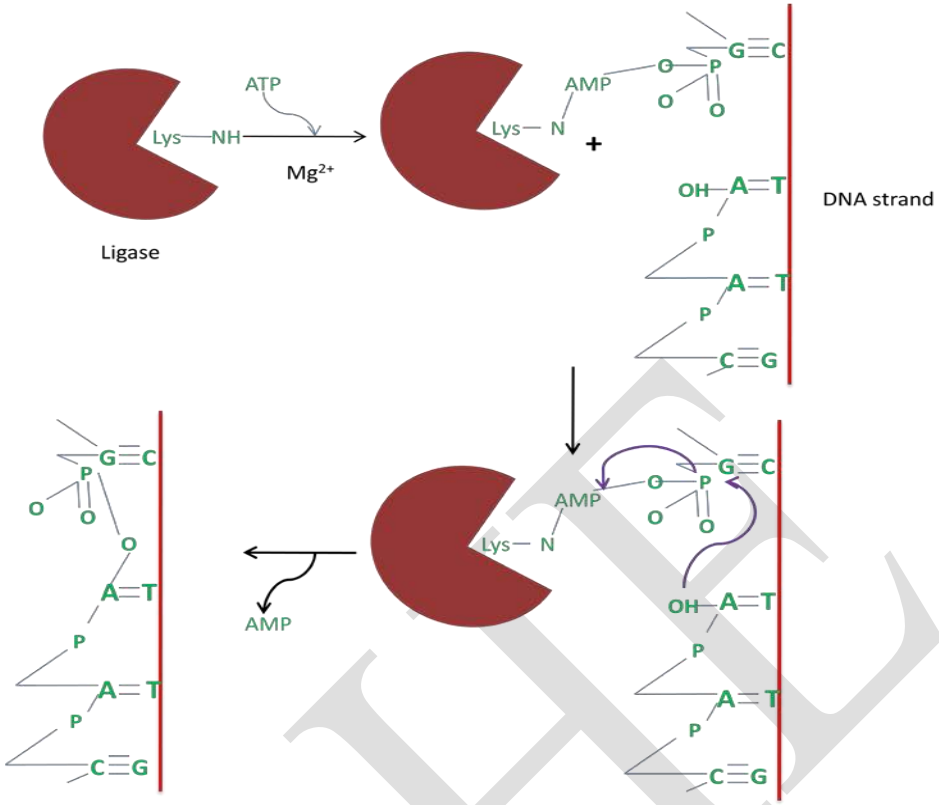


Fig 2-4.1.1: The mechanism of DNA joining by DNA ligase.

Application:

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

- Cloning of restricted DNA to vector to construct recombinant vector.

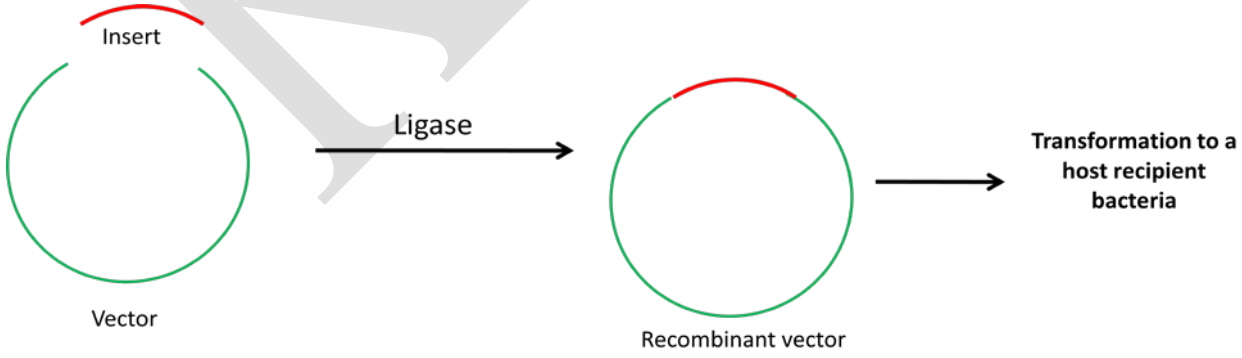


Fig 2-4.1.2: Ligation of a gene fragment into the vector and transformation of the cell.

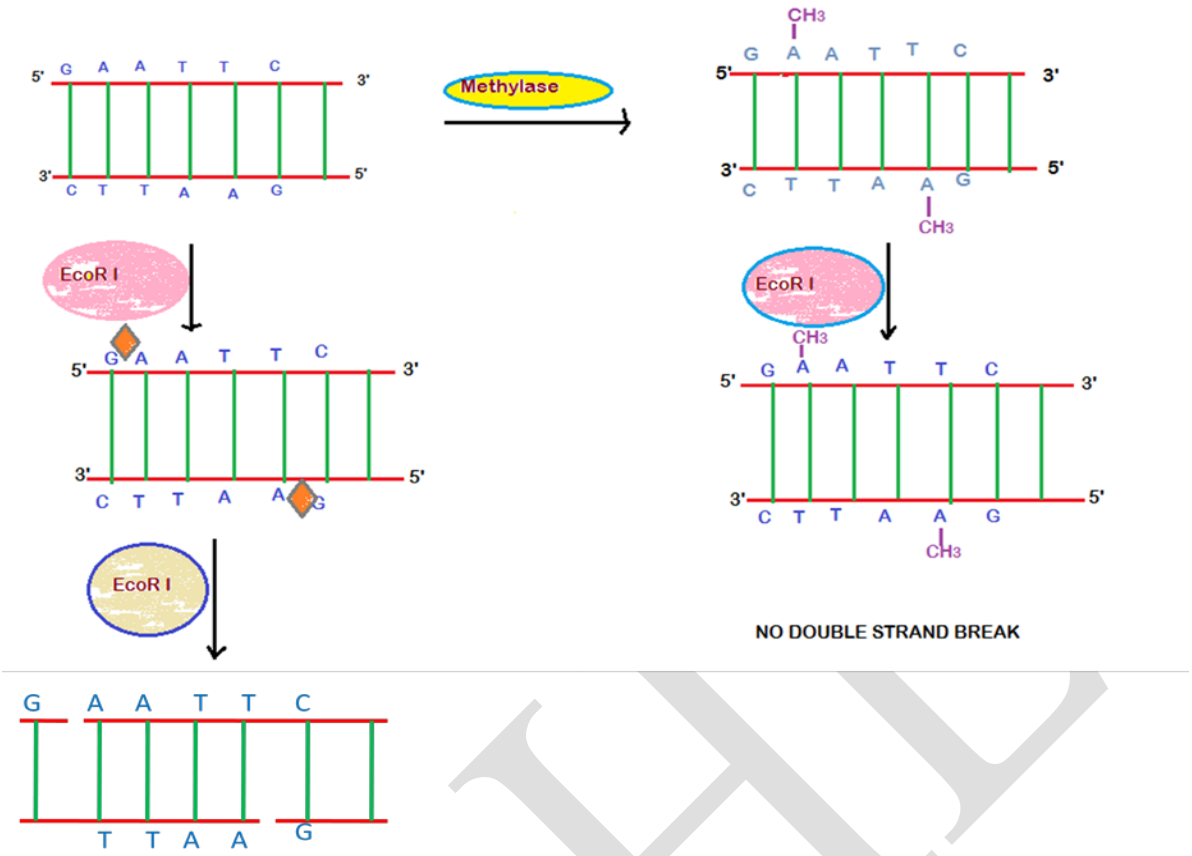


Fig 2-3.2: Activity of restriction and methylase enzymes

Joining of DNA fragments with vector

DNA ligation is an important technique in molecular cloning and in the generation of recombinant DNA (Figure-1). DNA ligation is the act of joining together DNA strands with covalent bonds with the aim of making new viable DNA or plasmids. The enzyme that catalyzes the reaction is called DNA ligase. DNA ligase used in genetic engineering is usually purified from E.coli bacteria that have been infected with T4 phage.

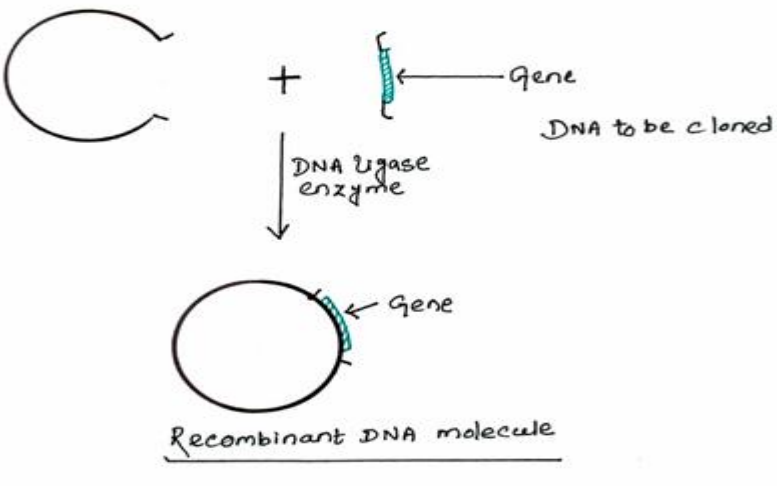


Figure 1: Construction of a recombinant DNA molecule by DNA ligase

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"). ATP is required for the ligase reaction, which proceeds in three steps: (1) adenylation (addition of AMP) of a residue in the active center of the enzyme, pyrophosphate is released; (2) transfer of the AMP to the 5' phosphate of the so-called donor, formation of a pyrophosphate bond; (3) formation of a phosphodiester bond between the 5' phosphate of the donor and the 3' hydroxyl of the acceptor (Figure-2). Within the cell the enzyme carries out the very important function of repairing any discontinuities that may arise in one of the strands of a double stranded molecule. Ligases play several vital roles in the cell.

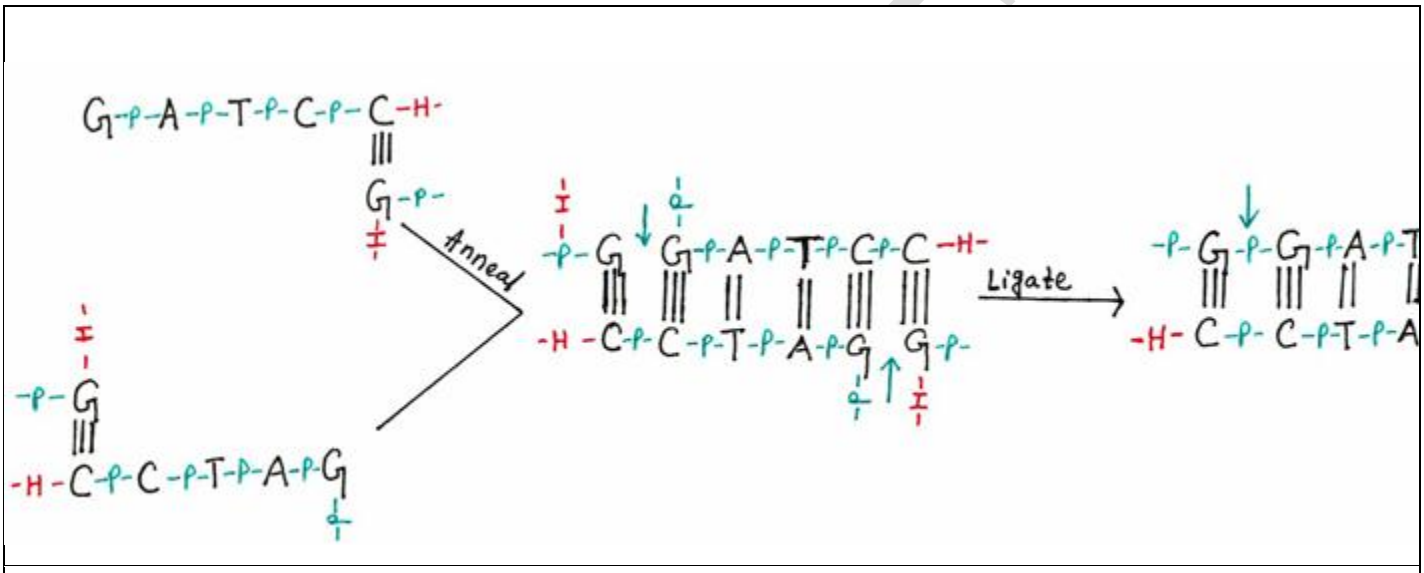


Figure 2: Formation of phosphodiester bonds during ligation

Ligation of complementary sticky ends is more efficient than blunt end ligation, because compatible sticky ends can base-pair with one another by hydrogen bonding forming a relatively stable structure for the enzyme to work on. For optimal ligation efficiency with cohesive-ended fragments ("sticky ends"), the optimal enzyme temperature needs to be balanced with the **melting temperature Tm** (also the annealing temperature) of the sticky ends being ligated. If the ambient temperature exceeds Tm, the homologous pairing of the sticky ends would not be stable because the high temperature disrupts hydrogen bonding. Ligation reaction is most efficient when the sticky ends are already stably annealed, disruption of the annealing ends would therefore results in low ligation efficiency. Since blunt-ended DNA fragments have no cohesive ends to anneal, the melting temperature is not a factor to consider within the normal temperature range of the ligation reaction. However, the higher the temperature, the less chance that the ends to be joined will be aligned to allow ligation (molecules move around the solution more at higher temperatures). The limiting factor in blunt end ligation is not the activity of the ligase but rather the number of alignments between DNA fragment ends that occur. The most efficient ligation temperature for blunt-ended DNA would therefore be the temperature at which the greatest

number of alignments can occur. Under the situation where the vector molecule has sticky ends, but the DNA fragments to be cloned are blunt ended, there the use of linkers and adapters can put the correct sticky ends on to the DNA fragments.

Linkers are short pieces of chemically synthesized, self complementary double stranded oligo-nucleotides which contain within them one or more restriction sites (Figure-3).

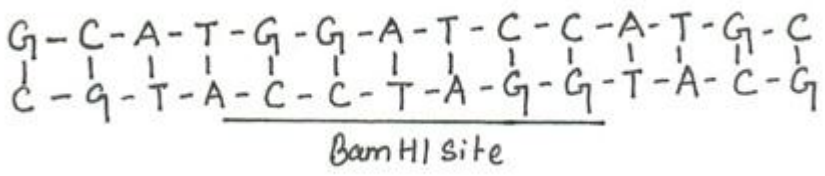


Figure 3: Structure of a linker

DNA ligase will attach linkers to the ends of larger blunt-end ligation. Although a blund end ligation this reaction can be performed very efficiently because synthetic oligonucleotides, such as linkers can be made in very large amounts and added into the ligation mixture at a high concentration (Figure-4). More than one linker will attach to each end of the DNA molecule, producing a chain structure. But digestion with *BamHI* cleaves the chains at the recognition sequences, producing a large number of cleaved linkers and the original DNA fragment, with *BamHI* sticky ends. Now this modified fragment can be ligated into a cloning vector restricted with *BamHI*.

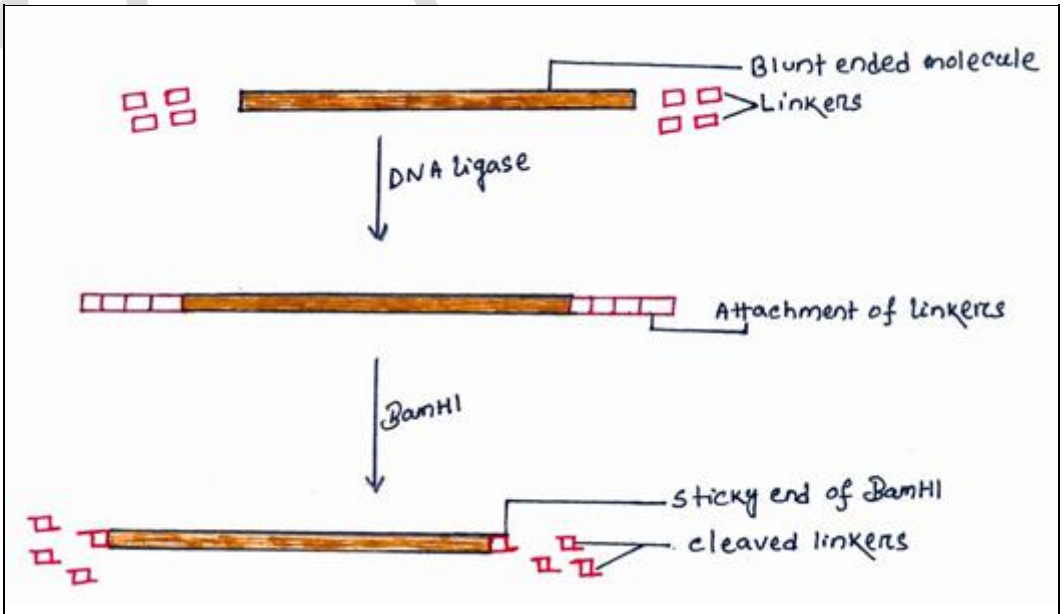


Figure 4: The attachment of linkers to a blunt-ended molecule

An adapter or linker in genetic engineering is a short, chemically synthesized, single-stranded or double-

stranded oligonucleotide that can be ligated to the ends of other DNA or RNA molecules. Double stranded adapters can be synthesized to have blunt ends to both terminals or to have sticky end at one end and blunt end at the other. For instance, a double stranded DNA adapter can be used to link the ends of two other DNA molecules (i.e., ends that do not have "sticky ends", that is complementary protruding single strands by themselves). It may be used to add sticky ends to cDNA allowing it to be ligated into the plasmid much more efficiently. Two adapters could base pair to each other to form dimers. A conversion adapter is used to join a DNA insert cut with one restriction enzyme, say EcoRl, with a vector opened with another enzyme, Bam Hl. This adapter can be used to convert the cohesive end produced by Bam Hl to one produced by Eco Rl or vice versa. One of its applications is ligating cDNA into a plasmid[1] or other vectors instead of using Terminal deoxynucleotide Transferase enzyme to add poly A to the cDNA fragment.

Adaptors, are short, chemically synthesized oligonucleotides which can be used to link the ends of two DNA molecules which have different sequences at their ends, when used in conjuction with linkers or other adaptor molecules. An adaptor is synthesized so that it already has one sticky end (Figure-5).

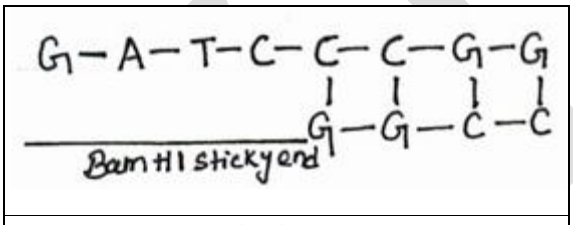


Figure 5: A typical adaptor

During ligation the blunt end of the adaptor ligates to the blunt ends of the DNA fragment to produce a new molecule with sticky ends. But practically, the sticky ends of individual adaptors could base pair with themselves to form dimers (Figure-6) and the new DNA molecule remains blunt-ended.

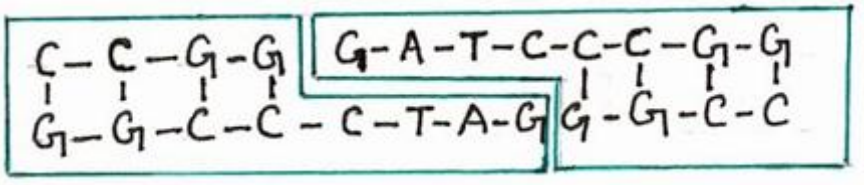


Figure 6: Ligation of two adaptors

For a solution to this problem, adaptor molecules are synthesized so that the blunt end is the same as natural DNA but the sticky end is different. The 3'-OH terminus of the sticky end is unchanged, but the 5' terminus is modified from 5'-P to 5'-OH terminus (Figure-7).

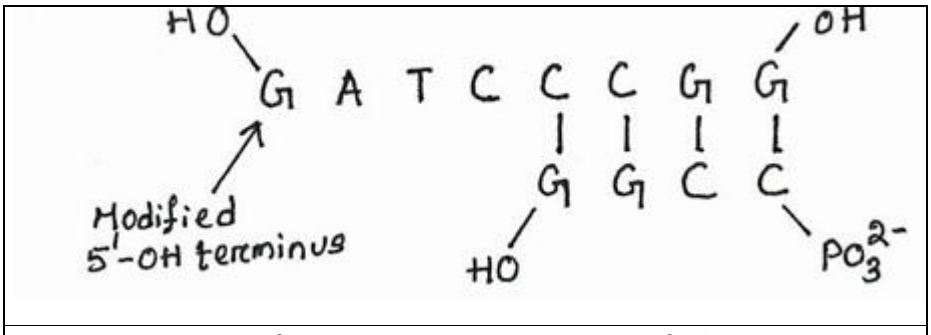


Figure 7: Structure of an adaptor showing the modified 5'-OH terminus

Therefore DNA ligase can't form a phosphodiester bond between 5'-OH and 3'-OH ends. So the base pairing between the sticky ends of adaptor molecules is never stabilized by ligation (Figure-8). Adaptors therefore can be ligated to a DNA molecule but not to themselves. Adaptor molecules alter their 5' terminus (From 5'-P to 5'-OH) by an enzymatic treatment of the enzyme Polynucleotide kinase.

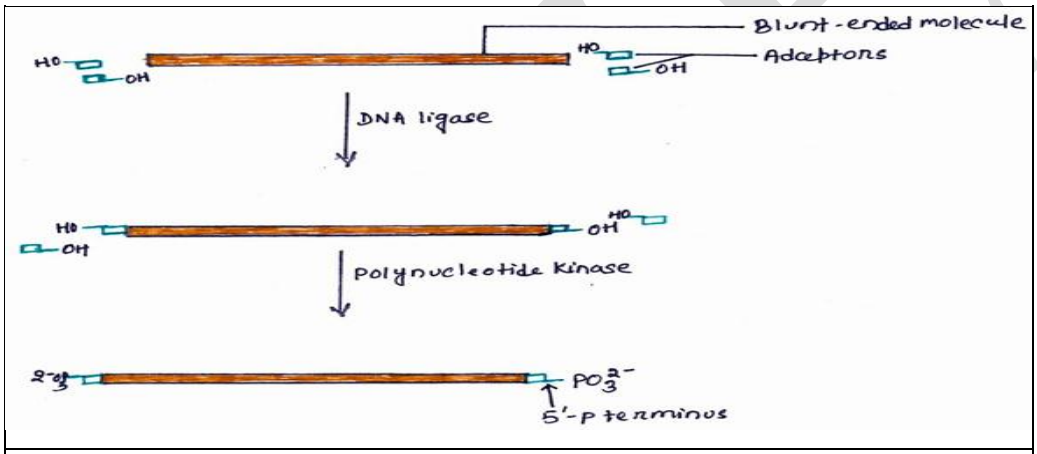


Figure 8: Blunt-end is converted to sticky end by the attachment of adaptors

Homopolymer tailing

This method of joining DNA molecules makes use of the annealing of complementary homopolymer sequences. Thus by adding oligo (dA) sequences to the 3' ends of one population of DNA molecules and oligo (dT) blocks to the 3' ends of another population, the two types of molecules can be annealed to form mixed dimeric circles. An enzyme purified from calf thymus, terminal deoynucleotidyl transferase, provides the means by which homopolymeric extensions can be synthesized.

It is a technique by which sticky ends can be produced on a blunt-ended DNA molecule. In a homopolymer, all the subunits are same. A DNA strand made up entirely of deoxyguanosine is an example of homopolymer, and is referred to as polydeoxyguanosine or poly(dG). Tailing invoves using the enzyme terminal deoxynucleotidyl transferase to add a series of nucleotides on to the 3'-OH termini of a double-stranded DNA molecule. The reaction when carried out in the presence of just one deoxynucleotide, then a homopolymer tail will be produced (Figure-9).

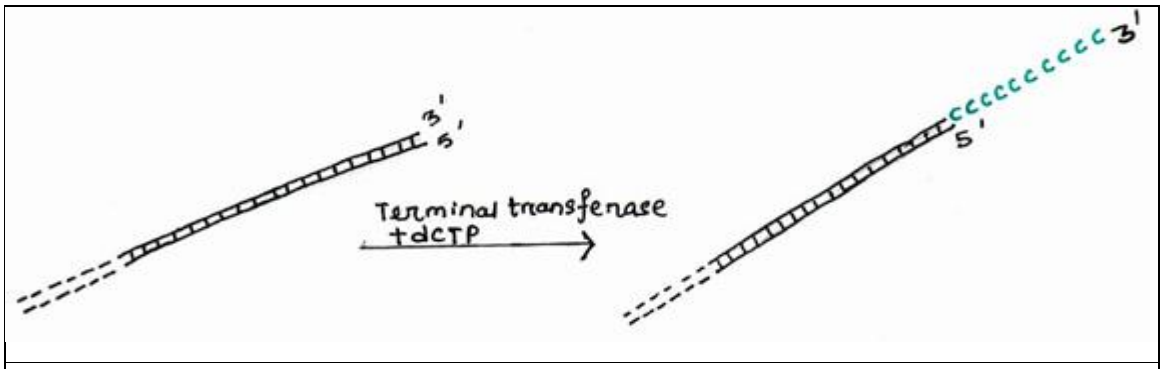


Figure 9: Synthesis of a homopolymer tail

For ligation of two tailed molecules, the homopolymers must be complementary. Frequently poly(dc) tails are attached to the vector and poly(dg) to the DNA to be cloned. Base pairing between the two occurs when the DNA molecules are mixed (Figure-10).

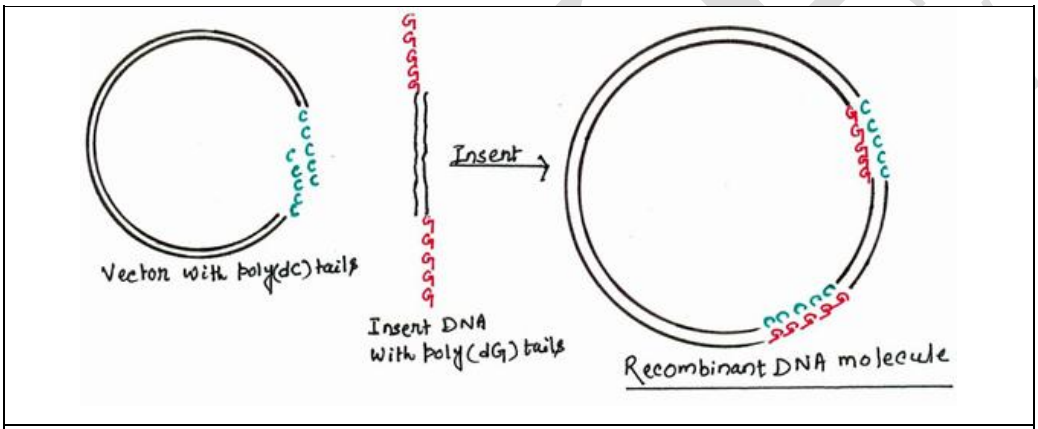


Figure 10: Construction of a recombinant DNA molecule from a tailed vector

A recombinant DNA molecule, held together by base pairing will often be stable enough to be introduced into the host cell. The repair of the recombinant DNA molecule is then done by using the enzyme Klenow polymerase to fill the nicks followed by DNA ligase to synthesize the final phosphodiester bonds (Figure-11).

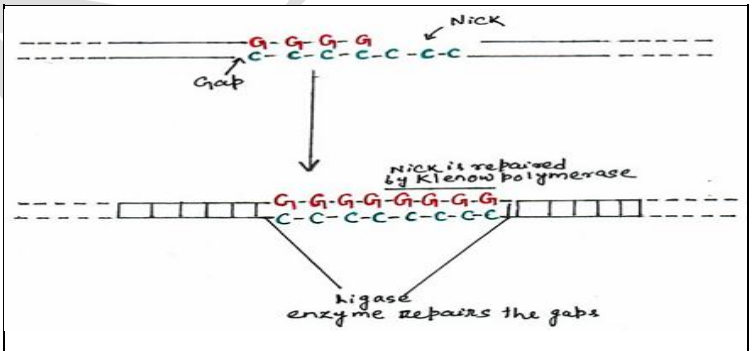


Figure 11: Repair of the recombinant DNA molecule

The main distinction between a living and non-living entity is the ability to replicate and reproduce similar offsprings. Nucleic acid molecules (DNA and RNA) present in a living organism acts as a genetic template to pass the hereditary information from one generation to the next. Nucleic acid molecules are organised as genes which code for a particular phenotype via specific proteins and the expression of a gene is

regulated by both external and internal factors which aid the developmental process of an organism. This relation between genes and proteins forms the "central dogma of life".

A gene can be defined as the region of DNA (or RNA in case of virus) that controls a discrete hereditary characteristic, usually corresponding to a single protein or RNA. This includes the entire functional unit, encompassing coding (exons) and noncoding sequences (introns and regulatory sequences).

- Exons and introns which represent the coding and noncoding regions are present in a eukaryotic gene. Introns are absent in prokaryotes.
- The introns are removed by splicing and the exons are translated in tandem to yield the functional polypeptide that further undergoes post translational modification to become functional. These functional polypeptides (proteins) are targeted to various organelles in the cell or exported out of the cell for carrying out various intracellular and extracellular processes respectively.

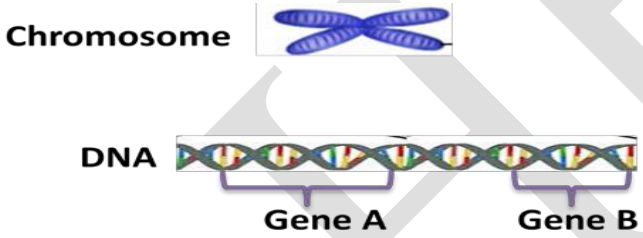


Fig 1-1.2: Organization of genes in DNA of a chromosome

Genome:

Genome is the complete set of genetic information of a cell or an organism; in particular, the complete sequence of DNA/RNA that carries this information. In diploid organisms, it refers to the haploid set of chromosomes present in a cell. Depending on its localization, genome may be nuclear or organellar. Organellar genomes are again of two types: mitochondrial and chloroplast genome. Genome size of organisms differs significantly between different species. The size of the genome governs the size and complexity of an organism. However, many small sized organisms, in fact have bigger genomes than their larger counterparts.

Various organisms have different sized genome as can be seen in the table below.

Species	**Organism**	**Genome Size (Mb)**
Triticum aestivum	Plant	16000
Homo sapiens	Mammal	3200
Arabidopsis thaliana	Plant	125
Drosophila melanogaster	Insect	180
Caenorhabditis elegans	Nematode worm	97
Saccharomyces cerevisiae	Yeast	12.1

Escherichia coli	Bacterium	4.64
Haemophilus influenzae	Bacterium	1.83
Mycoplasma genitalium	Bacterium	0.58

- The genome contains all the genes present in the nucleus of a cell. Gene varies in size from a few hundred DNA/RNA bases to more than few thousand bases.
- The haploid set of chromosome contains the total genome of the organism.
- The bacterium *Mycoplasma genitalium* has a small genome size of 0.58Mb and the plant *Triticum aestivim* has a large genome size of 16000Mb. The genome size in human is 3200Mb.

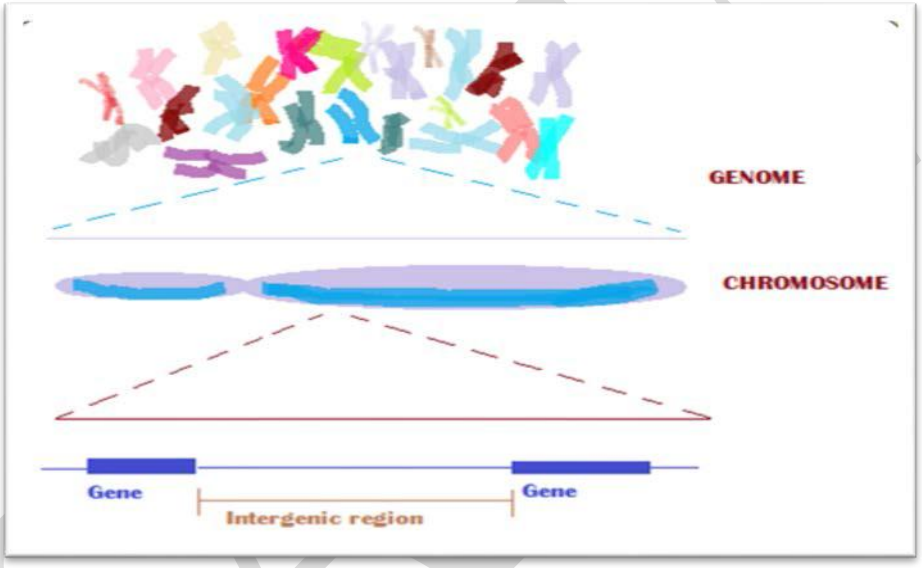


Fig 1-1.3: Illustration of genome, from genome to a chromosome, from a chromosome to gene

Role of genes within cells:

- Genes contain the instructions for each cell to make proteins and RNAs. Genes are made up of DNA fragments.
- Within the cell the DNA performs two tasks:
  - Act as information repository including instructions in making the component molecules of the cells.
  - Pass on the information to the next generation.
- The mere presence of DNA does not implicate a cell to be alive and functional. Mammalian red blood cells (RBCs) discard nucleus during developmental process and thus lacks DNA in mature state.
- Genes are transcribed to RNA which are processed to various forms like mRNA, tRNA, rRNA etc. mRNA are translated to proteins depending on the regulatory signals. tRNA and rRNA serve as the components of translational machinery.

- New functions of RNA are also being discovered like regulatory (miRNA, siRNA etc) and catalytic (ribozymes) functions.
- Proteins are structural components of cells, enzymes, hormones, various signalling molecules, receptors and other factors which are involved in performing the chemistry of life and are essential for the normal body function, for example, sugar conversion to energy and metabolite (small molecules) production in cell.

Genetic code:

The genetic code is the set of instructions that translates the information encoded in genetic material (mRNA or DNA sequences) into proteins (amino acid sequences) by living cells.

- The genetic code is a triplet code (i.e. a group of three adjacent nucleotides) called codon. This three nucleotide codon in nucleic acid sequence specifies a single amino acid. However, genetic code in human mitochondria differs from the standard nuclear genetic code.
- Epigenetic effects, however is not stored using the genetic code. Besides all organisms have DNA that contains regulatory sequences, intergenic segments, chromosomal structural areas and other non-coding DNA that can contribute greatly to phenotype.
- These codons are always written with the 5′-terminal nucleotide to the left.
- The code is unambiguous i.e. each triplet specifies only a single amino acid.
- The genetic code is degenerate i.e. more than one triplet codon can code for a single amino acid. (61 codons code for 20 amino acids)
- Three codons do not specify any amino acid but acts as terminal sites (stop codons), signalling the end of protein coding sequence. They are namely UAA (Ochre), UAG (Amber) and UGA (Opal).
- AUG is an initiation codon that signals the initiation of translation and also codes for methionine. In some mRNA, GUG and UUG also act as initiation codon.
- The genetic codes for each 20 amino acids were defined by pioneering works of Marshall W. Nirenberg and Har Gobind Khorana in 1964.

Gene expression:

Let us now understand the central dogma of life. You now know that most of the genes contain the information needed to make functional molecules called proteins. However, some genes produce other molecule (tRNA, rRNA, microRNA etc) that assist or regulates the protein expression and assembly. These complex events within each cell consist of two main steps: transcription and translation. In prokaryotes where there is no nuclear membrane, both transcription and translation occur in the cytoplasm whereas in eukaryotes, transcription occurs inside the nucleus and the translation occurs in the cytoplasm.

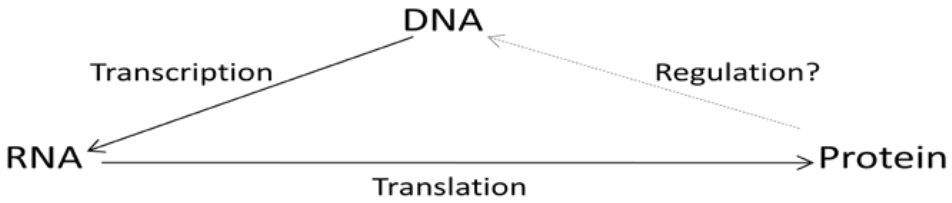


Fig1-1.5: Central dogma of life

Events involved in gene expression:

The events involved in genome expression in higher organisms with respect to protein coding genes are discussed below. The genes that give rise to non-coding RNAs are transcribed and processed but are never translated.

- **Access to gene: G**enes are inaccessible as they are buried deep within the highly packaged chromosomes. The initial step involves a preparative process that opens the chromatin structure and positions of the nucleosome in the region of genome containing active genes.
- **Formation of transcription initiation complex** involves the assembly of a set of proteins into a complex that copy DNA into RNA. This is a highly regulated process as the transcription initiation complex must be constructed at the precise position in the genome, adjacent to active genes to form a RNA copy.

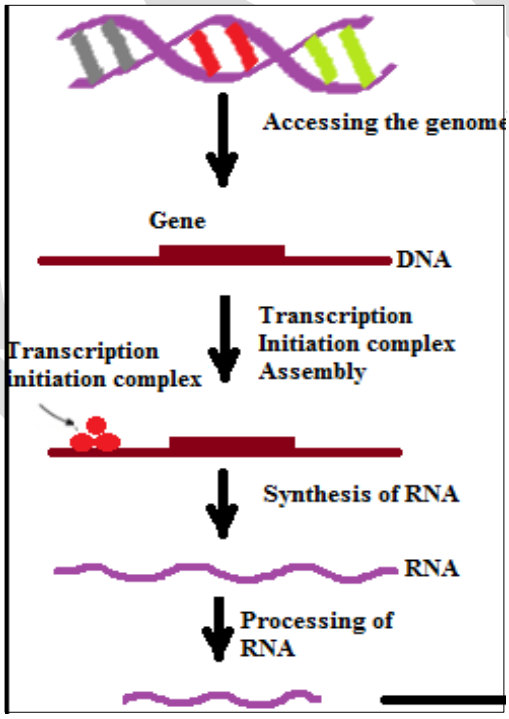


Fig: 1-1.5.1: Steps involved in Gene Expression

- **RNA Synthesis** involves the transcription of a gene into RNA molecule and it occurs in the nucleus.
- **RNA Processing** comprises of post transcriptional modification/alterations of the RNA molecule and its

chemical structure required for the RNA to be translated into protein or non-coding RNA (rRNA, tRNA, miRNA). RNA splicing (deletion of introns and combination of exons), 5' capping, polyadenylation etc are commonly occurred RNA processing steps in eukaryotes. However, prokaryotic organisms do not have a well-developed RNA processing machinery.

- **Degradation of RNA** is the controlled turnover of RNA molecules and should not be viewed simply as a mean of getting rid of unwanted RNAs. It determines the makeup of the transcriptome and is considered as an important step in genome expression. Different ribonucleases (RNases) plays the prime role in this process and multiple cofactors like small RNA (siRNA, miRNA etc), molecular cheparons (Lsm1-7, Lsm2-8, Hfq etc) regulate this process.
- **Protein synthesis** is initiated after the assembly of the translation initiation complex near the 5' termini of a mRNA molecule. It involves translation of RNA molecules into proteins.
- **Protein folding and protein processing** may occur together after protein synthesis. Post translation events like folding involve the protein attaining its correct three dimentional configuration. Processing (phosphorylation, glycosylation, carboxylation etc) involves the modification of the protein by addition of chemical groups and removal of one or more functional units of the protein.

Types of gene expression:

- **Constitutive expression:** Housekeeping genes are essential and necessary for sustaining life, and are therefore continuously expressed. *gapdh* (glyceraldehydes 3 phosphate dehydrogenase), sdha *(*succinate dehydrogenase) etc are human housekeeping genes which are expressed throughout the development.
- **Induction and repression:** The expression levels of some genes fluctuate in response to external signals. Also, under a certain situation, some genes show higher expression level, while others show lower expression levels. The former is called induced expression and the latter is called repressed expression.

Control of gene expression:

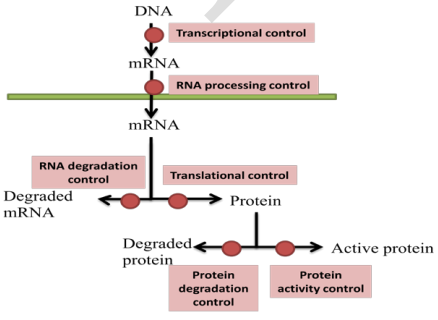


Fig1-1.6: Points of control of gene expression

The eukaryotic gene expressions in a cell are controlled at about six stages as shown in the figure 1-1.6.

- Transcriptional control decides when and how frequently a given gene be transcribed. It enables the cell to check any discrepancy in the quantity of transcriptomes generated.
- RNA processing regulates how the RNA transcript is spliced or otherwise processed (applicable only to eukaryotes), which have split genes consisting of exons and introns.
- Translational control decides which mRNAs in the cytoplasm are to be translated by ribosomes.
- mRNA degradation control selectively destabilizes some mRNA molecules in the cytoplasm.
- Protein activity control selectively activates, inactivates, degrades, or compartmentalizes specific protein molecules after they have been synthesized. (applicable only to eukaryotes). Regulatory elements:

Gene expression is a complex multi-step process. Amongst various steps involved, transcription initiation is a vital key point in controlling gene expression.

Fundamental elements that regulate the process of transcription are:

- cis-acting elements comprising of special DNA sequences.
- trans-acting elements comprising of regulatory proteins.
- DNA –protein interaction.
- Protein-protein interaction
- RNA polymerase

cis-acting elements:

In Latin “cis”, means "same side as". Cis acting elements are thus a region of DNA or RNA that regulates the expression of genes located on the same molecule. The cis-regulatory elements are often binding sites for one or more trans-acting factors. Cis-element may be located upstream to the coding sequence of the gene it controls (in the promoter region or further upstream 5'), in an intron, or 3' to the gene's coding sequence, either in the untranslated or untranscribed region.

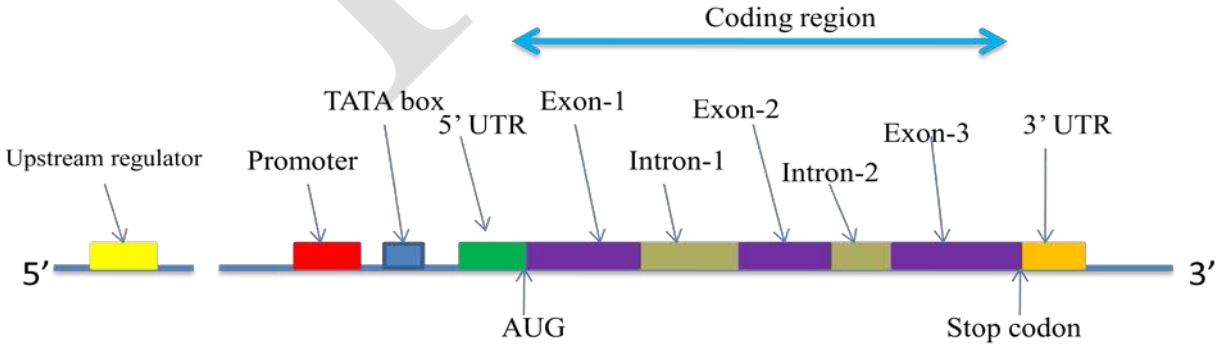


Fig1-1.6.1: Structure of eukaryotic gene with upstream regulatory elements

Examples of cis-acting elements:

Prokaryotic systems:

- Promoter is a DNA sequence where RNA polymerase binds to initiate transcription. There are two promoter sequences in prokaryotic systems known as -10 (Pribnow box or Pribnow-Schaller box) and -35 sequences.
- Operator is regulatory sequence of DNA located immediately upstream of the structural gene that controls transcription of an operon.
- Inducers are located upstream of promoter region.
- Downstream regulatory sequences comprise of GC-rich inverted repeats followed by four adenine (A) residues signal the termination of transcription.

Trans-acting elements:

The protein factors which regulate the expression of gene by binding to cis acting DNA sequence are termed as trans-acting elements. Trans-acting molecules generally have two domains: DNA binding domain (which binds to cis elements) and protein binding domain (required for activation or suppression of transcription). Transcription initiation is a tightly regulated process controlled by trans-acting elements both in prokaryotes as well as eukaryotes.

DNA – Protein interactions:

Gene regulatory proteins and the transcription factors are capable of binding to the DNA based on the interaction of amino acids of the protein with the nucleotides of the DNA. The regulation is implemented through various interactions between cis-acting elements and trans-acting factors. Examples of some DNA binding proteins are:

- Eukaryotic TATA-binding protein
- σ subunit of bacterial RNA polymerase etc.

There are four types of structures of DNA binding proteins,

1. Zinc finger proteins
2. Helix loop Helix protein
3. Leucine zipper proteins
4. Homeodomain proteins

Protein – Protein interactions:

Protein-protein interaction is present in both prokaryotes and eukaryotes. The external signals affect the gene expression with the help of such interaction. Proteins interact with each other to form a homo or hetero-dimers before binding to DNA molecules. Eg. transcription initiation factors interacts with TATA box binding proteins (TBP) to activate transcription of a gene.

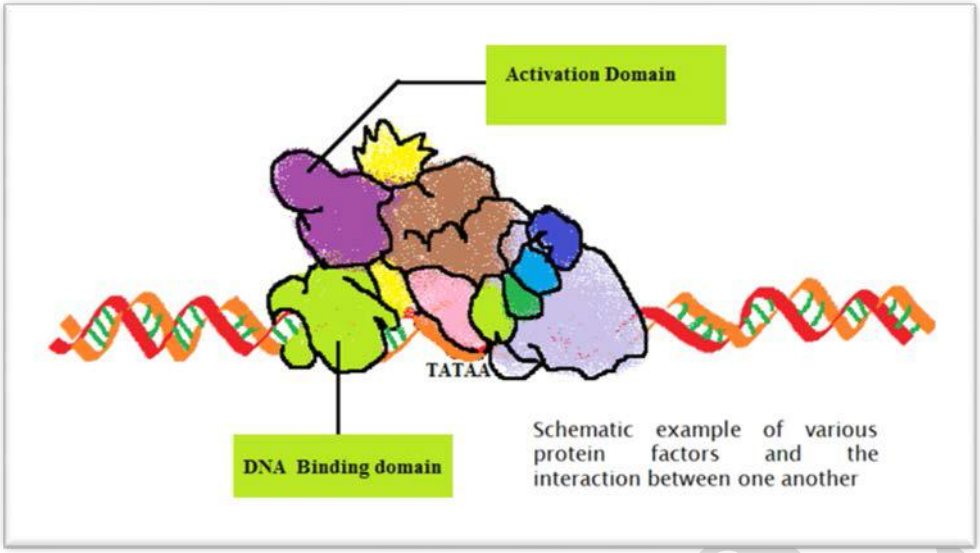


Fig1-1.6.2: Interaction between various protein factors

RNA Polymerase:

- A single RNA polymerase is responsible for transcribing all types of RNA in prokaryotic system.
- However, eukaryotes have three different RNA polymerases, which have been found to specialize in the synthesis of various types of RNA:
- RNA polymerase I (Pol I) - transcribes rRNA (ribosomal RNA) genes.
- RNA polymerase II (Pol II) - transcribes protein-coding genes or mRNA (messenger RNA).
- RNA polymerase III (Pol III) - transcribes other functional RNA genes (e.g., tRNA).
- In eukaryotes, transcription occurs inside the nucleus. All the enzymes responsible for translation are present in the cytosol therefore the transcripts formed then move out of the nucleus through nuclear pores into the cytosol (the liquid phase of the cytoplasm), where translation occurs. Organelle genomes (like the mitochondrial genome) are transcribed within the organelle (the mitochondria) and translation is also within the organelle (the mitochondria).

Since prokaryotes have no nucleus, the step involving the movement of transcripts from nucleus to cytoplasm does not take place, and translation can take place immediately in the cytoplasm, directly on the growing transcript.

Cloning vectors: Plasmid as Cloning Vectors, Binary vectors and co-integrate vectors. Plasmids types in Gram negative bacteria - *E. coli* - PBR322. Bacteriophage λ and M13, Cosmids - phagemid, Yeast vectors, Shuttle vectors, Ti – plasmid and Ri plasmid. Prokaryotic hosts: *E. coli*, Eukaryotic hosts - yeast cells.

Cloning vectors are designed for efficient transfer of foreign DNA into the host. Expression vectors have efficient machinery for cloning and expression of foreign gene in the host system. Selection of a vector depends upon various criteria decided by the experimental goal.

CloningVector:

A cloning vector is defined as a vector used for replication of a cloned DNA fragment in a host cell. These vectors are frequently engineered to contain "ori" – origin of replication sites particular to the host organism. Examples of commonly used cloning vectors are: pUC18, pUC19, pBluescript vectors etc.

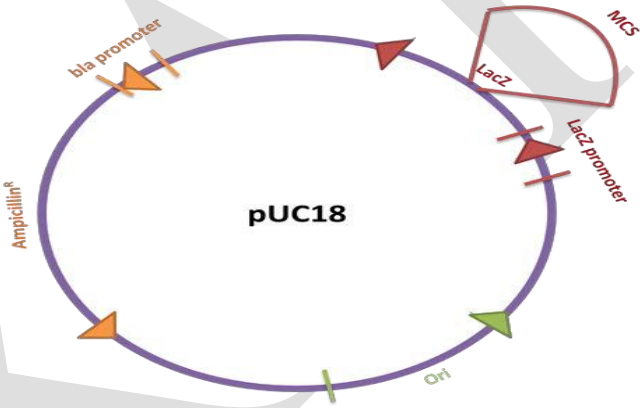


Fig 1-3.5: Cloning vector

Important features of a cloning vector used to carry DNA molecules are as follows:-

- Stability in host cell: Vectors should be stabile in host cell after introduction and should not get lost in subsequent generations. This permits replication of vectors producing large copies of gene of interest.
- Ability to control their own replication: This property enables them to multiply and exist in high copy number.
- Small size: Ideal vector should be less than or equal to 10kb. The small size is essential for

easy introduction in cell by transformation, transduction and electroporation.

- Multiple cloning sites: This property permits the insertion of gene of interest and plasmid re-circularization.

- Should not be transferred by conjugation: This property of vector molecule prevents recombinant DNA to escape to natural population of bacteria.

- Selectable make gene: Vector molecules should have some detectable traits. These traits enable the transformed cells to be identified among the non- transformed ones. eg. antibiotic resistance gene.

Types of Cloning Vectors:

- Cloning vectors extensively used in molecular cloning experiments can be considered under following types: **plasmid**, **phage vector** and **cosmid.**

- Different vectors have different insert size and also vary in mode of replication inside the host.

- Mammalian genes are usually too large (~100 kb) and thus suffer from restrictions in complete inclusion with the conventional cloning vectors having limited insert size.

- Vectors engineered more recently, known as artificial chromosomes, have overcome this problem by mimicking the properties of host cell chromosomes. They have much larger insert size than other vectors.

Vector	Insert size	Source	Application
Plasmid	≤ 15 kb	Bacteria	Subcloning and downstream manipulation, cDNA cloning and expression assays
Phage	5-20 kb	Bacteriophage λ	Genomic DNA cloning, cDNA cloning and expression library
Cosmid	35-45 kb	Plasmid containing a bacteriophage λ *cos* site	Genomic library construction
BAC (bacterial artificial chromosome)	75-300 kb	Plasmid ocntaining *ori* from *E.coli* F- plasmid	Analysis of large genomes

YAC (yeast artificial chromosome)	100-1000 kb (1 Mb)	*Saccharomyces cerevisiae* centromere, telomere and autonomously replicating sequence	Analysis of large genome, YAC transgenic mice
MAC (mammalian artificial chromosome)	100 kb to > 1 Mb	Mammalian centromere, telomere and origin of replication	Under development for use in animal biotechnology and human gene therapy

Table 1-3.6: Different type of cloning vectors

Examples of Cloning Vector:

pBR322

- pBR322 is a widely-used *E. coli* cloning vector. It was created in 1977 in the laboratory of Herbert Boyer at the University of California San Francisco. The *p* stands for "**plasmid**" and *BR* for "**Bolivar**" and "**Rodriguez**", researchers who constructed it.
- pBR322 is 4361 base pairs in length.
- pBR322 plasmid has the following elements:

  □□"*rep*" replicon from plasmid pMB1 which is responsible for replication of the plasmid.

  □□"*rop*" gene encoding Rop protein. Rop proteins are associated with stability of RNAI-RNAII complex and also decrease copy number. The source of "*rop*" gene is pMB1plasmid.

  □ "*tet*" gene encoding tetracycline resistance derived from pSC101 plasmid.

□□"*bla*" gene encoding β lactamase which provide ampicillin resistance (source: transposon Tn3).

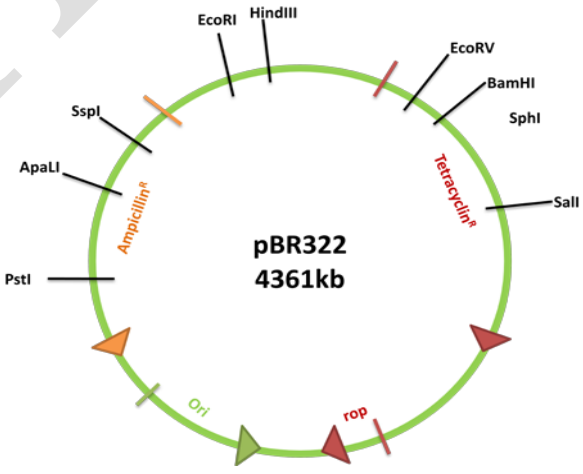


Fig 1-3.7.1.A: Plasmid pBR322.

pUC plasmids:

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

  pMB1 *"rep*" replicon region derived from plasmid pBR322 with single point mutation (to increase copy number).

  "*bla*" gene encoding β lactamase which provide ampicillin resistance which is derived from pBR322. This site is different from pBR322 by two point mutations.

  E.coli lac operon system.

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

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

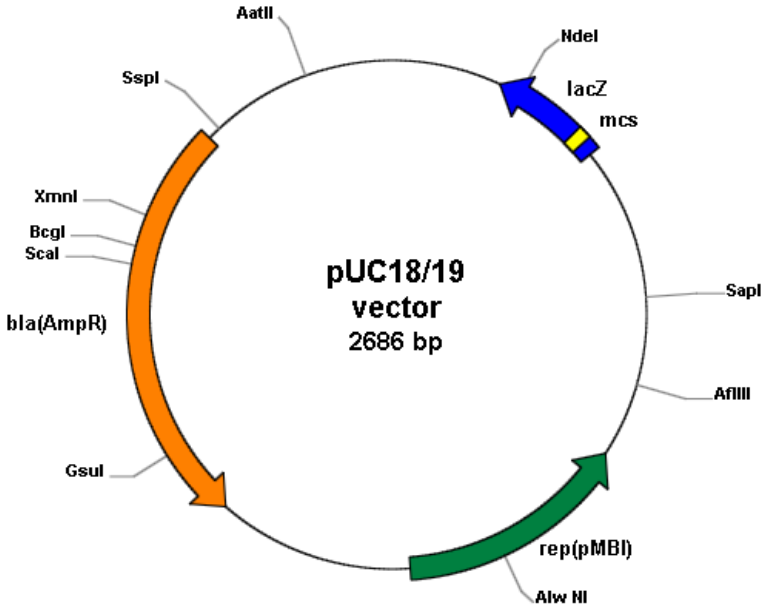


Fig 1-3.7.1.B: pUC plasmid

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

- **Prokaryotic systems** such as *E. coli* are commonly used due to various advantages like, They have-
  - Well studied expression system,
  - Compact genome,
  - Versatile,
  - Easy to transform,
  - Widely available, and
  - Rapid growth of recombinant organisms with minimal equipment.

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

- **Eukaryotic systems** are difficult to handle in contrast to bacterial hosts. They are favoured for

expression of recombinant proteins which require post translational modification and only if they can grow easily in continuous culture.

Choice of vector:

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

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

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

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

Choice of host organism:

A good host should have the following properties:

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

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- High copy number

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

List of different vectors

- **Plasmids** are circular DNA molecules that exist independently of chromosomal DNA and can replicate autonomously. Plasmids carry one or more genes which mostly code for useful characteristic of host. All plasmids have sequence that can act as origin of replication. Plasmids of different sizes and possessing different copy number are present.
- **Phage vectors** are consist of mainly DNA molecule (sometimes RNA); that carrys large number

of genes and are surrounded by a protein coat called as capsid. They can be used as vehicles for carrying DNA insert after modification to remove pathogenic genes and minimizing the size.

- **Cosmids** are hybrid between a phage DNA and bacterial plasmid. They have *cos* sites which are essentially required for packaging lambda (λ) into phage protein coat. They can carry large DNA insert.
- **Fosmids** are cosmid like plasmid but they are based on F-plasmid.
- **Phagemids** are plasmids having a part of M13 genome.
- **Artificial chromosomes** are artificially constructed DNA construct used for transferring DNA.

Preparation of vector DNA:

The vector DNA is cleaved by restriction endonucleases at the site where foreign DNA is desired to be inserted. The restriction enzyme is selected to generate a configuration at the cleavage site compatible with the ends of the foreign DNA. This can be achieved either by cleaving the foreign DNA and vector DNA with the same restriction enzyme or by adding adaptors/ linkers to both the ends of the insert DNA.

Preparation of DNA to be cloned:

DNA to be cloned can be obtained by:

1. Cutting using restriction enzyme from genomic or organellar DNA,
2. PCR based amplification,
3. Chemical synthesis.

The DNA to be cloned is isolated and treated with restriction enzymes to generate random fragments with ends capable of being linked to those of the vector. While choosing the restriction enzyme to cut the desired gene, care should be taken so that the restriction enzyme does not cut in the middle of the gene, but only at the ends. PCR based methods are used to obtain DNA segments, using either genomic DNA or mRNA as template sequences through reverse transcription. Short length sequences can be artificially synthesized *in vitro*. If necessary, linkers or adapters containing desired restriction sites are added to create the ends which are compatible with the vector. The complementary sticky ends result in an efficient ligation due to the formation a stable structure.

Creation of recombinant DNA by ligation

- The vector DNA, foreign DNA and DNA ligase enzyme are added together at appropriate concentrations which results in the covalent linkage between the ends of DNA fragments.

- DNA ligase recognizes the ends of linear DNA molecules and gives a complex mixture of DNA molecules with randomly joined ends.

- The resulting recombinant DNA vector is then introduced into the host organism.

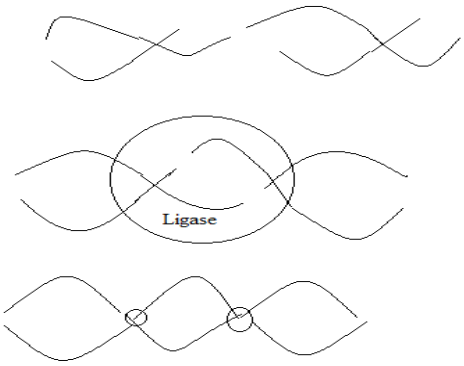

Fig 1-2.2.5: Ligation using ligase enzyme

- In addition to desired recombinant DNA, complex mixture containing self ligated vector DNA, foreign DNA linked with other sequences and several other combinations of vector and foreign DNA also appear in the reaction mixture.

- Sorting of the complex mixture is done by agarose gel electrophoresis based on size of the recombinant vector.

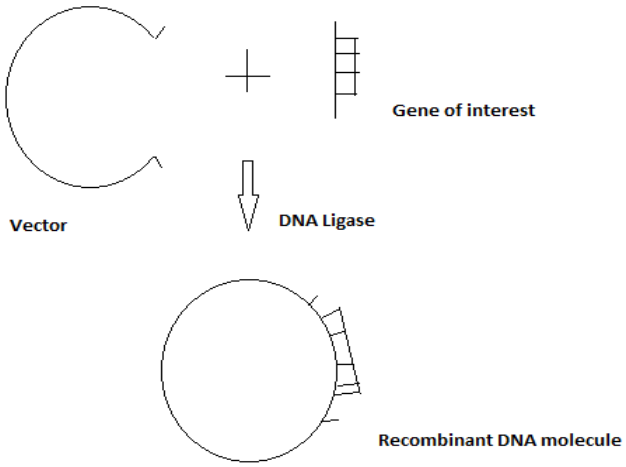

Fig 1-2.2.5.1: Preparation of recombinant DNA

Introduction of recombinant DNA into host organism:

- For the propagation of a cloned gene, the recombinant DNA molecules have to be introduced into a host.

- Numerous methods of gene transfer are available to meet the diverse requirement and compatibility with the host (e.g. transformation, transduction, transfection, electroporation etc.).

- **Transformation** is the process in which microorganisms are able to take up the DNA from their surrounding via plasma membrane and express it. Cells should be competent to take up the foreign DNA.

- DNA transfer into mammalian cells cultured in vitro using non-viral vectors is termed as **transfection**.

- **Transduction** is the process of transfer of DNA molecule using viruses.

- Both transformation and transfection requires preparation of the cells through a specific growth condition and chemical treatment process that varies with the specific species and cell types to be used. For example – Calcium chloride is used for preparation of competent *E.coli* cells.

- Electroporation uses electrical pulses to create transient holes in the cell membrane through which DNA is translocated across the cell membrane. Cell wall has to be previously removed in plants to increase the rate of transfer. Electroporation is usually done by two methods:
    - High voltage for short time,
    - Low voltage for long time.

- Electroporation and transduction are very efficient methods to transfer DNA into cells.

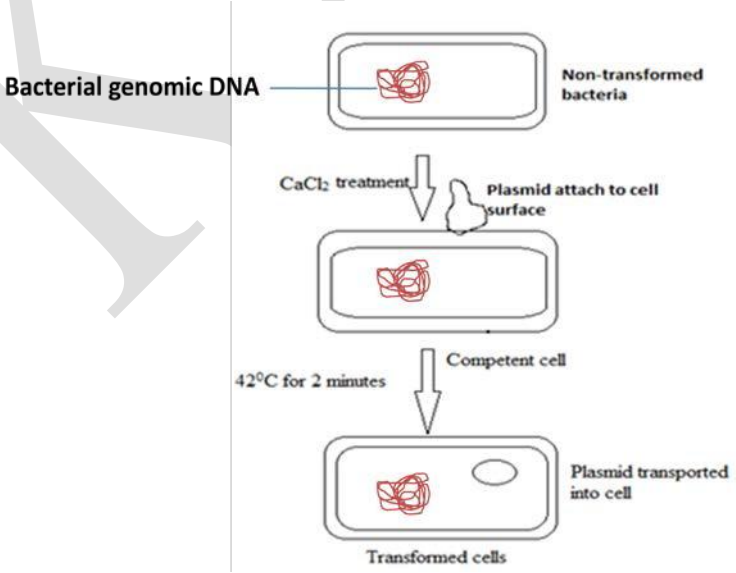

Fig 1-2.2.6: Preparation of competent cells

Selection of host cells/ organism containing vector sequences:

Selection of the transformed cells from the non-transformed population is done by using selectable marker genes that confers resistance to antibiotics. Hence, cells only having the vector with the resistance gene for the antibiotic would grow in the selection media containing the antibiotic (ampicillin, tetracycline etc.); while the non-transformed cells would die.

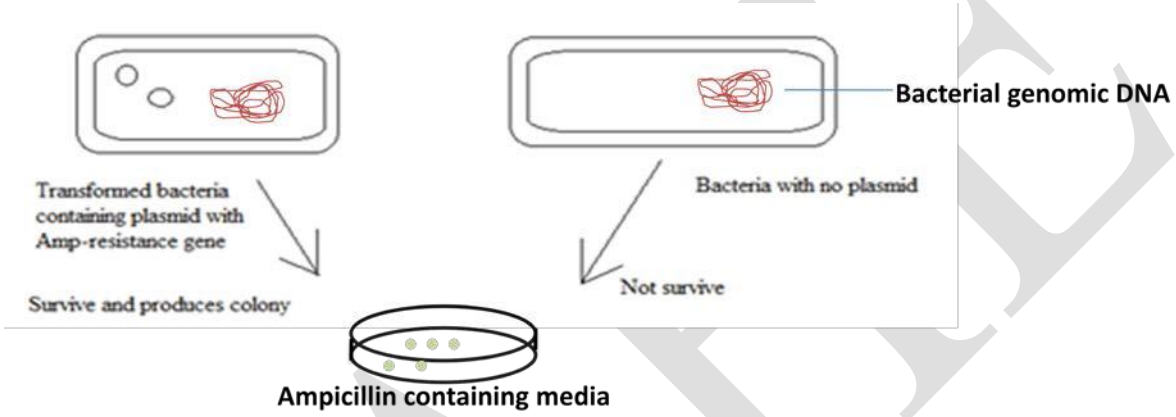

Fig 1-2.2.7: Selection of transformed cells

Screening clones having desired DNA inserts with the help of biological properties:

- After selection of colonies having the vector, the next step is to screen the colonies having the recombinant vector (vector containing foreign DNA insert).
- Bacterial cloning vectors (e.g. pUC19, pGEM vectors) use the blue-white screening system based on *lacZ* system to distinguish transgenic cells from those that contain the parental vector (i.e. vector DNA with no recombinant sequence inserted). The recombinant colonies are grown in presence of X-gal.
- In these vectors, foreign DNA is inserted into a sequence that encodes an essential part of beta-galactosidase (an enzyme which cleaves galactose). Its activity results in formation of a blue-colour colony on the culture medium.
- Insertion of the foreign DNA into the beta-galactosidase coding sequence disrupts the function of the enzyme, and colonies containing recombinant plasmids give no blue colour (white).

- Using this colour phenotype, transgenic bacterial clones can be easily identified from those that do not contain recombinant DNA.

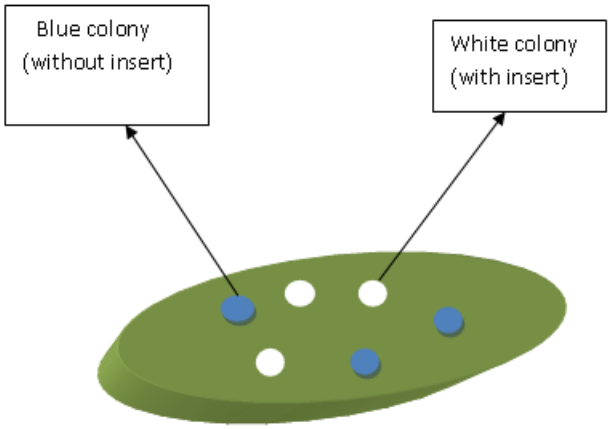

Fig 1-2.2.8: Screening for clones with desired DNA inserts

Insertional inactivation of antibiotic gene can also be used for the selection of recombinant cells.

- A vector is chosen where restriction sites are available for cloning within the antibiotic gene. Insertion of a foreign gene in the restriction site will lead to the loss of activity of the selectable marker (antibiotic) gene. For example-pBR322 have several restriction sites. *BamH1* cuts at a one position within genes that code for tetracycline resistance. Thus recombinant pBR322 carrying foreign DNA at *BamH1* site will not confer resistance to tetracycline, but are still resistant to ampicillin, which remains elsewhere.
- These recombinant cells are selected by replica plating method. The transformed cells are first plated on ampicillin containing medium and after the selection of transformed from non-transformed; the colonies are replica plated on medium containing tetracycline for screening of recombinant clones. After incubation, the viable colonies carrying pBR322 without DNA insert will appear and the positions in plate where the non-viable recombinant clones are present can be easily identified. Using the original master plate, these recombinant clones are picked up and subcultured using the same procedure to obtain a pure recombinant clone.

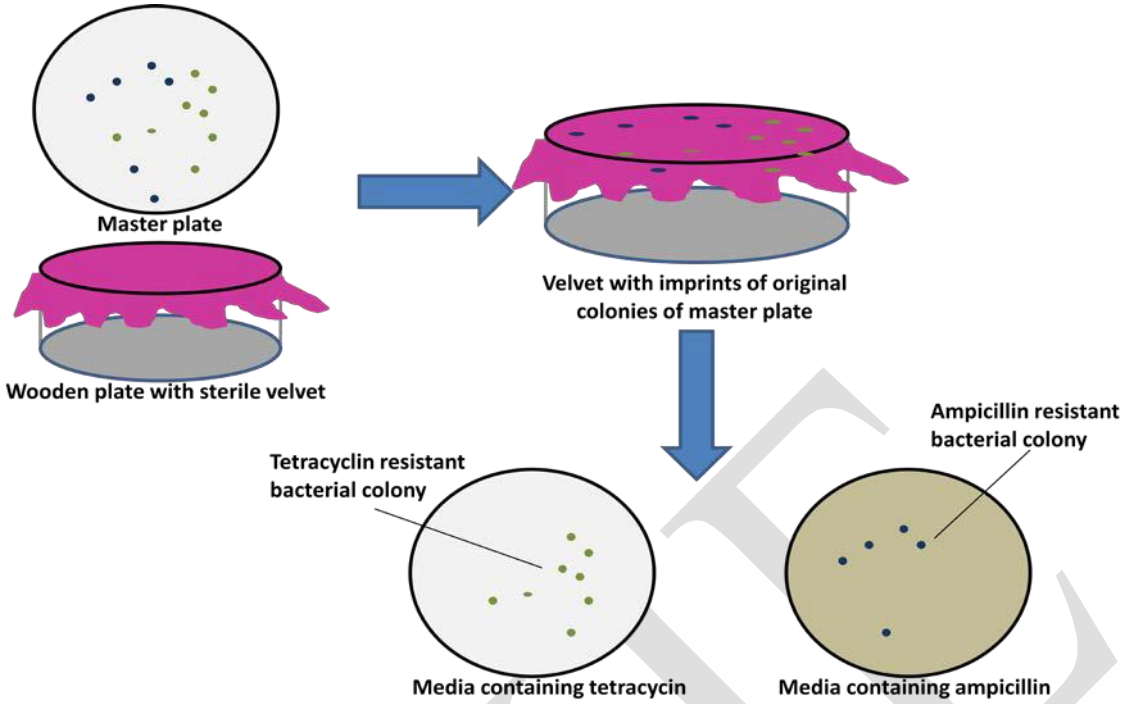

Fig 1-2.2.8.1: Selection through recombinant bacteria by replica plating

Screening for expression:

- Gene expression involves the synthesis of mRNA through transcription followed by synthesis of protein through translation. If the purpose of cloning is to express a foreign gene, it is necessary to check the expression both at the mRNA and protein level in terms of quality and quantity.
- Screening for foreign gene mRNA transcript can be done by:

Northern blotting: It involves the electrophoresis for the separation of RNA on the basis of size, and then transfer of RNA from the electrophoresis gel to the blotting membrane. It is then detected by a hybridization probe complementary to target sequence.

Reverse transcriptase PCR: The RNA strand is reverse transcribed into its DNA complement (cDNA) using the enzyme reverse transcriptase and the resulting cDNA are then amplified using routine PCR method.

- Screening for foreign protein is done by:

Western blotting: It involves the process of electrophoretic separation of cellular proteins followed by transfer to a blotting membrane, incubation with a complimentary primary antibody probe and

detection with a labelled secondary antibody.

- **Activity/functional testing assay or staining**: The proteins which have specific functionality or activity can be tested in- vitro to confirm the presence of the same. For example, protease activity testing or staining.

Quantitative evaluation of the expression levels of the protein is necessary to achieve desired amount of protein.

Applications:

Molecular cloning serves as a tool for developing of various recombinant cells and organisms which has application both in basic and applied biological science as discussed below.

- Production of recombinant proteins: Genes encoding proteins with diagnostic, therapeutic or commercial value can be cloned, expressed and purified to obtain recombinant proteins in bulk with limited space and lesser time. Examples include Humulin – the human insulin expressed in *E. coli.*
- Gene therapy is used for correcting a disorder or deficiency.
- To study the structure and function of a particular gene using a model host organism.
- To study the regulation of gene expression during developmental stages.
- Complete genome sequence of an organism having large genome size can be facilitated by BAC or YAC library formation using rDNA technology.

TYPES, BIOLOGY AND SALIENT FEATURES OF VECTORS IN RECOMBINANT DNA TECHNOLOGY – PLASMID

DNA molecule used for carrying an exogenous DNA into a host organism and facilitates stable integration and replication inside the host system is termed as **Vector**. Molecular cloning involves series of sequential steps which includes restriction digestion of DNA fragments both target DNA and vector, ligation of the target DNA with the vector and introduction into a host organism for multiplication. Then the fragments resulted after digestion with restriction enzymes are ligated to other DNA molecules that serve as vectors.

In general, vectors should have following characteristics:

- Capable of replicating inside the host.
- Have compatible restriction site for insertion of DNA molecule (insert).
- Capable of autonomous replication inside the host (*ori* site).
- Smaller in size and able to incorporate larger insert size.
- Have a selectable marker for screening of recombinant organism.

Plasmids:

- Plasmids are naturally occurring extra chromosomal double-stranded circular DNA molecules which can autonomously replicate inside bacterial cells. Plasmids range in size from about 1.0 kb to over 250 kb.
- Plasmids encode only few proteins required for their own replication (replication proteins) and these proteins encoding genes are located very close to the *ori*. All the other proteins required for replication, e.g. DNA polymerases, DNA ligase, helicase, etc., are provided by the host cell. Thus, only a small region surrounding the *ori* site is required for replication. Other parts of the plasmid can be deleted and foreign sequences can be added to the plasmid without compromising replication.
- The host range of a plasmid is determined by its *ori* region. Plasmids whose *ori* region is derived from plasmid Col E1 have a restricted host range. They only replicate in enteric bacteria, such as *E. coli*, *Salmonella*, etc. Plasmids of the RP4 type will replicate in most gram negative bacteria, to which they are readily transmitted by conjugation. Plasmids like RSF1010 are not conjugative but can be transformed into a wide range of gram -ve bacteria. Plasmids isolated from *Staphylococcus aureus* have a broad host range and can replicate in many other gram-positive bacteria.

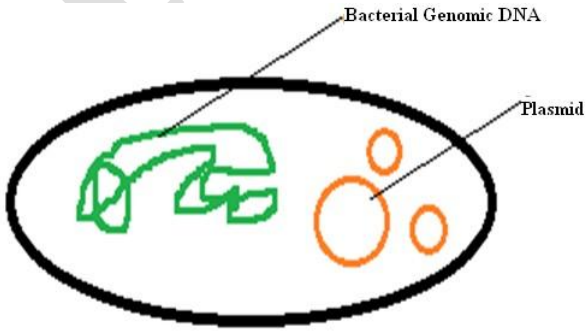

Fig 1-3.2: Bacterial Genomic DNA with plasmid

Some of the phenotypes which the naturally occurring plasmids confer on their host cells:

- Antibiotic resistance
- Antibiotic production
- Degradation of aromatic compounds
- Haemolysin production
- Sugar fermentation
- Enterotoxin production
- Heavy metal resistance
- Bacteriocin production
- Induction of plant tumors
- Hydrogen sulphide production

Most plasmids exist as double-stranded circular DNA molecules. However, the inter- conversion of super coiled, relaxed covalently closed circular DNA and open circular DNA is possible. Not all plasmids exist as circular molecules. Linear plasmids have been found in a variety of bacteria, e.g. *Streptomyces sp.* and *Borrelia burgdorferi.*

However, few types of plasmids are also able to replicate by integrating into bacterial chromosomal DNA; these are known as integrative plasmids or episomes. They are found mainly in prokaryotes but some eukaryotes are also found to harbour them. In prokaryotes they are found in *Escherichia coli***,** *Pseudomonas* species, *Agrobacterium* species etc. In eukaryotes they are mainly found in *Saccharomyces cerevisiae.*

Types of Plasmids

The plasmids are divided into 6 major classes as described below depending on the phenotype:

i) **Resistance or R plasmids** carry genes which give resistance to the bacteria from one or more chemical agents, such as antibacterial agents. R plasmids are very important in clinical microbiology as they can have profound consequences in the treatment of bacterial infections. Eg: RP4 plasmid, which is commonly found in *Pseudomonas* and in many other bacteria.

ii) **Fertility or F plasmids** are conjugative plasmid found in F^+ bacterium with higher frequency of conjugation. F plasmid carries transfer gene (*tra)* and has the ability to form Conjugation Bridge (F

pilus) with F^- bacterium. Eg: F plasmid of *E. coli*.

iii) **Col plasmids** have genes that code for colicins, proteins that kill other bacteria. Eg: ColE1 of *E. coli*.

iv) **Degradative plasmids** allow the host bacterium to metabolize unusual molecules such as toluene and salicylic acid. Eg TOL of *Pseudomonas putida*.

v) **Virulence plasmids** confer pathogenicity on the host bacterium. Eg: Ti plasmids of *Agrobacterium tumefaciens*, which induce crown gall disease on dicotyledonous plants.

vi) **Cryptic Plasmids** do not have any apparent effect on the phenotype of the cell harboring them. They just code for enzymes required for their replication and maintenance in the host cell.

Based on the origin or source of plasmids, they have been divided into two major classes: such as natural and artificial.

i) **Natural plasmids**: They occur naturally in prokaryotes or eukaryotes. Example*:* ColE1.

ii) **Artificial plasmids**: They are constructed *in-vitro* by re-combining selected segments of two or more other plasmids (natural or artificial). Example: pBR322.

Natural Plasmids

Few examples of naturally occurring plasmids and their characteristics are listed in table below

Plasmid	**Size (kb)**	**Origin**	**Host range**	**Antibiotic resistance**	**Additional marker genes showing insertional**
RSF1010	8.6	*E.coli* (strain K-	Broad host range	Streptomycin and sulfonamides.	None
ColE1	6.6	*E.coli*	Narrow host range	None	Immunity to colicin E1
R100	94.2	*E.coli*	*E.coli* K-12, *Shigella flexneri* 2b	Streptomycin, chloramphenycol, tetracycline	Mercuric (ion) reductase, putative ethidium bromide (EtBr) resistant

Table 1-3.3: Characteristics of natural plasmids

RSF1010

- It is a naturally occurring plasmid isolated from *E.coli K-12*.
- This plasmid has broad host range in gram negative bacteria.
- The size of plasmid is 8694bp.
- Antibiotic resistance genes for Streptomycin and sulfonamides have present.
- The replication of RSF1010 starts either bi- or uni-directionally from unique *ori-V* region (2347-2742).
- It cannot initiate transformation independently but can be transferred to host bacterium in presence of helper plasmid.
- Genebank accession no. M28829.

ColE1:

- It is a naturally occurring multicopy plasmid obtained from *E.coli (*copy number is around 40).
- The size of this natural plasmid is 6646bp.
- It forms the basis of many artificial vectors used in molecular cloning.
- The natural ColE1 plasmid has genes for colicin E1 production. Colicin is an antibacterial toxin produced under stressed condition. Cells harboring the plasmid will have resistance against the toxin.
- For using in molecular cloning experiment, colicin genes are replaced with selection marker (antibiotic resistant) gene.
- Genebank accession no. M33100.

Artificial Plasmids:

Naturally occurring plasmids has several limitations; for example, some are stringent and not relaxed (pSC101), some has poor marker genes (ColE1), and some are too large (RSF2124)**.** To overcome the limitations of natural vectors, artificial plasmid are designed by combining different elements from diverse sources.

Artificial plasmids vectors are classified into two broad types based on their use:

1. Cloning vector
2. Expression vector

Apart from the following, there is another class of vectors known as **shuttle vector.** Shuttle vectors can be propagated in two or more different host species (both in prokaryotes and eukaryotes). Hence, inserted DNA can be manipulated and replicated in two different cellular systems.

Cloning vectors are designed for efficient transfer of foreign DNA into the host. Expression vectors have efficient machinery for cloning and expression of foreign gene in the host system. Selection of a vector depends upon various criteria decided by the experimental goal.

CloningVector:

A cloning vector is defined as a vector used for replication of a cloned DNA fragment in a host cell. These vectors are frequently engineered to contain "ori" – origin of replication sites particular to the host organism. Examples of commonly used cloning vectors are: pUC18, pUC19, pBluescript vectors etc.

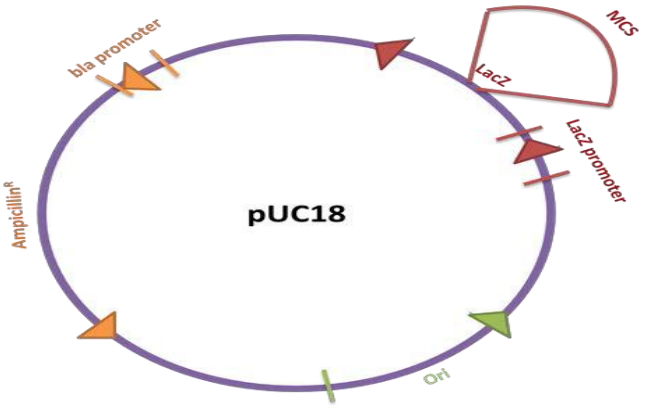

Fig 1-3.5: Cloning vector

Important features of a cloning vector used to carry DNA molecules are as follows:-

- Stability in host cell: Vectors should be stabile in host cell after introduction and should not get lost in subsequent generations. This permits replication of vectors producing large copies of gene of interest.

- Ability to control their own replication: This property enables them to multiply and exist in high copy number.

- Small size: Ideal vector should be less than or equal to 10kb. The small size is essential for easy introduction in cell by transformation, transduction and electroporation.

- Multiple cloning sites: This property permits the insertion of gene of interest and plasmid re-circularization.

- Should not be transferred by conjugation: This property of vector molecule prevents recombinant DNA to escape to natural population of bacteria.

- Selectable make gene: Vector molecules should have some detectable traits. These traits enable the transformed cells to be identified among the non- transformed ones. eg. antibiotic resistance gene.

Types of Cloning Vectors:

- Cloning vectors extensively used in molecular cloning experiments can be considered under following types: **plasmid**, **phage vector** and **cosmid.**

- Different vectors have different insert size and also vary in mode of replication inside the host.

- Mammalian genes are usually too large (~100 kb) and thus suffer from restrictions in complete inclusion with the conventional cloning vectors having limited insert size.

- Vectors engineered more recently, known as artificial chromosomes, have overcome this problem by mimicking the properties of host cell chromosomes. They have much larger insert size than other vectors.

Vector	Insert size	Source	Application
Plasmid	≤ 15 kb	Bacteria	Subcloning and downstream manipulation, cDNA cloning and expression assays
Phage	5-20 kb	Bacteriophage λ	Genomic DNA cloning, cDNA cloning and expression library
Cosmid	35-45 kb	Plasmid containing a bacteriophage λ *cos* site	Genomic library construction
BAC (bacterial artificial chromosome)	75-300 kb	Plasmid ocntaining *ori* from *E.coli* F- plasmid	Analysis of large genomes
YAC (yeast artificial chromosome)	100-1000 kb (1 Mb)	*Saccharomyces cerevisiae* centromere, telomere and autonomously replicating sequence	Analysis of large genome, YAC transgenic mice
MAC (mammalian artificial chromosome)	100 kb to > 1 Mb	Mammalian centromere, telomere and origin of replication	Under development for use in animal biotechnology and human gene therapy

Table 1-3.6: Different type of cloning vectors

Examples of Cloning Vector: **pBR322**

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Fig 1-3.7.1.A: Plasmid pBR322.

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E.coli lac operon system.

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

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

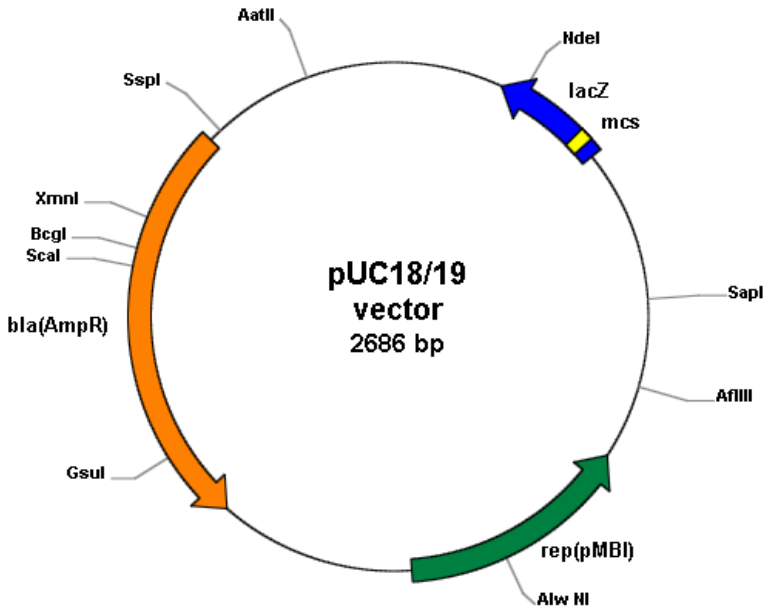


Fig 1-3.7.1.B: pUC plasmid

Expression Vector:

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.

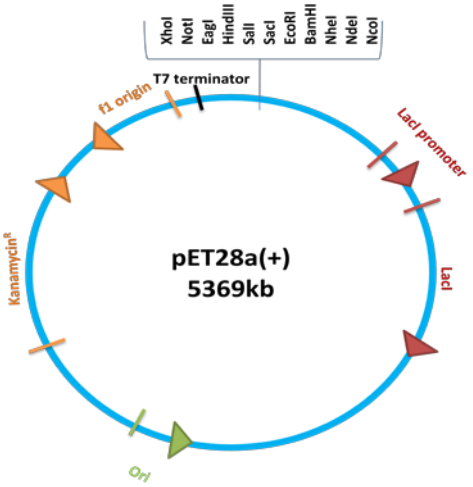


Fig 1-3.8: Expression vector

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 of gene ends. Terminator is short nucleotide sequences which can base pair with itself to form hair pin loop.
- 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.

Examples of Expression Vector:

pET vector:

- pET vector system is a cloning and expression vector system for recombinant protein production in *E.coli*. This product is registered under trademark of Novagen Inc.
- The original pET vector system was constructed by Studier and colleagues. That

plasmid is developed at Novagen with enhanced characteristics.

- Target genes are cloned under strong T7 bacteriophage promoter.
- The expression of the target protein is inducible by providing T7 RNA polymerase in the host cell as an inducing signal.
- Target gene is initially cloned to host cell that do not contain T7 RNA Polymerase, thus increasing plasmid stability.
- Once stabilized in a non-expression host, the recombinant plasmid is transferred to a expression host having T7 RNA Polymerase gene in the genome.
- Ampicillin and kannamycin resistance genes are available in pET vectors as selection marker.
- pET28 and pET32 are the most commonly used pET vectors.

Specialized Expression Vectors:

In molecular biology, vectors are generally designed for cloning a foreign gene into a host genome so that the host produces proteins which are normally not produced by host. But, apart from these applications, different specialized vectors have been constructed to achieve different application in genetic and molecular biology studies. The vectors constructed for thus specialized functions are termed as specialized vectors. Molecular and genetics study of a gene or protein can be aided by specialized vectors. Some of the applications of specialized vectors have been discussed below-

Promoter Probe Vectors:

Specialized vectors used for identification of efficient promoter region in a DNA segment are termed promoter probe vectors. Promoter-less reported genes (lacZ, GFP etc) are used for construction of promoter probe vectors. The expression of the reporter genes can be monitored and quantified easily using various biochemical or fluorescent techniques. Fusion of DNA fragment containing a promoter region upstream of the reporter gene drives the expression of the reported gene. However, there is no guarantee that the DNA sequence that behaves as promoter in recombinant host can behave in the same way in its native host (Pseudo- promoter). Further characterization is necessary to define a true novel promoter. Some of the widely used promoter probe vectors families are: pOT (eg. pRU1161, pRU1097 etc) and pJP2 (eg. pRU1156, pRU1157 etc). pOT vectors have higher copy number but lower stability as compared to pJP2 vectors.

Gene Fusion Vectors:

Fusion of one gene to another gene in order to produce a fusion protein is widely used in molecular biology studies. Fusion proteins are generated by cloning two or more target genes with a reporter gene (*His-tag, gfp, rfp, lacZ* etc) by using gene fusion vectors. Fusion proteins may provide improved properties like easy isolation and purification of target protein (His-tag), easy monitoring of gene expression level (GFP, RFP, *lacZ*), intracellular protein localization studies (GFP, RFP, LUC) etc. The target gene is cloned downstream of the promoter region present in the vector. Depending on the requirement, the target protein can be cloned either to the N-terminal or C-terminal of the reporter protein. Different vectors have been commercially available to provide such flexibility in cloning site and reporter gene.

Viral Vectors:

In recombinant molecular biology, virus particles has been modified to use as a carrier of nucleic acid into a cell, termed as viral vectors. Viral vectors are highly efficient in transferring target DNA/RNA segment to the host cells with high specificity. Wild type virus are modified by deleting the non-essential genes and incorporating exogenous nucleic acid segments to construct a viral vector. Viral vectors have wide application in gene therapy and targeted drug delivery systems. Main advantages of viral vectors are-high transfer efficiency and high cell specificity. Although, there are certain safety issues associated with viral vectors and careful handing is essential during the experimental procedure.

Commonly used viral vectors are- Adenovirus, retrovirus, lentivirus, adeno associated virus (AAV), herpes simplex virus (HSV) etc. The properties of different viral vectors are summarized in the table below.

Viral vector	Insert type	Insert size	Immunogenicity	Host genome
Adeno virus	DNA	2-8 kb	Very high	Non
Retro virus	RNA	2-8 kb	Low	Integrating
Lentivirus	RNA	7-18 kb	Low	Integrating
Adeno associated virus (AAV)	DNA	4.5 kb	Low	Non
Herpes simplex virus (HSV)	DNA	>30kb	Low	Non

Table 1-3.10: Types of viral vectors

Simian Virus 40 (SV40):

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.

Baculoviral 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.

Yeast Vector System:

Cloning and expression of a gene using yeast system has several advantages over E.coli system due to presence of eukaryotic post-translational modification machinery. Expression of complex proteins with proper modification and folding can be achieved by yeast eukaryotic system. These vectors have yeast origin of replication (ARS) for replication and maintenance in the yeast system and bacterial ori for maintaining inside a bacterial system.

Different types of yeast vector include YIp (yeast integrative plasmid), YEp (yeast episomal plasmid), YRp (yeast replicating plasmid), YCp (yeast centromere plasmid) etc.

YIp (yeast integrative plasmid) can integrate to the host genome by homologous recombination. This generally yields a single copy of recombinant vector DNA integrated to the host genome.

YEp (yeast episomal plasmid) can be maintained in the yeast system as an autonomously replicating episomal plasmid. This vector contains a part of 2μ plasmid which is essential for autonomous replication of the vector inside the yeast.

YRp (yeast replicating plasmid) are used to obtain a high copy number inside the host (upto 100 copy number).

YCp (yeast centromere plasmid) possess a centromeric region in addition to ARS which facilitates the mitotic segregation of the linear plasmid during replication. The copy number of this vector is essentially one per cell.

PHAGES, COSMIDS, FOSMIDS, PHAGMIDS AND ARTIFICIAL CHROMOSOMES

You now know that in recombinant DNA technology, vectors are used as carrier of foreign DNA into the host organism. Apart from bacterial plasmids, several other modified vectors are constructed using molecular tools. These "hybrid" vectors are designed by combining different components from various origins (bacteriophage, F plasmid etc) to create a capacity to load larger insert size and higher transfection efficiency.

Phage Vectors:

To insert DNA fragments of more than 10 kb, normally plasmids are not the suitable vehicles, as large inserts may trigger plasmid rearrangement or affect plasmid replication. This leads to development of a new class of vectors based on bacteriophages. Amongst various bacteriophages available such as λ, T4, T5, and T7 phages; the λ phage gained favourable attention due to its unique life cycle.

λ phage

Bacteriophage λ contains ~49kb of DNA and has a very efficient mechanism for delivering its genome into a bacterium. Two key features contribute to its utility as a vector to clone larger DNA fragments:

1. One-third λ genome is nonessential and could be replaced with foreign DNA. Approximately 24.6kb of λ genome can be deleted, hence maximum insert size could be upto 26 kb.

2. Packing of DNA in phage could only take place if the size is between 40 and 52 kb long, a constraint that can be used to ensure packaging.

Two problems had to be addressed before λ -based cloning vectors could be developed:

- The size limitation of the insert is determined by the genome size of phage λ (distance between the cos sites). The size range of the modified genome size should be within the range between 78-105% of the genome size for proper packaging. If >2.4kb is inserted to full length λ vector, the packaging efficiency is reduced. Hence λ vector should be smaller in size than wild type λ genome.
- The large λ genome has a few unique recognition sequences for bacterial restriction endonuclease. Bacterial restriction digestion system may target the

modified vector and cleave the λ DNA molecule. This limitation can be overcome by replacing or mutating the restriction sites.

Two types of vector have been developed using λ genome:

1) Insertion vectors:

- Foreign DNA sequence is inserted into the λ genome without any significant change of the wild type genome.
- Smaller insert size (upto ~10kb).
- They may contain a multiple cloning site inserted in *lacZ* system for screening of recombinant bacterial colonies.
- Can be used to clone smaller DNA molecule.
- Eg: λ ZAP, λ gt etc.

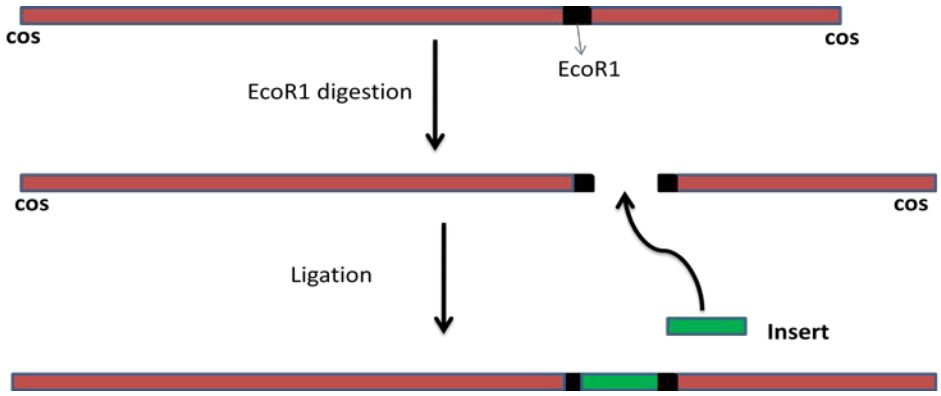


Fig 1-4.2.1: Insertion vector

2) Replacement vectors:

- Full length λ molecule having two identical restriction sites flanked by "stuffer fragment".
- Stuffer fragment is replaced by foreign DNA during restriction cloning.
- The vector without the foreign insert cannot be packaged due to the size limitation (smaller than the required).
- Insert size ranges between 10-23 kb.
- Example: λ EMBL 3, λ EMBL 4, λ DASH etc.

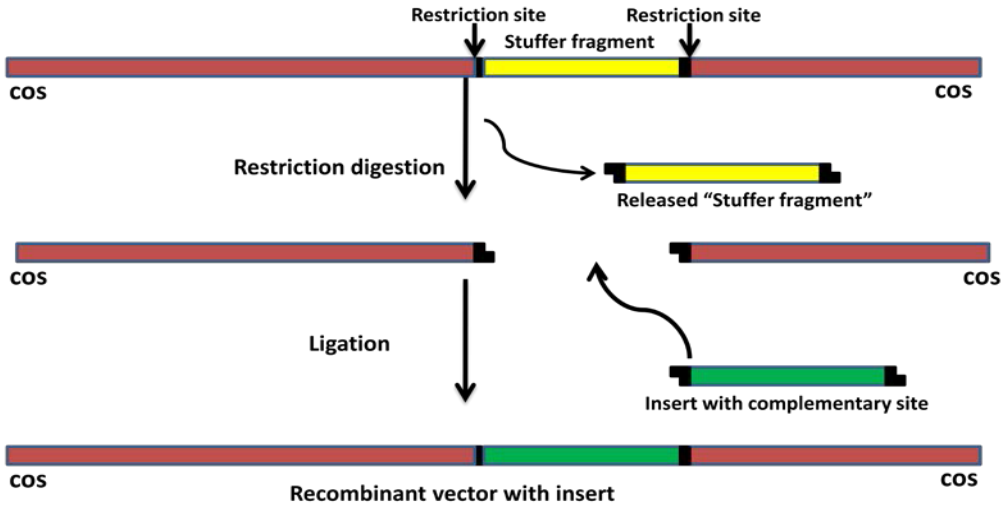


Fig 1-4.2.2:

Replacement vector

Features of λ Phage Vectors:

The λ genome is linear, but in the ends has 12-nucleotides overhangs, termed as ***cos*** sites, which are complementary to each other. A λ cloning vector can be circularized using *cos* site which can be manipulated and replicated inside *E. coli* via the process of transfection.

Alternatively, a more efficient uptake system called in vitro packaging can be utilized. Treatment with the appropriate restriction endonuclease followed by ligation in presence of insert DNA, produces 'left arm-new DNA-right arm' concatemers that are then added to an *in vitro* packaging mix to form λ phage particles. These phages are then co-incubated with *E. coli* cells, and the infection process naturally transports the vector plus new DNA into the bacteria. Bacteria that are infected with the packaged cloning vector die within about 20 minutes and several rounds of phage replication and bacterial lysis forms a zone of clearing, called a plaque.

M13 Phage Vectors:

M13 phage is filamentous phage that infects *E. coli* via F-pilus. The genome is a single stranded circular DNA of size ~6.4kb surrounded by a proteinaceous coat. The DNA strand present in phage is called plus (+) strand. After entering to *E. coli* host, it converts

into double stranded DNA molecule called **replicative form (RF)** by utilizing bacterial machinery. M13 phage as cloning vector can be obtained in both single stranded as well as double stranded form. Replicative form double stranded vector are modified and replicated inside *E. coli* host similar to a plasmid vector. Single stranded vectors can be isolated by collecting M13 phage. M13 vectors have useful application in following areas:

- DNA sequencing
- Mutagenesis study
- probe generation
- Phage display

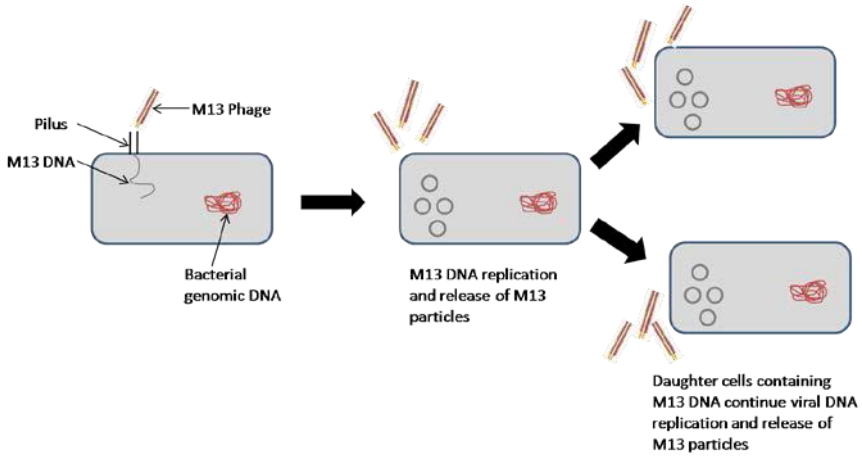


Fig1-4.2.2: M13 Phage Vectors

Cosmid:

- 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.
- *Cos* site is the sequence required by a DNA molecule in order to be recognized as a 'λ genome' by the proteins that package DNA into λ phage particles.
- Cosmid DNA containing particles are as transmittable as real λ phages, but once inside the cell, the cosmid cannot control synthesis of new phage particles and instead replicates as a plasmid.
- Recombinant DNA is therefore obtained from colonies rather than plaques. They

frequently also contain a gene for selection such as antibiotic resistance.

- They are able to load 37 to 52 kb of DNA, while normal plasmids are able to carry only 1–26 kb.
- Sometimes helper phage is used to assist in packaging of cosmid inside phage. Helper phage provides the essential proteins required for packaging which are lacked by cosmid vector.
- For packaging into a phage, concatemer formation is required (cosmid-insert-cosmid). This is generated by using two cos sites flanked by the insertion site for foreign DNA. Providing the inserted DNA in the right size, *in vitro* packaging cleaves the *cos* sites and replaces the recombinant cosmids in mature phage particles.
- Recombinant λ phages are used to infect an *E. coli* culture. Infected cells are plated on a selective medium and antibiotic-resistant colonies are grown. All colonies are recombinants, as non-recombinant linear cosmids are too small to be packaged into λ heads.
- Cosmids are widely exploited to build genomic libraries. The upper limit for the length of the cloned DNA is set by the space available within the λ phage particle. New DNA insert of size up to 44 kb can be inserted before the packaging limit of the λ phage particle is reached.

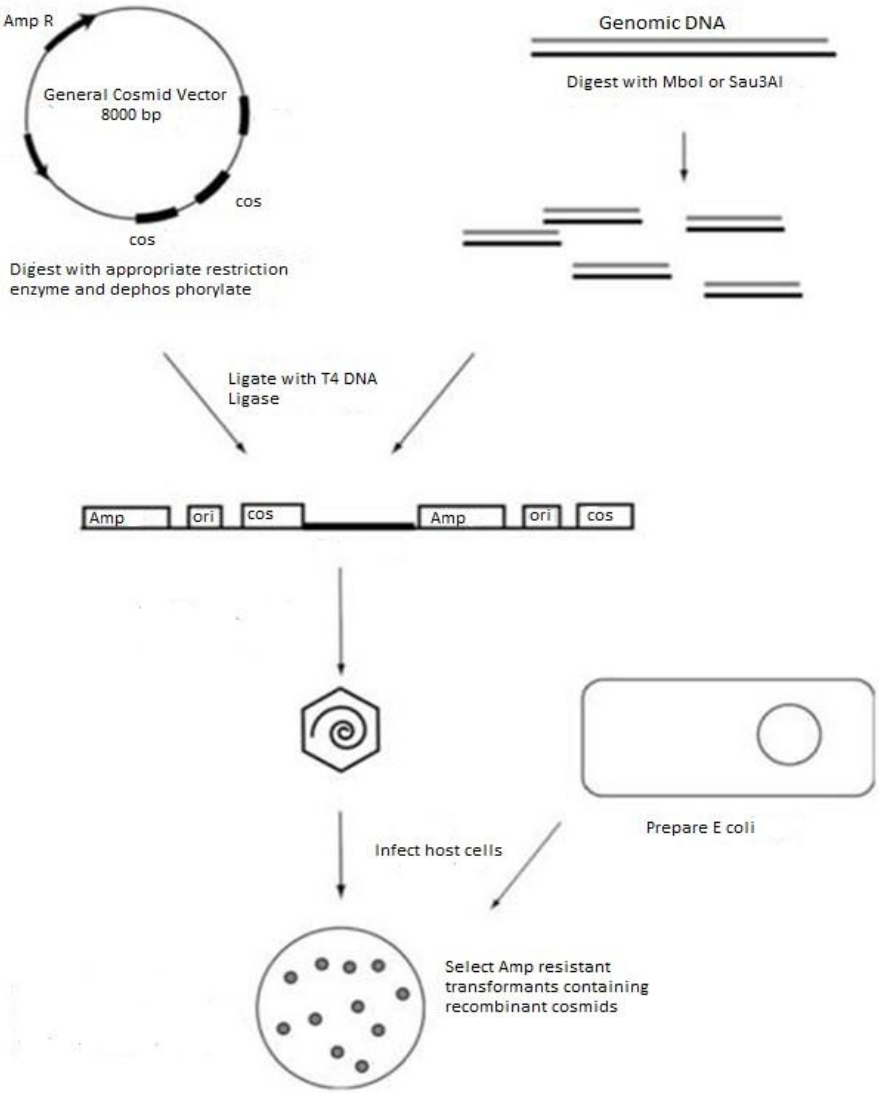


Fig 1-4.3: Schematics for Cosmid Library Construction

Limitation of Cosmid vector:

- Slower replication
- Higher frequency of recombination inside bacterial host.
- Unstable inside *E.coli* host and thus easy to lose vector.

Example:

pJB8 is 5.4 kb in size and carries the ampicillin-resistance gene (amp ^{R}), a segment of λ DNA containing the *cos* site, and an *Escherichia coli* origin of replication (ori).

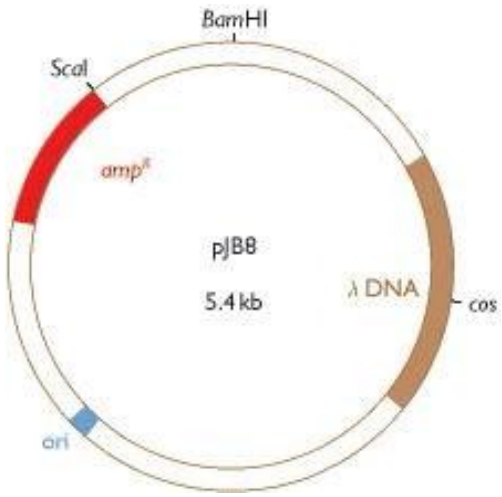


Fig 1-4.3.1: Schematic representation of pJB8

Fosmid:

- Fosmids are similar to cosmids, however they are primarily based on bacterial F-plasmid.
- **Simon and co-workers**, in the year 1992, first developed F-factor based vector named as **pFOS** for stable propagation of cosmid sized human genomic DNA inserts.
- They carry the F plasmid origin of replication and a λ *cos* site.
- Fosmids can carry up to 40 kb of insert DNA.
- The cloning vector is limited, as a host (usually *E. coli*) can only contain one fosmid molecule. Low copy number offers higher stability as compared to high

copy number cosmids.

- Fosmids have high structural stability and have been found to maintain human DNA effectively even after 100 generations of growth. It is ideal to use a fosmid vectors for constructing genomic and meta-genomic libraries.

Fosmids contain several functional elements as discussed below,

□□OriT (Origin of Transfer): The sequence which marks the starting point of conjugative transfer.

□□OriV (Origin of Replication): The sequence starting with which the plasmid-DNA will be replicated in the recipient cell.

□ *tra*-region (transfer genes): Genes coding the F-Pilus and DNA transfer process.

- IS (Insertion Elements): so-called "selfish genes" (sequence fragments which can integrate copies of themselves at different locations).

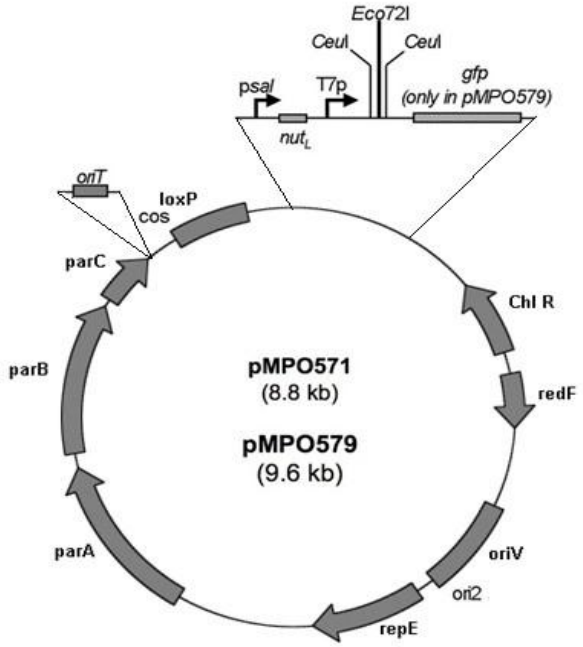


Fig 1-4.4: Schematic diagram of fosmids derived from pCC1FOS-CeuI

Phagemid

Although M13 vectors are very useful for the production of single-stranded recombinant genes, they have certain disadvantages. There is a limit to the size of DNA fragment that can be cloned in an M13vector, with 1.5 kb being the ideal capacity, although fragments up to 3 kb have occasionally been cloned. To overcome this limitation, phagemid vectors were developed by combining a part of the M13 genome with plasmid DNA.

- Phagemids are cloning vectors developed as a hybrid of the filamentous phage M13 and plasmids to produce a vector that can get packed as a phage particle but also can propagate as a plasmid.
- They contain an origin of replication (ori) for double stranded replication inside *E. coli* host, as well as an "f1 ori" to enable single stranded replication and packaging into phage particles. Many commonly used plasmids contain an f1 ori and are thus phagemids.
- Phagemid generally encode no or only one of the capsid proteins of virus. Other structural and functional proteins necessary for phage lifecycle are provided by the helper phage.
- The components present in a phagemid vector are:
    - Origin of replication (ori) of a plasmid.
    - Intergenic region (IG region) which contains the packaging signal for the phage particle and also has replication origin inside phage.
    - A gene encoding phage coat protein.
    - A selection marker.
    - Restriction enzyme recognition sites.

Phagemid vectors are commonly used for "phage display technology" by which a broad range of proteins and peptides can be expressed as fusions to phage coat proteins and displayed on the viral surface. The advantage of phagemid vectors is that double stranded phagemid vectors can be converted into single stranded vectors and packaged into virion particles by infecting the cells with helper phage.

- Phagemid has certain advantages over phage vectors:
  - The carrying capacity of phagemid is higher than phage vectors.
  - Phagemid has higher efficiency in transformation than phage vectors.
  - Phagemids are genetically more stable than recombinant phage vectors.
  - Phagemids can be exploited to generate single stranded DNA template for sequencing purposes.
  - Single stranded phagemid vectors inside the phage can be targeted for site-directed mutagenesis.
  - Single stranded vectors can be used to generate hybridization probes for mRNA or cDNA.

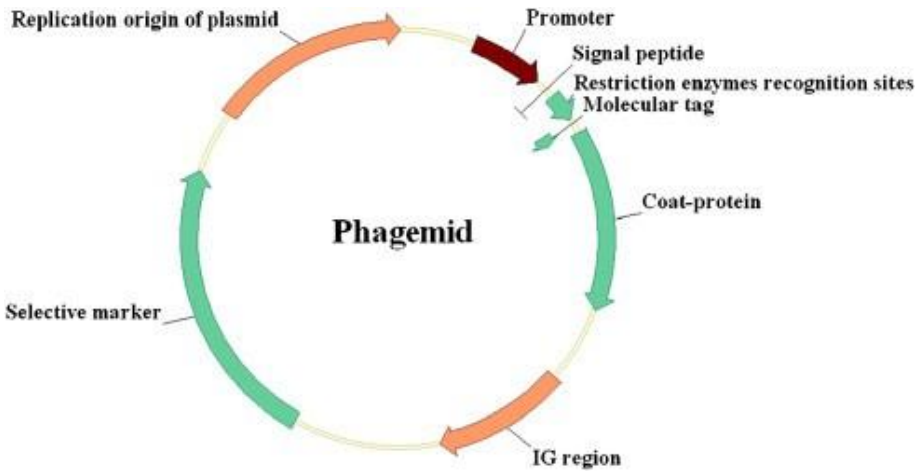


Fig1-4.5: Phagemid vector for phage display

[***Source***: Huan Qi, Haiqin Lu, Hua-Ji Qiu, Valery Petrenko and Aihua Liu (2012); Phagemid Vectors for Phage Display: Properties, Characteristics and Construction; *J.Mol. Bio* (417), 129-143]

Examples of Phagemid:

pEMBL

One of the first hybrid phagmid vectors was pEMBL constructed in 1983. They are characterized by the presence of –

1) The *bla* gene as selectable marker for ampicillin resistance.
2) A short segment coding for the alpha-peptide of beta-galactosidase (lacZ) and containing a MCS.
3) The intragenic (IG) region of phage F1.

These vectors have been used successfully for DNA sequencing with the dideoxy method, and can be used for other purposes for which M13 derivatives are used. However, the pEMBL plasmids have the advantage of being smaller than M13 vectors, and the purification of DNA is simpler. In addition, long inserts have a higher stability in pEMBL plasmids than M13 vectors.

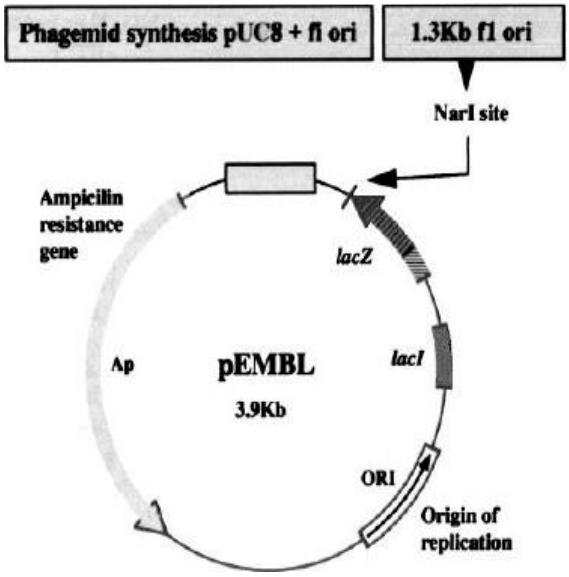


Fig: 1-4.5.1.A pEMBL vector

Artificial Chromosomes:

Artificial chromosomes are DNA molecules assembled *in vitro* from defined constituents that can function like natural chromosomes.

Types of artificial chromosomes:

i) BACs: Bacterial artificial chromosomes
ii) YACs: Yeast artificial chromosomes
iii) MACs: Mammalian artificial chromosomes
iv) HACs: Human artificial chromosomes
v) PACs: P1-derived artificial chromosomes

Some of the artificial chromosomes are discussed below-

Bacterial Artificial Chromosomes: BAC:

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.

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.

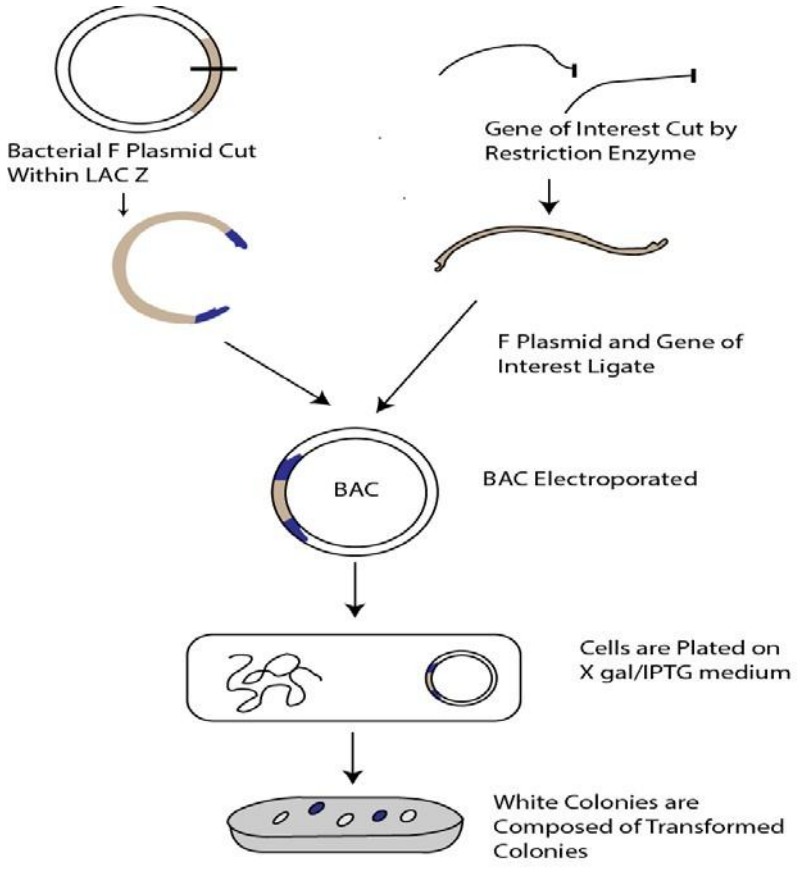


Fig 1-4.6.1: Transforming a Bacterium Using a BAC Vector

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*).

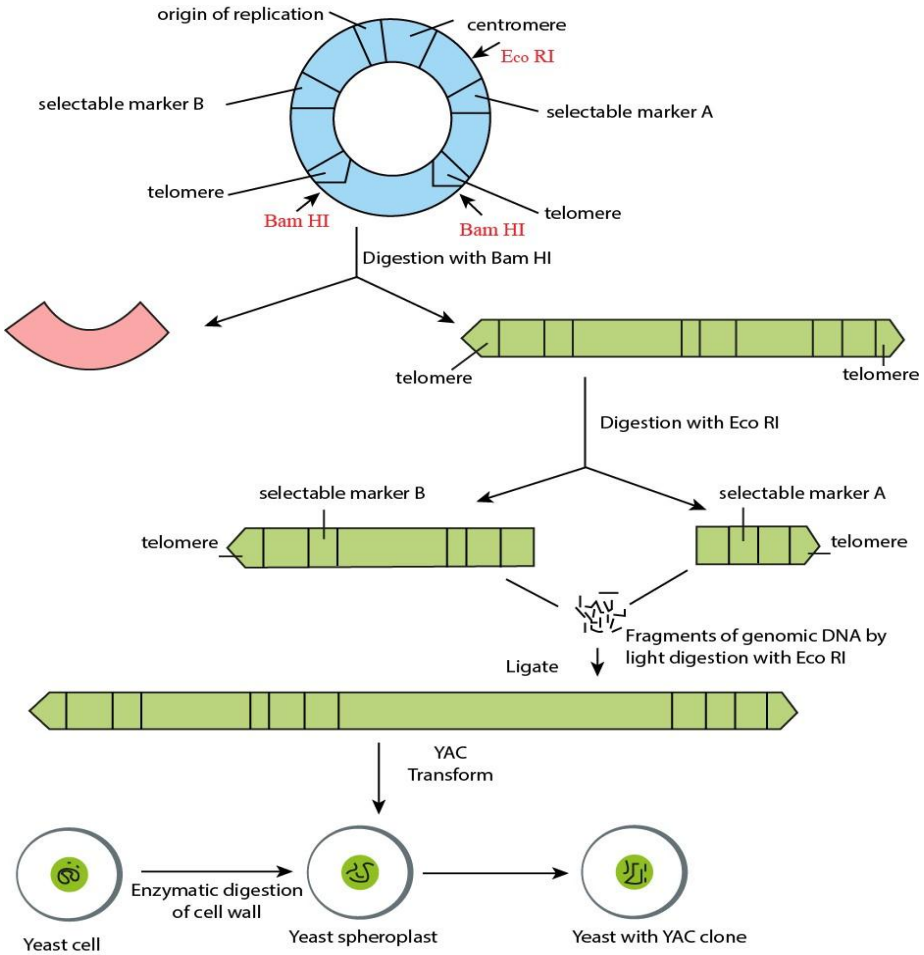


Fig 1-4.6.2: YAC vector

Mammalian Artificial Chromosomes: MAC

MACs or mammalian artificial chromosomes, like YACs, rely on the presence of centromeric and telomeric sequences and origin of DNA replication. They involve autonomous replication and segregation in mammalian cells, as opposed to random integration into chromosomes (as for other vectors). They can be modified for their use as expression systems of large genes, including not only the coding region but can contain control elements. Two principal procedures exist for the generation of MACs.

1) In one method, telomere-directed fragmentation of natural chromosomes is used. For example, a human artificial chromosome (HAC) has been derived from chromosome 21 using this method.
2) Another method involves *de novo* assembly of cloned centromeric, telomeric, and replication origins *in vitro*.

MAC vectors are difficult to assemble as compared to YAC vectors. Mammalian DNA has higher degree of repetition and larger centromere and telomere regions. Also the sequences necessary for chromosome replication in mammalian system are not well defined till now. MAC vectors have application in the field of gene therapy and eukaryotic protein expression and production.

DNA ligation

DNA ligation is an important technique in molecular cloning and in the generation of recombinant DNA (Figure-1). DNA ligation is the act of joining together DNA strands with covalent bonds with the aim of making new viable DNA or plasmids. The enzyme that catalyzes the reaction is called DNA ligase. DNA ligase used in genetic engineering is usually purified from E.coli bacteria that have been infected with T4 phage. 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"). ATP is required for the ligase reaction, which proceeds in three steps: (1) adenylation (addition of AMP) of a residue in the active center of the enzyme, pyrophosphate is released; (2) transfer of the AMP to the 5' phosphate of the so-called donor, formation of a pyrophosphate bond; (3) formation of a phosphodiester bond between the 5' phosphate of the donor and the 3' hydroxyl of the

acceptor (Figure-2). Within the cell the enzyme carries out the very important function of repairing any discontinuities that may arise in one of the strands of a double stranded molecule. Ligases play several vital roles in the cell.

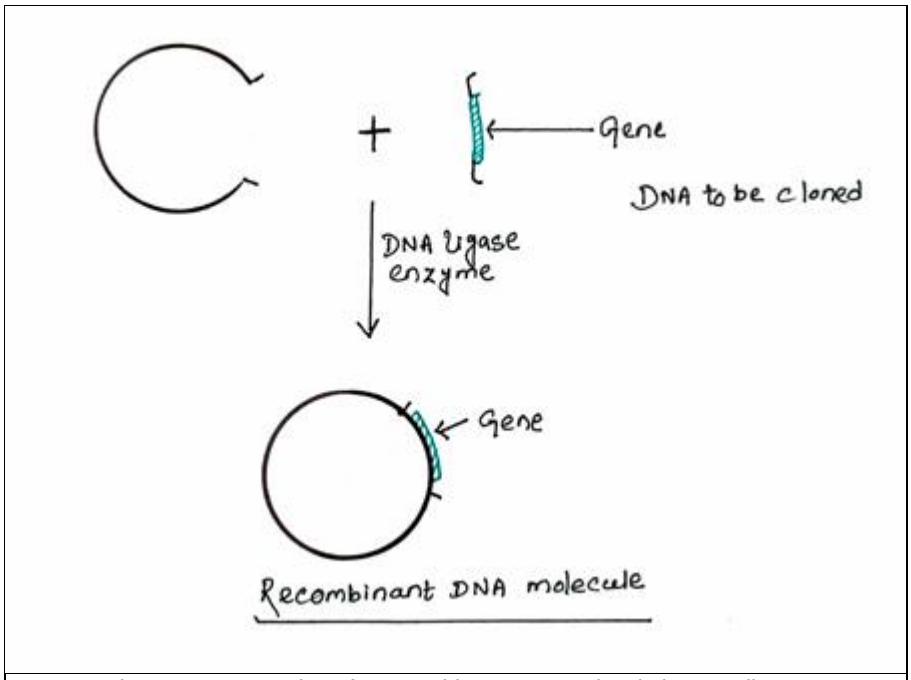


Figure 1: Construction of a recombinant DNA molecule by DNA ligase

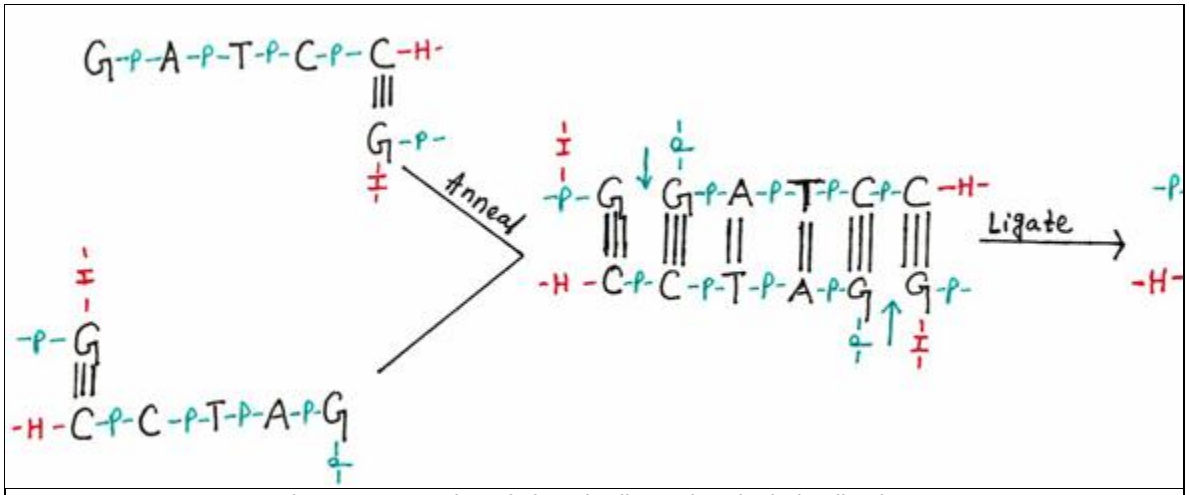


Figure 2: Formation of phosphodiester bonds during ligation

Ligation of complementary sticky ends is more efficient than blunt end ligation, because compatible sticky ends can base-pair with one another by hydrogen bonding forming a relatively stable structure for the enzyme to work on. For optimal ligation efficiency with cohesive-ended fragments ("sticky ends"), the optimal enzyme temperature needs to be

balanced with the **melting temperature Tm** (also the annealing temperature) of the sticky ends being ligated. If the ambient temperature exceeds Tm, the homologous pairing of the sticky ends would not be stable because the high temperature disrupts hydrogen bonding. Ligation reaction is most efficient when the sticky ends are already stably annealed, disruption of the annealing ends would therefore results in low ligation efficiency. Since blunt-ended DNA fragments have no cohesive ends to anneal, the melting temperature is not a factor to consider within the normal temperature range of the ligation reaction. However, the higher the temperature, the less chance that the ends to be joined will be aligned to allow ligation (molecules move around the solution more at higher temperatures). The limiting factor in blunt end ligation is not the activity of the ligase but rather the number of alignments between DNA fragment ends that occur. The most efficient ligation temperature for blunt-ended DNA would therefore be the temperature at which the greatest number of alignments can occur. Under the situation where the vector molecule has sticky ends, but the DNA fragments to be cloned are blunt ended, there the use of linkers and adapters can put the correct sticky ends on to the DNA fragments.

Linkers and adaptors

Linkers are short pieces of chemically synthesized, self complementary double stranded oligo-nucleotides which contain within them one or more restriction sites (Figure-3).

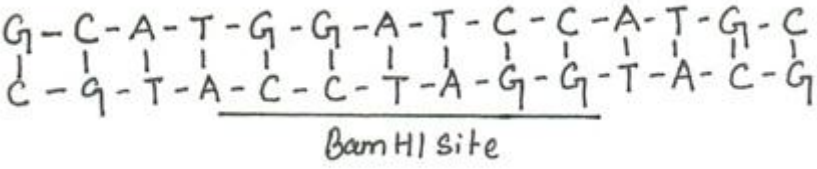


Figure 3: Structure of a linker

DNA ligase will attach linkers to the ends of larger blunt-end ligation. Although a blund end ligation this reaction can be performed very efficiently because synthetic oligonucleotides, such as linkers can be made in very large amounts and added into the ligation mixture at a high concentration (Figure-4). More than one linker will attach to each end of the DNA molecule, producing a chain structure. But digestion with *BamHI* cleaves the chains at the recognition sequences, producing a large number of cleaved linkers and the original DNA

fragment, with *BamHI* sticky ends. Now this modified fragment can be ligated into a cloning vector restricted with *BamHI*.

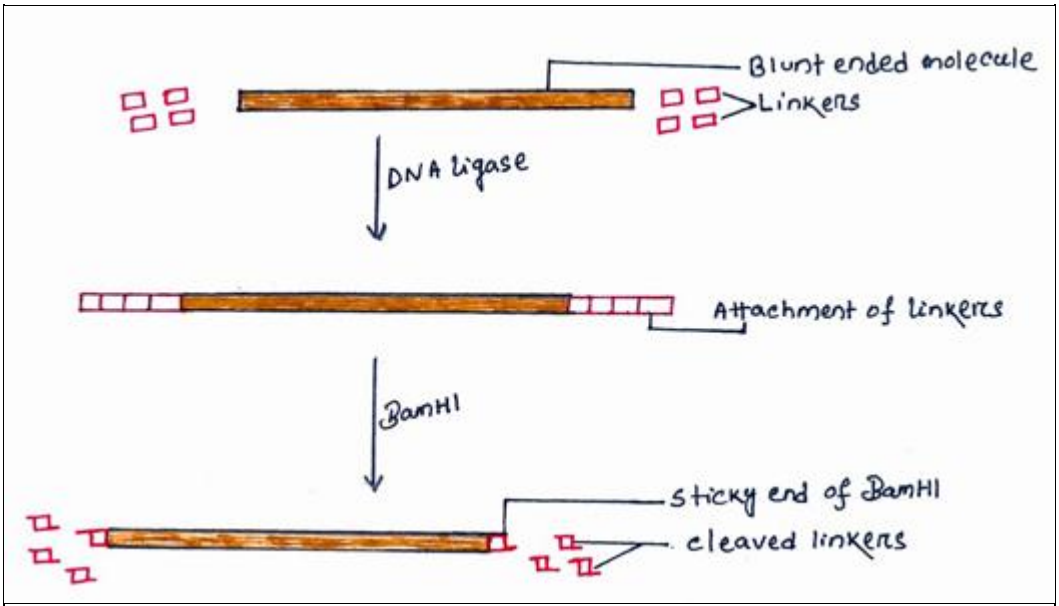


Figure 4: The attachment of linkers to a blunt-ended molecule

Adaptors, are short, chemically synthesized oligonucleotides which can be used to link the ends of two DNA molecules which have different sequences at their ends, when used in conjuction with linkers or other adaptor molecules. An adaptor is synthesized so that it already has one sticky end (Figure-5).

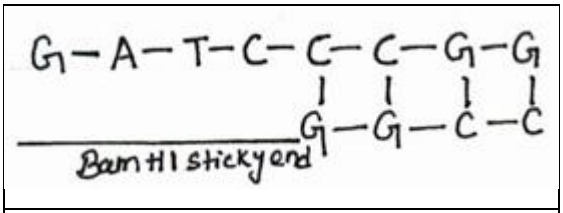


Figure 5: A typical adaptor

During ligation the blunt end of the adaptor ligates to the blunt ends of the DNA fragment to produce a new molecule with sticky ends. But practically, the sticky ends of individual adaptors could base pair with themselves to form dimers (Figure-6) and the new DNA molecule remains blunt-ended.

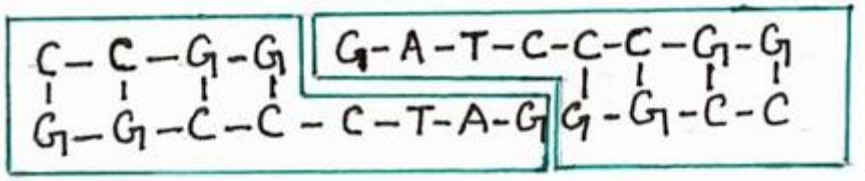


Figure 6: Ligation of two adaptors

For a solution to this problem, adaptor molecules are synthesized so that the blunt end is the same as natural DNA but the sticky end is different. The 3'-OH terminus of the sticky end is unchanged, but the 5' terminus is modified from 5'-P to 5'-OH terminus (Figure-7).

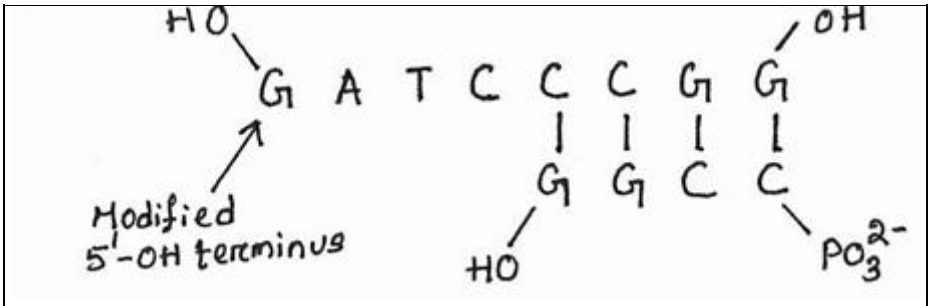


Figure 7: Structure of an adaptor showing the modified 5'-OH terminus

Therefore DNA ligase can't form a phosphodiester bond between 5'-OH and 3'-OH ends. So the base pairing between the sticky ends of adaptor molecules is never stabilized by ligation (Figure-8). Adaptors therefore can be ligated to a DNA molecule but not to themselves. Adaptor molecules alter their 5' terminus(From 5'-P to 5'-OH) by an enzymatic treatment of the enzyme Polynucleotide kinase.

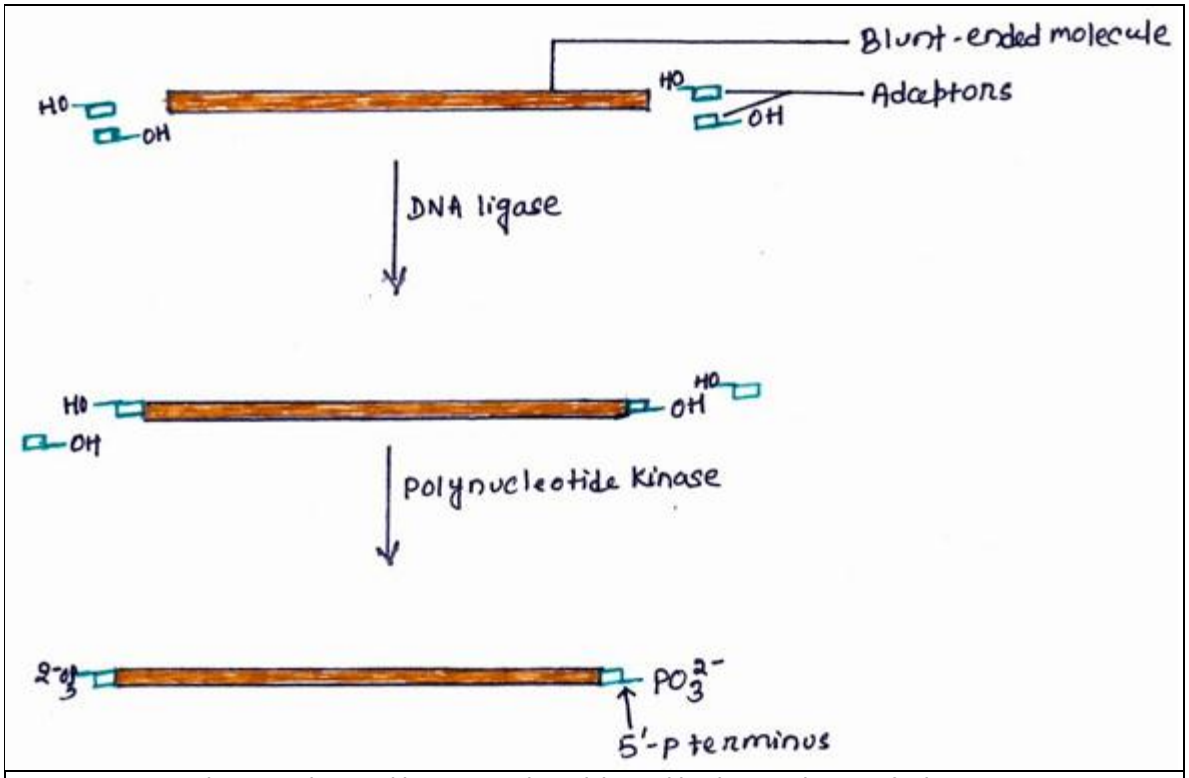


Figure 8: Blunt-end is converted to sticky end by the attachment of adaptors

Homopolymer tailing

It is a technique by which sticky ends can be produced on a blunt-ended DNA molecule. In a homopolymer, all the subunits are same. A DNA strand made up entirely of deoxyguanosine is an example of homopolymer, and is referred to as polydeoxyguanosine or poly(dG). Tailing invoves using the enzyme terminal deoxynucleotidyl transferase to add a series of nucleotides on to the 3'-OH termini of a double-stranded DNA molecule. The reaction when carried out in the presence of just one deoxynucleotide, then a homopolymer tail will be produced (Figure-9).

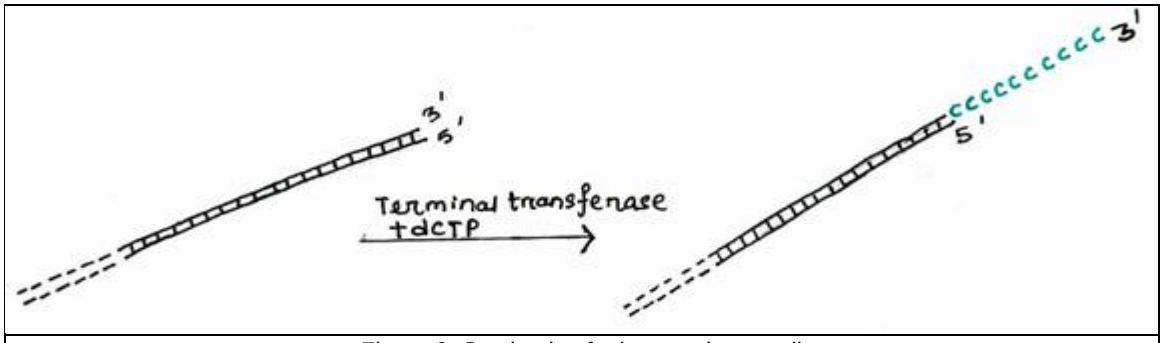


Figure 9: Synthesis of a homopolymer tail

For ligation of two tailed molecules, the homopolymers must be complementary. Frequently poly(dc) tails are attached to the vector and poly(dg) to the DNA to be cloned. Base pairing between the two occurs when the DNA molecules are mixed (Figure-10).

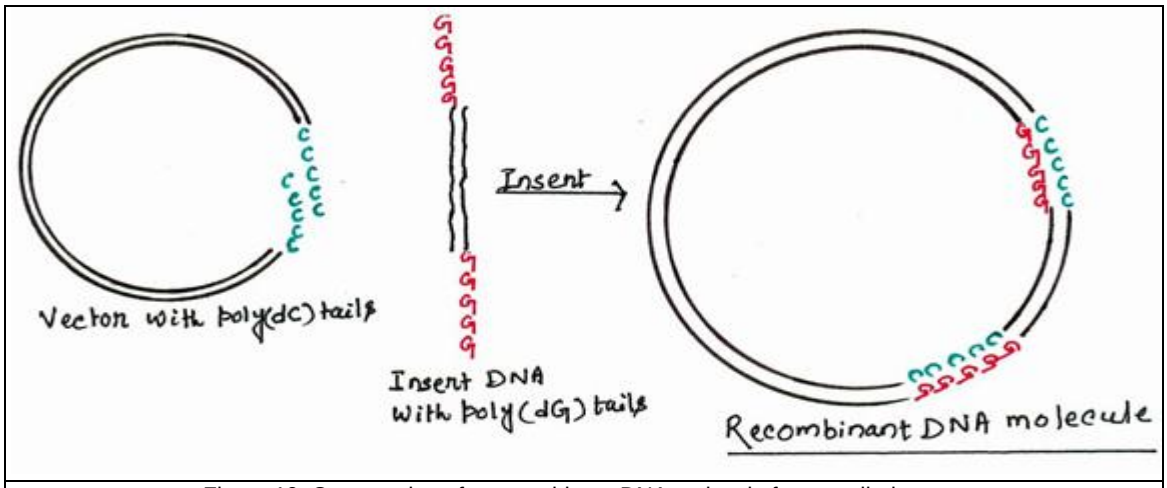


Figure 10: Construction of a recombinant DNA molecule from a tailed vector

A recombinant DNA molecule, held together by base pairing will often be stable enough to be introduced into the host cell. The repair of the recombinant DNA molecule is then done by using the enzyme Klenow polymerase to fill the nicks followed by DNA ligase to synthesize the final phosphodiester bonds (Figure-11).

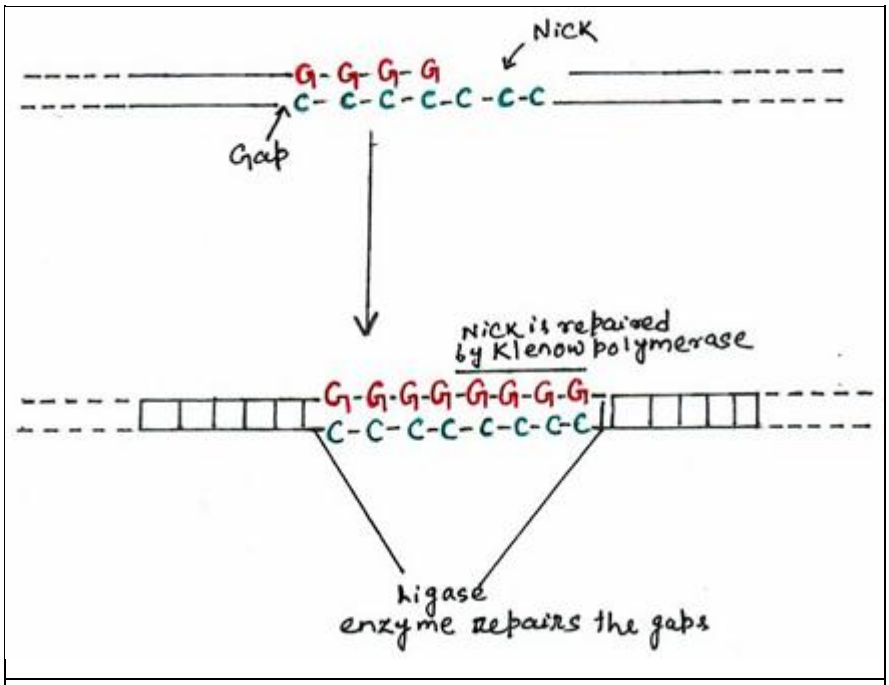


Figure 11: Repair of the recombinant DNA molecule

Recombinant DNA molecules

After DNA ligation, the DNA fragment containing the gene of interest is inserted into a plasmid vector. The cloning vector is treated with a restriction endonuclease to cleave the DNA at the site where foreign DNA will be inserted. The restriction enzyme is chosen to generate a configuration at the cleavage site that is compatible with that at the ends of the foreign DNA . The DNA molecule containing the gene of interest is known as recombinant DNA molecule. Cloning allows a large no of recombinant DNA molecules to be produced from a limited number of starting material. The ligation mixture may contain, in addition to the desired recombinant molecule, any number of unligated vector molecules, unligated DNA fragments, vector molecules that have recircularized without new DNA fragments or the recombinant DNA molecules that carry the wrong inserted DNA fragment (Figure-12). Purification of the desired molecule can achieved through cloning, because it is extremely unusual for any cell to take up more than one DNA molecule.

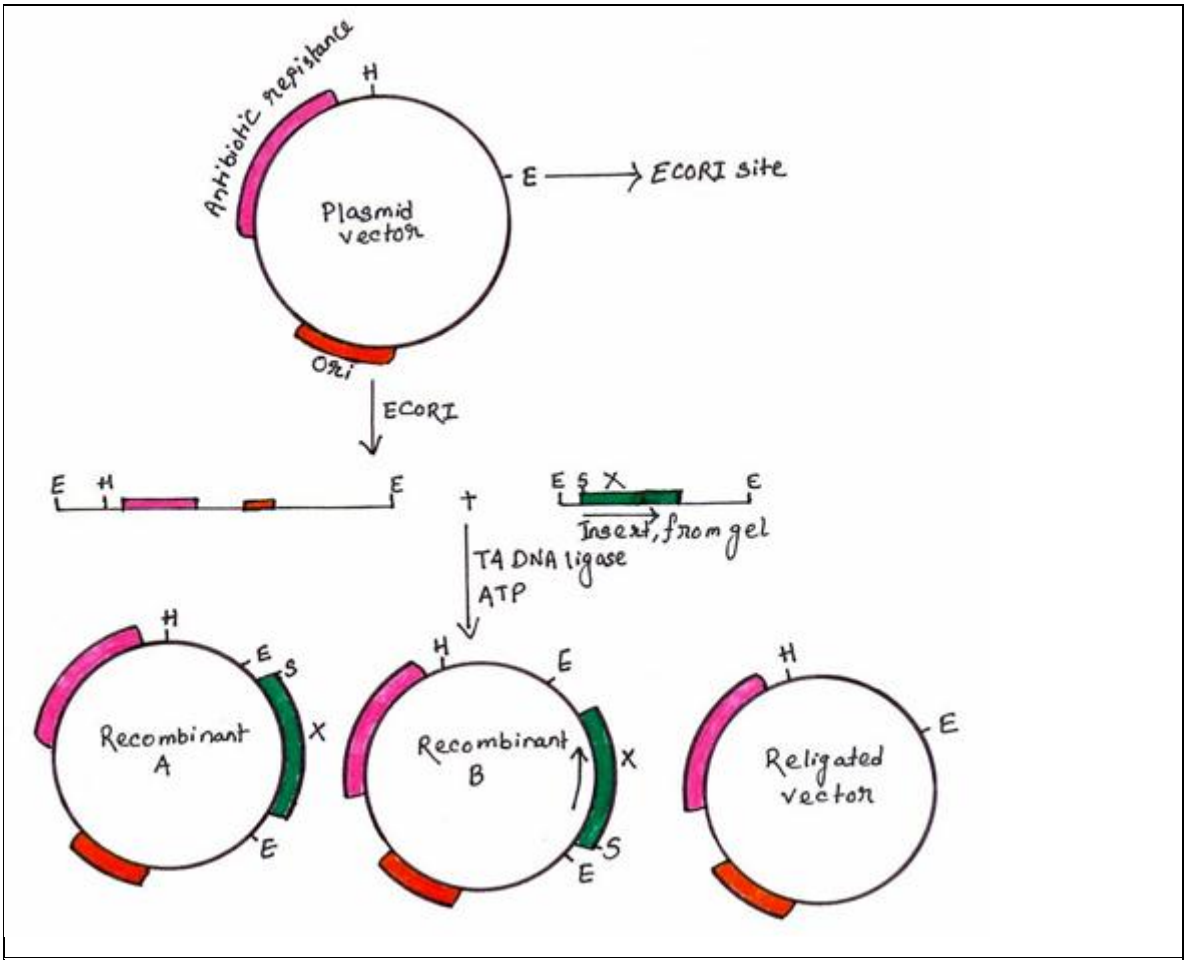


Figure 12: The ligation of vector and insert to form recombinant and non-recombinant products

Transformation

Transformation is the uptake of any DNA molecule by any type of cell, regardless of whether the uptake results in a detectable change in the cell, or whether the cell involved is bacterial, fungal, animal or plant. Bacteria that are capable of being transformed, whether naturally or artificially, are called competent. Transformation is one of three processes by which exogenous genetic material may be introduced into a bacterial cell, the other two being conjugation (transfer of genetic material between two bacterial cells in direct contact), and transduction (injection of foreign DNA by a bacteriophage virus into the host bacterium).

Preparation of competent *E coli* cells

There are two main methods for transformation of competent bacterial cells, the calcium chloride and the electroporation method. In the calcium chloride method, *E.coli* cells treated with solutions containing Ca^{2+} ions were rendered susceptible to take up exogenous DNA, the process known as transformation. Competent cells, are the cells which are pretreated with Ca^{2+} ions for taking up the DNA. In transformation of *E.coli* , a mixture of molecules formed in a ligation reaction, or a solution of plasmid molecules is combined with a suspension of competent cells for a period of time, to allow the DNA to be taken up. The mixture is then given a heat-shock treatment at 42°C for 2 minutes (Figure-13). This induces enzymes, involved in the repair of DNA and other cellular components to allow the cells to recover from the unusual conditions of the transformation process, and increases the efficiency of the process.

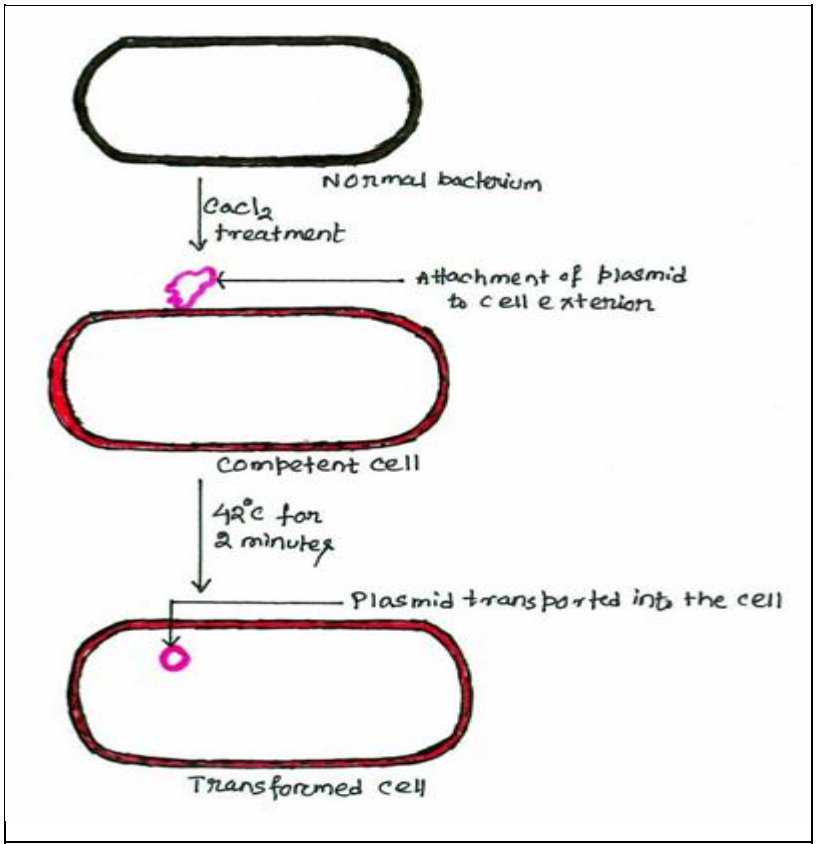


Figure 13: The uptake of DNA by a competent bacterial cell

The cells are then incubated in a growth medium and after a period of incubation they are finally spread on an agar plate and incubated until single colonies of bacteria grow(Figure-14). All the cells within a colony originate from division of a single individual. Thus all the cells will have the same genotype, barring spontaneous mutations, including the presence of any plasmid introduced in the transformation step.

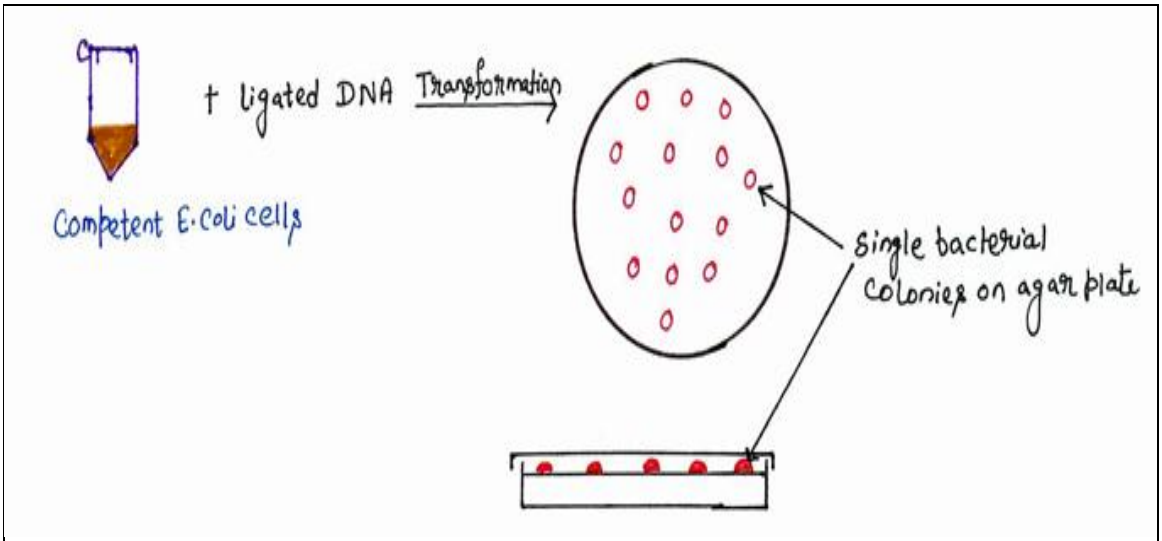


Figure 14: Formation of single colonies after transformation of *E coli*

Selection of transformed cells

Transformation of competent cells is an inefficient procedure; most of the resultant colonies would not contain a plasmid molecule. To make a way for the selection of clones containing plasmid,a selectable marker can be used carried by the plasmid. A selectable marker is a gene, e.g., an antibiotic resistance gene, which provides a transformed cell with a new characteristic which is not possessed by a non-transformant. A plasmid vector having an ampicillin resistance gene, after a transformation experiment shows only those cells that have taken up a plasmid will be ampR and able to to form colonies on an agar medium that contains ampicillin. The non-transformants, will not produce colonies on the selective medium.

Transformation efficiency

Transformation efficiency helps to measure the quality of a given preparation of competent cells. It can be determined as the number of colonies formed on a selective plate per microgram of input DNA(pure plasmid). A transformation efficiency of around 105 per µg would be adequate for a simple cloning experiment.

Screening of the transformants

Once a set of transformant clones has been produced in a cloning experiment, the first requirement is to know which clones contain a recombinant plasmid, with inserted target fragment. With most cloning vectors insertion of a DNA fragment into the plasmid destroys the integrity of one of the genes present on the molecule. Recombinants can therefore be identified because the characteristic coded by the inactivated gene is no longer displayed by the host cells.

Recombinant selection with pBR322

pBR322 has several unique restriction sites that can be used to open up the vector before insertion of a new DNA fragment. *BamHI*, cuts pBR322 at just one position, within the cluster of genes that code for resistance to tetracycline. A recombinant pBR322 molecule, one that carries an extra piece of DNA(new DNA) in the *BamHI* site is no longer able to confer tetracycline resistance on its host, because of the disruption of the necessary genes by the inserted DNA. Cells containing this recombinant pBR322 molecule will still be resistant to ampicillin, but sensitive to tetracycline($amp^R tet^S$) (Figure-15).

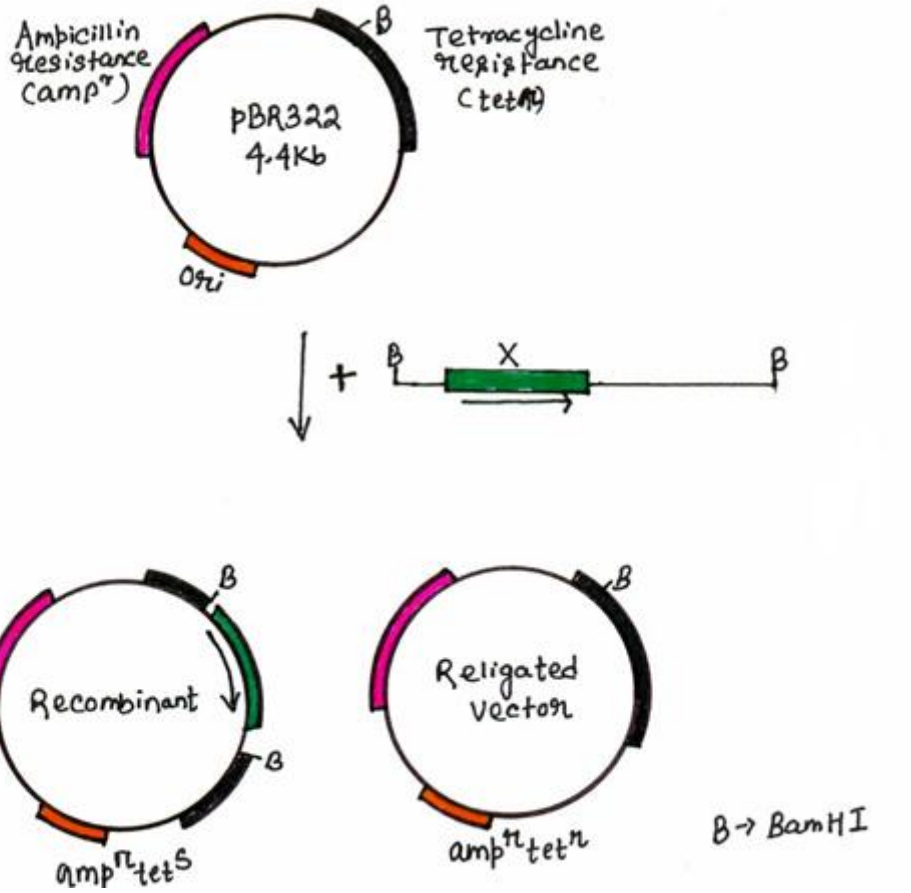


Figure 15: Recombinant selection with pBR322 by insertional inactivation of an antibiotic resistance gene

After transformation the cells are plated on to ampicillin medium and incubated until colonies appear. All of these colonies are transformants , but only a few contain recombinant pBR322 molecules, most contain the normal, self ligated plasmid. To idenify the recombinants the colonies are replica-plated (Figure-16) on to agar medium that contains tetracycline. After incubation, those colonies that will regrow consist of cells that carry the normal pBR322 with no inserted DNA and therefore having a functional tetracycline resistance gene cluster ($amp^R tet^R$). The colonies that do not grow on tetracycline agar are recombinants($amp^R tet^S$).

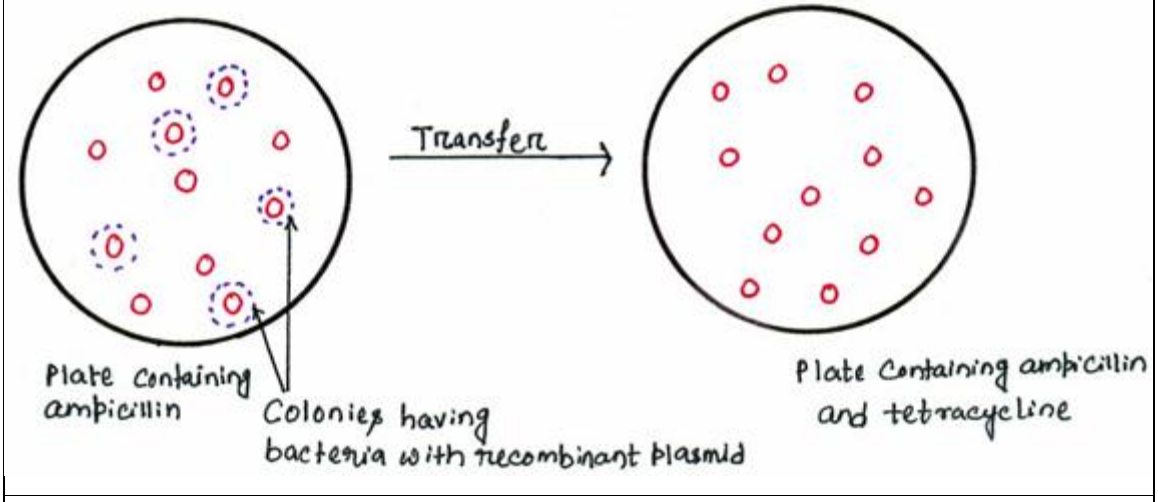


Figure 16: Replica plating

Cloning with pUC8

pUC8 plasmid carries the ampicillin resistance gene and a gene called *lacZ'*, which codes for part of the enzyme β-galactosidase (Figure-17). Cloning with pUC8 involves insertional inactivation of the *lacZ'* gene, and the recombinants are unable to synthesize β-galactosidase for which they can be easily idenified. β-galactosidase is the enzyme involved in the breakdown of lactose into glucose and galactose . It is normally coded by the gene *lacZ*, which resides on the *E. coli* chromosome. Some strains of *E.coli* have a modified *lacZ* gene, one that lacs the segment referred to as *lacZ'* and coding for the α-peptide portion of β-galactosidase. These mutants can synthesize the enzyme only when they hold a plasmid, such as pUC8, that carries the missing *lacZ'*segment of the gene. The *lacZ'* gene has multiple restriction enzyme sites within the first part of the coding region of the gene. This region is known as the multiple cloning site(MCS); insertion of target DNA in any of these sites or between any pair, inactivates the *lacZ'* gene, to give a white colony on an appropriate plate. The use of an MCS allows flexibility in the choice of a restriction enzyme or enzymes for cloning.

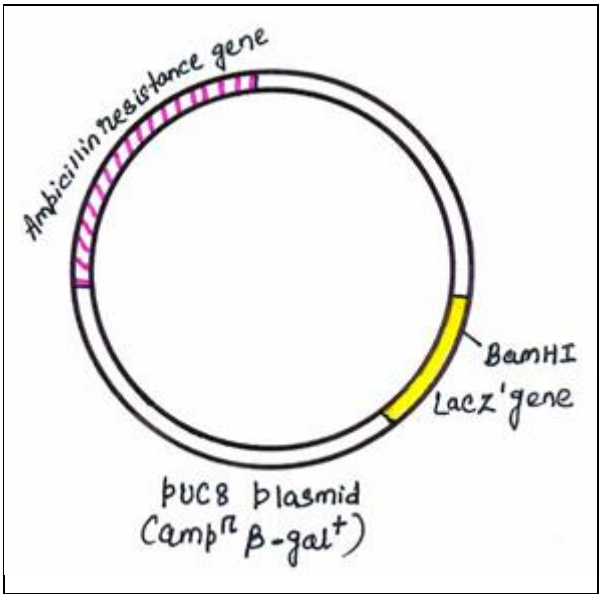


Figure 17: The normal pUC8 cloning vector

In a cloning experiment with pUC8 plasmid, transformants are selected on ampicillin agar medium. The recombinants are identified by screening for β-galactosidase activity. Cells that hold a normal pUC8 plasmid are amp^R and able to synthesize β-galactosidase; whereas recombinants are also amp^R but unable to make β-galactosidase (Figure-18).

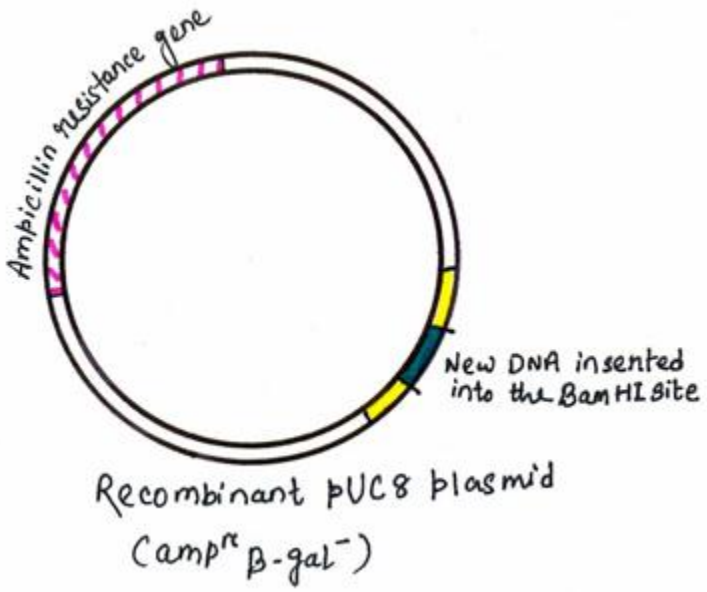


Figure 18: A recombinant pUC8 cloning vector containing an extra piece of DNA inserted into the *Bam*HI site

Screening for β-galactosidase presence or absence is done by involving a lactose analogue called **X-gal(5-bromo-4-chloro-3-indolyl- β-D galactopyranoside)** which is broken down by β-galactosidase to a product that is coloured deep blue. If X-gal with an inducer of the enzyme isopropyl-thiogalactoside(IPTG), is added to the agar, along with ampicillin, then the non- recombinant colonies, the cells of which synthesize β-galactosidase, will be coloured blue, whereas recombinants with a disrupted *lacZ'* gene and unable to make β-galactosidase, will be white (Figure-19).

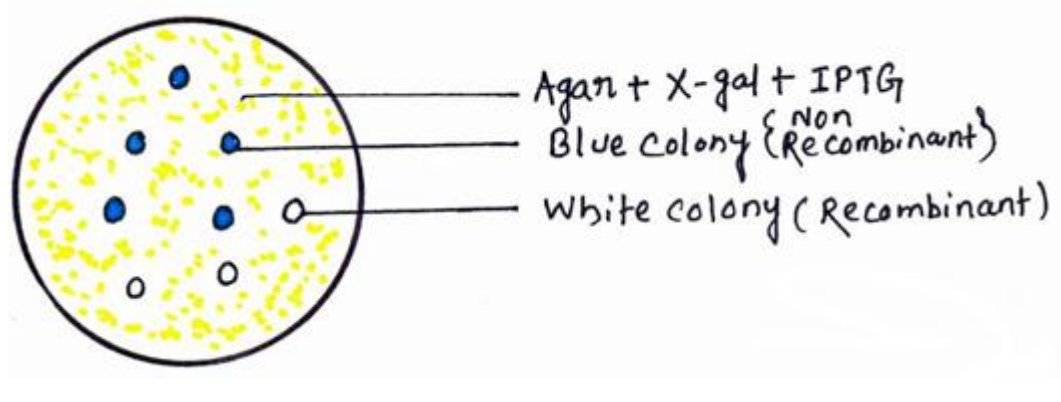


Figure 19: Screening for pUC8 recombinants

Transfection

Transfection is same as transformation but here instead of plasmid, phage DNA is involved. Here also like plasmid DNA the purified phage DNA, or recombinant phage molecule is mixed with competent E.coli cells and DNA uptake is induced by a heat shock treatment.

Visualization of phage infection

Cell lysis is the final stage of phage infection. Immediately after transfection with phage DNA, when infected cells are spread on to a solid agar medium then cell lysis can be visualized as plaques on a lawn of bacteria (Figure-20). Each plaque is a zone of clearing produced as the phage lyse the cells and move on to infect and eventually lyse the neighbouring bacteria. Plaques are formed by both λ phage and M13 phage. True plaques are formed by λ whereas M13 does not form true plaques as λ phage, because it doesn't lyse the host cell. M13 instead causes a decrease in the growth rate of infected cells sufficient to produce a zone of relative clearing on a bacterial lawn.

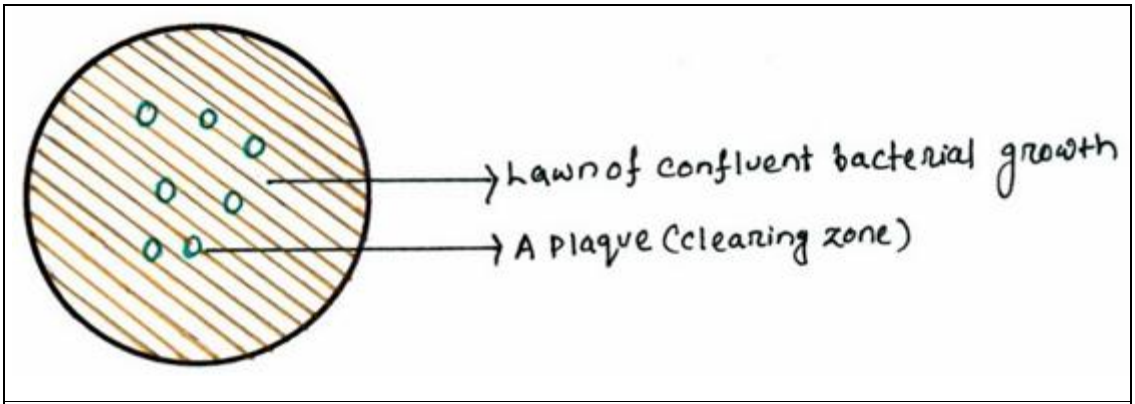


Figure 20: Formation of plaques on a lawn of bacteria

The end result of a gene cloning experiment using a λ or M13 vector is therefore an agar plate covered in phage plaques. Each plaque is derived from a single transfected or infected cell and therefore contains identical phage particles.

M13 phage vectors

The filamentous phage partcles contain a 6.7kb circular single stranded DNA. After infection of a sensitive *E.coli* host, the complementary strand is synthesized, and the double stranded DNA is known as **replicative form(RF)** with about 100 copies per cell. The cells are not lysed by M13, but continues to grow slowly, and single stranded forms are continuously packaged and released from the cells as new phage particles. The same single strand of the complementary pair is always present in the phage particle.

The useful properties of M13 as a vector are that the RF can be purified and manipulated exactly like a plasmid, but the same DNA may be isolated in a single-stranded form from phage particles in the medium.

Identification of recombinant phages

Insertional inactivation of a lacZ' gene carried by the phage vector

All M13 cloning vectors as well as several λ vectors, carry a copy of the *lacZ'* gene. Insertion of new DNA into this gene inactivates β-galactosidase synthesis, same as with the plasmid vector pUC18. Recombinants are distinguished by plating cells on to X-gal agar, plaques of normal phage are blue in colour while recombinant plaques are clear (Figure-21) or white in colour.

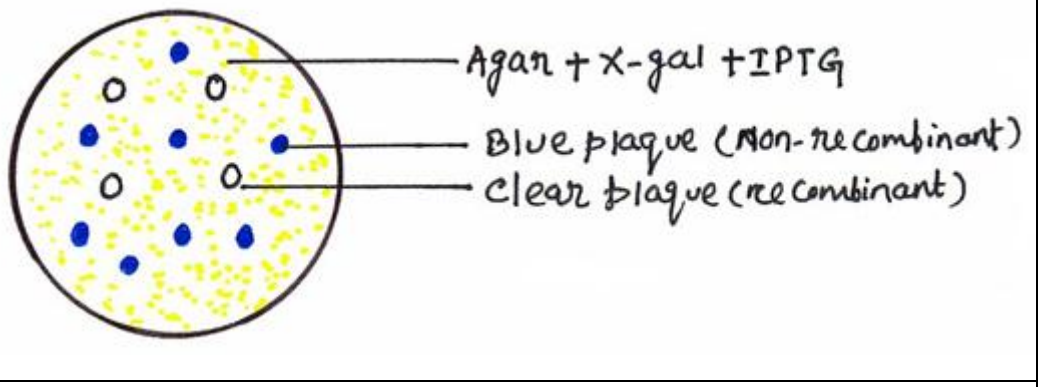


Figure 21: Insertional inactivation of the lacz' gene

Insertional inactivation of the λ cI gene

Several types of λ cloning vector have unique restriction sites in the cI gene. Insertional inactivation of this gene causes a change in plaque morphology. Normal plaques appear 'turbid' whereas recombinants with a disrupted cI gene are clear in appearance (Figure-22).

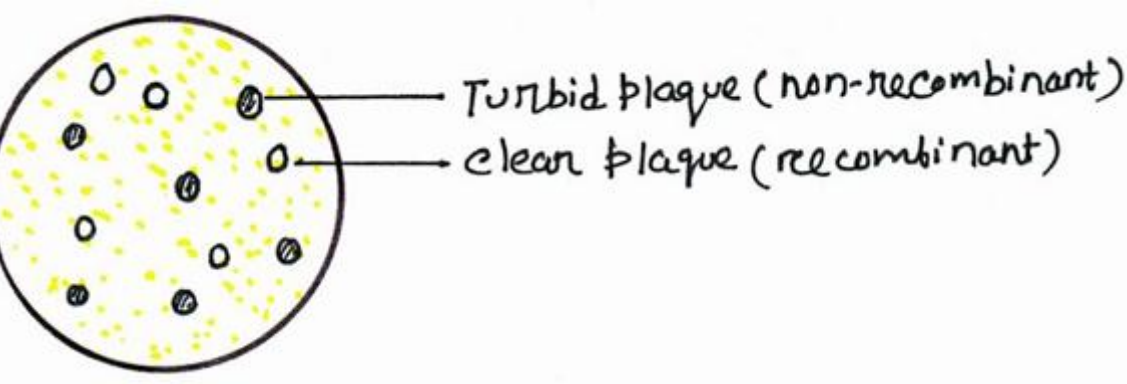


Figure 22: Insertional inactivation of the λ cI gene

Selection using the Spi phenotype

λ phage can't normally infect E.coli cells that possess an integrated form of a related phage called P2. λ is therfore called Spi^+ which is sensitive to P2 prophage inhibition. Some λ cloning vectors are designed so that insertion of new DNA causes a change from Spi^+ to Spi^-, thus the recombinants can infect cells that carry P2 prophages.

Selection on the basis of λ genome size

The λ packaging system, which assembles the mature phage particles, can only insert DNA molecules of between 37 and 52 kb into the head structure. Many λ vectors have been constructed by deleting large segments of the λ DNA molecules and so are less than 37kb in length. These only package into mature phage particles after extra DNA has been inserted, bringing the total genome size up to 37 kb or more. So only recombinant phage are able to replcate with these vectors (Figure-23).

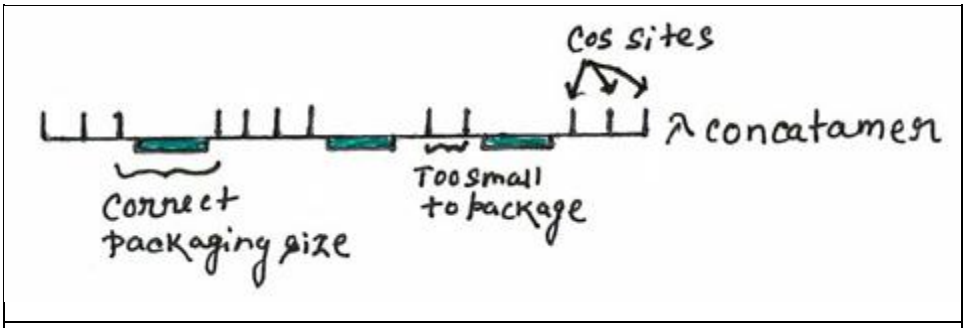


Figure 23: Selection on the basis of λ genome size

Gene cloning - basic steps in cloning, construction of cDNA and genomic libraries-selection and screening method of recombinants. Cloning strategies – transformation techniques and transfection. Blotting techniques: Southern, Northern and Western. PCR – methods and application. Finger printing.

Gene Cloning Step # 1.

Isolation of DNA (Gene of Interest) Fragments to be Cloned:

Before we carry out the operation of gene cloning we need two basic things in their purified state – the gene of our interest (GI) and the vector. A GI is a fragment of gene whose product (a protein, enzyme or a hormone) interests us. For example, gene encoding for the hormone insulin.

Similarly, the vector is a carrier molecule which can carry our GI into a host, replicate there along with the GI making its multiple copies. In this state the GI can also be expressed in the host cell producing the product of the gene which is needed by us.

Gene Cloning Step # 2.

Insertion of Isolated DNA into a Suitable Vector to Form the Recombinant DNA:

Once the ingredients are ready we can start the operation. Our next step will be to cut both the vectors as well as the GI by using a special type of enzyme, called restriction endonuclease. A restriction endonuclease is an enzyme that cuts double-stranded or single-stranded DNA at specific recognition nucleotide sequences known as restriction sites towards the inner region (hence endonuclease).

They are also regarded as molecular scissors as they cut open the DNA strands. After this cutting step we move to pasting. Here the GI is taken and pasted to the cut vector. This procedure also needs an enzyme, called DNA ligase. They are also considered as molecular glue.

The resulting DNA molecule is a hybrid of two DNA molecules – our GI and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination (which naturally takes place in the prophase 1 of meiosis 1). Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and this technology is called recombinant DNA technology (RDT).

Gene Cloning Step # 3.

Introduction of the Recombinant DNA into a Suitable Organism known as Host:

When our recombinant DNA molecule is ready we need to introduce it into a living system known as host.

This is done either for one or both of the following reasons:

(a) To replicate the recombinant DNA molecule in order to get the multiple copies of our GI.

(b) To let our GI get express and produce the protein which is needed by us.

Introduction of the recombinant DNA into the host cell is done by various ways and strictly depends upon the size of the DNA molecule and the nature of GI. Some of the methods followed to carry out this step includes electroporation, micro-injection, lipofection, etc.

When we carry out this process some of the host cells will take up the recombinant DNA and some will not. The host cells which have taken up the recombinant DNA are called transformed cells and the process is called transformation.

Gene Cloning Step # 4.

Selection of the Transformed Host Cells and Identification of the Clone Containing the Gene of Interest:

The transformation process generates a mixed population of transformed and non-trans- formed host cells. As we are interested only in transformed host cells it becomes necessary to filter them out. This is exactly what is done in the selection process. There are many existing selection strategies some of which include taking the help of reporter genes, colony hybridization technique, etc.

Gene Cloning Step # 5.

Multiplication/Expression of the Introduced Gene in the Host:

Once we have purified our transformed host cells by the screening process; it is now our job to provide them optimum parameters to grow and multiply. In this step the transformed host cells are introduced into fresh culture media which provide them rich nourishment followed by an incubation in the oven at right tempera- ture.

At this stage the host cells divide and re-divide along with the replication of the recombinant DNA carried by them. Now at this point we have two choices.

When the aim of the cloning process is to generate a gene library, then our target will be obtaining numerous copies of GI. So with this plan in our mind we will simply go for the replication of the recombinant DNA and not beyond that.

If the aim of the cloning experiment is to obtain the product of GI, then we will go for a step ahead where we will provide favourable conditions to the host cells in which the GI sitting in the vector can express our product of interest (PI).

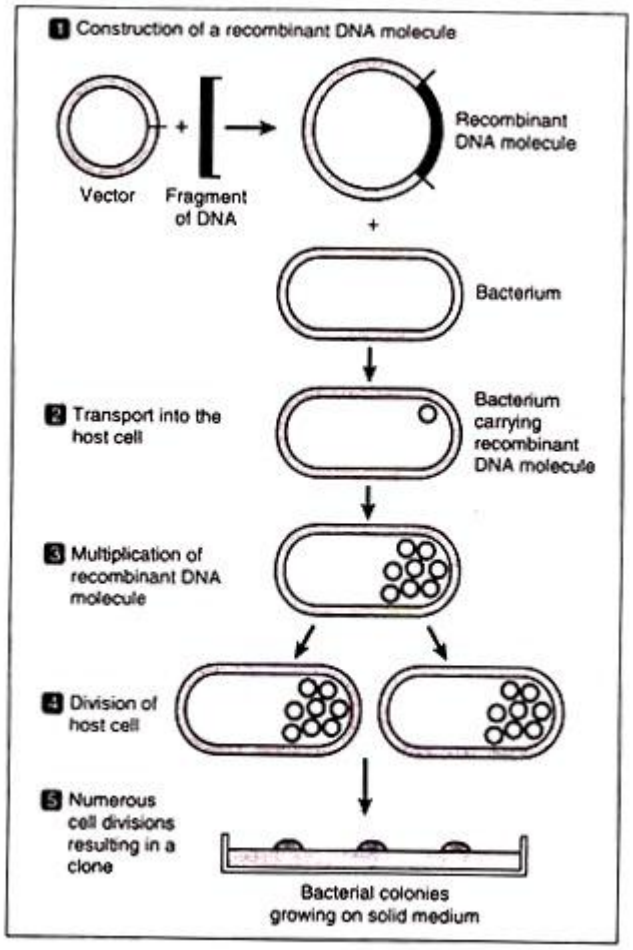


Fig. 2.2: Basic steps of gene cloning

Gene Cloning Step # 6.

Isolation of the Multiplied Gene Copies/Protein Expressed by the Introduced Gene:

In this step we isolate our multiplied GI which is present attached with the vector or the protein encoded by it. This can be rightly compared with the process of harvesting where we collect the crop from the field. There are many processes of isolation, the selection of which varies from case to case.

Gene Cloning Step # 7.

Purification of the Isolated Gene Copy/Protein:

After the harvesting of the isolated gene copy or the protein it is now our job to purify them.

ISOLATION AND PURIFICATION OF NUCLEIC ACIDS (GENOMIC/PLASMID DNA AND RNA)

Introduction

Every gene manipulation procedure requires genetic material like DNA and RNA. Nucleic acids occur naturally in association with proteins and lipoprotein organelles. The dissociation of a nucleoprotein into nucleic acid and protein moieties and their subsequent separation, are the essential steps in the isolation of all species of nucleic acids. Isolation of nucleic acids is followed by quantitation of nucleic acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture. Isolating the genetic material (DNA) from cells (bacterial, viral, plant or animal) involves three basic steps-

- Rupturing of cell membrane to release the cellular components and DNA
- Separation of the nucleic acids from other cellular components
- Purification of nucleic acids

Isolation and Purification of Genomic DNA

Genomic DNA is found in the nucleus of all living cells with the structure of double- stranded DNA remaining unchanged (helical ribbon). The isolation of genomic DNA differs in animals and plant cells. DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells. The amount and purity of extracted DNA depends on the nature of the cell.

The method of isolation of genomic DNA from a bacterium comprises following steps (Figure 4-1.2.)-

1. Bacterial culture growth and harvest.
2. Cell wall rupture and cell extract preparation.
3. DNA Purification from the cell extract.
4. Concentration of DNA solution.

Growth and harvest of bacterial culture

Bacterial cell culture is more convenient than any other microbe, as it requires only liquid medium (broth) containing essential nutrients at optimal concentrations, for the growth and division of bacterial cells. The bacterial cells are usually grown on a complex medium like Luria-Bertani (LB), in which the medium composition is difficult to decipher. Later, the cells are separated by centrifugation and resuspended in 1% or less of the initial culture volume.

Preparation of cell extract

Bacterial cell is surrounded by an additional layer called cell wall, apart from plasma membrane with some species of *E. coli* comprising multilayered cell wall. The lysis of cell wall to release the genetic material i.e. DNA can be achieved by following ways-

- Physical method by mechanical forces.
- Chemical method by metal chelating agents i.e. EDTA and surfactant i.e. SDS or enzyme (e.g. lysozyme).

Lysozyme

- present in egg-white, salivary secretion and tears.
- catalyzes the breakdown of cell wall i.e. the peptidoglycan layer.

EDTA (Ethylene diamine tetra-acetic acid)

- a chelating agent necessary for destabilizing the integrity of cell wall.
- inhibits the cellular enzymes that degrade DNA.

SDS (Sodium dodecyl sulphate)

- helps in removal of lipid molecules and denaturation of membrane proteins.

Generally, a mixture of EDTA and lysozyme is used. Cell lysis is followed by centrifugation to pellet down the cell wall fractions leaving a clear supernatant containing cell extract.

Purification of DNA

In addition to DNA, a cell extract contains significant quantities of protein and RNA which can be further purified by following methods-

Organic extraction and enzymatic digestion for the removal of contaminants

It involves the addition of a mixture of phenol and chloroform (1:1) to the cell lysate for protein separation. The proteins aggregate as a white mass in between the aqueous phase containing DNA and RNA, and the organic layer. Treatment of lysate with pronase or protease, in addition to phenol/chloroform, ensures complete removal of proteins from the extract. The RNA can be effectively removed by using Ribonuclease, an enzyme which rapidly degrades RNA into its ribonucleotide subunits. Repeated phenol extraction is not desirable, as it damages the DNA.

Using ion-exchange chromatography

This involves the separation of ions and polar molecules (proteins, small nucleotides and amino acids) based on their charge. DNA carrying negative charge binds to the cationic resin or matrix which can be eluted from the column by salt gradient. Gradual increase in salt concentration detaches molecules from the resin one after another.

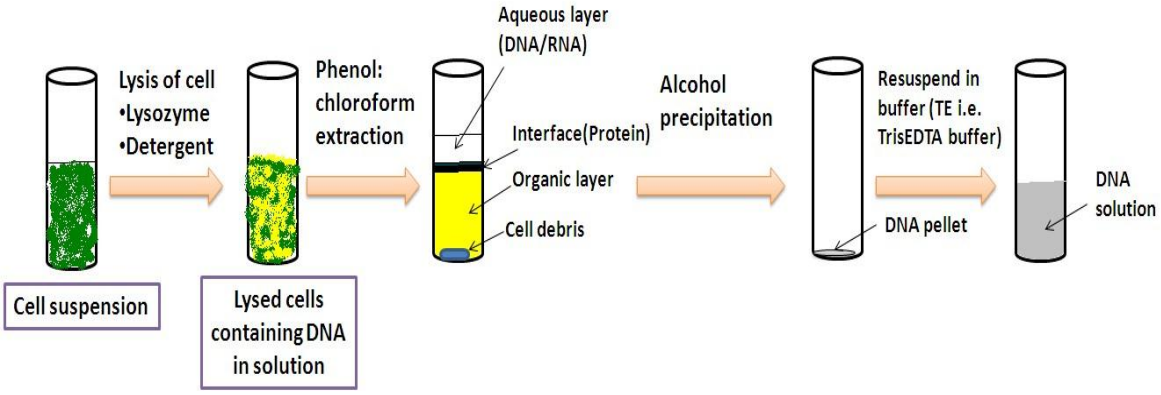


Figure 4-1.2. Preparation of genomic DNA

Concentration of DNA samples

Concentration of DNA can be done using ethanol along with salts such as sodium acetate, potassium acetate etc. These salts provide metal ions like sodium ions (Na+), potassium ions (K+) which help in aggregation and hence precipitation of DNA molecules.

Advantage

It leaves short-chain and monomeric nucleic acid components in solution. Ribonucleotides produced by the ribonuclease treatment are separated from DNA.

Isolation and Purification of Plasmid DNA

Plasmids are circular, double stranded extra cellular DNA molecules of bacterium and most commonly used in recombinant DNA technology. The isolation of plasmid DNA involves three major steps-

1. Growth of the bacterial cell.
2. Harvesting and lysis of the bacteria.
3. Purification of the plasmid DNA.

Growth of the bacterial cell

It involves growth of the bacterial cells in a media containing essential nutrients.

Harvest and lysis of bacteria

Lysis of bacteria results in the precipitation of DNA and cellular proteins. Addition of acetate-containing neutralization buffer results in the precipitation of large and less supercoiled chromosomal DNA and proteins leaving the small bacterial DNA plasmids in solution.

Purification of Plasmid DNA

This step is same for both plasmid and genomic but former involves an additional step i.e. e separation of plasmid DNA from the large bacterial chromosomal DNA.

Methods for separation of plasmid DNA

Separation of plasmid DNA is based on the several features like size and conformation of plasmid DNA and bacterial DNA. Plasmids are much smaller than the bacterial main chromosomes, the largest plasmids being only 8% of the size of the *E. coli* chromosome. The separation of small molecules (i.e. plasmids) from larger ones (i.e. bacterial chromosome) is based on the fact that plasmids and the bacterial chromosomes are circular but bacterial chromosomes break into linear fragments during the preparation of the cell extract resulting in separation of pure plasmids. The methods of separation of plasmid DNA are described as below-

Separation based on size difference

- It involves lysis of cells with lysozyme and EDTA (function as described above in point) in the presence of sucrose (prevents the immediate bursting of cell).
- Cells with partially degraded cell walls are formed that retain an intact cytoplasmic membrane called as sphaeroplasts.
- Cell lysis is then induced by the addition of a non-ionic detergent (e.g. Triton X-100) or ionic detergents (e.g. SDS) causing chromosomal breakage.
- Bacterial chromosome attached to cell membrane, upon lysis gets removed with the cell debris.
- A cleared lysate consisting almost entirely of plasmid DNA is formed with very little breakage of the bacterial DNA. (Figure 1).

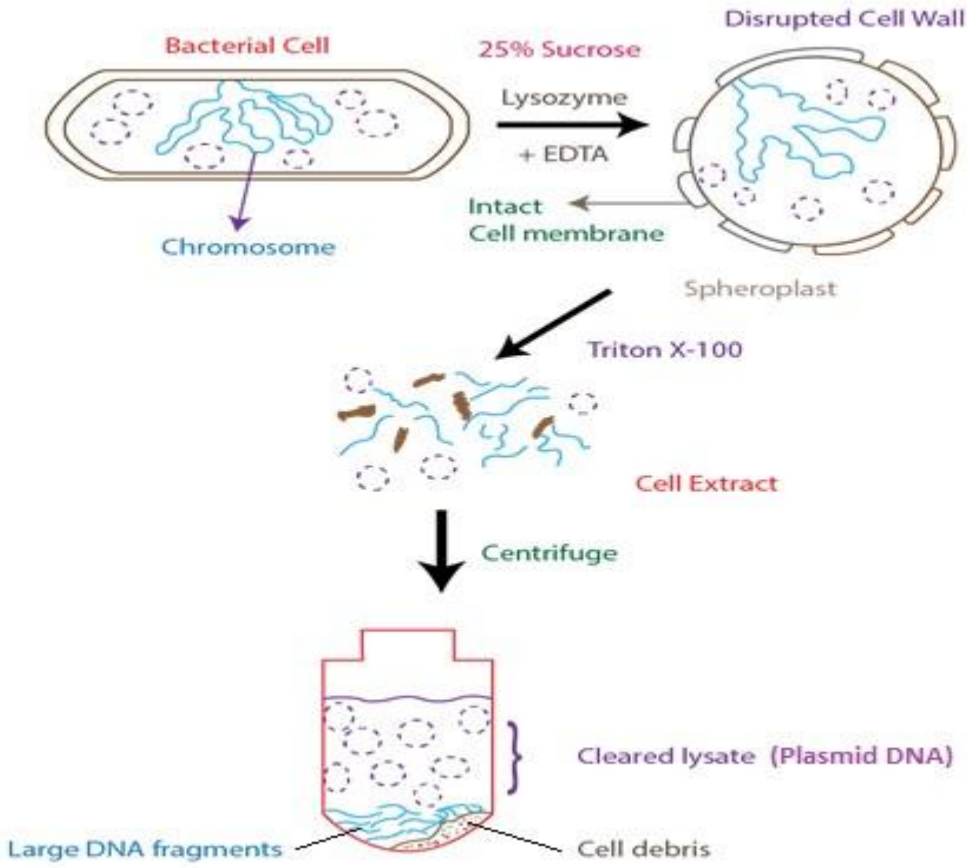


Figure 1. Separation of plasmid DNA on the basis of size.

Separation based on conformation

Plasmids are supercoiled molecules formed by partial unwinding of double helix of the plasmid DNA during the plasmid replication process by enzymes called topoisomerases. The supercoiled conformation can be maintained when both polynucleotide strands are intact, hence called covalently closed-circular (ccc) DNA. If one of the polynucleotide strands is broken, the double helix reverts to its normal relaxed state taking an alternative conformation, called open-circular (oc). Super coiling is important in plasmid preparation due to the easy separation of supercoiled molecules from non-supercoiled ones.

The commonly used methods of separation based on conformation are as follows-

Alkaline denaturation method

- This method is based on maintaining a very narrow pH range for the denaturation of non-supercoiled DNA but not the supercoiled plasmid (Figure 4-1.3.3.1.2(a).).
- Addition of sodium hydroxide to cell extract or cleared lysate (pH12.0-12.5) results in disruption of the hydrogen bonds of non-supercoiled DNA molecules.
- As a result, the double helix unwinds and two polynucleotide chains separate.
- Further addition of acid causes the aggregation of these denatured bacterial DNA strands into a tangled mass which can be pelleted by centrifugation, leaving plasmid DNA in the supernatant.

Advantage

- Most of the RNA and protein under defined conditions (specifically cell lysis by SDS and neutralization with sodium acetate) can be removed by the centrifugation.
- No requirement of organic extraction.

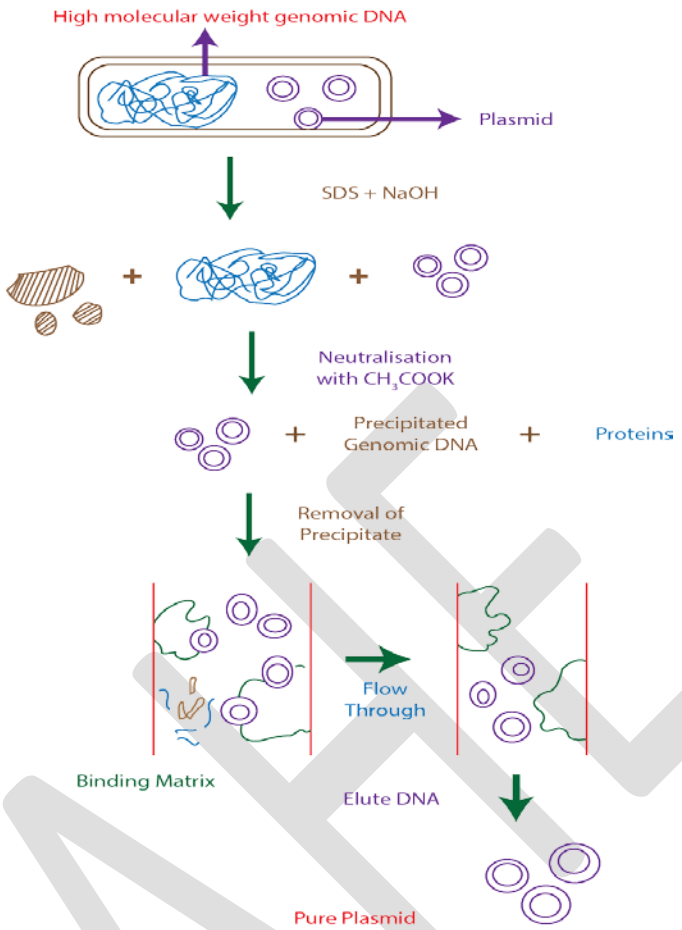


Figure 2. Separation of plasmid DNA by Alkaline denaturation method

Ethidium bromide-cesium chloride density gradient centrifugation

- Density gradient centrifugation can separate DNA, RNA and protein. It is a very efficient method for obtaining pure plasmid DNA.
- A density gradient is produced by centrifuging a solution of cesium chloride at a very high speed which pulls the CsCl ions towards the bottom. This process is referred as isopycnic centrifugation.
- The DNA migrates to the point at which it has density similar to that of CsCl i.e.1.7 g/cm^3 in the gradient.
- In contrast, protein molecules having lower buoyant densities float at the top of the tube whereas RNA gets pelleted at the bottom.

Density gradient centrifugation in the presence of ethidium bromide (EtBr) can be used to separate supercoiled DNA from non-super coiled molecules. Ethidium bromide is an intercalating dye that binds to DNA molecules causing partial unwinding of the double helix. Supercoiled DNA have very little freedom to unwind due to absence of free ends and bind to a limited amount of EtBr resulting in very less decrease in buoyant density g/cm^3) than that of linear DNA (0.125 g/cm^3). As a result, they form a distinct band separated from the linear bacterial DNA. The EtBr bound to DNA is then extracted by n-butanol and the CsCl is removed by dialysis.

Isolation and Purification of RNA

RNA (Ribonucleic acid) is a polymeric substance consisting of a long single-stranded chain of phosphate and ribose units with the nitrogen bases adenine, guanine, cytosine and uracil bonded to the ribose sugar present in living cells and many viruses. The steps for preparation of RNA involve homogenization, phase separation, RNA precipitation, washing and re-dissolving RNA.

The method for isolation and purification of RNA are as follows-

1) Organic extraction method
2) Filter-based, spin basket formats
3) Magnetic particle methods
4) Direct lysis method.

Organic extraction method

This method involves phase separation by addition and centrifugation of a mixture of a solution containing phenol, chloroform and a chaotropic agent (guanidinium thiocyanate) and aqueous sample. Guanidium thiocyanate results in the denaturation of proteins and RNases, separating rRNA from ribosomes. Addition of chloroform forms a colorless upper aqueous phase containing RNA, an interphase containing DNA and a lower phenol-chloroform phase containing protein. RNA is collected from the upper aqueous phase by alcohol (2-propanol or ethanol) precipitation followed by rehydration.

One of the advantages of this method is the stabilization of RNA and rapid denaturation of nucleases. Besides advantages, it has several drawbacks such as it is difficult to automate, needs labor and manual intensive processing, and use of chlorinated organic reagents

Direct lysis methods

This method involves use of lysis buffer under specified conditions for the disruption of sample and stabilization of nucleic acids. If desired, samples can also be purified from stabilized lysates. This method eliminates the need of binding and elution from solid surfaces and thus avoids bias and recovery efficiency effects.

Advantages

- Extremely fast and easy.
- Highest ability for precise RNA representation.
- Easy to work on very small samples.
- Amenable to simple automation.

Drawbacks

- Unable to perform traditional analytical methods (e.g. spectrophotometric method).
- Dilution-based (most useful with concentrated samples).
- Potential for suboptimal performance unless developed/optimized with downstream analysis.
- Potential for residual RNase activity if lysates are not handled properly.

QUANTIFICATION AND STORAGE OF NUCLEIC ACIDS

Quantification of nucleic acids

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

(i) Spectrophotometric analysis
(ii) Nanodrop
(iii) Fluorescence based method
(iv) Fluorescence in situ hybridization (FISH)

4-2.1.1. Spectrophotometric analysis

It is a simple and accurate method to assess the concentration and purity of nucleic acids based on their absorption at different wavelengths (described in detail in Module 3-Lecture 1). According to Beer Lambert's Law, when light is passed through a substance of concentration c at a pathlength l (typically 1 cm), the absorbance is directly proportional to the concentration of the substance and the pathlength i.e.

$$A = \in c\ell$$

Where, A= absorbance of nucleic acids at a particular wavelength

∈=Molar extinction coefficient (M^{-1} cm^{-1}) or specific absorption coefficient ($\mu g/ml)^{-1}$ cm^{-1} that measures how strongly a substance absorbs light at a particular wavelength.

l = Pathlength of the spectrophotometer cuvette

c= concentration of a substance

The value of ∈ for ss-DNA is 0.027; ds-DNA is 0.020 and ss-RNA is 0.025.

The concentration of nucleic acids can be calculated by the given equations-

DNA concentration (µg/ml) = OD_{260} x 100 (dilution factor) x 50 µg/ml

100

For ssDNA, c (pmol/µl) = A_{260}/10 x S

c(µg/ml)= A_{260}/0.027

For dsDNA, c (pmol/µl) = A_{260}/13.2 x S

c (µg/ml)=A_{260}/0.020

For ssRNA, c (µg/ml)=A_{260}/0.025

For **Oligonucleotide,** absorbance is significantly affected by composition of oligonucleotide bases.c (pmol/µl) = A260 x 100/1.5 N_A+ 0.71 N_C + 1.20 N_G + 0.84 N_T

where S denotes the size of DNA in Kb and N represents the number of bases A, G, C or T. The value of ∈ for the DNA bases is listed in the table below.

Bases	**∈ (at 260 nm) (**M^{-1} $cm^{-1)}$
Adenine (A)	15,200
Cytosine (C)	7,050
Guanine (G)	12,010
Thymine (T)	8,400

The purity of the nucleic acids can be determined by A260/A280 ratio described in detail

Nanodrop method

Unlike spectrophotometric method requiring 1-2 ml of the sample, this technique involves microvolume (1-2 µl) quantification of nucleic acid sample. The principle of NanoDrop method is described in detail in Module 3- Lecture 1. The nucleic acids having the concentration range from 2-15000 ng/µl can be assessed by this method. For this, 1-2µl of sample is loaded between the two optical surfaces and the software automatically calculates the concentration and purity of the nucleic acid and displays sample quality (purity) as a spectral output. Generally the pathlength is taken as 1 cm even though in

case of Hellma tray cell cuvette pathlength ranges from 0.2-1.0 mm during measurement cycle. Sensitivity measured by sample retention system of NanoDrop is increased by many folds compared to a standard spectrophotometer. Automatic pathlength adjustment facilitates the direct quantification of concentrated samples avoiding the need for sample dilutions.

Quantification using fluorescent dyes

This method is simple and more sensitive than spectrophotometric method which measures the fluorescence intensity of the dyes that fluoresce upon interaction with the nucleic acids. Various fluorescent dyes such as EtBr, Hoechst 33258, picogreen, DAPI can be used for quantification of nucleic acids described in detail in Module 3-Lecture 1. The sensitivity range of these dyes for the quantification is listed in Table 4-2.1.3. Quantification of nucleic acids separated by gel electrophoresis can be done by comparing the stained nucleic acids with stained standards of known concentration separated on the same gel. The dye: DNA complex shows greater fluorescence than the unbound dye by intercalating between the stacked base pairs. The fluorescence intensity of the band estimates the concentration/amount of DNA. The intensity of the stain is dependent at least in part on the base pair composition of the nucleic acid.

Nucleic acid	**UV Absorbance (A_{260})**	**Fluorescence methods**		
		Hoechst 33258	**EtBr**	**Picogreen**
DNA	1–50 µg/mL	0.01–15 µg/mL	0.1–10 µg/mL	0.025–1000 ng/mL
RNA	1–40 µg/mL	Not applicable	1–40 µg/mL	Minimal sensitivity
Ratio of signal (DNA/RNA)	0.8	400	2.2	>100

Table 4. Sensitivity range of quantification of nucleic acids by UV spectrophotometry and fluorescence-based methods.

Fluorescence in situ hybridization (FISH)

In a cytometric system, quantification of fluorescence ISH signals and accurate estimation of fluorescence intensity of specific DNA sequences can be performed using an epi-fluorescence microscope with a multi-wavelength illuminator, fitted with a cooled charge couple device (CCD) camera. The principle of this method is described in detail in Module 3- Lecture 1. Quantitation by FISH was first applied as a basis for rudimentary cytogenetic assays. Detection using CCD camera enables the quantitative analysis of mRNA as well. The two factors affecting the fluorescence assay are reproducibility and signal irregularity as well as background noise which may vary from sample to sample and cell to cell. Various approaches have been developed to reduce background noise such as use of reducing agents e.g. sodium borohydride and pre-treatment by light irradiation, and image analysis methods. The former cannot be possible and effective always so the later are now employed for analysis. The output thus consist of a true signal and various noise components, the profile for each of them can be estimated and deleted by digital methods like independent component analysis. Several computational methods have been developed to overcome the differential intensity and color overlap caused by multi-color image analysis.

Further, FISH can be employed for interpretation using various approaches such as multi-color cytometry algotithms, dot-counting approaches, use of diagnostic probe sets etc.

Storage of Nucleic Acids

The purified DNA can be stored at -20°C or -70°C under slightly basic conditions (e.g., Tris- Cl, pH 8.0) as acidic conditions result in hydrolysis of DNA.

Diluted solutions of nucleic acids can be stored in aliquots and thawed once only. RNA preservation under frozen conditions is helpful.

Purified RNA can be stored at -20°C or -80°C in RNase-free solution such as-

- The RNA Storage Solution (1 mM sodium citrate, pH 6.4 ± 0.2): It is a buffer that delivers greater RNA stability than 0.1 mM EDTA or TE. The presence of sodium

citrate and low pH minimizes base hydrolysis of RNA. Sodium citrate acts both as a chelating and buffering agent.

- 0.1 mM EDTA
- TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0)

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

CONSTRUCTION OF cDNA LIBRARY

Introduction

In higher eukaryotes, gene expression is tissue-specific. Only certain cell types show moderate to high expression of a single gene or a group of genes. For example, the genes encoding globin proteins are expressed only in erythrocyte precursor cells, called reticulocytes. Using this information a target gene can be cloned by isolating the mRNA from a specific tissue. The specific DNA sequences are synthesized as copies from mRNAs of a particular cell type, and cloned into bacteriophage vectors. cDNA (complementary DNA) is produced from a fully transcribed mRNA which contains only the expressed genes of an organism. Clones of such DNA copies of mRNAs are called cDNA clones.

A cDNA library is a combination of cloned cDNA fragments constituting some portion of the transcriptome of an organism which are inserted into a number of host cells. In eukaryotic cells, the mRNA is spliced before translation into protein. The DNA synthesized from the spliced mRNA doesn't have introns or non-coding regions of the gene. As a result, the protein under expression can be sequenced from the DNA which is the main advantage of cDNA cloning over genomic DNA cloning.

Construction of a cDNA Library

The construction of cDNA library involves following steps-

1. Isolation of mRNA

2. Synthesis of first and second strand of cDNA
3. Incorporation of cDNA into a vector
4. Cloning of cDNAs

Isolation of mRNA

It involves the isolation of total mRNA from a cell type or tissue of interest. The amount of desired mRNA can be increased by following ways-

- Chromatographic purification of mRNA using oligo-dT column, which retains mRNA molecules, resulting in their enrichment.
- Spinning down mRNA by density gradient centrifugation.
- mRNA preparation from specialized cell types, e.g. developing seeds, chicken oviduct, erythrocytes, β cells of pancreas etc.

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.

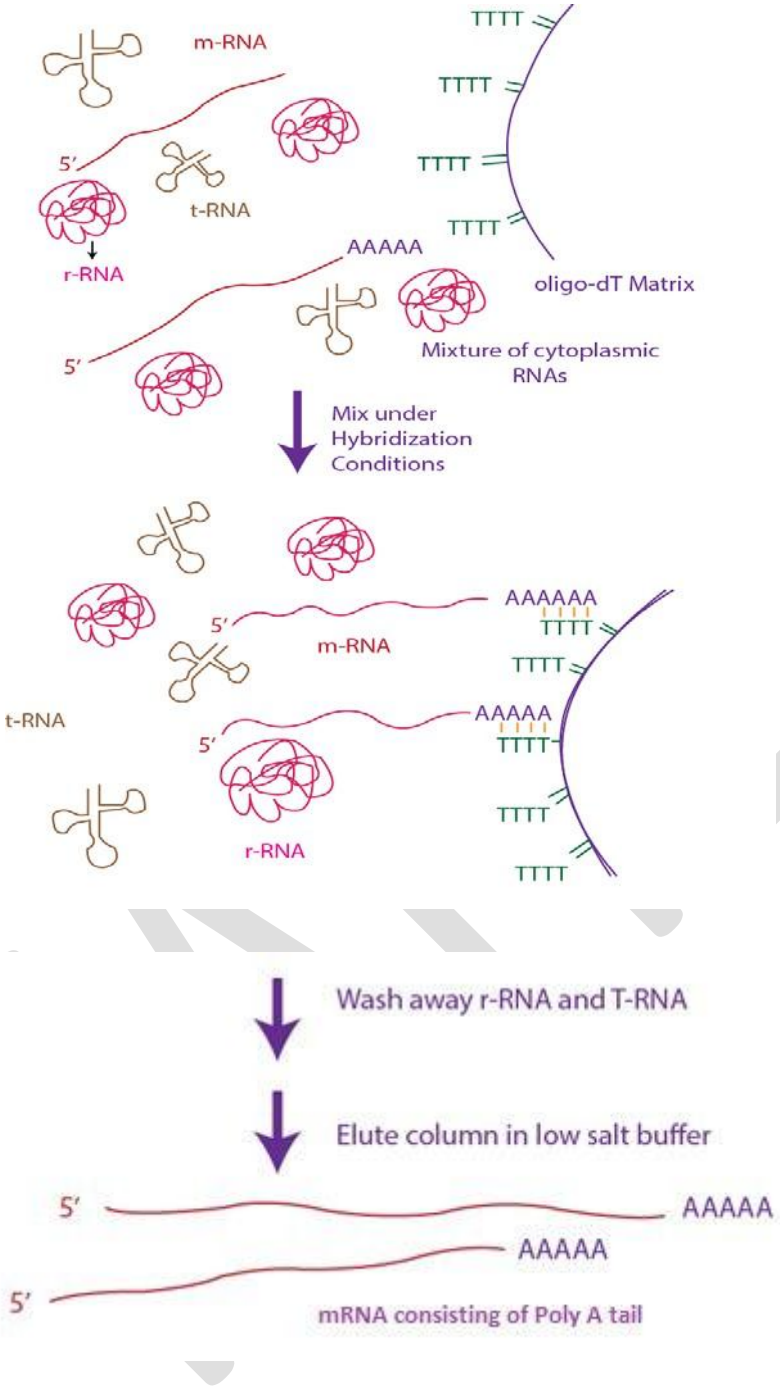


Figure 4-3.2.1. Isolation of mRNA using oligo-dT column chromatography.

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.

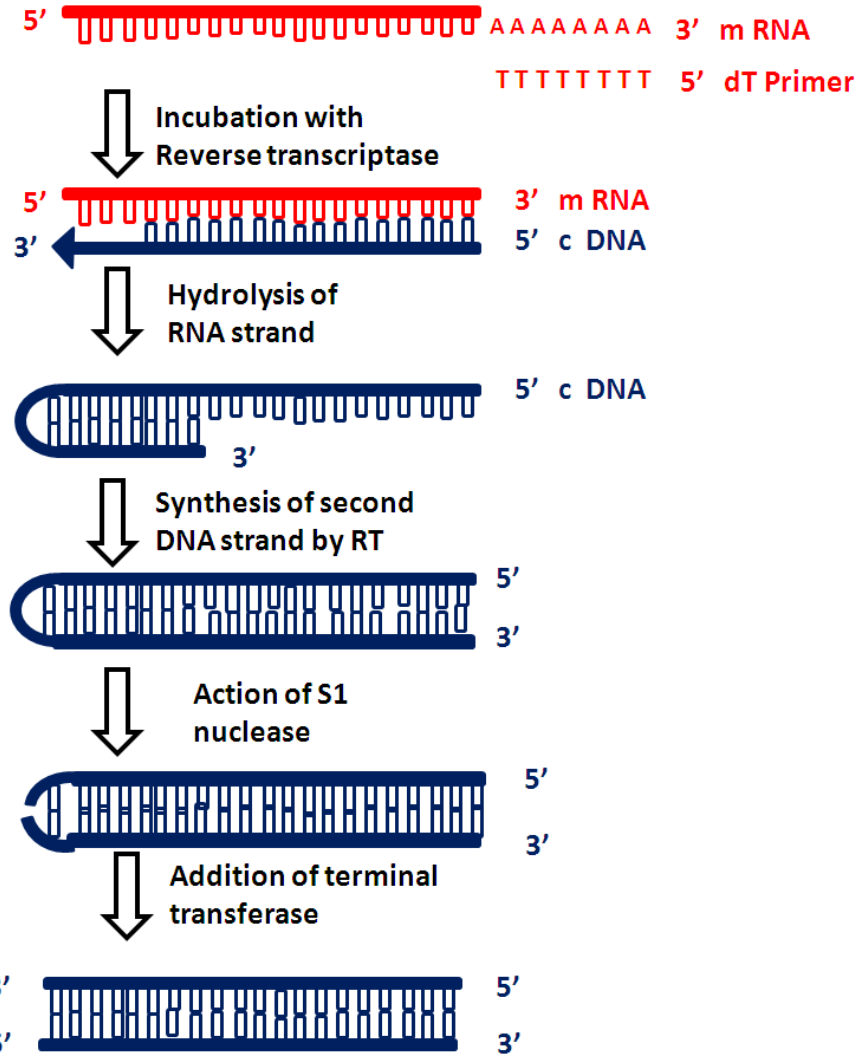


Figure 4-3.2.2. Synthesis of first and second strand of cDNA.

4-3.2.3. 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.

Linker

It is a double-stranded DNA segment with a recognition site for a particular restriction enzyme. It is 10-12 base pairs long prepared by hybridizing chemically synthesized complementary oligonucleotides. The blunt ended ds-DNAs are ligated with the linkers by the DNA ligase from T4 Bacteriophage.

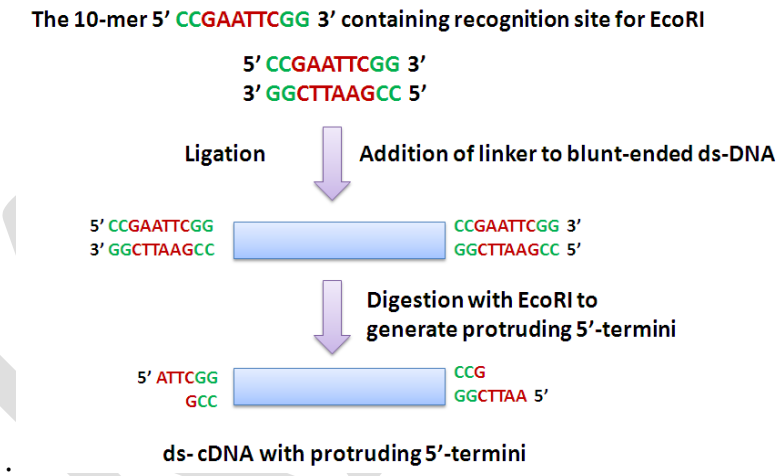


Figure 4-3.2.3.1. Modification of cDNA termini using linkers.

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. Problems arise, when cDNA itself has a site for the restriction enzyme cleaving the linkers. This can be overcome using an appropriate modification enzyme (methylase) to protect any internal recognition site from digestion which methylates specific bases within the restriction-site sequence, thereby, preventing the restriction enzyme binding.

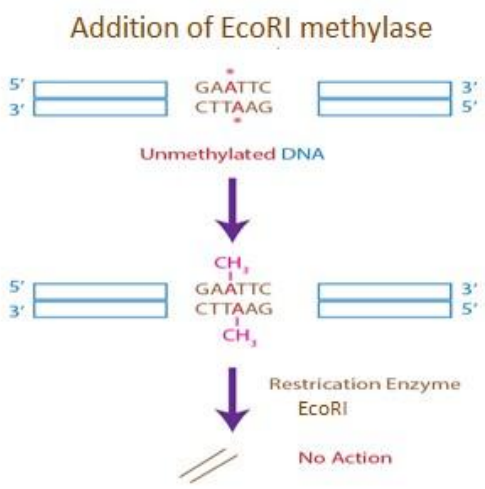


Figure 4-3.2.3.2. Action of methylases

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.

4-3.2.4. 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.

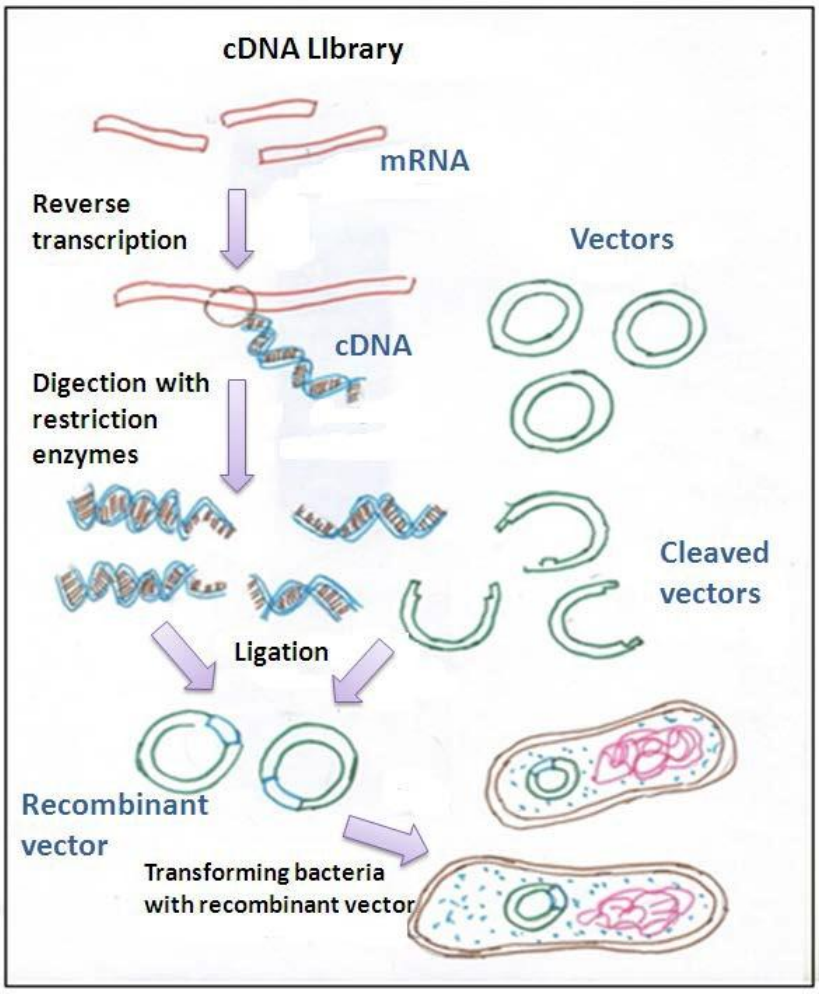


Figure 4-3.2.4. Cloning of cDNAs

4-3.3. Commonly used vectors for cDNA cloning and expression

Vector	**Features**
Lambda gt10, Lambda gt11	DNA inserts of 7.6 kb and 7.2 kb, respectively, inserted at a unique *EcoRI* cloning site; recombinant Lambda gt10 selected on the basis of plaque morphology ; Lambda gt11 has *E. coli LacZ* gene: *LacZ* and cDNA encoded protein is expressed as fusion protein.

Lambda ZAP series (phasmids)	Up to 10 kb DNA insert; therefore, most cDNAs can be cloned; polylinker has six cloning site; T3 and T7 RNA polymerase sites flank the polylinker so that riboprobes of both strands can be prepared; these features are contained in plasmid vector *pBluescript*, which is inserted into the phage genome; the plasmid containing cDNA recovered simply by co-infecting the bacteria with a helper f1 phage that helps excise from the phage genome.

4-3.4. Problems in cDNA preparation

- Large mRNA sequence results in inefficient synthesis of full- length cDNA. This cause problems during expression as it may not contain the entire coding sequence of the gene. This arises because of the poor processivity of RTase purified from avian myeloblastosis virus (AVM) or produced in *E.coli* from the gene of Moloney murine leukemia virus (MMLV).
- Use of S1 nuclease, the enzyme used to trim the ds cDNA, may remove some important 5´ sequences.

4-3.5. Strategies to overcome the limitations in cDNA preparation

Strategies that can be employed to overcome the above limitations are listed as follows-

- A specially designed *E. coli* vector can be used to avoid incomplete copying of the RNA.
- The use of single strand specific nuclease can be avoided by adding a poly-C tail to the 3´-end of the ss-cDNA produced by copying of the mRNA by the enzyme terminal deoxynucleotidyl transferase. Complementary oligonucleotide (Poly-G) is now used as a primer for the synthesis of complementary strand to yield ds-cDNA without a hairpin loop enhancing the full-length cDNA production.

4-3.6. Applications of cDNA libraries/cloning

- 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.

4-3.7. Disadvantages of cDNA libraries

- 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.

CONSTRUCTION OF GENOMIC LIBRARY

4-4.1. Introduction

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.

4-4.2. Construction of genomic library

Construction of a genomic DNA library involves isolation, purification and fragmentation of genomic DNA followed by cloning of the fragmented DNA using suitable vectors. The eukaryotic cell nuclei are purified by digestion with protease and organic (phenol-chloroform) extraction. The derived genomic DNA is too large to

incorporate into a vector and needs to be broken up into desirable fragment sizes. Fragmentation of DNA can be achieved by physical method and enzymatic method. The library created contains representative copies of all DNA fragments present within the genome.

4-4.2.1. Mechanisms for cleaving DNA

(a) Physical method

It involves mechanical shearing of genomic DNA using a narrow-gauge syringe needle or sonication to break up the DNA into suitable size fragments that can be cloned. Typically, an average DNA fragment size of about 20 kb is desirable for cloning into λ based vectors. DNA fragmentation is random which may result in variable sized DNA fragments. This method requires large quantities of DNA.

(b) Enzymatic method

- It involves use of restriction enzyme for the fragmentation of purified DNA.
- This method is limited by distribution probability of site prone to the action of restriction enzymes which will generate shorter DNA fragments than the desired size.
- If, a gene to be cloned contains multiple recognition sites for a particular restriction enzyme, the complete digestion will generate fragments that are generally too small to clone. As a consequence, the gene may not be represented within a library.
- To overcome this problem, partial digestion of the DNA molecule is usually carried out using known quantity of restriction enzyme to obtain fragments of ideal size.
- The two factors which govern the selection of the restriction enzymes are- type of ends (blunt or sticky) generated by the enzyme action and susceptibility of the enzyme to chemical modification of bases like methylation which can inhibit the enzyme activity.
- The fragments of desired size can be recovered by either agarose gel electrophoresis or sucrose gradient technique and ligated to suitable vectors.

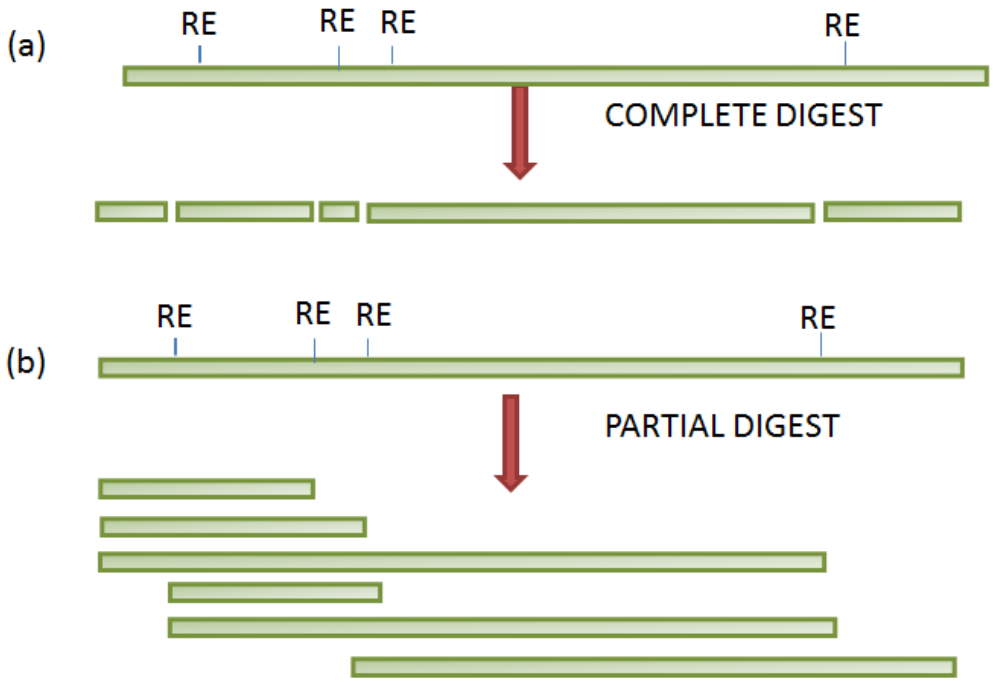


Figure4-4.2.1. The complete (a) and partial (b) digestion of a DNA fragment using restriction enzymes.

(Adapted from Reece RJ. 2000. Analysis of Genes and Genomes. John Wiley & Sons, U.K.)

Partial restriction digestion is achieved using restriction enzymes that produce blunt or sticky ends as described below-

i. Restriction enzymes generating blunt ends

The genomic DNA can be digested using restriction enzymes that generate blunt ends e.g. *HaeIII* and *AluI.*

```
HaeIII: 5'-GG|CC-3'    AluI: 5'-AG|CT-3'
        3'-CC|GG-5'          3'-TC|GA-5'
```

Blunt ends are converted into sticky ends prior to cloning. These blunt ended DNA fragments can be ligated to oligonucleotides that contain the recognition sequence for a restriction enzyme called linkers or possess an overhanging sticky end for cloning into particular restriction sites called adaptors.

Linkers

Linkers are short stretches of double stranded DNA of length 8-14 bp that 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 cohesive ends.

ii. Restriction enzymes that generate sticky ends

Genomic DNA can be digested with commonly available restriction enzymes that generate sticky ends. For example, digestion of genomic DNA with the restriction enzyme *Sau3AI* (recognition sequence 5'-GATC-3') generates DNA fragments that are compatible with the sticky end produced by *BamHI* (recognition sequence 5'-GGATCC-3') cleavage of a vector. Once the DNA fragments are produced, they are cloned into a suitable vector.

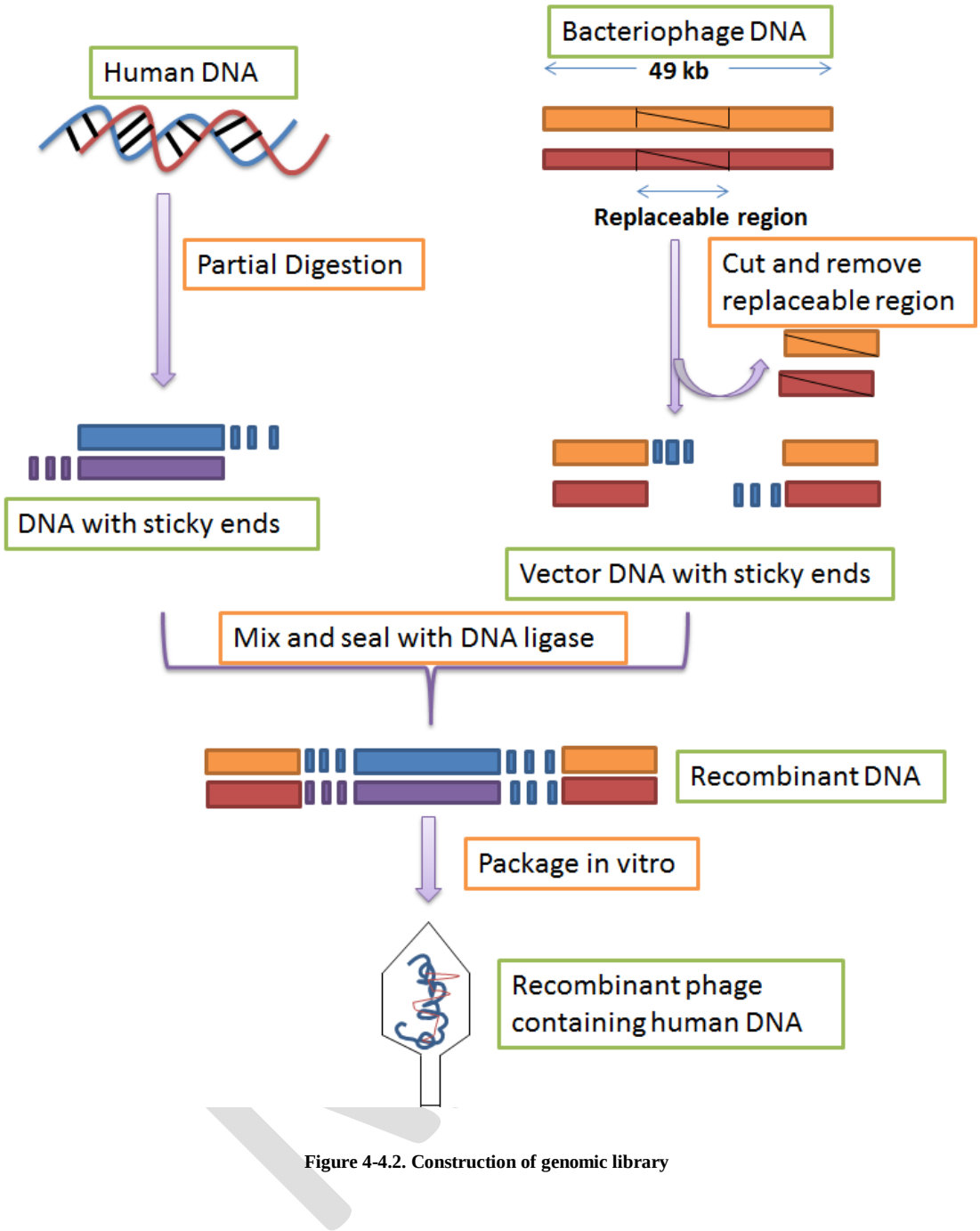


Figure 4-4.2. Construction of genomic library

4-4.2.2. Cloning of genomic DNA

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 λ replacement vectors like *λDASH* and *EMBL3* are preferred for construction of genomic DNA library. T4 DNA ligase is used to ligate the selected DNA sequence into the vector.

(1) λ replacement vectors

The *λEMBL* series of vectors are widely used for genomic library construction. The multiple cloning sites of these vectors flanking the stuffer fragment contain opposed promoters for the T3 and T7 RNA polymerases. The restriction digestion of the recombinant vector generates short fragments of insert DNA left attached to these promoters. This generates RNA probes for the ends of the DNA insert. These vectors can be made conveniently, directly from the vector, without recourse to sub-cloning.

(2) High-capacity vectors

The high capacity cloning vectors used for the construction of genomic libraries are cosmids, bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs) and yeast artificial chromosomes (YACs). They are designed to handle longer DNA inserts, much larger than for λ replacement vectors. So they require lower number of recombinants to be screened for identification of a particular gene of interest.

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)

Table 4-4.2.2. Vectors used for cloning genomic libraries.

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.

Number of clones required for a library

The number of clones to be pooled depends upon the size of the genome *f* and average size of the cloned DNA.

Let (*f*) be the fraction of the genome size compared to the average individual cloned fragment size, would represent the lowest possible number of clones that the library must contain.

The minimum number of clones required can be calculated as-

***f*= genome size/ fragment size**

For the *E. coli* genome (4.6 Mb) with an average cloned fragment size of 5 kb, *f* will be 920.

The number of independent recombinants required in the library must be greater than *f,* as sampling variation leads to the several times inclusion and exclusion of some sequences in a library of just *f* recombinants. In 1976, Clarke and Carbon derived a formula to calculate probability (P) of including any DNA sequence in a random library of N independent recombinants.

The actual number of clones required can be calculated as-

$$N = \frac{\ln (1-P)}{\ln (1-1/f)}$$

where N= number of clones and P= probability that a given gene will be present.

Bigger the library better will be the chance of finding the gene of interest. The pooling together of either recombinant plaques or bacterial colonies generates a primary library.

4-4.3. Amplified library

- The primary library created is usually of a low titer and unstable. The stability and titer can be increased by amplification. For this, the phages or bacterial colonies are plated out several times and the resulting progenies are collected to form an amplified library.
- The amplified library can then be stored almost indefinitely due to longshelf-life of phages.
- It usually has a much larger volume than the primary library, and consequently may be screened several times.
- It is possible that the amplification process will result in the composition of the amplified library not truly reflecting the primary one.
- Certain DNA sequences may be relatively toxic to *E. coli* cells. As a consequence bacteria harboring such clones will grow more slowly than other bacteria harboring non-toxic DNA sequences. Such problematic DNA sequences present in the primary library may be lost or under-represented after the growth phase required to produce the amplified library.

4-4.4. Subgenomic library

Subgenomic library is a library which represents only a fraction of the genome. Enhancing the fold of purification of target DNA is crucial for subgenomic DNA libraries which can be achieved by multiple, sequential digestion when information of the restriction map of the sequences of interest is known. After initial purification of a given fragment, the purification can further be increased by redigestion with another enzyme generating a smaller (clonable) fragment relative to original DNA.

4-4.5. Advantages of genomic libraries

- 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.

4-4.6. Disadvantages of genomic library

- 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.

4-4.7. Applications

- To determine the complete genome sequence of a given organism.
- To study and generate transgenic animals through genetic engineering, serving as a source of genomic sequence.
- To study the function of regulatory sequences *in vitro.*
- To study the genetic mutations.
- Used for genome mapping, sequencing and the assembly of clone contigs.

4-4.8 Comparison of Genomic and cDNA Libraries

cDNA library has revolutionized the field of molecular genetics and recombinant DNA technology. It consists of a population of bacterial transformants or phage lysates in which each mRNA isolated from an organism is represented as its cDNA insertion in a vector. cDNA libraries are used to express eukaryotic genes in prokaryotes. In addition, cDNAs are used to generate expressed sequence tags (ESTs) and splices variant analysis. Some of the differences of cDNA library with genomic library are presented in Table below

Feature	Genomic library	cDNA library
Sequences present	Ideally, all genomic sequences	Only structural genes that are transcribed
Contents affected by :		
(a) Developmental stage	No	Yes
(b) Cell type	No	Yes
Features of DNA insert(s) representing a gene:		
(a) Size	As present in genome	Ordinarily, much smaller
(b) Introns	Present	Absent
(c) 5´- and 3´- regulatory sequences	Present	Absent
As compared to the genome		
(a) Enrichment of sequences	In amplified genomic libraries	For abundant mRNAs
(b) Redundancy in frequency	In amplified libraries	For rare mRNA species
(c) Variant forms of a gene	Not possible	For such genes, whose RNA transcripts are alternatively spliced

Table 4-4.8. Features of genomic and cDNA library

SCREENING AND PRESERVATION OF DNA LIBRARIES

4-5.1. Introduction

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.

4-5.2. Methods for screening based on detecting a DNA sequence

4-5.2.1. Screening by hybridization

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

The commonly used methods of hybridization are,

a) Colony hybridization
b) Plaque hybridization.

4-5.2.1(a). Colony hybridization

Colony hybridization, also known as replica plating, allows the screening of colonies plated at high density using radioactive DNA probes. This method can be used to screen plasmid or cosmid based libraries (Explained in detail in Module 3-Lecture 4 as point **3-4.4)**.

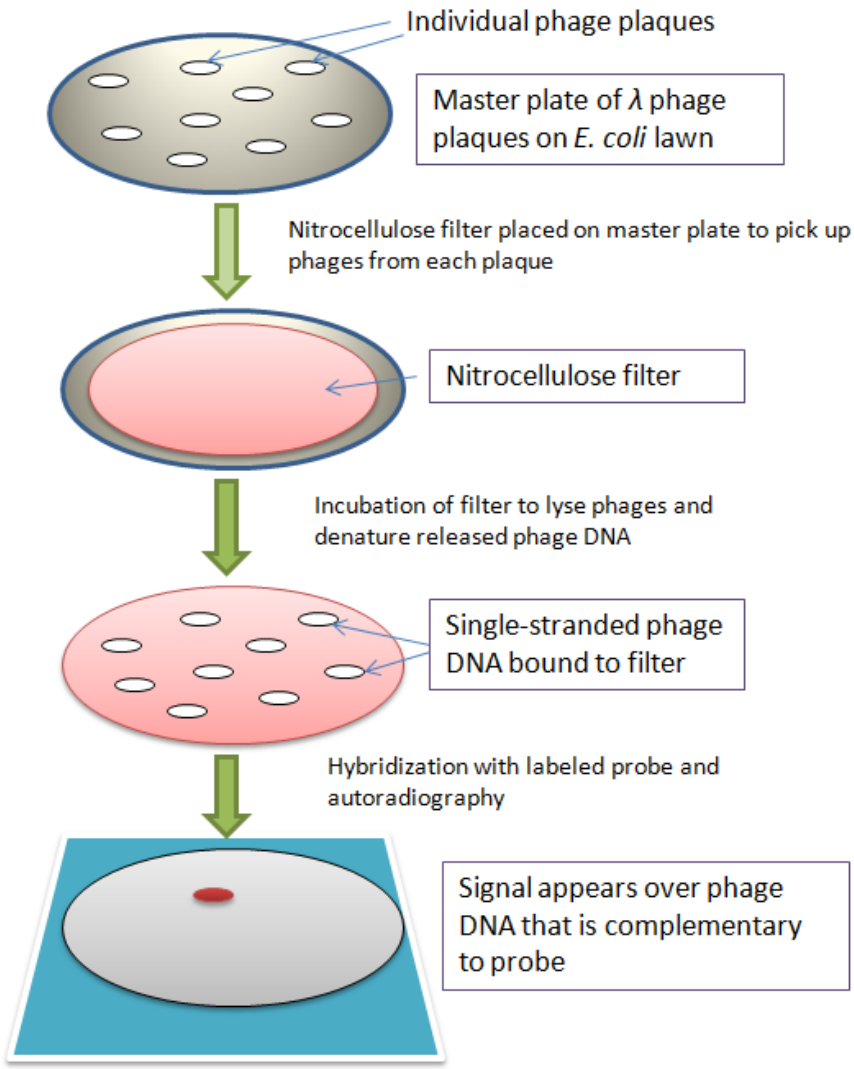
4-5.2.1(b). Plaque hybridization

Plaque hybridization, also known as *Plaque lift*, was developed by Benton and Davis in 1977 and employs a filter lift method applied to phage plaques. This procedure is successfully applied to the isolation of recombinant phage by nucleic acid hybridization and probably is the most widely applied method of library screening. The method of screening library by plaque hybridization is described below-

- The nitrocellulose filter is applied to the upper surface of agar plates, making a direct contact between plaques and filter.
- The plaques contain phage particles, as well as a considerable amount of unpackaged recombinant DNA which bind to the filter.
- The DNA is denatured, fixed to the filter, hybridized with radioactive probes and assayed by autoradiography.

Advantages

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



4-5.2.1(b). Schematic process for screening libraries by Plaque hybridization.

Probes used for hybridization

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

respectively.

4-5.2.2. Screening by PCR

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

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

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

4-5.3. Screening methods based on gene expression

4-5.3.1. Immunological screening

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

Earlier immunoscreening methods employed radio-labeled primary antibodies to detect antibody binding to the nitrocellulose sheet (Figure 4-5.3.1(a).). It is now superseded by antibody sandwiches resulting in highly amplified signals. The secondary antibody recognizes the constant region of the primary antibody and is, additionally, conjugated to an easily assayable enzyme (*e.g.* horseradish peroxidase or alkaline

phosphatase) which can be assayed using colorimetric change or emission of light using X-ray film (Figure 4-5.3.1(b).).

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

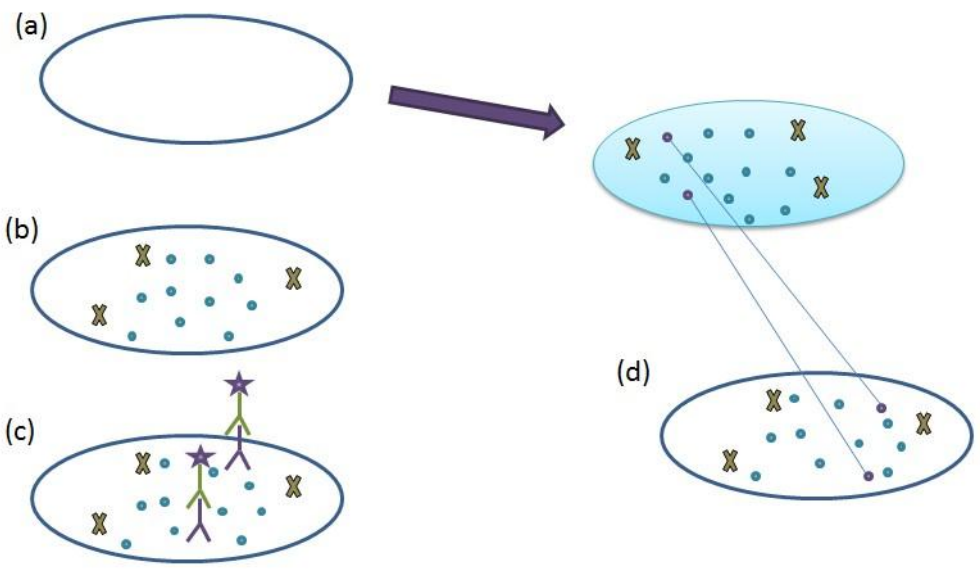


Figure 4-5.3.1(a). Schematic process of immunological screening (a) a nitrocellulose disk is placed onto the surface of an agar plate containing the phage library. Both agar plate and disk are marked so as to realign them later. (b) When the nitrocellulose disk is lifted off again, proteins released from the bacteria by phage lysis bind to the disk. (c) These proteins bind to specific antibody. (d) Plaques formed by bacteriophage that express the protein bound to the antibody will be detected by emission of light. The positive clones can be identified by realignment.

(Adapted from Lodge J. 2007.Gene cloning: principles and applications. Taylor & Francis Group)

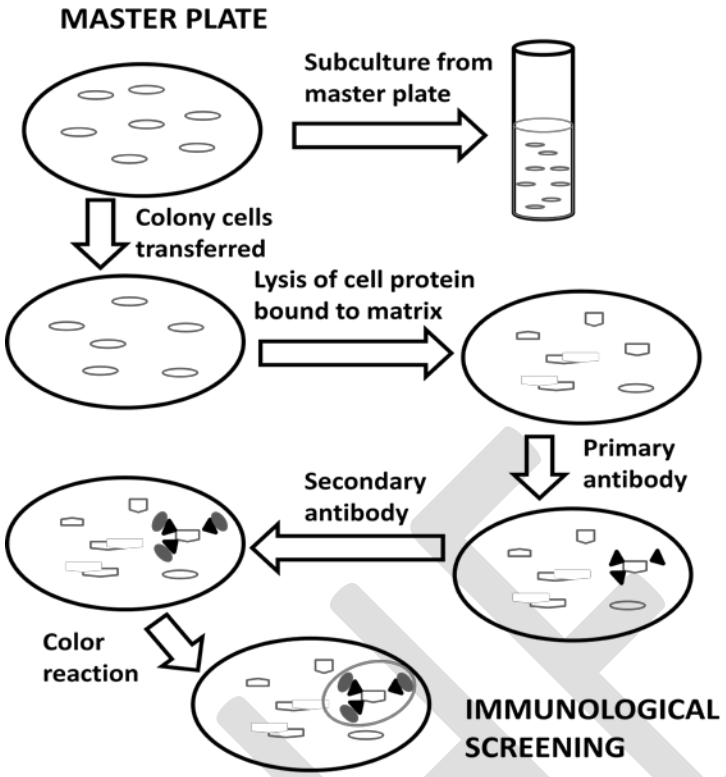


Figure 4-5.3.1(b). Schematic process of immunological screening using antibody sandwich.

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

4-5.3.2. Screening by functional complementation

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

For example, the functional complementation in transgenic mice for the isolation of *Shaker-2* gene applied by Probst *et al* in1988 shown in Figure 4-5.3.2.

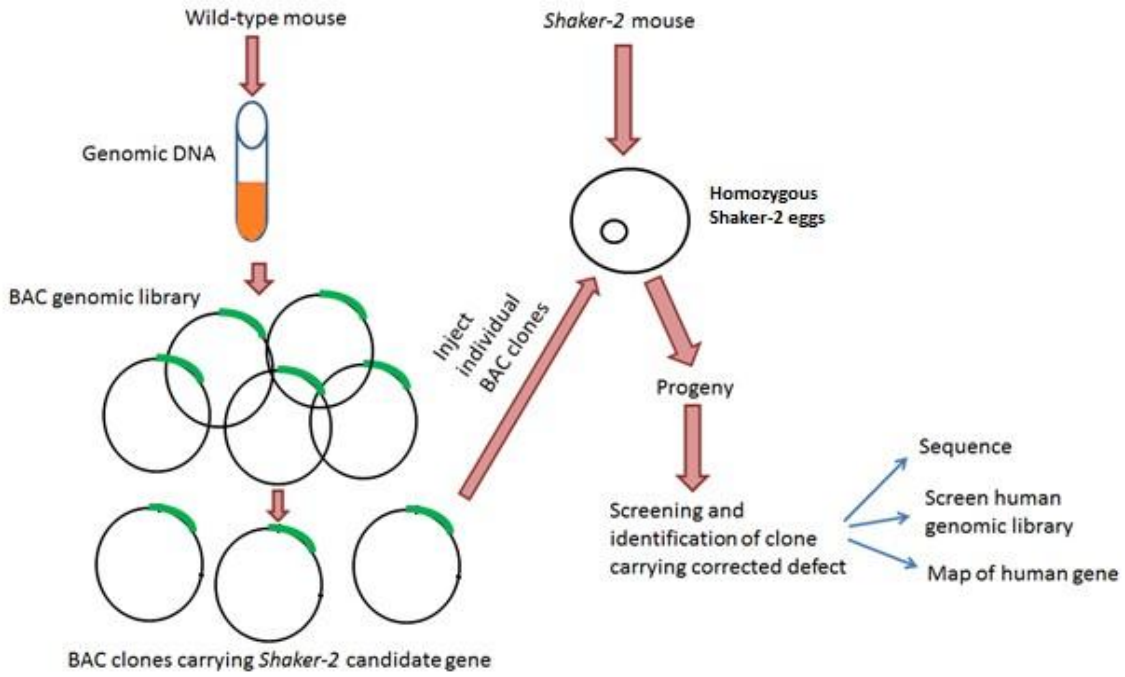


Figure 4-5.3.2. Functional complementation in transgenic mice for isolation of *Shaker-2* gene.

(Adapted from Primrose SB, Twyman RM. 2006. Principles of gene manipulation and genomics.7th ed. Blackwell Publishing.)

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

Drawbacks

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

Applications

- This method can be used for the isolation of higher-eukaryotic genes (e.g. Drosophila topoisomerase II gene, a number of human RNA polymerase II transcription factors) from an organism.

- It can also be possible in transgenic animals and plants to clone a specific gene from its functional homologue.

DNA SEQUENCING AND CLONING STATEGIES

DNA SEQUENCING

Sequencing is the method of resolving the order of the nucleotide bases in a DNA molecule (genomic DNA, cDNA, or organellar DNA). It is a primary step in assessing regulatory sequences, coding and non-coding regions. For past few years, DNA sequencing has been a solid foundation for various research fields such as taxonomy, phylogeny, ecology and genetic studies.

There are two classical methods of sequencing described as below-

1. Sanger dideoxy sequencing
2. Maxam-Gilbert sequencing

4-6.1.1 Sanger dideoxy sequencing

The Sanger or chain termination method was first developed by Fred Sanger and colleagues in the mid-1970s. It involves *in vitro* synthesis of DNA on a single-stranded template by using a primer, a set of labelled deoxynucleotide triphosphates (dNTPs), and dideoxynucleotide triphosphates (ddNTPs). Each ddNTP is fluorescently-labeled and impedes chain elongation. It lacks hydroxyl group at the third position of the sugar component which is required for attachment of the next nucleotide.

The template DNA to be sequenced can be obtained by in vivo or in vitro cloning. It can be elongated by incorporation of deoxynucleotides at random positions. The ratio of dNTP todideoxyNTP should be such that an individual strand can be polymerized for a significant distance before addition of dideoxyNTP molecule. The reaction terminates at the position where the ddNTP rather than dNTP incorporates into the growing DNA chain. This process results in the generation of amplicons of different sizes each ending in dideoxyNTP.

The next step involves separation of these fragments which can be achieved by acrylamide gel electrophoresis to obtain a sequence of up to a few hundred bases. Four parallel reactions can be carried out in parallel, one for each base. The base sequence can then be read by autoradiography of the banding patterns.

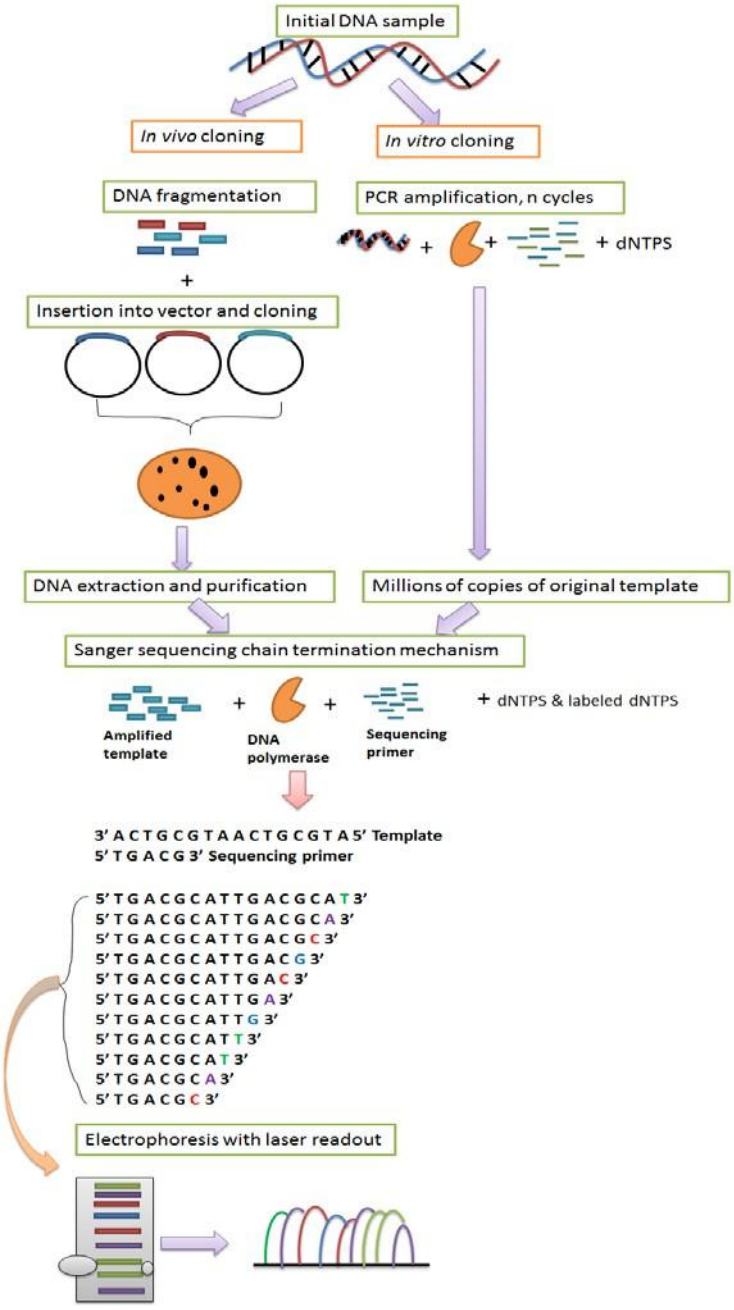


Figure4-6.1.1: Schematic representation of Sanger sequencing workflow.

(Adapted and modified from Hoy MA. 2013. DNA Sequencing and the Evolution of the "-Omics". Insect Molecular Genetics. 3rd Edn. Academic Press, San Diego, 251-305.)

4-6.1.2 Maxam-Gilbert Sequencing

This method was developed by Maxam and Gilbert in 1977 and is also known as chemical degradation method. In this method, double-stranded DNA to be sequenced is radioactively labeled at the 5′ end and undergoes selective fragmentation for the breakdown of specific base-pairing and dissociation of DNA. The resulting fragments are then loaded onto a polyacrylamide gel. Depending on the sizes of the fragments, the radioactively labelled fragments can be separated by electrophoresis, and the sequences are identified by autoradiography.

In contrast to Sanger sequencing, the samples to be sequenced need less complex preparation, but shorter reads (maximum 100 bp). This method itself is technically complex and utilizes hazardous chemicals.

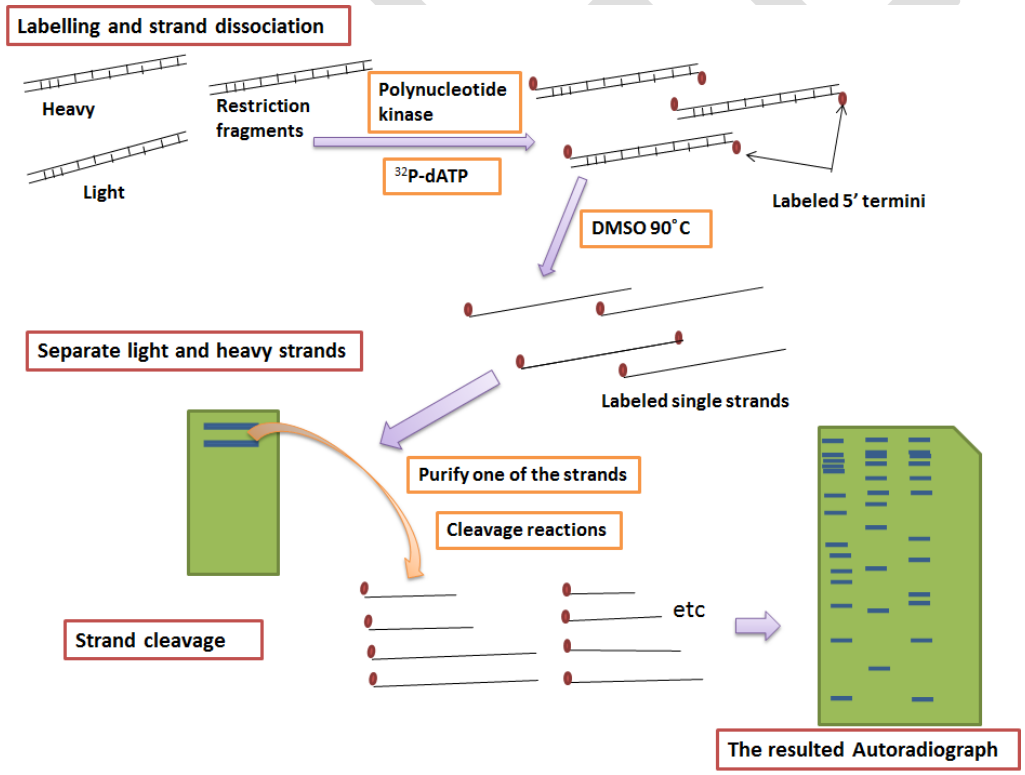


Figure 4-6.1.2 Schematic representation of Maxam-Gilbert sequencing workflow.

(Adapted and modified from Brown TA. 2006. Gene cloning and DNA analysis: an introduction.5th ed. Blackwell Scientific.)

4-6.2 CLONING STRATEGIES

The cloning of DNA fragment into a vector is routinely used method in recombinant DNA technology. Following are some strategies that are commonly used for cloning DNA fragment-

1. TA cloning
2. Gateway cloning

4-6.2.1 TA Cloning

It is a simple and more efficient cloning method without any requirement of restriction enzymes. It involves the use of terminal transferase activity of *Taq* polymerase which preferentially adds adenosine at 3' end of the PCR amplified DNA molecule. To clone PCR products having 3' overhangs, T-vector having 3'-T overhangs at both its ends can be used. This method is based on the complementary base pairing between the 3'-A overhangs of PCR product and 3'-T overhang of a vector molecule.

A single T-vector can be employed for easy and convenient cloning of any PCR amplified double-stranded DNA as well as blunt or sticky ended DNA molecules through certain minor modifications in TA cloning method. This technique, known as Universal TA cloning method, is useful in sub-cloning of DNA fragments when there are no compatible restriction sites available without compromising the cloning efficiency. Directional TA cloning can be possible by appropriate hemi-phosphorylation of the T-vectors and the inserts.

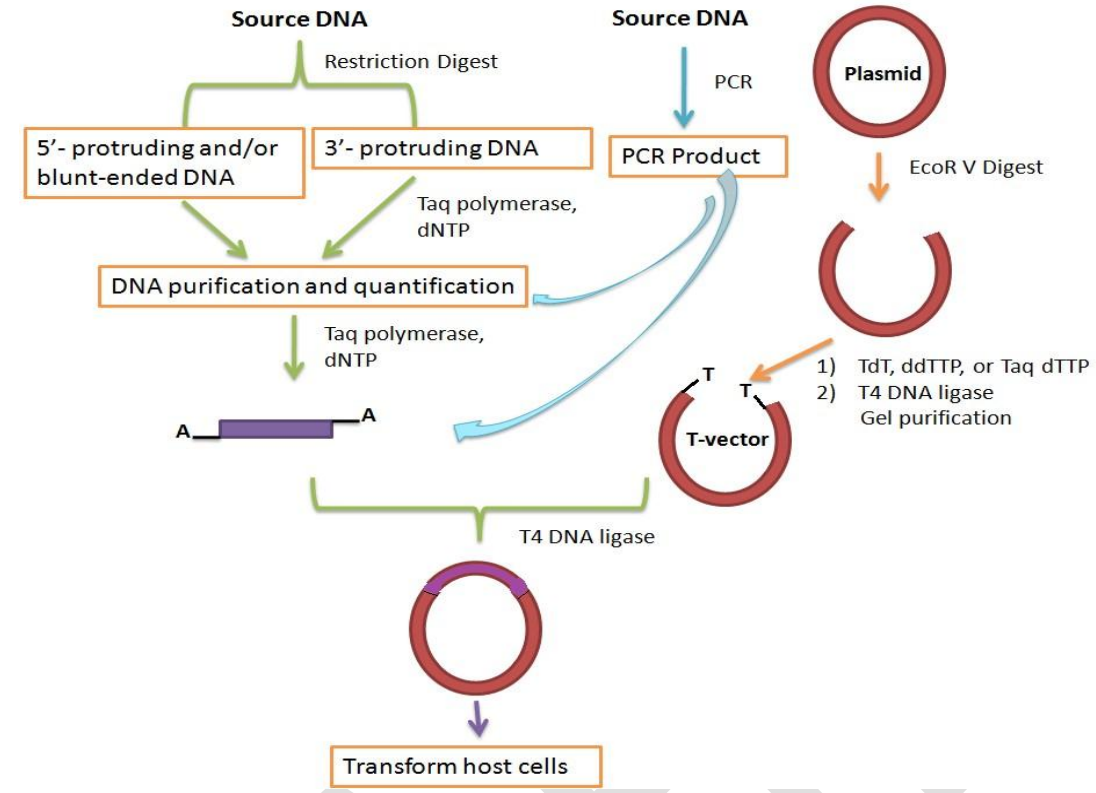


Figure 4-6.2.1(a) Schematic representation of the cloning procedures using the universal T-vectors

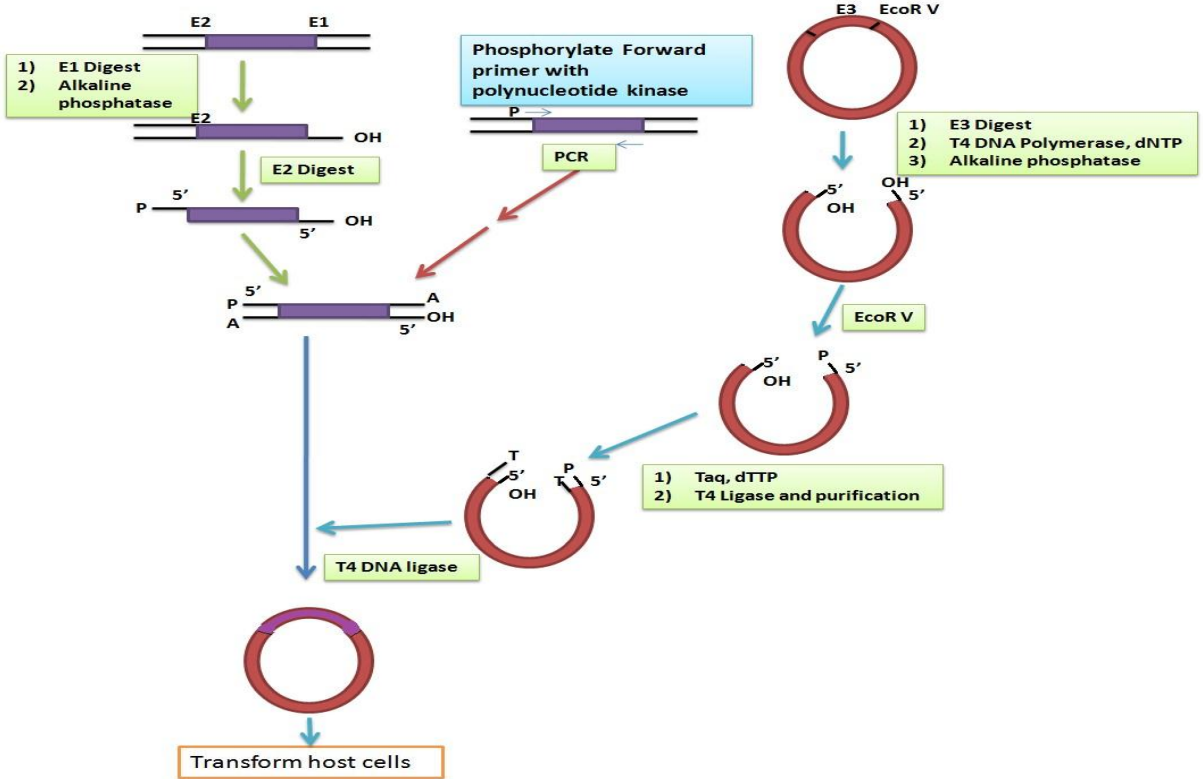


Figure 4-6.2.1(b) Schematic diagram demonstrating the strategies for directional TA cloning (E1, E2 and E3 - restriction enzymes, E3 - Generates blunt ends)

4-6.2.2 Gateway Cloning

The Gateway technology addresses the complications of efficiency, adaptability and compatibility of the traditional cloning procedures. It allows the cloning, combination and transfer of DNA fragments between various expression platforms in a high-throughput mode. It maintains the orientation and reading frame of the fragment/s of interest.

It is based on the site specific recombination method that mediates both integration and excision of bacteriophage λ. These events occur by recombination at specific attachment site on the phage DNA (*attp*) and bacterial chromosome (*attb*). The recombination begins when the phage encoded integrase and the bacterial integration host factor binds to the *attp* sequence and now this complex couples with the *attb* in the bacterial chromosome. Staggered DNA nicks are produced at the ends of *attp* and *attb* sequence followed by strand exchange between them. This results in the formation of a tiny heteroduplex joint at the point of exchange due to the presence of a short region (7 bp) of sequence homology in the two joined sequences. The holiday junction is then resolved and the prophage becomes flanked by two-hybrid *att* sites, called *attL* (for 'left') and *attR* (for 'right'). This initial reaction is referred as BP reaction. Although recombination reaction is reversible, different reaction condition prevail for each reaction ensuring that integration is not reversed by excision. Excision requires excisionase (Xis) and this reaction is known as LR reaction.

4-6.2.2.1 LR and BP Reactions

- The core of the Gateway is the *entry clone* which consists of a plasmid where the fragment of interest is flanked by *attL* sequences.
- Once the entry clone is obtained, the fragment of interest can be transferred to a secondary plasmid, the *destination vector* which is application-specific vector.
- This reaction is accomplished by mixing, *in vitro*, the entry clone (that usually carries *attL* sequences) with the destination vector of choice (that carries *attR* sequences) in the presence of a blend of the recombination proteins (Int, IHF and Xis), the LR Clonase™ enzyme mix.

- The product of this 'LR reaction' is the *expression clone*, which carries the DNA of interest and the donor vector, which is considered as a reaction byproduct.
- An aliquot of this reaction is used to transform competent *E. coli*.
- Two selection schemes are imposed to select the cells harboring the expression clone only.
    - First, the entry clone and the destination vector contain different antibiotic resistance genes (kanamycin and ampicillin in the as shown in Figure 4-6.2.2.1).
    - Second, the destination vector contains a counter selectable marker, the *ccdB* gene, which kills the cells by inducing gyrase-mediated double-stranded DNA breakage.
- Thus, transformants selected for ampicillin resistance contain only the expression clone.

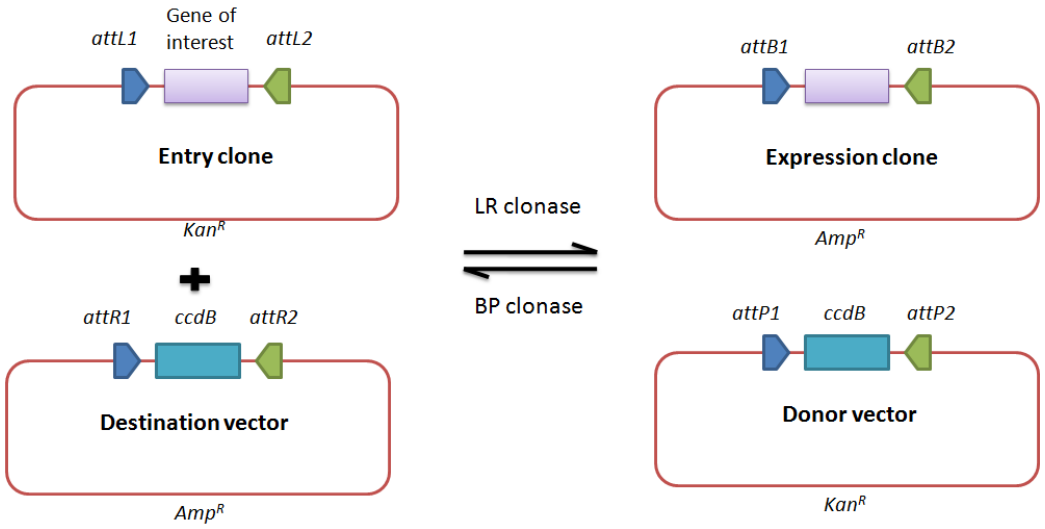


Figure 4-6.2.2.1 The Gateway reactions. The scheme shows the four types of plasmids and enzyme mixtures involved in Gateway cloning reactions. Red arrows represent the fragment of interest.

The Entry Clone

- The main door to enter a Gateway technology is the entry clone.
- There are 3 ways to generate this construct.
- First strategy is based on BP reaction.

- The second method is to ligate a PCR product into a TOPO- adapter entry clone. In this approach a single enzyme called vaccinia DNA topoisomerase having the ability to both cleave and rejoin DNA strands with extreme specificity at each step is used.
- Linearized vectors covalently attaches to the enzyme at 3' end to efficiently ligate the PCR product in a 5 minute reaction.
- The third method is the standard restriction cloning.

Destination Vectors

- A blunt-ended cassette comprising *attR1*-chloramphenicol resistance gene (CmR)-*ccdB-attR2* is inserted to any vector which results in a destination vector.
- Then the destination vector can be used in LR reaction to generate expression clone.
- Expression clones can also be generated by TOPO-cloning a PCR fragment into a TOPO-adapted expression vector.

Patenting – fundamental requirements – patenting multicellular organisms – patenting and fundamental research. Patenting the genes, patenting biological materials and biotechnology. Discrepencies in biotechnology / chemical patenting. Patenting Process.

PATENTING AND THE PROCEDURES INVOLVED IN THE APPLICATION FOR GRANTING OF A PATENT

Overview of the Patenting Process

A patent is an exclusive right of its owner to exclude others from making, using, or selling the invention as defined in the claims of the patent for a period of time, which in the United States is 20 years from the date of filing the patent application.

There are three types of patents:

1. Utility Patents may be granted to anyone who invents or discovers any new and useful process, machine, article of manufacture, or composition of matter, or any new and useful improvement thereof;

2. Design Patents may be granted to anyone who invents a new, original, and ornamental design for an article of manufacture; and

3. Plant Patents may be granted to anyone who invents or discovers and asexually reproduces any distinct and new variety of plant.

STEPS TO A PATENT

Introduction

There are several steps that help in securing a patent. The steps begin in the lab and move through the legal process of patent prosecution and maintenance. This section describes the steps that you should follow to help maximize the value of your invention and protect your intellectual property rights.

(*a*) Patent success starts in the lab

Documentation is the beginning of strong patents because it authenticates with whom a theory originates (conception) and the steps taken to test and produce results (diligent reduction to practice). These documents are scrutinized to determine inventorship, reduction to practice, and to support a patent's validity.

Well-maintained laboratory records can document the date of conception of an invention and also establish diligence in developing an idea. Such documentation is needed in case of a question about which inventor should be entitled to pursue a patent. Records can be more useful in this regard if the following steps are followed:

• Records have more value if they are meaningful to others. Entries should be complete, accurate, and legible.

• Preface the record of each experiment with a brief purpose or statement of the problem.
• Use a permanently bound notebook with numbered pages.
• Make frequent entries (daily is best) in ink, and design and date each page.
• Start a new page for each new experiment.
• Draw a continuous diagonal line through unused portions of pages remaining at the end of an experiment.
• Don't erase. Instead, where necessary, cross out with a single line.
• Initial and date all major changes.
• Record observations of physical results even if they are not fully appreciated or understood at the time.
• Have work corroborated by having notebooks witnessed by dated signature of an associate who understands the content, but not a co-worker or one who collaborates in the research area and who could be a joint inventor.
• Think carefully before destroying any samples, run sheets, or records related to any inventions.

(*b*) Plan to both publish and patent

Although the timing of publications may sometimes prohibit patenting, planning allows the inventor to both publish and patent. Disclosing the idea to the Office of Technology Transfer as soon as the invention is clearly conceptualized, or at least before submitting abstracts or manuscripts disclosing the invention, allows time to complete a patentability and commercialization assessment before being barred from a patent.

In the United States, an inventor has a grace period of one year to file a patent application after disclosure through publication. However, if an invention is publicly disclosed before a U.S. patent application is filed, patent rights in most other countries are lost. What constitutes publication? Articles in newspapers, newsletters, bulletins, textbooks, journals, theses, reports, and even letters to the editor all qualify as publications. Oral presentations may constitute publication, as would distribution of a paper at a public meeting. Some legal experts also think that disclosure through electronic communications, such as e-mail, may be considered publication. The key test is that the publication be enabling it must describe the invention in sufficient detail that it could be duplicated or put into use.

(*c*) Disclosing an invention

The technology transfer process actually begins when the inventor discloses his or her potential invention to the Office of Technology Transfer a step required by IU's intellectual property policy.

Discussions between the inventor and office staff members can help determine whether an invention has been made and whether a formal disclosure should be completed. The office staff supplies inventors with a formal disclosure form and assists in its preparation. An invention disclosure is a written record of an invention

containing a complete description of the invention, the inventor's dated signature, and dated signatures of witnesses who fully understand the invention (but are not joint inventors).

(*d*) Invention evaluation

When the completed disclosure form has been reviewed by the Office of Technology Transfer and discussed with the inventor, recommendations are formulated on ownership, patenting, and licensing. Inventions are evaluated for novelty, likelihood of patentability, potential market, usefulness, projected development time, and cost. The most effective way to bring the invention's benefits to the public will be determined, whether through patent, copyright, or placing the invention in the public domain (usually through publication).

The probability for an invention's economic success may be roughly gauged by these questions:

• How big is the potential market for the invention? Can the invention be sold to a large section of the public or a large number of manufacturers? Can it be sold to different industries?

• What development will be required before the invention can be sold? How long will development take? What will it cost? What regulatory requirements must be satisfied?

• How will the product be marketed? Can it be distributed through normal commercial channels?

• What is the demand for the invention? Does it fill a real need and not just replace satisfactory article? Does it contribute to the interest?

In most cases, before a patent application is prepared, the Office of Technology Transfer staff will search for potential licensees to determine the level of industrial interest in the technology. (A license is essentially an agreement by the patent owner not to sue the licensee for infringement as long as the licensee abides by the agreement. Licensing is typically the way the university realizes an invention's commercial potential). In most cases, commercial organizations will underwrite patent expenses in return for the right to a license.

(*e*) Patent prosecution (process of obtaining a patent)

The process of obtaining a patent is called patent prosecution. It consists of preparing and filing the patent application, then filing responses and amendments to the objections of the patent examiner. Patent prosecution will result in either the issuance of a published patent or the rejection or abandonment of the application.

Under U.S. law, individual inventors are allowed to prosecute their own patent applications. However, because the Patent and Trademark Office has specific and often complex rules about the content and examination of applications and because patents are interpreted and enforced in court, inventors should be represented by a patent attorney or agent. To qualify as a patent attorney, an individual must have a law degree and a degree in a technical area, and the person must pass the rigorous patent bar exam. To become a patent agent, a person still

must pass the patent bar exam, but a law degree is not required. The patent application is prepared by the patent attorney with the help of the inventor and is similar in many respects to a detailed scientific paper (the specifications and drawings) accompanied by one or more claims, which make-up the legal definition of the invention. The patent application must make a full disclosure of the invention to teach others how to make and use the invention and to clearly define the borders of the patent protection. Accomplishing both these objectives requires the close collaboration of the inventor and the patent attorney. Although the make-up of patent applications varies considerably, it is commonly divided into the following headings:

• **Field of the invention —** This describes the general technological field and the broad nature of the invention.

• **Background of the invention —** The background describes the technological problem to be solved and gives a brief description of present technology and its limitations.

• **Objectives of the invention —** The objectives indicate the nature of the improvements the invention seeks to provide.

• **Summary of the invention —** The summary states the essential elements of the invention in broad terms and often introduces the terminology to be used in the main claims of the patent.

• **Detailed description of the invention and/or description of the drawings —**

These details include the experimental data, given by way of example, describing the methodology of the invention and the apparatus used (if any).

• **Claims —** Claims define the invention in one or more single-sentence paragraphs and serve as the legal definition of the invention. When received in the Patent and Trademark Office, the application package is assigned to a patent examiner with expertise in the invention's technical field. Although work loads and response times vary, usually six months or more pass before the application's initial examination. In the initial examination, the examiner searches both the scientific and the patent literature to determine whether the application discloses and claims new and patentable subject matter, and the examiner judges the allowability of each claim. Most applications are initially rejected. The basis for rejection is most often prior patents or publications which, in the examiner's view, render the new invention obvious. The patent attorney, with the assistance of the inventor, responds to the examiner with arguments about why the invention is patentable. The cycle can be repeated several times and the patent application can be amended, restricted, divided, or continued in the process. On average, the examination of a patent application takes two years in the United States, where the application is considered confidential throughout the process. In many other countries, patent applications are published after a given period of time. The Patent and Trademark Office allows, or approves, around 90,000 patents a year. The total number of patents issued now exceeds 5 million. When the Patent and Trademark Office gives notice of allowance, and the issue fee is paid, the patent is issued, or published in the Patent Gazette.

The cost of the typical U.S. patent prosecution for university, conducted by outside legal counsel, is $15,000 or more.

Summary of Legal Process from the Inventor's Perspective

Filing a Patent Application

Office of Technology Transfer (OTT) submits the invention disclosure to a patent attorney

↓

Patent attorney determines patentability and with the aid of the inventor(s), drafts a application for review

↓

Application is filed along with an assignment, declaration, and power of attorney

↓

Provisional *Regular*

After 1 year the application must be Converted to a PCT application

After 1 year the foreign placeholder called a PCT application is filed

↓

18 months after the first US filing, the PCT will publish

↓

18 months after the PCT is filed, the PCT must be converted to a separate application per each foreign country ("nationalization"). Because nationalization is extremely expensive, the university does not nationalize without corporate sponsorship.

Examination of a Patent Application

Patent offices worldwide correspond with applicants through official "office actions". The patent attorney and ARTI work with the inventor(s) to answer each office action the time allotted (typically 3 months from the date the examiner issues the action).

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A successful prosecution results in a "notice of allowance" from the patent office indicating that the examiner has accepted claims that will "issue" in a patent.

Maintenance of a Patent

Maintenance fees must be paid 3.5, 7.5, and 11.5 years after the patent is issued.

↓

All patent applications filed after June 8, 1995 expire 20 years from the filing, while applications filed before this date expire 17 years from the date the patent was issued.

COMPULSORY LICENSES

Compulsory licensing system has become a typical feature of patent laws; it has also been widely adopted in other areas of intellectual property rights. Developed countries have largely relied on such licenses in order to limit exclusive rights and prevent or remedy abusive practices in several areas. The study reveals there is a broad range of grounds under which compulsory licenses may be granted in both developed and developing countries. The grounds and conditions on which compulsory licenses have been regulated and granted in developed countries illustrate the flexibility and potential of the compulsory licensing system to address a multiplicity of public interests and concerns. The evidence also indicates that arguments often voiced by the developed countries' business community and governments against compulsory licenses as a deviation from acceptable standards for intellectual property rights, are not reflected in the policies actually applied in such countries.

Three Main Conclusions Particularly Relevant for Developing Countries may be Drawn from the Previous Analysis.

• Compulsory licenses should be considered as an essential element in patent laws and other intellectual property regimes. Developing countries should disregard any attempts by developed countries to limit under bilateral or other agreements the scope of and grounds for compulsory licensing.

• The grounds and conditions for compulsory licenses should be carefully determined by national laws. The extent to which such licenses would be available and effective depend on the provisions of national legislation and on its adequate administration by informed national authorities.

• Developing countries should preserve the maximum possible freedom under international rules to design their compulsory licensing systems, according to their own interests and needs, including in such areas as the protection of health and the environment, and the promotion of transfer of technology and local industrialization. Should the issue of compulsory licenses be included in the agenda of possible future negotiations in WTO, developing countries should seek to clarify the scope for the granting of such licenses in certain cases (*e.g.,* of non-exploitation), as well as to remove some of the restrictive conditions imposed by the said TRIPs Agreement.

EXAMPLES OF PATENTS IN BIOTECHNOLOGY

In recent years, society has witnessed the explosive growth of biotechnology research. Much of this research has had a profound effect on our perception of the fundamental fabric of life itself. However, because of the

complex nature of these discoveries, commercialization is often a long and expensive process. As a result of the need to achieve a proprietary position over this technology and the investment entailed in its commercialization, the field has seen phenomenal growth in the number of biotechnology patent applications which have been filed. Intellectual property is a term used to describe property that is not tangible, but which instead originates through the creative effort of the inventor. Such property can be further characterized as a trademark, a copyright, a trade secret, or a patent. These divergent areas have in common a highly abstract concept of property.

(*a*) **Trademarks** include words, names, slogans, logos, and symbols which are used to *indicate the source* of a product or service. A trademark owner has the right to stop the commercialization of competitive goods having trademarks which are *confusingly similar* to those of the trademark owner.

(*b*) A **copyright** is the right to the exclusive publication, reproduction, adaptation, display, and performance of an original work which is fixed in a tangible medium of expression. A copyright protects the expression of an idea, but not the idea itself.

(*c*) A **trade secret** can be patentable and, unlike patents, is potentially indefinite in duration. However, the value of a trade secret is lost once it is disclosed. Also, the holder of a trade secret has no cause of action against those who independently discover the trade secret. From a societal standpoint, probably the greatest disadvantage of a trade secret is that, by its very nature, the trade secret may die with the owner. For example, the methods of treating wood employed by the famous violin maker, Stradivarius, have been lost forever. It was partly in response to the drawbacks of trade secret protection, as embodied by the Guild System of Medieval Europe, that the **patent system** was established.

The public policy behind the patent system is to encourage inventors to share their discoveries with the general public and thereby advance the general status of technology. The advancement is accomplished by encouraging innovation through giving the inventor the right of exclusive commercial use, and by encouraging competitors to design around the invention. Thus, the knowledge of the inventor is preserved for the benefit of society and future generations. In the U.S., patent can be further categorized as design, plant, and utility patents. A **design patent** can be obtained for a new, original, and ornamental design; the invention covered by the design patent must not be for a characteristic which is primarily functional. For example, a design patent application might be filed for a pitcher which has a particularly attractive handle. If it were found that the handle of the pitcher were primarily functional (for example, it provided a better grip), then a design patent would not be appropriate.

Under the **Plant Patent** Act (PPA), patent protection is available for new varieties of asexually-reproduced plants. Protection under the PPA is very narrow; only one claim is permitted, and it covers only the whole plant. Thus, under the PPA, it is not possible to claim seeds, fruit, cells, or any other part of a plant which may be of commercial value. Also, because the PPA covers only plants which are asexually reproduced, such as roses and other ornamental plants, many agriculturally important plants cannot be protected under the PPA. The most important type of patent, from a scientific and commercial standpoint, is the **utility patent.** From the standpoint of the inventor, the purpose in trying to obtain a utility patent is to secure the exclusive right to make, use and sell the patented invention. Those exclusive rights exist for the term of the patent. Patent terms were recently changed: (1) for applications filed after June 8, 1995, the term of any patent that issues will be 20 years from the first effective United States filing date of the application; (2) for applications on file on or before June 8, 1995, and for patents in force on June 8, 1995, the term of the patent is the longer of 20 years from the date of filing or 17 years from issuance of the patent.

The first thing which should be considered in determining whether a utility application should be filed is whether the subject matter of the invention is proper under the patent laws. One can address this issue by first considering what types of subject matter are not patentable.

Non-patent subject matter includes:

1. Printed matter, where the invention resides solely within the printed matter and does not involve any mechanical features;

2. Naturally occurring articles, where there has been *no human intervention***;**

3. Methods of doing business, such as book-keeping and accounting techniques; and

4. Scientific principles, such as theories and formulas.

Utility patent protection is available for:

(*i*) processes (*i.e.*, methods);

(*ii*) machines;

(*iii*) articles of manufacture; and

(*iv*) compositions of matter.

Examples of process-type inventions that may be patented include chemical processes, such as the manufacture of chemicals, and methods of treating or diagnosing disease. Also included in the category are methods of using previously-known drugs. The terms “machines” and “articles of manufacture” should be fairly self-explanatory.

A. In biotechnology, some of the most valuable types of patents fall into the *composition of matter* category. The term "composition of matter" includes mixtures of chemicals, pure chemical compounds, polymers (such as plastics), and *purified products* not pure in nature. Examples of this latter category are antibiotics, enzymes, and lymphokines. More specifically, patents can be obtained for:

(*i*) DNA

(*ii*) proteins

(*iii*) antibodies (*e.g.*, monoclonal antibodies)

(*iv*) pure cultures of microorganisms and viruses

(*v*) transgenic plants and animals.

Assuming an invention is patentable in terms of subject matter, there then remain three statutory requirements which the invention must meet: the invention must be useful, novel, and non-obvious.

"Useful" means the invention must be of some (even if small) benefit. Thus, the statute precludes obtaining a patent on an invention which is merely a curiosity, or which is illegal or immoral. For example, a machine useful solely for producing counterfeit money is unpatentable. The requirement that the invention be useful does **not** mean it must rise to the level of being *commercially* useful. Many inexperienced applicants believe they must delay filing a patent application until they have developed the invention to the point where it is a commercial product.

For example, where a new anti-cancer drug has been discovered, inventors sometime believe, wrongly, that human data meeting the requirements of the FDA must be obtained in order to file a patent application, whereas it is usually sufficient to have *in vitro* data showing inhibition of a cancer cell line. In fact, delaying filing a patent application in order to generate elaborate experimental results carries with it the very real risk that another inventor will file ahead of you.

Another statutory requirement of the patent law is that the invention be novel. The events which can prevent an invention from being considered novel are events which occur before the date of invention, and more than 12 months before the filing date of the patent application.

Thus, a patent cannot be obtained if, before the date of invention, the invention was: publicly known or used by others in this country, or patented or described in a printed publication anywhere in the world. Further, a patent cannot be obtained if, more than 12 months before the filing of the patent application, the invention was patented or described in a printed publication anywhere in the world, or in public use or on sale in this country. It is

important to keep in mind that the 12-month publication grace period is unique to U.S. law; most foreign countries have a different rule, known as *absolute novelty*. (Absolute novelty means patent protection is lost by sale or publication of the invention prior to the filing of the patent application).

Non-obviousness of an invention, like novelty, involves a comparison of the invention with the prior art. But, unlike novelty, which only considers prior art which is the same as the invention, obviousness considers the prior art with respect to what the next obvious step would have been. In evaluating obviousness, it is necessary to evaluate the so-called subjective and objective indicia of obviousness. The subjective factors relating to obviousness have been defined by the U.S. Supreme Court: the scope and content of the prior art; the differences between the invention and the prior art; and the level of ordinary skill in the art.

The objective obviousness factors, developed by the courts over several decades, are: commercial success; long-felt need; failure of others; unexpected results; skepticism by others; and teaching away in the literature.

A patent application also must teach one of the ordinary skill in the field how to make and use the invention. This is known as the *enablement requirement*. If the patent application is so inexact as to require substantial experimentation for success, the invention may be unpatentable. The patent statute also requires that the inventor disclose the *best mode* for making and using the invention. Thus, where an inventor has developed two different processes for synthesizing a compound, but one of the processes is less expensive or simpler, then the patent application must teach that preferred process.

B. In biotechnology, the enablement and best mode requirements have resulted in what has become known as the *deposit requirement.* When life forms are an essential part of a patent application, special problems arise with respect to satisfying the enablement requirement. Sometimes these types of inventions cannot be reproduced by following a written description. For example, new antibiotics made by microorganisms not generally available to the public raise the issue of whether merely describing the microorganisms, and where and how they were found, satisfies the enablement requirement. It was on this basis that the Patent Office established the policy of requiring inventors to place these rare organisms in depositories accessible to the public.

Investorship: Another issue which must be addressed when filing a patent application is that of inventorship. In the U.S., unlike many foreign countries, the patent application must be filed in the name of the true inventor or inventors. Joint inventorship requires that each inventor contribute to the *conception* of the invention. Conception is the mental formulation of an idea complete enough so as to enable one of ordinary skill in the

field to reduce the concept to practice without undue experimentation. Contribution to the actual reduction to practice is irrelevant for purposes of determining inventorship.

Although it is not necessary for joint inventors to physically work together, there must be some degree of collaboration among them. Further, an individual who merely follows the instructions of another is not a joint inventor. For example, a lab technician who carries out experiments under someone else's instructions and records the results is not an inventor. Finally, an individual does not become a joint inventor by suggesting a desirable end or result without suggesting the means of accomplishing the result.

I. Biotechnology has increased the availability of many natural biological products useful in treating various diseases. These products, such as human growth hormone (hGH), often exist in such minute quantities that isolation from natural sources is impractical or extremely expensive. Sometimes, such as in the case of insulin, the product can be isolated from alternative natural sources (*e.g.*, pig), but may be less effective or accompanied by undesired side effects.

II. Biotechnology has also been used in disease detection. For example, HIV diagnostic tests have been developed using recombinant DNA technology.

III. Biotechnology research is extremely risky and expensive and often involves time-consuming, resource-intensive characterization of the genes that encode the desired biological product. Sometimes, the product itself has to be characterized to determine which gene is the "right" one. Organizations who do biotechnology research are very interested in protecting their investment by obtaining patents to prevent others from freely practicing the "fruits" of this research. What "fruits" of biotechnology research can be patented? This list is almost endless. For example, patents can be (and have been) obtained on the isolated gene, modifications of the gene, purified and modified biological products encoded by the gene, methods for making the gene and its encoded products, as well as methods for using the gene and its encoded products.

The ability to obtain patents on these "fruits" has been aided by the perception of biotechnology as an "unpredictable" art. One of the requirements for getting patent coverage is that the invention not be obvious to one of ordinary skill in the art. The perception of biotechnology as an unpredictable art tends to negate obviousness, as reflected in many court cases that have upheld the validity of biotechnology patents.

IV. As owners of biotechnology patents have unfortunately found out, the perception of biotechnology as unpredictable is a two-edged sword. To be valid, the patent must also contain a written description of the invention "in such full, clear, concise, and exact terms as to enable any person skilled in the art" to make and use the invention ("enablement requirement"). The predictability as to what will (and will not) work greatly determines how much of the patented invention is enabled. The enablement requirement has proven to be a significant barrier to enforcing broad biotechnology patents. Many broad biotechnology patents claim the invention in terms of its functional characteristics, rather than its chemical structure, to obtain broader coverage. For example, some biotechnology patents claim the gene in terms of its ability to encode a class of proteins that are functionally analogous to a particular biological protein. Other broad biotechnology patents claim the biological protein (*e.g.*, hormone) in terms of its activity. These broad "functionalized" biotechnology patent claims have not faired well in court. The courts have applied a fairly stringent enablement standard to such broad claims because of the perceived unpredictability of biotechnology. This stringent standard has been difficult to satisfy, especially since the litigated patents usually have had only one or a few working examples of the gene or product.

The difficulty in satisfying the enablement requirement in the biotechnology area has led alternatively to claims limited to genes or encoded products that are specifically exemplified in the patent. However, as the litigated patents show, a competitor may slightly alter the gene or encoded product, and thus avoid infringing such narrow patent claims.

So how does one get broad biotechnology patent claims that will satisfy the enablement requirement? Here are some suggestions:

(*a*) Exemplify as much as possible in the patent disclosure the scope of the biotechnology being claimed. This includes how to make and how to use the claimed biotechnology. It is also important to understand as much as possible the operative limits of the claimed biotechnology and to put that knowledge into the patent disclosure. Be careful in relying on "illustrative" or "prophetic" examples without actually testing a representative selection of such examples to see if they do work. The litigated patents have shown the danger of relying on such examples when they later turn out not to work. Also, make sure each term, competent and step recited in the patent claim is defined in sufficient detail. As one 1996 court case painfully demonstrates, reliance on general teachings in the art can be extremely risky in the biotechnology area.

(*b*) Augment the initial patent disclosure by filing continuation or provisional patent applications to include new working examples or learnings. To preserve patent rights in "first to file" countries such as Europe, it is not unusual for a biotechnology patent to be filed with broad claims, but with only a few or possibly only one working example disclosed. Rather than rely on what later may be held to be inadequate enablement, consider filing continuation or provisional patent applications to include new working examples or learnings. Provisionals are especially valuable for doing multiple filings (at relatively low cost) to augment the initial patent disclosure. For example, five U.S. provisional applications can be filed for less than the cost of one traditional U.S. patent application.

(*c*) Where possible, pursue broad claims to methods for making the gene or biological product. Do not overlook the value getting patent coverage on the method of or making the gene or biological product. Under appropriate circumstances, importation of the gene, and more importantly the biological product, into the U.S. can be prevented if the gene or biological product is made by a patented method. Indeed, one court case that prevented importation of hGH made by a patented method involving recombinant DNA suggests broad method claims for making genes or biological products may be less likely to run afoul of the enablement requirement. The perceived unpredictability of biotechnology has certainly made it more difficult to get enforceable broad claims on the "fruits" of biotechnology research. However, as the courts have made clear, broad patent coverage on these "fruits" is not precluded. The challenge is to craft patent disclosures that satisfy the more stringent enablement standard applied by the courts to the biotechnology area.

SPECIAL APPLICATION OF PATENTS IN BIOTECHNOLOGY

Patenting in drugs, filings by Indians/Indian companies

Indian scientists and technologists have been quick in responding to the post TRIPS challenge by filing substantially more patent applications. The filing by Indians has gone up by 155% over the applications published in 1996 as compared to an increase of 25% in the filing by foreigners/foreign companies. However, the former is now about 33% of the latter as against 16% observed in the earlier analysis, a significant improvement to be noted. Further, now there are seven companies/institutions which have filed 5 or more applications as against two observed in the preceding analysis, showing a growth of about 250%. It has been noted that almost 30% of the applications relate to herbal and ayurvedic medicines; such applications have even been filed by companies like Lupin and JB Chemicals. However, this is only the beginning of a triumphant journey, a successful end will be determined by how many of these are finally accepted and how many of these are picked up for commercialization. A good beginning portends good results.

Of the Indian applications filed, 114 applications were filed by Indian companies and the rest 52 by individuals. CSIR with 19 patent applications, ranks first among Indian companies and enjoys an overall 8th position jointly with Merck Patent. The CSIR applications relate to inventions for processes producing the following: oxindole as intermediate for producing the tenidap, an anti-inflammatory agent, analgesic analogous to enkephalin, vaginal contraceptive, 17-ketosteroids, dihydroasperoside and dihudrosiredloside, artmesnin, antibiotic from fermented froths, ciprofloxacin, organotin useful as cytotoxic agents, L-alanyl-l lysyl derivatives useful as antiasthmatic/antiallergic, pyridines as potent cardiovascular agents, codeinone, chloropropane and 2-piperidone useful as potential hypotensive agents, pyridine and 3-picoline, lipopolysaccharide (LPS), 7 methoxy deoxyvasicinone, 4-arylamino/alkylamino-4- demethylpodophyllotoxins as potential anticancer agents: and epichloromydrin. Three of the **Lupin's** applications concern anti-tubercular composition. Other applications include process for purification of atenolol (2-4-hydroxy-3-1) phenyl acetamide, process for extraction of hydroxycitric acid from fruit and garcinia species, process for the manufacture of ceftazidime, process for isomerization of N-7 isomer to N-9 isomer, process for the manufacture of 3-hydroxy- 3-cephem derivatives, process for manufacture of cephalosporin antibiotics such as cefazolin, a regiospecific process for synthesis of acyclic nucleosides and ayurvedic formulation from *Amla* and *Ritha.* **Neam Herbal Remedies** has focused on pre-cooked ayurvedic medicinal food and all the applications fall in this area. The applications from **Dr. Reddy's Foundation** relate to new heterocyclic compounds having antidiabetic, hypolipidaemic, antihypertensive properties, their preparation and pharmaceutical compositions containing them; benximidazole derivatives as antiulcer agents; novel heterocyclic compounds for treating diabetics and related problems; 4-hydroxy-10-deacetylbaccatin III derivatives; azolidinediones useful for the treatment of diabetes dyslipidemia and hypertension; podophyllotoxin analogues and their derivatives as anti-cancer and anti-viral agents. Applications filed by Hindustan Lever are not included under the Indian companies as all of these are convention applications. All the seven applications filed by the Indian citizens, **P.B. Mathur** et al, are ayurvedic medicines for reducing cholesterol and treating chronic diseases such as cough, acidity and gastritis, piles, sinusitis, and cold. Two of the **Sun Pharmaceuticals'** applications relate to antihypertensive fixed-dose combination products while the other two are on topical antibacterial anaesthetic combinations. The remaining two applications are entitled 'a process for the recovery of tramadol as cis-hydrochloride in asymptotically quantitative amount from mixtures of diastereomers of tramadol' and 'an improved process for the preparation of 1-(2,3-epoxypropyl)- 5-nitroimidazole'.

A few other Indian companies and institutions who have filed more than one application with the no. of applications filed given in brackets are Panacea Biotech (4), Ranbaxy Laboratories (4), Raptakos Brett & Co.

(4), Tablets (India) Ltd. (4), National Institute of Immunology (3), Osmania University (3), Dabur Research Foundation (2), Hindustan Antibiotics (2), IIT (2), Sonic Biochem Extraction Pvt. Ltd. (2), Sree Chitra Tirunal Institute for Medical Science and Technology (2), and Themis Chemicals (2).

PATENTING OF LIVING ORGANISMS

Patenting Life? — An Introduction to the Issues

Once upon a time, we knew that animals were products of nature. We used them and "owned" them, but it was different from owning a pair of shoes. Animals could get up and walk away; shoes couldn't. And unlike patent leather, you couldn't patent a cow. Patents are about inventions, and since when had human beings invented an animal? Since 1984, if you believe Harvard University and the US Patent Office. For that waswhen Harvard applied for a patent on a genetically modified mouse, which was granted 4 years later, causing a big bang of controversy which soon reached the shores of Europe and whose ripples are still very much in evidence. For this was the first time it was officially decreed that an animal could indeed be classed as an invention. Moreover, it was a mouse specifically engineered to have an increased probability of suffering malignant tumours — for use as a "model" for studying human cancers and carcinogens.

Controversy in Europe

The combination of these two factors has raised human hackles far and wide. It generates surprisingly heated arguments wherever the issue is debated. The question of patenting "animate matter" has given long term headaches to the European Patent Office in Munich, and in March 1995, it led to the first ever rejection by the European Parliament of a European Commission Directive. A new draft EC Directive on patenting is currently being discussed in the early committee stages of the European Parliament, and is again the subject of deep seated controversy between industry proponents and many diverse groups which include church groups, NGO's, environmental and animal welfare organisations, and also many doctors, farmers and ethicists.

Biotechnological Inventions — Products of Nature or Products of Industry?

You cannot patent a mere discovery. It must have a non-obvious "inventive step", and some specified practical application. Patent law was framed in an industrial context, and typically applied to objects, chemicals, designs and processes. Agriculture was seen as lying outside this realm. You could patent a mouse trap, but not a mouse. But, with the rise of biotechnology, a shift has occurred, partly in technical sense, and partly in our perceptions. Once it became possible to alter the genetic make up of living things, researchers could genuinely claim an "inventive step" in the organism itself. And since such research is expensive and easily copied, organisations wanted to patenting genetically modified organisms to protect their valuable investment. The key case

concerned a micro-organism, perhaps only a small step from patenting biochemical products. It went right up to the US Supreme Court, who in 1980 ruled that "anything under the sun that is made by man" was patentable subject matter, which turned it into the giant leap which has set the trend ever since. But it was not until its implications began to extend from micro-organisms to warm and furry animals that the fundamental question dawned on people generally: were they right? Oncomice, transgenic sheep, or whatever: should we be patenting our fellow creatures at all? Isn't this violating something rather basic in our attitudes to nature, implying they are nothing more than machines for our use? We say we "own" animals, but what does this really mean? They have their own lives and freedom which we are surely to respect – simply as parts of nature alongside us, and, from a Christian perspective, as God's creatures each of inherent worth. For many, the heart of the problem is that to patent an animal includes it in the same category as mere mechanical objects. Is that symbolic association sending ourselves and our society entirely the wrong kind of signal? Patent expert Stephen Crespi suggests that living things are now regarded as "products of manufacture" and agriculture to be a kind of industry.

The Guidelines for Labelling of Genetically-Modified Foods

Pre-packed food must state in the list of ingredients for any soya or maize, which has been genetically-modified *e.g.,* "produced from genetically-modified soya" or "produced from genetically modified maize" or, those words may display in footnote to the list of ingredients related by means of an asterisk (*) to the ingredient concerned *e.g.,* soya * flour. *genetically-modified.

Some approaches proposed by the European Commission that leads to the following labelling:

1. Voluntary labelling (*e.g.*, "this does not contain GMO…)" for certified non-GMO produce.
2. Mandatory labelling (*e.g.,* "this contains GMO…") for produce known to be of GMO origin or "this may contain…" in cases where material of GMO origin cannot be excluded but where no evidence of such material is available).

In order to make an educated decision on the matter, one needs to see the both sides of the issue.

Disadvantages

(*a*) Difficult to trace every use of GM technology. Unless you follow the farmer step by step, it is difficult to assess what has been modified, especially when the ingredients come from various farms.

(*b*) On what level should labeling be done? *i.e.,* do you label beef as being GM if the grass the cow ate was sprayed with GM pesticides?

(*c*) Not economical for farmers to segregate their GM crops from conventional ones.

(*d*) Would cause trade barriers between countries. *i.e.,* if Canada wanted to export foodstuffs to a country that had strict mandatory labeling on all GM foods, the receiving country may not accept the products if they have not been labeled appropriately.

Advantages

(*a*) Consumer knowledge of potential health concerns. *i.e.*, allergens.

(*b*) Increased customer awareness. *i.e.,* give them the choice of what to buy and eat such as for religious reasons or vegetarianism, etc.

(*c*) Economical for retailers to make two-tiered system of products : conventional and nonconventional. Can charge higher prices for conventional foods if there is a demand for it.

Social and Political Issues

Food is of particular interest when considering biotechnology. Because we take it into our bodies, we have a fundamental right to know what it is, how it was processed and that it is safe. About 60 percent of our processed foods are genetically engineered. Therefore, it is important to concern about the pros and the cons sides of labeling genetically-modified foods.

Pros

European Union states that labeling must be applied to novel foods and their ingredients produced by means of genetic engineering when there is no substantial equivalence between a novel food and its original counterpart; when materials present in the novel food are not present in an equivalent non-modified product and may have consequences for the health of certain groups of people; when the novel food contains biotechnologically-derived material that may present ethical problems; and when living Genetically-Modified Organisms (GMOs) are present in the novel food (Nature biotechnology 16(10), 889, 1998 Oct).

In an Environics poll conducted in August 1999, 80 percent of respondents said they wanted labeling which told them what foods were Genetically Modified. Government and industry have responded with a voluntary labeling plan. That has some consumers dissatisfied. “I don’t want to take the risk I would like to have them labeled, so that I can decide what I’m going to buy and not going to buy”, “You hear about plants being altered with animal genetic material and *vice versa*. I don’t know how that works but I’m really apprehensive about it”. Therefore, consumer have the right to choose whether they want to buy GM food or not (Marketplace “Labeling Genetically Modified Foods” Dec. 7, 1999).

Lawsuit also argues that the Food and Drug Administration (FDA) should be treating genetic modifications as new food additives, which need to be tested for safety and approved before being sold. Moreover, the lawsuit claims that the agency should be treating all genetic modifications as additives (Nature "Lawsuit demands labels for modified foods", June 4, 1998). Consumers International (CI) says that labeling is not just an issue of health and safety, they are labeled to enable consumer choice. CI says that surveys from many countries indicate widespread public support for comprehensive labeling of GM foods, 92% of respondents to survey by the UK Consumers' Association wanted GM food to be labeled, regardless of the presence of a GM ingredient in the final produce (Nature "GM foods debate needs a recipe for restoring trust", 22 April, 1999).

Genetically-Modified Foods are inherently allergenic and/or harmful. By labeling, consumers have knowledge about the potential allergens and other health risks of the GM food. (UCT "The Genetically-Modified Foods debate in South Africa" 22 May, 1999). The Government is determined that all food which contain Genetically-Modified material should be clearly labeled. Food will also require labeling if there are any health or ethical concerns or if it contains a labeling of ingredients derived from GM soya and maize. Committee on the Ethics of Genetic Modification and Food Use recommended that a GM food should be labeled if it contains a gene derived from a human, or from an animal which is the subject of religious dietary restrictions; or if is plant or microbial material containing a gene derived from an animal. These recommendations are now a legal requirement, having been implemented under the Novel Foods Regulation 1997.

Cons

Labeling requires the availability of a technique that can guarantee the detection of transgenic DNA and protein, however, the detection of transgenic DNA or protein is not an easy task, and currently there is no officially validated protocol available for use. Currently, a few private companies and public laboratories are offering a PCR-based method for the detection of traces of specific transgenic genes in soya and maize. It would also be necessary to establish threshold levels above which labeling should be mandatory.

CLASS: III BSc MB COURSE NAME: MICROBIAL TECHNOLOGIES AND INTELLECTUAL PROPERTY RIGHTS
COURSE CODE: 15MBU602 UNIT: V

Intellectual Property Rights – historical perspective – recent developments, IPR in India, IPR and the rights of farmers in developing countries.

Intellectual Property Rights

Intellectual property, often known as IP, allows people to own their creativity and innovation in the same way that they can own physical property. The owner of IP can control and be rewarded for its use, and this encourages further innovation and creativity to the benefit of us all.

In some cases, IP gives rise to protection for ideas but in other areas, there will have to be more elaboration of an idea before protection can arise. It will often not be possible to protect IP and gain IP rights (or IPRs) unless, they have been applied for and granted, but some IP protection such as copyright arises automatically, without any registration, as soon as there is a record in some form of what has been created.

The four main types of IP are:

• Patents for inventions—new and improved products and processes that are capable of industrial application

• Trade marks for brand identity—of goods and services allowing distinctions to be made between different traders

• Designs for product appearance—of the whole or a part of a product resulting from the features of, in particular, the lines, contours, colours, shape, texture or materials of the product itself or its ornamentation

• Copyright for material—literary and artistic material, music, films, sound recordings and broadcasts, including software and multimedia. However, IP is much broader than this extending to trade secrets, plant varieties, geographical indications, performers rights and so on. To understand exactly what can be protected by IP, you will need to check the four main areas of copyright, designs, patents and trade marks as well as other IP. Often, more than one type of IP may apply to the same creation.

Patent

A patent gives an inventor the right for a limited period to stop others from making, using or selling an invention without the permission of the inventor. It is a deal between an inventor and the state in which the inventor is allowed a short-term monopoly in return for allowing the invention to be made public.

Patents are about functional and technical aspects of products and processes. Most patents are for incremental improvements in known technology—evolution rather than revolution. The technology does not have to be complex.

• Specific conditions must be fulfilled to get a patent. Major ones are that the invention must be new. The invention must not form part of the "state of the art". The state of the art is everything that has been made available to the public before the date of applying for the patent. This includes published documents and articles, but can also include use, display, spoken description, or any other way in which information is made available to the public.

• Involve an inventive step, as well as being new, the invention must not be obvious from the state of the art. Obviousness is from the viewpoint of a person skilled in the area of technology that the invention is in.

• Be industrially applicable. This condition requires that the invention can be made or used in any kind of industry.

A patented invention is recorded in a patent document. A patent document must have

• description of the invention, possibly with drawings, with enough details for a person skilled in the area of technology to perform the invention.

• claims to define the scope of the protection. The description is taken into account while interpreting the claims.

The original patent document of a patent application is published by a patent office. The application then adds to the state of the art for later applications and anyone can comment on the application. Often the patent document needs altering or amending to meet the conditions above before a patent can be granted. The final version of the granted patent document is then republished. If more information about the state of the art is discovered after grant, the patent document can be amended and republished again. Patent rights are territorial; a UK patent does not give rights outside of the UK. Patent rights last for up to 20 years in the UK. Some patents, such as those for medicinal products, may be eligible for a further 5 years protection with a Supplementary Protection Certificate.

A patent can be of value to an inventor—as well as protecting his business, patents can be bought, sold, mortgaged, or licenced to others. They also benefit people other than the inventor since large amounts of information can be learnt from other peoples patents — they can stop you from reinventing things or you can monitor what your competitors are doing. Patents also spur you or others on to develop your idea further, and once the term of the patent expires it can be freely performed by anyone which benefits the public and the economy.

TRADEMARK

A trademark is any sign which can distinguish the goods and services of one trader from those of another. A sign includes words, logos, colours, slogans, three-dimensional shapes and sometimes sounds and gestures. A

trademark is therefore a "badge" of trade origin. It is used as a marketing tool so that customers can recognize the product of a particular trader. To be registrable in the UK it must also be capable of being represented graphically, that is, in words and/ or pictures.

DESIGN

A design refers to the appearance of the whole or a part of a product resulting from the features of, in particular, the lines, contours, colours, shape, texture or materials of the product or its ornamentation.

In the United Kingdom, designs are protected by three legal rights:

(a) Registered designs rights

• gives the owner a monopoly on their product design

• brings the right to take legal action against others who might be infringing the design and to claim damages

• may deter a potential infringement.

• also brings the exclusive right to make, offer, put on the market import, export, use or stock any product to which the design has been applied or is incorporated or to let others use the design under the terms agreed with the registered owner, in the UK and the Isle of Man.

Design registration gives the owner a monopoly on their product design, *i.e.*, the right for a limited period to stop others from making, using or selling a product to which the design has been applied, or in which it has been incorporated without their permission and is additional to any design right or copyright protection that may exist automatically in the design.

(*b*) Unregistered design right.

Is not a monopoly right but a right to prevent deliberate copying, and lasts until 10 years after first marketing articles made to the design, subject to an overall limit of 15 years from creation of the design. Unlike design registration, you do not have to apply to register design right. A design right is a property that, like any other business commodity, may be bought, sold or licensed.

(*c*) Artistic copyright.

Work can only be original if it is the result of independent creative effort. It will not be original if it has been copied from something that already exists. If it is similar to something that already exists but there has been no copying from the existing work either directly or indirectly, then it may be original.

The term "original" also involves a test of substantiality—literary, dramatic, musical and artistic works will not be original if there has not been sufficient skill and labour expended in their creation. But, sometimes significant investment of resources without significant intellectual input can still count as sufficient skill and labour.

Ultimately, only the courts can decide whether something is original, but there is much case law indicating, for example, that names and titles do not have sufficient substantiality to be original and that, where an existing work is widely known, it will be difficult to convince a court that there has been no copying if your work is very similar or identical. Sound recordings, films and published editions do not have to be original but they will not be new copyright works if they have been copied from existing sound recordings, films and published editions. Broadcasts do not have to be original, but there will be no copyright, if, or to the extent that, they infringe copyright in another broadcast.

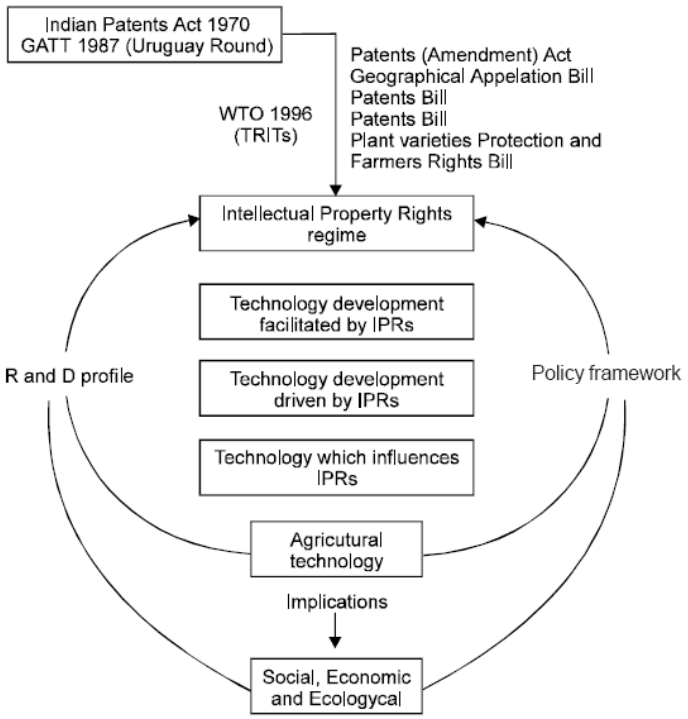
IMPLICATIONS OF IPRs AND AGRICULTURAL TECHNOLOGY

The dynamics and interplay of IPRs and technological innovations have multiple impacts. These can be categorized into social, economic and ecological. Due to peculiarities of Indian agriculture, the magnitude of these impacts will be manifold. The IPR regime not only influences research portfolio but also the contours of technology development. Primarily, the underlying motive of protection is to share profits with innovators. Therefore, the economic implications are not only predominant but also most obvious. The other two implications of access to newer technologies are on social and ecological dimensions. These three impacts are not mutually exclusive and often overlap.

Social Implications

Social impact of new technologies is manifested in terms of its influence on equity. Other important issue pertains to "scale effect". These issues can be explained by the illustration of Green Revolution. This seed-fertiliser technology was predominantly applicable in the areas with assured irrigation. These technologies contributed to the widening of the regional disparity. Viewed from a macro-perspective, however, the revolution was a great success that helped realize cherished goal of self-sufficiency in food grains. Therefore, the magnitude and nature of social implications vary according to the category of the technology (Table). Knowledge-based technologies and technologies concerning conservation of natural resources have positive impact on the society. Because of their nature (public good), the net social welfare increases manifold. Certain technologies like HYVs and hybrids require intensive input use and therefore have a mixed impact on the society. The predominant positive impact (+ + –) clouds the negative effects. Yield enhancement by conventional breeding is an ideal example. By the same yardstick, if conventional breeding aims at preventing yield loss (pest- and disease-resistant varieties) it becomes cost-reducing and has no negative impact (+). There are technologies where the negative component impact is marked (– +). Current levels of technologies (and its

costs) in farm machinery and power precludes their accessibility to small and marginal farmers. There is a distinct possibility that in the near future farm machinery is tailor-made to suit small holdings?



Economic Implications

Most technologies, excluding agricultural biotechnology and crop protection chemicals have a net positive impact on the economy. There are also implicit benefits like savings from potential losses due to pests and diseases. Newer techniques invariably shift production functions thereby improving income of individuals and that of the nation. Research in the public domain will concentrate in cost-reducing technologies that are helpful to the weaker sections. Conservation of genetic resources have huge positive externalities (both intra and inter generational). Considering the market structure of crop varieties and crop protection chemicals and the nature of potential technologies, the scope for market malpractice such as monopoly and cartelisation is real. Generally embodied technologies are likely to have relatively more apparent impacts. Active presence of the public sector is vital for the provision of disembodied technologies.

Ecological implications

Increased use of agrochemicals will accelerate environmental degradation (– – –).Though biotechnological innovations minimise the use of agrochemicals to some extent (+ –), they are feared for their contribution to

gene pollution (– ? ?). Development of such resistant varieties by conventional breeding has no negative impacts (++). Any technology encouraging the use of improved varieties is likely to contribute to narrowing of genetic base (–).

Increasingly, the use of antibiotics, hormones, unconventional feeds and genetic engineering in livestock and fisheries have raised questions about health hazards and animal biodiversity (– –). Destruction of soil structure and groundwater depletion are serious ecological risks associated with the excessive use of technologies associated with farm machinery and power. Technological advancements in the conservation of soil, water and genetic resources have profound positive impacts on the ecology (+++). Being locally evolved and practice based, knowledge based technologies optimise resource use thereby imparting positive externalities to the environment.

WORLD TRADE ORGANISATION (WTO)

In brief, the World Trade Organisation (WTO) is the only international organisation dealing with the global rules of trade between nations. Its main function is to ensure that trade flows as smoothly, predictably and freely as possible.

Location: Geneva, Switzerland

Established: 1 January 1995

Created by: Uruguay Round negotiations (1986-94)

Membership: 146 countries (as of April 2003)

Budget: 155 million Swiss francs for 2003

Secretariat staff: 560

Head: Director-General, Supachai Panitchpakdi

Functions:

• Administering WTO trade agreements

• Forum for trade negotiations

• Handling trade disputes

• Monitoring national trade policies

• Technical assistance and training for developing countries

• Cooperation with other international organizations

The result is assurance. Consumers and producers know that they can enjoy secure supplies and greater choice of the finished products, components, raw materials and services that they use. Producers and exporters know

that foreign markets will remain open to them. The result is also a more prosperous, peaceful and accountable economic world. Decisions in the WTO are typically taken by consensus among all member countries and they are ratified by members' parliaments. Trade friction is channeled into the WTO's dispute settlement process where the focus is on interpreting agreements and commitments, and how to ensure that countries' trade policies confirm with them. That way, the risk of disputes spilling over into political or military conflict is reduced. By lowering trade barriers, the WTO's system also breaks down other barriers between peoples and nations.

At the heart of the system—known as the multilateral trading system—are the WTO's agreements, negotiated and signed by a large majority of the world's trading nations, and gratified in their parliaments. These agreements are the legal ground-rules for international commerce. Essentially, they are contracts, guaranteeing member countries important trade rights. They also bind governments to keep their trade policies within agreed limits to everybody's benefit. The agreements are negotiated and signed by governments. But their purpose is to help producers of goods and services, exporters, and importers conduct their business. **The goal** is to improve the welfare of the people of the member countries.

A Closer Look at These Principles

1. ***Trade without Discrimination***

(*a*) Most-favoured-nation (MFN): Treating other people equally. Under the WTO agreements, countries cannot normally discriminate between their trading partners. Grant someone a special favour (such as a lower customs duty rate for one of their products) and you have to do the same for all other WTO members. This principle is known as Most-Favoured-Nation (MFN) treatment. It is so important that it is the first article of the General Agreement on Tariffs and Trade (GATT), which governs trade in goods. MFN is also a priority in the General Agreement on Trade in Services (GATS) and the Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS), although in each agreement the principle is handled slightly differently. Together, these three agreement cover all the three main areas of trade handled by the WTO. Some exceptions are allowed. For example, countries can set up a free-trade agreement that applies only to goods traded within the group—discriminating against goods from outside. Or they can give developing countries special access to their markets. Or a country can raise barriers against products that are considered to be traded unfairly from specific countries. And in services, countries are allowed, in limited circumstances, to discriminate. But the agreements permit these exceptions only under strict conditions. In general, MFN means that every time a country lowers a trade barrier or opens up a market, it has to do so for the same goods or services from all its trading partners—whether rich or poor, weak or strong.

(*b*) National treatment: Treating foreigners and locals equally. Imported and locallyproduced goods should be treated equally—at least after the foreign goods have entered the market. The same should apply to foreign and domestic services, and to foreign and local trademarks, copyrights and patents. This principle of "national treatment" (giving others the same treatment as one's own nationals) is also found in all the three main WTO agreements (Article 3 of GATT, Article 17 of GATS and Article of 3 TRIPS), although once again the principle is handled slightly differently in each of these.

National treatment only applies once a product, service or item of intellectual property has entered the market. Therefore, charging customs duty on an import is not a violation of national treatment even if locally-produced products are not charged an equivalent tax.

2. ***Free Trade: Gradually, Through Negotiation***

Lowering trade barriers is one of the most obvious means of encouraging trade. The barriers concerned include customs duties (or tariffs) and measures such as import bans or quotas that restrict quantities selectively. From time to time other issues such as red tape and exchange rate policies have also been discussed.

Since GATT's creation in 1947-48 there have been eight rounds of trade negotiations. A ninth round, under the Doha Development Agenda, is now underway. At first, these are focused on lowering tariffs (customs duties) on imported goods. As a result of the negotiations, by the mid-1990s industrial countries' tariff rates on industrial goods had fallen steadily to less than 4%.

But by the 1980s, the negotiations had expanded to cover non-tariff barriers on goods, and to the new areas such as services and intellectual property. Opening markets can be beneficial, but it also requires adjustment. The WTO agreements allow countries to introduce changes gradually, through "progressive liberalization". Developing countries are usually given longer time period to fulfil their obligations.

3. ***Predictability: Through Binding and Transparency***

Sometimes, promising not to raise a trade barrier can be as important as lowering one, because the promise gives business a clearer view of their future opportunities. With stability and predictability, investment is encouraged, jobs are created and consumers can fully enjoy the benefits of competition—choice and lower prices. The multilateral trading system is an attempt to governments to make the business environment stable and predictable.

In the WTO, when countries agree to open their markets for goods or services, they "bind" their commitments. For goods, these bindings amount to ceilings on customs tariff rates. Sometimes countries tax imports at rates that are lower than the bound rates. Frequently, this is the case in developing countries. In developed countries, the rates actually charged and the bound rates tend to be the same.

A country can change its bindings, but only after negotiating with its trading partners, which could mean compensating them for loss of trade. One of the achievements of the Uruguay Round of multilateral trade talks was to increase the amount of trade under binding commitments. In agriculture, 100% of products now have bound tariffs. The result of all this: a substantially higher degree of market security for traders and investors. The system tries to improve predictability and stability in other ways as well. One way is to discourage the use of quotas and other measures used to set limits on quantities of imports administering quotas can lead to more red-tape and accusations of unfair play. Another is to make countries' trade rules as clear and public (transparent) as possible. Many WTO agreements require governments to disclose their policies and practices publicly within the country or by notifying the WTO. The regular surveillance of national trade policies through the Trade Policy Review Mechanism provides a further means of encouraging transparency both domestically and at the multilateral level.

4. ***Promoting Fair Competition***

The WTO is sometimes described as a "free trade" institution, but that is not entirely accurate. The system does allow tariffs and, in limited circumstances, other forms of protection. More accurately, it is a system of rules dedicated to open, fair and undistorted competition. The rules on non-discrimination—MFN and national treatment – are designed to secure fair conditions of trade. So too are those on dumping (exporting at below cost to gain market share) and subsidies. The issues are complex, and the rules try to establish what is fair or unfair, and how governments can respond, in particular by charging additional import duties calculated to compensate for damage caused by unfair trade.

Many of the other WTO agreements aim to support fair competition: in agriculture, intellectual property, services, for example. The agreement on government procurement (a "plurilateral" agreement because it is signed by only a few WTO members) extends competition rules to purchases by thousands of government entities in many countries, and so on.

5. ***Encouraging Development and Economic Reform***

The WTO system contributes to development. On the other hand, developing countries need flexibility in the time they take to implement the system's agreements. And the agreements themselves inherit the earlier provisions of GATT that allow for special assistance and trade concessions for developing countries. Over three quarters of WTO members are developing countries and countries in transition to market economies. During the seven and a half years of the Uruguay Round, over 60 of these countries implemented trade liberalization programmes autonomously. At the same time, developing countries and transition economies were much more active and influential in the Uruguay Round negotiations than in any previous round, and they are even more, so in the current Doha Development Agenda. At the end of the Uruguay Round, developing countries were prepared to take on most of the obligations that are required of developed countries. But the agreements did give them transition periods to adjust to the more unfamiliar and, perhaps, difficult WTO provisions—particularly so for the poorest, "least-developed" countries. A ministerial decision adopted at the end of the round says better-off countries should accelerate implementing market access commitments on goods exported by the least-developed countries, and it seeks increased technical assistance for them. More recently, developed countries have started to allow duty-free and quota-free imports for almost all products from least-developed countries. On all of this, the WTO and its members are still going through a learning process. The current Doha Development Agenda includes developing countries' concern about the difficulties they face in implementing the Uruguay Round agreements.

DEVELOPING COUNTRIES DEVELOPMENT AND TRADE

Over three quarters of WTO members are developing or least-developed countries. All WTO agreements contain special provision for them, including longer time periods to implement agreement and commitments, measures to increase their trading opportunities, provisions requiring all WTO members to safeguard their trade interests, and support to help them build the infrastructure for WTO work, handle disputes, and implement technical standards. The 2001 Ministerial Conference in Doha set out tasks, including negotiations, for a wide range of issues concerning developing countries. Some people call the new negotiations the Doha Development Round.

Before that part in 1997, a high-level meeting on trade initiatives and technical assistance for least-developed countries resulted in an "integrated framework" involving six intergovernmental agencies, to help least-developed countries increase their ability to trade, and some additional preferential market access agreements.

A WTO committee on trade and development, assisted by a sub-committee on least-developed countries, looks at developing countries' special needs. Its responsibility includes implementation of the agreements, technical cooperation, and the increased participation of developing countries in the global trading system.

TECHNICAL ASSISTANCE AND TRAINING

The WTO organizes around 100 technical cooperation missions to developing countries annually. It holds on average three-trade policy courses each year in Geneva for government officials. Regional seminars are held regularly in all regions of the world with a special emphasis on African countries. Training courses are also organized in Geneva for officials from countries in transition from central planning to market economies. The WTO set up reference centres in over 100 trade ministries and regional organizations in capitals of developing and least-developed countries, providing computers and internet-access to enable ministry officials to keep abreast of events in the WTO in Geneva through online access to the WTO's immense database of official documents and other material.

• Assisting developing countries in trade policy issues, through technical assistance and training programmes.

• Cooperating with other international organizations.

THE ORGANIZATION FUNCTIONS

The WTO's overriding objective is to help trade flow smoothly, freely, fairly and predictably. It does this by:

• administering trade agreements;

• acting as a forum for trade negotiations;

• settling trade disputes;

• reviewing national trade policies.

STRUCTURE

The WTO has nearly 150 members, accounting for over 97% of world trade. Around 30 others are negotiating membership. Decisions are made by the entire membership. This is typically byconsensus. A majority vote is also possible but it has never been used in the WTO, and was extremely rare under the WTO's predecessor, GATT. The WTO's agreements have been ratified in all members' parliaments.

The WTO's top level decision-making body is the **Ministerial Conference** which meets at least once every two years. The Fifth WTO Ministerial Conference was held in Cancun, Mexico from 10 to 14 September, 2003.

Below this is the **General Council** (normally ambassadors and heads of delegation in Geneva, but sometimes officials sent from members' capitals) which meets several times a year in the Geneva headquarters. The General Council also meets as the Trade Policy Review Body and the Dispute Settlement Body.

At the next level, the **Goods Council, Services Council and Intellectual Property (TRIPS) Council** report to the General Council. Numerous **specialized committees, working groups and working parties** deal with the individual agreements and other areas such as environment, development, membership applications and regional trade agreements.

SECRETARIAT

The WTO Secretariat, based in Geneva has around 560 staff and is headed by a director general. It does not have branch offices outside Geneva. Since decisions are taken by the members themselves, the Secretariat does not have the decision-making role those other international bureaucracies are given. The secretariat's main duties are to supply technical support for the various councils and committees and the ministerial conferences, to provide technical assistance for developing countries, to analyze world trade, and to explain WTO affairs to the public and media. The Secretariat also provides some forms of legal assistance in the dispute settlement process and advises governments wishing to become members of the WTO. The annual budget is roughly 155 million Swiss francs. relied on such licenses in order to limit exclusive rights and prevent or remedy abusive practices in several areas. The study reveals there is a broad range of grounds under which compulsory licenses may be granted in both developed and developing countries. The grounds and conditions on which compulsory licenses have been regulated and granted in developed countries illustrate the flexibility and potential of the compulsory licensing system to address a multiplicity of public interests and concerns. The evidence also indicates that arguments— often voiced by the developed countries' business community and governments— against compulsory licenses as a deviation from acceptable standards for intellectual property rights, are not reflected in the policies actually applied in such countries.

Three Main Conclusions Particularly Relevant for Developing Countries may be Drawn from the Previous Analysis.

• Compulsory licenses should be considered as an essential element in patent laws and other intellectual property regimes. Developing countries should disregard any attempts by developed countries to limit under bilateral or other agreements the scope of and grounds for compulsory licensing.

• The grounds and conditions for compulsory licenses should be carefully determined by national laws. The extent to which such licenses would be available and effective depend on the provisions of national legislation and on its adequate administration by informed national authorities.

• Developing countries should preserve the maximum possible freedom under international rules to design their compulsory licensing systems, according to their own interests and needs, including in such areas as the protection of health and the environment, and the promotion of transfer of technology and local industrialization. Should the issue of compulsory licenses be included in the agenda of possible future negotiations in WTO, developing countries should seek to clarify the scope for the granting of such licenses in certain cases (*e.g.,* of non-exploitation), as well as to remove some of the restrictive conditions imposed by the said TRIPs Agreement.

KARPAGAM ACADEMY OF HIGHER EDUCATION					
DEPARTMENT OF MICROBIOLOGY					
MTIPR - [17MBU211]					
UNIT I	**Option A**	**Option B**	**Option C**	**Option D**	**ANSWERS**
---------- is commonly used as DNA hybridization probe.	Mononucleotide	Dinucleotide	Polynucleotide	Oligonucleotide	Oligonucleotide
_____ are the group of enzymes which mediate annealing, sealing or joining of DNA fragments	RNases	Ligases	Nucleases	Polymerases	Ligases
_____ enzymes enable the breakage in internal phosphodiester bonds within a DNA molecule	Endonuclease	Exonucleases	DNaseI	S1 nucleases	Endonucleases
DNA ligases are isolated from	Bovine pancreas	Avian mycloblastosis	E.coli infected with phage T4	sheep	E.coli infected with phage T4
_____ are also known as DNA ligases	Klenow fragment	Molecular sutures	Molecular scissors	Holo enzyme	Molecular sutures
Southern blotting technique helps in detecting fragments of	RNA	DNA & RNA	DNA	Potein	DNA
The southern blotting technique can be used for the	Separation of DNA	Screening of recombinants	Denaturation of DNA	DNA sequencing	Screening of recombinants
Northern blotting is used for _____	Detection of RNA	Detection of DNA	Detection of protein	Detection of plasmid	Detection of RNA
_____ is the restriction site of E.coRI.	5’ AAGCTT 3’ 3’ TTCGAA 5’	5’GAATTC 3’ 3’CTTAAG 5’	5’ CCCGGG 3’ 3’ GGGCCC 5’	5’ GATC 3’ 3’ CTAG 5’	5’GAATTC 3’ 3’CTTAAG 5’
_____ enzyme mediates Nick translation	DNA Pol I	DNA Pol II	. DNA Pol III	RNA Pol	DNA Pol I
Western Blotting technique was described by ----------	Towbin et.al	Alwin et.al.,	E. M. Southern	None of the above	Towbin et.al

Western blotting involves ----------	DNA probe	RNA probe	. Protein probe	Antibody probe	Antibody probe
…….. gel electrophoresis is used for the separation of DNA fragments	Agarose	PAGE	SDS-PAGE	Agarose & SDS-PAGE	Agarose
Enzyme used to remove unannealed regions of RNA from DNA:RNA hybrids	Exonuclease III	Ribonuclease T1	Phosphatase	Endonuclease	Ribonuclease T1
RNA is tightly associated with	L ipids	Amino acids	Proteins	carbohydrates	Proteins
Precipitation of RNA can be taken place by	Ethanol	Alcohol	Formaldehyde	methane	Ethanol
Commonly used reagent in RNA extraction is	EDTA	Guanadinium Thiocyanate	NAOH	SDS	Guanadinium Thiocyanate
The process by which the foreign DNA escapes host restriction is	Cloning	Host control restriction & modification	Sequencing	Blotting	Host control restriction & modification
_____ are the enzymes are used to cut the target DNA fragments.	Ligases	Restriction endonucleases	Methylases	Exo Nucleases	Restriction endonucleases
Alkali treatment of DNA fragment results in the ……	Disruption	Denaturation	depurination	none of the above	Denaturation
The blotted filter paper is baked at …….	90 °C	82 °C	100 °C	80 °C	80 °C
In southern blotting, the kind of filter paper used for blotting is ……..	Whatmann No.1	Nitrocellulose filter paper	Aminobenzyloxymethyl	Whatmann No.509	Nitrocellulose filter paper
Western blotting is used for the identification of	DNA fragment	RNA fragment	Antibodies	Protein	Protein
Solvent used in western blotting involve	SDS	Ethanol	Chloroform	None of the above	SDS

Western blotting is based on the principle	Ag-Ab reaction	Electrophoresis	Hybridisation	Translocation	Ag-Ab reaction
The Type I restriction enzyme need ……..	Methyl gps	Sulfur gps	Fe+	S	Methyl gps
Western blotting detects protein even at	low as 5 ug	low as 10 ug	less than or equal to 15 ug	less than 0.5 ug	low as 5 ug
The cutting of DNA takes place with the enzyme at _____ sites.	identification site	Cleavage site	Restriction site	clear site	Restriction site
Site specific clearage is carried out by	Type III	Type I	Type II	Type IV	Type II
……. are the enzymes are used to cut the target DNA fragments	Ligases	Methylases	Restriction endonucleases	Exo Nucleases	Restriction endonucleases
The cleavage site of Type III enzymes is about _____ away from the recognition site.	30 kbp	24 – 26 kbp	24 – 28 kbp	40 kbp	24 – 26 kbp
The symbol for type I restriction systems	Mod	hsd	Res	sap	Hsd
Cofactor for type II system	mn 2+	ca 2+	mg 2+	fe 3+	mg 2+
The presence of restriction enzyme was postulated by	Werner Arber	Watson	Smith	Nathan	Nathan
Restriction enzymes mostly preferred for genetic engineering are of type	Type I	Type II	Type III	Type IV	Type II
Use of only one single enzyme for DNA digestion during the construction of restriction map is called	Single digestion	Double digestion	restrictive digestion	end labeling	Single digestion
Altering the optimal conditions for the activity of restriction enzymes as to skip some of	Single digestion	Double digestion	Partial digestion	End labeling	Partial digestion

their restriction sites during the construction of restriction maps is called					
Two restriction enztmes which have the same recognition sequence but leave at different sites ……	Neoschizomers	Isoschizomers	Epimers	Isomers.	Neoschizomers
The recognition / cleavage site of Type II enzymes have ___	Same site	Different site	Adjacent site	modified site	Same site
In supercoiled DNA, if both polynucleotide strands are intact, they are describes as	CAD	Open circular DNA	Covalently closed circle DNA	supercoiled DNA	Open circular DNA
CsCI2 density gradient centrifugation is to separate	nucleus	DNA	aminoacid	membrane	DNA
Mostly density gradient centrifugation can be used to separate supercoiled DNA from non-supercoiled DNA by using intercalating agent is	EtBr(ethidium bromide)	CsCI2	EtrBr- CsCI2	CsNo2	EtBr(ethidium bromide)
_____ will yield multiple copies of plasmid	Plasmid amplification	Plasmid purification	plasmid denaturation	plasmid multiplication	Plasmid amplification
Radiolabelling of nucleic acid is done by	Horse radish peroxidose system	DIG labelling system	Nick translation	Biotin-Streptavidin labelling system	Horse radish peroxidose system
In vitro labeling of nucleic acid is done by	Probe preparation by PCR	Using 32 P	DIG labelling system	Horse radish peroxidose system	DIG labelling system
Gel electrophoresis separates DNA molecules according to their	Shape	Size	Volume	Structure	Size

Stain used in gel electrophoresis for the detection of DNA	Ethidium bromide	Crystal violet	Malachite green	Bromothymol blue	Ethidium bromide
DNA molecules in gel electrophoresis is labeled by radioactive isotope of	Phosphorous	Sulfur	Carbon	Iodine	Phosphorous
For good seperation of DNA fragments is gel electrophoresis, the voltage applied should be not more than	5V / cm	10V / cm	15V / cm	20V / cm	5V / cm
Which is the effective methodology for separation of intact proteins by molecular weight	1D-SDS PAGE	2D-PAGE	Agarose gel electrophoresis	Nanospray – MS/MS	1D-SDS PAGE
The most commonly used stain for protein detection is	Methylene blue	Coomassic Brilliant blue	Ethidium bromide	Crystal violet.	Coomassic Brilliant blue
Photochemical polymerization of polyacrylamids gel is initiated by	Riboflavin	Vitamin C	Thymine	Vitamin A	Riboflavin
Which is the tracking dye used in SDS-PAGE	Bromophenol blue	Coomassic Brilliant blue	Ethidium bromide	Crystal violet	Bromophenol blue
What is the role of SDS in SDS-PAGE	Denative the proteins	Separate the proteins	Stain the protein	None of the above	Denative the proteins
Agarose is made up of	Glucose	Lactose	Agarobiose	Polyarylamide	Agarobiose
The polymerization of acrylamide is initiated by the addition of	Ammonium persulfate	Ammonium persulfate and TEMED	Riboflavin	None of the above	Ammonium persulfate and TEMED
PH of stacking gel is	7.8	6.8	5.8	4.8	6.8
UNIT II	**Option A**	**Option B**	**Option C**	**Option D**	**ANSWERS**

Phages were first discovered by	Fredrick Twort	Edward Tautum	Flein d' Hericcle	Griffith	Fredrick Twort
A typical example for a head and tail phage	Phage M13	pHC79	lambda Vectors	pUC118	lambda Vectors
What are the reasons for considering the yeast as a vector?	length	short doubling time (90 minutes)	can be grown on complex media	tough selection	short doubling time (90 minutes)
Which are the shuttle vectors?	YE ps	PBR 322	LE u2	pUC118	YE ps
Which is the yeast chromosomal gene that codes for isopropyl malate dehydrogenase?	2um plasmid	pBR322	YEPs	LEU2	LEU2
Which is the one of the enzyme involved in the conversion of pyuvie acid to leucine?	Malate dehydrozenase	Isopropyl kinase	Isopropyl malate dehydrozenase	Pyruvate carboxylase	Isopropyl malate dehydrozenase
Origin of Replication in yeast chromosomes having 100 bp	LEU2	ARS	2mm plasmid	ANS	ARS
Bacterial plasmids carrying a yeast gene	YRPs	2 mm plasmid	YIPs	YAC	2 mm plasmid
The cloned genes are expressed in ----------	Periplasm	Recombinant cell.	Host cell	Donor cell	Recombinant cell
The smallest known phage is	Phage M13	PUC 118	PUC 119	PHC 79	Phage M13
……. Can be used as an selectable marker in certain experiment to ensure the presence of plasmid present in a bacteria in a culture	DNA probe	Radiolabelled RNA	Antibiotics resistant plasmid	Radiolabelled DNA.	Antibiotics resistant plasmid
Origin of replication in yeast chromosomes having 100 bp	Minichromosome vectors	LEU2	ARS	2um plasmid	ARS
The insertion of DNA fragment is accompanied with deletion	Okazaki fragment	Stuffer fragment	Coding region	Non coding region	Stuffer fragment

of all or the major part of non essential region of genome, the deleted region is called					
Which plasmid is referred to as the 'work house' of gene cloning	pBR 322	Col El Plasmid DNA	Col El Amp Plasmid DNA	pBR 325 Plasmid DNA	pBR 322
The ampicillin resistant gene of pBR 322 was derived from	pSC 101	Ti Plasmid	pBR 313	RSF 2124	RSF 2124
The size of pBR 322 is	4,363 bp	6,600 bp	10,900 bp	5,300 bp	4,363 bp
A derivative of pBR 322 which confers resistance to ampicillin, tetracycline and chloramphenicol	pBR 313	Col El Plasmid DNA	pBR 325	Col El Amp Plasmid DNA	pBR 325
In Rhizobium leguminosarum, the genes for nitrogen fixation and module formations are located in	Plasmid	DNA	Bacteriophage	RNA	Plasmid
In EcoRI the first two letters are known as ____	Genus & specific name (species)	Genus name	Specific name	Inventor name	Genus & specific name (species)
Bacterial plasmids carrying a yeast gene	YRPs	2 um plasmid	YIPs	YAC	2 um plasmid
YAC has a approximate of …… DNA frequent	30 kbp	100 kbp	200 kbp	20kbp	30 kbp
Which is not the difference between YAC & BAC	BAC is circular and YAC is linear	YAC has telomere & BAC wont have	YAC has centromere and BAC wont	YAV would be bound to histone but BAC wont	BAC contain much larger DNA insert than a YAC
Vectors are ……..	Extrachromosomal DNA molecule	protein	BAC	YAC	Extrachromosomal DNA molecule
YAC differs from typical cloning vector is having	Several multiple cloning stes	A centromere sequence at each end	More than one origin of replication	Tolemere sequence	Several antibiotic resistance gene

Retroviral infection can be applied to introduce the gene into	fish	mice	plant	bacteria	Mice
Which of the following is an insertion vertor	λ EHBL 4	Charon 16A	λ GEM 12	Charon 4a.	Charon 16A
Among these which one is the replacement vector	Charon 16A	λ ZAP II	λ ZAP II	λ EHBL 4	λ EHBL 4
Enzyme used to cleave the appropriate site of the λ for the insertion of vector is	Endonucleases	lyases	Ligases	Translocase	Endonucleases
vector is involved in _____	cloning	cutting	joiing	screening	Cloning
restriction enzymes mostly preferred for genetic engineering are of type	blue colour	green colour	colourless	greenish yellow	Colourless
Achieving same copy by	blotting	electrophoresis	cloning	joining	Cloning
The other name of r DNA is	Ribosomal DNA	Chimeric DNA	Bactriophage	Chromosomal DNA	Chimeric DNA
In the method of identification of recombinants the method usually used is	Primary screening	Replica Plating	Secondary screening	Auxano graphy	Replica Plating
A lactose analogue which is involved in the screening of β galactosidase	Y-gal	X-gal	B-galactosidase	B-galactoside permease	X-gal
When X-gal added to the agar the cells of which synthesize β-galactosidase will be coloured	Yellow	Red	Blue	Black	Blue
Baculo virus is a	Parasite	Obligate parasite	Saprophyte	Pathogen	Obligate parasite
RNA4 which encodes the ----------	Virus coat protein	cell wall protein	Viral core protein	Glyco protein	Virus coat protein

Introduce the gene in to E.coli by _____	Transduction	Particle bambrtment	Transformation	Micro injection	Transformation
Cells containing pBR will be resistant to …….. and sensitive to ……	Resistant to Ampicillin sensitive to tetracyclin	Resistant to methronine and sensitive to Arginine	Resistant to tetracycline and sensitive to ampicillin	Resistant Argmine and sensitive to methronine	Resistant to Ampicillin sensitive to tetracycline
Bacteriophage P 1 resembles bacteriophage _____.	λ	T7	φ	T3	Λ
Name the animal virus used as vectors	SV 40 Virus	HIV	Rabbies	polio virus	SV 40 Virus
SV40 vectors are grown and manipulated using _____ as the host	Plant cells	E.coli	Bacteria	Animal cells	E.coli
cDNA clones are ligated to suitable vector are	m13 vectorand phage vector	Yac vector	Plasmid Vector		m13 vector and phage vector
λ phage lacks	Icosahedral	Base plate	Flexiblr fluid	Contractile sheath	Contractile sheath
…….. phage display system is powerful technology for engineering proteins such as functional mutant proteins and peptides	φ×174	Coliphage	M13 Phage	d)λ phage	M13 Phage
Ti plasmid is in …… size.	~ 200 kb	100 kb	50 kb	150 kb	~ 200 kb
The molecular weight of the cloning vector should ideally around …… kbp	100	10	20	40	10
pBR 322 is first identified and developed by	A.Chan and N.cohen	Elinst Berlimer	T. Bolival ond Rodrigues	Ishiwata	T. Bolival ond Rodrigues
whch organism is infected by M13 bacteriophage	Pseudomonas	E.coli	Bacillus	Klebsiella	E.coli

In pBR 322 ,322 is stands for	No of genes	No of Restriction sites	No of base pairs	Number used to distinguish from other plasmid	Number used to distinguish from other plasmid
ARS is …….	Autonomous replicating plasmid	Automatic replicating sequence	Automatic reproducing sequence	Autonomous reproducing sequence.	Autonomous replicating plasmid
Advantage of lamda phage vector	Transformation efficiency	Easy to grow	low cost	Self treplication	Transformation efficiency
Most commenly used plasmid vector for cloning	pBR 322	pUC8	F plasmid	Ri plasmid	pBR 322
DNA ligase is synthesized from	E.coli and bacteriophage	E.coli & Staphylococcus	Klebsiella & bacteriophage	Bacteriophage	E.coli and bacteriophage
Plasmid vector can carry upto ……kb of fragment	40kb	8kb	20kb	75kb	8kb
Baculoviruses infect	Animal cells	Human cells	Insect cells	Plant cells	Insect cells
The digested DNA molecule are run agarose gel for	identify the change	Purification	Suitable range of length of DNA.	to remove impurities	Suitable range of length of DNA.
Transfer vector is a ……vector	Shuttle Vector	Plasmid Vector	Binary Vector	Cointegrate Vector	Shuttle Vector
Transfer vector contains __ cloning site	Single	Multiple	Double	Triple	Multiple
UNIT III	**Option A**	**Option B**	**Option C**	**Option D**	**ANSWERS**
.----------converts the protoxin into active toxin.	Nuclease	Urease	Protease.	Lipase.	Protease
Convertion of protoxin to active toxin require both protease and ----------	Alkaline pH	Urease	Acid pH	Lipase.	Alkaline pH
Parasporal crystals sensitive to ----------	Sunlight	Chemicals	Acid pH	Lipase.	Sunlight

Most effective and most often utilized microbial insecticides are toxins synthesized from .	.B.amyloliquefaciens	B.thuringiensis	B.subtilis	B.licheniform	B.thuringiensis
B.thuringienis subspecies kurstaki is toxic to	bugs	worms	cabbage worm	small worms	cabbage worm
B.thuringiensis subspecies israelensis kills .	cabbage worm	black flies	lepidopteron larvae	none of the above	black flies
_____ is also known as sandeigo.	B.thuringiensis subsp israelensis	B.thuringiensis subsp kurstaki	B.thuringiensis	B.thuringiensis stubs tenebrionis	B.thuringiensis stubs tenebrionis
The parasporal crystal is .	not the active form	a protoxin	precursor of active toxin	all the above	all the above
The insecticidal activity of B.thuringiensis is contained within a very large structure called _____ .	parasporal crystal	parabasal crystal	perisporal crystal	sporal crystal	parasporal crystal
The subunits of the parasporal crystal can be dissociated invitro by treatment with	alcohol	ethylene	β-mercaptoethanol	.xylene	β-mercaptoethanol
Parasporal crystals are lived in the environment	short	long	.moderately	limited	short
To enhance production rate for B.thuringiensis are used.	promoters active in all phase	promoters active in sporulation	promoters active in vegitation	promoters active in one phase	promoters active in all phase
When tenebrionis toxin gene is transformed into B.thuringiensis subsp israelensis the transformants was toxic to .	brassicae	cabbage white butterfly	catterpiller	butterfly	cabbage white butterfly

The parasporal crystals of B.thuringiensis subsp israelensis shows as an insecticide	more efficacy	sinks rapidly when sprayed	sinks slowly when sprayed	medium efficacy	sinks rapidly when sprayed
The alternative bacterium used for B.thuringiensis toxin is	Caulobacter crescentus	Zymomonas	A. niger	none of the above	Caulobacter crescentus
Which one of the following can proliferate well in water surface near mosquito larvae?	Synechocystis	Synechovibrio	both a and b	Synechococcus6	both a and b
Which one of the following can be used as a biocontrol agent?	Baculovirus	retrovirus	A. niger	Penicillium sp	Baculovirus
Baculovirus are pathogenic to	Neuroptera	Trichoptera	Diptera	all the above	all the above
. The gene that encodes insect specific neurotoxin was produced by	Androctonus australis	Synechocystis	Caulobacter	Synechovibrio	Androctonus australis
The insect specific neurotoxin disrupts the .	normal life cycle of insect	flow of Na ions	sporulation	flow of chloride ions	flow of Na ions
When the parasporal crystal is ingested by a target insect, the protoxin is activated by .	alkaline pH	specific digestive protein	acidic pH	non spesific priotein	alkaline pH
The mode of action of B.thuringiensis toxins imposes certain constraints in application. They are	for killing the insect it must be ingested	insects of plant roots are less likely ingesting Bt toxin.	kills insects during a non specific developmental stage	for killing the insect must not be ingested	for killing the insect it must be ingested
Chemical insecticides has the following disadvantage	specificity	insects become sensitive easily	beneficial insects being killed	simple degradation	beneficial insects being killed

The steps taken to kill insects in plant roots is	to inject Bt toxin into roots	to introduce Bt gene into cells of root	introduce Bt toxin gene into bacterial species of rhizosphere	spray the Bt toxin	introduce Bt toxin gene into bacterial species of rhizosphere
Methods for biological protection of plants	transgenic plants	chemical insecticides	trimming of plants	avoid plant damage	transgenic plants
Baculo virus is a	Parasite	Obligate parasite	Saprophyte	Pathogen	Obligate parasite
Biological insecticides are usually_____ for a number of insect species.	less specific	Highly specific	resistant	highly resistant	highly resistant
The biological insecticides are to humans and other animals	hazardous	.useless	non-economical	non-hazardous	non-hazardous
Bt toxin is safe because	persist in the environment	hazardous to mammals	does not persist in the environment	non degradable	does not persist in the environment
gene transfer to animal by ----------	Transformation efficiency	microinjection	vector Ti	transduction	Microinjection
Site directed mutagenesis refer to	change of whole genome	changes in a single base	change of whole DNA sequence	none	changes in a single base
The transgenic plant transformed with highly modified synthetic protoxin gene had level of expression than wild type.	10 fold	100 fold	1000 fold	10000	100 fold
What is antisense RNA?	RNA molecule complementary to gene transcript (mRNA)	RNA molecule complementary to DNA	DNA molecule complementary to RNA.	Type of RNA	RNA molecule complementary to gene transcript (mRNA)
Sense RNA is	the translate m RNA	the translate DNA	both a and b	none	the translate m RNA
Chemical herbicides are .	No side effects	discriminate weeds from crop	persist in the environment	all of the above	persist in the environment.

The different biological manipulations that would cause a crop plant to be herbicide resistant are	overproduction of herbicide sensitive target protein	improve the ability of herbicide	resistant protein to bind to herbicide	metabolic activation of herbicide	overproduction of herbicide sensitive target protein
Glyphosate is _____ .	environment friendly	hazardous	toxic to living beings	none of the above	environment friendly
The EPSPS plays important role in the synthesis of amino acids in both bacteria and plants	aromatic	aliphatic	non - aliphatic	non- aromatic	Aromatic
Possible attractive host for the expression of mosquitocidal Cry genes ----------	Bacillus sphacericus	B.thuringinsis	B.rhizogenes	Asticcacaulis excentricus	B.thuringinsis
Protoxin is activated within the -----	Gut	Lungs	Respiratory tract	Stomach	Gut
Antisense therapy is .	prevents the expression of the defective gene	delivery of a remedial gene into organ	cells taken from organ after the correction it transplanted back	remedial gene is introduced into an embryo.	prevents the expression of the defective gene
Testing of newborn for children for genetic disease is	parental screening	postnatal screening	antenatal screening	prenatal genetic screening	postnatal screening
The microinjected transgene construct is in form and free of vector DNA sequences	linear and prokaryotic	Circle and prokaryotic	Circle and eukaryotic	linear and eukaryotic	linear and prokaryotic
UNIT IV	**Option A**	**Option B**	**Option C**	**Option D**	**ANSWERS**
Scienticfic theories, mathematical methods and therapeutic treatments are	Easily patentable	Non patentable	Un copyrightable	long time processing	Non patentable
The legal characterization and treatment of trade related	FDA	IPR	EPO	WISO	IPR

biotechnological process and products popularly described as ----------					
Development of crop varities are protected through ----------	Plant Breeder's Right	Plant biotech rights	Plant biotech regulations	Prevent breeders right	Plant Breeder's Right
According to USA ----------means grant of right to exclude others from making using or selling an invention for a 17 years period	IPR	PBR	Patents	FDA	Patent
The Indian patent Act include ----------but not ----------	Product and invention	process patents and product patents	inventions and discoveries	FDA but not IPR	process patents and product patents
The duration of indian patent is ----------	5 years	6 months	1 years	10 years	5 years
Before the release of genetically engineered microbial pesticide it should be approved by ----------	FDA	TRIP	GATT	EPA	EPA
Grant specification and claims are the parts of	Copy right	IPR	Patent	intelectual right	Patent
Which is the famous convention of London	European	paris	london	Budapest treaty	Paris
Which are patentable	Inventions	Discoveries	Scienticfic throries	Treatments	Inventions
The first patent for living organism was awared in	1978	1988	1999	1965	1988
The Indian patent Act was formed in	1976	1878	1960	1970	1970
EMR stands for	Exclusive Market Rights	Exclusive Medical Rights	Exclusive Market Rate	External Market Rights	Exclusive Market Rights

A patent should contain	Name of the inventor	Name of the Patntence	Description of patent	All the three in needed	All the three in needed
Legal documents are	Pantents	Cliams	Description	Inventions	Cliams
Pseudomonas was patented by	TJ.C.Bose	Khorana	Dubey	Anand chakraboty	Anand chakraboty
Requirements of patents are	A New useful invention	Invention	A useful product	Previously known product	A New useful invention
When the Paris convention for protection of industrial property signed	1884	1890	1883	1863	1883
Paris convention is for the protection of	industrial property	personal property	institutes property	eqiupments	industrial property
How many years USPTO can issue a patent	4	2	1	3	3
How many types of patents avilable	2	3	4	5	3
Duration of patent	5years	10years	20years	1years	20years
Which company has the patent for tissue plasmogen activator	Sigma	biocon	Genetech	Genetech	Genetech
PCT came effective in India from	1999	1998	1997	1995	1998
To patent a product or process it must satisfy	3 Fundamental requirements	2 Fundamental requirements	5 Fundamental requirements	6Fundamental requirements	3 Fundamental requirements
How many claims present in the original patent application	10	20	30	5	20
In which country Genetech applied to get patent	france	Japan	USA	UK	UK
The colonical theory was proposed by	John	Haeckel	J.C.Bose	Anand chakraboty	Haeckel

The colonical theory was proposed in the year	1874	1875	1876	1884	1874
The patenting of multicellular organisms constitute to raise	Ethical and social concern	Ethical concern	social concern	trede concern	Ethical and social concern
Indian patent Act allow to patent	products	process	preparation		Process
In USA the maximum limit of monoply is for	10years	5 years	17 years	2 years	17 years
The word Patent derived from	Latin word patere	paten	pantor	patentor	Latin word patere
PCT is	Patent Cooperation Treaty	Patent Control Term	Public Cooperation Team	Private Cooperation Team	Patent Cooperation Treaty
PCT is an agreement for cooperation on patenting	National	Local	International	State	International
What is the mode of revocation of patent	State government	Central government	Union territories	UN	Central government
Who got the patent for Psuedomonas	Robert Koch	Louis Pastuer	Dr. Chakrabarty	Edward Jenner	Dr. Chakrabarty
The process of collecting biological samples for medical and scientific research is	Bioprospecting	Bioprojects	Biogenesis	Bioprocessing	Bioprospecting
refers to the illegal commercial development of naturally occuring biological material	Bio patenting	Bio piracy	Bio projects	Piracy	Bio piracy
is the form of protection of plant related inventions	Plant Variety Protection	Plant Protection Act	Plant Patent Act	Plant Utility Act	Plant Patent Act
investigation about cell bared therapies to treat disease	Plant cell research	Stem cell research	Animal tissue research	Plant enzymes	Stem cell research

is done to produce pure antibodies by fusing cells	Monoclonal antibody technique	Immunoglobulins	Antigens	Antigen antibody complex	Monoclonal antibody technique
Plant patents have been granted by	American Patent Office	England Patent Office	Japan Patent Office	European Patent Office	European Patent Office
The patentable product involving R-DNA technology	Genes	Vaccines	Transposons	Base pairs	Vaccines
is the patentable process involving R-DNA technology	Modifying genomic sequences	Enzyme sequences	Gene sequences	Plant genes	Modifying genomic sequences
The product is kept as a safety guarded and termed as	Tread mark	Trade secrete	patent	IPR	Trade secrete
protection is only form of expression of ideas	Trade mark	Patend	Copyright	Trade secrete	Copyright
In which year copyright was amended	1944	1994	1942	1960	1994
In which year copyright was brought enforced	1940	1990	1999	2002	1999
.Indian copy right Act was published in	1967	1957	1937	1977	1957
In which year Copy right was amended	1994	1944	1984	1997	1994
Which symbol was used to distiguish one treade to another?	Copy right	Patend	Treade mark	Treade secrete	Treade mark
In which year industrial design bill was passed	1940	1980	1999	1950	1999
In which year Treade mark bill was passed in India	1948	1978	1990	1958	1958
UNIT V	**Option A**	**Option B**	**Option C**	**Option D**	**ANSWERS**

include the rights arising from conserving, improving and making	Agricultural act	Plant breeder's act	Farmer's right	Copyright	Farmer's right
IPR are usually limited to	Non-rival goods	Rival goods	Imported goods	Food products	Non-rival goods
Modern usage of the term IPR began in	1987	1977	1967	1955	1967
In 1994 which act was amended	Tread mark	trade secrete	patent	copy right	copy right
In 1957 which agreement was approved for copy right act	TRIPS	PPVFR	IPR	PCT	TRIPS
The maximum limit of 17 years for monoply was given by	Paris	UK	USA	India	USA
The meaning for patent	property	To lay open	Protection	To open	To lay open
Haeckel proposed the theory of	Colonical	gem theory	Theory of koch	Theory of Pasteur	Colonical
There is a 20 claims present in	original patent application	New developed patent application	patent application	IPR application	original patent application
In 1988 the first patent was given to	Living organism	hybride plant	plant	Fungi	Living organism
In 1883 which convention for production of industrial property was signed	Paris	US	UK	USSR	Paris
Three years of patent was issued by	TRIPS	PPVFR	IPR	USPTO	USPTO
According to PPVFR Act How many rights have been given to farmers	6	7	10	9	9
_____is classified into two categories	PBR	IPR	FDA	USR	IPR

Trademark uses symbols in _____	Intellectual committee	Intellectual property	Indian committee	Indian property	Intellectual property
Geographical indications protects the quality,reputation of products originated from _____	Histarical area	Seasonal area	developmental area	Geographical area	Geographical area
Tade secrets protects _____ of industries	Trade information	confidential information	Machinery information	Tribunal information	confidential information
Copyrights prevents copying and _____	Reproduction	Construction	Development	Tradition	Reproduction
IPR develops and protects ___resources.	Physical	chemical	biological	academic	Biological
For breeder's right, ____ Act is in practice.	PPR	PPVFR	PPVR	PPFR	PPVFR
In which year patent Act was published in India	1945	1990	1999	2000	1999
In which year patent bill was introduced to upper part of india parliament	1970	1980	1960	1990	1970
When copyright Act got TRIPS agreement	1950	1960	1957	1980	1957
When did geographical indication good bill got published	1920	1960	1999	2004	1999
Which year Act was replaced in 1999	1920	1999	1990	1911	1911
Stem cell research investigation about--- therapies to treat disease	cell bared therapies	cell therapies	Plant enzymes	Plant cell research	cell bared therapies
Trade secrecy applicable rather than patents in ----------	Fermentation	drugs	chemicals	invitro fertilization	Fermentation

Which can be protected using patents	micropropagation	tissue	organ culture techniques	method for reducing pathogenecity	method for reducing pathogenicity
Not possible to get patents for ----------	Plastic surgery	modified plants	DNA sequences	. modified Microorganisms	Plastic surgery
The existing patent law does not allow ----------	Process patent	Product patent	Live forms	Inventions	Product patent
In India the patent law is based on ----------	Indian Patent Act of 1970	Indian Patent Act of 1911	. Indian Patent Act of 1991	. Indian Patent Act of 1811	Indian Patent Act of 1970
The main instrument of international collaboration for intellectual property is the ----------	GATT	WIPO	OECD	Court	WIPO
Patents are granted by ----------	GATT	WIPO	OECD	Court.	Court.
The provision of _____ enables the developing countries to paten GM crops.	TRIPS-GATT	WHO	. UN	UNESCO	TRIPS-GATT
The TRIPS final negotiation schedule was proposed in	Jan-00	02-Jan	01-Jan	Jan-99	Jan-00
Monopoly rights were granted in -------	.USA	Japan	Astralia	Europe.	Europe.
In USA period of patents ----------	25 years	12 years	10 years	17 years	17 years
In TRIPS, appellations of origin are covered in	Patents	Trade marks	Geographical indications	Copy rights	Geographical indications
Trade Related Aspects of Intellectual Property Rights (TRIPS) is administered by	WHO	WTO	. FAO	UNDP	WTO
_____are the rights given to people over the creations of their minds.	Intellectual property rights	Human rights	Right to speech	Right to speech	Intellectual property rights

International patent protection for Biotechnology was published by	WIPO	EC	.OECD	EPC.	OECD
TRIP Strands for ----------	Trade related intellectual property.	Trademark related intellectual property.	Trend related intellectual property.	Trade related international property.	Trade related intellectual property.
WIPO Strands for ----------	Word intellectual property organization	World intellectual property organization	Word intellectual property origin	World intellectual property origin	World intellectual property organization
_____Comprise of private information about specific technical procedures and formulations.	copy right	trade secret	trade mark	patent	trade secret
. _____ is a milk clotting proteolytic enzyme that hydrolyses the K-casein protein of milk.	chymosin	.renin	both	Casein	Chymosin
---------- is the key component of the rennet	Chymosin	Lipin	Casein	Rennin	Chymosin
Milk clotting activity found in ----------	Chymosin	Lipin	Casein	Ptotein	.Chymosin
Mastitis is the ----------	viral infection of milk gland	Parasitic infection of milk gland	Fungal infection of milk gland	Bacterial infection of milk gland	Bacterial infection of milk gland
EBT contaminate the ----------	Methonine	Lipin	Alanin	Tryptophan	Tryptophan
. _____ is an important example for gene piracy.	. GM Pseudomonas	Pentacliplandra brazzeana	Clostridium	Bacillus	Pentacliplandra brazzeana
In India, _____ is an important example biopiracy	Dalbergia	Neem	Ginger	Onion	Neem

---------- Serve as a source of transplanted organs for humans.	Transgenic pigs	Transgenic rabbits	Transgenic goats	Transgenic sheep	Transgenic pigs
Trade secrete comprise the information about ----------	Specific technical procedures	symbol	particular product	document.	Specific technical procedures
Recombinant DNA technology,were known by several phasses such as	techniques	manipulation of life	man made earth	ecosystem	manipulation of life
The original guide line providede by NIH was modified in need by……committee	NIH-RAC	GEO's	FDA	none of the above	NIH-RAC
3.earlier, most prominently used strain/host organism in RD	klebsiella.spp	E.coli k-12	E.coli	Pseudomonas aeruginosa	E.coli k-12
……is responsible for the regulating the introduction of foods,drugs,pharmaceutical and medical devices into the market place	FDA	WHO	NIH	FSI	FDA
…..is an enzyme approved by FDA in making cheese	chymosin	tryptophan	streptomycin	Penicillin	Chymosin
The following can not be exploited by assigning or by licensing the rights to others.	Patents	Designs	Trade mark	Food products	Trade mark
The patentable product involving R-DNA technology	Genes	Vaccines	Transposons	Base pairs	Vaccines
Intellectual Property Rights (IPR) protect the use of information and ideas that are of	Ethical value	Moral value	Commercial value	Social value	Commercial value

Requirements of patents are	A New useful invention	Invention	A useful product	Previously known product	A New useful invention
patent protection last for	13	17	16	20	20
.Indian copy right Act was published in	1967	1957	1937	1977	1957
IPR clasified in to	4 catagories	5 catagories	2 catagories	3 catagories	2 catagories
PCT came effective in India from	1999	1998	1997	1995	1998
In 'quid-pro-quo', quo stands for	knowledge disclosed to the public	a. monopoly granted for the term of the patent	selling and using the invention	food stuffs	. monopoly granted for the term of the patent
A request for restoration of patent can be filed within	12 months	16 months	18 months	20 months	18 months
TRIPS stands for	Trade related Aspects of Intellectual Property Rights	Trade random Aspects of Intellectual Property Rights	Trade random Aspects of Intellectual Principle Rights	Trade regulatory Aspects of Intellectual Principle Rights	Trade random Aspects of Intellectual Property Rights
In USA the maximum limit of monoply is for	10years	5 years	17 years	2 years	17 years
is the form of protection of plant related inventions	Plant Variety Protection	Plant Protection Act	Plant Patent Act	Plant Utility Act	Plant Patent Act
The World Intellectual Property Organization (WIPO) was established by a convention of	14 July 1967	17-Oct-66	18-Jul-87	08-Oct-86	14 July 1967
The meaning for patent	property	To lay open	Protection	To open	To lay open
.Indian copy right Act was published in	1967	1957	1937	1977	1957

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: I BSc MB **COURSE NAME: MTIPR**

COURSE CODE: 15MBU602 **POSSIBLE QUESTIONS**

1. Describe the host-controlled restriction.
2. What are adaptors and its application?
3. Plasmid as a cloning vector - justify.
4. Explain the shuttle vectors and give example.
5. Give a brief note on construction of cDNA library.
6. Explain the application of finger printing.
7. Write a detailed note on patenting biological materials.
8. Write a short note on patenting and fundamental research.
9. Write shortly the role of IPR on trade secrets.
10. Give a detail note on IPR and the rights of farmers in developing countries.
11. Explain the principle, properties and application of Type – II Restriction enzymes.
12. Explain methods of joining the DNA fragments.
13. Give a detailed note on the development of pBR322.
14. Give a short note on cloning vectors.
15. Describe the transformation in cloning process.
16. Explain briefly the construction of genomic DNA library.
17. Write about the chemical patenting and patenting process.
18. Give a brief on patenting multicellular organisms.
19. Explain the recent developments of IPR.
20. Patenting in other countries – Explain.
21. Explain in detail about the cutting and joining of DNA fragments
22. What are restriction enzymes? Explain various types of restriction enzymes
23. Write the application of M13 vector
24. Give short note on Ti plasmid
25. Detail account on blotting techniques
26. What is cDNA? Explain in detail about the synthesis and applications
27. What constitutes a patent? Briefly explain
28. Write a short note on patenting and fundamental research
29. Write shortly the role of IPR on trade secrets
30. Explain the patenting of biological material

31. Write the different types of restriction enzymes and its application

32. What are adaptors and explain its application
33. What is binary vectors? Give the example.
34. Plasmid as a cloning vector – justify.
35. Lambda vector – what is its role in microbial technology?
36. Give short note on Ri plasmid.
37. Give a brief on patenting multicellular organisms.
38. Write about the chemical patenting and patenting process.
39. Give a detail note on IPR and the rights of farmers in developing countries.
40. IPR – Explain its role in India.
41. Describe the host-controlled restriction system
42. Write the various types of restriction enzymes and its application.
43. Give a detailed note on the development of pBR322.
44. Plasmid as a cloning vector – justify.
45. Give a brief note on construction of cDNA library.
46. Give short note on Ri plasmid.
47. write about the chemical patenting and patenting process.
48. Write a short note on patenting and fundamental research.
49. Give a detail note on IPR and the rights of farmers in developing countries.
50. Patenting in other countries – Explain.