DR. UMAYAL RAMANATHAN COLLEGE
FOR WOMEN, KARAIKUDI – 3
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EDUCATIONAL TRUST

URCW

DEPARTMENT OF BIOTECHNOLOGY

RECOMBINANT DNA TECHNOLOGY

7BBT6C1

DR.J.CHITRA

VI	Recombinant DNA technology	7BBT6C1	CO1	Discuss various Molecular tools and applications
			CO2	Discuss various cloning vectors-plasmid derived vectors, bacteriophage derived vectors hybrid vectors & high capacity vectors with suitable examples.
			CO3	Discuss the Regulation of gene expression in Prokaryotes
			CO4	Describe the Basic principles of construction of genomic and cDNA libraries.
			CO5	Describe the principles of nucleic acid hybridization techniques- Southern blot hybridization, Northern blot hybridization and insitu hybridization

III YEAR – VI SEMESTER

COURSE CODE: 7BBT6C1

CORE COURSE - XII – RECOMBINANT DNA TECHNOLOGY

Unit - I

Molecular tools and applications: Restriction enzymes. DNA Polymerases, DNA dependent RNA polymerases. DNA ligases, alkaline phosphatase.

Unit - II

Cloning vectors and their applications: Bacterial plasmids, pBR322, pUC18, pUC19. Cosmids, Phagemids. Construction, Principle and uses of YAC, BAC.

Unit - III

Regulation of gene expression in Prokaryotes – Lac operon and trp operon & attenuation. Regulation in eukaryotes: gene loss, gene amplification, gene rearrangement.

Unit - IV

Basic principles of construction of genomic and cDNA libraries. PCR based cloning approach (TA cloning).

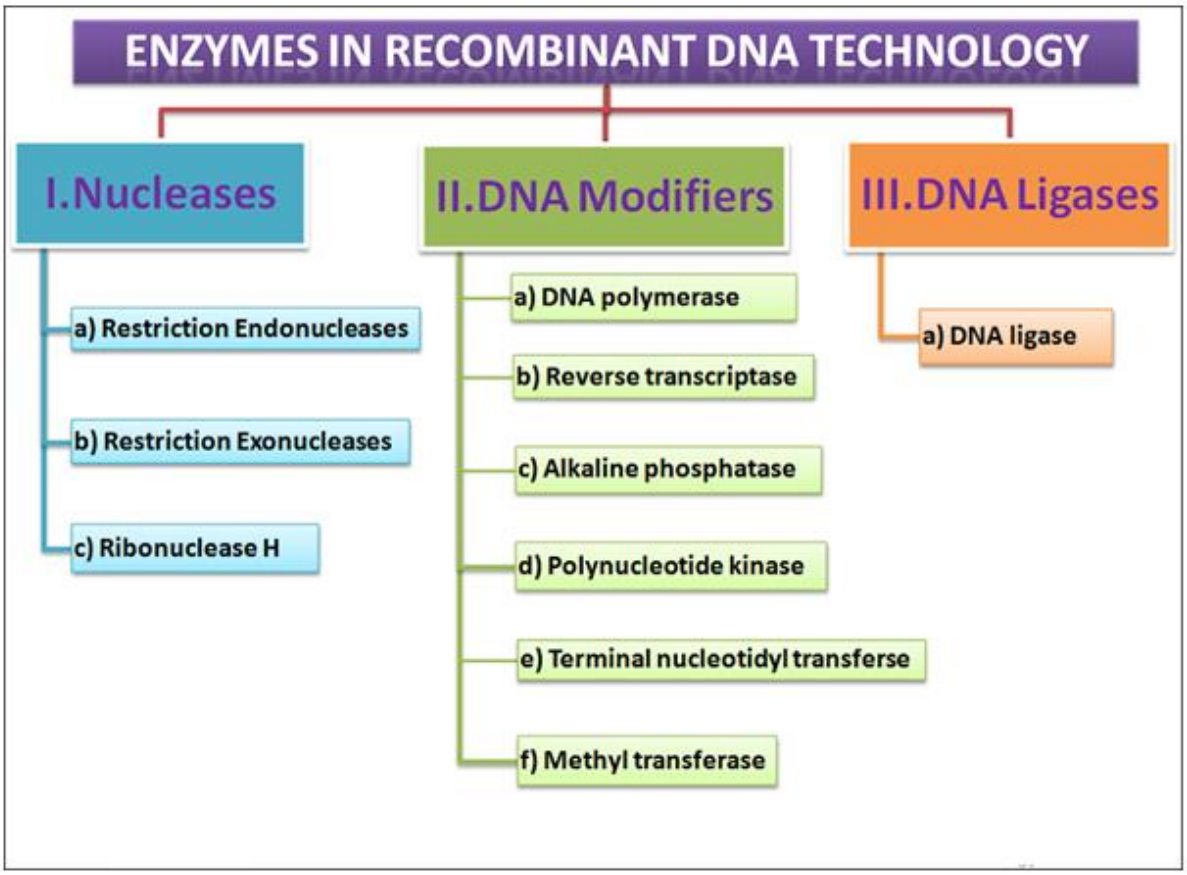
Unit - V

Analysis of cloned genes. Southern hybridization – Preparation of radiolabelled / non radiolabelled DNA & RNA probes, hybridization and autoradiography.

Unit - I

Molecular tools and applications

Mind Map



I. Restriction enzymes

Restriction Endonucleases:

A commonly used tool in molecular biology is restriction endonucleases. Restriction endonucleases, otherwise known as restriction enzymes, are molecular scissors that can cut double-stranded DNA at a specific base-pair sequence.

Each type of restriction enzyme recognizes a characteristic sequence of nucleotides that is known as its recognition site. Researchers can use these enzymes to cut DNA in a predictable and precise manner.

Discovery of Restriction Endonucleases:

Scientific discoveries often have their origin in seemingly unimportant observation that receive little attention by researchers before their general significance is appreciated. In case

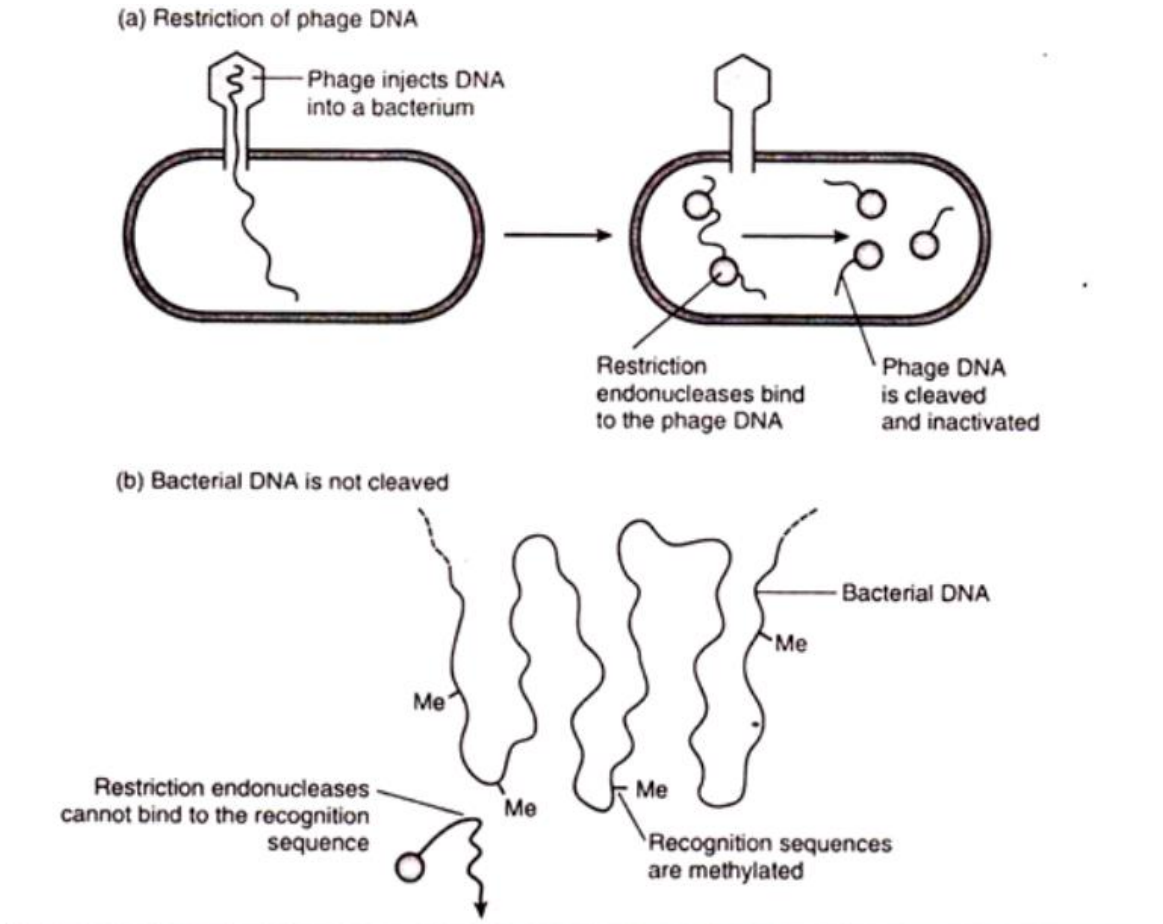
of genetic engineering, the original observation was that bacteria use enzymes to defend themselves against viruses.

Most organisms eventually evolve means of defending themselves from predators and parasites, and bacteria are no exception. Among the natural enemies of bacteria are bacteriophages, viruses that infect bacteria and multiply within them.

At some point, they cause the bacterial cells to burst, releasing thousands more viruses. Through natural selection, some types of bacteria have acquired powerful weapons against these viruses; they contain enzymes called restriction endonucleases that fragment the viral DNA as soon as it enters the bacterial cell.

Many restriction endonucleases recognize specific nucleotide sequences in a DNA strand, bind to the DNA at those sequences, and cleave the DNA at a particular place within the recognition sequence. Why don't restriction endonucleases cleave the bacterial cells' own DNA as well as that of the viruses?

The answer to this question is that bacteria modify their own DNA, using other enzymes known as methylases to add methyl (—CH_3) groups to some of the nucleotides in the bacterial DNA. When nucleotides within a restriction endonuclease's recognition sequence have been methylated, the endonuclease cannot bind to that sequence.
Consequently, the bacterial DNA is protected from being degraded at that site. Viral DNA, on the other hand, has not been methylated and, therefore, is not protected from enzymatic cleavage.



The function of a restriction endonuclease in bacterial cell: (a) phage DNA is cleaved, but (b) bacterial DNA is not cleaved due to methylation

Types of Restriction Endonucleases:

All restriction enzymes fall into one of three classes, basing upon their molecular structure and need for specific co-factors.

I. Class I Endonucleases:

These have a molecular weight around 300,000 Daltons, Eire composed of non-identical subunits, and require Mg^{2+}, ATP (adenosine triphosphate), and SAM (S-adenosylmethionine) as cofactors for activity. Not used in RDT experiments.

II. Class II Endonucleases:

These are much smaller, with molecular weights in the range of 20,000 to 100,000 Daltons. They have identical sub-units and require only Mg^{2+} as a cofactor (Nathans and Smith, 1975). Only class II endonucleases are used in RDT experiments due to their site specific cleavage action.

III. Class III Endonucleases:

These are large molecules, with a molecular weight of around 200,000 Daltons, composed of non- identical sub-units. These enzymes differ from enzymes of other two classes in that they require both Mg^{2+} and ATP but not SAM as co-factors. Class III endonucleases are the rarest of three types. Not used in RDT experiments.

Nomenclature:

As a large number of restriction enzymes have been discovered, a uniform nomenclature system is adopted to avoid confusion.

Characteristics of restriction and modification systems

	Class I	Class II	Class III
Composition	Multienzyme complex with R (endonuclease), M (methylase), and S (specificity) subunits, e.g., R_2M_2S	Separate enzymes: endonuclease is a homodimer, methylase a monomer	M subunit provides specificity; on its own, functions as methylase; as heaterodimer with R subunit, functions as methylase endonuclease
Cofactors[a]	Mg^{2+}, ATP, SAM (needed for cleavage and methylation)	Mg^{2+}, SAM (for methylation only)	Mg^{2+}, ATP (for cleavage), SAM (needed for methylation; stimulates cleavage)
Recognition sites	Asymmetric, bipartite, may be degenerate, e.g., EcoK ($AACN_6GTGC$)	Symmetric, may be bipartite, may be degenerate (Table 1.2)	Asymmetric uninterrupted 5-6 nt long, e.g. *Eco*PI5-CAGCAG. Two copies in opposite orientation, but not necessarily adjacent, needed for cleavage; one for methylation.
Cleavage	Variable distance (100–1000 nt) from recognition site	Within recognition site, except for Class IIs (shifted cleavage), which cleaves outside, at a defined distance	25–27 nt from recognition site
Number of systems characterized	Several, grouped into a few families, e.g., K, includes *Eco*B, *Eco*D, *Eco*K, and others	Hundreds	Few

[a]ATP: adenosine triphosphate; SAM: S-adenosyl methionine

This nomenclature was first proposed by Smith and Nattens in 1973.

1. The first letter of restriction enzymes (RE) should be from first letter of the species name of organism from which the enzyme is isolated.

The letter should be written in capitals and italics, e.g., RE from E. coli will have E as starting letter.

2. The second and third letters of RE should be from the first and second letters of genus name of the organism. The letter should be written in lower case and should be in italics, e.g., RE from E coli will have Eco as starting words.

3. If the RE is isolated from particular strain of an organism, then that should be written as fourth letter. It should be in capitals and not in italics. For example, RE from E. coli R strain will be written as Eco R.

4. If the RE isolated is the first of its kind from that particular organism, then the number I should be given. If already two REs are isolated, then number III should be given for new restriction enzymes. The number should be written in roman, e.g., the first E. coli RE should be written as Eco RI whereas the third restriction enzyme isolated from E. coli R strains should be written as Eco RIII.

Recognition Sequences:

The recognition sequences for class II endonucleases form palindromes with rotational symmetry. In a palindrome, the base sequence in the second half of a DNA strand is the mirror image of sequence in its first half (Fig. 4.2). But in a palindrome with rotational symmetry, the base sequence in the first half of one strand of a DNA double helix is the mirror image of second half of its complementary strand (Fig 4.3).

Thus in such palindromes, the base sequence in both the strands of a DNA duplex reads the same when read from the same end (either 5′ or 3′) of both the strands.

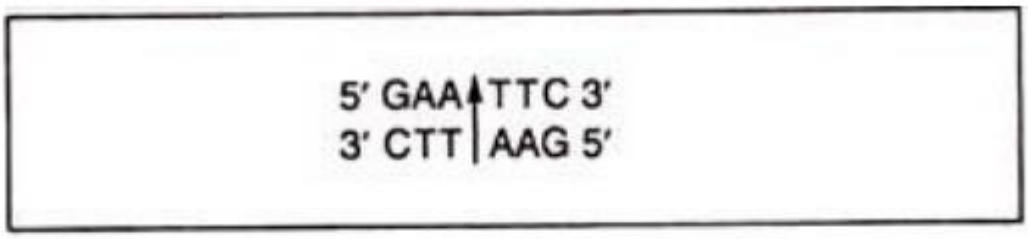
Examples of Restriction endonucleases used in Recombinant DNA experiments.

Enzyme	Microorganism from which enzyme is isolated	Recognition sequence	Type of fragment end produced
*Bam*HI	*Bacillus amyloliquefaciens*	5′–GGATCC–3′ 3′–CCTAGG–5′	Cohesive
*Cof*I	*Clostridium formicoaceticum*	5′–GCGC–3′ 3′–CGCG–5′	Cohesive
*Dra*I	*Deinococcus radiophilus*	5′–TTTAAA–3′ 3′–AAATTT–5′	Blunt
*Eco*RI	*Escherichia coli*	5′–GAATTC–3′ 3′–CTTAAG–5′	Cohesive
*Eco*RII	*Escherichia coli*	5′–CCAGG–3′ 3′–GGTCC–5′	Cohesive
*Hae*III	*Haemophilus aegyptius*	5′–GGCC–3′ 3′–CCGG–5′	Blunt
*Hind*III	*Haemophilus influenczae*	5′–AAGCTT–3′ 3′–TTCGAA–5′	Cohesive
*Hpa*II	*Haemophilus parainfluenzae*	5′–CCGG–3′ 3′–GGCC–5′	Cohesive
*Not*I	*Nocardia otitidis-caviarum*	5′–GCGGCCGC–3′ 3′–CGCCGGCG–5′	Cohesive
*Pst*I	*Providencia stuartii*	5′–CTGCAG–3′ 3′–GACGTC–5′	Cohesive
*Pvu*II	*Proteus vulgaris*	5′–CAGCTG–3′ 3′–GTCGAC–5′	Blunt
*Sma*I	*Serratia marcescens*	5′–CCCGGG–3′ 3′–GGGCCC–5′	Blunt

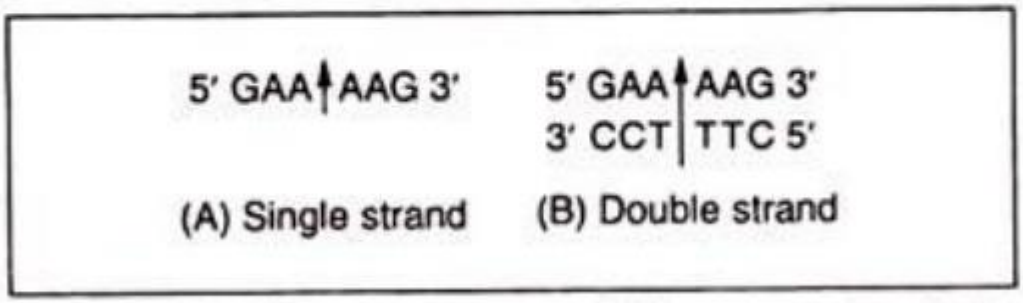
Mechanism of Action of Restriction Endonucleases:

EcoRI can be taken as an example of class II endonucleases and its works can be seen. When the restriction endonuclease encounters its respective restriction site sequence (5′ GAATTC 3′), it cleaves each backbone between the G and the closest A base residues.

Once the cuts have been made, the resulting fragments are held together only by relatively weak hydrogen bonds that hold the complementary bases to each other. The weakness of these bonds allows the DNA fragments to separate from each other. Each resulting fragment has a protruding 5′ end composed of unpaired bases.



A palindrome with rotational symmetry



A palindrome sequence. (A) sequence in a single DNA strand. (B) Sequence in a double helix. The arrow represents the axis of symmetry

Other enzymes also create cuts in the DNA backbone in the same manner, which results in protruding 3′ ends. Protruding ends—both 3′ and 5'—are sometimes called 'sticky ends' because they tend to bond with complementary sequences of bases.

In other words, if an unpaired length of bases (5′ A A T T 3′) encounters another unpaired length with the sequence (3′ T T A A 5′) they will bond to each other—they are 'sticky' for each other. Ligase enzymes are then used to join the phosphate backbones of two molecules.

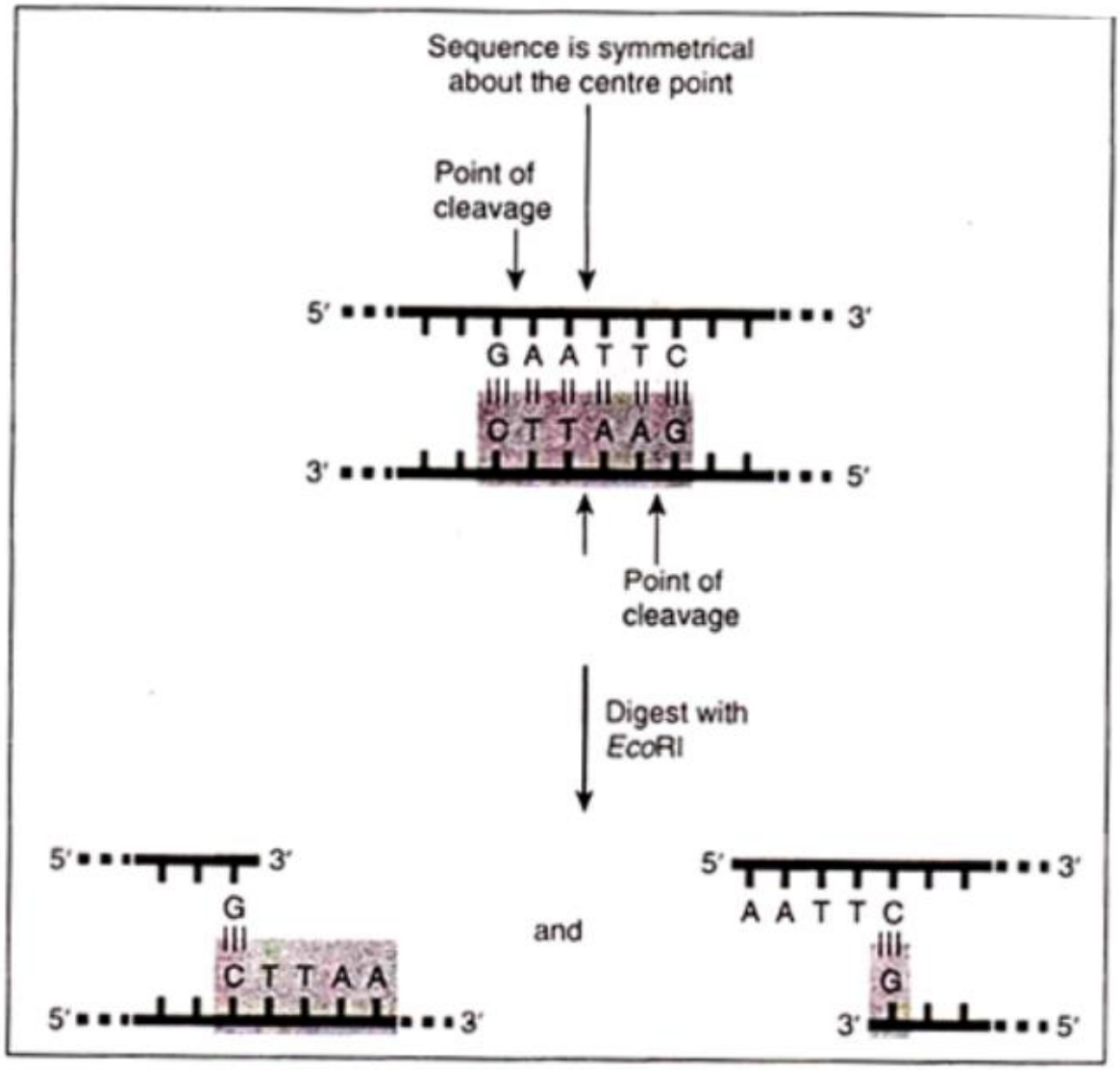
The cellular origin, or even the species origin, of the sticky ends does not affect their stickiness. Any pair of complementary sequences will tend to bond, even if one of the sequences comes from a length of human DNA, and the other comes from a length of bacterial DNA.

In fact, it is this quality of stickiness that allows the production of recombinant DNA molecules (molecules which are composed of DNA from different sources and have given birth to a powerful technology and industry) the genetic engineering or the recombinant DNA.

Examples of some other restriction enzymes, their mode of cutting and generation of 5′ overhangs and 3′ overhangs, are illustrated in (Fig. 4.5). Sticky ends (also called cohesive ends or overhanging heads) are useful for DNA cloning because complementary sequences anneal and can be ligated directly by DNA ligase.

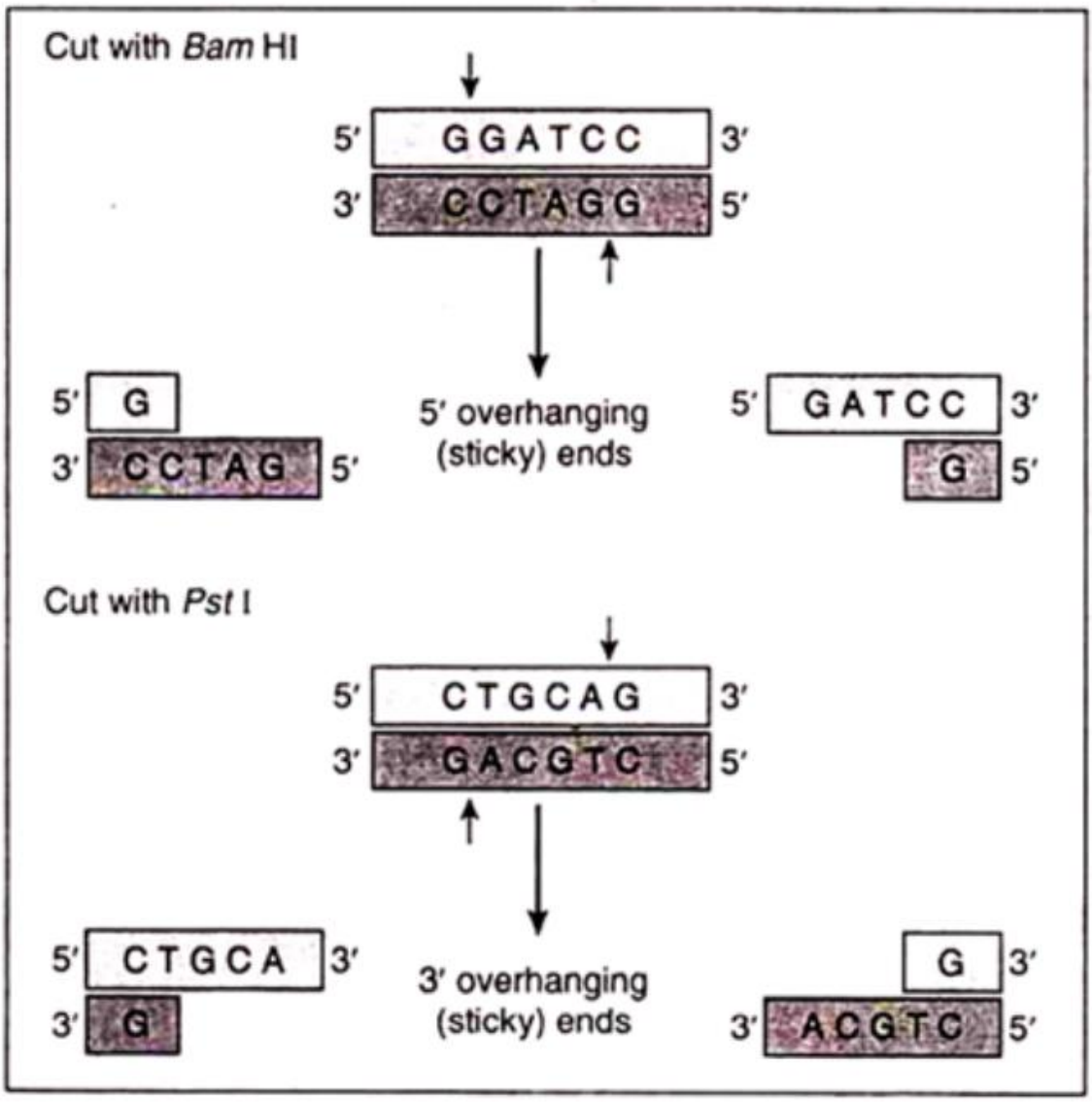
If two different DNA samples cleaved with the same type of restriction enzymes are mixed together in the presence of DNA ligase, a recombinant DNA molecule can be generated. This is possible because of the presence of same type of sticky ends.

The complementary sequences of sticky ends from the unrelated DNA samples will anneal together and are finally joined by the DNA ligase enzyme to form the recombinant DNA molecule.



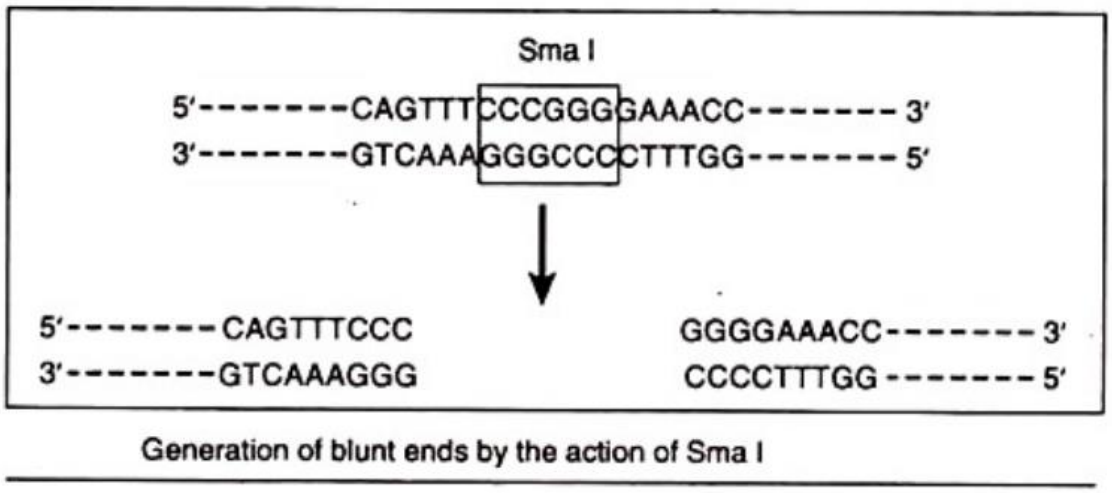
The restriction site sequence of *Eco*RI is a palindrome of a hexamer and it cuts between the bases G and A on both the strands resulting in sticky ends or cohesive ends

Some restriction enzymes, on the other hand, cut both the strands of a DNA molecule at the same site so that the resulting termini or ends have blunt or flush ends (Fig. 4.6) in which the two strands end at the same point. The blunt ends also can effectively be utilized as recombinants followed by some end point modifications.



Restriction digestion with *Bam* HI resulting in 5′ overhanging ends and with *Pst* I resulting in 3′ overhanging ends

As the list of restriction enzymes grew and their recognition sequences were identified, it was found in some cases that more than one enzyme could recognize the same sequence. RJ Roberts conferred the term isoschizomer (same cutter) on restriction enzymes that recognized the same DNA sequence.



Generation of blunt ends by the action of Sma I

Star Activity:

Sometimes restriction enzymes recognize and cleave the DNA strand at the recognition site with asymmetrical palindromic sequence; for example, Bam HI cuts at the sequence GA TCC, but under extreme conditions, as in low ionic strength it will cleave in any of the following sequences NGA TCC, GPOA TCC, GGNTCC. Such an activity of the restriction endonucleases is called star activity.

Uses of Restriction Endonucleases:

Restriction enzymes have been used for sequence analysis, cloning and amplifying DNA. DNA from animal viruses bacteriophages contains 5,000 to 50,000 base pairs. It is important to know the primary structure of DNA, i.e., the sequence of bases, for decoding the information stored in genes, for understanding gene structure and regulation at molecular level.

The discovery of restriction enzymes was a major breakthrough in sequence analysis of DNA. By using combinations of different restriction enzymes it is possible to hydrolyse large DNA molecules into fragments less than 300 base pairs in length.

These fragments can then be used for sequence analysis and are arranged into a physical map of the chromosome. This is a slow and laborious process. The mapping of entire 5,000 base pair DNA of the virus SV40 into some 100 fragments has taken several years. Complete sequence analysis of the fragments would take much longer.

The DNA fragments produced by restriction endonucleases can covalently be linked in vitro to linear plasmid DNA or to lambda phage DNA. The recombinant DNA species produced can be inserted into E. coli by transformation.

Each transformed cell can then be grown as a separate clone. By this method a complex genome can be broken down into thousands or millions of pieces, and each piece is isolated to form a separate clone.

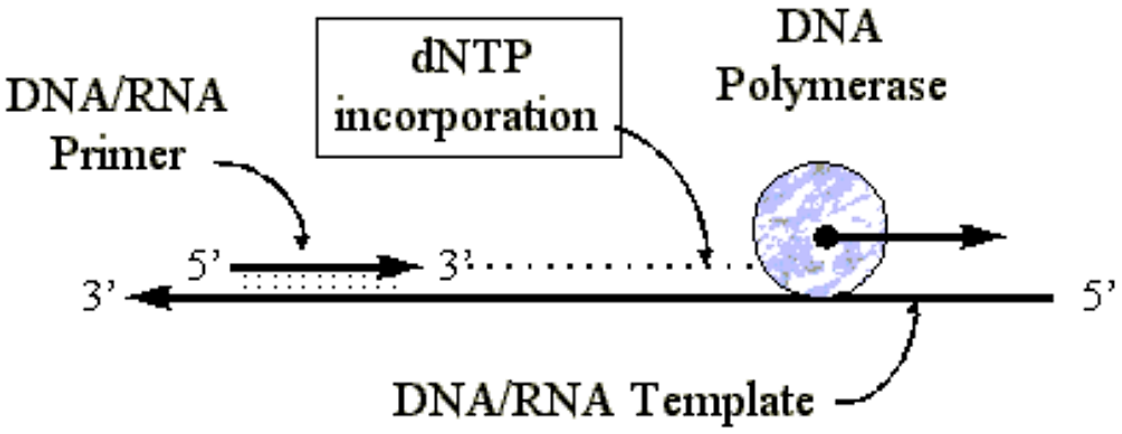
II. DNA Polymerases

- DNA polymerase is a complex enzyme which synthesize nucleotide complementary to template strand.
- It adds nucleotide to free 3′ OH end and help in elongation of strand
- It also helps to fill gap in double stranded DNA.
- DNA polymerase-I isolated from E. coli is commonly used in gene cloning
- Taq polymerase isolated from *Thermus aquaticus* is used in PCR

These are mostly used when we are carrying out the cloning of the recombinant DNA in the prokaryotic host cells like E. coli. Then we fill the gaps in duplexes by stepwise addition of nucleotide to 3′ ends.

A wide variety of polymerases have been characterized and are commercially available. All DNA polymerases share two general characteristics:

1. They add nucleotides to the 3'-OH end of a primer
2. The order of the nucleotides in the nascent polynucleotide is template directed



In addition to the 5'->3' polymerase activity, polymerases can contain exonuclease activity. This exonuclease activity can proceed either in the 5'->3'direction, or in the 3'->5' direction.

- Exonuclease activity in the 3'->5' direction allows the polymerase to correct a mistake if it incorporates an incorrect nucleotide (so called "***error correction activity***"). *It can also slowly degrade the 3' end of the primer*.
- Exonuclease activity in the 5'->3' direction will allow it to degrade any other hybridized primer it may encounter. Without 5'->3' exonuclease activity, obstructing primers may or may not be physically deplaced, depending on the polymerase being used.

Different polymerases have differing error rates of misincorporation, and different rates of polymerization.

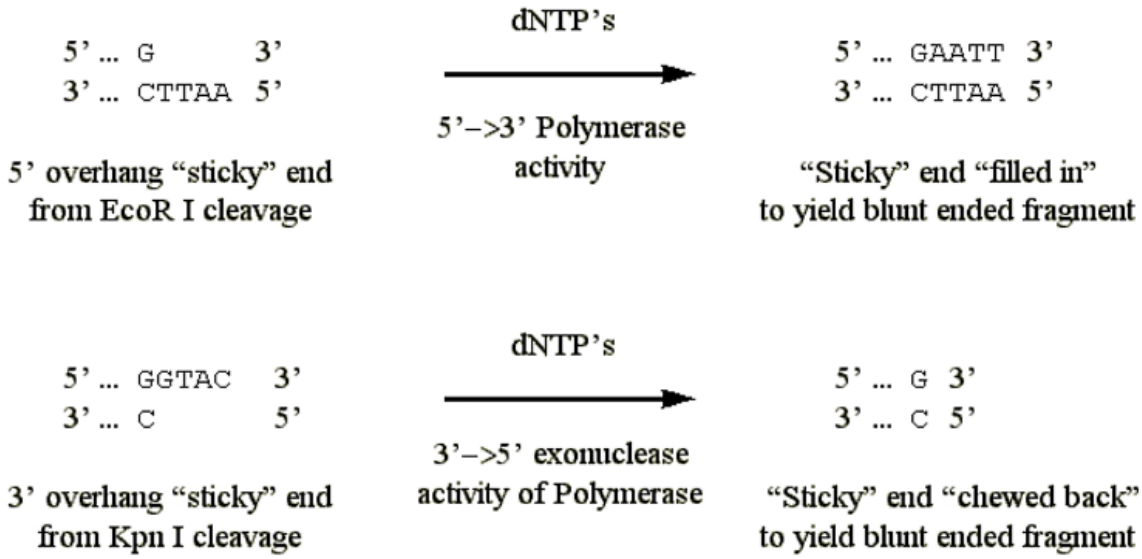
E. coli DNA polymerase I	E. coli DNA polymerase I - Klenow Fragment	T4 DNA polymerase	T7 DNA polymerase	Taq DNA polymerase	M-MuLV Reverse Transcriptase
5'->3' exonuclease activity	*				*

3'->5' exonuclease activity	*	*	*	*	
Error Rate ($x10^{-6}$)	9	40	<1	15	285
Strand Displacement		*			
Heat Inactivation	*	*	*	*	

Uses of polymerases

The various activities of the different polymerases lend them to a variety of applications. For example, restriction endonucleases can yield fragments of DNA with either 3' or 5' nucleotide "overhangs".

- In the case of 5' overhangs, the 5'->3' polymerase activity can fill these in to make **blunt ends**.
- In the case of 3' overhangs, the 3'->5' exonuclease activity present in some polymerases (especially T4 DNA polymerase) can "chew back" these ends to also make blunt-ended DNA fragments.

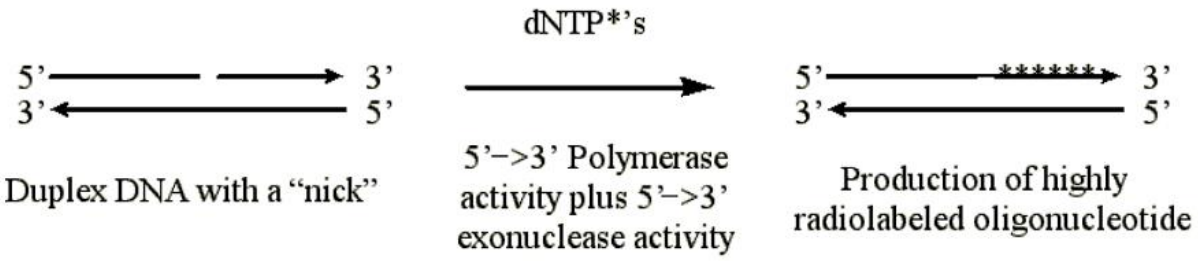


Polymerase activity

"Nick-translation"

This method is used to obtain highly radiolabeled single strand DNA fragments, which makes use of 5'->3' exonuclease activity present in some polymerases (*E. coli* DNA polymerase I, for example).

- In this method a DNA duplex of interest is "nicked" (i.e. one of the strands is cut; see DNAse I).
- Then DNA pol I is added along with radiolabeled nucleotides. The 5'->3' exonuclease activity chews away the 5' end at the "nick" site and the polymerase activity incorporates the radiolabeled nucleotides. The resulting polynucleotide will be highly radiolabeled and will hybridize to the DNA sequence of interest.



: *Nick-translation*

- Thermostable polymerases have the ability to remain functional at temperature ranges where the DNA duplex will actually "melt" and become separated. This has allowed the development of the **"Polymerase Chain Reaction"** technique (PCR), which has had a profound impact on modern Biotechnology. We will discuss this method at a later date.
- The incorporation of dideoxy bases (i.e. no hydroxyl groups on either the 2' or 3' carbon of the ribose sugar) leads to **termination of the polymerase reaction**. This will be discussed in greater detail later. However, this chain termination by incorporation of dideoxynucleotides is the basis of the Sanger method of DNA sequencing, as well as therapies to try to inhibit viral replication.

III. RNA dependent DNA polymerases (reverse transcriptase)

Reverse transcriptase enzyme is also called RNA dependent DNA polymerase. These enzymes are present in most of the RNA tumour viruses and retroviruses.

Reverse transcriptases have been identified in many organisms, including viruses, bacteria, animals, and plants. In these organisms, the general role of reverse transcriptase is to convert RNA sequences to cDNA sequences that are capable of inserting into different areas of the genome. In this manner, reverse transcription contributes to:

- Propagation of retroviruses—e.g., human immunodeficiency virus (HIV), Moloney murine leukemia virus (M-MuLV), and avian myeloblastosis virus (AMV)
- Genetic diversity in eukaryotes via mobile transposable elements called retrotransposons
- Replication of chromosomal ends called telomeres
- Synthesis of extrachromosomal DNA/RNA chimeric elements called multicopy single-stranded DNA (msDNA) in bacteria

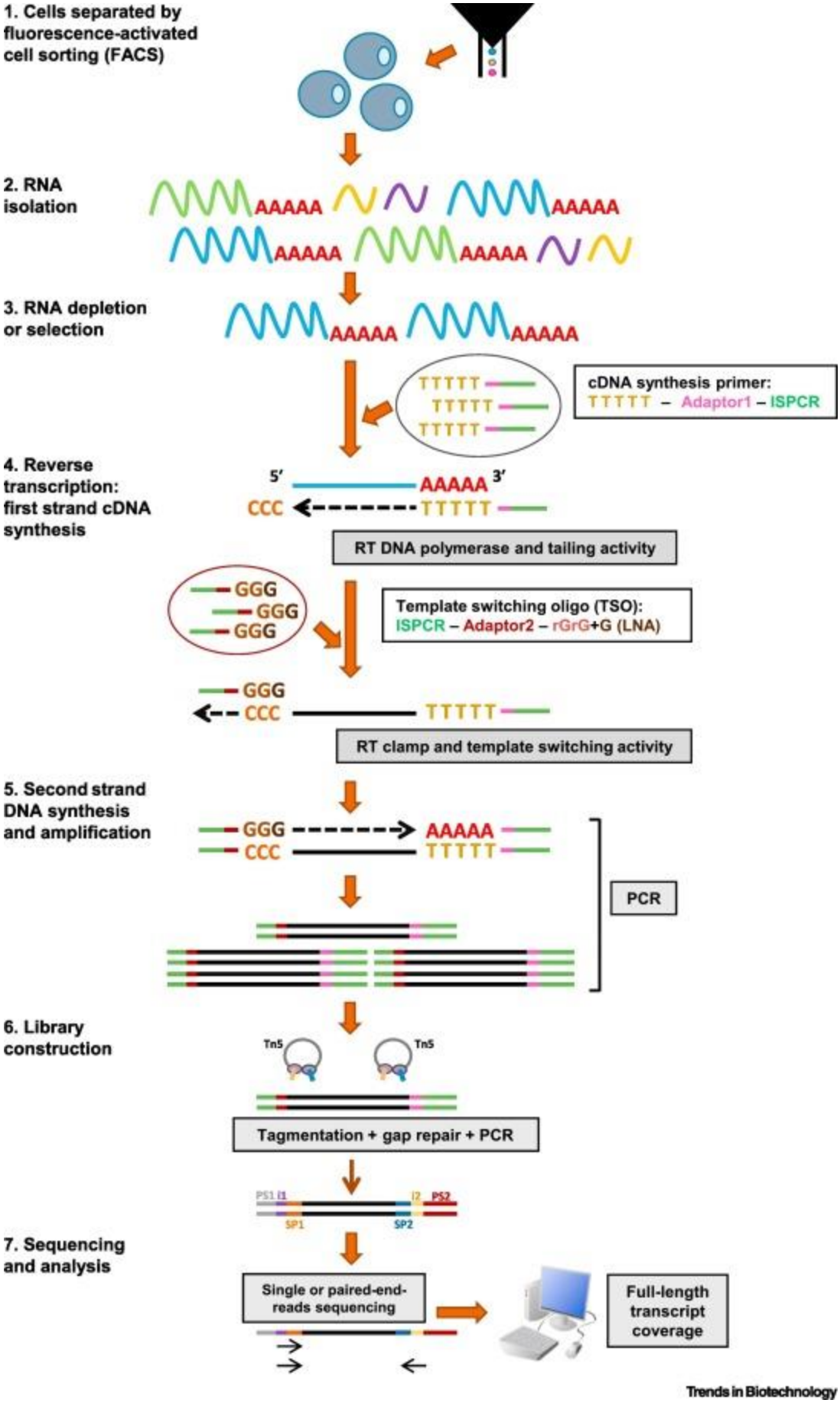
Manytimes we do not get our gene of interest rather its mRNA. In this case reverse transcriptase enzyme can be used to prepare a double stranded DNA (our gene of interest) from the available single-stranded mRNA (template) by a process called reverse transcription.

While reverse transcriptases have functional roles in biological systems, they also serve as important tools for studying RNA populations. One of the first molecular biology protocols utilizing reverse transcriptases was for the production of cDNA to build libraries that

contained DNA copies of mRNA from cells and tissues. These cDNA libraries aid in understanding actively expressed genes and their functions at a specific time point.

Although the creation of cDNA libraries was an important step forward in characterizing expressed genes, challenges remained for the study of low-abundance RNAs. These were subsequently addressed with the development of the polymerase chain reaction (PCR), a technique to amplify small amounts of genetic material. Reverse transcription combined with PCR, or reverse transcription PCR (RT-PCR), allows detection of RNA even at very low levels of gene expression and paves the way for detection of circulating RNA, RNA viruses, and cancerous gene fusions in molecular diagnostics.

In addition, cDNAs serve as templates in applications such as microarray and RNA sequencing to characterize unknown RNAs in a high-throughput manner


1. Cells separated by fluorescence-activated cell sorting (FACS)
2. RNA isolation
AAAAA
3. RNA depletion or selection
TTTTT
cDNA synthesis primer:
TTTTT – Adaptor1 – ISPCR
4. Reverse transcription: first strand cDNA synthesis
5'
3'
CCC
RT DNA polymerase and tailing activity
GGG
Template switching oligo (TSO):
ISPCR – Adaptor2 – rGrG+G (LNA)
RT clamp and template switching activity
5. Second strand DNA synthesis and amplification
PCR
6. Library construction
Tn5
Tagmentation + gap repair + PCR
PS1
i1
i2
PS2
SP1
SP2
7. Sequencing and analysis
Single or paired-end-reads sequencing
Full-length transcript coverage
Trends in Biotechnology

IV. DNA ligases

This enzyme is responsible for the formation of the phosphodiester linkage between two adjacent nucleotides and thus joins two double-stranded DNA fragments. The phosphodiester linkage is formed between the 3′OH group (hydroxyl group at the 3′ carbon) and 5′ PO4 group of the pentose sugar of adjacent nucleotides of two different DNA fragments or nicks created in a double-stranded DNA molecule. In rDNA experiments, DNA ligase is used to join two different DNA fragments (plasmid/vector and the foreign DNA) that are annealed by the sticky ends. There are different types of ligase enzymes from different sources. But, the most frequently used one is the T4 DNA ligase produced by T4 bacteriophage.

Recombinant DNA experiments require the joining of two different DNA segments or fragments in vitro. The ends generated by some RE will be either cohesive (sticky) or blunt. The cohesive ends will anneal (join) themselves by forming hydrogen bonds. But the segments annealed thus are weak and do not withstand experimental conditions.

To get a stable joining, the DNA should be joined by using an enzyme called ligase. In the case of blunt ends we use linker or adaptors for successful ligation.

There are two types of DNA ligases:

(a) T4 DNA Ligase:

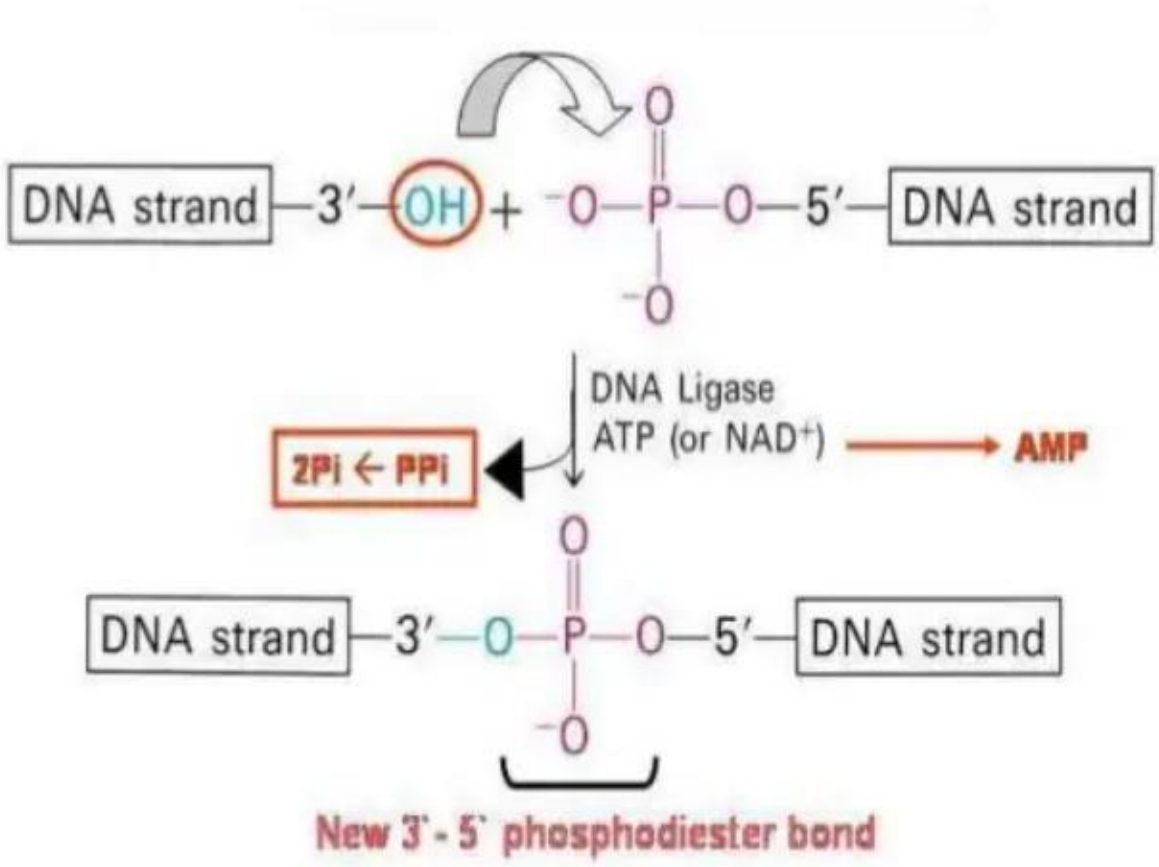
Naturally coded by T4 bacteriophage. The catalytic activity of the enzyme requires the presence of ATP as cofactor and Mg^{++}. This is predominantly used in RDT experiments.

(b) NAD^{+} dependent DNA Ligase:

Naturally found in E. coli. Uses NAD+ as a co-factor and only found in bacteria.

Mechanism of Action:

The cofactor is first spited (ATP→ AMP + 2Pi) and then AMP binds to the enzyme to form the enzyme-AMP complex. This complex then binds to the nick or break (with 5′ $-PO_4$ and 3′ −OH) and makes a covalent bond in the phosphodiester chain. The ligase reaction is carried out at 4°C for better results.



V. Alkaline phosphatase.

Alkaline phosphatase is a glycoprotein with two identical subunits. The cohesive ends of broken plasmids, instead of joining with foreign DNA, join the cohesive end of the same DNA molecules and get re-circularized. To overcome this problem the restricted plasmid is treated with an enzyme, alkaline phosphatase, that digests the terminal phosphoryl group.

The restriction fragments of the foreign DNA to be cloned are not treated with alkaline phosphatase.

Therefore, the 5′ end of foreign DNA fragment can covalently join to 3′ end of the plasmid. The recombinant DNA thus obtained has a nick with 3′ and 5′ P hydroxy ends. Ligase will only join 3′ and 5′ ends of recombinant DNA together if the 5′ end is phosphorylated.

Thus, alkaline phosphatase and ligase prevent re-circularization of the vector and increase the frequency of production of recombinant DNA molecules. The nicks between two 3′ ends fragment and vector DNA are repaired inside the bacterial cells during the transformation.

In rDNA experiments there is always the problem of self-ligation and the reformation of the original plasmid or the vector. This reduces the efficiency of the recombinant DNA formation. Self-ligation is the process of annealing the sticky ends of the linearized vector without inserting the foreign DNA fragment. On ligation it will result in the original vector without any insert DNA. This can be prevented by using alkaline phosphatase. We know that the presence of 3′OH group and 5′ PO4 group is a prerequisite for the ligase enzyme to act. If any one group of this is absent, the DNA ligase cannot function. Alkaline phosphatase can remove the phosphate group from the 5′ end of the DNA molecule resulting in a 5′OH group. Now, the only chance of ligation is between the vector and the foreign DNA resulting in a recombinant DNA molecule as the insert DNA has the 5′PO4 group. Thus, self-ligation can be prevented and the efficiency of rDNA formation can be increased. This enzyme is isolated from some bacteria (bacterial alkaline phosphatase—BAP) or calf intestine (calf intestine alkaline phosphatase—CAP).

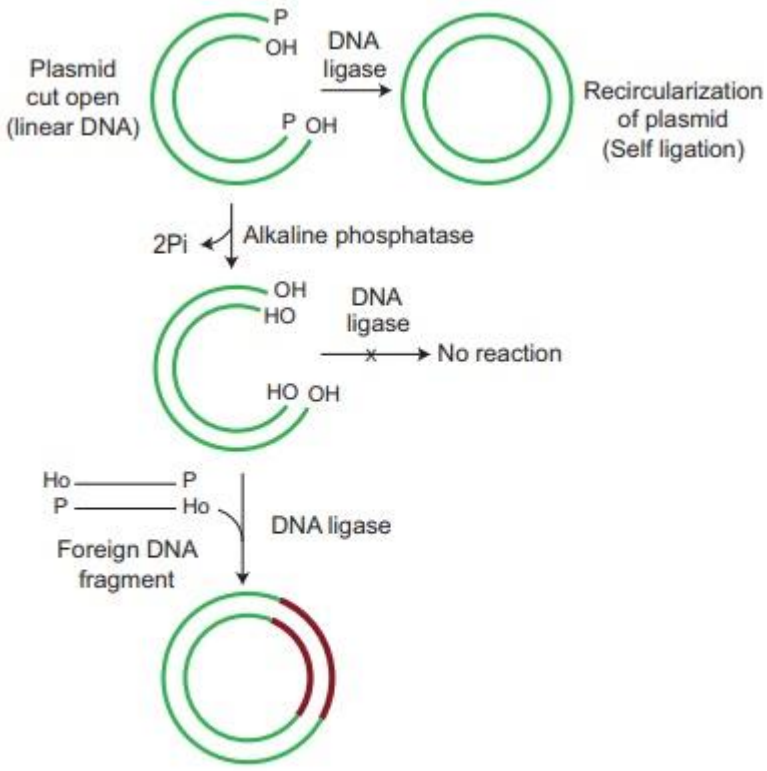
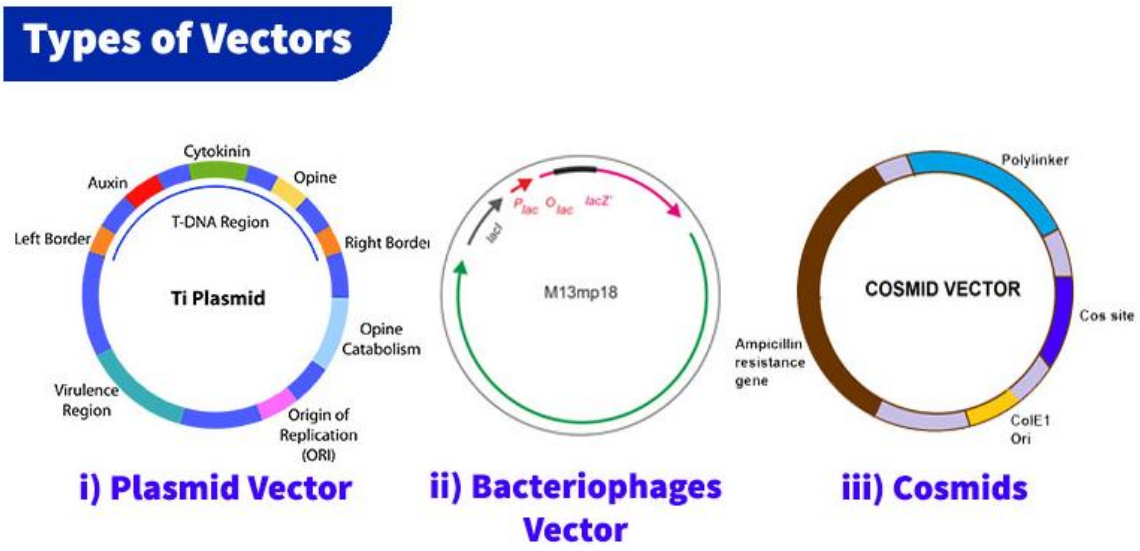


Figure 4.7: Action of Alkaline Phosphatase

Unit II

Mind Map



I. Cloning vectors and their applications

- Genetic vectors are vehicles for delivering foreign **DNA** into recipient cells.
- In molecular cloning, a vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed.
- Vectors can replicate autonomously and typically include features to facilitate the manipulation of DNA as well as a genetic marker for their selective recognition.
- The different types of vectors available for cloning are **plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs)** and **mammalian artificial chromosomes (MACs).**
- The cloning vectors are limited to the size of insert that they can carry. Depending on the size and the application of the insert the suitable vector is selected for a particular purpose.

Essential Characteristics of Cloning Vectors

Regardless of the selection of a vector, all vectors are carrier DNA molecules. These carrier molecules should have few common features in general such as:

- It must be self-replicating inside host cell.
- It must possess a unique restriction site for RE enzymes.
- Introduction of donor DNA fragment must not interfere with replication property of the vector.

- It must possess some marker gene such that it can be used for later identification of recombinant cell (usually an antibiotic resistance gene that is absent in the host cell).
- They should be easily isolated from host cell.

Plasmids

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

Bacteriophage

- The viruses that infect bacteria are called bacteriophage. These are intracellular obligate parasites that multiply inside bacterial cell by making use of some or all of the host enzymes.
- Bacteriophages have a very high significant mechanism for delivering its genome into bacterial cell. Hence it can be used as a cloning vector to deliver larger DNA segments.
- Most of the bacteriophage genome is non-essential and can be replaced with foreign DNA.
- Using bacteriophage as a vector, a DNA fragment of size up to 20 kb can be transformed.

Bacterial artificial chromosomes (BACs)

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

- Several hundred thousand base pair DNA fragments can be cloned using BACs.

Yeast artificial chromosomes (YACs)

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

Human artificial chromosomes (HACs)

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

Other Types of Vectors

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

Expression Vectors

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

Transcription Vectors

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

Uses of Vectors

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

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

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

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

II. Bacterial 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.

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

• Antibiotic resistance

• Antibiotic production

• Degradation of aromatic compounds

• Hemolysis production

• Sugar fermentation

• Enterotoxin production

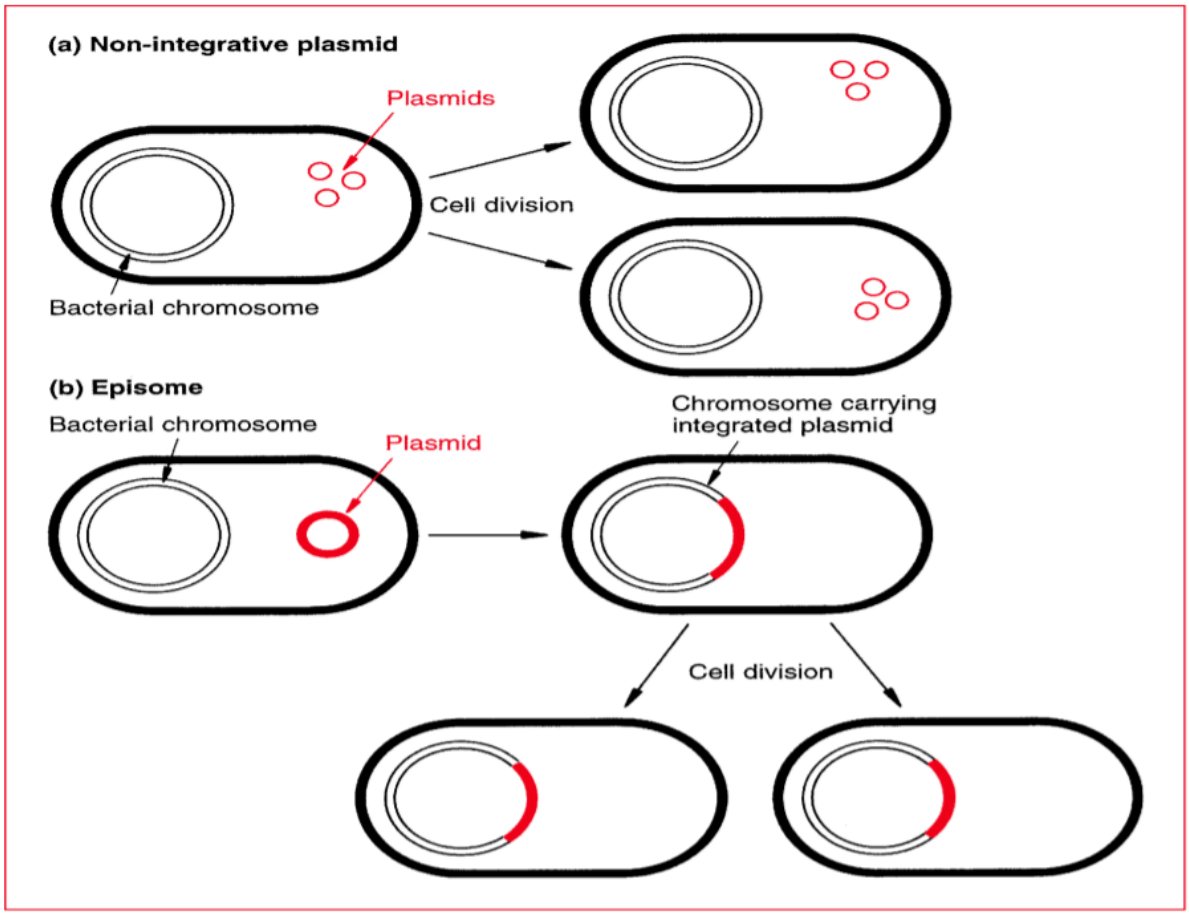
• Heavy metal resistance

• Bacteriocin production

• Induction of plant tumors

• Hydrogen sulphide production

- Plasmid transfer by conjugation between bacterial cells. The donor and recipient cells attach to each other by a pilus, a hollow appendage present on the surface of the donor cell. A copy of the plasmid is then passed to the recipient cell. Transfer is thought to occur through the pilus, but this has not been proven and transfer by some other means (e.g. directly across the bacterial cell walls) remains a possibility.
- 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.



Plasmid classification

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

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

Plasmid	Size (kb)	Origin	Host range	Antibiotic resistance	Additional marker genes showing insertional inactivation
RSF1010	8.6	*E.coli* (strain K-12)	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 protein.

Characteristics of ideal plasmid vectors

- Size: plasmid must be small in size. The small is helpful for easy uptake of cDNA by host cells and for the isolation of plasmid without damage. 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.
- Copy number: the plasmid must be present in multiple copies.
- Genetic markers: plasmid must have one or few genetic markers. These markers help us for the selection of organism that has recombinant DNA
- Origin of replication: the plasmid must have its own orogin of replication and regulatory genes for the self-replication.
- Unique restriction sites: the plasmid must have unique restriction sites common restriction enzymes in use.
- Multiple cloning sites: This property permits the insertion of gene of interest and plasmid recircularization.
- Insertional inactivation: the plasmid must have unique sites for restriction enzymes in marker genes. This will help us for the selection of recombination by insertional inactivation method. 8. Pathogenicity: the plasmid should not have any pathogenic property.
- 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.

Multiple cloning sites (MCS)

- MCS is a synthetic DNA segment that has a cluster of unique sites for restriction enzymes . It is inserted into a gene cloning vector with a view to increasing the number of gene cloning sites.
- The size of MCS usually from 60 bp to 84 bp. The number and arrangement of restriction sites varies from MCS to MCS in different vectors.
- As MCS is a cluster of many restriction sites, it is also known as polylinker or polylinker sequence.

USES:

- MCS are used to increase gene cloning sites in vector DNAs.
- As they have unique sites for many restriction enzymes, DNA segments with different types of cut -ends can be inserted into the vector.
- Restriction enzymes of choice can be used to insert a gene into the vector.

Properties of good host

A good host has the following features:

- Be easy to transform
- Support the replication of recombinant DNA
- Be free from elements that interfere with replication of recombinant DNA
- Lack active restriction enzymes, e.g., E.coli K12 substrain HB 101.
- Should not have methylases, since, these enzymes would methylate the replicated recombinant DNA which, as a result, would become resistant to useful restriction enzymes.
- Be deficient in normal recombinant function, so, that, the DNA insert is not altered by recombination events.

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.

Types of Cloning Vectors:

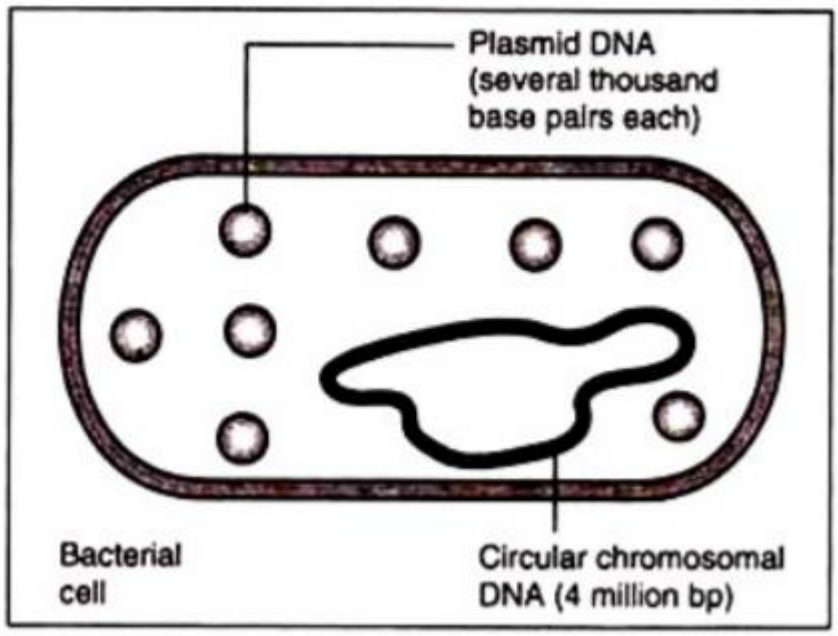
- Cloning vectors extensively used in molecular cloning experiments can be considered under following types: plasmid, phage vector and cosmid.
- Different vectors have different insert size and also vary in mode of replication inside the host.
- Mammalian genes are usually too large (~100 kb), and thus 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.

Different type of cloning vectors

Vector	Insert size	Source	Application
Plasmid	≤ 15 kb	Bacteria	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

Plasmid Vectors

Plasmid Vectors are the most common vectors for the prokaryotic host cells. Bacteria are able to ex press foreign genes inserted into plasmids. Plasmids are small, circular, double-stranded DNA molecules lacking protein coat that naturally exists in the cytoplasm of many strains of bacteria. Some of the examples of naturally occurring plasmids are Ti plasmids, F factors, R-factors, Co/E1 plasmid, etc. Plasmids are independent of the chromosome of bacterial cell and range in size from 1000 to 200 000 base pairs. Using the enzymes and 70s ribosomes that the bacterial cell houses, DNA contained in plasmids can be replicated and expressed.



The bacterial cells benefit from the presence of plasmids, which often carry genes that express proteins able to confer antibiotic resistance. These also protect bacteria by carrying genes for resistance to toxic heavy metals, such as mercury, lead, or cadmium.

In addition, some bacteria carry plasmids possessing genes that enable bacteria to break down herbicides, certain industrial chemicals, or the components of petroleum. The relationship between bacteria and plasmids is endosymbiotic; both the bacteria and plasmids benefit from mutual arrangement. Plasmids also possess characteristic copy number. The higher the copy number, higher is the number of individual plasmids in a host bacterial cell. If more copies of plasmid exist, more protein will be synthesized because of the larger number of gene copies carried by the plasmid. The number of copies plays a role in phenotypic manifestation of a gene. For example, the more copies of an antibiotic-resistance gene there are, the higher the resistance to the antibiotic. It is very important to note that naturally occur ring plasmids do not have all necessary sequences which are required by a DNA molecule to act as a profitable vector. Due to this, natural plasmids are extracted and modified by inserting suitable DNA segments and a complete vector DNA molecule is made. Plasmid-cloning vectors are derived from bacterial plasmids and are the most widely used, versatile, and easily manipulated ones.

III. 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. ‘322’ distinguishes this

plasmid from others developed in the same laboratory (there are also plasmids called pBR325, pBR327, pBR328, etc.).

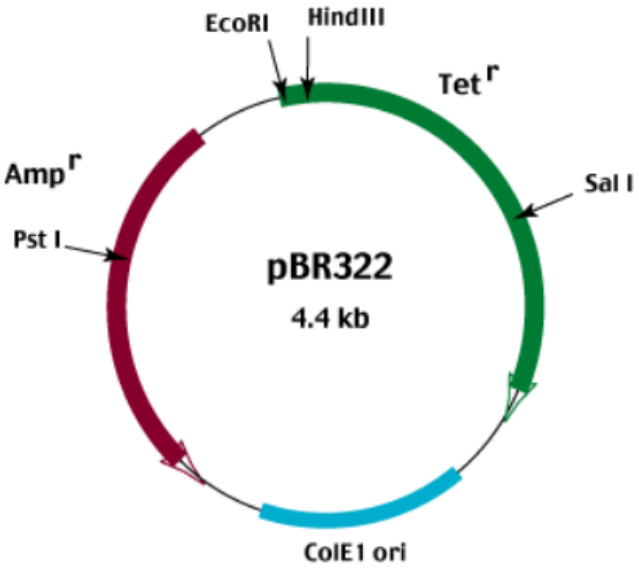
• pBR322 is 4363 base pairs in length.

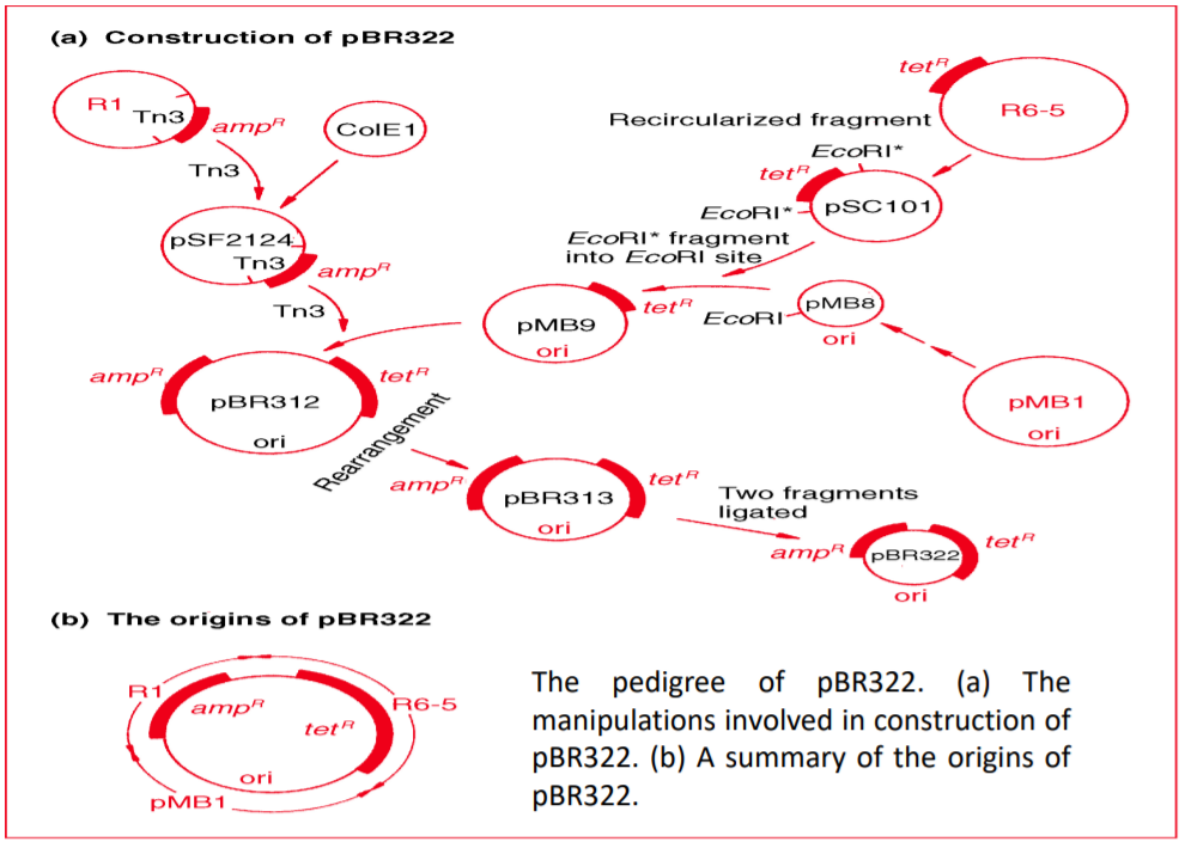
• pBR322 plasmid has the following elements:

- "rep" replicon from plasmid pMB1 which is responsible for replication of the plasmid.
- "rop" gene encoding Rop protein, are associated with stability of plasmid and also controls copy number (increase 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).

Useful features of pBR322:

- The first useful feature of pBR322 is its size. pBR322 is 4363 bp, which means that not only can the vector itself be purified with ease, but so too can any recombinant DNA molecules constructed with it. Even with 6 kb of additional DNA, a recombinant pBR322 molecule is still of a manageable size.
- The second feature of pBR322 is that, it carries two sets of antibiotic resistance genes. Either ampicillin or tetracycline resistance can be used as a selectable marker for cells containing the plasmid, and each marker gene contains unique restriction sites that can be used in cloning experiments.
- A third advantage of pBR322 is that it has a reasonably high copy number. Generally, there are about 15 molecules present in a transformed E. coli cell, but this number can be increased up to between 1000 and 3000 by plasmid amplification in the presence of a protein synthesis inhibitor such as chloramphenicol. An E. coli culture therefore provides a good yield of recombinant pBR322 molecules.
- It has 528 restriction sites for 66 restriction enzymes. Among them 20 restriction enzymes cut it at unique restriction sites. Tetracycline has 6 unique sites for 6 restriction enzymes. Ampicillin gene has 3 unique restriction site.
- The sequences other than Tet and Amp genes, have unique sites for 1 restriction enzymes. There is no restriction inactivation when gene is inserted into any one of these sites





The pedigree of pBR322. (a) The manipulations involved in construction of pBR322. (b) A summary of the origins of pBR322.

- The amp R gene originally resided on the plasmid R1, a typical antibiotic resistance plasmid that occurs in natural populations of E. coli.
- The tet R gene is derived from R6 - 5, a second antibiotic resistance plasmid.
- The replication origin of pBR322, which directs multiplication of the vector in host cells, is originally from pMB 1, which is closely related to the colicin -producing plasmid ColE 1.

1. **Origin of Replication:**

   It carries a fragment of plasmid pMB1 that acts as an ori gin for DNA replication and thus ensures multiplication of the vector.

2. **Selectable Marker:**

   It carries two antibiotic resistance genes—ampicillin and tetracycline.

3. **Cloning Sites:**

   It carries a number of unique restriction sites. Some of these are located in one of the antibiotic resistance genes (e.g., sites for Pst I, Pvu I, and Sac I are found in Amp^r and BamHI and Hind III in Tet^r). Cloning into one of these sites inactivates the gene allowing recombinants to be differentiated from nonrecombinants known as insertional inactivation.

4. **Pedigree of pBR322:**

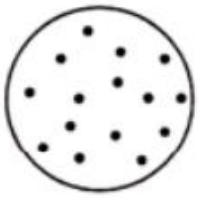
   By pedigree we understand the origin of pBR322. pBR322 is not a naturally occurring plasmid. It is important to note that pBR322 comprises DNA derived from three different naturally occur ring plasmids. The amp gene originally resided on the plasmid R1 (a naturally occur ring antibiotic resistant plasmid in E. coli), the tet is derived from R6-5 (a second antibiotic resistant plasmid) and the origin of replication is derived from pMB1, which is closely related to the Colicin producing plasmid ColE1.

5. Recombinant selection with pBR322 – in- sectional inactivation of an antibiotic resistance gene. When we have introduced our recombinant DNA (vector + gene of interest) into the host cell (by a process called transformation and the host cells that takes up the recombinant DNA are called transformed host cells) then we have to screen the entire host population in order to select the transformed cells (with recombinant DNA) from the non-transformed one (without recombinant DNA).

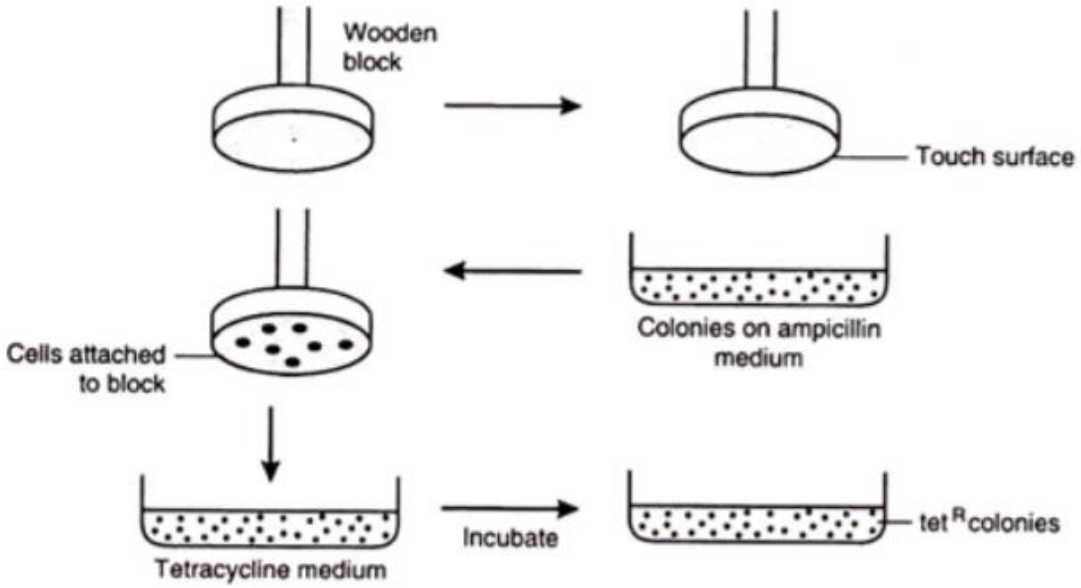
6. pBR322 has several unique restriction sites that can be used to open up the vector before insertion of a new DNA fragment. BamHl, for example, cuts pBR322 at just one position, within the cluster of genes that code for resistance to tetracycline. A recombinant pBR322 molecule, one that carries an extra piece of DNA in the BamHl site is no longer able to confer tetracycline resistance on its host, as one of the necessary genes is now disrupted by the inserted DNA. Cells containing this recombinant pBR322 molecule are still resistant to ampicillin, but sensitive to tetracycline (amp^R tet^S). Screening for pBR322 recombinants is performed in the following way. After transformation the cells are plated onto ampicillin medium and incubated until colonies appear [Fig. (a)]. All of these colonies are trans-formants (remember, untransformed cells are amp and so do not produce colonies on the selective medium), but only a few contain recombinant pBR322 molecules: most con tain the normal, self-ligated plasmid. To identify the recombinants the colonies are replica plated onto agar medium that contains tetracycline [Fig. (b)]. After incubation, some of the original colonies regrow, but others do not [Fig. (c)]. Those that do grow consist of cells that carry the normal pBR322 with no inserted DNA and, therefore, a functional tetracycline resistance gene cluster (amp^R tet^R).

7. The colonies that do not grow on tetracycline agar are recombinants (amp^R tet^S); once their positions are known, samples for further study can be recovered from the original ampicillin agar plate.

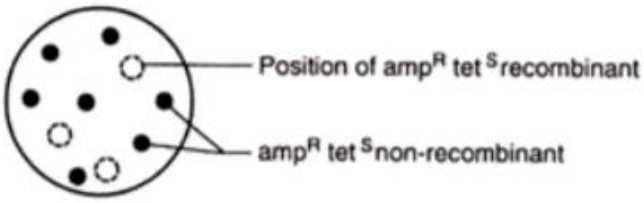
(a) Colonies on ampicillin medium



(b) Replica plating



(c) amp^R tet ^{R}colonies grew on tetracycline medium



Screening for pBR322 recombinants by insertional inactivation of the tetracycline resistance gene. (a) Cells are plated onto ampicillin agar: all the transformants produce colonies. (b) The colonies are replica plated onto tetracycline medium. (c) The colonies that grow on tetracycline medium are *amp^R tet^R* and, therefore, non-recombinants. Recombinants *(amp^R tet^S)* do not grow, but their position on the ampicillin plate is now known

8. **Uses of pBR322:**

   It is widely used as a cloning vector. In addition to this, it has been widely used as a model system for study of prokaryotic transcription and translation.

9. **Advantages of pBR322:**

   - Small size (~ 4.4 kb) enables easy purifi cation and manipulation.
   - 2. Two selectable markers (amp and tet) al low easy selection of recombinant DNA.
   - 3. It can be amplified up to 1000-3000 copies per cell when protein synthesis is blocked by the application of chloramphenicol.

10. **Disadvantages of pBR322:**

    - It has very high mobility i.e it can move to another cell in the presence of a conjugative plasmid like F-factor. The nic-bom (bom=basis of mobility) region of pBR322 is responsible for this feature. Due to this, the vector may get lost in a population of mixed host cells.
    - There is a limitation in the size of the gene of interest that it can accommodate.
    - Not a very high copy number is present as is expected from a good vector.
    - Although insertional inactivation of an antibiotic resistance gene provides an effective means of recombinant identification, the method is made inconvenient by the need to carry out two screenings, one with the antibiotic that selects for trans-formants, followed by the second screen, after replica plating, with the antibiotic which distinguishes recombinants. This makes the screening process time- consuming and laborious.

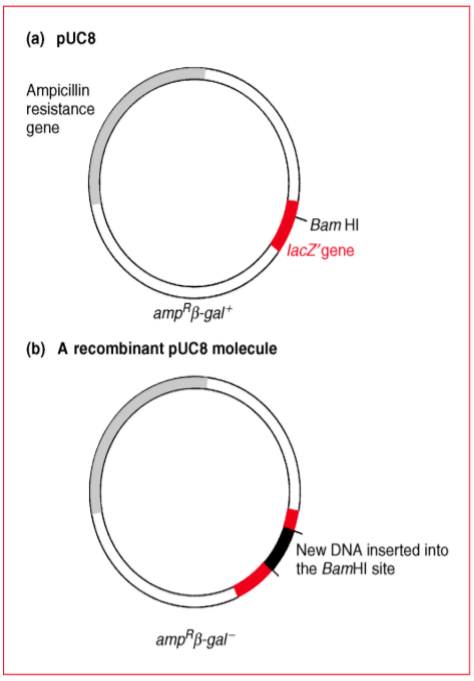
Another vector pBR327 was derived from pBR322, by deletion of nucleotides between 1,427 to 2,516. These nucleotides are deleted to reduce the size of the vector and to eliminate sequences that were known to interfere with the expression of cloned DNA in eukaryotic cells. pBR327 still contains genes for resistance against two antibiotics (tetracycline and ampicillin).

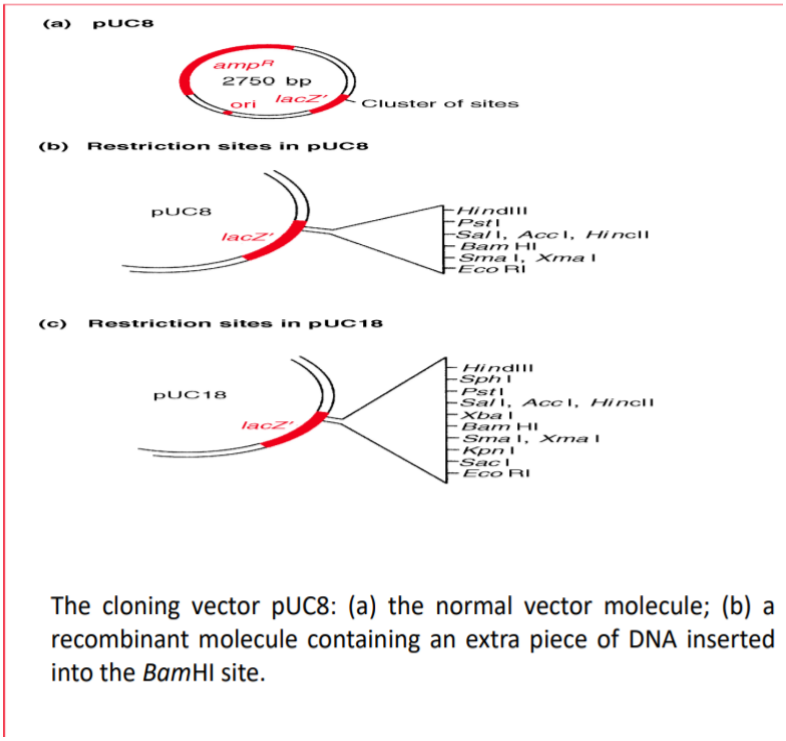
IV. pUC18 and pUC19

pUC plasmids are small, high copy number plasmids of size 2686bp.

1. 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.
2. 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.
3. pUC18 and pUC19 vectors are identical apart from the fact that the MCS is arranged in opposite orientation.
4. 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.
5. "rop" gene is removed from this vector which leads to an increase in copy number.

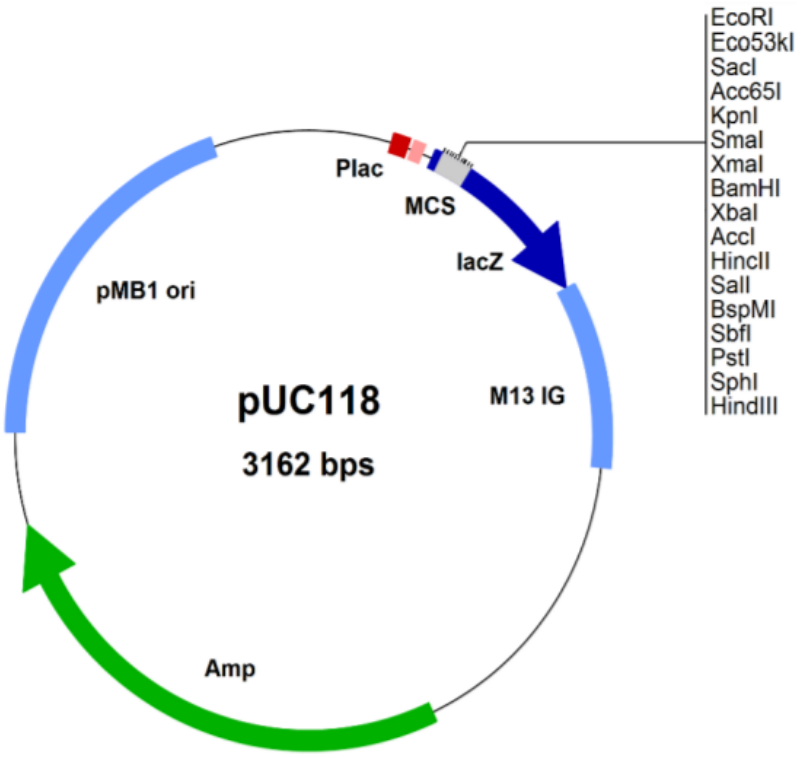
pUC8: A Lac selection plasmid





The cloning vector pUC8: (a) the normal vector molecule; (b) a recombinant molecule containing an extra piece of DNA inserted into the *Bam*HI site.

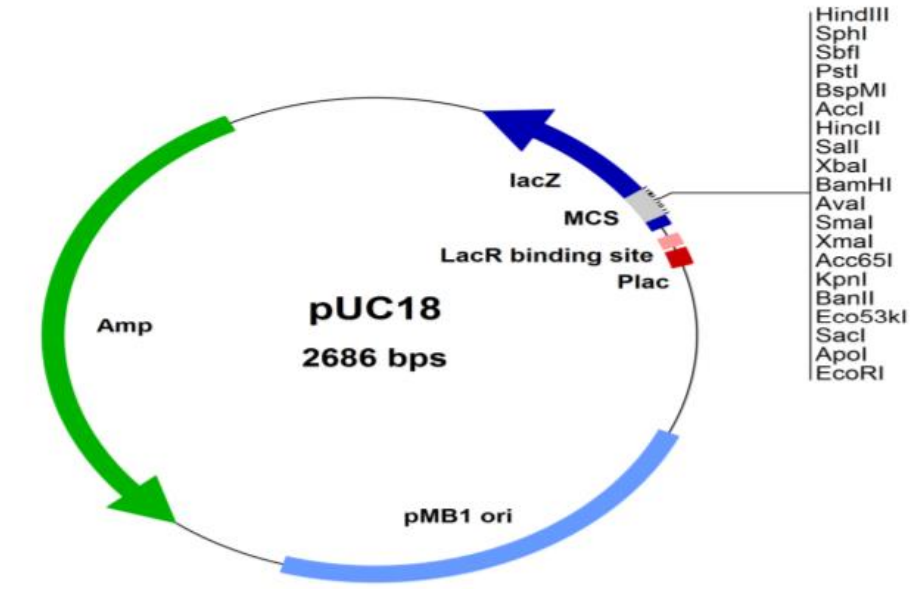
- An MCS is a short DNA sequence consisting of restriction sites for many different restriction endonucleases.
- The MCS is inserted into the lacZ sequence, which encodes the promoter and the α-peptide of β-galactosidase.
- Insertion of the MCS into the lacZ fragment does not affect the ability of the 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.
- The native lacZ promoter (Plac) is situated just upstream of the cloned gene, allowing expression of genes on inserts that are correctly oriented. Most of the nonessential DNA has been removed to provide the ability to clone larger fragments. An ampicillin marker is included for selection of transformants.

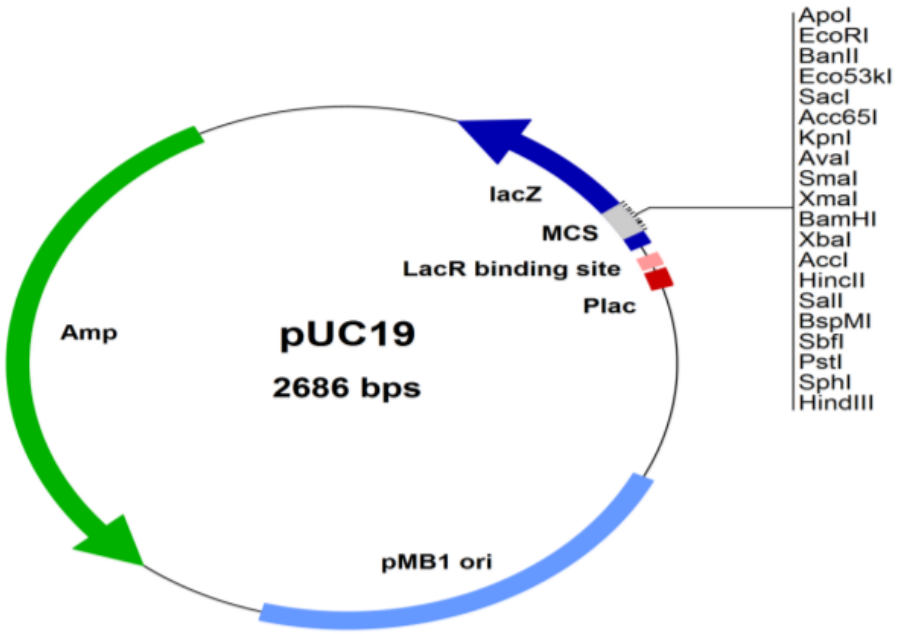


The pUC plasmids were engineered from the pBR322 origin of replication to include the alpha portion of the beta-galactosidase gene (lacZ'), complete with its promoter. The beta portion of lacZ was included in the chromosome of the host, so that the host containing the plasmid was Lac+.

The restriction enzyme sites (6 in pUC8/9; 10 in pUC18/19) are clustered together on an oligonucleotide (called the polylinker, or multiple cloning site), which is in-frame within the

first few amino acids of the lacZ‘ reporter gene (thus providing insertional inactivation, Lac+ → Lac-).





Recombinant selection with pUC vectors:

Alpha complementation/ blue-white screening / Insertional inactivation

a. The lac-Z gene product (β-galactosidase) is a tetramer, and each monomer is made of two parts – lacZ-alpha, and lacZ- omega.

b. If the alpha fragment was deleted, the omega fragment is non-functional; however, alpha fragment functionality can be restored in transformation via plasmid. Hence, then name alpha-complementation.

c. The E. coli enzyme β-galactosidase is a homo-tetramer of the protein product of the lacZ gene.

d. Certain mutations in the 5' region of lacZ prevent subunit association.

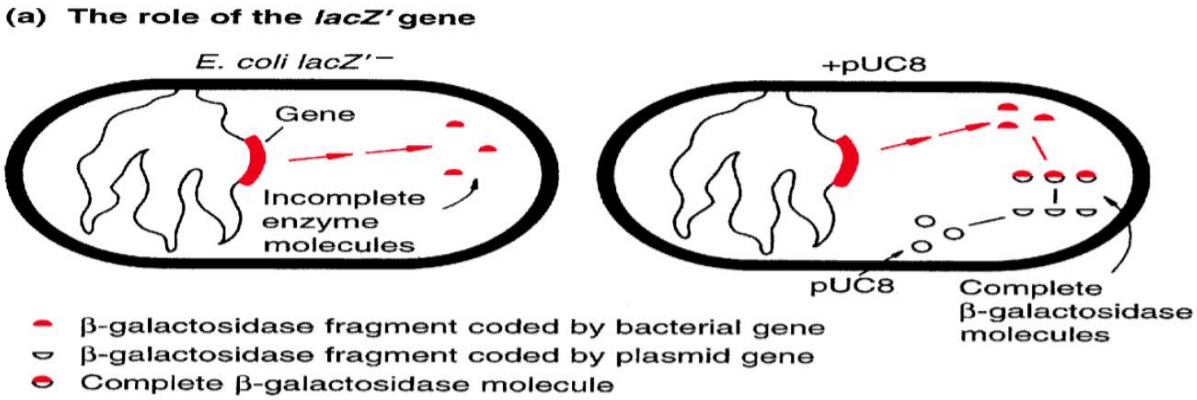
e. Because monomers lack enzyme activity, the failure to assemble leads to a Lac- phenotype.

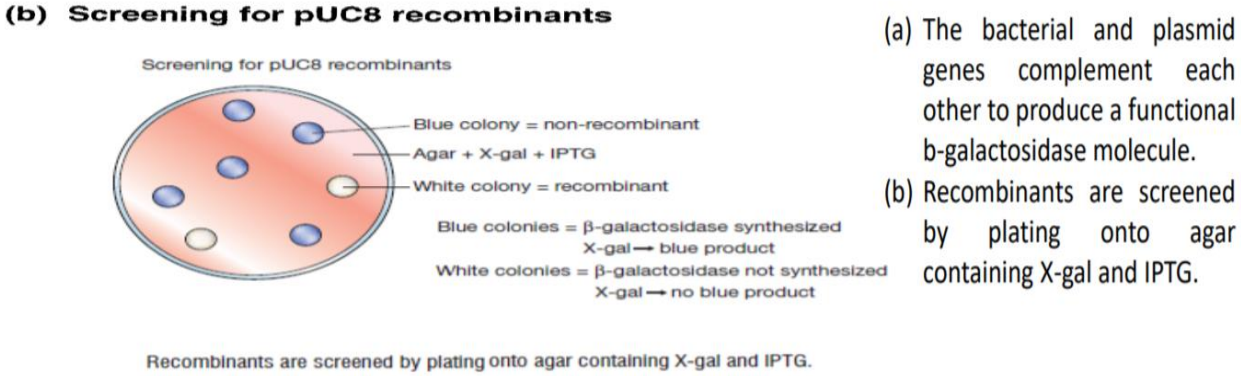
f. The activity of the enzyme β –galactosidase is easily monitored by inducing in the growth medium the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β–galactoside (Xgal).

g. This compound is colorless, but on cleavage, releases a blue indolyl derivative.

h. On solid medium, colonies that are expressing active β –galactosidase are blue in colour, while those without the activity are white in colour.

i. This is often referred as blue/white screening.





Uses of pUC Vectors:

pUC vectors can be used both as cloning vec tor and expression vector. When used as an expression vector its sequences are slightly modified to meet necessary requirements.

Advantages of pUC Vectors:

The pUC vectors offer following major advantages over pBR322 vectors:

(a) High copy number of 500-600 copies per cell.

(b) Easy and single step selection.

(c) The unique restriction sites used for cloning are clustered within the MCS. This allows cloning of a DNA fragment having two different sticky ends.

Disadvantages of pUC:

It cannot accommodate a gene of interest larger than 15kb.

2. Cosmids

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. • It is formed by joining ends of a linearized plasmid DNA with cos-site of lambda DNA. • It is a derived vector.

- The cosmid DNA can be packed in a capsid of lambda phage in vitro to form recombinant phage particles.
- It is linear inside the phage capsid.
- The cosmid gets circularized and behaves like a plasmid.
- Cosmid has an origin of replication, selectable markers, and gene cloning sites of plasmid DNA.
- They lack structural and regulatory genes of lambda DNA.
- Hence there is no lysis and integration of cosmid DNA in the host cell.
- Examples: Col EI cosmid, pHC 79, pJB8, pWE cosmid, etc.

COSMID

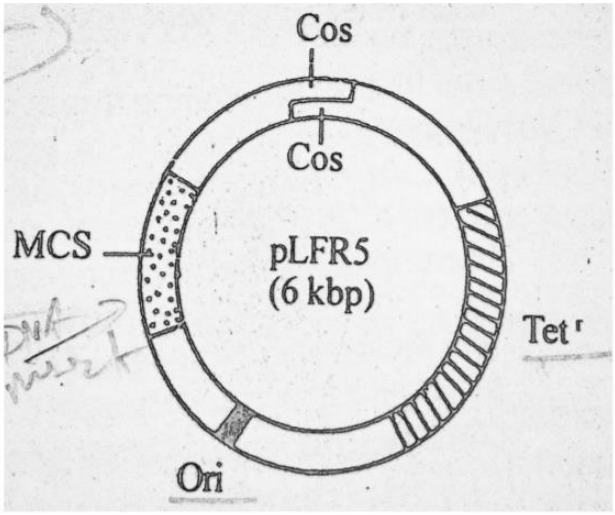
a. Cosmid is a circular ds DNA
b. It has two complementary single-stranded regions at both ends of a plasmid DNA. The two cos-ends form a duplex by base pairing.
c. The cosmid DNA does not code for phage proteins and host cell lysis.
d. It does not involve in ,multiplication of phage particles.
e. It has an origin of replication from plasmid DNA for independent replication.
f. It has selectable marker genes and gene cloning sites of plasmid DNA
g. The cosmid DNA is packed within protein coat of bacteriophage to form inactive phage particles. Cos-site is a prerequsites for invitro packaging of cosmid in phage protein coat.
h. After infection, the cosmid DNA does not integrate into host chromosomal DNA. It exits as a definite extra chromosomal DNA and replicates independently.

SALIENT FEATURES OF COSMIDS

COSMID pLFR5

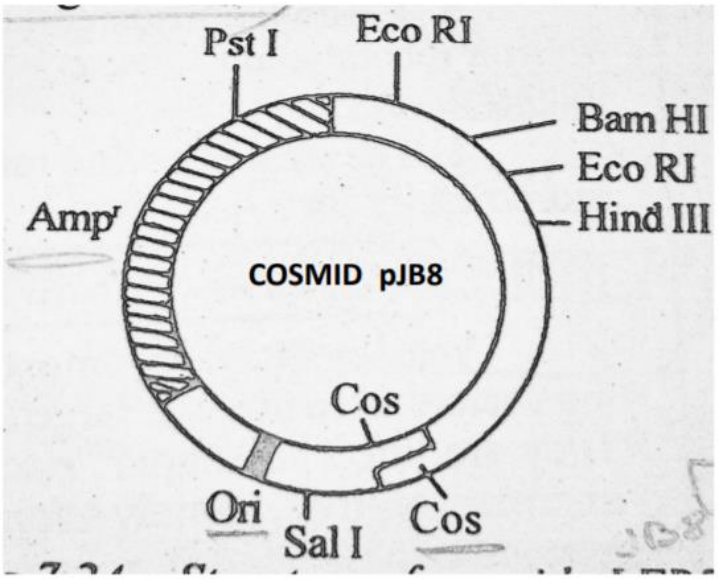
- pLFR5 is the commonly used cloning vector suitable for cloning large DNA fragments upto 45 kbp.
- It is 6 kbp in size.

- It is constructed from E.coli plasmid pBR322 and two cos-ends of lambda DNA.
- The plasmid derived portion contributes an origin of replication (Ori) and tetracycline resistance gene.
- There is a MCS between the orogin of replication and cos-site.
- A foreign DNA of upto 45 kbp is inserted into the MCS of pLR5 and the rDNA is packed into bacteriophage head in vitro.
- The phage thus formed delivers the DNA into E.coli while infecting the cell.



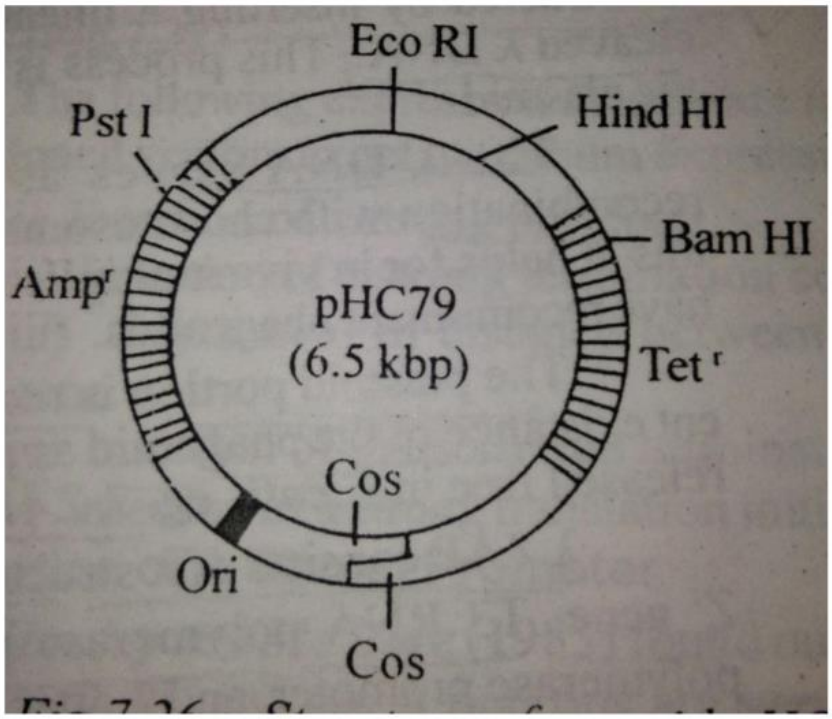
COSMID pJB8

- pJB8 is constructed from the plasmid pBR322 and cos sites of lambda DNA.
- It is 5.4 kbp in size.
- It has an origin of replication and ampicillin resistance gene derived from pBR322 and two cos-ends from lambda DNA.
- A foreign DNA of about 45 kbp is inserted into BamHI or RcoRI or HindIII restriction site of the cosmid.
- The recombinant cosmid is packaged into lambda phage head to form an infective phage particle.
- The phage delivers its rDNA into E.coli while infecting the cell.



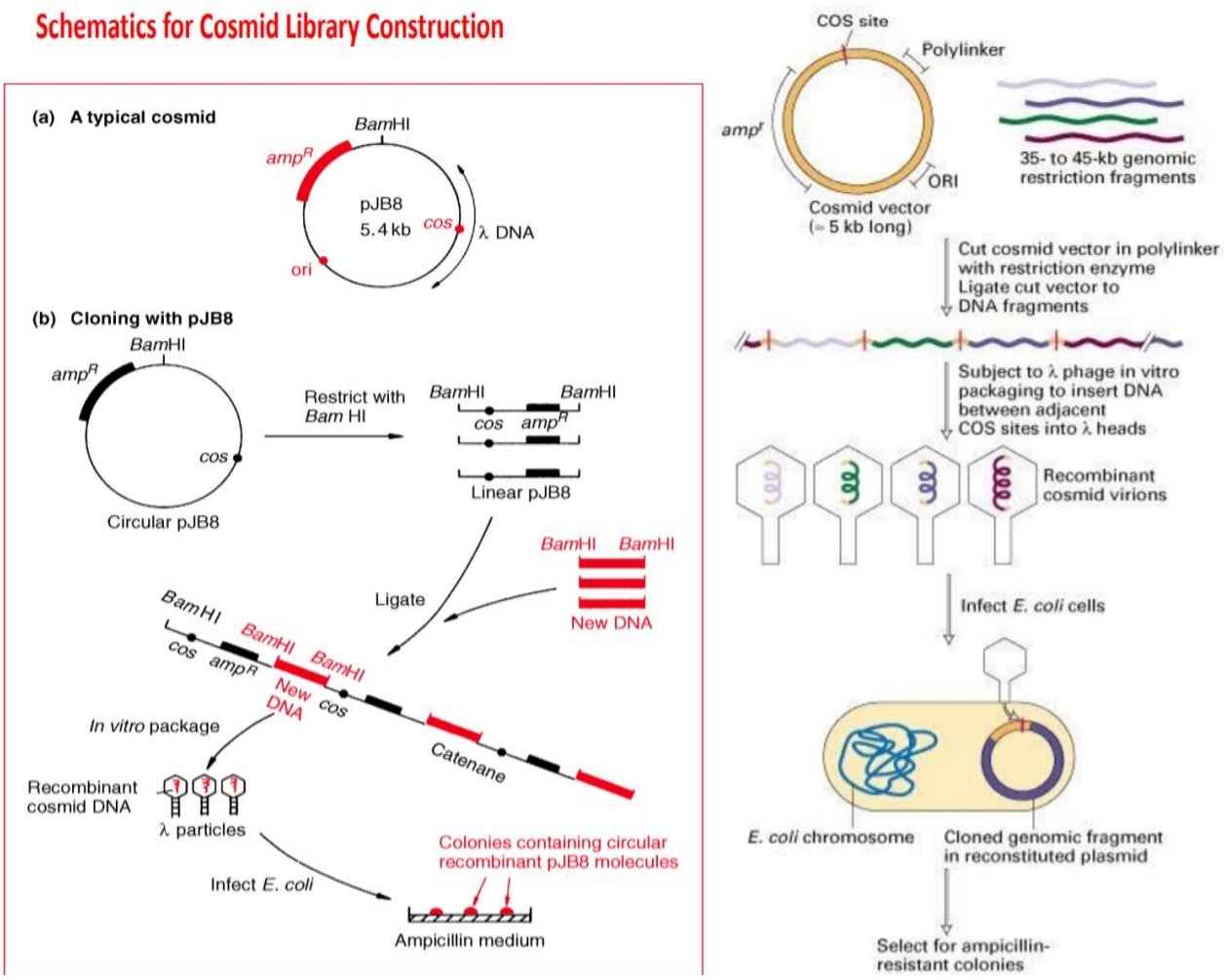
COSMID pHC79

- pHC79 is constructed from pBR322 and cos-sites of lambda DNA.
- It is 6.5 kbp in size. • It can carry DNA fragments upto 40 kbp.
- The derivative of pBR322 has an origin of replication and two marker genes- ampicillin resistance gene and tetracycline resistance gene.
- The derivative of the cosmid into lambda phage head.



Schematics for Cosmid Library Construction

1. Cosmid pick up relatively larger DNA fragments than the plasmid do.
2. As cosmids pick up large DNA fragments, they are used to establish gene libraries
3. Gene cloning through cosmids helps in the study of non-sence sequences in the genome of organisms.
4. Some cosmids are constructed by joining a linearized plasmid DNA with DNA fragments of p1 bacteriophage that have cos-ends. The P1 bacteriophage has the genome of 115 kbp. So, a DNA of 85 kbp can be packaged into the head of P1 phage. These cosmids help to clone large genes and gene clusters in bacteria.



COSMID: ADVANTAGES

- Cosmid pick up relatively larger DNA fragments than the plasmid do.
- As cosmids pick up large DNA fragments, they are used to establish gene libraries
- Gene cloning through cosmids helps in the study of non-sence sequences in the genome of organisms.

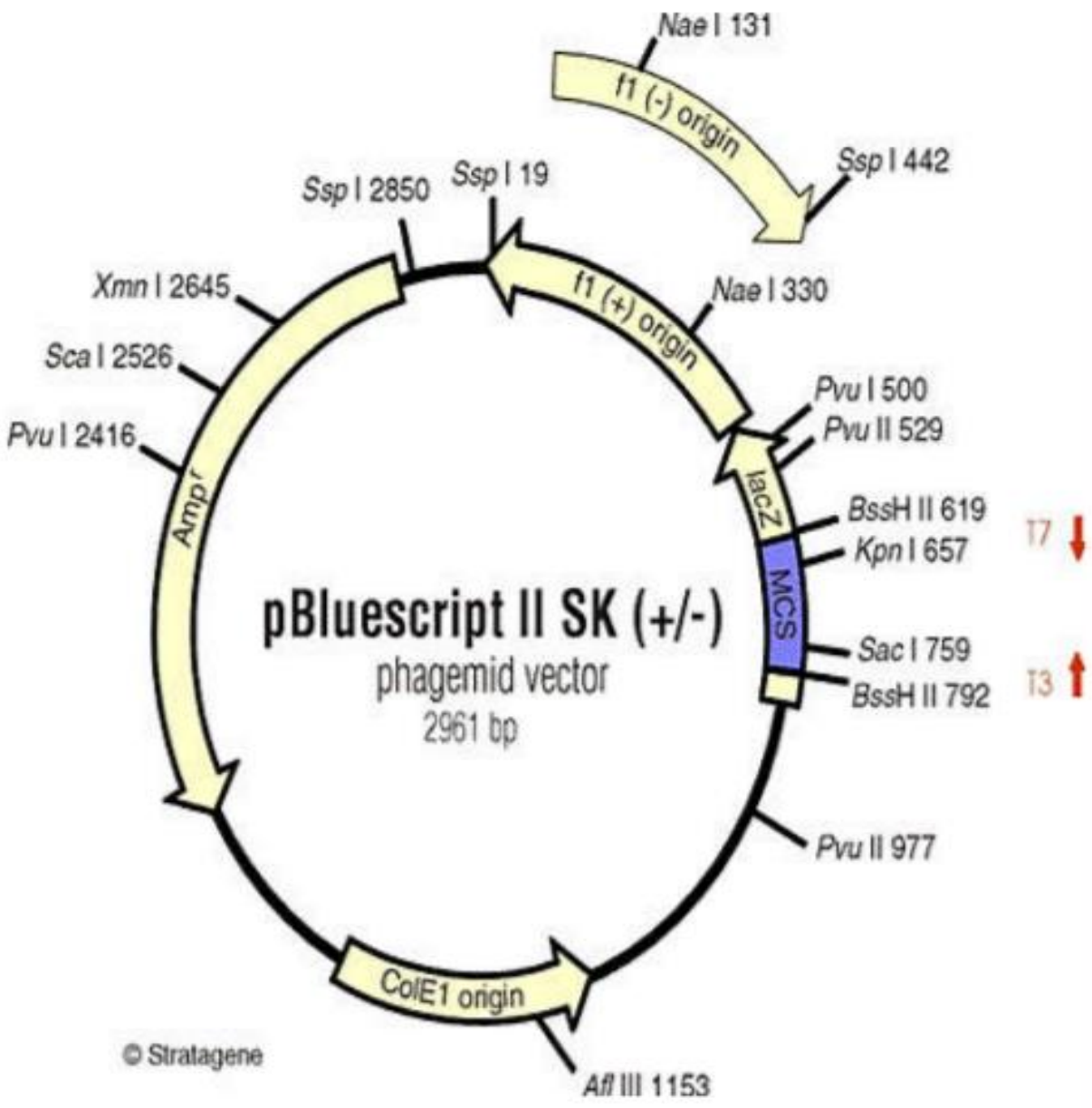
- Some cosmids are constructed by joining a linearized plasmid DNA with DNA fragments of p1 bacteriophage that have cos-ends. The P1 bacteriophage has the genome of 115 kbp. So, a DNA of 85 kbp can be packaged into the head of P1 phage. These cosmids help to clone large genes and gene clusters in bacteria

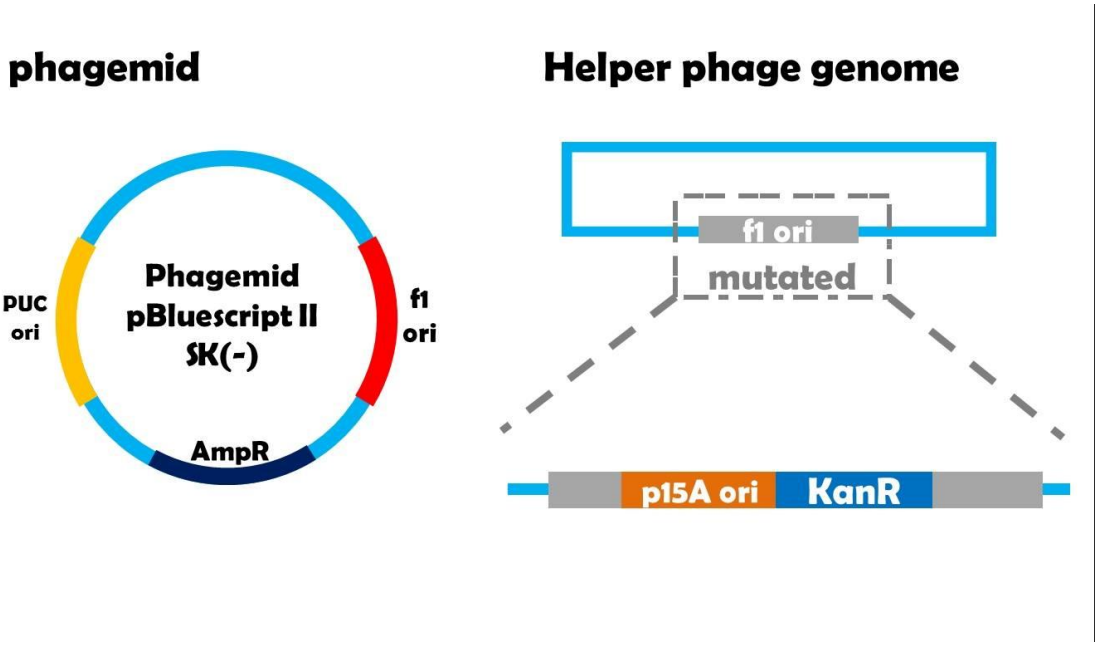
COSMID: DISADVANTAGES

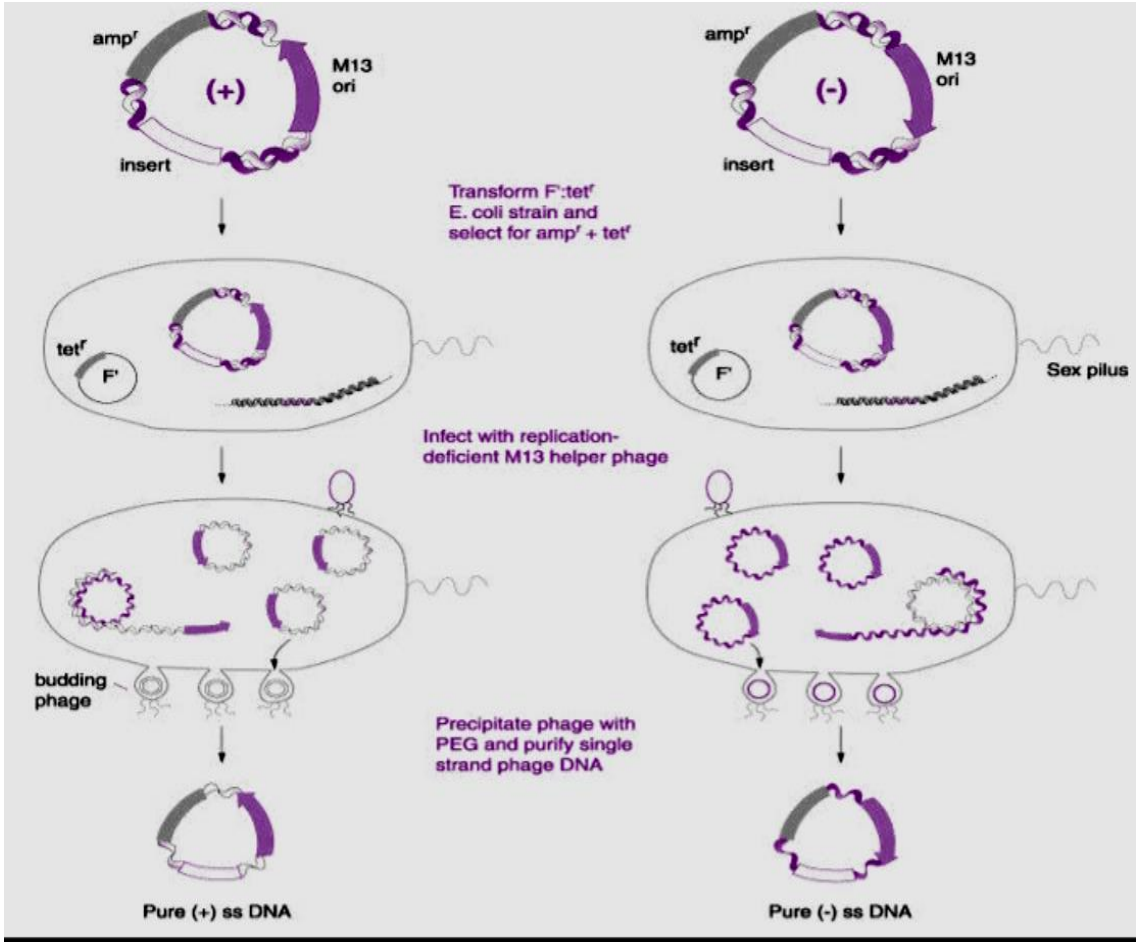
- The packaging enzyme fails to pack recombinant cosmids into the phage head, if any one of the two cos-ends is missing.
- Sometimes more than one recombinant cosmid join together to form a large DNA. If so, the packaging enzyme fails to pack the DNA into the phage head.
- Slower replication
- Higher frequency of recombination inside bacterial host.
- Unstable inside E.coli host and thus easy to lose vector.

V. Phagemids

- A phagemid or phasmid is a plasmid that contains an f1 origin of replication from an f1 phage.
- It can be used as a type of cloning vectorin combination with filamentous phage M13.
- A phagemid can be replicated as a plasmid, and also be packaged as single stranded DNA in viral particles.
- Phagemids contain an origin of replication (ori) for double stranded replication, as well as an f1 ori to enable single stranded replication and packaging into phage particles. transformation and electroporation.
- Many commonly used plasmids contain an f1 ori and are thus phagemids.
- Similarly to a plasmid, a phagemid can be used to clone DNA fragments and be introduced into a bacterial host by a range of techniques, such as transformation and electroporation.
- However, infection of a bacterial host containing a phagemid with a 'helper' phage, for example VCSM13 or M13K07, provides the necessary viral components to enable single stranded DNA replication and packaging of the phagemid DNA into phage particles. into the cytoplasm of the host cell.
- The 'helper' phage infects the bacterial host by first attaching to the host cell's pilus and then, after attachment, transporting the phage genome
- Inside the cell, the phage genome triggers production of single stranded phagemid DNA in the cytoplasm. This phagemid DNA is then packaged into phage particles.
- The phage particles containihngyb ri ds ssDNA are released from the bacterial host cell into the extracellular environment.
- Filamentous bacterial phages growth retard but, contrasting with the lambda phage and the T7 phage, are not generally lytic.
- Helper phages are usually engineered to package less efficiently (via a defective phage origin of replication) than the phagemid so that the resultant phage particles contain predominantly phagemid DNA.
- F1 Filamentous phage infection requires the presence of a pilus so only bacterial hosts containing the F-plasmid or its derivatives can be used to generate phage particles.


Nae I 131
f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
Xmn I 2645
f1 (+) origin
Nae I 330
Sca I 2526
Pvu I 500
Pvu II 529
Pvu I 2416
Amp r
lacZ
BssH II 619
Kpn I 657
T7
MCS
pBluescript II SK (+/-)
phagemid vector
2961 bp
Sac I 759
BssH II 792
T3
Pvu II 977
ColE1 origin
© Stratagene
Afl III 1153


phagemid
Helper phage genome
Phagemid
pBluescript II
SK(-)
PUC
ori
f1
ori
AmpR
f1 ori
mutated
p15A ori
KanR


amp^r
M13
ori
(+)
insert
Transform F':tet^r
E. coli strain and
select for amp^r + tet^r
tet^r
F'
Infect with replication-
deficient M13 helper phage
budding
phage
Precipitate phage with
PEG and purify single
strand phage DNA
Pure (+) ss DNA
amp^r
M13
ori
(-)
insert
tet^r
F'
Sex pilus
Pure (-) ss DNA

VI. Construction, Principle and uses of YAC

Yeast Artificial Chromosomes (YAC): A YAC can be considered as a functional artificial chromosome, since it includes three specific DNA sequences that enable it to propagate from one yeast cell to its offspring:

- The Telomere (TEL): The telomere which is located at each chromosome end, protects the linear DNA from degradation by Nucleases.
- The Centromere (CEN): The centromere which is the attachment site for mitotic spindle fibres, "pulls" one copy of each du plicated chromosome into each new daughter cell.
- Origin of Replication (OR): Replication origin sequences which are specific DNA sequences that allow the DNA replication machinery to assemble on the DNA and move at the replication forks.
- A and B: Selectable markers that allow easy isolation of yeast cells that have taken up artificial chromosome.
- Recognition Site: Recognition Site for two restriction en zymes EcoRI and BamHl.

Cloning experiment using a YAC vector:

a. Large DNA fragments are obtained by car rying out restriction digestion using EcoRI.
b. The YAC is digested by two restriction enzymes EcoRI and Bam HI.
c. Those two elements recombine at the EcoRI sites and are covalently linked by DNA ligase.
d. A recombinant YAC vector, a yeast artificial chromosome with genomic DNA inserted, is produced. This vector can be used to infect yeast cells and generate an un limited number of copies.

Uses of YAC Vectors:

- YAC can be used to study various aspects of chromosome structure and behaviour; for instance, to examine the segregation of chromosomes during meiosis.
- YAC cloning system can take DNA insert greater than l00kb. Due to this they can be used to study the functions and modes of expression of genes that had previously been intractable to analysis by recombinant DNA techniques.
- YACs can be propagated in mammalian cells, enabling the functional analysis to be carried out in the organism in which the gene normally resides. Thus by using them we can learn about the true form of gene expression in vivo conditions.

- Yeast artificial chromosomes are very helpful in the production of gene libraries. E. coli vectors can take DNA insert maxi mum up to 300kb. Due to these some 30000 clones are needed for a human gene library if we use them as cloning vector.

However, YAC vectors are routinely used to clone 600 kb fragments, and special types are able to handle DNA up to 1400 kb in length, the latter bringing the size of a human gene library down to just 6500 clones.

Sometimes YAC is seen with problem of lacking insert stability, the cloned DNA sometimes becoming rearranged by intra-molecular recombination. Nevertheless, YACs have been of immense value in providing long pieces of cloned DNA for use in large scale DNA sequencing projects. Example of YAC is pYAC3.

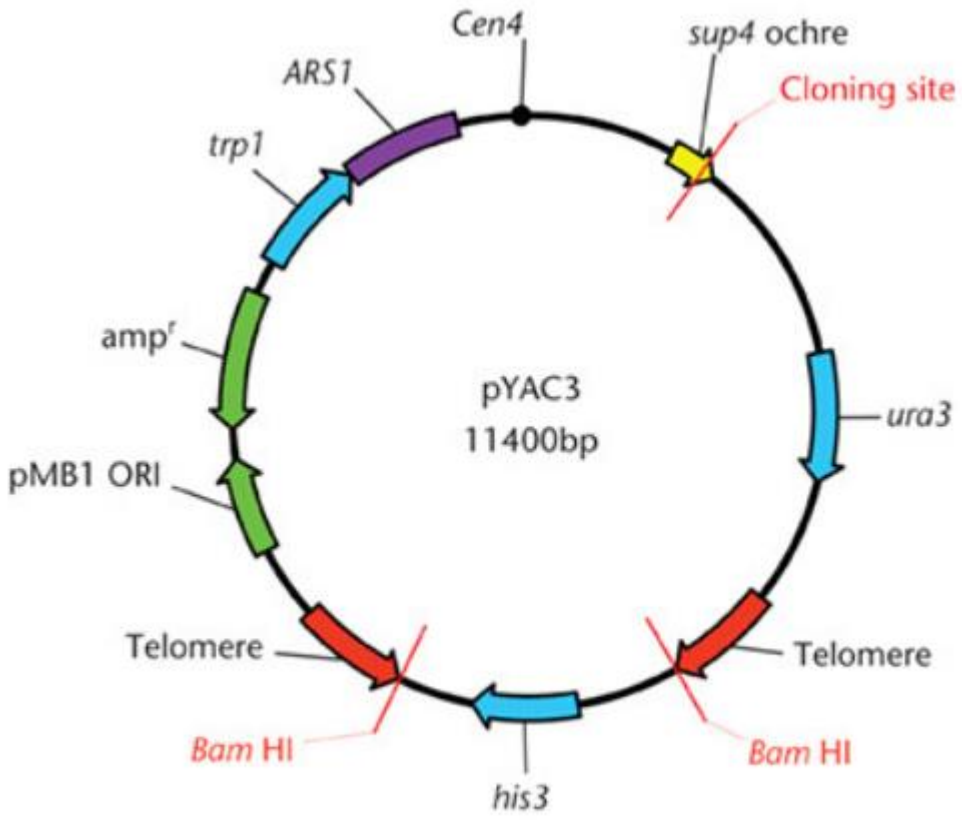
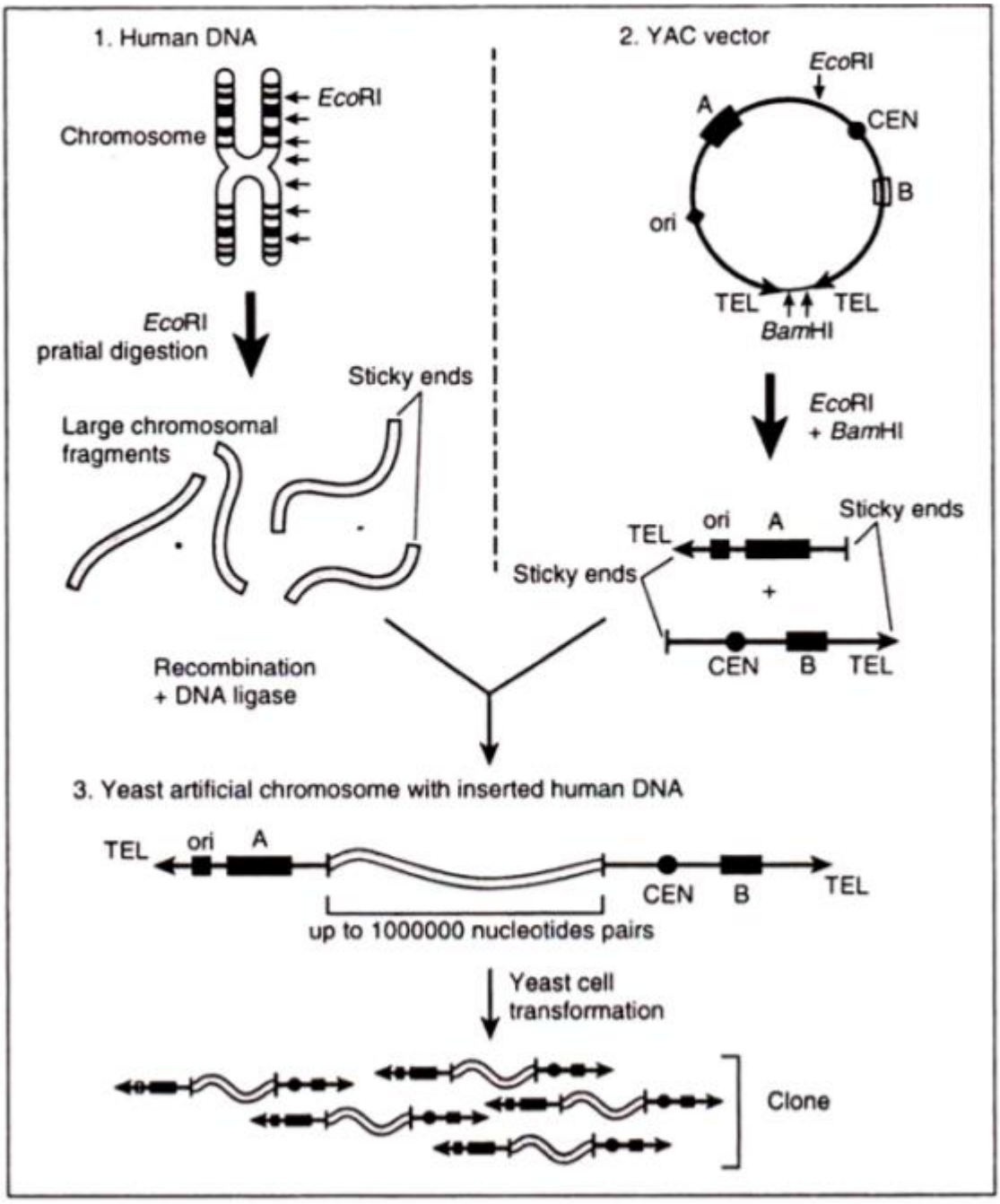


figure: pYAC3



Steps involved in cloning large human DNA in YAC.

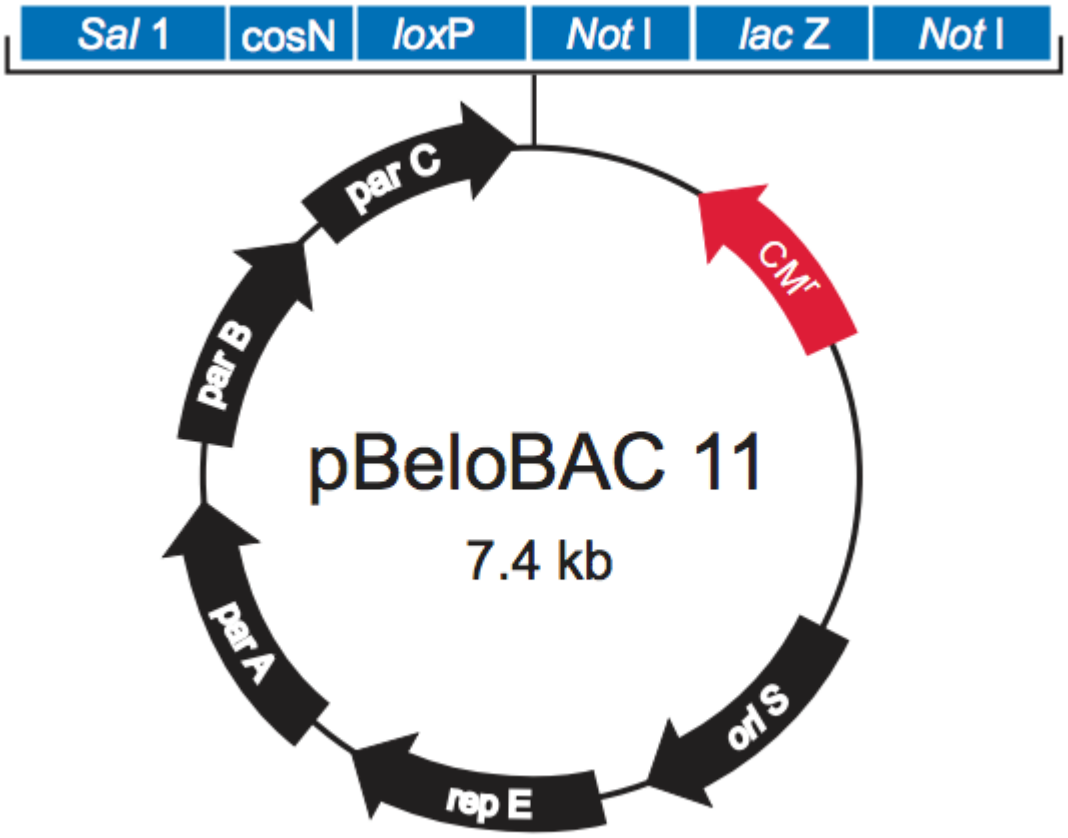
VII. BAC

Bacterial Artificial Chromosomes (BACs) are cloning vectors based on the extra-chromosomal plasmids of E.coli, called F factor or fertility factor. These vectors enable the construction of artificial chromosomes, which can be cloned in E.coli. This vector is useful for cloning DNA fragments up to 350 kb, but can be handled like regular bacterial plasmid vectors, and is very useful for sequencing large stretches of chromosomal DNA. Like any other vector, BACs contain ori sequences derived from E. coli plasmid F factor, multiple cloning sites (MCS) having unique restriction sites, and suitable selectable markers. The genomes of several large DNA viruses and RNA vi ruses have been cloned as BACs. These constructs are referred to as "infectious clones", as transfection of the BAC construct into host cells is sufficient to initiate viral infection. The infectious property of these BACs has made the study of many viruses such as herpes viruses, poxviruses and coronaviruses more accessible. BACs are now being utilized to a greater extent in modelling genetic diseases, often alongside transgenic mice. BACs have been used in this field as complex genes may have several regulatory sequences upstream of the encoding sequence, including various promoter sequences that will govern a gene's expression level. BACs have been used to some degree of success with mice when studying neurological diseases such as Alzheimer's dis ease or as in the case of aneuploidy associated with Down's syndrome. Examples of BAC are pBACe3.6, pBeloBAC11 etc.

Useful for cloning up to 200-300 kb, but can be handled like regular bacterial plasmid vectors. ¬ Useful for sequencing large stretches of chromosomal DNA; frequently used in genome sequencing projects. F factor.

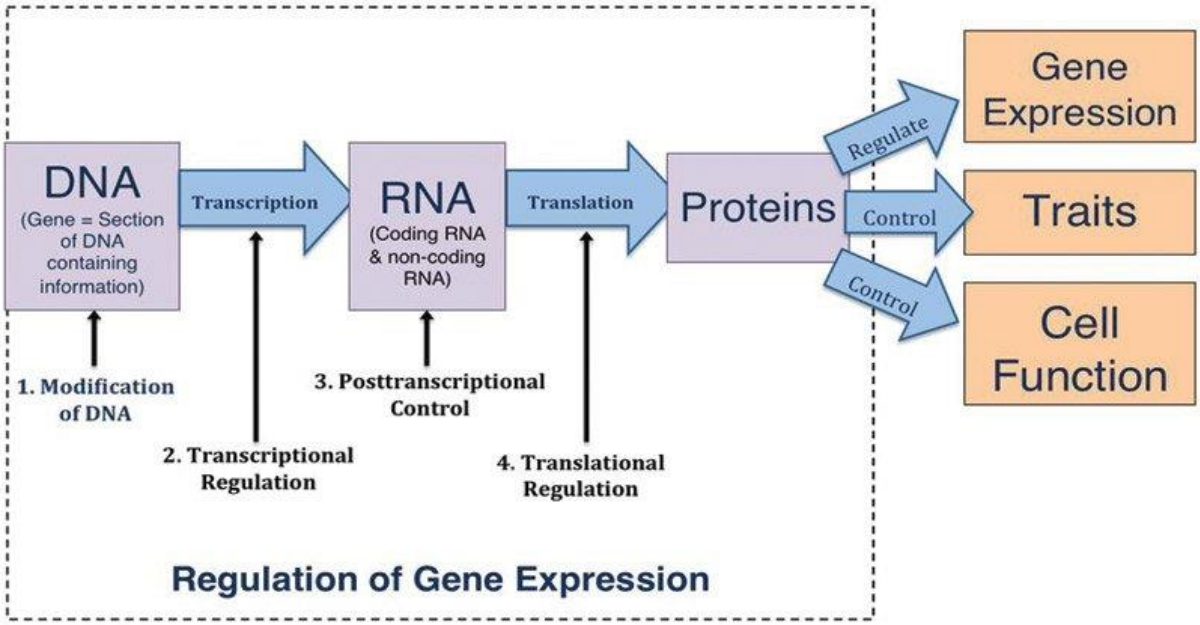
Like other vectors, BACs contain:

- Origin (ori) sequence derived from an E. coli plasmid called the F factor
- Multiple cloning sites (restriction sites).
- Selectable markers (antibiotic resistance).
- Based on the F factor of E. coli: 100 kb plasmid, propagates through conjugation
- low copy number (1-2 copies per cell)
- 2 genes (parA and parB): accurate partitioning during cell division
- BACs: just have par genes, replication ori, cloning sites, selectable marker
- Can propagate very large pieces of DNA: up to 300 kb
- Relatively easy to manipulate: move into cells by transformation (electroporation)


Sal 1
cosN
loxP
Not I
lac Z
Not I
par C
CMr
par B
pBeloBAC 11
7.4 kb
par A
ori S
rep E

Unit - III

Mind Map

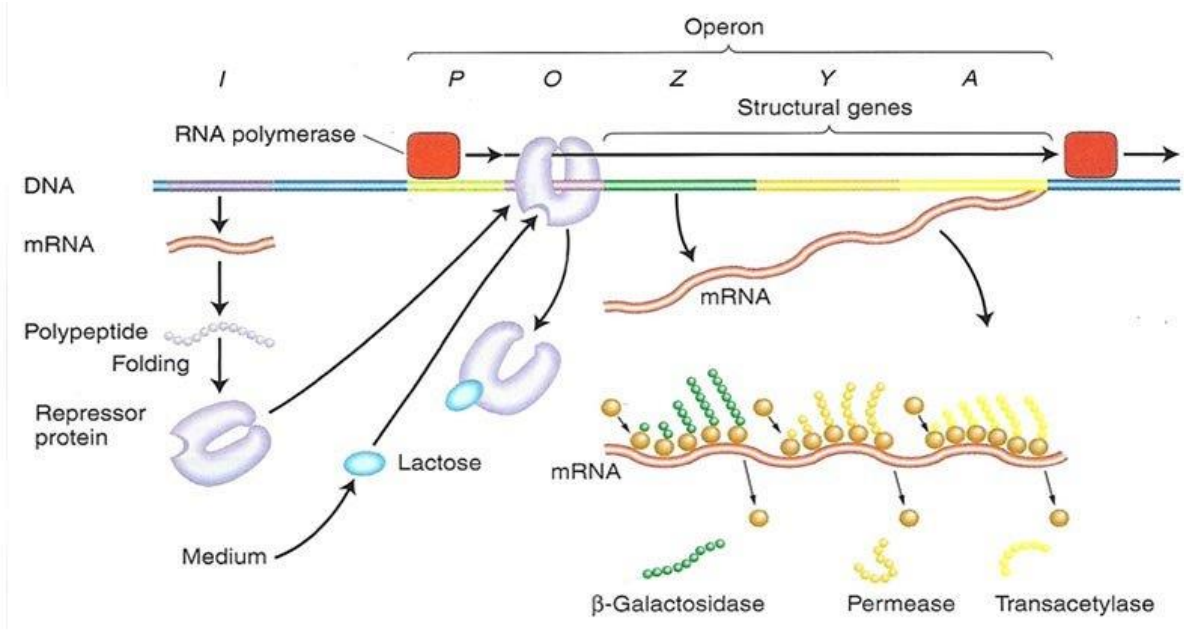


Regulation of gene expression in Prokaryotes – Lac operon

- The lactose or lac operon of *Escherichia coli* is a cluster of three structural genes encoding proteins involved in lactose metabolism and the sites on the **DNA** involved in the regulation of the operon.
- Many **protein-coding** genes in bacteria are clustered together in operons which serve as transcriptional units that are coordinately regulated.
- It was Jacob and Monod in 1961 who proposed the operon model for the regulation of transcription.

The operon model proposes three elements:

- A set of structural genes (i.e. genes encoding the proteins to be regulated);
- An operator site, which is a DNA sequence that regulates transcription of the structural genes;
- A regulator gene which encodes a protein that recognizes the operator sequence.



Lac operon

- One of the most studied operons is the lac operon in *E. coli*.
- This codes for key enzymes involved in lactose metabolism:

1. **Galactoside permease** (also known as lactose permease).

Transports lactose into the cell across the cell membrane.

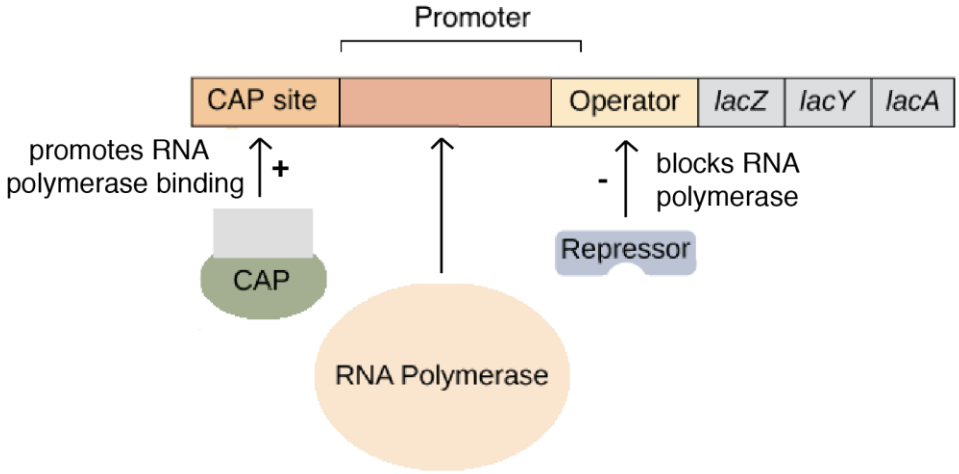
2. **Galactosidase**

Hydrolyzes lactose to glucose and galactose.

3. **Thiogalactoside transacetylase**

Structure of Lac operon

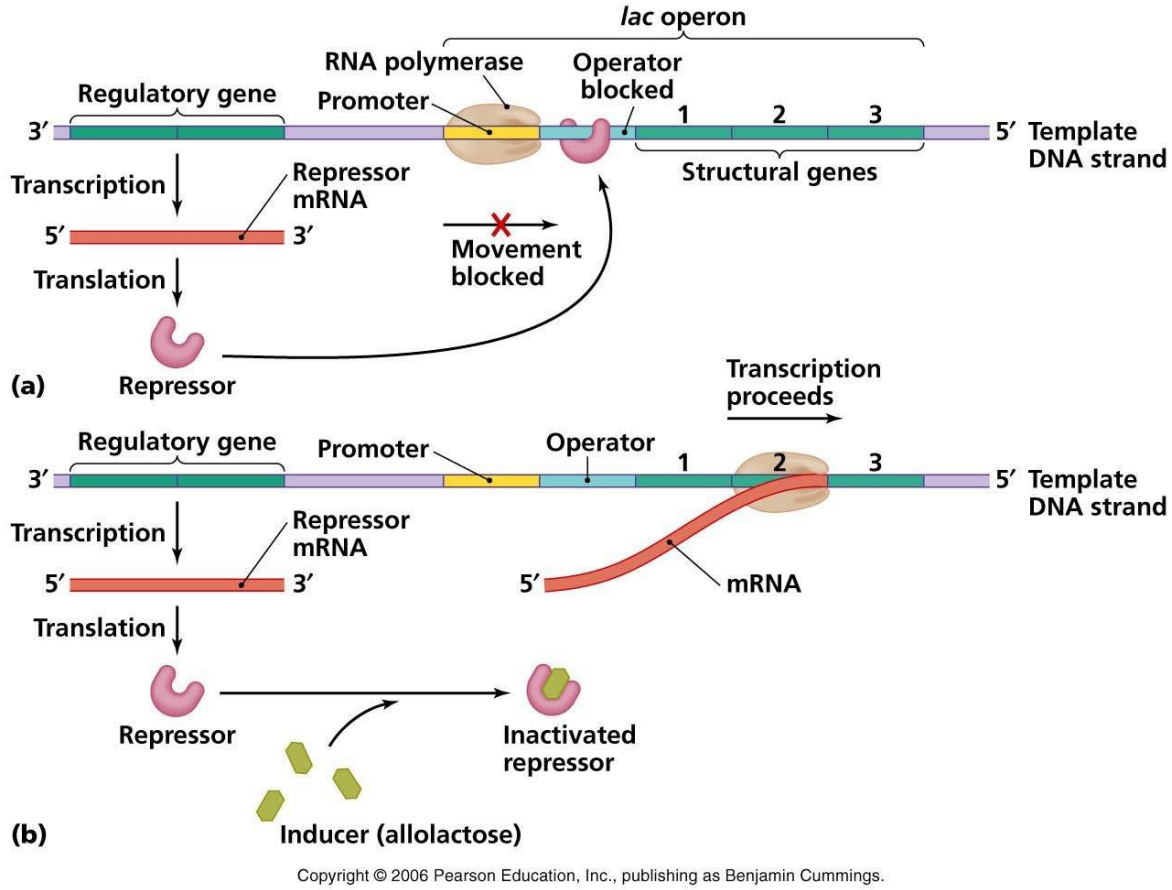
The *lac* operon:



- In the lac operon, the structural genes are the lacZ, lacY and lacA genes encoding _-galactosidase, the permease, and the transacetylase, respectively.
- Transcription occurs from a single promoter (P_{lac}) that lies upstream of these structural genes and binds RNA polymerase.
- However, also present are an operator site (O_{lac}) between the promoter and the structural genes, and a lacI gene that codes for the lac repressor protein.
- The lacI gene has its own promoter (PlacI) that binds RNA polymerase and leads to transcription of lac repressor mRNA and hence the production of lac repressor protein monomers.
- Four identical repressor monomers come together to form the active tetramer which can bind tightly to the lac operator site, O_{lac}.

Inducers and the Induction of Lac operon

- Normally, *E. coli* cells make very little of any of these three proteins but when lactose is available it, causes a large and coordinated increase in the amount of each enzyme.
- Thus each enzyme is an inducible enzyme and the process is called **induction**.
- The mechanism is that the few molecules of ß-galactosidase in the cell before induction convert the lactose to allolactose which then turns on the transcription of these three genes in the lac operon.
- Thus allolactose is an inducer.
- Another inducer of the lac operon is isopropylthiogalactoside (IPTG). Unlike allolactose, this inducer is not metabolized by *E. coli* and so, is useful for experimental studies of induction only.



Lac Operon in absence of Inducers

- In the absence of an inducer such as allolactose or IPTG, the lacI gene is transcribed and the resulting repressor protein binds to the operator site of the lac operon, Olac, and prevents transcription of the lacZ, lacY and lacA genes.

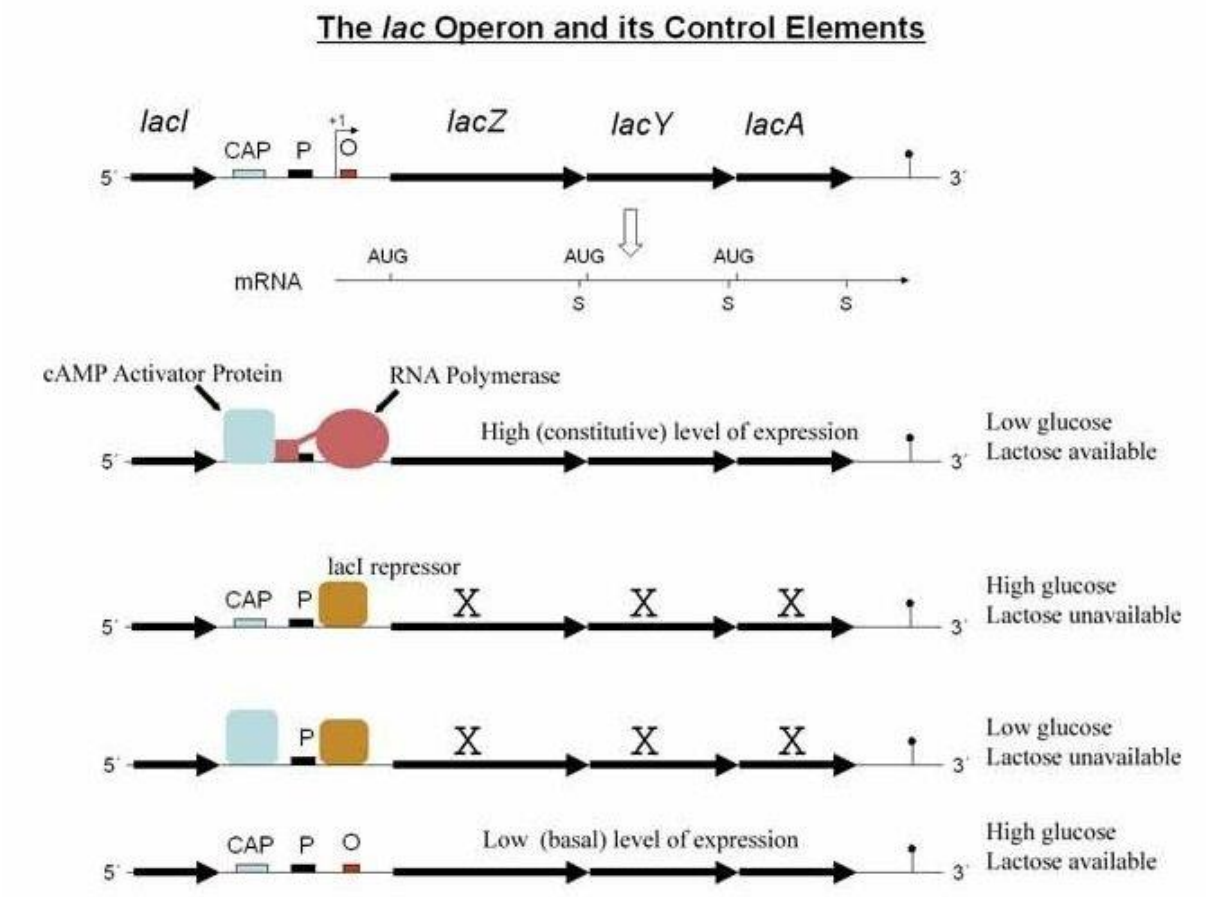
Lac Operon in presence of Inducers

- During induction, the inducer binds to the repressor.
- This causes a change in the conformation of the repressor that greatly reduces its affinity for the lac operator site.
- The lac repressor now dissociates from the operator site and allows the RNA polymerase (already in place on the adjacent promoter site) to begin transcribing the lacZ, lacY and lacA genes.
- They are transcribed to yield a single polycistronic mRNA that is then translated to produce all three enzymes in large amounts.
- The existence of a polycistronic mRNA ensures that the amounts of all three gene products are regulated coordinately.

- If the inducer is removed, the lac repressor rapidly binds to the lac operator site and transcription is inhibited almost immediately.

CRP/CAP

- High-level transcription of the lac operon requires the presence of a specific activator protein called catabolite activator protein (CAP), also called cAMP receptor protein (CRP).
- This protein, which is a dimer, cannot bind to DNA unless it is complexed with 3’5′ cyclic AMP (cAMP).
- The CRP–cAMP complex binds to the lac promoter just upstream from the binding site for RNA polymerase.
- It increases the binding of RNA polymerase and so stimulates transcription of the lac operon.
- Whether or not the CRP protein is able to bind to the lac promoter depends on the carbon source available to the bacterium.



Lac operon in the presence of Glucose

- When glucose is present, *E. coli* does not need to use lactose as a carbon source and so the lac operon does not need to be active.
- Thus the system has evolved to be responsive to glucose.
- Glucose inhibits adenylate cyclase, the enzyme that synthesizes cAMP from ATP.
- Thus, in the presence of glucose the intracellular level of cAMP falls, so CRP cannot bind to the lac promoter, and the lac operon is only weakly active (even in the presence of lactose).

Lac operon in the absence of Glucose

- When glucose is absent, adenylate cyclase is not inhibited, the level of intracellular cAMP rises and binds to CRP.
- Therefore, when glucose is absent but lactose is present, the CRP–cAMP complex stimulates transcription of the lac operon and allows the lactose to be used as an alternative carbon source.
- In the absence of lactose, the lac repressor, of course, ensures that the lac operon remains inactive.
- These combined controls ensure that the lacZ, lacY and lacA genes are transcribed strongly only if glucose is absent and lactose is present.

Positive and Negative Regulation of Lac Operon

- The lac operon is a good example of the negative control (negative regulation) of gene expression in that bound repressor prevents transcription of the structural genes.
- Positive control or regulation of gene expression is when the regulatory protein binds to DNA and increases the rate of transcription.
- In this case, the regulatory protein is called an activator. The CAP/CRP involved in regulating the lac operon is a good example of an activator.
- Thus the lac operon is subject to both negative and positive control.

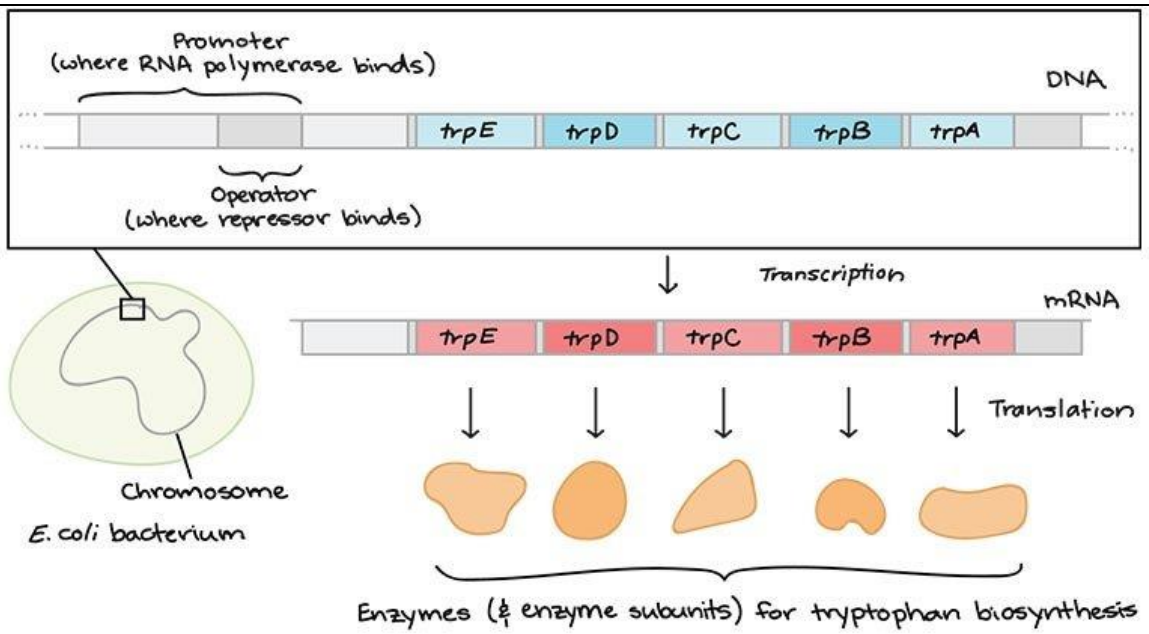
II. Tryptophan (trp operon) & attenuation.

Tryptophan (Trp) Operon

- Many protein-coding genes in bacteria are clustered together in operons which serve as transcriptional units that are coordinately regulated.
- It was Jacob and Monod in 1961 who proposed the operon model for the regulation of transcription.

- The operon model proposes three elements:
    - A set of structural genes (i.e. genes encoding the proteins to be regulated);
    - An operator site, which is a DNA sequence that regulates transcription of the structural genes.
    - A regulator gene which encodes a protein that recognizes the operator sequence.
- Operons are thus clusters of structural genes under the control of a single operator site and regulator gene which ensures that expression of the structural genes is coordinately controlled.

Trp Operon



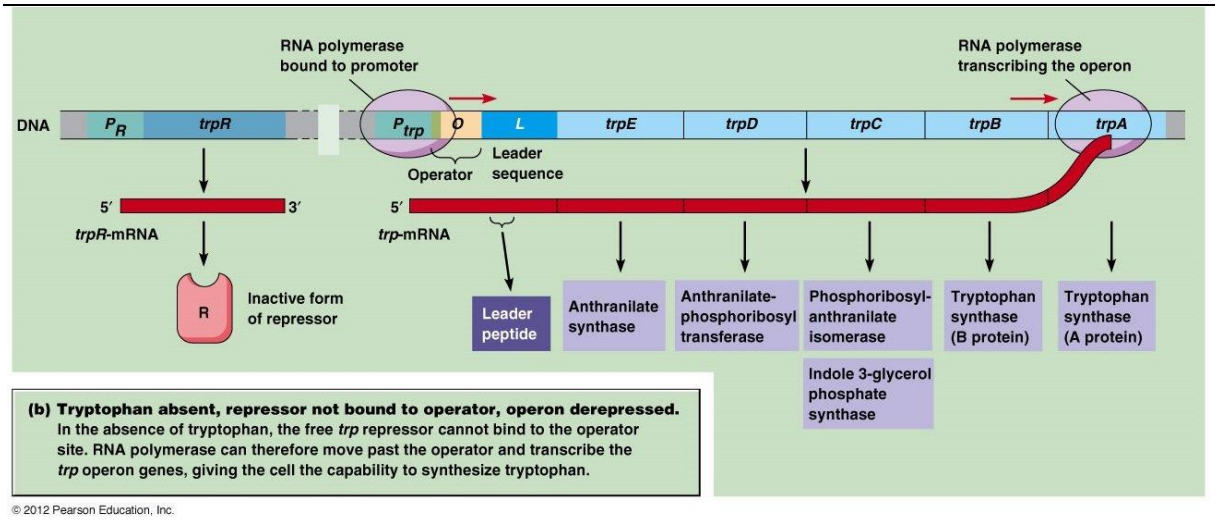
- The tryptophan operon is the regulation of transcription of the gene responsible for biosynthesis of tryptophan.
- The tryptophan (trp) operon contains five structural genes encoding enzymes for tryptophan biosynthesis with an upstream trp promoter (Ptrp) and trp operator sequence (Otrp).
- Structural genes are TrpE, TrpD, TrpC, TrpB and TrpA

1. trpE: It enodes the enzyme Anthranilate synthase I
2. trpD: It encodes the enzyme Anthranilate synthase II
3. trpC: It encodes the enzyme N-5'-Phosphoribosyl anthranilate isomerase and Indole-3-glycerolphosphate synthase
4. trpB: It encodes the enzyme tryptophan synthase-B sub unit
5. trpA: It encode the enzyme tryptophan synthase-A sub unit

- The trp operator region partly overlaps the trp promoter.
- The operon is regulated such that transcription occurs when tryptophan in the cell is in short supply.

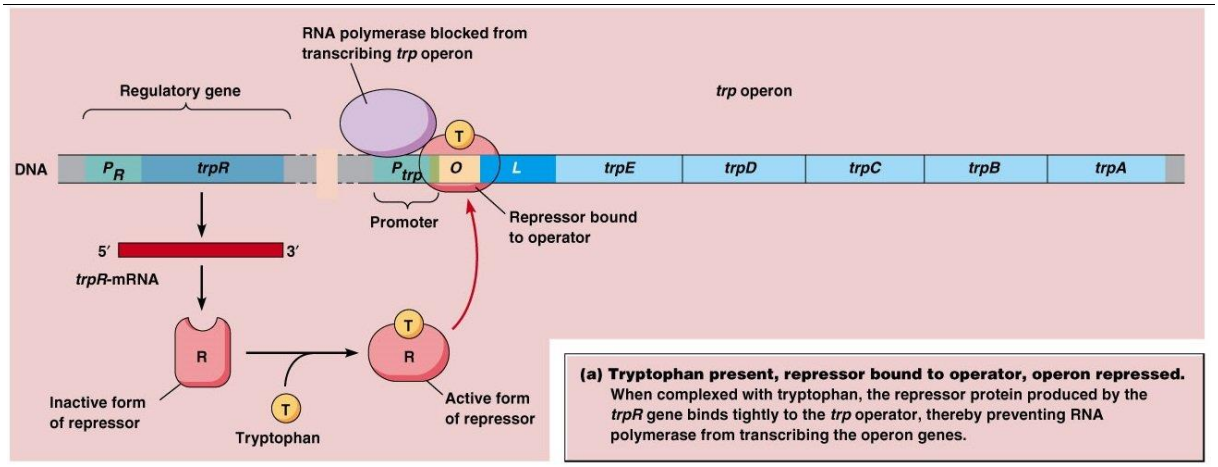
Scientists Say Alien Life Could Be

In the Absence of Tryptophan



- In the absence of tryptophan, a trp repressor protein encoded by a separate operon, trpR, is synthesized and forms a dimer.
- However, this is inactive and so is unable to bind to the trp operator and the structural genes of the trp operon are transcribed.

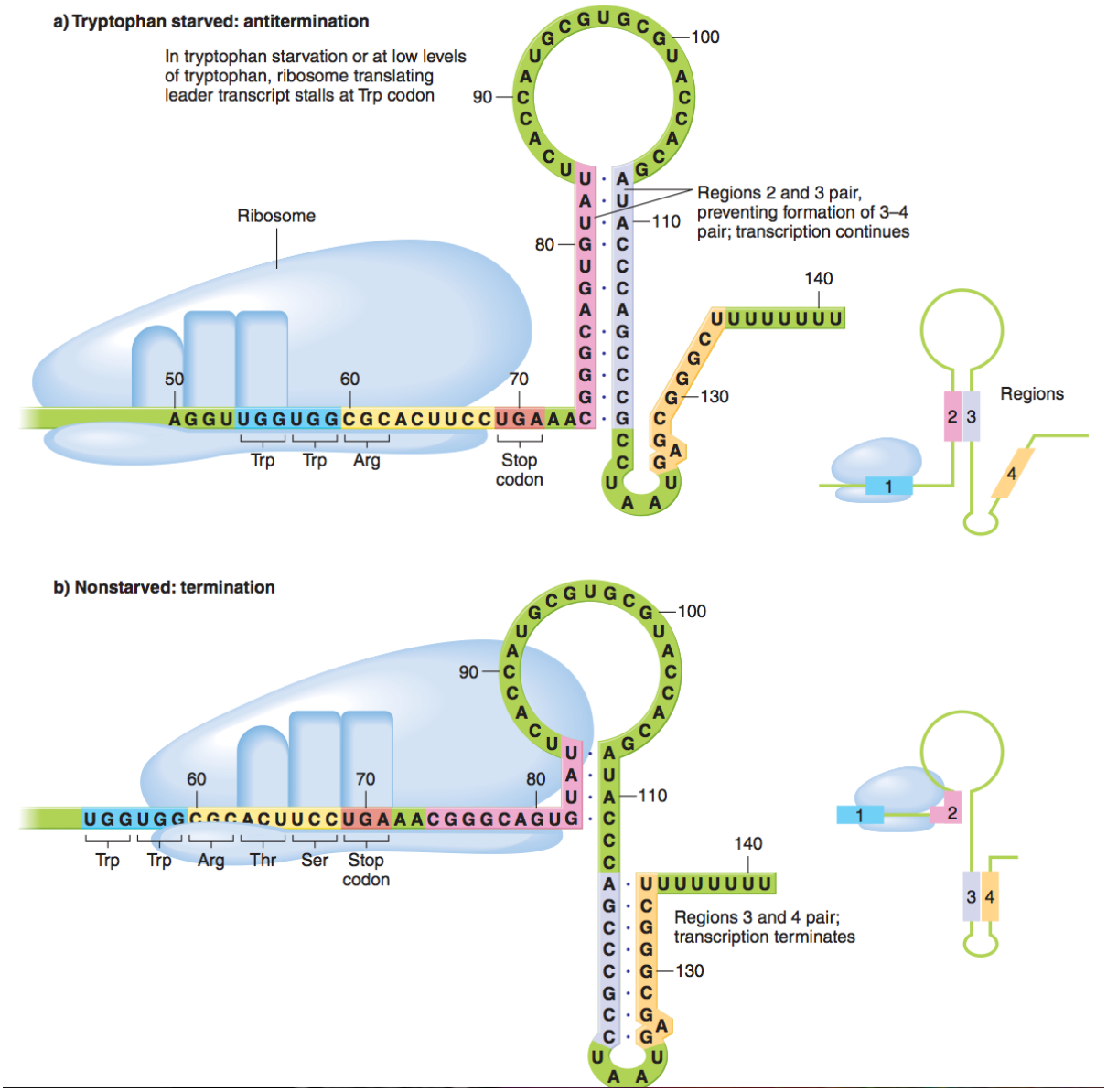
In the Presence of Tryptophan



- When tryptophan is present, the enzymes for tryptophan biosynthesis are not needed and so expression of these genes is turned off.
- This is achieved by tryptophan binding to the repressor to activate it so that it now binds to the operator and stops transcription of the structural genes.
- Binding of repressor protein to operator overlaps the promoter, so RNA polymerase cannot bind to the prometer. Hence transcription is halted.

- In this role, tryptophan is said to be a co-repressor. This is negative control, because the bound repressor prevents transcription.

Trp Operon Attenuation



- A second mechanism, called attenuation, is also used to control expression of the trp operon.
- The 5′ end of the polycistronic mRNA transcribed from the trp operon has a leader sequence upstream of the coding region of the trpE structural gene.
- This **leader sequence** encodes a 14 amino acid leader peptide containing two tryptophan residues.

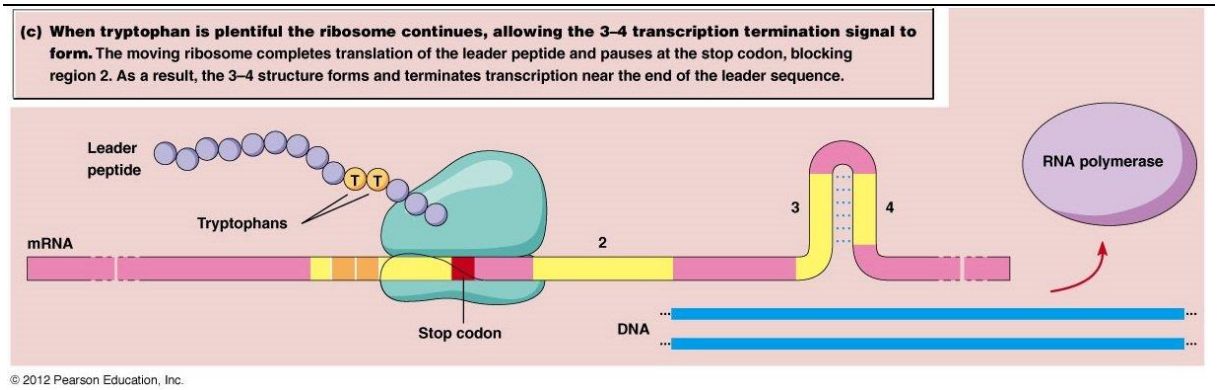
- The function of the leader sequence is to fine tune expression of the trp operon based on the availability of tryptophan inside the cell.

The leader sequence contains four regions (numbered 1–4) that can form a variety of base paired stem-loop (‘hairpin’) secondary structures.

- The regions are: Region 1, region 2, region 3 and Region 4. Region 3 is complementary to both region 2 and region 4.
- If region 3 and region 4 base pair with each other, they form a loop like structure called attenuator and it function as transcriptional termination. If pairing occur between region 3 and region 2, then no such attenuator form so that transcription continues.

Attenuation depends on the fact that, in bacteria, ribosomes attach to mRNA as it is being synthesized and so translation starts even before transcription of the whole mRNA is complete.

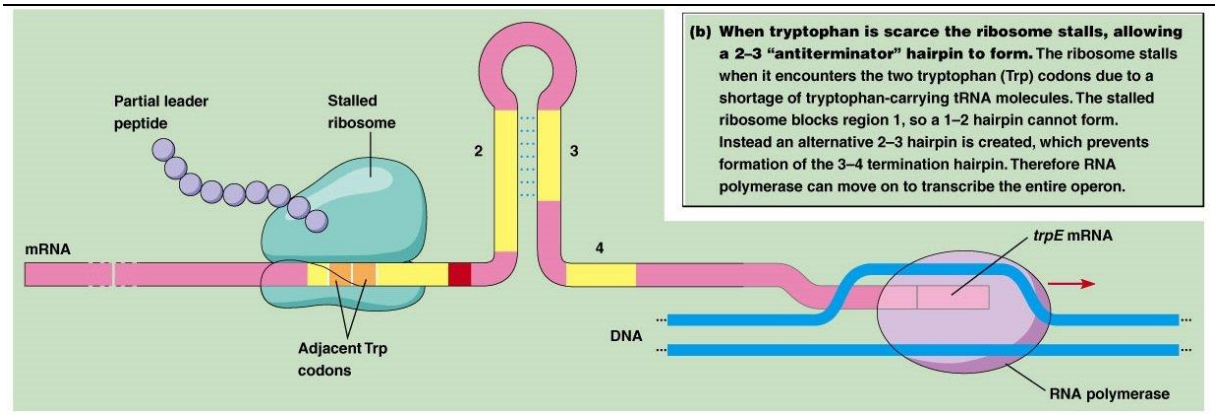
When Trypophan is abundant



- When tryptophan is abundant, ribosomes bind to the trp polycistronic mRNA that is being transcribed and begin to translate the leader sequence.
- Now, the two trp codons for the leader peptide lie within sequence 1, and the translational Stop codon lies between sequence 1 and 2.
- During translation, the ribosomes follow very closely behind the RNA polymerase and synthesize the leader peptide, with translation stopping eventually between sequences 1 and 2.
- At this point, the position of the ribosome prevents sequence 2 from interacting with sequence 3.
- Instead sequence 3 base pairs with sequence 4 to form a 3:4 stem loop which acts as a transcription terminator.

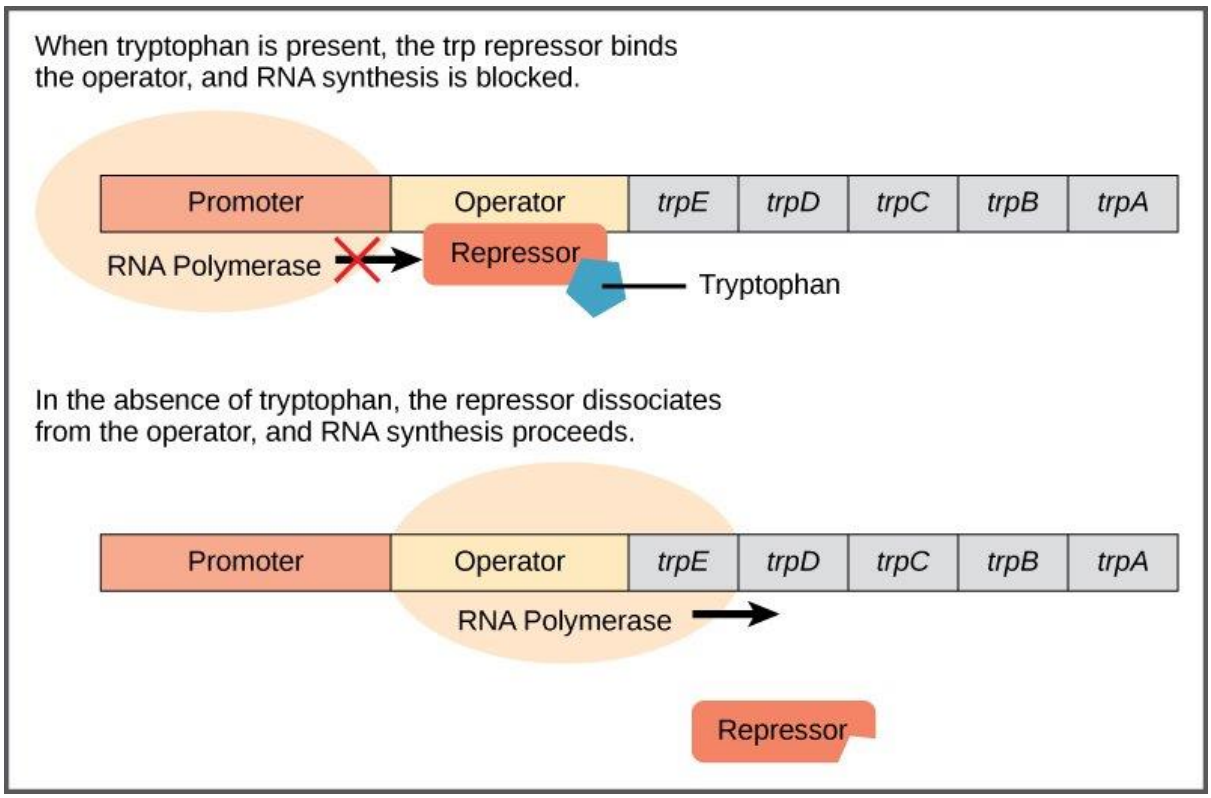
- Therefore, when tryptophan is present, further transcription of the trp operon is prevented.

When Trypophan is scarce



- If, however, tryptophan is in short supply, the ribosome will pause at the two trp codons contained within sequence 1.
- This leaves sequence 2 free to base pair with sequence 3 to form a 2:3 structure (also called the anti-terminator), so the 3:4 structure cannot form and transcription continues to the end of the trp operon.

Hence the availability of tryptophan controls whether transcription of this operon will stop early (attenuation) or continue to synthesize a complete polycistronic mRNA.



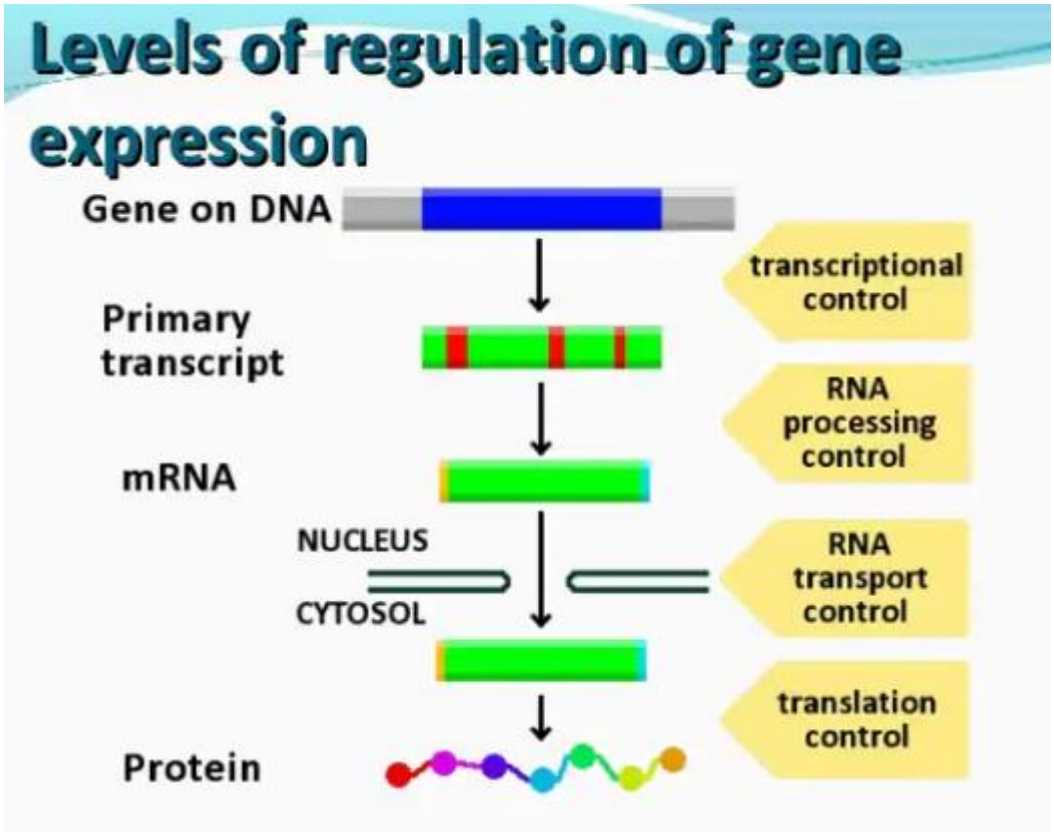
Regulation of Trp Operon

Overall, for the trp operon, repression via the trp repressor determines whether transcription will occur or not and attenuation then fine tunes transcription.

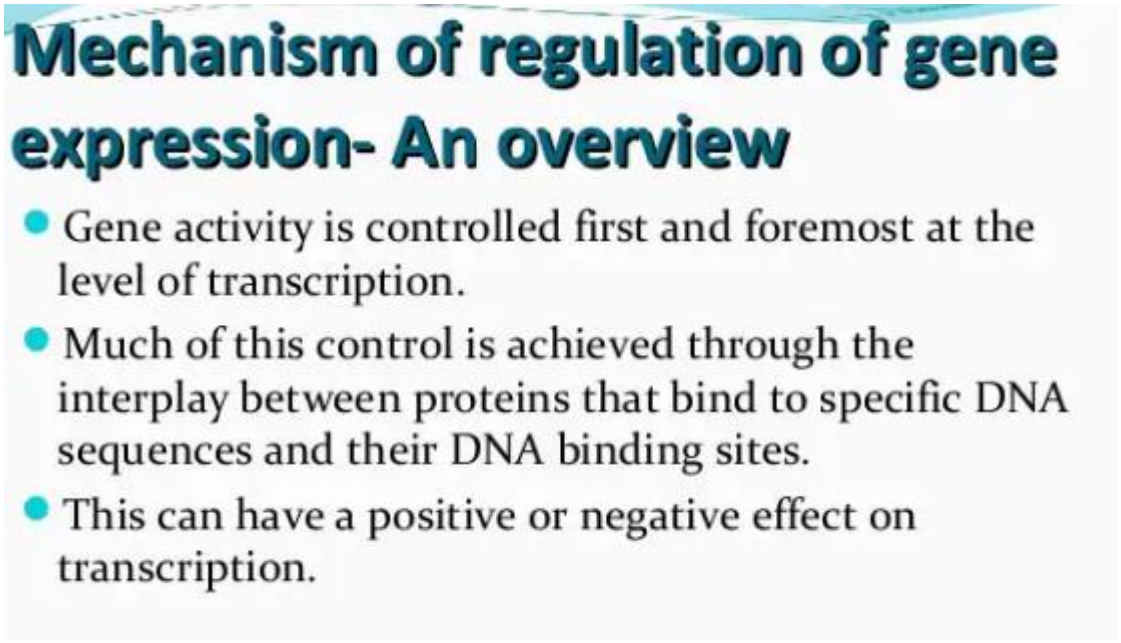
III. Regulation in eukaryotes:

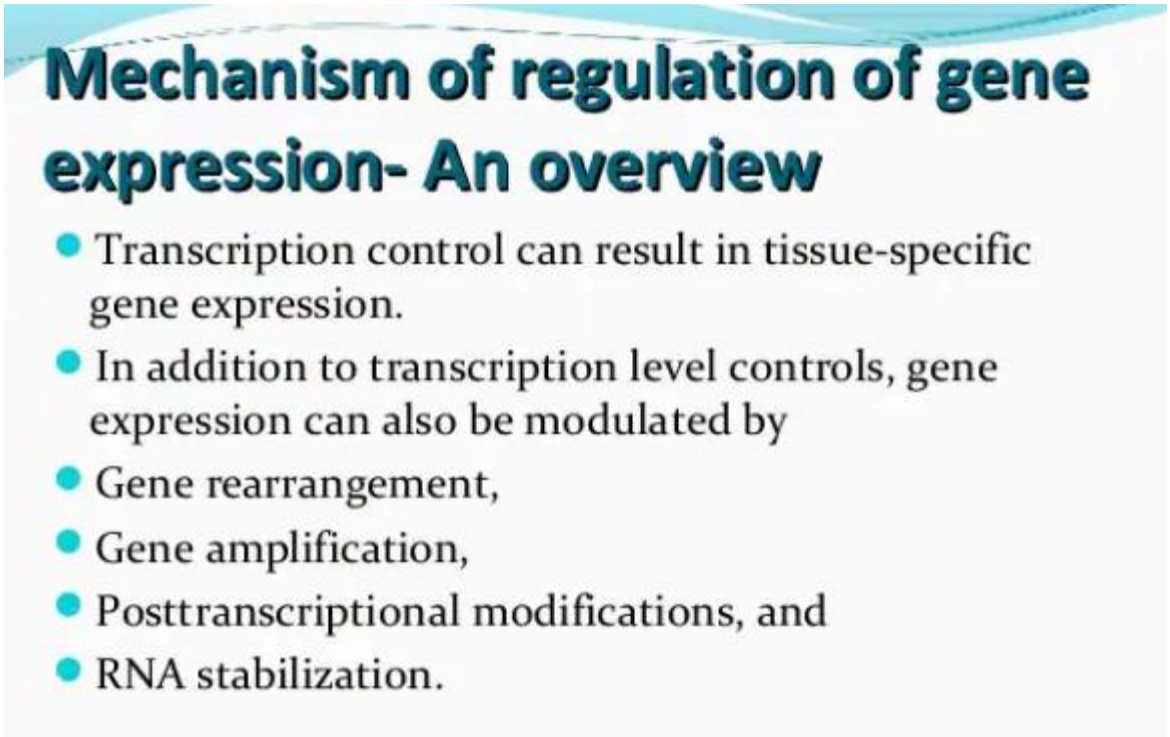
Gene expression is the combined process of :

- the transcription of a gene into mRNA,
- the processing of that mRNA, and
- its translation into protein (for protein-encoding genes).



I. Gene loss, Gene amplification, Gene rearrangement





II. Gene Amplification

Gene amplification was recognized as a physiological process during the development of *Drosophila melanogaster*. Intriguingly, mammalian cells use this mechanism to overexpress particular genes for survival under stress, such as during exposure to cytotoxic drugs. One well-known example is the amplification of the dihydrofolate reductase gene observed in methotrexate-resistant cells. Four models have been proposed for the generation

of amplifications: extrareplication and recombination, the breakage-fusion-bridge cycle, double rolling-circle replication, and replication fork stalling and template switching. Gene amplification is a typical genetic alteration in cancer, and historically many oncogenes have been identified in the amplified regions

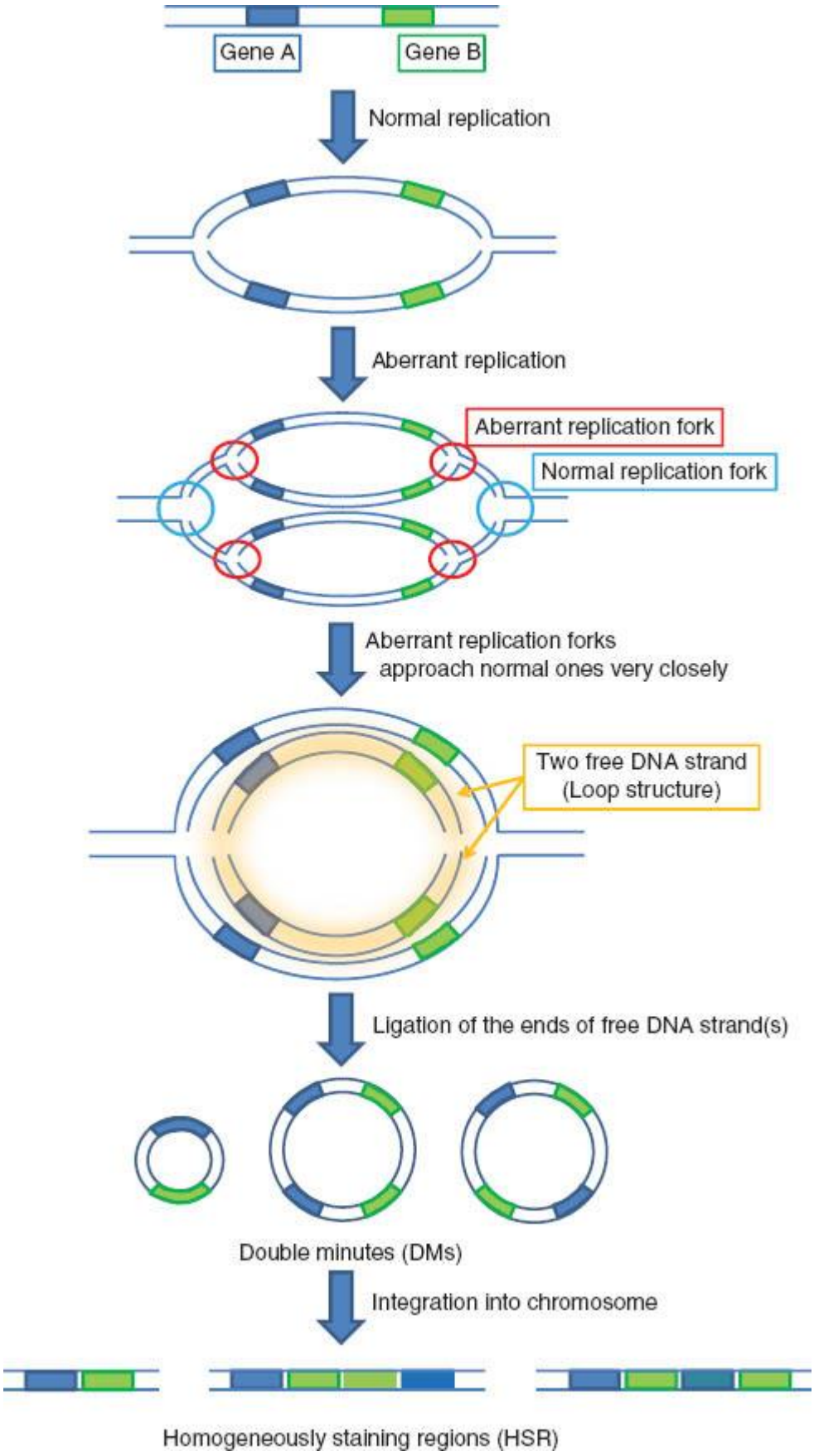
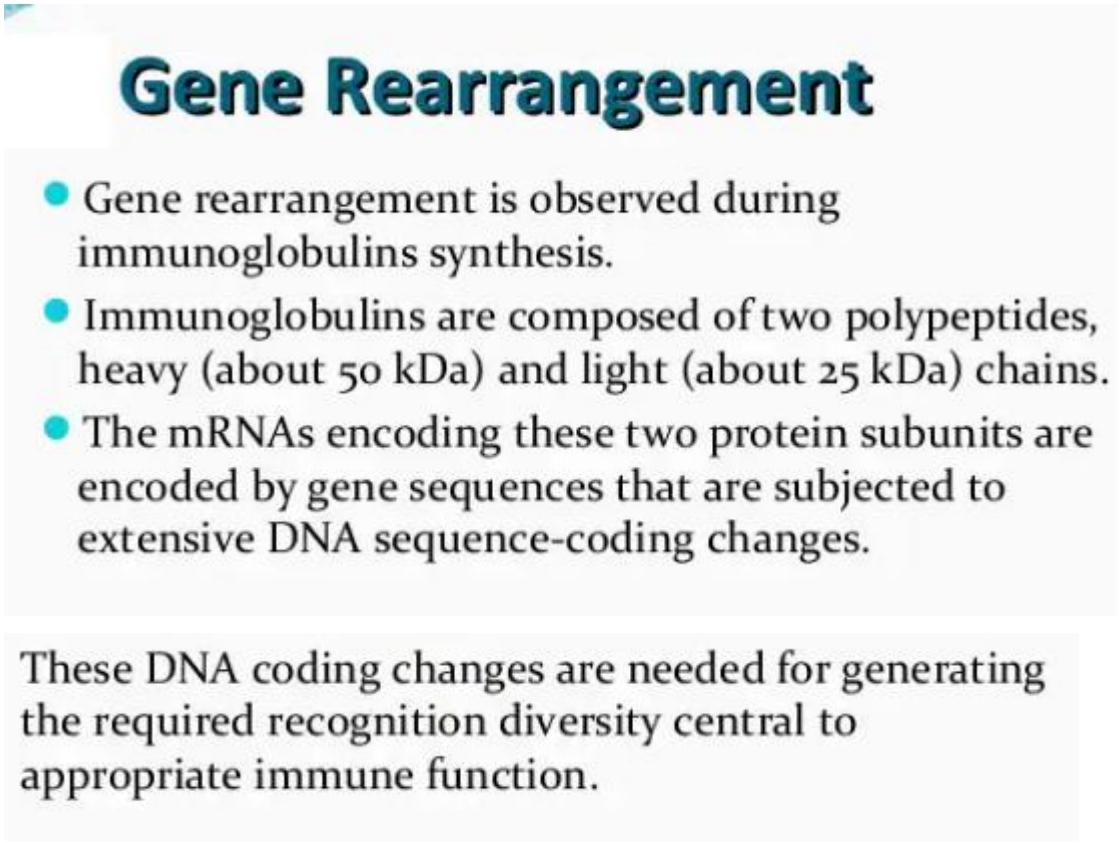
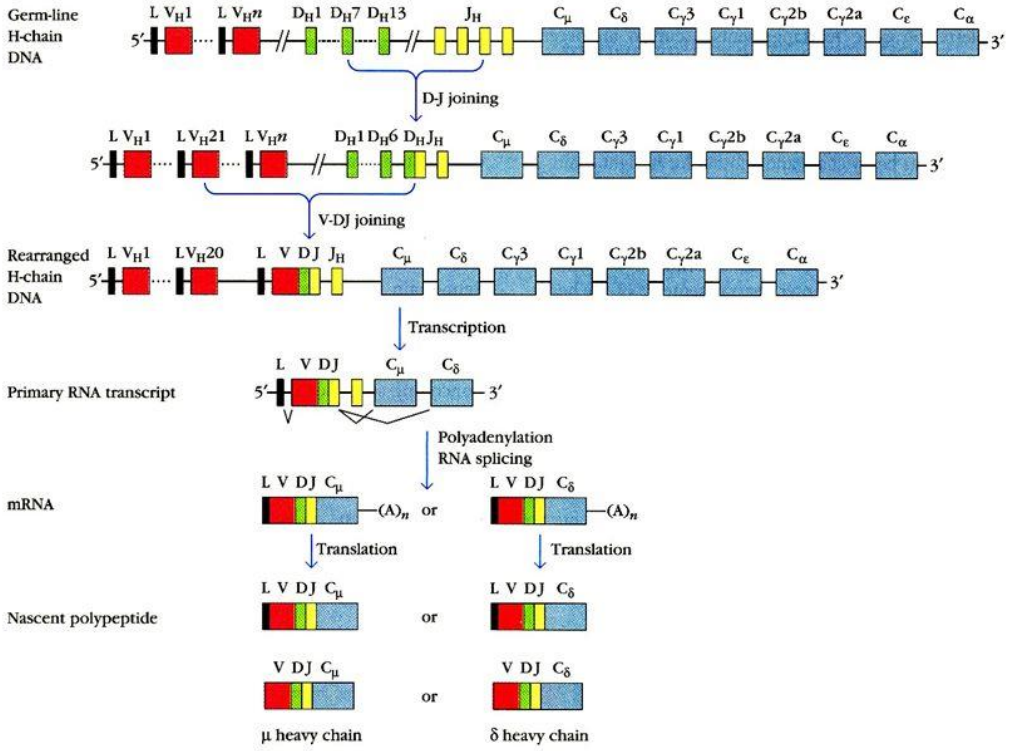


Figure: Model of gene amplification generated by extrareplication and recombination

This type of amplification is triggered by aberrant replication in a replication fork, resulting from a single additional initiation of replication within the replication fork. In this replication fork, there is a loop structure including the two free DNA strands. These strands can form three types of double minute chromosome by ligase. Some of these circular DNAs would be recombined into chromosomes, generating HSRs.



Heavy Chain Rearrangement



Gene Rearrangement

- The IgG light chain is composed of variable (V_L), joining (J_L), and constant (C_L) domains or segments.
- For particular subsets of IgG light chains, there are roughly 300 tandemly repeated V_L gene coding segments, five tandemly arranged J_L coding sequences, and roughly ten C_L gene coding segments.
- All of these multiple, distinct coding regions are located in the same region of the same chromosome.

Gene Rearrangement

- However, a given functional IgG light chain transcription unit contains only the coding sequences for a single protein.
- Thus, before a particular IgG light chain can be expressed, *single* V_L, J_L, and C_L coding sequences must be recombined to generate a *single,* contiguous transcription unit excluding the multiple nonutilized segments.

Gene Rearrangement

- This deletion of unused genetic information is accomplished by selective DNA recombination that removes the unwanted coding DNA while retaining the required coding sequences: one V_L, one J_L, and one C_L sequence.

(5). These experiments lead to the conclusion that at least some somatic cells in these organisms have not undergone an irreversible loss or change of essential genetic material. However, the number of instances of successful nuclear transplantation is too small to rule out programmed genome alteration as a widespread phenomenon in differentiation. Even if obvious gene loss is restricted to a few species, the biological event with which it is related is a general one. I refer to the early determination of germ cells and their virtual segregation from somatic cells in cleaving embryos (6). It could be construed that something dramatic is happening to the genome of somatic cells from which germ cells must be protected. It is the similarity in the biology of germ cell determination, regardless of whether gene loss has been detected, that suggests a search for less obvious genome changes in other animals might be fruitful.

The protozoan *Oxytrichia* undergoes gene loss and is especially tractable for detailed biochemical examination (7). A micronucleus retains germ line continuity, while a macronucleus is responsible for all of the cell's RNA synthesis. The macronucleus is formed from the micronucleus by a process involving cleavage of the DNA, elimination of most of the DNA, and replication (polytenization) of the remaining fragments. These remaining DNA fragments are not replicated uniformly. A remarkable feature about this process is that most fragments in the macronucleus have the same DNA sequence at each end (8). This terminal repetition could be a cleavage site for some restriction-like enzyme, a site involved in DNA replication, or a promoter for RNA synthesis.

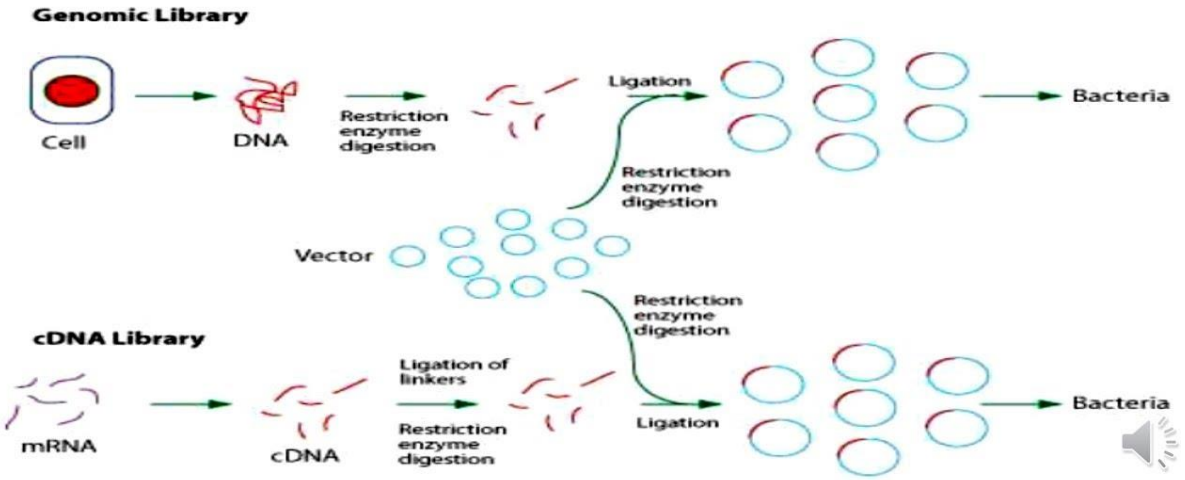
Gene Loss and Germ Cell Determination

Somatic cells of some animals are altered extensively during development by diminution of their chromatin or elimination of certain chromosomes (3). This event occurs in early cleavage in progenitors of somatic cells so that only the germ cells maintain their genome intact. The discarded DNA is presumed to contain genes required for germ cell differentiation since the cells that lose DNA are incapable of becoming germ cells. Chromosome diminution has been observed in some nematodes, protozoa, crustaceans, and insects. The inability to detect diminution in other eukaryotes has led most investigators to believe that it is not a mechanism of general significance for gene control although, if small amounts of chromosomal material are lost from presumptive somatic cells, this loss could have escaped detection. The best evidence against loss of DNA is the totipotency of some nuclei from some differentiated frog cells (4) and plant cells

Unit – IV

Mind map

Genomic library & cDNA library & its screening

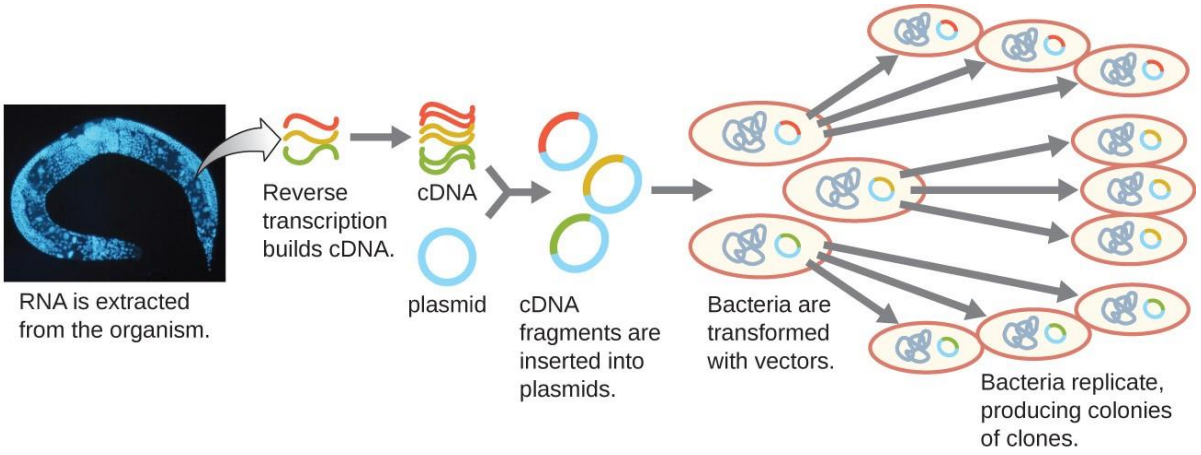


I. cDNA Library

A cDNA library is defined as a collection of cDNA fragments, each of which has been cloned into a separate vector molecule.

To focus on the expressed genes in an organism or even a tissue, researchers construct libraries using the organism's messenger RNA (mRNA) rather than its genomic DNA. Whereas all cells in a single organism will have the same genomic DNA, different tissues express different genes, producing different complements of **mRNA**. For example, all human cells' genomic DNA contains the gene for insulin, but only cells in the pancreas express mRNA directing the production of insulin. Because mRNA cannot be cloned directly, in the laboratory mRNA must be used as a template by the retroviral enzyme **reverse transcriptase** to make **complementary DNA (cDNA)**. A cell's full complement of mRNA can be reverse-transcribed into cDNA molecules, which can be used as a template for DNA polymerase to make double-stranded DNA copies; these fragments can subsequently be ligated into either plasmid vectors or bacteriophage to produce a cDNA library. The benefit of a cDNA library is that it contains DNA from only the expressed genes in the cell. This means that the introns, control sequences such as promoters, and DNA not destined to be translated into proteins are not represented in the library. The focus on translated sequences

means that the library cannot be used to study the sequence and structure of the genome in its entirety. The construction of a cDNA genomic library is shown in Figure.



Complementary DNA (cDNA) is made from mRNA by the retroviral enzyme reverse transcriptase, converted into double-stranded copies, and inserted into either plasmid vectors or bacteriophage, producing a cDNA library.

Principle of cDNA Library:

In the case of cDNA libraries we produce DNA copies of the RNA sequences (usually the mRNA) of an organism and clone them. It is called a cDNA library because all the DNA in this library is complementary to mRNA and are produced by the reverse transcription of the latter.

Much of eukaryotic DNA consists of repetitive sequences that are not transcribed into mRNA and the sequences are not represented in a cDNA library. It must be noted that prokaryotes and lower eukaryotes do not contain introns, and preparation of cDNA is generally unnecessary for these organisms. Hence, cDNA libraries are produced only from higher eukaryotes.

Vectors used in the Construction of cDNA Library:

Both the bacterial and bacteriophage DNA are used as vectors in the construction of cDNA library.

Table 6.2: Vectors used in the construction of cDNA library

Vectors	Insert size	Remarks
λ-phages	Up to 20-30kb (for replacement vectors) and 10-15kb (for insertion vectors)	• Maximum size of mRNA is about 8kb. Hence the capicity of DNA insert is not a major conern • Insertion vector system is usually employed • Useful for study of individual genes and their putative functions • Efficient packaging system, easy for gene transfer into *E. coli*, more representative than plasmid libraries, subcloning and subsequent DNA manipulation process are less convenient than plasmid systems
Bacterial plasmids	Up to 10-15kb	• Relatively easy to transform *E. coli* cells although may not be efficient as the λ-phage system for large scale gene transfer • Less representative than λ-phage libraries, subcloning and subsequent DNA manipulation processes are more convenient than the λ-pahge systems.

Procedure in the Construction of cDNA Library:

The steps involved in the construction of a cDNA library are as follows:

1. Extraction of mRNA from the eukaryotic Cell:

Firstly, the mRNA is obtained and purified from the rest of the RNAs. Several methods exist for purifying RNA such as trizol extraction and column purification. Column purification is done by using oligomeric dT nucleotide coated resins where only the mRNA having the poly-A tail will bind.

The rest of the RNAs are eluted out. The mRNA is eluted by using eluting buffer and some heat to separate the mRNA strands from oligo-dT.

2. Construction of cDNA from the Extracted mRNA:

There are different strategies for the construction of a cDNA. These are discussed as follows:

(a) The RNase Method:

The principle of this method is that a complementary DNA strand is synthesized using reverse transcriptase to make an RNA: DNA duplex. The RNA strand is then nicked and replaced by DNA. In this method the first step is to anneal a chemically synthesized oligo-dT primer to the 3′ polyA-tail of the RNA.

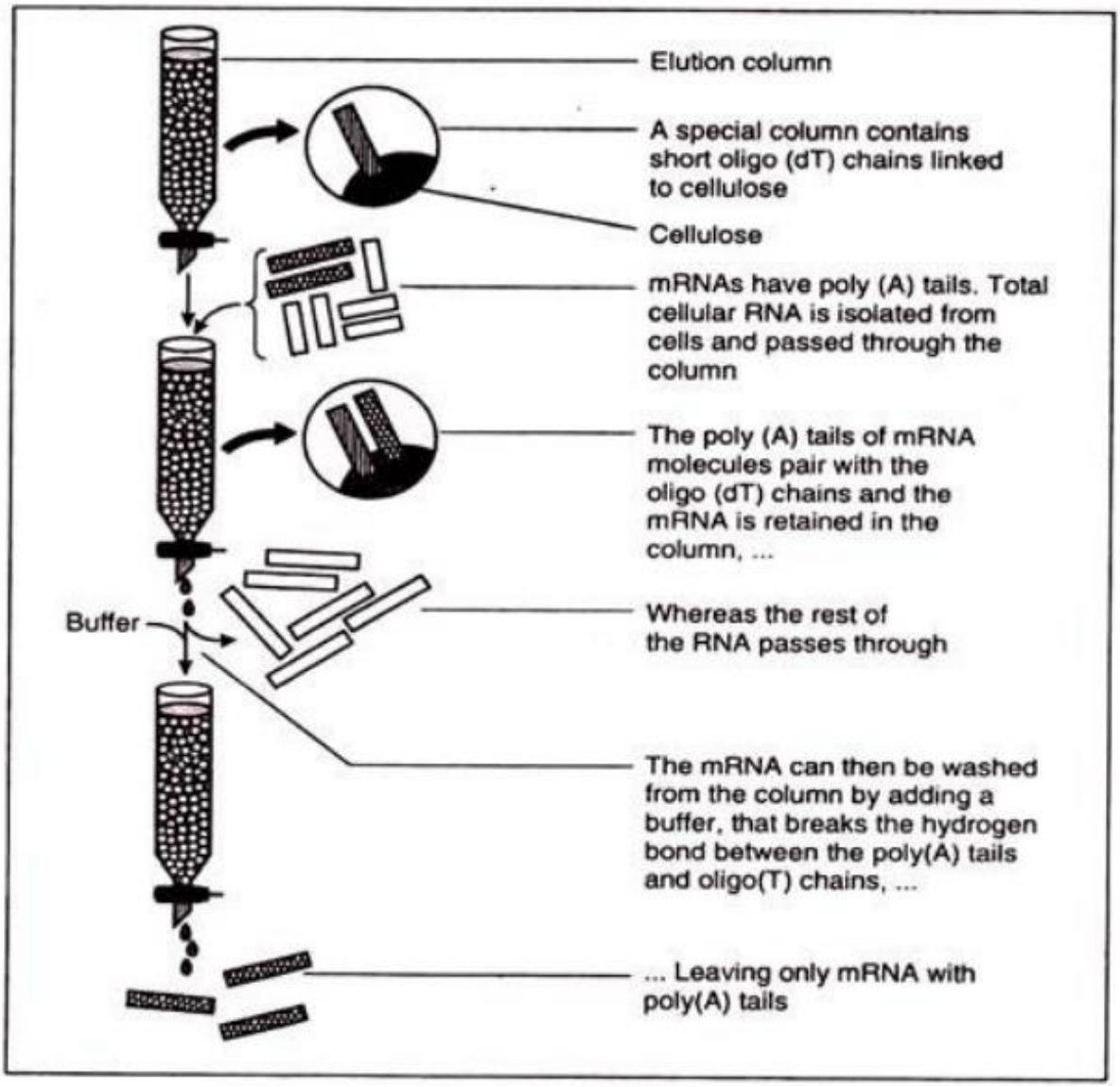
The primer is typically 10-15 residues long, and it primes (by providing a free 3′ end) the synthesis of the first DNA strand in the presence of reverse transcriptase and deoxyribonucleotides. This leaves an RNA: DNA duplex.

The next step is to replace the RNA strand with a DNA strand. This is done by using RNase H enzyme which removes the RNA from RNA: DNA duplex. The DNA strand thus left behind is then considered as the template and the second DNA strand is synthesized by the action of DNA polymerase II.

(b) The Self-Priming method:

This involved the use of an oligo-dT primer annealing at the polyadenylate tail of the mRNA to prime first DNA strand synthesis against the mRNA. This cDNA thus formed has the tendency to transiently fold back on itself, forming a hairpin loop. This results in the self-priming of the second strand.

After the synthesis of the second DNA strand, this loop must be cleaved with a single-strand-specific nuclease, e.g., SI nuclease, to allow insertion into the cloning vector. This method has a serious disadvantage. The cleavage with SI nuclease results in the loss of a certain amount of sequence at the 5′ end of the clone.



Extraction of mRNA from Eukaryotic cell

(c) Land et al. Strategy:

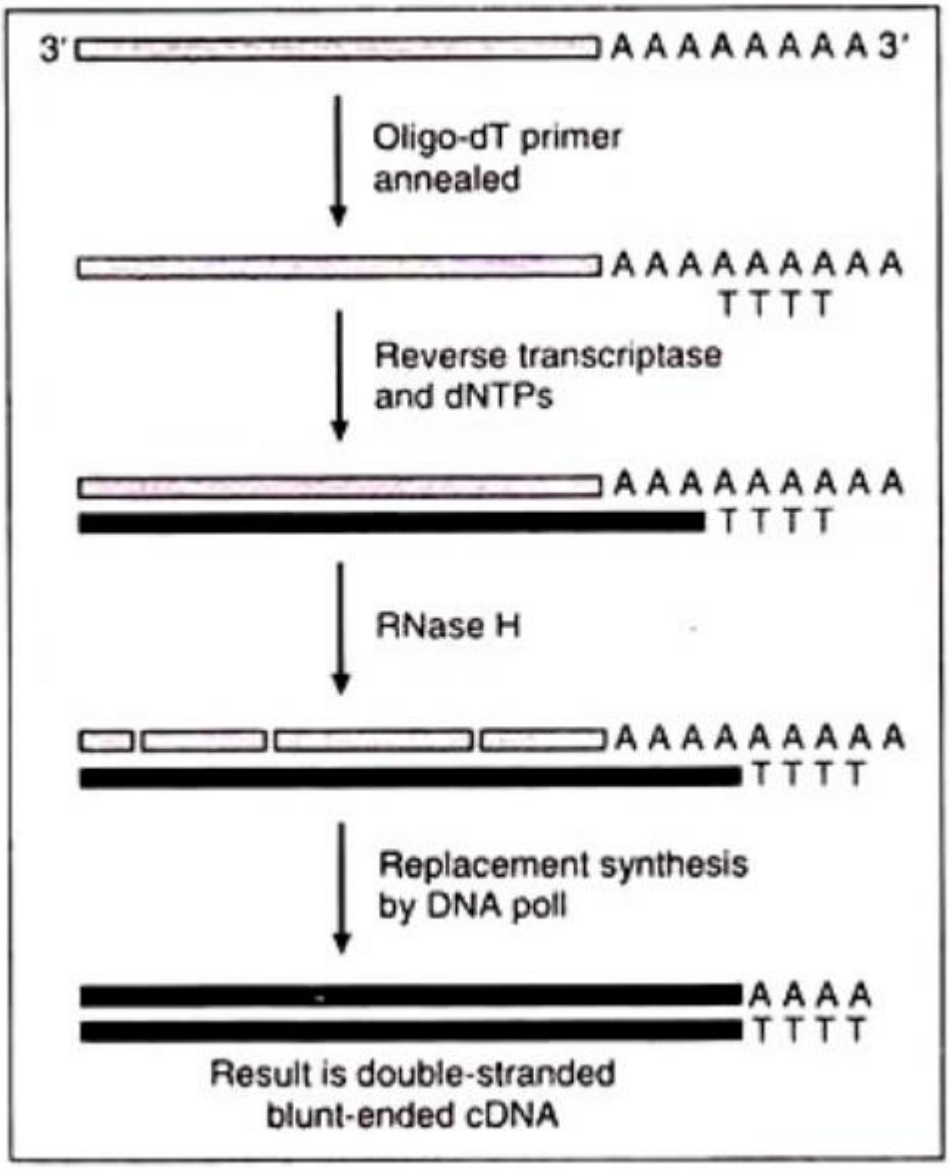
After first-strand synthesis, which is primed with an oligo- dT primer as usual, the cDNA is tailed with a string of cytidine residues using the enzyme terminal transferase. This artificial oligo-dC tail is then used as an annealing site for a synthetic oligo-dG primer, allowing synthesis of the second strand.

(d) Homopolymer Tailing:

This approach uses the enzyme terminal transferase, which can polymerize nucleotides onto the 3′-hydroxyl of both DNA and RNA molecules. We carry out the synthesis of the first DNA strand essentially as before, to produce an RNA: DNA hybrid.

We then use terminal transferase and a single deoxyribonucleotide to add tails of that nucleotide to the 3′ ends of both RNA and DNA strands. The result of this is that the DNA strand now has a known sequence at its 3′ end Typically, dCTP or dATP are used.

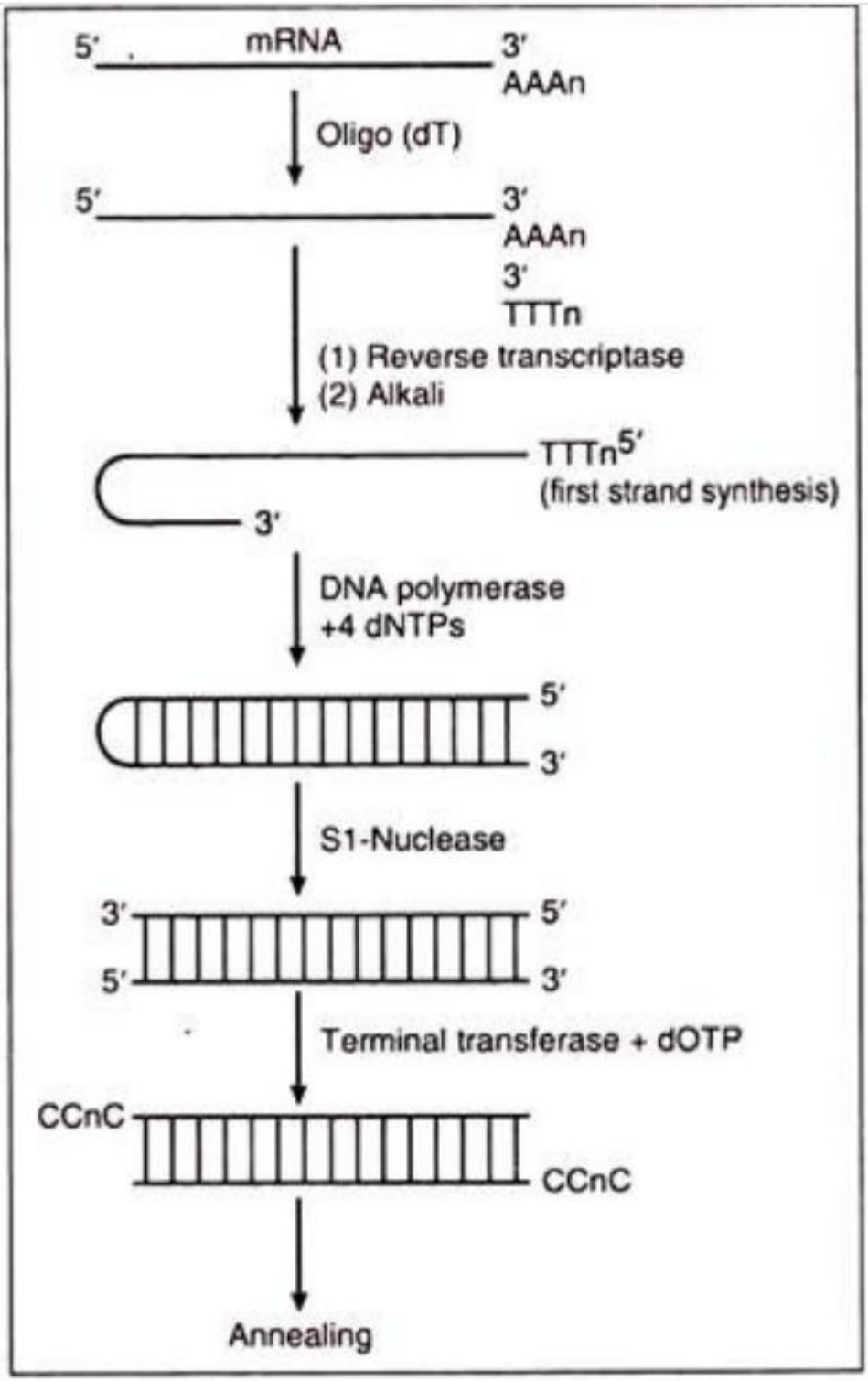
A complementary oligomer (synthesized chemically) can now be annealed and used as a primer to direct second strand synthesis. This oligomer (and also the one used for first strand synthesis) may additionally incorporate a restriction site, to help in cloning the resulting double- stranded cDNA.



The RNase H method of c-DNA synthesis

(e) Rapid Amplification of cDNA Ends (RACE):

It is sometimes the case that we wish to clone a particular cDNA for which we already have some sequence data, but with particular emphasis on the integrity of the 5′ or 3′ ends. RACE techniques (Rapid Amplification of cDNA Ends) are available for this. The RACE methods are divided into 3'RACE and 5'RACE, according to which end of the cDNA we are interested in.



Self-priming method of c-DNA synthesis

(a) 3'RACE:

In this type of RACE, reverse transcriptase synthesis of a first DNA strand is carried out using a modified oligo-dT primer. This primer comprises a stretch of unique adaptor se-

quence followed by an oligo-dT stretch. The first strand synthesis is followed by a second strand synthesis using a primer internal to the coding sequence of interest.

This is followed by PCR using

(i) The same internal primer and ‘

(ii) The adaptor sequence (i.e., omitting the oligo-dT). Although in theory it should be possible to use a simple oligo- dT primer throughout instead of the adaptor-oligo-dT and adaptor combination, the low melting temperature for an oligo-dT primer may interfere with the subsequent rounds of PCR.

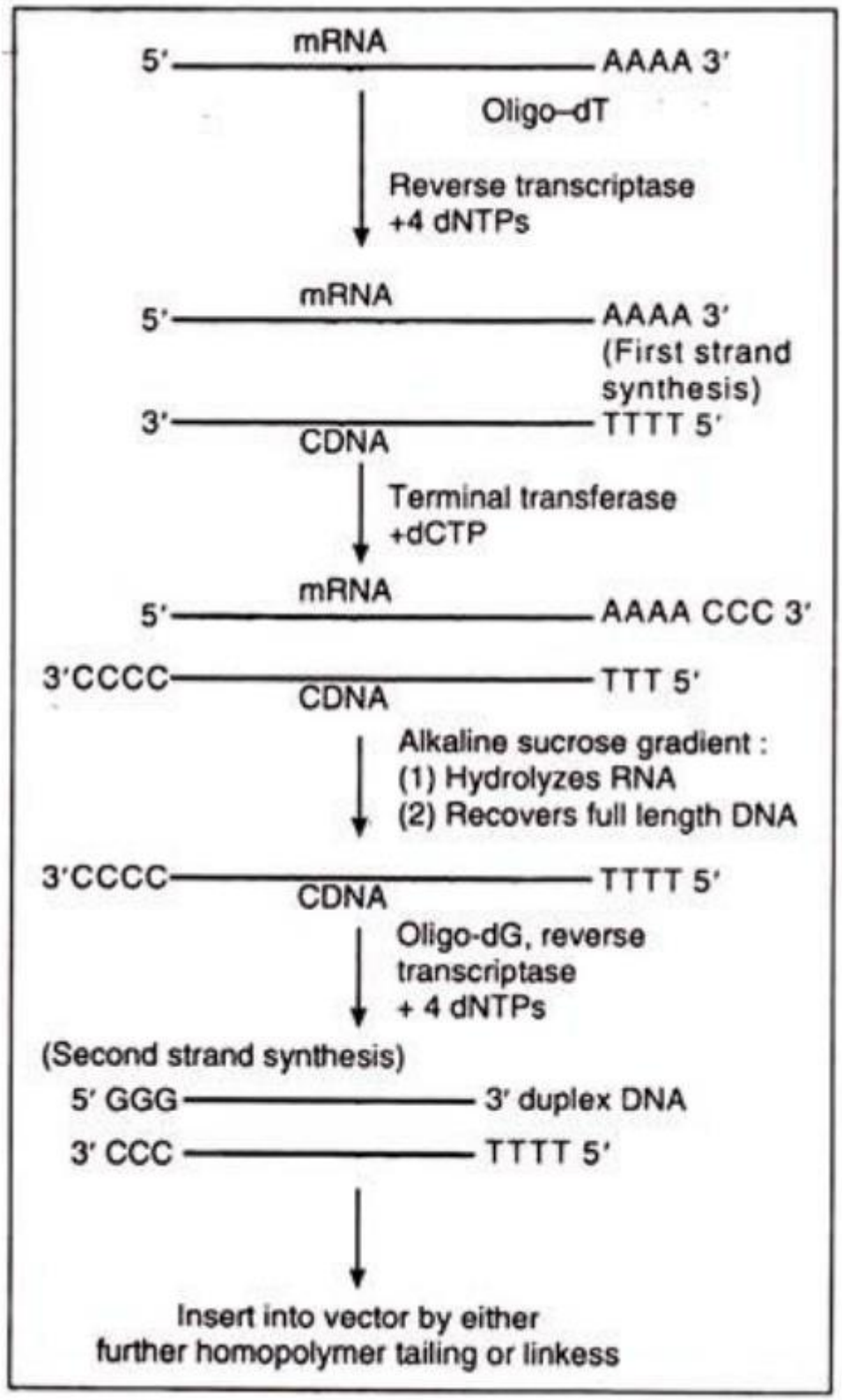
(b) 5’RACE:

In this type of RACE first cDNA strand is synthesized with re- verse transcriptase and a primer from within the coding sequence. Unincorporated primer is removed and the cDNA strands are tailed with oligo-dA. A second cDNA strand is then synthesized with an adaptor-oligo-dT primer.

The resulting double-stranded molecules are then subject to PCR using

(i) A primer nested within the coding region and

(ii) The adaptor sequence. A nested primer is used in the final PCR to improve specificity. The adaptor sequence is used in the PCR because of the low melting temperature of a simple oligo-dT primer, as in 3’RACE above. A number of kits for RACE are commercially available.



Land et al. strategy

3. Cloning the c-DNA:

(a) Linkers:

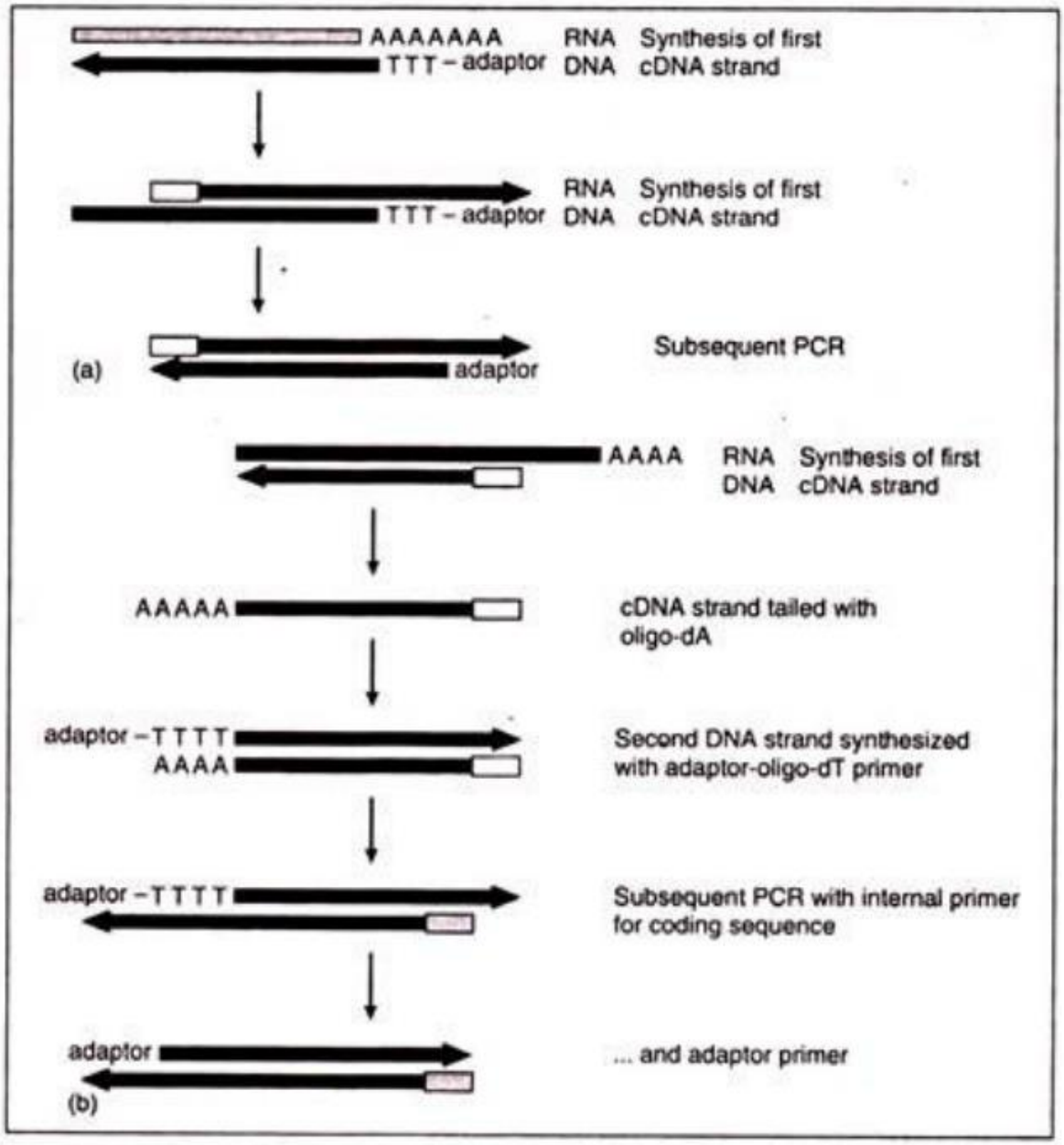
The RNaseH and homopolymer tailing methods ultimately generate a collection of double-stranded, blunt-ended cDNA molecules. They must now be attached to the vector molecules. This could be done by blunt-ended ligation, or by the addition of linkers, digestion with the relevant enzyme and ligation into vector.

(b) Incorporation of Restriction Sites:

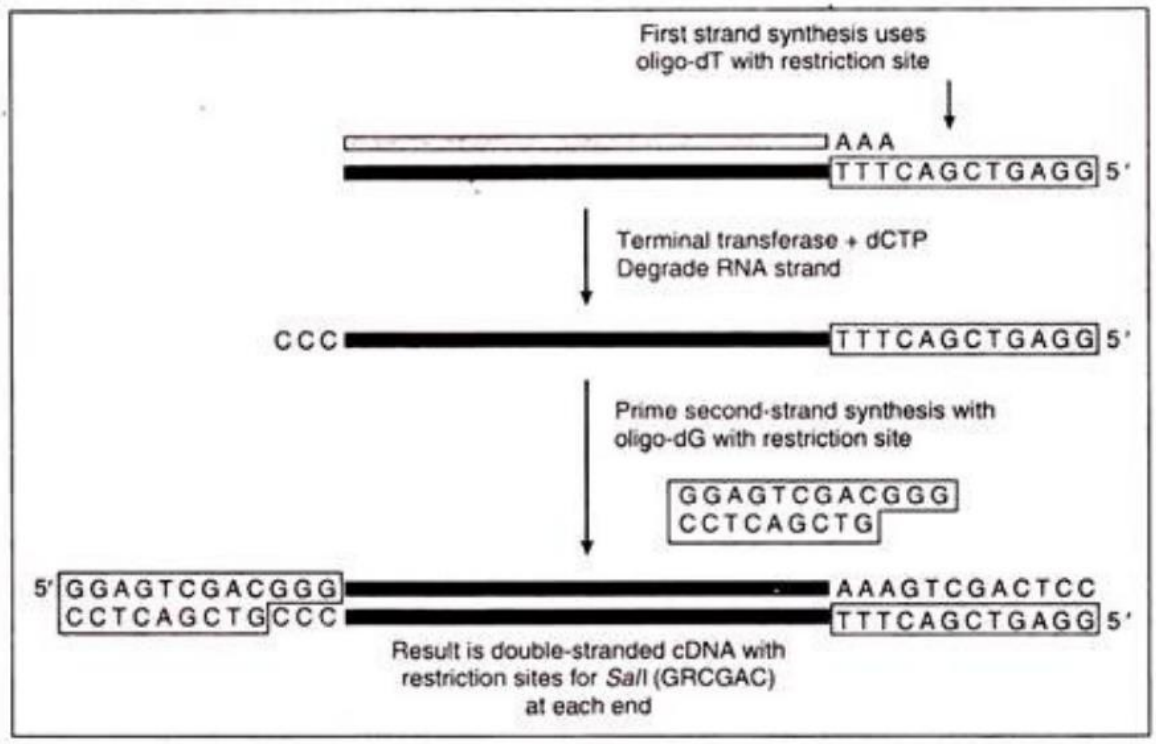
It is possible to adapt the homopolymer tailing method by using primers that are modified to incorporate restriction. In the diagram shown next page, the oligo-dT primer is modified to contain a restriction site (in the figure, a Sail site GTCGAC).

The 3′ end of the newly synthesized first cDNA strand is tailed with C's. An oligo-dG primer, again preceded by a Sail site within a short double-stranded region of the oligonucleotide, is then used for second-strand synthesis.

Note that this method requires the use of an oligonucleotide containing a double-stranded region. Such oligonucleotides are made by synthesizing the two strands separately and then allowing them to anneal to one another.



RACE. (a) 3′RACE. The first primer is the oligo-dT-adaptor molecule. The second primer (open box) is internal to the coding sequence of interest. This is used in conjunction with the adaptor primer (rather than the oligo-dT-adaptor primer) in subsequent PCR. (b) 5′RACE. Synthesis of the first cDNA strand uses a primer within the coding region (open box). The first cDNA strand is tailed with oligo-dA. A second DNA strand is synthesized with an adaptor-oligo-dT primer. This is followed by PCR with (i) a primer nested within the coding sequence (shaded box) and (ii) the adaptor.



Modification of homopolymer tailing, incorporating restriction sites

Advantages of cDNA Library:

A cDNA library has two additional advantages. First, it is enriched with fragments from actively transcribed genes. Second, introns do not interrupt the cloned sequences; introns would pose a problem when the goal is to produce a eukaryotic protein in bacteria, because most bacteria have no means of removing the introns.

Disadvantages of cDNA Library:

The disadvantage of a cDNA library is that it contains only sequences that are present in mature mRNA. Introns and any other sequences that are altered after transcription are not present; sequences, such as promoters and enhancers, that are not transcribed into RNA also are not present in a cDNA library.

It is also important to note that the cDNA library represents only those gene sequences expressed in the tissue from which the RNA was isolated. Furthermore, the frequency of a particular DNA sequence in a cDNA library depends on the abundance of the corresponding mRNA in the given tissue. In contrast, almost all genes are present at the same frequency in a genomic DNA library.

Applications of cDNA Library:

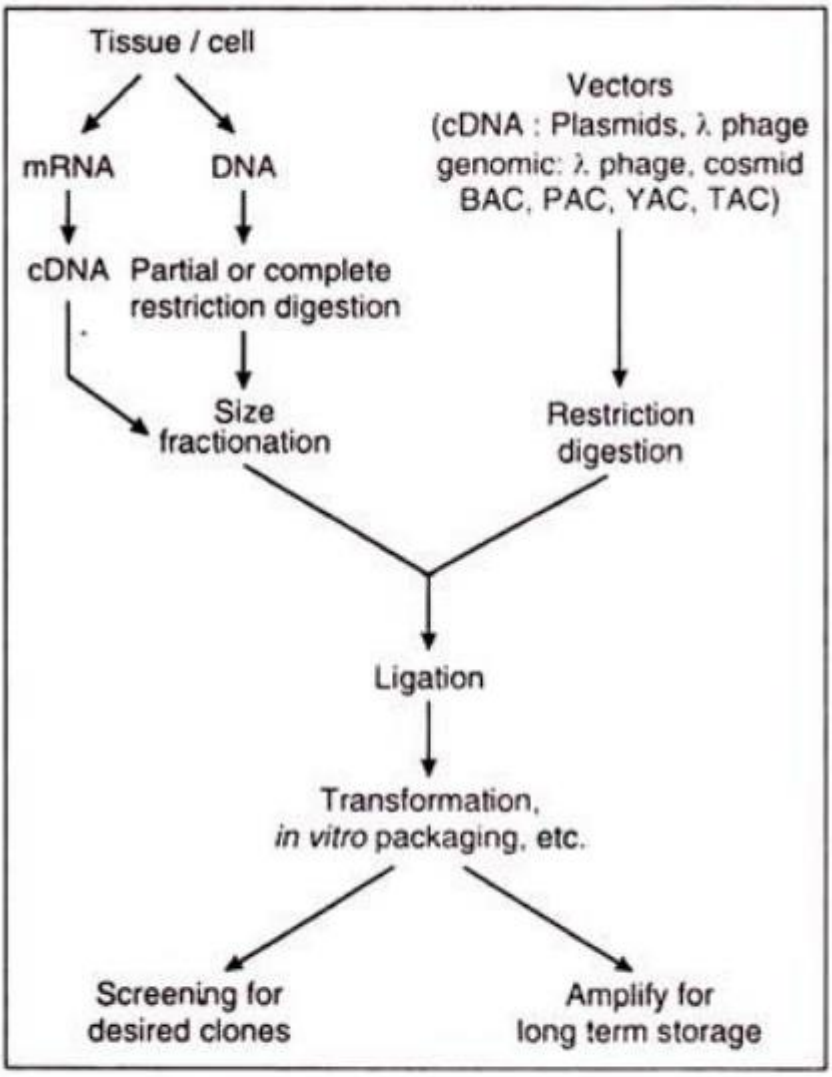
Following are the applications of cDNA libraries:

1. Discovery of novel genes.

2. Cloning of full-length cDNA molecules for in vitro study of gene function.

3. Study of the repertoire of mRNAs expressed in different cells or tissues.

4. Study of alternative splicing in different cells or tissues.



Flow chart showing the construction of genomic and cDNA library

II. Genomic Libraries

Genomic libraries are libraries of genomic DNA sequences. These can be produced using DNA from any organism.

Table 6.1: Vectors used in the construction of genomic library

Vector	Size	Remarks
BAC (bacterial artificial chromosome)	Up to 300 kb Average: 100 kb	• Plasmid vector containing the F factor replicon • One copy per bacterial cell.
Bacteriophage P1	Maximum about 100 kb	• Deletion version of natural phage genome. • P1 phage genome is about 100 kb • Efficient packaging system • pac cleavage site for recognition • P1 plasmid replican and inducible P1 lytic replican • lox P site for cre action
PAC (P1 derived artificial chromosome)	Similar to BAC	• A combintion of BAC and P1 features
TAC (transformable artificial chromosome)	Similar to P1	• With P1 plasmid replican (single copy in *E. coli*) and Ri-plasmid replican (single copy is A. tumefaciens) • With T-DNA border and can transform plant directly
YAC (Yeast artificial chromosome)	230-1700 kb (length of natural yeast chromosome) Average: 400-700 kb	• Propagate in *S. cerevisiae* • Three major elements: → Centromere for nuclear division → Telomere for marking the end of chromosme → Origin of replication for initiation of new DNA synthesis when the chromosome divides • An improtant tool to map complex genomes • Problems: Chimera, instability (rearrangement)
λ phages	Up to 20-30 kb	• Genome size is about 47 kb • Packaging system is efficient and can handle total size of 78-105% of the λ-genome • Replacement vector system is usually employed • Pre-digested arms are commercially available for library constructions • Useful for study of individual genes.
Cosmid	35-45 kb	• Plasmid contain the cos site of λ phage and hence can use λ phage packaging system • Propagate in *E. coli* as plasmids • Useful for subcloning of DNA inserts from YAC, BAC, PAC, etc.
Fosmids	Similar to cosmid	• Contain F plasmid origin of replication and λ cos site • Low copy number and hence more stable

2. Principle of Genomic Libraries:

A genomic library contains all the sequences present in the genome of an organism (apart from any sequences, such as telomeres that cannot be readily cloned). It is a collection of cloned, restriction-enzyme-digested DNA fragments containing at least one copy of every DNA sequence in a genome. The entire genome of an organism is represented as a set of DNA fragments inserted into a vector molecule.

3. Vectors used for the Construction of Genomic Library:

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

- The size of the DNA insert that these vectors can accommodate.
- The size of the library that is necessary to obtain a reasonably complete representation of the entire genome.
- The total size of the genome of the target organism.
- In the case of organism with small genomic sizes, such as E. coli, a genomic library could be constructed by using a plasmid vector. In this case only 5000 clones (of average DNA insert size 5kb) would give a greater than 99% chance of cloning the entire genome (4.6 $x10^6$ bp).
- Most libraries from organisms with larger genomes are constructed using lambda phage, BAC or YAC vectors. These accept DNA inserts of approximately 23,45,350 and 1000kb respectively. Due to this, fewer recombinants are needed for complete genome coverage in comparison to the use of plasmids.

Size of Genomic Library:

- It is possible to calculate the number (N) of recombinants (plaques or colonies) that must be in a genomic library to give a particular probability of obtaining a given sequence.

The formula is:

N = In (1 – P)/ln (1 – f),

where 'P' is the desired probability and 'f is the fraction of the genome in one insert. For example, for a probability of 0.99 with insert sizes of 20kb this values for the E. coli (4.6 x 10^6 bp) and human (3 x 10^9 bp) genomes are:

$N_{g\ coli}$ = In (1 – 0.99) / In [1 – (2 x 10^4/4.6 x 10^6)] = 1.1 x 10^3

N_{human} = In (1 – 0.99)/ In [1 – (2 x 10^4/3 x 10^9)] = 6.9 x 10^5

These values explain why it is possible to make good genomic libraries from prokaryotes in plasmids where the insert size is 5-10 kb, as only a few thousand recombinants will be needed.

5. Types of Genomic Libraries:

Depending on the source of DNA used forced construction of genomic library it is of following two types:

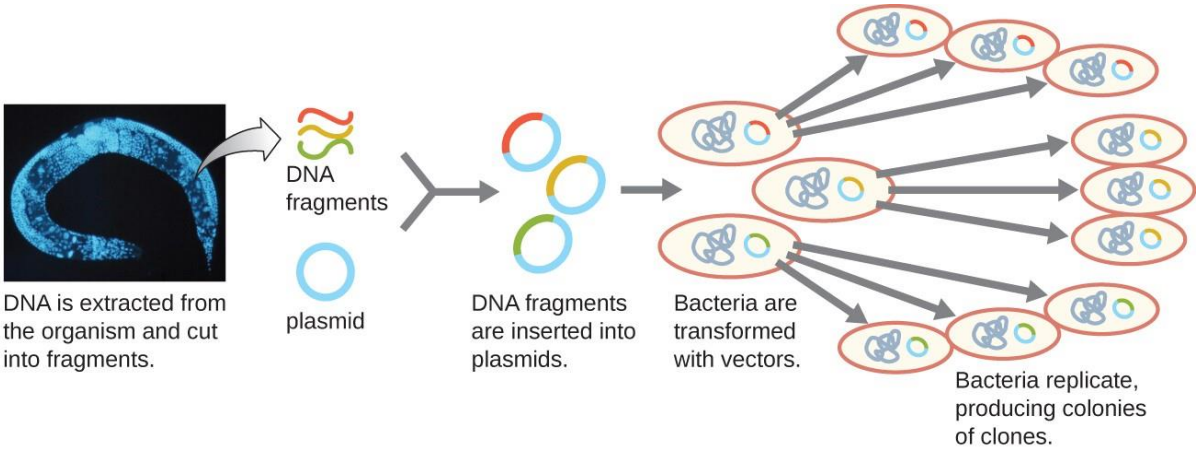
(a) Nuclear Genomic Library:

This is genomic library which includes the total DNA content of the nucleus. While making such a library we specifically extract the nuclear DNA and use it for the making of the library.

(b) Organelle Genomic Library:

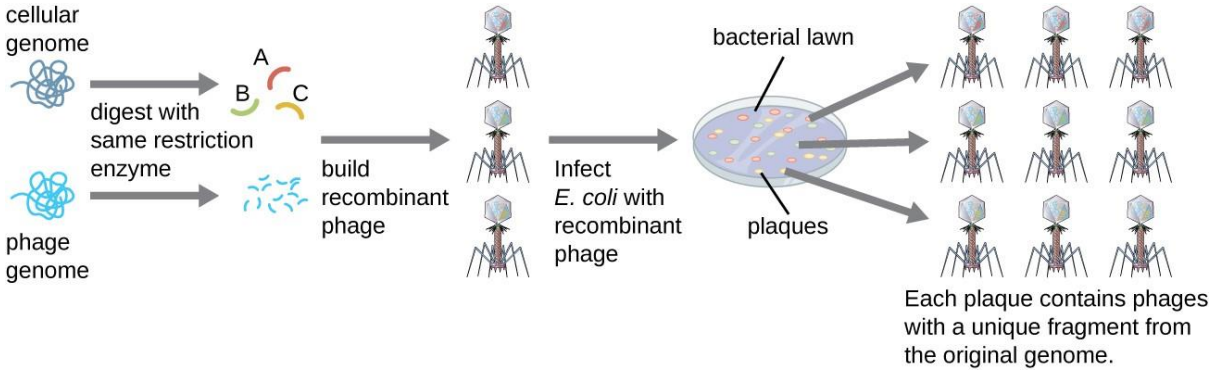
In this case we exclude the nuclear DNA and targets the total DNA of either mitochondria, chloroplast or both.

One method for generating a genomic library is to ligate individual restriction enzyme-digested genomic fragments into plasmid vectors cut with the same restriction enzyme (Figure). After transformation into a bacterial host, each transformed bacterial cell takes up a single recombinant plasmid and grows into a colony of cells. All of the cells in this colony are identical **clones** and carry the same recombinant plasmid. The resulting library is a collection of colonies, each of which contains a fragment of the original organism's genome, that are each separate and distinct and can each be used for further study. This makes it possible for researchers to screen these different clones to discover the one containing a gene of interest from the original organism's genome.



The generation of a genomic library facilitates the discovery of the genomic DNA fragment that contains a gene of interest

To construct a genomic library using larger fragments of genomic DNA, an *E. coli* bacteriophage, such as **lambda**, can be used as a host (Figure 6). Genomic DNA can be sheared or enzymatically digested and ligated into a pre-digested bacteriophage lambda DNA vector. Then, these recombinant phage DNA molecules can be packaged into phage particles and used to infect *E. coli* host cells on a plate. During infection within each cell, each recombinant phage will make many copies of itself and lyse the *E. coli* lawn, forming a plaque. Thus, each plaque from a phage library represents a unique recombinant phage containing a distinct genomic DNA fragment. Plaques can then be screened further to look for genes of interest. One advantage to producing a library using phages instead of plasmids is that a phage particle holds a much larger insert of foreign DNA compared with a plasmid vector, thus requiring a much smaller number of cultures to fully represent the entire genome of the original organism.



Storage of Genomic Library:

Once a genomic library has been made it forms a useful resource for subsequent experiments as well as for the initial purpose for which it was produced. Therefore, it is necessary to store it safely for future use. A random library will consist of a test tube containing a suspension of bacteriophage particle (for a phage vector).

The libraries are stored at – 80°C. Bacterial cells in a plasmid library are protected from the adverse effects of freezing by glycerol, while phage libraries are cryoprotected by dimethyl sulfoxide (DMSO).

2. Applications of Genomic Library:

Genomic library has following applications:

1. It helps in the determination of the complete genome sequence of a given organism.

2. It serves as a source of genomic sequence for generation of transgenic animals through genetic engineering.

3. It helps in the study of the function of regulatory sequences in vitro.

4. It helps in the study of genetic mutations in cancer tissues.

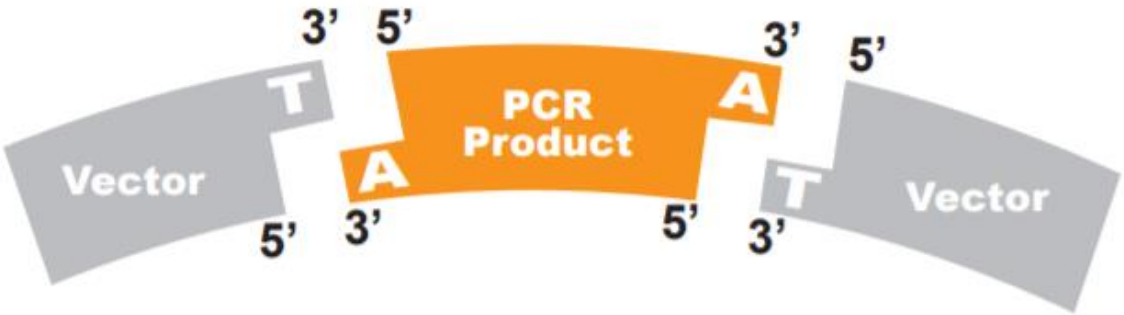
5. Genomic library helps in identification of the novel pharmaceutical important genes.

6. It helps us in understanding the complexity of genomes.

III. PCR based cloning approach (TA cloning).

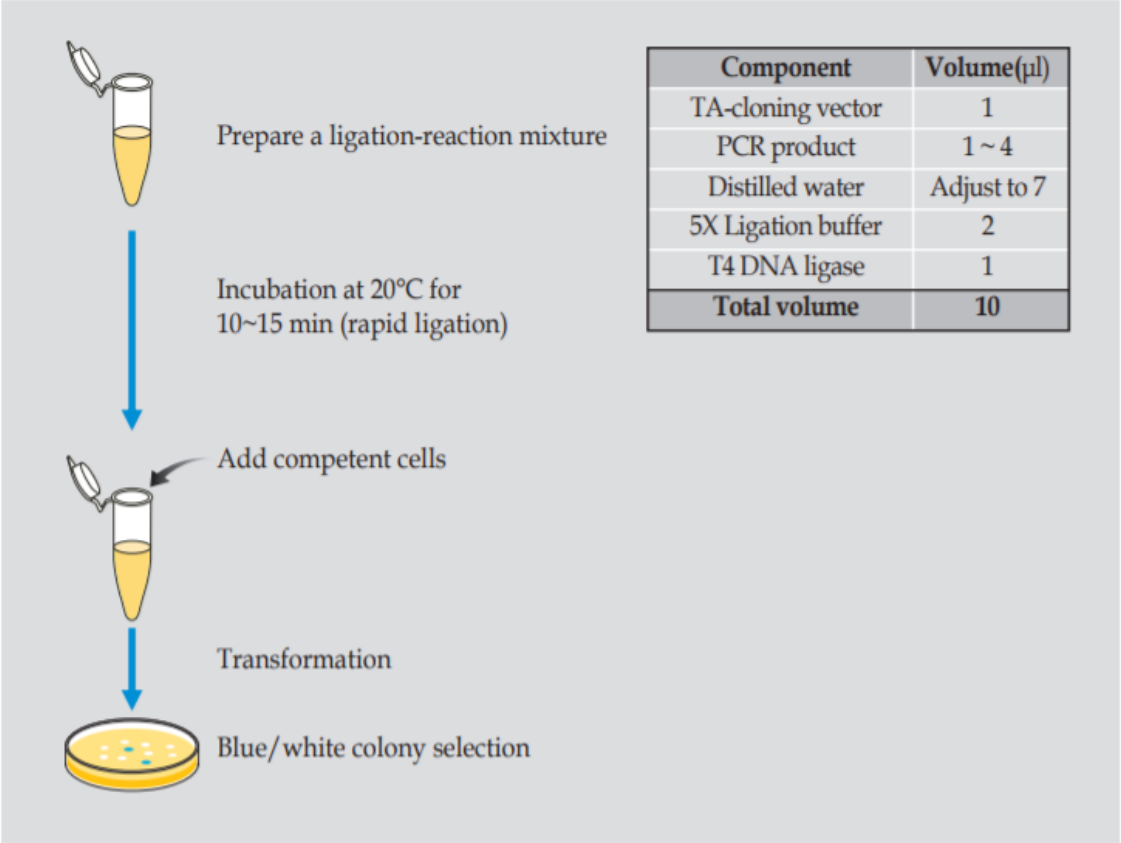
TA-cloning technology exploits the terminal transferase activity of some DNA polymerases such as Taq DNA polymerase and other non-proofreading DNA polymerase. These enzymes preferentially add a 3'-end A-overhang to PCR products. This allows the direct insertion of such PCR products into the prelinearized cloning vector, which has a T overhang on each 3'-end. This eliminates the need for restriction enzyme digestion of the vector or insert, primers with built-in restriction sites, or specially designed adapters, resulting in a much more efficient and robust cloning procedure. This technique is especially useful when compatible restriction sites are not available for the subcloning of DNA fragments.

The PCR product and TA-cloning vector with a T-overhang on each 3'-end can be ligated under the action of T4 DNA ligase. The mechanism of T4 DNA ligase is to form covalent phosphodiester bonds between 3'-hydroxyl ends of Aoverhang in PCR product with 5'-phosphate ends of T-overhang in TA-cloning vector. ATP is required for the ligase reaction.



Schematic diagram for TA-cloning

Flow-chart of TA-cloning

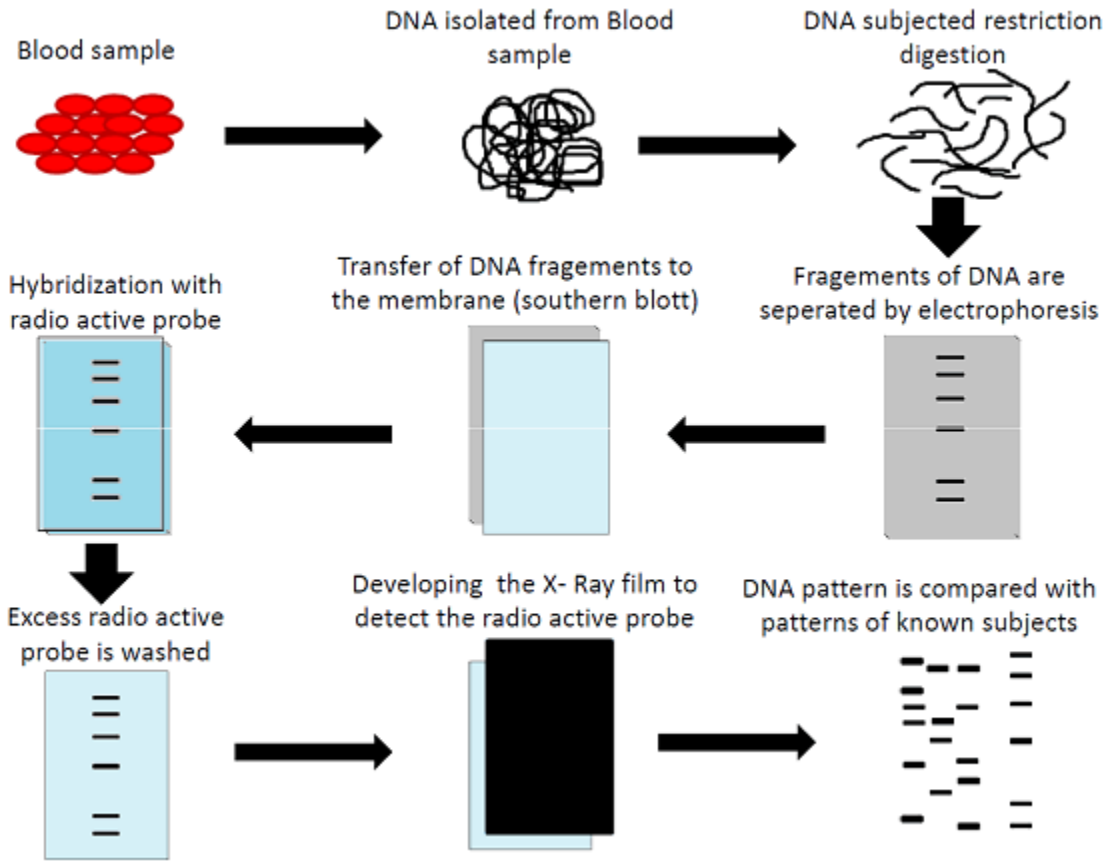


Component	Volume(μl)
TA-cloning vector	1
PCR product	1 ~ 4
Distilled water	Adjust to 7
5X Ligation buffer	2
T4 DNA ligase	1
Total volume	**10**

Overview of TA-cloning

Unit V

Mind Map



Blotting

The first blotting technique described was the Southern blot; published in 1975 by E. M. Southern. He described a technique for detecting specific DNA fragments after electrophoresis so a specific gene could be isolated from a complex DNA mixture. Since then, electrophoresis/blotting has been done with RNA (northern blots) and proteins (western blots). There has been lots of speculation on how you would run an eastern, but no one has solved it to date. [Note that the only technique that earns a capital initial is Southern!] Blots done without electrophoresis are called dot assays or dot blots or slot blots if a linear slot was used – often with filter paper migration of the target towards the probe. All blotting assays can be qualitative or quantitative depending on the fine details of the protocol. Detection of the DNA and RNA bands on the membrane is accomplished by the incorporation of some type of label in a specific probe. Probes will be described below. Western blots and protein dot blots are detected using specific antibodies, antibody conjugates and substrates. Western blots differ from DNA/RNA blots in that electrophoresis is used to accomplish the transfer of

the proteins from the acrylamide gel to the membrane. Western blotting will be explored in detail in Module III, Protein Techniques.

In Southern/northern blotting, the DNA fragments/RNA molecules are separated according to size by gel electrophoresis in agarose gel. The technique is identical for RNA from DNA except for the reagents used and the sampling precautions of working with RNA (Module 2). Northern gels contain formamide – a potent inhibitor of RNases, so your sample is safe once it is in the gel. The next step in the Southern/northern blot technique is to transfer the bands of DNA to an inert membrane. When the membrane is being prepared for testing with a DNA probe, the DNA must be denatured to single stranded DNA before being fixed to the membrane. This may be accomplished by soaking the gel in sodium hydroxide or denaturing the DNA after it is blotted on the inert membrane.

Nitrocellulose or positively-charged nylon filters are used for blotting. The agarose gel is placed between several sheets of filter paper soaked in buffer, the nitrocellulose filter sits on top of this and paper towels sit on top of the nitrocellulose. As the buffer is drawn thought the agarose gel, it carries the DNA to the nitrocellulose or nylon filter, where it sticks. The blotting procedure may be accelerated by transmitting a vacuum through the gel and filter paper (vacuum blotting). Once the DNA is transferred to the filter, the DNA is permanently fixed the membrane if 1) it is the right kind of charged membrane, 2) the filter is heated several hours in a vacuum oven or seconds in a microwave, or 3) crosslinked by UV light (special UV linkers or your gel transilluminator).

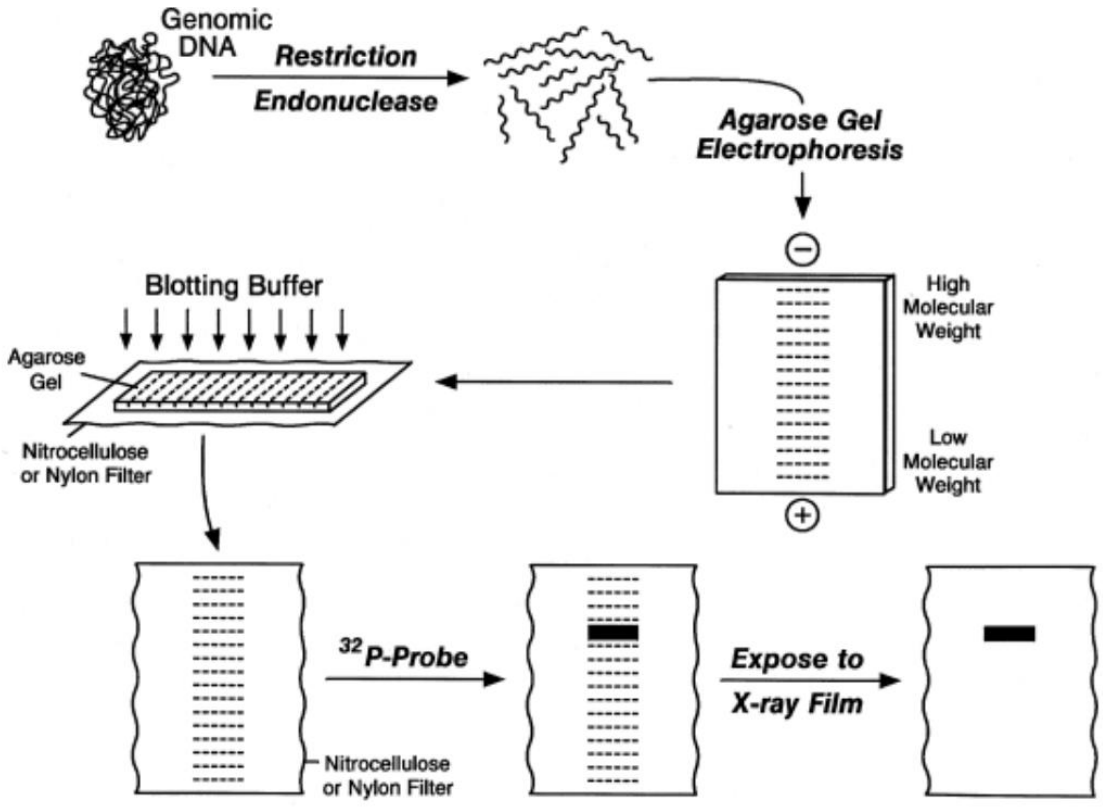
I. Southern hybridization

- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.

- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/ Steps

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



Step I: Restriction digest

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

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

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

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

Step IV: Blotting

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

Step V: Baking and blocking

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

Step VI: Hybridization with labelled probes

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

Step VII: Visualization by Autoradiogram

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

Application of Southern blotting:

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

II. Preparation of radiolabelled and non-radiolabelled DNA & RNA probes

Nucleic acids may be modified with tags that enable detection or purification. The resulting nucleic acid probes can be used to identify or recover other interacting molecules. Common labels used to generate nucleic acid probes include radioactive phosphates, biotin, fluorophores and enzymes. In addition, the bioconjugation methods used for nucleic acid probe generation may be adapted for attaching nucleic acids to other molecules or surfaces to facilitate targeted delivery or immobilization, respectively.

Nucleic acid probes can be labeled with tags or other modifications during synthesis. However, purchasing custom oligonucleotide probes (especially RNA) can be quite expensive depending on the modification and whether costly purification services are required. Additionally, the minimum order quantity for modified oligonucleotides is typically much higher than unmodified versions and may be excessive compared to the amount required for the intended application. Because of this, many researchers may choose in-house methods or labeling kits for probe generation.

Numerous reagents are available for quick and efficient benchtop oligonucleotide labeling, and they are most useful for making small amounts of probe or when many different probes with the same label are required (i.e., for mutational analysis). For small-scale probe generation needs, enzymatic methods are an economical method for labeling probes. In contrast, chemical methods are amenable to larger scale reactions. There are enzymatic and chemical methods for creating probes labeled at either the 5′ or 3′ ends of the oligonucleotide as well as randomly incorporated throughout the sequence. The choice of method needed is determined in part by the degree of labeling required and whether the modification will cause steric hindrance that prevents the desired interactions. Typically, nucleic acids hybridization reactions (i.e., northern blotting) benefit from the high specific activity gained through random incorporation of label into a probe. However, assays requiring protein interactions (i.e., gel shift and pull-down assays) require end-labeling to allow protein binding.

Summary of nucleic acid labeling methods

Method		Effective for	Labeling site	Recommended for
Enzyme	TdT	ssDNA	3′	modified nucleotide incorporation
	T4 RNA ligase	ssDNA, RNA	3′	modified nucleotide incorporation (including isotopes)
	T4 PNK	ssDNA, RNA	5′	phosphate isotopes
	DNA polymerase	DNA, RNA[3]	5′[1],3′[2], random	modified nucleotide incorporation (including isotopes)
	RNA polymerase	RNA	random	modified nucleotide incorporation (including isotopes)
Chemical	Periodate	RNA	3′	amine- or hydrazide-modified tag addition
	EDC	DNA, RNA	5′	amine- or hydrazide-modified tag addition
	Nonspecific crosslinkers	DNA, RNA	random	psoralen-, phenyl azide-, or ULS-modified tag addition

1. 5′ end-labeled primers can be used with this method in order to add a 5′ modification to a DNA probe.
2. Modified nucleotides can be added to the 3′ recessed-end of double-stranded DNA during fill-in reactions.
3. Modified nucleotides can be added to the 3′ recessed-end of RNA when hybridized to a complementary DNA oligonucleotide producing a 5′ overhang.

a) Terminal deoxynucleotidyl transferase (TdT)

Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase enzyme expressed in certain populations of lymphoid cells. TdT typically adds numerous deoxynucleotides to the 3′ terminus of a DNA strand, but reaction conditions can be optimized such that only 1–3 incorporation events occur. TdT is template independent and not significantly affected by DNA sequence, but DNA structure is important. TdT has the highest activity towards the 3′ end of single-stranded DNA but can also modify the 3′ overhang of double-stranded DNA with lower efficiency. TdT has poor activity towards double-stranded DNA with blunt ends or 5′ overhangs. Common sources of DNA templates modified with TdT include unlabeled, single-stranded PCR primers and double-stranded restriction endonuclease fragments with 3′ overhangs (“sticky ends”, 5′ recessed ends).

TdT is often used to label DNA probes for RACE (Rapid Amplification of cDNA Ends), TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) assays and as a method for adding 3′ overhangs to DNA fragments to facilitate cloning. TdT can also be used

to label the 3′ end of DNA probes with radioactive and nonradioactive tags for a variety of detection and affinity applications. For example, TdT addition of biotin-11-UTP to the 3′ end of complementary DNA probes is an effective way of creating probes for use in nonradioactive electrophoretic mobility shift assays (EMSA) and DNA pull-down assays. The following graph shows a comparison of TdT incorporation of several different modified nucleotides.

b) T4 RNA ligase

T4 RNA ligase is an enzyme coded for in the genome of the T4 bacteriophage. T4 RNA ligase catalyzes the attachment of a terminal 5′-phosphate to a terminal 3′-hydroxyl group on RNA. T4 RNA ligase is template independent but requires single-stranded RNA and ATP. Though the primary substrate for T4 RNA ligase is RNA, reaction conditions can be optimized for single-stranded DNA molecules; however, efficiency is very low.

T4 RNA ligase is used for labeling the 3′ end of RNA with [5′ ^{32}P]pCp (cytidine-3',5'-bis-phosphate), modifying mRNA for cDNA library generation and performing 5′-RACE. T4 RNA ligase can also be used to 3′ end-label RNA with nonradioactive tags using an appropriately modified nucleoside 3',5'-bisphosphate.

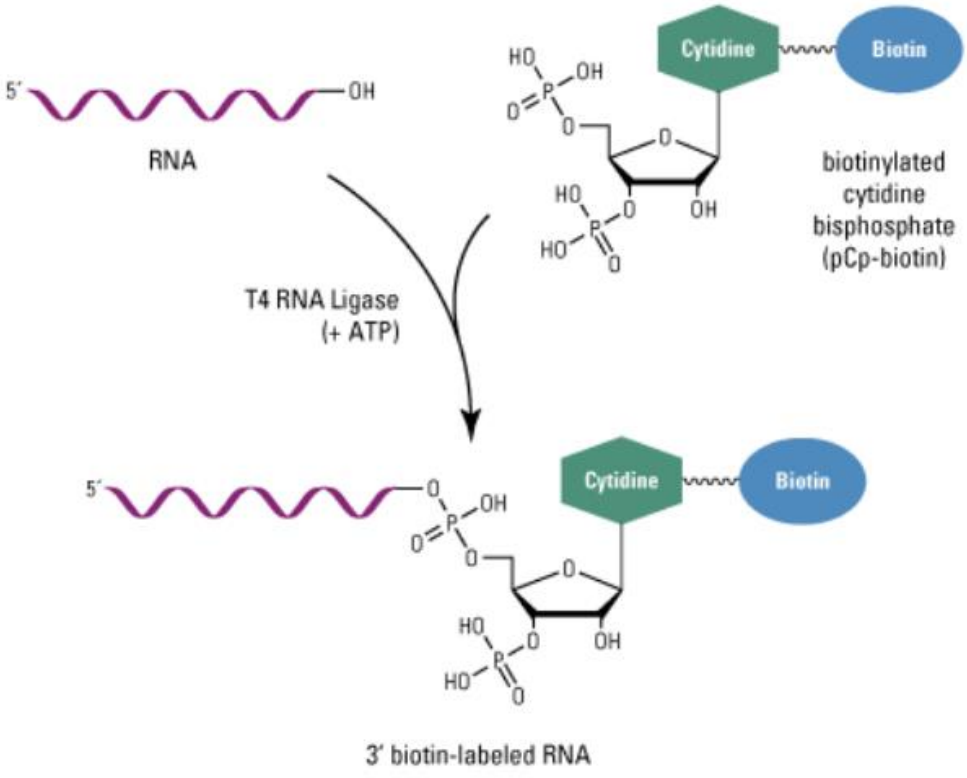


Fig. Chemical process for labeling the 3′ end of RNA using T4 RNA ligase.

c) T4 polynucleotide kinase (PNK)

T4 polynucleotide kinase (T4 PNK) is an enzyme coded for in the genome of the T4 bacteriophage. T4 PNK transfers an organic phosphate from the gamma position on ATP to the 5′-hydroxyl group of DNA and RNA. The wild-type enzyme also has 3′-phosphatase activity. T4 PNK ligase is template independent and modifies single-stranded polynucleotides and 5′ overhangs efficiently. Blunt-ended and 5′ recessed ends can be modified with reduced efficiency.

T4 PNK is used primarily for labeling the 5′ ends of polynucleotides with radioactive phosphate from isotope-modified ATP. PNK is more efficient at modifying short overhangs and blunt-end fragments than TdT or T4 RNA ligase. While it is possible to perform phosphate-exchange reactions, PNK labeling is most efficient when the 5′ end of the target molecule has been dephosphorylated. Another common use of PNK is the 5′ phosphorylation of synthetic polynucleotides (i.e., DNA primers) to facilitate cloning. The Invitrogen KinaseMax 5' End-Labeling Kit allows the efficient end-labeling of DNA or RNA to high specific activity with T4 polynucleotide kinase and [gamma-^{32}P] ATP, or quantitative phosphorylation of 5' ends using unlabeled ATP. The kit includes sufficient reagents for 30 reactions.

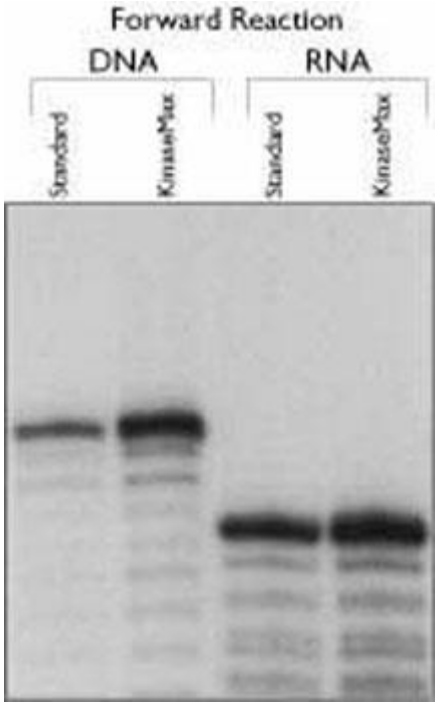


Fig: 5’ end-labeling reactions with T4 PNK. Comparison of 5’ end-labeling reactions

d) DNA polymerase

DNA polymerases are a family of enzymes that create deoxyribonucleic acid polymers by catalyzing the joining of the 5′-phosphorylated end of a deoxyribonucleotide (monomer) to the 3′-hydroxyl end of an existing DNA strand (DNA elongation) or primer (primer extension). DNA polymerases are template dependent but not sequence dependent. To synthesize DNA, the 3′-OH end of an existing DNA strand must be annealed to a complementary strand of DNA. Viral reverse transcriptase enzymes are an exception to this rule and make use of DNA or RNA primers and a RNA template for the synthesis of complementary DNA. The DNA polymerase will synthesize a new DNA strand through elongation of the existing 3′-OH end, adding individual nucleotides complementary to the template strand being read. DNA polymerases are used for a variety of lab purposes from cloning to sequencing. Applications requiring the amplification of a specific fragment of DNA are performed with the aid of heat stable enzymes cloned from thermophilic organisms (e.g., *Thermus aquaticus* (*Taq*), *Bacillus stearothermophilus* (*Bst*), *Thermococcus litoralis* (Vent)) using the polymerase chain reaction (PCR). For applications in which amplification is not required, DNA polymerases from mesophilic organisms (e.g., *E. coli* (Klenow) and bacteriophage (T4, T7)) may be used depending on the length of the DNA to be synthesized and the degree of replication fidelity that is required.

Probes generated with the aid of DNA polymerase are most commonly made by the random incorporation of modified nucleotides during the DNA replication process, which can be done by PCR or simple primer extension reactions. Probes generated in this manner have high specific activity and allow the detection of small quantities of target. Traditionally, this method required radioactive nucleotides to generate probes; however, biotin, fluorophores and other nonradioactive tags can be used if the modification does not interfere with the polymerase elongation reaction (except for terminator sequencing applications). For applications requiring a lower specific activity or when targeted labeling is desired, DNA polymerase can be used to specifically label the ends of a DNA probe. To label the 5′-end of a DNA probe, a 5′ end-modified primer must be used. This method is particularly useful when adding affinity tags or fluorophores to DNA probes that are too long for efficient

automated synthesis. This method is ideal for 5′ end-labeling by PCR or simple primer extension reactions.

To label DNA probes at or near the 3′-end, DNA polymerase can be used to incorporate one or more modified nucleotides into the end of a double-stranded probe with a recessed 3′-end. This "fill-in" reaction can be performed with staggered annealed primers, restriction fragments or any other double-stranded DNA molecule in which a complementary DNA sequence can be annealed and used as the template for the extension of a 3′-end. Klenow fragment (*E. coli* DNA polymerase I) is commonly used for fill-in reactions and can even be used to 3′ end-label RNA molecules when hybridized to a DNA primer producing a 3′ recessed-end.

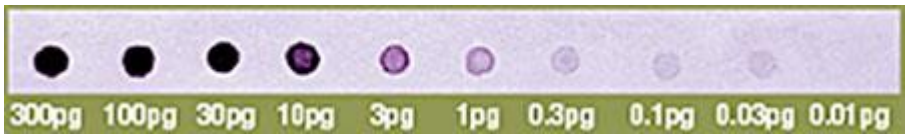


Fig: Dot blot with biotin-labeled DNA using Klenow fragment.

e) RNA polymerase

RNA polymerases are a family of enzymes that create ribonucleic acid polymers by catalyzing the joining of the 5′-phosphorylated end of a ribonucleotide (monomer) to the 3′-hydroxyl end of a previously incorporated ribonucleotide. RNA polymerases are template dependent and sequence dependent, requiring a promoter sequence within the template DNA in order to initiate binding of the enzyme and, depending on the host system, various cofactors are required for RNA transcription to proceed on the single-stranded template.

RNA polymerases are used for a variety of lab purposes, from the *in vitro* synthesis of mRNA to the generation of probes for hybridization and binding assays. Probes generated with the aid of RNA polymerase are made by the random incorporation of modified nucleotides during the transcription process. This is typically done by cloning a cDNA or other template sequence into a plasmid containing one or more RNA polymerase promoter sequences. RNA probes generated in this manner have high specific activity and are most commonly made with radioactive nucleotides, although biotin and other tags are also available. Thermo Scientific Bacteriophage T7 RNA polymerase is a DNA-dependent RNA polymerase with strict specificity for its respective double-stranded promoters. It catalyzes

the 5'-to-3' synthesis of RNA on either single-stranded DNA or double-stranded DNA downstream from its promoter.

Fig: T7 RNA polymerase promoter sequence. A linear DNA template with this promoter sequence can be used with T7 RNA polymerase to *in vitro* transcribe labeled RNA probes. The +1 position indicates the first nucleotide that is incorporated into the RNA during transcription. The bases at positions +1 through +3 are critical for transcription and must be G and 2 purine bases, respectively. Use of T7 RNA polymerase with this promoter sequence can result in the generation of labeled RNA probes.

f) Chemical methods for nucleic acid labeling

Periodate oxidation of RNA

Meta- and *ortho*-periodates (IO_4 and IO_6, respectively) are anions formed from iodine and oxygen and commonly found as salts with potassium (e.g., KIO_4) or sodium (e.g., $NaIO_4$). In solution, periodate cleaves the bonds between adjacent carbon atoms having hydroxyl groups (vicinal diols or *cis*-glycols), creating two aldehydes groups. The resulting aldehyde groups are spontaneously reactive toward primary amine-containing molecules and surfaces. Aldehydes can be used in two types of coupling reactions with either primary amine- or hydrazide-activated tags. Primary amines react with aldehydes to form Schiff bases, which readily hydrolyze and must be stabilized through their reduction to secondary amine bonds with sodium cyanoborohydride ($NaBH_4$). Hydrazide-modified molecules also spontaneously react with aldehydes but form fairly stable hydrazone linkages, making the reaction much more efficient. The addition of sodium cyanoborohydride will increase the reaction efficiency further and increase bond stability over time and changes in pH.

Because of its mild oxidative properties, sodium *meta*-periodate is often used as the oxidizer of protein carbohydrates to generate reactive aldehyde groups for either detection or chemical conjugation procedures. The vicinal diols of ribose in RNA nucleotides can also be cleaved with periodate, enabling this method to be used to add a single 3′-end label to RNA but not DNA.

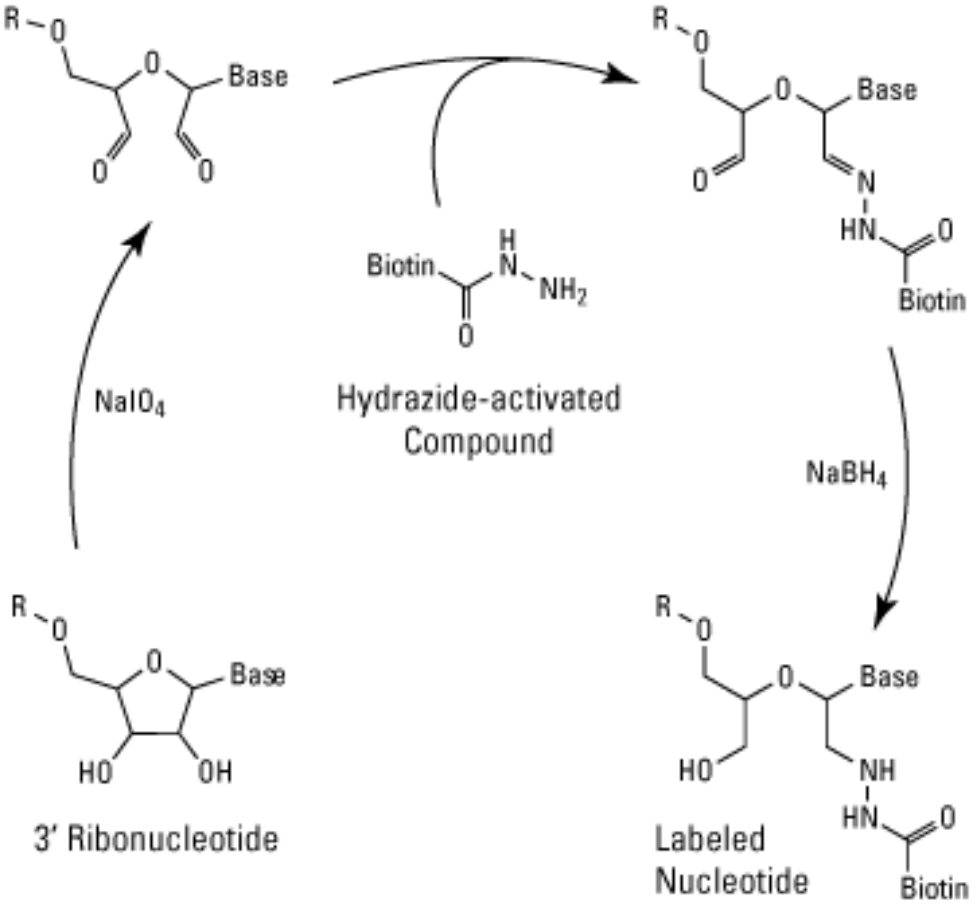


Fig: Cleavage of RNA by sodium meta-periodate.

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g) EDC activation of 5′ phosphate

Carbodiimides are functional groups (RN=C=NR) that are typically used in organic synthesis to activate the formation of amide or phosphoramidate linkages between primary amines (RNH_2) and carboxylate (RCOOR′)- or phosphate ($R\text{-}PO_4$)-containing molecules, respectively. Unlike most crosslinkers, carbodiimides do not become part of the final crosslink between the molecules and thus do not add any additional chemical structure to the resulting products. EDC (EDAC, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) is a water-soluble carbodiimide preferred for use in aqueous reactions in the 4.0 to 6.0 pH range, where the reaction byproducts can be easily removed by dialysis or precipitation of the conjugation products.

In reactions with carboxylic acid groups, EDC forms an active *O*-acylisourea intermediate that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture. The same reaction can be performed with phosphates (i.e., 5′ phosphate of oligonucleotides), although imidazole must be included in the reaction to obtain efficient conjugation. Because the 5′ phosphate is required, synthetic oligonucleotides must first be treated with a kinase. With that minor exception, EDC-mediated conjugations are an economical means for coupling both RNA and DNA to nearly any other primary amine-containing molecule or surface.

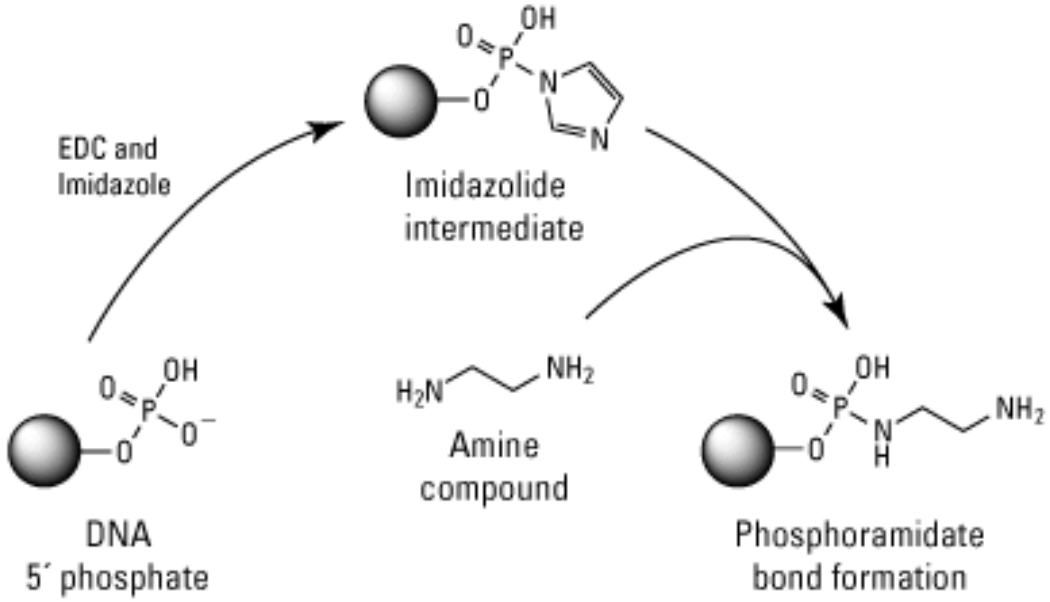

Fig: 5′ phosphate activation by EDC and imidazole.

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h) Chemical random-labeling

Random chemical labeling of nucleic acids can be accomplished by various means. Because these methods label at random sites along the length of a DNA or RNA molecule, they allow a higher degree of labeling to be achieved than end-labeling techniques. However, one disadvantage of these methods is that the nucleotide bases are directly modified, which will reduce or prevent base-pairing between complementary strands during hybridization experiments. Therefore, it may be necessary to balance the degree of labeling with the hybridization efficiency of the probes in certain experiments.

Two types of photoreactive label reagents are used for nucleic acids: phenylazide- and psoralen-based. When the phenylazide functional group is exposed to UV light, it forms a labile nitrene that can insert nonspecifically into double bonds and C-H and N-H sites via addition reactions, provided that more reactive nucleophilic (e.g., primary amines) are not present. Molecules containing the psoralen functional group can be used to label double-stranded DNA or RNA. The psoralen ring structure effectively intercalates into the double-stranded portions, and exposure to UV light causes a cyclo-addition product to be formed with the 5,6-double bond in thymine residues. The ULS labeling reagents contain the proprietary temperature-activated platinum-based moiety which reacts with guanine bases in RNA and DNA and the side chains of methionine, cysteine and histidine residues of proteins.

DNA fragment probes

Probes can be made from any sequence of DNA. The only requirement is that it is complementary to the target. Fragments of known size and sequence can be isolated from agarose or acrylamide gels and purified, then labeled for use as a probe. Alternately, cloned fragments can be cut out from plasmids and purified from the rest of the plasmid also by gel electrophoresis. Once the probe is purified, labeling is performed (labeling will be discussed in a section below). dsDNA (double-stranded) probes made in any fashion need to be denatured just before use into single strands. This is usually done by boiling the probes just before use.

Probe synthesis

Special phage techniques can make ssDNA probes, or PCR can be used to amplify a region, and the amplicons used as probes. In synthesis reactions, the label is commonly incorporated during polymerizeation. RNA probes or “riboprobes” are prepared by transcribing (as compared to PCR – replicating) a double stranded DNA template that has been cloned into a vector. The labeled RNA probe is single stranded and will combine with either DNA or RNA. To bind mRNA, the antisense (opposite strand to mRNA) riboprobe must be prepared.

Oligonucleotide Probes

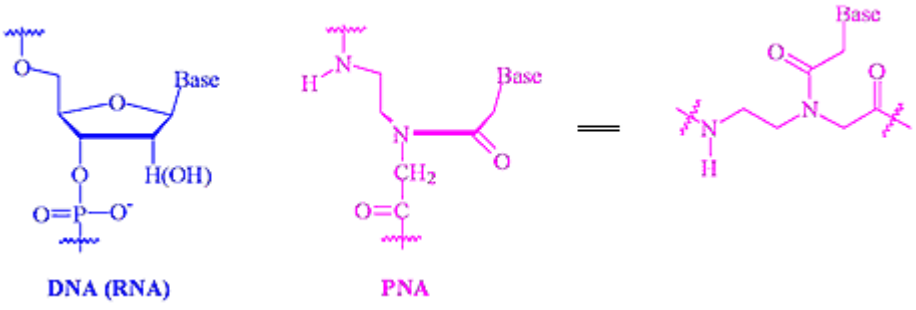
An oligonucleotide is a short fragment (10-100 nucleotides) of single stranded DNA prepared by a series of chemical reactions producing a known sequence of nucleotides. For the oligonucleotide to be used as a probe, the specific nucleotide sequence must be

known before synthesis begins. This information may be obtained directly from analysis of the target DNA or RNA. Less desirably, it is obtained indirectly from the amino acid sequence in the target protein. Each amino acid in a peptide chain is coded by a specific sequence of three nucleotide bases. Once the amino sequence in the protein is worked out, the corresponding nucleotide sequences can be determined.

Oligonucleotide probes are rapidly and inexpensively produced without the need for cloning vectors. The probes are exquisitely specific and a change of a single base pair in the patient sample may prevent binding. Hybridization reactions are faster than those using longer, less specific probes, but amplification may be needed to detect if reactions have taken place. Since the probes are smaller, there are less detection molecules in the test system. Test procedures for oligonucleotide probes will differ from those conventional probes and must be rigidly adhered to.

PNA Probes

Peptide Nucleic Acid probes is a nucleic acid analog with a peptide rather than sugar-phosphate backbone. Without a sugar-phosphate backbone, PNAs carry no charge, thereby exhibiting higher thermal stability (at least 1°C per base as compared to DNA/DNA hybrids) presumably due to the lack of charge repulsion within the duplex. This feature also greatly increases the rate of hybridization, some 50,000 times the rate of equivalent DNA probes. PNA/DNA duplexes also exhibit greater sensitivity to mismatches, a property that has facilitated the development of techniques to detect single base changes in DNA using PNA probes, thus PNA probes can be used as real-time PCR probes. Finally, PNA is not recognized by nucleases or proteases (yet another victim of the lack of electrostatic interactions) and hence is resistant to degradation by enzymes.

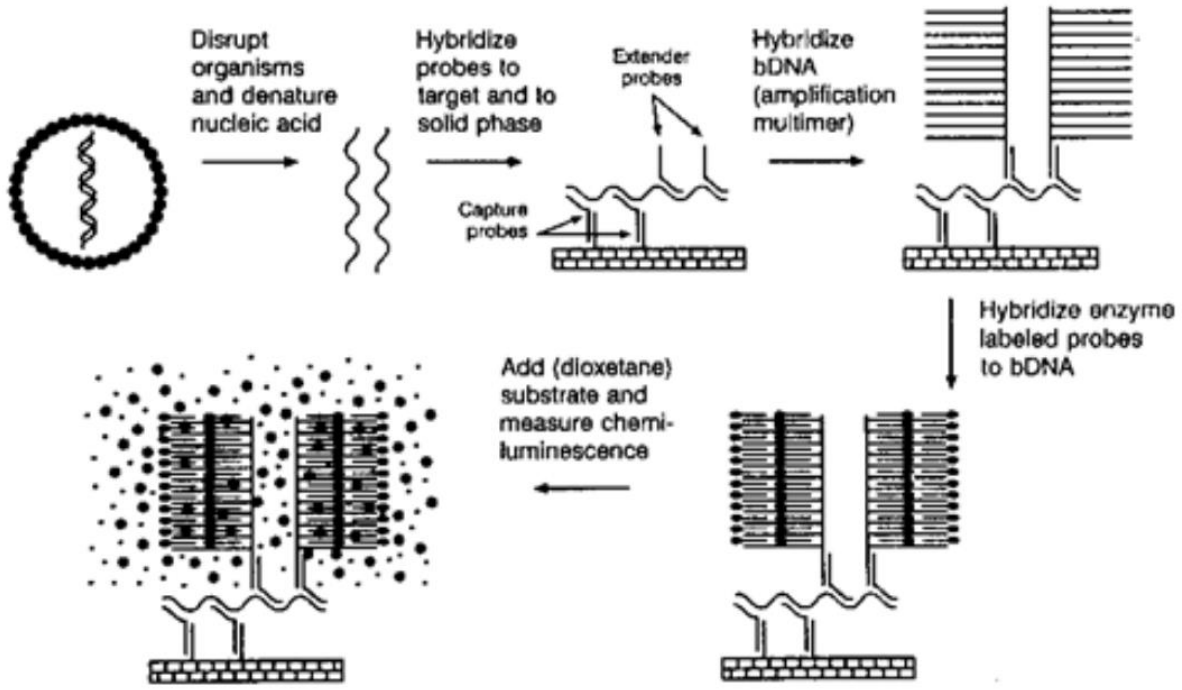


Because of the higher stability of the duplexes formed, smaller probes can be used (8-mers), which further increases the specificity. In addition, the greater efficiency of these molecules means that less probe can be used per experiment. PerSeptive (AP) Biosystems scientists have developed a technique that even takes a number of steps out of the Southern blotting and hybridization technique. By prehybridizing the DNA sample with fluorescent-PNA probes, hybrids that have been separated electrophoretically can be detected directly by fluorescence without any of the time-consuming blotting and hybridization steps. In addition, multiple probes can be run on the same gel, since the hybridization is conducted on the sample rather than on the gel. Even more astounding is the observation that PNA probes hybridize quantitatively in hybridization experiments in situ, an outcome virtually unheard of with this technique.

Signal Amplification

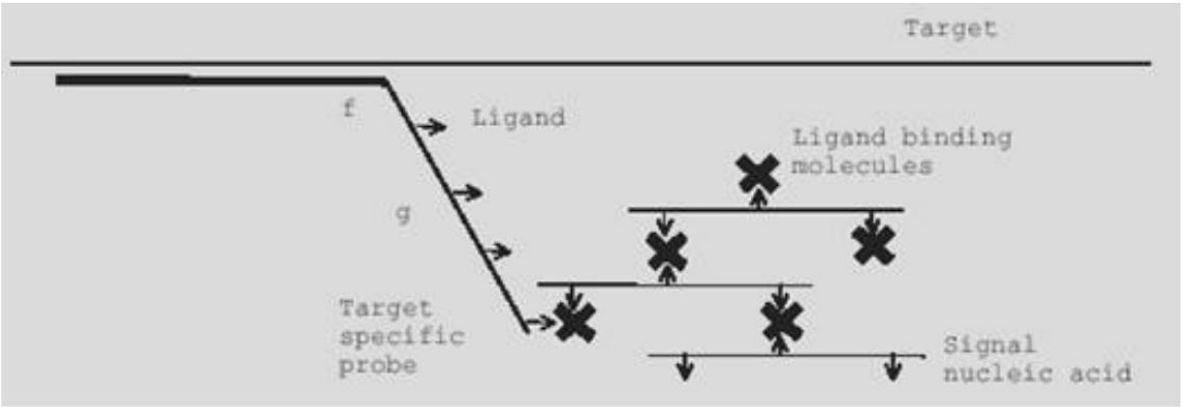
Branched DNA (bDNA technology)

Recently, novel ways of constructing oligonucleotide probes have been developed. For example, branched probes combine several probe sequences into one complex. Target nucleic acid is captured to a solid surface via multiple contiguous capture probes. Extender probes hybridize with adjacent target sequences and contain additional sequences homologous to the branched amplification multimer. Enzyme-labeled oligonucleotides bind to the bDNA via homologous base pairing and the enzyme-probe complex is measured by detection of chemiluminescence (incorporates the emission of light with the emission of limited heat as the result of a chemical reaction taking place). All hybridization reactions occur simultaneously. More detection molecules are incorporated into the probe compared to the methods below, thus increasing the strength of the reporter signal.



Ligand-Binding Probes

Probes are synthesized with biotin in at least 3 positions; at each end and in the middle. Streptavidin is used to cross link the probes, as one streptavidin molecule is able to bind 4 biotins, and labeled probes then used to detect the ligand-bound probes. More below.



Labeling the Probe

The probe must be labeled so it can be detected after hybridization has occurred under test conditions. If the DNA to be labeled as a probe is double stranded, it is heated to produce single stranded DNA. A variety of labeling systems are used.

Radioactive Isotopes

The traditional method of labeling dsDNA sequence probes has been to incorporate 32P into the phosphate portion of some of the nucleotides. This is accomplished by several types of reactions. Nick translation works by mixing the DNA, deoxyribonuclease (DNase), nucleotides containing 32P and DNA polymerase. The DNase breaks or nicks the DNA. New nucleotides containing 32P will be incorporated into the nicks by DNA polymerase. Alternately, end-labeling uses kinase enzyme to attach a 32P-labeled nucleotide to the 3'OH of the strands. Compared to nick translation, end labeling only incorporates one radioactive phosphate/probe. This is useful for Southern or northern blots where there is a good deal of target.

The inclusion of 32P in the nucleotides does not interfere with hybridization reactions. Sample DNA hybridized to the probe can be detected by using an overlay of X-ray film (autoradiography) or scintillation counters (radiation detection is triggered by a flash of light that permits the high-speed counting of particles and measurement of the energy of the radiation).

One of the main disadvantages of this method of labeling is the instability of the 32P. It has a half life of 14 days giving a very short shelf life to the probe. 32P is a high level β particle emitter, thus there are safety concerns to do with the handling and disposal of the radioactive material, but 32P-labeling remains the most inexpensive and sometimes the most sensitive labeling method. Many of the methods below have been combined with the amplification techniques described in module subset II-b and signal amplification above.

Biotin Streptavidin Labeling

Biotin is a water soluble vitamin that can be incorporated into the nucleic acid by using dUTP-biotin instead of the normal dTTP. This is accomplished without altering the ability of the bases to hybridize with complementary bases (dUTP bonds with dATP). After hybridization of the probe with sample nucleic acid, the probe is detected by adding streptavidin that has an enzyme such as horseradish peroxidase coupled to it. Streptavidin has an unusual affinity for biotin equal to that of covalent bonds and will combine with biotin containing probe bringing the peroxidase along with it. The next step in the procedure is to add a chromogenic substrate that changes color because of the action of peroxidase. The color change may be measured by standard procedures.

Solid Phase Labeling/Capture

When using probes to measure hybridization in membranes, the substrate must be one that becomes insoluble and sticks to the reaction site on the membrane for visualization. In-solution assays require the purification of the hybridized probe/target away from any unhybridized target. This can be accomplished by incorporating paramagnetic beads in the probe. A magnet can then be used to capture the hybridized complex. Alternately, one probe can be attached to a surface (a microtitre plate wall, a plastic bead), hybridization is performed and a second probe to a different region of the target is hybridized. This probe is labeled with a detection system label. In these assays, soluble color substrates such as those used in ELISA or chemiluminescent protocols can be used. Indeed, the technique for detection of hybridization using these techniques is very similar to an ELISA protocol. The ELISA (Enzyme-Linked Immunosorbant Assay) technique will be explored in detail in Module III, Protein Techniques.

Alkaline Phosphatase (AP)

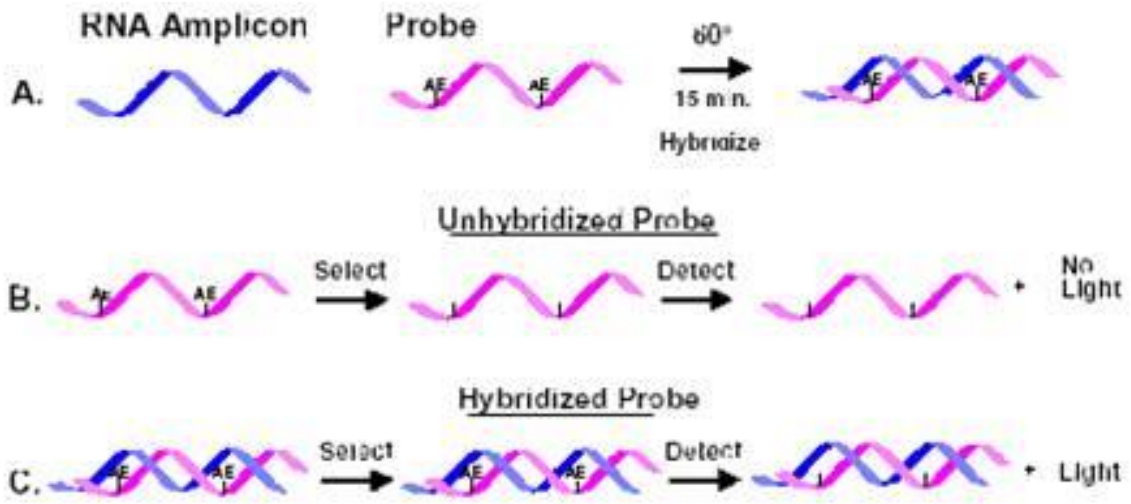
Alkaline phosphate enzyme may be added to a probe with a 12-atom linker arm. Once the probe is hybridized, it is detected by color change in an appropriate substrate caused by the phosphatase enzyme. This is usually used with an oligonucleotide probe and tends to give fast results (one hour).

Antibodies

A highly antigenic compound, digoxigenin, can be attached to the guanine bases of a probe. Hybridized probe can then be detected by adding anti-Dig antibodies. The antibodies are coupled with a detection system such as horseradish peroxidase (HRP). The hybridized probe coupled with antigen, antibody and enzyme can then be detected by standard methods for the specific enzyme used. For example, a chemiluminescent assay can be performed to detect the HRP enzyme coupled to the anitibody.

Chemiluminescence

Probes may be labeled with a chemiluminescent acridinium ester. This label will not emit light after hydrolysis. When the probe hybridizes to the sample DNA or RNA, it is protected from hydrolysis. The test sample is added to the probe and after hybridization has taken place, a hydrolyzing agent is added.



Ester that is not bound will hydrolyze and not emit light. The sample is placed in a luminometer and an increase in relative light units indicated hybridization of the probe. More commonly today, chemiluminescent substrates are used for detection of enzyme labeled probes, either AP or HRP. The major advantages to this technology is the rapid development of the signal (minutes versus hours or days for 32P), and signal strength equal to that of 32P. The substrates are moderately priced, but there are no safety concerns. Development of blots is done in a similar manner as autoradiography – expose the blot to X-ray film.

Fluorescence

Increasingly more common than all of the above labels is the fluorophores. There is an increasing number of colors available and detection methods. For direct detection of DNA without gel electrophoresis, Sybr Green is the most commonly used dye [for rtPCR, for example]. Ethidium bromide is the common one for gel electrophoresis. NOTE: Sybr green and EtBr are molecules that intercalate between the bases of DNA. They are thus known carcinogens and must be handled with gloves.
Cy3 (green) and Cy5 (red) are the most commonly used probe labels for DNA arrays, Southerns, or in-situ hybridization. For rtPCR, several other probe strategies use matched pairs of fluorophores where one excites the emission of the other if they are in close proximity, or pairs of fluorophore and quencher. If the quencher is removed, fluorescence increases. There will be increasing use of these probe types with rtPCR entering the clinical laboratory as multiple PCR reactions can be done in one tube and detection of specific genes by the probes shown by 4 or more colors of fluorescence. An example: Staphylococcus organisms seen in a blood culture bottle can be harvested, washed and lysed for rtPCR. The

rtPCR reaction can confirm that it is Staphylococcus, identify it as S. aureus, determine if it is MRSA, and even tell if it is highly or intermediately resistant. This can all be done in as little as 2 hours – certainly of clinical impact.

Hybridization Protection Assays

Hybridization protection of a label was described above in the chemiluminescence section. This idea is also the basis behind an RNA assay for expression of cyokines, the communication molecules of the immune system. The sample RNA is hybridized to DNA probes. Each cytokine has its own probe and each is of a different length. Once the hybridization has completed, RNase A is added. RNase will digest ssRNA, but not the RNA in a DNA/RNA hybrid. The hybrid fragments are separated on an acrylamide gel and then detected by detection system appropriate for the label on the DNA probe.

III. Hybridization

Nucleic Acid Hybridization/Annealing and Stringency

Hybridization or annealing is based on the ability of a single-stranded nucleic acid sequence (either DNA or RNA) to specifically bind (hybridize/anneal) to its/a complementary strand. If dsDNA is denatured or melted, it will hybridize when the denaturant or heat is removed. A few bases start the process and the rest of a matching sequence will hybridize much like a zipper closing. Hybridization in molecular methods uses single-stranded nucleic acid probes (either DNA or RNA) or oligonucleotides of defined sequence to hybridize to a target DNA or RNA of interest. Complementarity between the two strands is determined by the formation of specific hydrogen bonds between nucleotide bases of the probe/primer and target nucleic acid, such that only the base pairs adenine-thymine, adenine-uracil, and guanine-cytosine form hydrogen bonds, giving sequence specificity to the double stranded duplex.

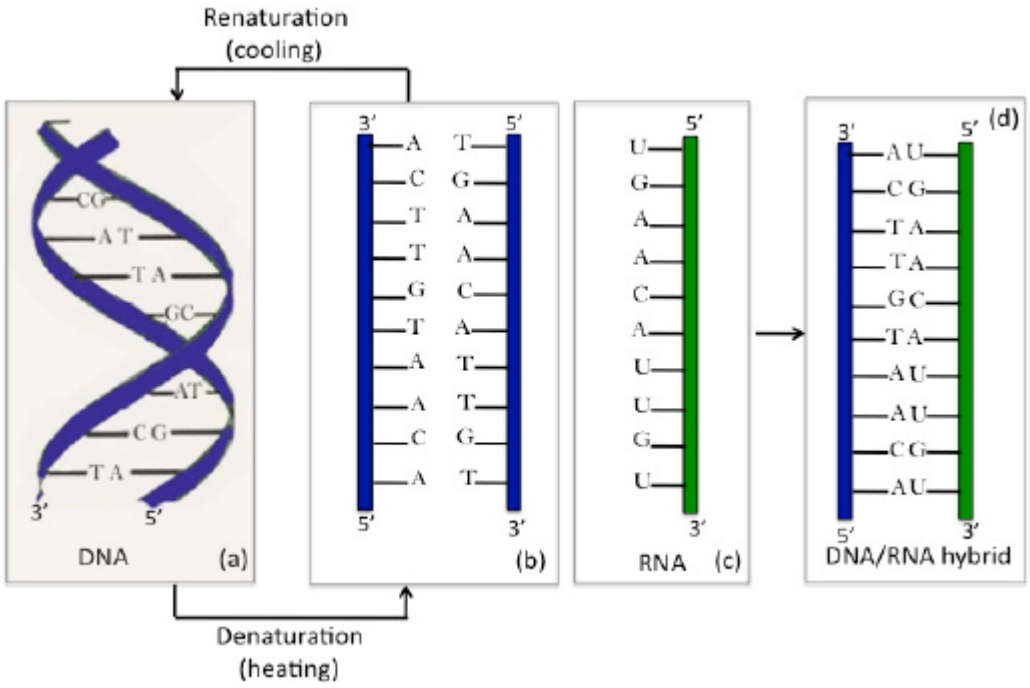
The degree of binding and specificity of the binding depends on a number of factors that control the strength of the hydrogen bonds between complementary nucleotide bases: temperature, pH, use of a denaturant such as formamide, salt concentration of buffer solutions. A certain number of mismatched base pairs can be tolerated in DNA hybridization. For example, the base adenine lining up opposite to guanine would be a mismatch. As long as a significant number of base pairs do match and form bonds, the strands will hybridize. The degree of mismatching tolerated in a hybridization reaction is called “stringency”. Under

conditions of high stringency (for example: high temperature), only exact matches of bases will anneal and stay together. Under conditions of low stringency (low temperature), strands that are only 80 – 90% homologous will bind and give a positive hybridization signal or be able to prime replication. Short probes and primers require more precise test conditions than do longer probes to give an acceptable level of stringency.

Another concept that goes with annealing is melting temperature. Recall from the Introduction Module I, that GC bp have 3 hydrogen bonds and AT bp have two. The more bonds, the higher the amount of heat required to disrupt them. In long pieces of DNA, temperatures >93°C are required and the more GC bonds present, the higher the temperature. For shorter oligonucleotide pieces like primers and some probes, lower temperatures are required to melt the primer or probe from the target. The melting temperature is determined by the GC and AT content of the primer/probe. To achieve stringency in the amplification techniques described below (esp. PCR and LCR), the annealing temperature of the primers/probes is usually 5°C less than the melting temperature. This should maintain high stringency in the reaction, ensuring that only the desired target is amplified.

Nucleic Acid Hybridization

As mentioned above, hybridization methods are based on the ability of a single-stranded nucleic acid probe (either DNA or RNA) of defined sequence to specifically bind (hybridize) to the target DNA or RNA of interest. The probe can be labeled with enzymes, chemiluminscent, radioisotopic, magnetic particles or fluorescent moieties that can be readily detected or captured by automated instruments. Briefly, after extraction of the DNA of interest from the clinical specimen or growing cultures, the DNA is then denatured (e.g. the two strands of the DNA molecule are separated using heat or alkali treatment). Since RNA is single-stranded, no denaturation is necessary if RNA is the target nucleic acid. A labeled single-stranded probe unique to the target being sought is added and hybridizes to one of the DNA strands of the specimen if it has a complementary sequence (i.e.: if the pathogen is present). After unbound probe is removed, bound probe can then be detected using one of the methods described above. Hybridization methods are currently in use for the detection and identification of pathogens directly from clinical specimens (e.g. Chlamydia trachomatis, Neisseria gonorrhoeae) or from growing cultures (e.g. Mycobacterium tuberculosis). Hybridization of PCR products to probes or vice versa can be used for HLA typing. A schematic diagram of hybridization of an RNA probe to a DNA target is shown below.



Once double stranded DNA is denatured to single stranded DNA, it is capable of binding or reassociating with complementary single stranded DNA or RNA. Once a probe is prepared, it must be brought into contact with sample nucleic acid to allow hybridization to take place.

In situ Hybridization

In situ hybridization brings a DNA or RNA probe into contact with the target DNA or mRNA in the tissue or cell under investigation. The technique finds use in histotechnology where tissue sections are probed for the presence of an infectious agent, often viral. It is also used to locate specific genes responsible for an abnormality or specific RNA for expression in certain tissues at certain times. Finally, this is the premise of FISH, or fluorescence in-situ hybridization, now used in cytogenetics. HPV–In Situ with FITC label.

The fixation and preparation of the tissue must preserve nucleic acid and the probe must have access to nucleic acid during the hybridization period. Formalin-fixed parafin-embedded tissues may be used for hybridization reactions. The tissue is fixed to the slide and the parafin removed by usual means. A digestion process with hydrochloric acid or protenase is necessary to remove histones and other proteins that may mask nucleic acid. The section is subjected to heat, about 85˚C for 10 minutes, to denature the nucleic acid to single strands. A solution containing the probe is added and the preparation allowed to sit overnight for hybridization to take place. Nonhydridized probe is removed by washing the slide in buffers.

Enzyme-labeled probes are common for this technique using the biotin-avidin horseradish peroxidase label or the alkaline phosphatase label. The appropriate chromogenic substrate is added to the preparation and labeled probe is located in tissue under microscopic examination. Fluorescent labeled probes are also extremely common, and only require a fluorescent microscope for detection. Digital images are taken in each color and then the images are combined.

IV. Autoradiography

Autoradiography is an imaging technique that uses radioactive sources contained *within* the exposed sample. *In vitro* autoradiography methods involve the isolation of cellular components such as DNA, RNA, proteins or lipids, followed by labeling with suitable radioisotopes. In *in vivo* autoradiography, radioisotopes are coupled with radioactive tracers and administered orally or via injection, and the distribution of radiation is evaluated in thin tissue or whole-body cryosections. *In vivo* autoradiography using laboratory animals is widely used in metabolic studies, disease monitoring and new drug development experiments. Common radioisotopes in autoradiography are sulfur-35, hydrogen-3, carbon-14, ^{125}-iodine or phosphorus-32 (^{35}S, ^{3}H, ^{14}C, ^{125}I and ^{32}P, respectively) which are used to determine the distribution of the radiolabeled molecules in tissues, cells or cellular organelles, but also in the study of protein modifications and DNA / RNA sequencing.

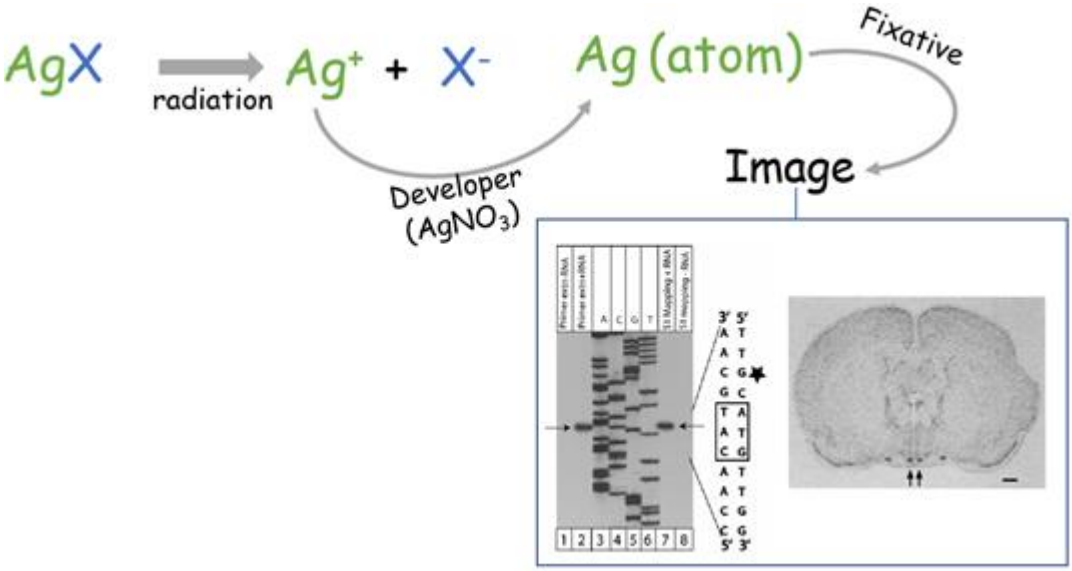


Figure: General principle of autoradiography. A silver halide (AgX) is ionized by the radiation emitted from radioisotopes, forming Ag^+ ions. Ag^+ is then reduced and converted to metallic Ag by a developer reagent (usually containing $AgNO_3$), which precipitates within

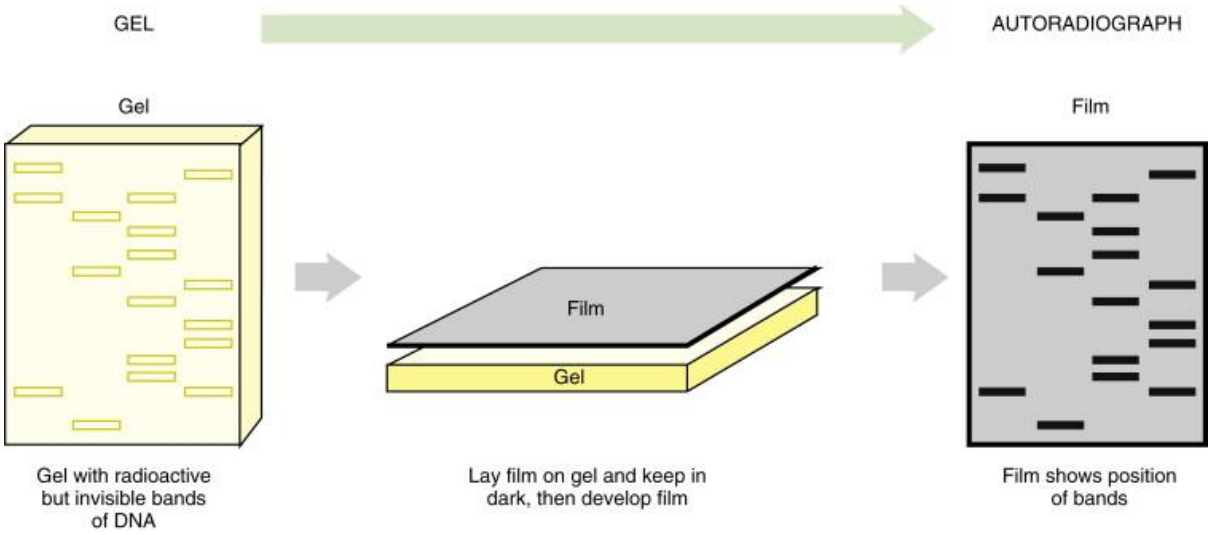
the gelatin emulsion of the X-ray film. The reduction and precipitation are stopped by emerging the film in a fixative solution forming the final image.

General Principle of Autoradiography

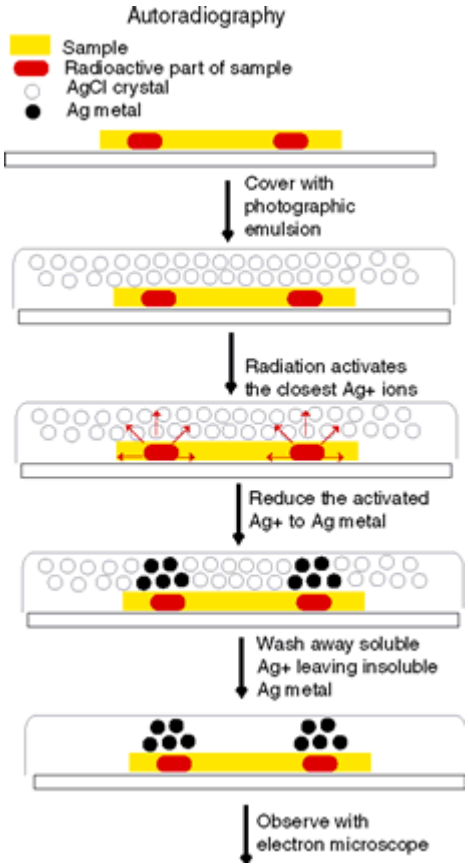
The principle of autoradiographic imaging is the precipitation of silver (Ag) atoms, resulting from the ionization of a silver halide (AgX – silver bromide, chloride, iodide, or fluoride – AgBr, AgCl, AgI, or AgF, respectively) by radiolabeled samples. AgX is light sensitive compound commonly used in photography. They are generally suspended in a gelatin photographic emulsion. Each AgX molecule is individually encapsulated in the gelatin, and functions as an independent detector of radioactive decay from the radiolabeled sample. Once radioactive particles hit the gelatin emulsion, AgX is reduced resulting in the production of insoluble silver crystals.

Gelatin photographic emulsions are used to coat photographic and X-ray films, which are made of a flexible base (usually cellulose acetate). When a radiolabeled sample is in contact within a coated X-ray film (exposure), it generates a latent (*hidden*) image corresponding to the radioactivity distribution within the sample. To make the image visible, the exposed photographic / X-ray film must be submerged in a developing reagent, a chemical mixture that converts the silver crystals into metallic silver, darkening the gelatin emulsion. Silver nitrate ($AgNO_3$) is highly efficient in the reduction of AgX molecules and is usually a component of developer solutions. The reaction is then stopped by a fixative reagent, which removes the excess AgX from the photographic / X-ray film. Highly radioactive areas (e.g. areas with a higher concentration of a radiolabeled drug, or with higher metabolic activity) reduce more AgX molecules, resulting in higher optical density in the film (darker areas). Thus, autoradiography must be avoided in samples that are homogeneously labeled. Although it can be quantitative, autoradiography can be a slow process, depending on the half-life of the radioisotopes used.

In a clinical context, advanced autoradiographic methods like positron-emission tomography (PET) and single-photon emission computerized tomography (SPECT) are used in the diagnosis, staging, and monitoring of disease. PET and SPECT rely on the same principle as classic autoradiography but use different radiotracers (suitable for human use) and radioactive decay is detected by gamma cameras (PET / SPECT detectors). Computer algorithms are then used to produce high-resolution 3D reconstructions (tomograms) of the whole body or parts of an organism.



Autoradiography: Methods



Classic autoradiography techniques are performed according to the following general sequential steps:

In vivo autoradiography

1. Radioactive labeling of biological samples. Labeling time depends on the type of radioisotope and the radiotracer molecule.
    1. Injection or oral administration of radioactive tracer in laboratory animals
2. Sample preparation
    1. Cryopreservation of euthanized animals and cryosection – whole-body or tissue sections (20-50 μm thick) for microscopy evaluation. Light or electron microscopy can be used, depending on the aim of the study.
    2. Whole-body or tissue sections are mounted into glass slides and embedded in the photographic emulsion to generate a latent image.
3. Image development. Here, the incubation time in the developer reagent depends only on the radioisotope used.
4. The arrest of image development by exposing the slide to a fixative reagent.

When analyzing histological sections, a counterstaining step can be added to the protocol, to identify specific tissue structures or cellular components. For example, hematoxylin and eosin staining can be used to define the nucleus and cytoplasm of cells in tissue sections, after autoradiographic detection.

In vitro autoradiography

1. Sample preparation
    1. Isolation of cellular components such as proteins, DNA or RNA
2. *In vitro* labeling of isolated cellular components (DNA, RNA, proteins, etc.)
3. Loading the sample in a suitable matrix for image development using a photographic / X-ray film. Radiolabeled proteins can be loaded into acrylamide gels, and transferred to a nitrocellulose membrane, which is then put in contact with the film
4. Exposure of the photographic / X-ray film to the matrix containing the radiolabeled sample. Exposure time depends on the radioisotope used
5. Development of the autoradiographic image by emerging the film in a developer reagent solution.
6. The arrest of image development by emerging the film in a fixative reagent to remove the excess silver halide in the photographic emulsion

In both *in vivo* and *in vitro* autoradiography, the exposure of the radiolabeled sample to AgX-containing photographic emulsion must always be performed in the dark (e.g., in a dark

room, or in closed boxes). Because the AgX is sensitive to light and radiation, this ensures that the only AgX molecules reduced are the ones where radioactivity is emitted from the sample. The resolution of the autoradiographic image is proportional to the ionization capacity of the radioisotopes used. Low energy radioisotopes such as ^{3}H reduce only neighboring silver halide molecules, producing a very sharp image, while high energy isotopes such as ^{125}I can reduce a lot more halide molecules, producing darker images with lower specificity.

Applications Of Autoradiography

Autoradiography In Preclinical Research

Whole-body autoradiography (WBA) and Microautoradiography (MARG) – *In vivo* autoradiography techniques used to determine the tissue distribution of radiolabeled compounds in laboratory animals. A radiotracer (usually labeled with ^{14}C and/or ^{3}H) is administered and incubated for specific time points. WBA and MARG are especially suitable for the study of receptor biology, as the radioisotopes can be coupled with ligands that are specific to cell membrane receptors. Thus, WBA and MARG provide data on the distribution and activity of cellular receptors, making them exceptional techniques in the study of cell biology and cell signaling mechanisms. In microbiology, the MARG method has been combined with fluorescence *in situ* hybridization (FISH), using specific oligonucleotides (DNA probes) to identify organisms.

WBA can be quantified (quantitative WBA) using dosimetry techniques, i.e., comparing the different radioactivity intensities with radioactive standards of known concentrations.

Autoradiography In The Clinical Context

Positron-emission tomography (PET) – Diagnostic imaging method used to observe biological processes in the body. In a PET scan, a radioisotope is coupled to a radioactive tracer and then introduced into the body. The radioactive decay from the radiotracer produces positrons: particles with the same mass as electrons, but with opposite charges (i.e., +1). When a positron and an electron collide, they annihilate each other, and two gamma rays are produced, which are detected by a gamma camera. Common radionuclides used in PET scans are listed in **Table**. Gamma cameras record several 2D images that correspond to the radioactivity in the capillary vessels near the target organ or in the whole body. Using

multiple projections of the acquired images, a computed tomogram, i.e, a 3D reconstruction, is generated. The 3D tomogram can then be digitally manipulated to analyze the radioactivity distribution in the sample.

Positron Isotope	Half-life (min)	Application
11-carbon (^{11}C)	20.4	Detection of tumor margins and metastasis
13-nitrogen (^{13}N)	9.96	Myocardial perfusion imaging
15-oxygen (^{15}O)	2.07	Hemodynamics and blood flow
18-fluor (^{18}F)	109.7	Tumor and metastasis detection
68-gallium (^{68}Ga)	68.3	Detection of neuroendocrine tumors

Proteins, lipids, sugars, and even patient cells can be used as radioactive tracers. For example, to determine the source of internal bleeding in a patient, it is possible to radiolabel the patient's red blood cells, inject them back into the body and then follow the accumulation of radioactivity in a specific part of the body using a PET scan.

Single-photon emission computerized tomography (SPECT) – Similar to PET scan but uses different radiotracers. While in PET scans image comes from the production of positrons, in SPECT imaging, the radioactive tracers emit gamma rays. A list of the most common SPECT radioisotopes is shown in following table.

Radioisotope	Half-life	Application
67-gallium (^{67}Ga)	78 hours	Bone disease
99-technetium (^{99m}Tc)	6 hours	Bone scan Myocardial perfusion scan Brain scan
111-indium (^{111}In)	2.81 days	White cell scan
123-iodine (^{123}I)	13 hours	Neuroendocrine or neurological tumor scan
131-iodine (^{131}I)	8.06 days	Neuroendocrine or neurological tumor scan

Autoradiography: Advantages And Disadvantages

The use of autoradiographic methods brought significant advances to preclinical research. Classic autoradiographic methods are technically straightforward and do not require much expertise from the operators, Moreover, the ability to couple radioisotopes with radiotracers that target radioactive decay to specific tissues made autoradiographic methods highly efficient. However, a major limitation of these techniques is the lack of standard methods to evaluate the binding specificity of radiotracers to their targets. Another limitation of autoradiographic methods is that detection is dependent on the radioactive decay of the radioisotopes used. In the case of long-lived radioisotopes, image development may require several hours of exposure, making it very time-consuming.

In the clinical setting, the controlled used of autoradiography by humans allowed for astonishing advances in human health. However, it should not be overlooked the fact that all the autoradiography methods detailed above make use of *ionizing radiation* to produce the

final images – autoradiographs. In PET and SPECT scans, the radiation to which patients are exposed is higher than in routine chest X-rays exams, due to the internal exposure to the radioactive source. As such, the prescription of PET and SPECT scans must consider health consequences, like increased cancer and neurodegenerative disease risks.

RECOMBINANT DNA TECHNOLOGY QUESTION BANK

UNIT – 1 (2MARK)

1. Write the function of DNA ligase.
2. What is the function of TAQ polymerase?
3. Polymerase.
4. Alkaline phosphate.
5. RNA polymerase.
6. DNA ligase.
7. Sticky ends
8. dNTPs.
9. Klenow fragment.
10. Recombinant DNA.
11. Write the function of restriction enzymes.
12. What is DNA ligation.

UNIT – 1 (5MARK)

1. Write short note on alkaline phosphatase.
2. Briefly describe about the important characters of type II restriction enzyme.
3. Write a short note on DNA polymerase.
4. Give an account on DNA ligase.
5. Write a short note on DNA dependent RNA polymerase.
6. Give the enzymology of DNA replication
7. Give in detail about inhibition of alkaline phosphate.
8. Give an account on restriction enzyme and their types with suitable example.
9. Write a note on DNA ligase and alkaline phosphate.
10. Write the pattern of DNA cutting by restriction enzymes.
11. Explain in detail about palindromic sequence.
12. Comment on DNA polymerase and its important.

UNIT –1 (10MARK)

1. Briefly explain the types and function of DNA polymerase.
2. Describe the molecular tools and their applications.
3. Give in detail on the application of restriction enzymes.
4. Describe about DNA dependent RNA polymerase and its application.
5. Write an essay to describe the structure and significance of different types of DNA polymerase.
6. Describe about DNA and RNA polymerases.
7. Write a note on restriction enzyme and its nomenclature.
8. List out the properties of PBR322.
9. Discuss in detail about restriction enzyme and its application.
10. Write in detail about role of DNA polymerase in DNA synthesis and its application.

UNIT – 2 (2MARK)

1. What is plasmid?
2. What is phagemid?
3. Vectors.
4. Cosmids.
5. Expression vector.
6. DNA ligase.
7. Promoter.
8. Strong promoter.
9. Cos – site.
10. Palindromic sequence.
11. PBR322.
12. List out the application of cloning.
13. Uses of BAC.
14. ORI region.
15. Stringent plasmid.

UNIT – 2 (5MARK)

1. Explain the character and function of expression vector.
2. Write the general characteristics of cloning vector.
3. Write a brief note on cosmids.
4. Give an account on the expression vector.
5. Give an account on phagemids.
6. Write a short note on cloning vectors.
7. Give the limitation of cloning with agro bacterium plasmids.
8. Give the types of yeast cloning vectors.
9. What do you mean by high and low copy number plasmid and explain how do the copy number is regulated?
10. With neat diagram write notes on cosmids and explain it significance.
11. Describe about yeast replicative plasmids.
12. Write down the properties of Puc18.
13. List out the uses of YAC.
14. List out the application of cloning vector.
15. Write about prokaryotic expression vector.

UNIT – 2 (10MARK)

1. Explain about prokaryotic and eukaryotic expression vector.
2. Write on account on the basic information of expression vectors.
3. Give an account on cloning vector and their application.
4. Explain in detail about site directed mutagenesis.
5. Define the term expression vector. with suitable diagram explain the salient features of an expression vector.
6. Explain in detail about baculoviruses as expression vector.
7. Write an essay on construction, principle and uses of cosmids.
8. Write about the plasmid vector and its copy number regulation.

UNIT – 3 (2MARK)

1. What is the technique used to send the DNA into the *E. coli*?
2. What are the gene components of YAC?

3. BAC.
4. Clone.
5. YAC.
6. Cloning.
7. Ti plasmid.
8. Telomere.
9. Centromere.
10. Sup 4 – gene of YAC.
11. Shuttle vector.
12. Taq DNA polymerase.
13. Histones.
14. Gene amplification.
15. Gene loss.
16. Cloning host.

UNIT – 3 (5MARK)

1. Briefly describe about the construction and uses of BAC vector.
2. Describe about the *E. coli* as the host for cloning process.
3. Give brief note on principle of YAC.
4. Give a brief account on construction of BAC.
5. Write an account on construction and uses of YAC.
6. Write the steps involved in DNA binding and cleavage by a type II restriction endonuclease.
7. Write the structure and uses of YAC vector.
8. Discuss about the specialized features of animal cells as a cloning host.
9. Give the major classes of restriction endonuclease.
10. Describe about the amplification and purification of recombinant plasmid DNA.
11. Write short note on gene losing.
12. Briefly explain the gene rearrangement.
13. Discuss about LAC operon.
14. Comment on importance on TRP operon.

15. Explain about animal cell as a host for cloning vector.

UNIT – 3 (10MARK).

1. Write about the construction, principle and uses of YAC vector.
2. Discuss on the specialized features of cloning host yeast.
3. Describe on the specialized features of cloning host *E. coli*.
4. What is YAC? With suitable diagram explain about its construction and its application.
5. Explain in detail about the constructing DNA libraries.
6. Write in detailed about gene amplification.
7. Explain about gene regulation in eukaryotes.
8. Explain in detail about construction, principle and application of BAC.

UNIT – 4 (2MARK)

1. 1.What is blunt ends in cloning?
2. 2.What are the important uses of cDNA library?
3. 3.Blunt ended DNA.
4. 4.CDNA.
5. 5.DNA ends.
6. 6.Thermal cycler
7. 7.Reverse transcriptase
8. 8.EcoRI.
9. 9.Blunt ends.
10. 10.Recognition site.
11. 11.Bacteriophage.
12. 12.Hybridization.
13. 13.Restriction maps.
14. 14.TA cloning.
15. CDNA library.
16. 16.Sticky ends in cloning.

17. Uses of CDNA.

UNIT – 4 (5MARK)

1. Explain about sticky and blunt ends in cloning process.
2. Write short notes on construction of genomic DNA library.
3. Write an account on CDNA libraries.
4. Write a note on PCR based cloning approach.
5. Write an account on TA cloning.
6. Write a note on blunt ended DNA.
7. Write the principle and applications of PCR.
8. With suitable example and diagram explain how sticky ends and blunt ends are formed and also explain their significance.
9. Describe about the first strand synthesis of CDNA.
10. Mention briefly about sticky ends and blunt ends.
11. Write short note on construction of genomic library.
12. Write a short note on the preparation of radiolabeled probes.
13. Comment on hybridization with an example.
14. What is the difference between sticky and blunt end ligation.
15. Write the application of genomic DNA library.

UNIT – 4 (10MARK)

1. Describe about the construction of cDNA library and its application.
2. Explain the basic principle of construction of genomic libraries.
3. How the genomic libraries are constructing using plasmids.
4. Discuss about complementary CDNA synthesis.
5. Write an essay based on PCR based cloning.

UNIT – 5 (2MARK)

1. What is radiolabeled probe?
2. What are the radioisotopes used to make radiolabeled probes?
3. Non radiolabeled RNA probe.

4. Hybridization.
5. Radiolabeled DNA probe.
6. Immobilize DNA.
7. Radio activity.
8. Probe DNA.
9. Biotin Streptavidin.
10. DNA polymerase.
11. Chemiluminescence.
12. Radioactive isotopes.
13. AGE.
14. Probes.
15. Autoradiography.
16. Write any two example for radiolabeled probe.
17. Write application for southern hybridization.

UNIT – 5 (5MARK)

1. Briefly explain about autoradiography and its applications.
2. Describe about the preparation of non-radiolabeled DNA probes.
3. Give a brief account on southern blotting.
4. Write a short note on radio labeled DNA probe.
5. Write short note on non-radio labeled RNA probe.
6. Explain in detail about selectable markers.
7. Mention briefly about the synthesis of a radiolabeled DNA probe.
8. What do you mean by non-radio labeled probe how it is prepared?
9. Describe briefly about multiple cloning site of PUC19.
10. Mention briefly about the synthesis of a radiolabeled RNA probe.
11. How to analyze the cloned genes.
12. Briefly explain the process of autoradiography.
13. Explain PCR based cloning.
14. Discuss about the Principle and construction of genomic library.

15. Describe briefly about the preparation of radiolabeled DNA probes.
16. Describe the type of probe used for southern hybridization.

UNIT – 5 (10 MARK)

1. Explain about the preparation of radio labelled probes and its application in southern hybridization.
2. Describe the principle and mechanism of autoradiography.
3. Explain the principle and method of southern blotting.
4. Write the principle, procedure and application of autoradiography.
5. Write an essay to explain the principle and the technique involved and the application of southern hybridization.
6. Discuss about the DNA microarray.
7. Explain in detail about the preparation of non-radiolabelled probes and its application in southern hybridization.