

DNA modifying enzymes

- in the preparation of RNA molecule, the vector DNA and the DNA to be cloned have to be cut at specific sites and joined in a particular manner.
 - DNA molecules have to be shortened, lengthened, copied into RNA or into new DNA, some are modified by adding or removing special chemical groups.
 - All these manipulations require purified enzymes.
- DNA manipulating enzymes are of ~~four~~ classes.
1. Nucleases
 2. DNA ligase.
 3. polymerases
 4. modifying enzymes (topoisomerase).

1. Nuclease.

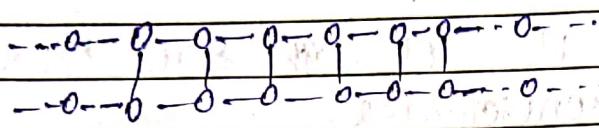
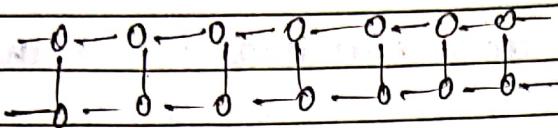
- Nuclease degrade DNA molecule by breaking the phosphodiester bond that link one nucleotide to the next in DNA strand.
- Some nucleases are specific for DNA & some for RNA.
- ~~there~~ it is of ~~is~~ two types.
 - 1. Exonucleases
 - 2. Endonucleases

1. Exonucleases:-

- They remove nucleotide one at a time from the end of a DNA molecule.
- The main distinction b/w different exonucleases lies in the number of strand are degraded when a double-stranded molecule is attacked.
 - (A) Bal 31 :- it removes nucleotide from both strands of a double - stranded molecule.
 - (B) E. coli exonuclease III :- it removes nucleotides only from 3' end of double - stranded molecule.

(C) Lambda exonucleases \rightarrow it remove nucleotides only from 5' end of double stranded molecule.

Bal 31 :-



E. coli exonuclease III



(ii) Endonucleases

• They are able to break internal phosphodiester bond within a DNA molecule.

(a) S1 endonuclease \rightarrow it cleaves single stranded DNA, including single stranded nicks in double stranded DNA.

(b) Deoxyribonuclease I (DNAse I) \rightarrow it cleaves both single and double stranded DNA.

- The special group of enzymes called as restriction endonuclease which cleaves double stranded DNA only at a specific recognition sites. It is also called molecular scissor.
 - These enzymes are found in bacteria & archae, & provide defense mechanism against invading viruses.
- Q. Tell us about Ligases
- DNA ligase is used to seal the nicks that remain in the DNA by forming a phosphodiester bond.
 - It is also called molecular glue.
 - e.g., T4 polynucleotide ligase is prepared from *E. coli* cells infected with T4 phage. It is involved in the replication of phage-DNA & is encoded by the T4 bacteriophage.
 - The reaction requires energy which is provided by adding ATP to the reaction mixture.

3.

polymerases

DNA polymerases are enzymes that synthesize a new strand of DNA complementary to an existing DNA or RNA template.

1) The basic polymerase reaction - it involves the synthesis of a new DNA strand along the DNA template in the 5' to 3' direction. Most polymerase require a double stranded primer region of the template to initiate synthesis.

In genetics engineering three types of polymerases are commonly used, DNA polymerase I, the Klenow polymerase and Reverse transcriptase.

(A) DNA polymerase I

- It has both polymerization (synthesis) and Nuclease (degradation) action.
- The dual activity is performed by different enzyme.
- It synthesizes a short strand to fill in gap in the nick region synthesizing a complementary DNA. It finally replace the existing ~~DNA~~ nucleotides by a process of DNA degradation immediately followed by DNA polymerization.

Transcription → Synthesis of RNA on DNA.

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(B) The Klenow polymerase or fragment of it is a part of the DNA polymerase enzyme that retains only polymerase activity but lacks the nucleic acid activity.

It is therefore used to fill in the nick by synthesizing a complementary DNA strand on a single stranded template. Since it cannot degrade nucleotides, it does not replace existing nucleotides.

(C) Reverse transcriptase:

- It synthesizes a new complementary DNA strand on the RNA template.
- This property is important for complementary DNA cloning.
- Reverse transcriptase is involved in the replication of several kinds of viruses.

4. DNA modifying enzymes:-

- There are numerous enzymes that modify DNA molecules by addition or removal of specific chemical groups.
- They are also called end-modification enzymes.
- The most important are as follows:-
 1. Alkaline phosphatase:- it removes phosphate group present at the 5' terminus end of DNA.
 - (ii) T4, polynucleotide kinase:- it adds phosphate group to the 5' terminus.
 - (iii) Terminal deoxynucleotidyl transferase:- it adds one or more deoxyribonucleotides to the 3' prime end of a single stranded DNA molecule. This is called tailing.
 - (iv) Topoisomerase:- it introduce or remove supercoils from covalently closed circular DNA.

1. Restriction enzymes

- Most used in molecular biology labs
it cuts the DNA at particular sites
within their recognition sequence.
- Postulated by Werner Arber while studying bacteriophages.

Biological role

- Most bacteria use restriction enzymes as a defense against bacteriophages.
- Restriction enzymes prevent the replication of the phage by cleaving its DNA at specific sites.
- The host DNA is protected by methylases which add methyl groups to adenine or cytosine bases within the recognition site thereby modifying the site and protecting the DNA.
- Term restriction enzyme originated from A phage, by Hamilton O. Smith from the bacterium *Haemophilus influenza*.

Restriction enzyme

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- Found by Hamilton O Smith and his colleagues.
- Restriction enzymes are:-
 - protein
 - DNA-cutting enzymes.
 - Also known as restriction endonucleases.
 - Isolated from bacteria and blue-green algae.
- One of the first enzyme from *E. coli*, was named after the organism isolated by **MCRT**.
- Cleave the phosphodiester bond of double stranded DNA.
- Cut at specific recognition sites.
- Recognition palindromic sequence of 4 to 8 base pair long.

Palindromic Sequence :- if we read from 5' to 3' and 3' to 5' it will read same called palindromic sequence.

Definition :- Restriction

Restriction enzymes are of three types.
Type I, II and III

Type I and III, are not much used:-

They have both restriction and modification activity. They cut DNA at sites some distance away from their recognition sequence. They need ATP for energy and lack predictability.

Type I enzymes are non specific in cleavage and hence type-I and type-III are restriction enzymes are not much used in genetic engineering.

Type II restriction enzymes are ideal for biotechnology:-

Restriction activity but no modification. Each cuts in a predictable and site specific manner at a site within or adjacent to recognition sequence.

They only require Mg²⁺ as a co-factor not ATP.

Nomenclature

- Enzyme designated by three letters are used abbreviation for host organism followed by fourth letter designated the strain.
- If required roman numerals are used to indicate different restriction-modification system, when more than one size is obtained from same organism. more often Roman numerals indicate the order of discovery.
- Of the three letters the first is the initial letter of the genus name of the organism from which the enzyme is isolated, while second & third letter are the initial letters of the species name.

Blunt and sticky ends

Blunt end ligation

(Joining of DNA molecules)

There are two major ways in which restriction enzymes cleave a DNA, it either creates blunt ends or stick ends.

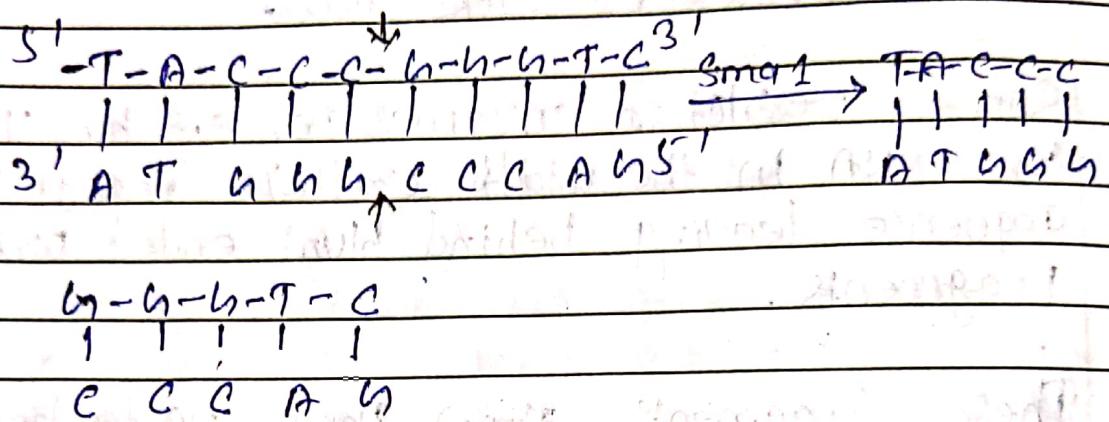
Some restriction enzymes for e.g Hind II, cut the DNA in the middle of their recognition sequence leaving behind blunt ended DNA fragments.

These fragments ~~can~~ show no tendency to stick together. The T4 DNA ligase enzyme can not ligate or join such blunt ended DNA molecules.

Their ligation can occur by chance. Further very high concentration of ligase and DNA.

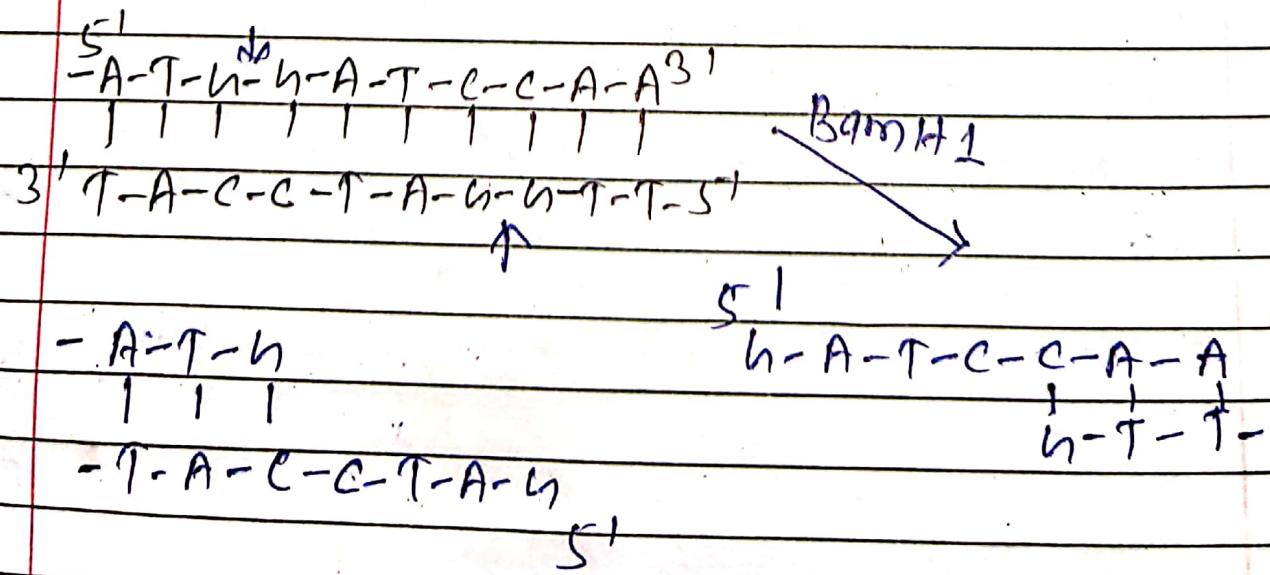
(Blunt ends)

Blunts:- Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs. Small is an example of an enzyme that generates blunt ends.



~~Sticky ends~~ or 5' overhangs.

The enzymes cuts asymmetrically within the recognition sites such that a short single stranded segment extends from the 5' ends. Bam HI cuts in this manner.



Blunt end ligation \rightarrow joining a sticky end to blunt end of DNA.

(A) In such a situation when R.E produces blunt ended DNA fragments the recombination frequency lowers due to non-availability of suitable sites on the DNA to be manipulated.

Since sticky or cohesive ends are absent on blunt ended DNA fragments, cleavage sites can be added as linkers, adaptor molecules or as homopolymer tails.

Linkers

- Linkers are chemically synthesized double stranded DNA oligonucleotides containing one or more restriction sites for cleavage by restriction enzymes. e.g. - EcoRI, HindIII, BamHI etc.
- Linkers are ligated to blunt end DNA by using DNA ligase.
- Both the vector and DNA are treated with restriction enzyme to develop sticky end.
- The DNA staggered cuts i.e. sticky ends are then ligated with T4 DNA ligase with very high efficiency to the termini of the vector and recombinant plasmid DNA molecules are produced.

Adapters

- Adapters are also short ds oligonucleotides strands having preformed sticky or cohesive ends.
- They are used when the RE has site within the Foreign DNA to be cloned.

Homopolymer tails

- The homopolymer are oligo d(A A A A A --- A A) sequences and oligo dT (T T T T --- T T) sequences.
- These sequences are added to the Foreign DNA fragments to be cloned as well as to the vector used, by the action of enzyme terminal transferase.
- The homopolymer tails are used in such a way that if Foreign DNA ll no add oligo d(A) (or poly d(A)) tail then the vector will be added with oligo d(T) tail.
- Consequently there develops poly d(A) and poly d(T) tails on Foreign DNA and vector respectively.
- When mixed together they unite to form the covalently closed circular rDNA molecule.

vectors and their types:-

- ~~vectors carry the fragment of foreign~~
- vectors are those DNA molecules that can carry a foreign DNA fragment when inserted into them, they are also called vehicles DNA because they act as carriers of genes to be cloned into a recipient cell or a cloning organism.
- There are many types of vectors that are used in genetic engineering experiment.

Many types of vectors

- Plasmid
- Bacteriophage
- cosmid
- phasmid
- shuttle vector
- Bacterial artificial chromosome (BAC)
- yeast artificial chromosome (YAC)

Characteristics of vectors:-

- (a) Small size (ideally less than 10 kb) as large DNA molecules tend to breakdown during purification.
- (b) It must be able to replicate in the host cell.
- (c) The vector should have several unique restriction sites for cloning foreign DNA fragment.
- (d) A vector should contain selectable marker gene like antibiotic resistance.
- (e) It should be high copy number plasmid.
- (f) Visual marker for selecting bacteria carrying plasmids with inserts.
- (g) Promoters for In vitro

Plasmid vector

- Plasmid is a covalently closed, circular, double stranded, extrachromosomal, self replicating DNA molecule present in bacterial cell.
- Plasmid contain very few genes, usually three or four.
- contains genes which offer them resistance to antibiotics.
- origin of replication is present (ORI).

- Plasmid can be considered suitable cloning vector if they bear the following features.

- (a) They can be isolated from cells but not found naturally.
- (b) They must posses at least one cleavage site for one or more restriction enzymes.
- (c) They must contain two or more marker genes (antibiotic resistance gene) which help in its identification.
- (d) Insertion of foreign DNA molecule at one of these sites does not alter its replication properties.
- (e) They can be introduced into a bacterial cell and cells carrying plasmid or without insert can be selected or identified.

- Moreover, the number of plasmid in a bacterial cell can be increased (amplified) to about 1000 per cell.
- Number of plasmid can be amplified by incubation of the host cell with the antibiotic chloramphenicol. It inhibits the replication of chromosome but do not inhibit plasmid replication.

1. PBR322 Plasmid

- (A) One of the original plasmid used and a popular cloning vehicle.
- The nomenclature of the PBR322 plasmid is as stated below; P - plasmid, BR - stand for Bolivar and Rodriguez who constructed this plasmid, 322 - is the strain number.
 - It has a length of 4863 bp which enable it to carry about 8 kb of foreign DNA.
 - Plasmid PBR322 has two resistance genes - ampicillin resistance (ampR) and tetracycline resistance (tetR). Which are considered useful for suitable markers.
 - Selected marker helps in selecting host cell which contain the transformed vector and eliminate the non-transformants.

- General unique restriction sites scattered throughout plasmid (some are within antibiotic resistance genes = means of screening for insert).
- it contains ori site derived from Col E1 plasmid of *E. coli*.
- plasmid is derived from pBR318 and pBR320 plasmid of *E. coli*.
- Plasmid pBR322 has a variety of unique recognition sites for restriction endonucleases.
- Unique site PstI is located within the ampR and BamHI and SalI are within the tetR gene.
- Insertion of the DNA fragment into the plasmid using enzyme PstI places the DNA insert within the gene ampR, which makes ampR non-functional.
- Bacterial cell containing such a recombinant pBR322 will be unable to grow in the presence of ampicillin but will grow on tetracycline.

Q. pUC 18

- Derivative of pBR 322.
- Advantages over pBR 322.
 - Smaller in size so can accommodate larger DNA fragment during cloning (5-10 kbp).
 - Higher copy number plasmid (500 per cell) = 5-10X more than pBR 322.
 - Multiple cloning sites clustered in same location - ~~polylinker~~ and location called polylinker.

Bacteriophage vectors

- Virus infecting bacteria are called bacteriophage.
- Bacteriophage are capable of cloning large pieces of DNA.
- Most commonly used bacteriophages are lambda phage and M13.

Bacteriophage Lambda

- Phage lambda is a bacteriophage or phage ie bacterial virus, that uses E. coli as a host.
- Its structure is that of a typical phage: head, tail, tail fibers.

Lambda viral genome :-

~~It~~ It is 48.5 kb linear ds DNA with a 12 base ss DNA sticky end at both ends. These ends are complementary in sequence and can hybridize to each other (this is the COB site: cohesive ends).

However there is a possibility to introduce a foreign DNA upto 25 kb in length into a genome, still retaining its ability to infect *E. coli* and replicate properly.

Infection :-

Lambda tail fibres adsorb to a cell surface receptor, the tail contracts, and the DNA is injected.

The DNA circularizes at the COB site, and Lambda begins its life cycle in the *E. coli* host.

Packaging of the DNA into the head does not require a complete length of wild type Lambda. It has been determined that a Lambda molecule that is between 98% and 105% of wild type length can be packaged. This is from 37 to 53 kb in length.

- Two types of phage cloning vectors have been constructed, insertion vector and replacement vector.
- Insertional vector → DNA is inserted into a specific site without removing any region of λ DNA.
- Replacement vector → Foreign DNA replaces a piece of DNA (stuffer fragment) of the vector, which is the central non-essential region.

A replacement vector

- This type of vector has a loading capacity of 10-25 kb and consist of full length λ DNA molecule with two identical restriction sites flanking a non-essential region called stuffer fragment.

The idea behind it is that the stuffer fragment is deleted and replaced by Foreign DNA.

Again a selection system is required to differentiate between wild type and recombinant phage.

This is done by placing relevant genes onto the stuffer fragment the genes of which gives rise to a detectable phenotypic signal.

1 insertion vector

Insertion vector are the simplest form of lambda cloning vectors.

The vector itself can be grown (therefore must contain at least 75% of the wild type genome length).

Foreign DNA fragments are inserted into a unique restriction site in the vector genome.

Packaging requirement thus limit insert fragment size to 10 kb - due to limitation on viral genome size.

(75% to 105% of the wild type length = 50 kb)

Advantage:

1. DNA can be packed in-vitro into the phage particles and transduced into E. coli with efficiency.
2. Foreign DNA upto 25 kb in length can be inserted into phage vector.
3. Screening and storage of recombinant DNA is easier.

Colloid vector

A colloid vector is a type of hybrid vector derived from plasmid that contains col sites of bacteriophage.

- First described by Collins and Hohn
- Colloid lack genes encoding viral proteins, therefore neither viral particles are formed within the host cell nor cell lysis occurs.
- Thus coloids always form colonies and not plaques.

→ Special features of coloids similar to plasmids are

- (i) A bacterial origin of replication (oriV)
- (2) an antibiotic selection marker
- (3) and a cleavage site for insertion of Foreign DNA (cloning site)

- Characteristics dissimilar to plasmid in the presence of one, or more recently two col sites derived from bacteriophage lambda.
- The colloid have a length of about 5 kb. the loading capacity of colloid varies depending on the size of vector itself but usually lies around 40-45 kb.

PURPOSE:

- 1. Clone large inserts of DNA: size \sim 45 kb. (Features)
- Cosmids are plasmid with one or ~~two~~ Lambda col sites.
- Presence of col site permits *in vitro* packaging of cosmid DNA into lambda particles.

DEFINITION:-

- Cosmids are plasmid vectors that contain col sites. The col sites is the only requirement for DNA to be packaged into a phage particle.
- To clone the DNA into the vector as you would with any plasmid.
- Introduce the DNA into bacterial cell via a phage particle.
- Propagate the plasmid.

Phagemid

- Phagemid or phasmid is hybrid of the filamentous phage M13 and plasmid.
- Phasmid contain the origin of replication and selectable marker from plasmid.
- Phasmid contain an intergenic sequence from filamentous phage, ~~is~~ bcz it is necessary for initiation and termination of viral DNA synthesis and for morphogenesis of phage particle.
- Phagemid replicate as a plasmid but when cells harbouring these plasmids are infected with a suitable filamentous phage (helper phage) phagemid genome behaves like that of a phage.
- Phasmid is thus a gene cloning vector consisting of an artificial combination of a plasmid with a phage such that its genome contain functional origin of replication or both; it may thus be propagated either as a plasmid or a phage in appropriate host strains.

- A phasmid is formed by insertion of one or more plasmid molecules into a phage genome.
- for example, A plasmid vector molecule designated p_λφ₈λ₁₁ has been constructed.
- The vector combines some useful features of plasmid and phage vector molecules lambda.
- p_λφ₈λ₁₁ is a hybrid of lambda 47-1 vector and pBR322 plasmid.
- The maximum size of fragments to be cloned is 21 kilobase pair.
- A library of *Escherichia coli* genes is constructed with the help of lambda p_λφ₈λ₁₁ as a vector molecule.

SHUTTLE VECTORS

- Shuttle vectors are the plasmids (hybrid molecule) designed for use in multiple cell types on different host system i.e. prokaryotes and eukaryotes.
- A shuttle vector is constructed by using bacterial origin of replication in a yeast plasmid.
- Thus the origin of replication of different host ~~enzymes~~ systems like *E. coli* and yeast are combined in one plasmid.
- Multiple ORI sites allow replication in both prokaryotic and eukaryotic host cells allowing transfer b/w diff. cell types.
Ex -
 - *E. coli* \rightarrow yeast cells
 - *E. coli* \rightarrow human cell lines.
- It contains selectable marker and cloning sites.

Thus shuttle vectors can replicate in two different organisms e.g. bacteria and yeast or mammalian cell and bacteria.

They have appropriate origins of replication.

Hence one ~~clone~~ can clone a gene in bacteria, may be modify it or mutate it in bacteria, and test its function by introducing it into yeast or animal cells.

These vectors can be maintained and shuttled between both host (say yeast and E. coli) and hence the name shuttle or binary vectors.

firstly it can express in mammal (yeast) & then can also express in bacterial (E. coli).

It can be used to express animal genes in bacterial cells.

It can be used to express bacterial genes in animal cells.

Yeast artificial chromosomes (YACs)

→ it is a hybrid molecule containing components of yeast, protozoa and bacterial plasmids.

Factors of

→ yeast: ORI = ARS (Autonomously replicating sequence).

- Selectable marker on each arm (TRP1 and URA3)
- Yeast chromosome, yeast plasmid
- it also contains certain characteristic of protozoa = tetrahymena.
- containing telomere sequence (Yeast telomerase may also be used).

Features taken from bacterial plasmid

- poly linker

- it can accommodate more than 1 Mb.
It is used in human cloning genome.

Definition:- Yeast artificial chromosome (YAC) is a human-engineered DNA molecules used to clone DNA sequence in yeast cells.

YACs are plasmid shuttle vectors capable of replicating and being selected in common bacterial host such as *Escherichia coli*, as well as in the budding yeast *Saccharomyces cerevisiae*.

Gene Cloning

- Production of multiple copies of a desired DNA in-vivo by constructing an recombinant DNA and introducing it into a bacterium, is called gene cloning.
- clone → A clone is exact copy or exact genetic replica of an organism.
- Gene cloning is the act of making copies of a single gene.
- Once a gene is identified clones can be used in many areas of biomedical and industrial research.
- Genetic engineering is the process of cloning genes into new organisms, or altering a genetic sequence to change the protein product.

- Essential components for cloning a gene:
- Enzyme of cutting DNA fragments: Restriction endonucleases (RE).
- DNA ligase
- vector
- gene libraries (DNA fragments)
- Selection
- Gene cloning: The insertion of a fragment DNA carrying a gene into a cloning vector and subsequent propagation of recombinant DNA molecules into many copies is known as gene cloning.
- Gene cloning involves using bacteria to make multiple copies of a gene.
- Foreign DNA is inserted into a plasmid, and the recombinant plasmid is inserted into a bacterial cell.
- Reproduction in bacterial cell results in cloning of the plasmid including the Foreign DNA.
- This results in the production of multiple copies of a single gene.

Fundamental steps for gene cloning

- Identification and isolation of the desired gene or DNA fragment to be cloned
- Insertion of the isolated gene in a suitable vector
- Introduction of this vector into suitable organism/cell called host
- The vector multiplies within the host cell producing numerous identical copies not only of itself but also of the gene that it carries.
- During the division of host cell, copies of the recombinant DNA molecules are passed to the progeny and further replication take place.
- After a large no of cell divisions, a colony, or clone of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule.

Note:- Plasmid containing foreign gene is called recombinant plasmid.

Gene cloning in E. coli

• Step B -

1. Construction of recombinant DNA (rDNA) -

- by joining Foreign DNA with vector DNA

- rDNA is also known as chimeric DNA.

It involves following steps:-

(A) Preparation of desired Foreign gene / DNA

(B) Preparation of vector DNA.

(C) Insertion of desired gene into plasmid.

(2) Introduction of rDNA into E. coli.

E. coli is highly permeable to negative

by λ cact and then recombinant plasmid

go easily into E. coli.