Recombinant DNA technology

Restriction endonuclease –types and roles

Restriction endonucleases (R.E.) are the enzymes which recognise and cut specific nucleotide sequence in DNA which are 4-8 nucleotides long. For cloning of DNA, often we need to cut the DNA at specific sites, which are recognised and cleaved by specific enzymes (endonuclease, which cleave DNA at interval sites), described as restriction enzymes. Each enzyme is named by a three letter (or four letter) abbreviation (letters are italicized) that identifies its origin. Roman numerals (I, II, III etc.) are added to distinguish several enzymes with same origin.

Microbial origin	Enzyme	Recognition site
1. *Escherichia coli*	EcoRI	5' G↓AA TTC 3' 3' CTT AA↑G 5'
2. *Hemophilus influenza*	HindIII	5' A↓AG CTT 3' 3' TTC GA↑A 5'
3. *Serratia marcescens*	SmaI	5' CCC↓GGG 3' 3' GGG↑CCC 5'
4. *Hemophilus parainfluenzae*	HpaII	5' C↓C GG 3' 3' GG C↑C 5'
5. *Hemophilus aegyptius*	HaeIII	5' GG↓CC 3' 3' CC↑GG 5'

Depending upon mode of action, it is of three categories – type I, type II and type III.

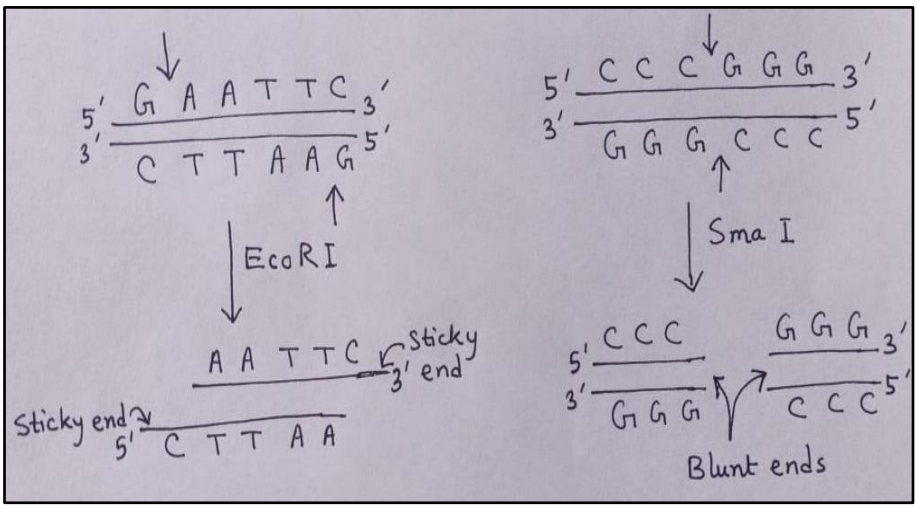
Type I and III have DNA modification (methylation) capacity in addition to DNA cleavage activity, but the two functions are controlled by two different sub-units of the same molecule. Type II have separate enzyme molecule for modification and restriction cleavage.

Type I recognises the specific base sequence in DNA but cut in DNA in a non-specific manner away from that sequence. Thus it is generally not used in Recombinant DNA technology. Example- EcoB, EcoK etc.

Type II and III recognises specific palindromic base sequence in DNA and make cut within that sequence. Type III needs ATP for its activity, while type II not. Thus type II is most

extensively used enzymes in Recombinant DNA technology. Example of type II – EcoRI, EcoRII, HindIII etc; type III- HpaI, MboII, FokI etc.

Some enzymes introduce staggered cuts, others generate blunt ends. Enzymes with 4bp target sites are used when frequent cuts are desired and those with 8bp are used when rare cuts are desired to get long DNA segments. Otherwise majority of enzymes used have 6bp target sites.



Vector (plasmid pBR 322)

Cloning of one DNA molecule is possible only with the help of another DNA molecule, which is capable of replicating in the host. This other DNA molecule is often used in the form of a vector, which could be a plasmid, a bacteriophage, a desired cosmid, a phagemid (phage+plasmid) or even a virus.

Properties of a DNA molecule to serve as a cloning vector:

1. Self replicating capacity of both inserted DNA and own DNA
2. Should have number of restriction sites
3. Should possess number of selectable marker sites for example different antibiotic resistant genes
4. Should be easily purifyable

Plasmid vector

Plasmid is extrachromosomal, circular dsDNA – naturally present in different bacteria, yeast cell, eukaryotic cell organelles like mitochondria. It's general length is 1-200 kb and it can replicate autonomously within host cell and thus can be accumulated upto 1000 copies.

The naturally occurring plasmids may not possess all the essential properties of a suitable cloning vector. Therefore they need to be restructured by inserting genes for relaxed replication and/or by genes for antibiotic resistance.

pBR 322 is the first artificial cloning vector developed by Boliver and Rodriguez in 1977 from *E. coli* plasmid ColE1. According to their name the plasmid name is given. It is 4362bp long and most widely used cloning vector. It have one site for origin of replication, two selectable marker gene (ampicillin and tetracycline resistant gene) and many unique restriction cleavage sites. It can accumulate within transformed *E. coli* cell upto 15-20 copies; its number may be even amplified more by incubating the cell in Chloramphenicol containing medium.

Ti plasmid (from *Agrobacterium tumefaciens*) and Ri plasmid (from *A. rhizogenes*) are used to gene transfer in higher plants.

Marker Genes

The genes which mark the transformed cells or tissues from the non-transformed one are called as marker gene. Selectable marker genes are those that usually linked to the gene of interest to facilitate its detection once inside the plant tissues. This enables the selection of cells that have been successfully incorporated with the gene of interest, thus saving considerable expense and effort. Genetic engineers used antibiotic resistance and herbicide resistance marker genes to detect cells that contain the inserted gene. Example- cat gene (chloramphenicol acetyl transferase gene), npt II gene (neomycin phosphotransferase gene), bnl gene (bromoxynil nitrilase gene) which provides resistance towards the antibiotic kanamycin, neomycin and towards the herbicide bromoxynil resprctively. In addition, alternative types of marker genes have been developed which are related to plant metabolism such as phosphomannose isomerase, xylose isomerase and others.

Marker genes fall into two categories: selectable marker genes and nonselectable (also referred to as scorable marker or reporter) genes.

Because only a few cells are transformed in a population of target cells in explants, there would be little chance of recovering transgenic cells without selectable markers.

Selectable marker genes fall into two separate families: one provide positive selection and another negative selection. Positive selectable marker genes confer a selective growth advantage on plant cells so that transformed cells can outgrow the nontransformed cells. The chemicals are designed to selectively kill nontransformed cells, whereas transformed cells are allowed to live through the resistance mechanisms encoded by the selectable marker genes. Negative selectable marker genes encode systems that selectively kill them. It is the positive selection systems that are used in plant biotechnology for the recovery of transgenic plants as the transformation process is seldom very efficient.

Cells that survive the addition of marker agents to the growth medium indicate the presence of the inserted gene.

For expression in plant cells selectable marker genes from bacteria must be extensively modified because the signals on the bacterial gene will not be correctly recognized by the plant gene expression machinery.

Reporter Genes

Whereas selectable marker genes help the researcher to select transgenic tissue, reporter genes usually report which cells are transgenic.

Reporter genes are cloned into the vector in close proximity to the gene of interest, to facilitate the identification of transformed cells as well as to determine the correct expression of the inserted gene. Reporter genes that have been used include the beta-glucuronidase gene (gusA gene) which acts on a particular substrate producing a blue product, hence making the transformed cells blue; the green fluorescent protein (gfp) which allows transformed cells to glow under a green light; and luciferase gene that allows cells to glow in the dark, among others.

Steps of cloning technique

There are basically four stages in any cloning experiment:

1. Generation of DNA fragments –

The DNA (cloned DNA, insert DNA, target DNA, or foreign DNA) from a desired donor organism is extracted, enzymatically cleaved (cut, or digested by restriction endonuclease) into smaller fragments with defined ends

2. Joining to a cloning vector –

   Desired DNA is joined (ligated) to another DNA entity (a cloning vector) to form a new, recombined DNA molecule (cloning vector– insert DNA construct, or DNA construct).

3. Propagation in a host cell –

   This cloning vector–insert DNA construct is transferred into and maintained within a host cell. The introduction of DNA into a bacterial host cell is called transformation. Copies of the vector will then harbour many different inserts of the genome.

4. Selection of the required sequence –

   Using information relating to specific molecular marker sequences and the desired phenotype, those host cells that take up the DNA construct (transformed cells) are identified and selected (separated, or isolated) from those that do not.