rDNA Technology

The exchange of genetic information between DNA segments of the same species is termed genetic recombination. Nonetheless, with the advancement of technology, one can transfer genes of one species to another artificially.

The technology of recombinant DNA was developed in 1973 by Boyer and Cohen.

It is the technology to produce an artificial DNA molecule by combining two or more fragments of DNA that are not necessarily associated with each other. Usually, such DNA fragments are obtained from several biological sources.

Recombinant DNA is DNA from two distinct species injected into a host organism to create new genetic combinations useful in science, medicine, agriculture, and industry. Laboratory geneticists' primary purpose is to identify, define, and modify genes, as the gene is the focus of all genetics. Consider that each human cell has about 2 meters (6 feet) of DNA. As a result, even a small piece of tissue can contain thousands of kilometers of DNA. Recombinant DNA technology, on the other hand, has made it possible to isolate a single gene or other section of DNA, allowing researchers to establish its nucleotide sequence, investigate its transcripts, alter it in extremely specific ways, and reintroduce the transformed sequence into a living creature.

Notably, several steps are followed to recombine DNA segments. Furthermore, under an ideal situation, a recombinant DNA molecule can replicate by entering a cell.

The said technology is also known as genetic engineering. In a broader sense, it is created through three different methods – transformation, non-bacterial transformation, and phage introduction.

Tools of rDNA Technology

Recombinant DNA Technology's Implementation Tools are as follows:

Restriction enzymes, for example, aid in cutting, polymerases aid in synthesising, and ligases aid in binding. In recombinant DNA technology, restriction enzymes play an important role in deciding where the desired gene is inserted into the vector genome. Endonucleases and Exonucleases are the two types of endonucleases.

Exonucleases remove the nucleotides off the ends of the strands, whereas Endonucleases cut within the DNA strand. Restriction endonucleases are sequence-specific enzymes that cleave DNA at specified locations and are frequently palindrome sequences. They examine the length of DNA and cut it at a specified location known as the restriction site. As a result, the sequence has sticky ends. The complimentary sticky notes are obtained by cutting the desired genes and vectors with the same restriction enzymes, enabling the work of the ligases to bind the desired gene to the vector much easier.

The vectors aid in the transport and integration of the desired gene. These are the ultimate vehicles that transfer the desired gene into the host organism, hence they are a crucial part of the recombinant DNA technology toolkit. Because of their large copy number, plasmids and bacteriophages are the most commonly employed vectors in recombinant DNA technology. The vectors are made up of an origin of replication, which is a nucleotide sequence from which replication begins, a selectable marker, which are genes that show antibiotic resistance, such as ampicillin resistance, and cloning sites, which are sites recognised by restriction enzymes where desired DNAs are inserted.

The recombinant DNA is injected into the host organism. The host is the most powerful instrument in recombinant DNA technology, as it uses enzymes to take in the vector that has been modified with the desired DNA.
These recombinant DNAs are injected into the host in a variety of ways, including microinjection, biolistics or gene gun, alternate cooling and heating, calcium ion usage, and soon.

Goals of rDNA Technology

Some of the goals of this technology are mentioned below:

- Isolation and characterization of genes.
- Desired modification in isolated genes.
- Artificial synthesis of new genes.

- Modification of organisms' genome.
- Interpretation of hereditary diseases and related cures.
- Enhancement of the human genome.

Steps of Recombinant DNA Technology

1. DNA Isolation

- DNA is isolated in its pure form, which means they are devoid of other macromolecules.
- In rDNA technology, the initial step is to extract the desired DNA in its purest form, that is, free of extraneous macromolecules.
- Because DNA coexists with other macromolecules such as RNA, polysaccharides, proteins, and lipids within the cell membrane, it must be separated and purified using enzymes such as lysozymes, cellulase, chitinase, ribonuclease, and proteases.
- Other enzymes or treatments can remove other macromolecules. The DNA eventually precipitates out as fine threads as a result of the presence of ethanol. After that, the pure DNA is spooled out.

2. Cutting of DNA/Restriction Enzyme Digestion

- For this step, the restriction enzymes are quite vital. It helps to identify the location wherein a designated gene is introduced into a vector genome. The said reaction is known as restriction enzyme digestions.
- They entail incubating pure DNA with a restriction enzyme of choice at conditions that are appropriate for that enzyme.
- The 'Agarose Gel Electrophoresis' technology displays the restriction enzyme digestion's progress.

- This method entails passing the DNA across an agarose gel. When current is applied, negatively charged DNA flows to the positive electrode and is divided into different sizes. This permits the digested DNA fragments to be separated and snipped out.
- The same method is used to process the vector DNA.

3. Amplifying of DNA

- Copies of genes are amplified through PCR or polymerase chain reaction. It is essentially a process to increase a single DNA copy into several copies after the desired gene of interest is cut with restriction enzymes.
- It allows a single copy or a few copies of DNA to be amplified into thousands or millions of copies.
- The following components are used in PCR reactions that are conducted on 'thermal cyclers':

    1. Template: DNA that has to be amplified.
    2. Primers: oligonucleotides are tiny, chemically produced oligohnucleotides that are complementary to a DNA region.
    3. Enzyme: DNA polymerase.
    4. Nucleotides: The enzyme is required to lengthen the primers.

- PCR can be used to amplify the cut DNA fragments, which can subsequently be ligated with the cut vector.

4. Joining DNA

- The vector and a section of DNA are joined in this step. It is achieved with the help of the enzyme DNA ligase.
- With the same restriction enzyme, the pure DNA and the vector of interest are cut.
- This yields the cut DNA fragment and the cut vector, both of which are now open.

- Ligation is the process of putting these two parts together with the enzyme 'DNA ligase.'
- The resulting DNA molecule is a hybrid of the interest molecule and the vector DNA molecules. Recombination is the term used in genetics to describe the merging of different DNA strands.
- As a result, this new hybrid DNA molecule is known as a recombinant DNA molecule, and the process is known as recombinant DNA technology.

5. Insertion of rDNA into a Host

- Here rDNA is added to the recipient host cell, and the entire process is called transformation. Post insertion, the recombinant DNA multiplies and manifests as manufactured protein under favorable conditions.
- The recombinant DNA is then transferred into a recipient host cell, most commonly a bacterial cell, in this stage. The term for this procedure is 'Transformation.'
- Bacterial cells have a hard time accepting foreign DNA. As a result, they are given treatments to make them 'capable' of accepting new DNA. Thermal shock, Ca++ ion therapy, electroporation, and other procedures may be applied.

6. Recombinant Cell Isolation

- A mixed population of converted and non-transformed host cells results from the transformation process.
- Only the transformed host cells are filtered during the selection procedure.
- The marker gene of the plasmid vector is used to distinguish recombinant cells from non-recombinant cells.
- PBR322 plasmid vector, for example, comprises two marker genes (Ampicillin resistant gene and Tetracycline resistant gene). When pst1 RE is utilised, it eliminates the Ampicillin resistance gene from the plasmid, causing the recombinant cell to become Ampicillin sensitive.

Application of Recombinant DNA Technology

Recombinant DNA technology has been widely used in medical science, industries, animal husbandry, and agriculture.

The following highlights the application of r DNA technology in brief -

- To produce recombinant HB vaccines.
- For producing human insulin.
- To facilitate better crop production.
- For producing growth hormones in humans to treat dwarfism.
- For better gene therapy.
- To acquire DNA fingerprinting.
- To diagnose several types of diseases.

What is DNA Cloning?

Typically, a clone is defined as a cluster of individual cells that come from a progenitor. A clone is genetically similar to its parent cell from which it replicates.

DNA cloning is initiated when DNA fragments are inserted into DNA molecules. The said replicating molecule is the carrier of DNA vectors. A clone is a group of individuals or cells that derive from a single progenitor. Clones are genetically identical because each time a cell replicates, it produces identical daughter cells. Scientists have discovered a way to make many copies of a single DNA fragment, a gene that can be used to make identical copies of a DNA clone. DNA cloning is accomplished by inserting DNA pieces into a small DNA molecule. This molecule is designed to multiply inside a living cell, such as a bacteria. The carrier of the DNA vector is a small replicating molecule. Plasmids, yeast cells, and viruses are among the prominent vectors of rDNA technology examples. Plasmids are circular DNA molecules that bacteria inject into the human body. They aren't a part of the cell's core genome. It carries genes that confer favourable traits on the host cell, such as the ability to mate and drug resistance. They can be easily controlled since they are small enough and can carry extra DNA that has been woven into them.

Importance of DNA Cloning:

- Agriculture is one of the fields where gene cloning is used. Nitrogen fixation is carried out by cyanobacteria, and desired genes can be used to boost crop output and improve health. As a result of this method, the consumption of fertilisers is reduced, and chemical-free products are produced.
- In the world of medicine, gene cloning is extremely significant. Hormones, vitamins, and medicines are all made with it.
- It can be used to identify and detect a clone that contains a specific gene that can be modified by growing in a controlled environment.
- It is utilised in gene therapy, in which a damaged gene is replaced with a healthy one. This technique can be used to cure diseases like leukaemia and sickle cell anaemia.

VECTORS

1. Vectors should be capable of replicating autonomously, which, in turn, depends on the presence of particular sequences in the vector that enables them to initiate replication and propagation within the host cell. Some vectors might even have sequences that allow the production of proteins essential for the inserted DNA, regulation of the process, and further transfer of the insert between different vectors.
2. The size of an ideal vector should also be small enough for it to be incorporated into the host genome. The small size of the vector also enables it to incorporate a large-sized insert for transfer.
3. Vectors should be easy to isolate and purify as these need to be recovered and reused for multiple processes.
4. For a vector to be effective, these should also have certain components that facilitate the process of determining whether the host cell has received the vector. Most vectors used in this process have a gene that either provides resistance to an antibiotic or produces a particular type of protein. These components are called marker genes.
5. Many vectors also require unique restriction enzyme recognition sites that enable the insertion of the vector DNA in the presence of specific restriction enzymes. However, many vectors have been designed with a series of restriction sites close to multiple cloning sites that increases the possible restriction enzymes that can be used to digest the sequence.
6. The introduction of vectors into the host cell should be easy, which depends on a number of factors.
7. In the case of gene transfer processes, it is important that the vector is capable of integrating itself or the recombinant DNA into the genome of the host cell.
8. It is important that the introduction of recombinant DNA into the vector doesn’t affect the replication cycle of the vector.

Types of vectors

Vectors can be classified into different groups depending on the purpose of the process and the type of particles used in the process. The following are the commonly studied group of vectors that are used for different purposes;

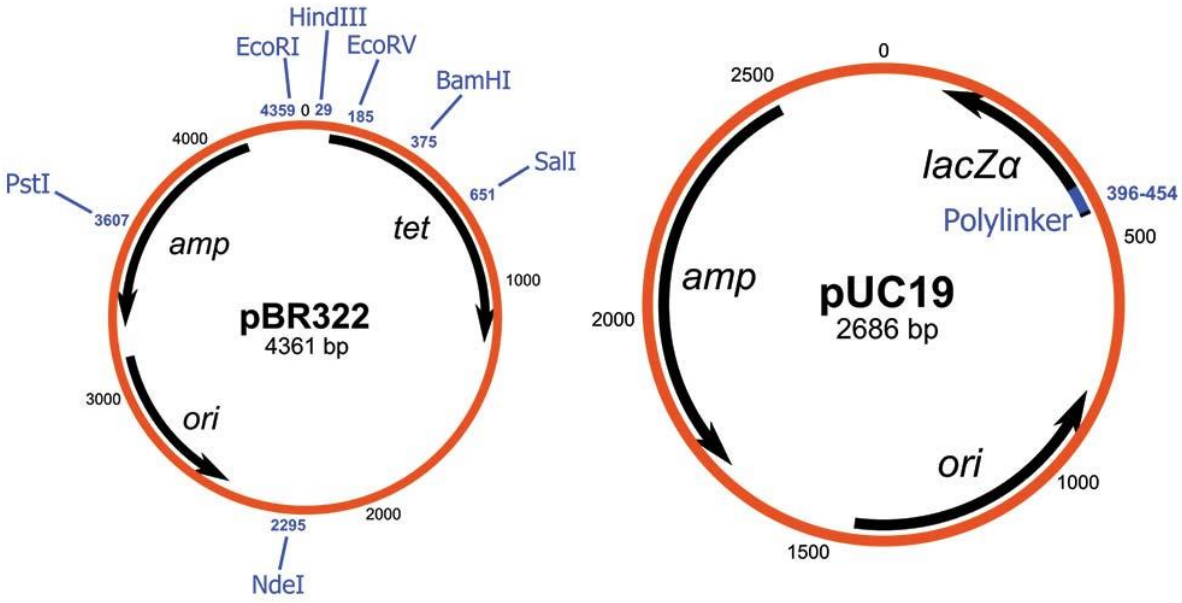
1. Cloning vectors

- Cloning vectors are vectors that are capable of replicating autonomously and thus are used for the replication of the recombinant DNA within the host cell.
- Cloning vectors are responsible for the determination of which host cells are appropriate for replicating a particular DNA segment.
- Cloning vectors are of further different types that are defined by different features unique to each type of vector.

a. Plasmid vector

- Plasmids are small extrachromosomal circular DNA molecules capable of replicating autonomously within the host cell.
- These are also termed as the workhorse cloning vector in recombinant DNA technology.
- Plasmids are widely used as vectors in all three domains of life; however, these are frequently used in bacteria and yeasts.
- The most important feature of plasmids that makes them one of the best vectors is their small size. The small size of the plasmid facilitates the separation of recombinant DNA from the host's genomic DNA.
- The size of plasmids ranges from a few thousand base pairs to more than 100 kilobases.
- Plasmids can carry insert DNA that is less than 20 kb as the cloning efficiency and plasmid stability decrease with the size of the vectors.
- The autonomous replication of plasmid is made possible by the presence of genes and sequences that can initiate plasmid replication independent of the host's replication cycle.
- Bacterial plasmids contain ori sequences that not only control plasmid replication but also determine the possibility of two plasmids coexisting within the same host cell.

- Different plasmids have different types of selective markers, but the most common markers include antibiotic resistance and the production of the β-galactosidase enzyme.
- Some of the most widely used plasmids are pBR322, pUC, and pBluescript vectors that use *E. coli* as the host.



pBR322

- Created in 1977 in the laboratory of Herbert Boyer at the University of California, San Francisco. Named after Bolivar and Rodriguez
- pBR322 is 4361 base pairs in length
- has two antibiotic resistance genes – the gene **bla** encoding the ampicillin resistance (AmpR) protein, and the gene **tetA** encoding the tetracycline resistance (TetR) protein
- contains the origin of replication of pMB1, and the *rop* gene, which encodes a restrictor of plasmid copy number
- has unique restriction sites for more than forty restriction enzymes.
- There are two sites for restriction enzymes HindIII and ClaI within the promoter of the Tet^R gene. There are six key restriction sites inside the Amp^R gene.
- A large number of other plasmids based on pBR322 have since been constructed specifically designed for a wide variety of purpose, eg. PUC series

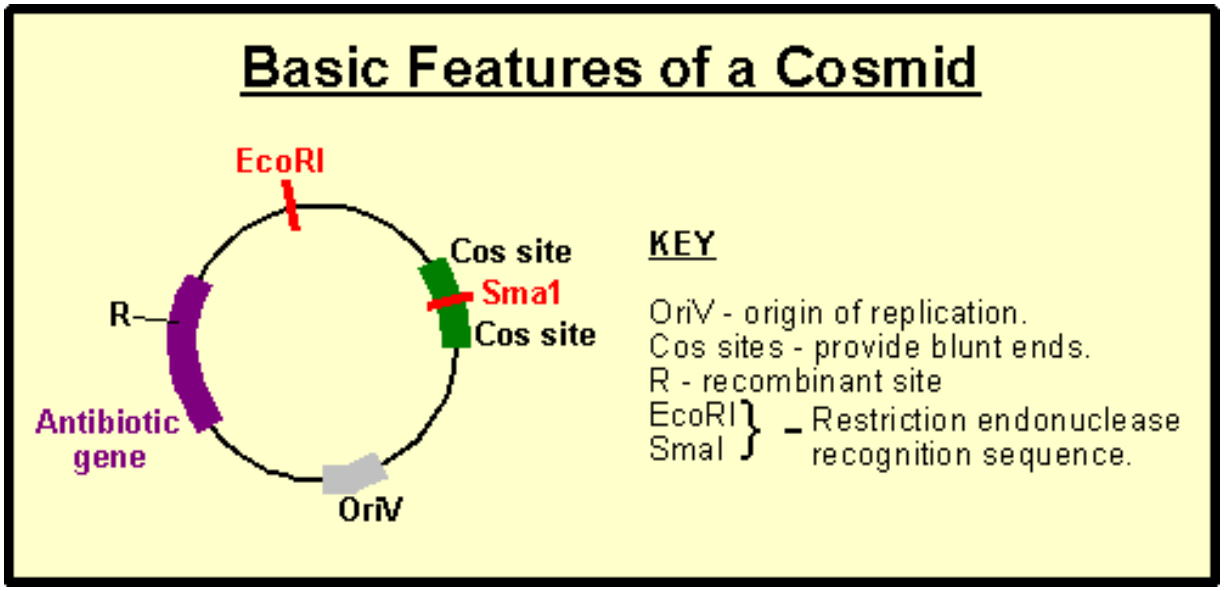
pUC series

- Developed from pBR322
- circular double stranded DNA and has 2686 base pairs
- pUC19 is one of the most widely used vector molecules as the recombinants, or the cells into which foreign DNA has been introduced, can be easily distinguished from the non-recombinants based on color differences of colonies on growth media.
- it has an N-terminal fragment of β-galactosidase (*lacZ*) gene of *E. coli*
- multiple cloning site (MCS) region is split into codons 6-7 of the lacZ gene, providing for many restriction endonucleases restriction sites
- pUC19 also encodes for an ampicillin resistance gene (amp^R), pUC19 is small but has a high copy number
- recognition sites for HindIII, SphI, PstI, SalI, XbaI, BamHI, SmaI, SacI and EcoRI restriction enzymes.

b. Cosmid

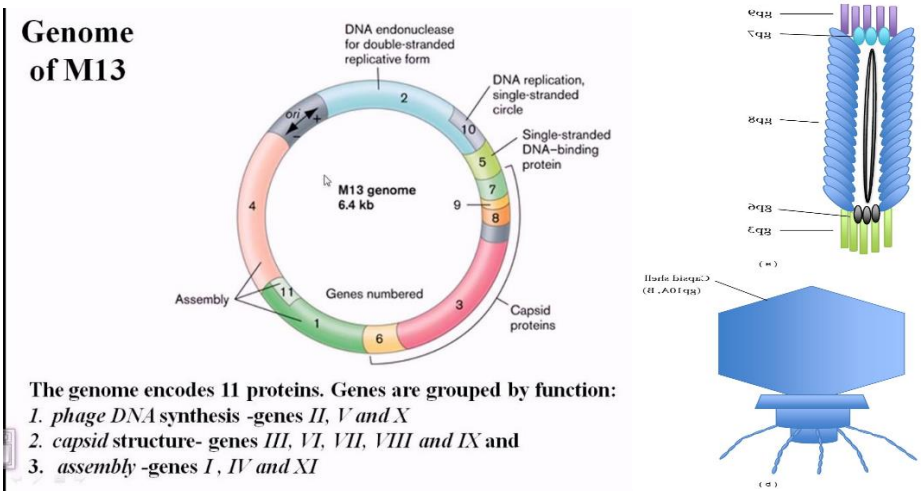
- Cosmid vectors are hybrid vectors composed of plasmid and cos sequence of phage λ vectors, capable of incorporating up to 42 kb of DNA.
- Cosmid vectors are prepared by the insertion of the cos region of the phage vector into the plasmid vectors.
- The cos site is a ≈200 bp long segment that is required to both initiate and terminate the packaging of a monomeric genome from concatemeric DNA (somewhat repeat seq.). The site where terminase introduces staggered nicks to generate the cohesive ends is called cosN
- Cosmid vectors are large-sized vectors with sizes ranging from 400 base pairs to 30 kb. These can carry DNA sequences having sizes ranging from 28 to 46 kb.
- Cosmid vectors are created in order to incorporate large-sized DNA molecules that cannot be carried by plasmids.
- Since these are hybrid vectors, these can replicate within the host cell like plasmids or remain packaged like a phage.

- Cosmid vectors do not have many phage characteristics except the signal sequences that promote phage-head stuffing.
- The hybrid structure of cosmid enables the phage heads to be incorporated within all donor DNA for transfer.
- The use and production of cosmid vectors have increased over the years as the packaged system is highly efficient and selective for the recovery of larger hybrids.
- One of the examples of the cosmid vectors prepared and used in practice are cosmid pHC79 which is a cos-containing derivative of the vector pBR322.



c. Bacteriophage vectors

- Bacteriophage vectors are viruses that only infect bacteria and transform them efficiently while carrying large inserts.
- Bacteriophages or phages have higher transformation efficiencies which increase the chances of recovering a clone containing the recombinant DNA segments.
- The most important feature of a phage is the packaging system which enables the incorporation of large eukaryotic genes and their regulatory elements.
- The use of phages also facilitates the isolation of larger quantities of DNA that can be used for the analysis of the insert.
- Even though there are a number of phages that can and have been used as vectors, phage λ is the most convenient cloning vector.
- It can selectively package a chromosome about 50 kb in length, and the size of the phage can be adjusted by removing the central part of the genome as it is not necessary for replication or the packaging of the donor DNA.
- The use of a bacteriophage vector that can incorporate larger DNA segments decreases the number of clones required to obtain a particular DNA library with the entire genome of the organism.
- Phage vectors are also effective as cloning vectors as the recombinant molecules formed after the cloning process are packaged into infective particles that can then be stored or handled efficiently.
- Some of the common phages used as vectors include M13 phages, λ phages, and P1 phages.



M13 phage:

- Contains 6.7kb circular ssDNA
- Used to insert single stranded DNA transcripts
- Do not have a full phage life cycle if developed as a hybrid along with plasmid. But can be used as an effective vector
- DNA sequencing and site directed mutagenesis

d. Bacterial artificial chromosome

- Bacterial artificial chromosomes are engineered DNA molecules that are used to clone DNA segments in bacteria cells (usually *E. coli*).
- These consist of a bacteria-derived F-factor replication origin which enables the propagation of large DNA fragments in a supercoiled circular form.
- Bacterial artificial chromosomes can carry a much larger size of insert DNA as compared to plasmid or phage vectors.
- These vectors are considered superior over other artificial chromosomes like yeast artificial chromosomes, and mammalian artificial chromosomes as the F-factor found in the bacteria reduces insert chimerism and instability that might arise during the process.
- These are highly efficient as DNA segments as large as 300,000 base pairs can be inserted into bacterial artificial chromosomes, which decreases the number of clones and cycles to be performed to obtain the desired result.

- BAC libraries have been used to generate large genomic DNA inserts for processes like positional cloning, physical mapping, and genome sequencing.
- BAC cloning system has been increasingly used in genetic engineering due to its stability and ease of use as compared to other similar vectors.
- **Drawback:** BACs have been associated with the random insertion of DNA fragments into the host genome resulting in unpredicted expression.

e. Yeast artificial chromosome

- Yeast artificial chromosomes are engineered DNA molecules that are used to clone DNA inserts within the yeast cells, particularly Saccharomyces cerevisiae.
- YACs have been developed in order to clone large sequences of DNA so as to increase the efficiency of the process.
- YACs can clone up to 1000-2000kb of DNA, which is much higher than most traditional cloning vectors.
- Even though these are frequently used as cloning vectors, they are also helpful in other genetic processes like DNA sequencing and analysis.
- These are also unique in their ability to clone the complete sequences of larger genomes that exceed the limits of traditional techniques.
- Since yeast cells are eukaryotic cells, YACs can be used for unstable sequences when cloned in prokaryotic systems.
- These consist of a mixture of functional units from different organisms, but once the insert DNA is cloned, these can function as normally replicating yeast chromosomes.
- There are some limitations with using YAC as vectors as these introduce a high degree of chimerism and insert rearrangement.
- Since these are eukaryotic cells, these are difficult to handle and have lower efficiencies as compared to bacterial artificial chromosomes.
- Different yeast artificial chromosomes have been created over the years that are then used for different purposes.

- One of the most commonly used examples of yeast artificial chromosomes includes pYAC4, which has been extensively used as a cloning vector.

f. Human artificial chromosome

- Human artificial chromosomes are extrachromosomal DNA fragments that act as a new chromosome within the human cell.
- The use of human artificial chromosomes has increased with advances in genetic engineering as it helps overcome problems commonly associated with traditional vector systems.
- HACs can exist as single copy episomes without integration into the host chromosomes allowing long-term stable maintenance.
- Besides, there is no upper limit in the size of the DNA insert to be incorporated into a HAC as entire genomic units can be used to mimic the natural gene expression.
- In spite of numerous advantages, HACs have only been used for studies related to the structure and function of human kinetochores.
- Limitations associated with HACs are due to technical difficulties during gene loading and ill-defined structures of the vectors.

2. Viral vectors

- Viral vectors are one of the most effective means of gene transfer to modify host cells or tissues and manipulate them to express different types of genes.
- The concept of using viruses as vectors arose from the fact that viruses are very effective in transducing their own genetic information into the host cell.
- During viral transduction, the non-essential viral genes are replaced with foreign DNA sequences of therapeutic interest in order to produce recombinant viral vectors.
- Currently, different groups of viruses have been studied for their possible use as viral vectors to deliver genes to provide transient or permanent transgene expression.
- The use of viral vectors also enables location specificity with unique injection technology within a specific time period.

- Some of the common virus groups considered for viral vectors are adenoviruses, retroviruses, poxviruses, and adeno-associated viruses.
- The choice of a particular virus as a vector depends on a number of factors that include efficiency of transgenic expression, ease of production, safety, and stability.
- Different clinical trials have been held with different potential viral vectors that are suitable for different purposes.
- Adenoviruses have been used for the transfer of tumor suppressor genes in cancer treatment, and retroviruses are studied for their potential use in tissue repair and engineering.

3. Expression vector

- Expression vectors are vectors that enable the expression of cloned genes in order to determine the successful cloning process.
- Usually, cloning vectors do not allow the expression of a cloned gene which is why the use of expression vectors is required.
- The use of expression vectors facilitates the processing of introns in prokaryotes as these are designed with restriction sites next to the regulatory region.
- The restriction sites on the vectors result in splicing of the cloned gene to permit the expression of the gene under the regulatory system.
- The regulatory system in expression vectors consists of a promoter sequence, a termination sequence, along a transcription termination sequence.
- The use of expression vectors is essential to determine the success of a cloning procedure and the efficiency of selective markers on the vectors.
- Expression vectors can be plasmid-based or viral-based that are introduced into the host cells in order to code for particular mRNAs.
- The expression vectors are often used for the production of proteins that can then be visualized by different methods depending on the complexity of the host cell.

- Expression vectors are of varying degrees of complexity depending on whether they are to be used in prokaryotic or eukaryotic cells.

4. Shuttle vector

- Shuttle vectors are that carry origins of replication from two different hosts, which enables them to ‘shuttle’ between the two hosts.
- These vectors contain DNA plasmids that can usually replicate in both mammalian cells as well as bacterial cells.
- Shuttle vectors function as hybrid vectors containing DNA sequences from bacterial plasmids and mammalian viruses.
- The vectors contain three functional DNA sequences involved in the cloning process; a viral replication origin, a bacterial replication origin, and a drug resistance gene.
- The presence of different replication sites and repair sequences enable the recovery and maintenance of these vectors in bacterial cells.
- There are three different shuttle vectors depending on the type of replication system utilized by the vectors.
- Transiently replicating shuttle vectors that need to be recognized by large T antigen in order to replicate in human cells.
- Episomal shuttle vectors work to establish cell lines that can replicate permanently in the form of plasmid DNA containing the DNA insert.
- Integrated shuttle vector undergoes replication only after fusion with particular cell types for gene expression.

Limitations of Vectors

The following are some of the limitations of vectors;

1. Vectors are not very stable due to changes in metabolic energy and changing pH and temperature in different hosts. The stability of vectors depends largely on the type of vector and host genotypes.
2. Overexpression of a particular type of genes in the host cell is a common problem associated with the use of vectors.
3. The use of a single type of vector might not be sufficient for a particular purpose. The use of multiple vectors is complex and results in difficulties during the process.
4. Even though a large number of studies are done in the field of molecular biology for the production of more efficient vectors, it is a time-consuming and expensive process.