BTY 402 GENETIC ENGINEERING & GENE TRANSFER TECHNIQUES

UNIT 1

Topic ;DNA Sequencing methods

What is DNA sequencing

DNA Sequencing is the method that determines the order of the four nucleotides bases (adenine, thymine, cytosine, and guanine) that make up the DNA molecule and convey important genetic information. In the DNAdoublehelix,thefour basesbondwiththespecificpartnertoform unitscalledbasepairs(bp).Adenine (A) pairs with thymine (T) and cytosine (C) pairs with guanine (G). The human genome contains around 3 billion basepairsthatprovidetheinstructions for thecreation andmaintenanceofa human being.Thebased-paired structuremakesDNAsequencewell suitedtothestorageofa vastamountofgeneticinformation.This complementarybase-pairingis thebasis for themechanism bywhich DNA molecules are copied, transcribed and translated, and the pairing also underlies most of DNA sequencing methods. Thanks to the tremendous improvement in DNA sequencingtechnologies andmethods, whole genome sequencinghas become possible and affordable.

Sanger sequencing method

TheSangermethodreliesonaprimerthat bindstoadenaturedDNAmoleculeandinitiatesthesynthesisofa single-stranded polynucleotide in the presence ofa DNA polymerase enzyme, usingthedenaturedDNAas a template. In most circumstances, the enzyme catalyzes the addition of a nucleotide. A covalent bond, therefore, forms between the 3′ carbon atom of the deoxyribose sugar molecule in one nucleotide and the 5′ carbon atom of the next. This image below shows how this bond is formed.

Asequencingreactionmixture,however, wouldhavea smallproportion ofmodifiednucleotidesthat cannot form this covalent bond due to the absence of a reactive hydroxyl group, giving rise to the term ‘dideoxyribonucleotides’, i.e., theydo not have a 2’ or 3’ oxygen atom when compared to the corresponding ribonucleotide. This would terminate the DNA polymerization reaction prematurely. At the end of multiple rounds of such polymerizations, a mixture of molecules of varying lengths would be created.In the earliest attempts at using the Sanger method, the DNA molecule was first amplified using a labeled primer and then splitintofour testtubes, eachhaving onlyonetypeofddNTP.Thatis,eachreaction mixturewouldhaveonly

one type of modified nucleotide that could cause chain termination. After the four reactions were completed, the mixture of DNA molecules created by chain termination would undergo electrophoresis on a polyacrylamide gel, and get separated according to their length.

Intheimageabove, a sequencingreactionwith ddATPwaselectrophoresedthroughthefirstcolumn.Each line represents a DNA molecule of a particular length, the result of a polymerization reaction that was terminated bytheaddition ofa ddATPnucleotide. Thesecond,thirdandfourth columnscontainedddTTP, ddGTP, and ddCTP respectively.

With time,thismethod wasmodified sothateach ddNTPhad adifferentfluorescentlabel.Theprimer wasno longer the source of theradiolabel or fluorescent tag. Also known as dye-terminator sequencing, thismethod used four dyes with non-overlapping emission spectra, one for each ddNTP.

Maxam–Gilbert sequencing method

Maxam–Gilbert sequencingisamethodofDNAsequencingdevelopedbyAllan MaxamandWalter Gilbert in 1976–1977. This method is based on nucleobase-specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides.

1. Themethodrequiresradioactivelabellingatoneendandpurification oftheDNAfragmenttobe sequenced.

2. Chemicaltreatment generatesbreaksatasmall proportionsofoneor twoofthefournucleotide based in each of four reactions (G,A+G, C, C+T).
3. Thusa seriesoflabelled fragmentsisgenerated,fromtheradiolabelled endtothefirst ‘cut’sitein each molecule.
4. Thefragmentsinthefourreactionsarearrangedsidebysidein gelelectrophoresisfor size separation.
5. Tovisualizethefragments,thegelisexposedtoX-rayfilm for autoradiography, yieldinga seriesof dark bands each corresponding to aradiolabelled DNA fragment, from which the sequence maybe inferred.

Topic: Enzymes used in genetic engineering

1. DNA ligase
2. Reverse transcriptase
3. Restriction endonuclease

4. Terminal transcriptase
5. Nuclease
6. DNA polymease
7. Ribonuclease-H
8. Alkaline phosphatase
9. Polynucleotide kinase

1. DNA ligase:

- DNA ligase is isolated from E.coli and Bacteriophage commercially and used in recombinant DNA technology.
- The enzyme DNA ligase joins the DNA fragments with cloning vector.

2. Reverse transcriptase:

- RT is used to synthesize complementary strand (cDNA) from mRNA template.
- It is also known as RNA dependent DNA polymerase
- It is isolated from retrovirus

3. Restriction endonuclease:

- Restriction endonuclease enzyme recognize and cut DNA strand at specific sequence called restriction site.
- These enzyme is isolated from wide variety of microorganisms. Endonuclease enzyme degrades foreign genome when enter inside microbial cell but the host cell own DNA is protected from its endonuclease by methylation of bases at restriction site.
- There are 3 types of restriction endonuclease:

Type I Restriction endonuclease:

- It has both methylation and endonuclease activity.
- It require ATP to cut the DNA
- It cuts DNA about 1000bp away from its restriction site
- eg. *Eco*KI

Type II Restriction endonuclease:

- It does not require ATP to cut DNA
- It cuts DNA at restriction site itself
- eg. *Eco*RI, *Hind* III

Type III Restriction endonuclease:

- It requires ATP to cut DNA
- It cuts DNA about 25bp away from restriction site.
- eg. *Eco*PI

4. Terminal transferase:

- It is the enzyme that converts blunt end of DNA fragments into sticky end.
- If the restriction enzyme cuts DNA forming blunt ends, then efficiency of ligation is very low. So theenzyme terminal transferase converts bunt end into sticky end.
- Terminal transferase enzyme synthesize short sequence of complementary nucleotide at free ends of DNA,so that blunt end is converted into sticky end.

5. Nuclease:

- The enzyme nucleases hydrolyses the phosphodiester bond on DNA strand creating **3'-OH** group and **5'-** It usually cut DNA on either side of distortion caused by thymine dimers or intercalating agents
- The gap is filled by DNA polymerase and strand is joined by DNA ligase
- Nucelase are of two types; endonuclease and exonuclease

6. DNA polymerase:

- 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

7. Ribonuclease-H (RNase H):

- RNase-H removes mRNA from DNA-RNA heteroduplex and that mRNA is used to synthesize cDNA
- It is isolated from retrovirus

8. Alkaline phosphatase:

- The enzyme Alkaline phosphatase helps in removal of terminal phosphate group from 5′ end
- It prevents self annealing of vector DNA soon after cut open by restriction endonuclease

9. Polynucleotide kinase:

- It adds phosphate group from ATP molecule to terminal 5'end after dephosphorylation by alkaline phosphatase.

UNIT-II

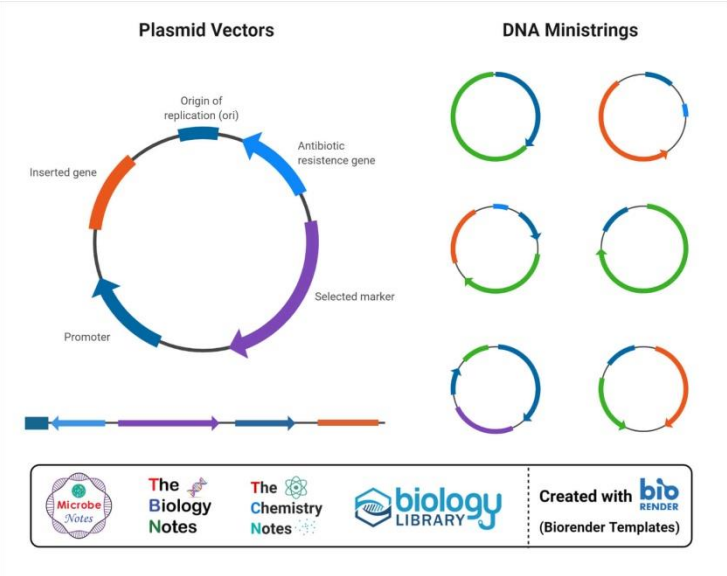
TOPIC : VECTORS TYPES

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.



DNA Cloning Vectors

a. Plasmid vector

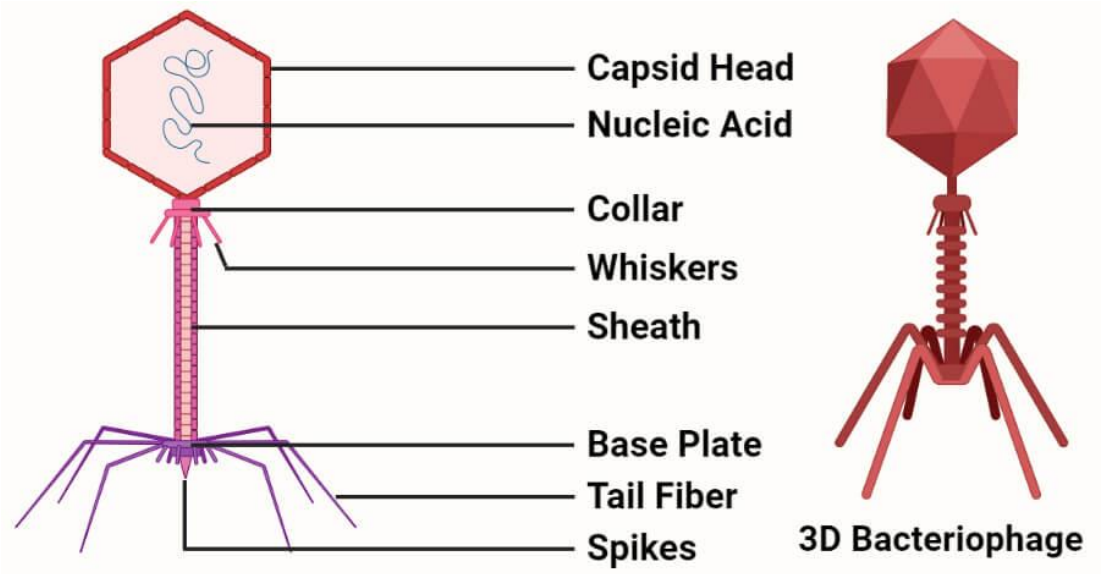
- Plasmids are small extrachromosomal circular DNA molecules capable of replicating autonomously within the host cell.
- These are also termed as the workhorse cloning vector in recombinant DNA technology.
- Plasmids are widely used as vectors in all three domains of life; however, these are frequently 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. The small size of the vector does, however, affect the maximum size of the insert DNA it can carry.
- 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.

b. Cosmid

- Cosmid vectors are hybrid vectors composed of plasmid and 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.
- 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 vector



- 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 handle efficiently.
- Some of the common phages used as vectors include M13 phages, λ phages, and P1 phages.

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.
- However, 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 500 kb 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.

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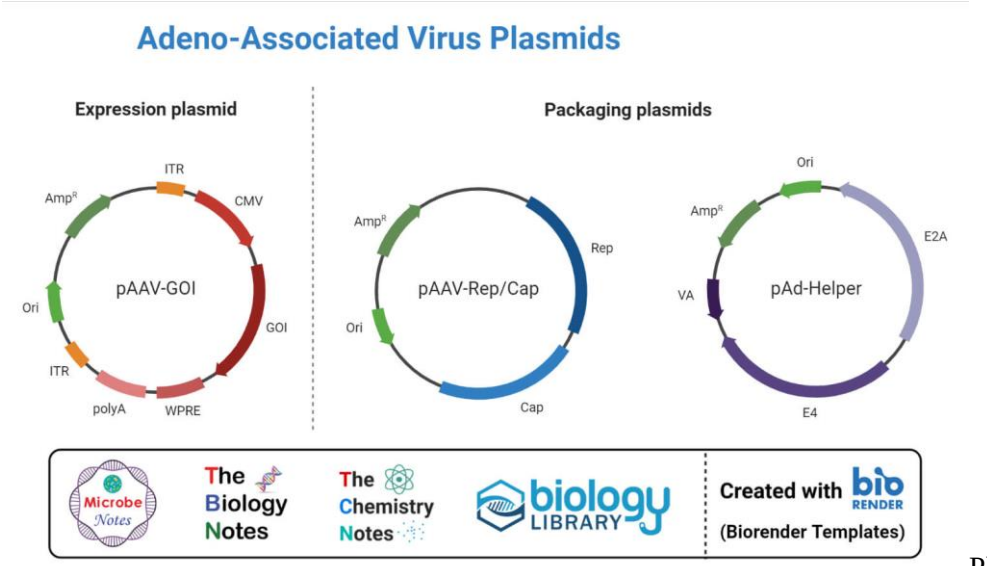
2. Viral vectors

- 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 doe 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.



Plasmids for Adeno-Associated Virus Vector Production.

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

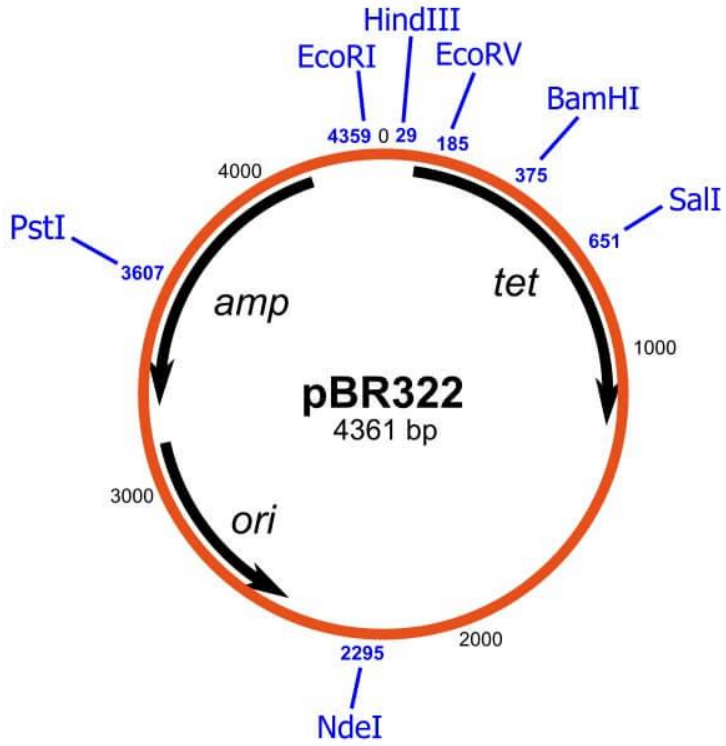
- Shuttle vectors are that carry origins of replication from two different hosts, which enables them to 'shuttle' between the two hosts.
- These vectors contain DNA plasmids that can usually replicate in both mammalian cells as well as bacterial cells.
- Shuttle vectors function as hybrid vectors containing 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.

5. Secretion vector

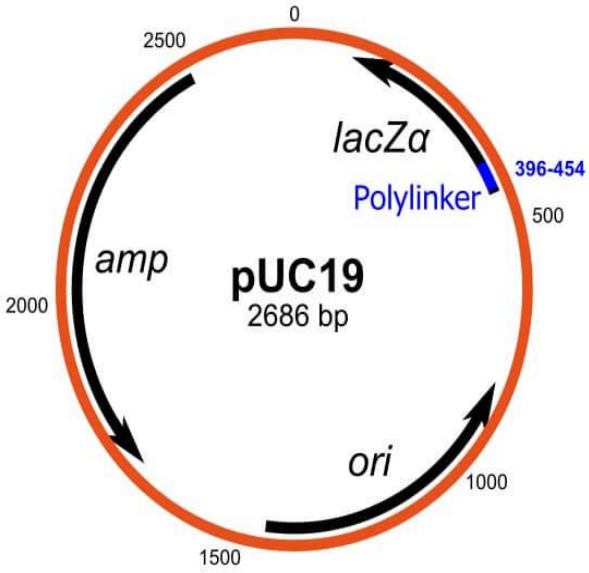
- Secretion vectors are a type of specialized expression vector that expresses the cloned genes in order to produce proteins at locations other than the cytoplasm.
- The transport of protein product from the cell is achieved by the fusion of the inset DNA with a nucleotide sequence encoding the peptide of an easily secreted protein.
- The use of secretion vector has many advantages like higher yield, simple purification process, and improved protein stability.
- Secretion vectors can be designed for more than one type of prokaryotes or eukaryotes, including mammals.
- A commonly associated problem with the incorporation of a protein of eukaryotic origin into a prokaryotic host is the overexpression of the protein. This problem is solved by the use of secretion vectors that alleviate the formation of inclusion bodies.
- Secretion vectors have replaced cloning vectors in processes focusing on the production of proteins and the expression of eukaryotic DNA fragments.

Examples of Vectors

The following are some of the examples of vectors that are commonly used for different genetic engineering processes;



- pBR322 is a commonly used plasmid cloning vector used in prokaryotes, primarily E. coli.
- The vector consists of an origin of replication from a ColE1-like plasmid, pMB1, an ApR gene (Ampicillin resistance gene) from the transposon, Tn3, and a TcR gene from pSC101.
- pBR322 was designed to overcome the limitations with pBR312 and pBR313, both of which have extraneous DNA sequences and restriction enzyme cleavage sites that affected their function as vectors.
- The structure of pBR322 was designed to maximize the number of restriction enzyme cleavage sites on the vector and to minimize its size.
- The vector contains twenty-one unique restriction enzyme cleavage sites, eleven of which are present in the TcR and ApR genes.
- The structure also facilitates a unique EcoRI cleavage site within the plasmid in order to increase the efficiency of the vector.
- The pBR322 family of vectors was initially created for general cloning purposes in *E. coli* and other similar prokaryotes; however, over the years, derivatives of the vector have been designed for cloning purposes that specific to a particular organism or a particular function.
- Even though pBR322 has been used for decades as an effective multipurpose cloning vector, it has some limitations.
- The vector might be lost in continuous culture in the absence of selective pressure, which might be a problem in large-scale fermentation of recombinant bacteria.



- pUC19 is also an example of a plasmid cloning vector that is used for the transfer of recombinant DNA fragments into a host cell.
- The name 'pUC19' is given o the vector where the 'p' indicates plasmid and 'UC' indicates the University of California' where the vector was designed and constructed.
- The vector has been extensively used for cloning purposes where the host cells containing the plasmid are distinguished from the ones that do not have it by the differences in the color of the colonies on the growth medium.
- The vector is a double-stranded DNA molecule with 2686 bp and a high copy number.
- pUC19 consists of a 54 base-pair cloning site polylinker that further contains 13 different hexanucleotide-specific restriction endonucleases.
- The colony screening after cloning with pUC19 is due to the presence of a selective marker that encodes for the N-terminal fragment of β-galactosidase.

λ phage

- λ-phage is an example of a bacteriophage that infects the bacterial species, *Escherichia coli* (*E. coli*).
- This vector is more effective than other plasmid vectors as it has a higher efficiency in entering bacterial cells so as to incorporate the recombinant DNA within the host genome.
- It is a double-stranded DNA bacteriophage that contains an ori sequence requires for replication and a number of DNA sequences encoding regulatory and replicative proteins.
- The phage DNA replicates by the combination of theta and rolling circle replication process to produce a linear dsDNA. It is then followed by the cos sequence, which enables the circularization of the genome after infection.
- The DNA sequences between the two arms of the vector are not essential which are then replaced with the recombinant DNA during cloning.

Applications of Vectors

The application of vectors in molecular biology and genetic engineering has increased with time due to the simplicity, cost-effectiveness, and rapidity of the process. The following are some of the major applications of vectors in molecular biology;

1. Cloning vectors are the most important group of vectors that are used for the transfer of foreign DNA into host cells for different purposes.
2. One of the most important applications of vectors is to generate engineered organisms for a particular function, like engineering *E. coli* bacteria for insulin production.
3. Vectors can be used to isolate a particular gene sequence within a genome and to determine its nucleotide sequence through DNA sequencing.
4. Vectors are one of the components in molecular biology which enable numerous studies related to cell structure, nucleic acid composition, and genetic engineering techniques.

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.

Unit-III

Topic: GENE TRANSFER TECHNIQUES

- Gene transfer or up takeof DNA refers to the processthat movesaspecific piece of DNA into cell.
- Thedirecteddesirablegenetransferfromoneorganismtoanotherandthesubsequentstable integration&expressionofforeigngeneintothegenomeisreferredasgenetictransformation.
- Thetransfeeredgeneisknownastransgeneandtheorganismthatdevelopafterasuccessfulgene transfer is known as transgenic.
- Transgenicplantaretheplantthatcarrythestablyintegratedforeign genes. Theseplantsmayalsobecalledtransformedplants.
- The transferred DNA may be expressed for only short period of time following the DNA transfer process and this is called transient expression.
- StabletransformationoccurwhenDNAisintegratedintotheplantnucleargenomeexpressionoccurs inregeneratedplantand is inheritedinsubsequent generations.

STEPS IN TRANSFORMATION

- Identification of useful genes; desirable genes located in

Wild species, unrelated plantspecies, unrelated organism and animals.

- Designing gene for insertion: The gene of interest is isolated from the donor source and cloned in the laboratory. The cloning is done generally using plasmid.
- Insertion of gene into target plant: The cloned gene i.e multiple copies of the gene of interest are inserted into host plant or the recipient plant.
- Identification of transgenic cell: Transformed cells are identified using selectable marker and are regenerated into whole plant in nutrient medium.
- Regenerate plant compared with plant variety. It should look like parent variety except gene of interest.

GENE TRANSFER METHODS

- Vector Mediated Gene Transfer
- Vector less or Direct Gene Transfer Methods

- VECTOR MEDIATED GENETRANSFER
  - Plant gene vectors being exploited for transfer of genes are plasmids of Agrobacterium, viruses & transposable elements.
  - Vectors: Small circular DNA molecule occurring in bacteria, which can exchange between different cells under natural condition.
  - Plasmids: Plasmids are the extra chromosomal self replicating & double stranded, closed & circular DNA molecule present in the bacterial cell.

AGROBACTERIUM MEDIATEDTRANSFORMATION

- Agrobacterium, system historically first successful plant transformation system,break through in1983.
- Break throughingene manipulation in plants came by characterizing and exploiting plasmids carried by the bacterial plant pathogens Agro bacterium tumefaciens & Agrobacterium rhizogenes.
- Bacteria of the genus Agrobacterium –gene vectors for plant cells. Agro bacterium–gram negative long to bacterial family Rhizobiaceae
- LargePlasmidsinthesesagrobacteriaarecalled Tumou rinducingplasmids(Ti)androotinducing plasmid(Ri)
- Diseases result from transfer and
- Functional integration of particular set of Ti or Ri plasmid in plant chromosome
- Features of Ti plasmid which make them attractive gene vector
- Ti plasmid integrates into plant genome and stably transmitted through division of mitosis and meiosis.
- Genes like nopaline synthase encoded y TDNA possess promoter that function in plant cells.
- Foreigngene/DNAinsertedinto'TDNA'regionisintegratedintoplantgenome.
- Agrobacterium has broad hostrange hence the gene of interest in the"T-DNA"can be transferred to wide range of plants.

- Transformation Technique Using Agrobacterium
- Important requirement for Agrobacterium mediated enter transferin plants:
- Plant explants must produce aceto syring one/
- Agro bacterium may be pre induced with synthetic acetosyring one.
- Agro bacteria should have access to cell that are competent for transformation. Transformation competent cells/tissue should be able to regenerate into whole plant
- Infection of wounded plant
- Seedlings decapitated and freshly cut surface wound is inoculated with over night culture of Agro bacterium
- Tumour produced excised out and grown as callus culture.
- Transforming callus are picked off & regenerated.

Electroporation

DNA is introduced into protoplasts and plant cells via the transformation procedure known as electroporation. This method makes use of an electric pulse with high voltage. Plant samples are incubated in a DNA-containing buffer solution. Then an electric pulse with a high voltage is applied to the solution. Plant cell membranes develop high voltage-driven pores that allow DNA to go within the cells and combine with the genomic DNA of plants. The plants used and the conditions under which they are treated determine how effective this procedure is.

Only 40-50% of cells obtain DNA when the transformation is performed using electroporation. Additionally, this approach only allows 50% of the transformed cells to survive. However, this procedure is simple to perform and does not modify the biological configuration or function of cells. It can also be applied to various cells.

Microinjection

The transformation method of microinjection is particularly efficient when inserting DNA into giant cells. The microinjection technique introduces DNA into animal cells (eggs, oocytes, and embryos) or plant protoplasts using a micropipette (fine-tipped glass needle). This technique is more appropriate for producing transgenic mice. This process involves incorporating DNA straight into the cytoplasm or nucleus.

Microinjection is a method of direct transformation, much like electroporation. Microinjection is carried out using a specialised microscope setup. The effectiveness of this technique has been improved by computerised control of the microscope stage, needle, holding pipette, and video technologies. A dye can be utilised to determine transformed cells after DNA injection.

This technique is very trustworthy and effective. However, this procedure is expensive, time-consuming, and labour-intensive. Furthermore, this technique can only be used to treat a few cells.

Nucleic Acid Probes

The following points highlight the three types of nucleic acid probes. The probes are: 1. Oligonucleotide Probes 2. DNA Probes 3. RNA Probes.

1. **Oligonucleotide Probes:**

These are synthesized chemically as oligonucleotides based on the information available on the amino acid sequence of the protein of interest. These oligo nucleotides can be used as a probe the identification of gene which encode for that particular protein.

However, due to degeneracy of the genetic code, construction of oligonucleotide is carried out with those that are rich in methionine or tryptophan residue or with only two codons. Generally oligonucleotide probe is used to screen cDNA libraries.

2. DNA Probes:

These are longer than the oligonucleotides. Thus, clones of longer DNA sequence is used as a DNA probes. The sequence obtained from cDNA library (cDNA clones) can be used to probe genomic library to identify genomic clones. The same probe can also be used to reprobe the same cDNA library to identify more cDNA clones. The genomic DNA clones are used to screen cDNA library or a genomic library.

Preparation of DNA Probe:

The DNA probe is prepared by random primer method as follows:

(i) In double stranded DNA containing the sequence that is to act as the probe is denatured and an oligonucleotide sample containing all possible sequences of six nucleotides is added (it is statistical certainty that some of the molecules of the oligonucleotide mixture will hybridize to the unlabelled, denatured probe DNA).

(ii) In the presence of klenow fragment and four deoxyribunucleotides, one of the four deoxyribonucleotides is labelled.

(iii) The bound oligonucleotides act as primers for DNA synthesis.

(iv) The synthesised DNA is labelled and can be used as a probe to detect the presence of a complementary DNA sequence in a source DNA sample

The gene for the phage polymerase and the phage promoter were both cloned in a vector, and a target gene sequence for transcription inserted downstream of the phage promoter. Cells transformed with recombinant DNA are supplied with RNA precursor nucleotides, of which one nucleotide is radio labelled. Transcription of the inserted gene is therefore labelled and may be used as RNA probes

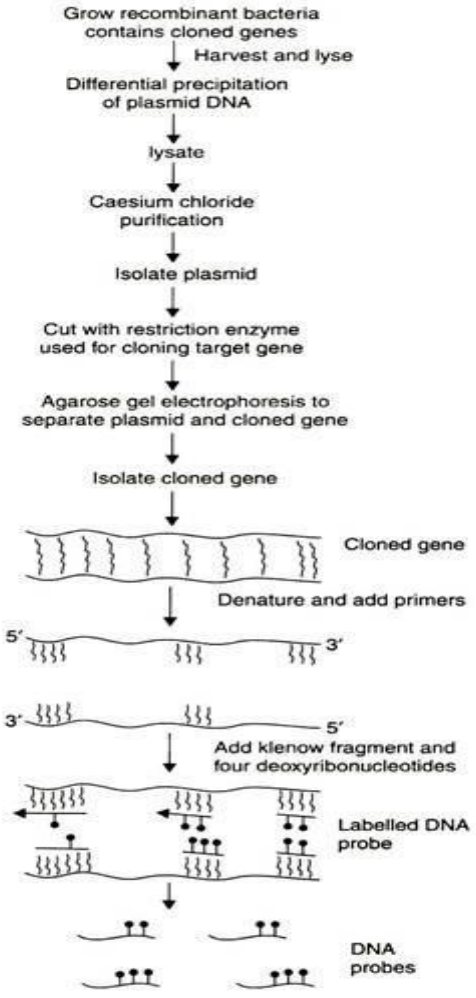


Fig. 13.8 Preparation of labelled probes

There are at least two possible sources of probes: one is from cloned DNA, second is the nucleotide sequence of a synthetic probe, based on the probable nucleotide sequence that is deduced from the known aminoacid sequence of the protein encoded by the target gene.

3. RNA Probes:

RNA probes used only under certain circumstances. Purification of particular RNA is generally used as a specific probe for the corresponding DNA specifies. By employing positive- negative screening or differential hybridized approach, often possible to identify clones for RNA in one population but not so in other population.

The colony is first probed with labelled RNA from one population. After the location of hybridized colonies, labelled RNA is then washed off from the membrane and the membrane is then probed with labelled RNA from the second population and hybridized colonies are identified.

The first RNA probes were mRNAs of a gene that are abundantly expressed in a cell, labelled with $^{32}po_4$. One of the efficient ways of preparing RNA probes involves transcription from a target gene cloned in a plasmid. Transcription of this gene is initiated from a promoter that is specifically recognised by an RNA polymerase.

Due to unspecificity of RNA pol recognition, specific promoter can be selected. The promoter of a bacteriophage is very specific for the phage polymerase. Some researchers have utilized phage (SP6) T7 promoter and the corresponding enzyme to transcribe a DNA (target gene) cloned in a plasmid vector.

The gene for the phage polymerase and the phage promoter were both cloned in a vector, and a target gene sequence for transcription inserted downstream of the phage promoter. Cells transformed with recombinant DNA are supplied with RNA precursor nucleotides, of which one nucleotide is radio labelled. Transcription of the inserted gene is therefore labelled and may be used as RNA probes

UNIT-4

Polymerase Chain Reaction

PCR or Polymerase Chain Reaction is a technique used in molecular biology to create several copies of a certain DNA segment. This technique was developed in 1983 by Kary Mullis, an American biochemist. PCR has made it possible to generate millions of copies of a small segment of DNA. This tool is commonly used in the molecular biology and biotechnology labs.

Principle of PCR

The PCR technique is based on the enzymatic replication of DNA. In PCR, a short segment of DNA is amplified using primer mediated enzymes. DNA Polymerase synthesises new strands of DNA complementary to the template DNA. The DNA polymerase can add a nucleotide to the pre-existing 3'-OH group only. Therefore, a primer is required. Thus, more nucleotides are added to the 3' prime end of the DNA polymerase.

Components Of PCR

Components Of PCR constitutes the following:

1. **DNA Template**– The DNA of interest from the sample.
2. **DNA Polymerase**– Taq Polymerase is used. It is thermostable and does not denature at very high temperatures.
3. **Oligonucleotide Primer**s- These are the short stretches of single-stranded DNA complementary to the 3' ends of sense and anti-sense strands.
4. **Deoxyribonucleotide triphosphate**– These provide energy for polymerization and are the building blocks for the synthesis of DNA. These are single units of bases.
5. **Buffer System**– Magnesium and Potassium provide optimum conditions for DNA denaturation and renaturation. It is also important for fidelity, polymerase activity, and stability.

Types of PCR

PCR is of the following types:

Real-time PCR

In this type, the DNA amplification is detected in real-time with the help of a fluorescent reporter. The signal strength of the fluorescent reporter is directly proportional to the number of amplified DNA molecules.

Nested PCR

This was designed to improve sensitivity and specificity. They reduce the non-specific binding of products due to the amplification of unexpected primer binding sites.

Multiplex PCR

This is used for the amplification of multiple targets in a single PCR experiment. It amplifies many different DNA sequences simultaneously.

Quantitative PCR

It uses the DNA amplification linearity to detect, characterize and quantify a known sequence in a sample.

Arbitrary Primed PCR

It is a DNA fingerprinting technique based on PCR. It uses primers the DNA sequence of which is chosen arbitrarily.

PCR Steps

The PCR involves three major cyclic reactions:

Denaturation

Denaturation occurs when the reaction mixture is heated to 94℃ for about 0.5 to 2 minutes. This breaks the hydrogen bonds between the two strands of DNA and converts it into a single-stranded DNA.

The single strands now act as a template for the production of new strands of DNA. The temperature should be provided for a longer time to ensure the separation of the two strands.

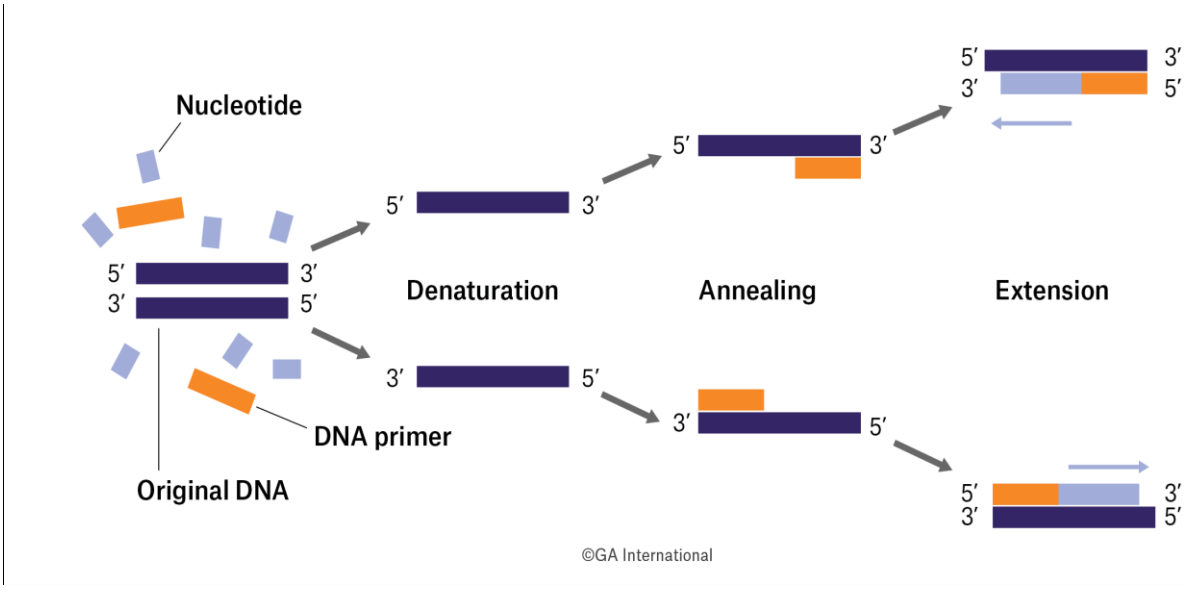
Annealing

The reaction temperature is lowered to 54-60℃ for around 20-40 seconds. Here, the primers bind to their complementary sequences on the template DNA.

Primers are single-strand sequences of DNA or RNA around 20 to 30 bases in length.

They serve as the starting point for the synthesis of DNA.

The two separated strands run in the opposite direction and consequently there are two primers- a forward primer and a reverse primer.



Elongation

At this step, the temperature is raised to 72-80℃. The bases are added to the 3' end of the primer by the Taq polymerase enzyme.

This elongates the DNA in the 5' to 3' direction. The DNA polymerase adds about 1000bp/minute under optimum conditions.

Taq Polymerase can tolerate very high temperatures. It attaches to the primer and adds DNA bases to the single strand. As a result, a double-stranded DNA molecule is obtained.

These three steps are repeated 20-40 times in order to obtain a number of sequences of DNA of interest in a very short time period.

Applications of PCR

The following are the applications of PCR :

Medicine

- Testing of genetic disease mutations.
- Monitoring the gene in gene therapy.
- Detecting disease-causing genes in the parents.

Forensic Science

- Used as a tool in genetic fingerprinting.
- Identifying the criminal from millions of people.
- Paternity tests

Research and Genetics

- Compare the genome of two organisms in genomic studies.
- In the phylogenetic analysis of DNA from any source such as fossils.
- Analysis of gene expression.
- Gene Mapping

DNA Microarray

DNA microarrays are solid supports, usually of glass or silicon, upon which DNA is attached in an organized, pre-determined grid fashion.

- Each spot of DNA, called a probe, represents a single gene.
- DNA microarrays can analyze the expression of tens of thousands of genes simultaneously.
- There are several synonyms for DNA microarrays, such as DNA chips, gene chips, DNA arrays, gene arrays, and biochips.

Principle of DNA Microarray Technique

The principle of DNA microarrays lies on the hybridization between the nucleic acid strands.

The property of complementary nucleic acid sequences is to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs.

For this, samples are labeled using fluorescent dyes.

At least two samples are hybridized to chip.

- Complementary nucleic acid sequences between the sample and the probe attached on the chip get paired via hydrogen bonds.
- The non-specific bonding sequences while remain unattached and washed out during the washing step of the process.
- Fluorescently labeled target sequences that bind to a probe sequence generate a signal.
- The signal depends on the hybridization conditions (ex: temperature), washing after hybridization etc while the total strength of the signal, depends upon the amount of target sample present.
- Using this technology the presence of one genomic or cDNA sequence in 1,00,000 or more sequences can be screened in a single hybridization.

Types of DNA Microarrays

There are 2 types of DNA Chips/Microarrays:

- cDNA based microarray
- Oligonucleotide based microarray
- Spotted DNA arrays ("cDNA arrays")
- Chips are prepared by using cDNA.
- Called cDNA chips or cDNA microarray or probe DNA.
- The cDNAs are amplified by using PCR.
- Then these immobilized on a solid support made up of nylon filtre of glass slide (1 x 3 inches). The probe DNA are loaded into a a spotting spin by capillary action.
- Small volume of this DNA preparation is spotted on solid surface making physical contact between these two.
- DNA is delivered mechanically or in a robotic manner.
- Oligonucleotide arrays (Gene Chips)
- In oligonucleotide microarrays, short DNA oligonucleotides are spotted onto the array.
- Small number of 20-25mers/gene.
- The main feature of oligonucleotide microarray is that each gene is normally represented by more than one probe.
- Enabled by photolithography from the computer industry

Requirements of DNA Microarray Technique

There are certain requirements for designing a DNA microarray system, viz:

- DNA Chip
- Target sample (Fluorescently labelled)
- Fluorescent dyes
- Probes
- Scanner

The reaction procedure of DNA microarray takes places in several steps:

- Collection of samples
- The sample may be a cell/tissue of the organism that we wish to conduct the study on.
- Two types of samples are collected: healthy cells and infected cells, for comparison and to obtain the results.
- Isolation of mRNA
- RNA is extracted from the sample using a column or solvent like phenol-chloroform.
- From the extracted RNA, mRNA is separated leaving behind rRNA and tRNA.
- As mRNA has a poly-A tail, column beads with poly-T-tails are used to bind mRNA.
- After the extraction, the column is rinsed with buffer to isolate mRNA from the beads.
- Creation of labeled cDNA
- To create cDNA (complementary DNA strand), reverse transcription of the mRNA is done.
- Both the samples are then incorporated with different fluorescent dyes for producing fluorescent cDNA strands. This helps in distinguishing the sample category of the cDNAs.
- Hybridization
- The labeled cDNAs from both the samples are placed in the DNA microarray so that each cDNA gets hybridized to its complementary strand; they are also thoroughly washed to remove unbounded sequences.

Collection and analysis

- The collection of data is done by using a microarray scanner.
- This scanner consists of a laser, a computer, and a camera. The laser excites fluorescence of the cDNA, generating signals.
- When the laser scans the array, the camera records the images produced.
- Then the computer stores the data and provides the results immediately. The data thus produced are then analyzed.

- The difference in the intensity of the colors for each spot determines the character of the gene in that particular spot.

Applications of DNA Microarray

- In humans, they can be used to determine how particular diseases affect the pattern of gene expression (the expression profile) in various tissues, or the identity (from the expression profile) of the infecting organism. Thus, in clinical medicine alone, DNA microarrays have huge potential for diagnosis.
- Besides, it has applications in many fields such as:
- Discovery of drugs
- Diagnostics and genetic engineering
- Alternative splicing detection
- Proteomics
- Functional genomics
- DNA sequencing
- Gene expression profiling
- Toxicological research (Toxicogenomics)

Advantages of DNA Microarray

- Provides data for thousands of genes in real time.
- Single experiment generates many results easily.
- Fast and easy to obtain results.
- Promising for discovering cures to diseases and cancer.
- Different parts of DNA can be used to study gene expression.

Disadvantages of DNA Microarray

- Expensive to create.
- The production of too many results at a time requires long time for analysis, which is quite complex in nature.
- The DNA chips do not have very long shelf life.