Chapter 11

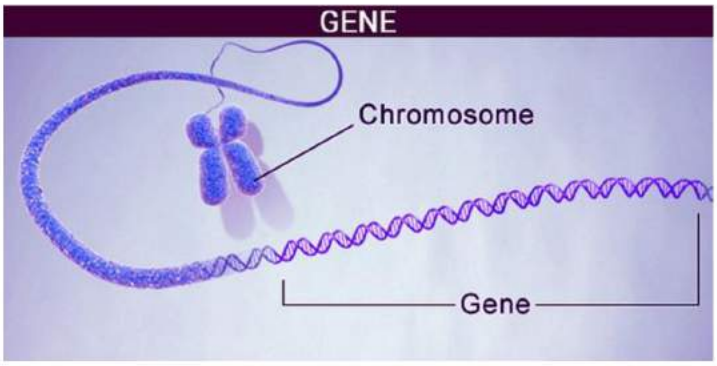
Biotechnology: Principles and Processes

Gene Transfer

What is Gene Transfer?

The insertion of unrelated genetic information in the form of DNA into cells.

The transfer of desired genes from one organism into another is an important aspect of genetic engineering.



Stages of Gene Transfer:

1. Isolating a useful DNA segment from the donor organism.
2. Inserting it into a suitable vector.
3. Splicing of these altered DNAs into a recipient organism or host cell.

The cell begins making the appropriate product and then device an economical method for its mass production.

Methods for Gene Transfer:

1. Indirect method through vectors or carriers and
2. Director vector less transfer method.

Gene Transfer Through Vectors:

Plasmids and viruses are commonly used vectors for the transfer of desired genes. The desired genes are first made to join suitable plasmid or virus which are then introduced into the target cells.

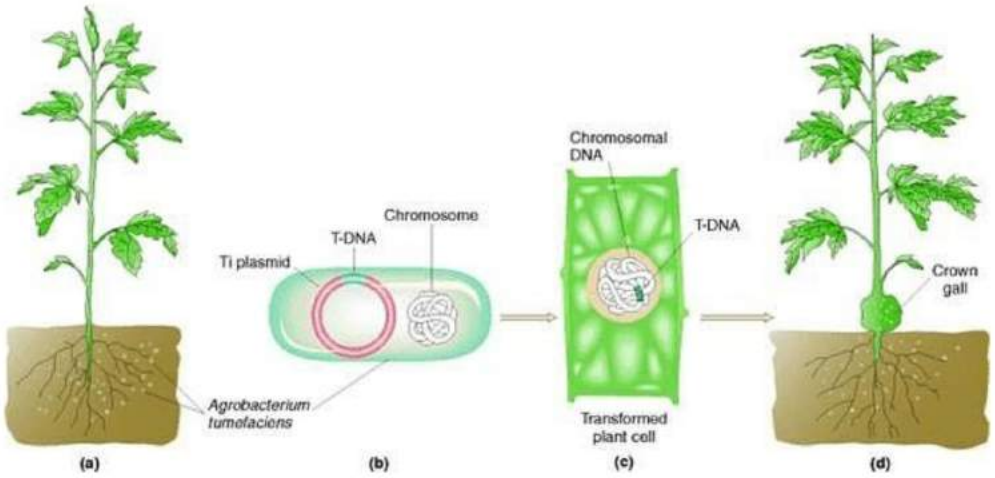


Fig: Steps involved in Agrobacterium-mediated Gene Transfer

- A plant pathogenic bacterium-Agrobacterium tumefaciens produces crown galls or plant tumours in almost all dicotyledonous plants.
- This bacterium infects all broad-leaved agricultural crops such as tomato, soybean, sunflower and cotton but not cereals.
- Tumour formation is induced by its plasmid which is therefore called Tiplasmid (Ti for tumour-inducing) Agrobacterium tumefaciens naturally transfers some part of Ti-plasmid into host plant DNA without any human effort so it is called natural genetic engineer of the plant.

In the transformation process, two essential components in Ti-plasmid are

- T-DNA – (Transferred DNA)
- Vir-region – (Virulence region)

1. Inside the host plant cell T-DNA is separated from Ti-plasmid, and integrated into host plant DNA that causes crown gall tumour.
2. Vir-region contains genes that are essential for T-DNA transfer and integration to host plant DNA. Vir-region contains 6-operons A, B, C, D, E, and G. (Acetosyringone is an inducer of Vir-operon).
3. When we use Ti plasmid as a vector, first we remove the tumour-causing gene from the T-DNA region. Then desired gene is inserted in place of it. Now, this plasmid is called a disarmed plasmid. Same as Ri plasmid of

A.rhizogenes (causing hairy root disease) also used as the vector for gene transfer to plant cell.

Vector less Gene Transfer

Foreign genes can also be transferred directly by the following methods:

1. **Electroporation:** It creates transients (temporary pores) in the plasma membrane to facilitate the entry of foreign DNA.

Fig: DNA

1. **Chemical mediated genetic transformation:** It involves certain chemicals such as polyethylene glycol (PEG), that help in the uptake of foreign DNA into host cells.
2. **Micro-injection:** It is the introduction of foreign genes into plant or animal cells using micro-pipettes or glass needles.
3. **Particle gun / Biolistic method:** It is a technique in which tungsten or gold particles coated with foreign DNA are bombarded into target cells to facilitate entry of the foreign genes.
4. **Liposome mediated gene transfer:** In this method, DNA encloses within lipid begs. These lipid begs fused with protoplast.

Gene Transfer In Animals

- In animals, the genes are transferred mostly through direct methods such as electroporation, micro-injection or using particle gun.
- The desired foreign genes can be introduced into fertilized eggs or embryos through micro-injection. These transgenic eggs or embryos can be implanted into the uterus of another female, called a surrogate (foster) mother for their further development.

- Now, since most of the human genes have been identified through the 'human genome project', it is hoped that a number of human genetic disorders such as Alzheimer, cancer, haemophilia, thalaessemia and cystic fibrosis can now be cured through the insertion of the correct genes into these patients.

Achievements of Genetic Engineering

DNA recombinant technology or genetic engineering provides great benefits for the advancement of science and society.

1. Gene Therapy: A new system of medicine gene therapy, may develop to treat hereditary diseases such as haemophilia. – It is the technique for curing genetic disease by replacing a "Faulty Gene" with a normal healthy functional gene.

- The first gene therapy used in severe combined immunodeficiency (SCID) patient.
- About 25% of an infant with SCID disorder lack the enzyme adenosine deaminase (ADA) – ADA enzyme involved in purine metabolism.
- These patients have no functioning T & B lymphocytes.
- The affected child of SCID develops recurrent infection early in life because they have no immune response against invading pathogen.
- The ideal approach would be to give the patient a functioning ADA by gene therapy.
- Thus, the genetic disorder can be overcome by introducing a specific gene.

2. Bacteria may be used as "Living factories" for synthesizing vitamins, hormones and antibodies.

- Human insulin (Humulin) was the first genetically engineered product produced by an American firm Eli Lilly – 5th July 1983.
- Charles Weismann of the University of Zurich obtained interferon through recombinant E.coli (1980).
- Microbes have been engineered to produce Human Growth Hormone (HGH) for curing dwarfism.
- Vaccines are produced by genetic engineering e.g., for Hepatitis-B and Herpes virus).
- Nitrogen fixation genes may be transferred from bacteria to the major food crops to boost food production without using expensive fertilizers.
- Transgenic plant obtained through recombinant DNA technology. The first transgenic plant was tobacco.

Fig: Tobacco

- It contains a resistant gene against weedicide (Glycophosate).
- The first transgenic animal was a mouse contain the gene for growth hormone.
- The first introduced transgenic crop in India (2002) is Bt-cotton.
- It is resistant for boll worm(Helicoverpa armigera - Larva of insect).
- It is formed by the transfer of a pest-resistant gene from Bacillus thuringiensis (bt-2 gene encoding Bt–toxin).
- Bacillus thuringiensis produces a toxic protein called crystal protein (Cry-Protein) this protein is toxic for the larva of a certain insect.

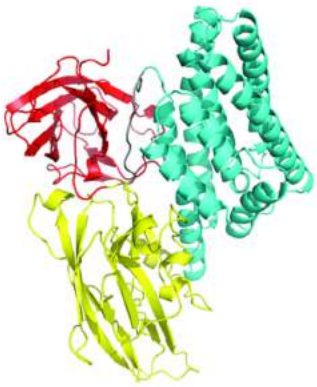


Fig: Cry-protein

- This protein kills the insect by inhibiting ion transport in the mid-gut (bt 2 genes is called cry -gene) – In pollution control, microbes have been engineered to break up the crude oil spills.
- Dr Ananda Mohan Chakraborty introduced plasmid from different strains into a single cell of pseudomonas putida. The result was a new

genetically engineered bacterium that would clean oil spills called "Superbug" (oil eating bug.)

3. Medical Diagnosis of Disease

- Recombinant DNA technology is one of the important tools for the diagnosis of several diseases. The diagnostic technique involves the construction of probes consisting of short segments of single-stranded DNA attached to a radioactive or fluorescent marker.
- Such probes are used to identify infections agents such as Salmonella (cause food poisoning), Staphylococcus (pus-forming bacterium), hepatitis virus, HIV; muscular dystrophy, cystic fibrosis and so on.
- Recombinant DNA technology can also be employed to predict the inheritance of genetic disorders from carrier parents. The chances of birth of an affected child can be predicted by testing the DNA of repetitive prospective genetic disorder carrier parents.

Application of Recombinant DNA Products

Medically useful recombinant products	**Applications**
Human insulin	Treatment of insulin - dependent diabetes
Human growth hormone	Treatment of rickets.
Calcitonin	Treatment of infertility.
Chorionic gonadotropin	Replacement of clotting factor missing in patients with Haemophilla A/B.
Blood clotting factor VIII/IX	Dissolving of blood clots after heart attacks and strokes.
Tissue Plasminogen activator (TPA)	Stimulation of the formation of erythrocytes (RBCs) for patients suffering from anaemia during dialysis or side effects of AIDS patients treated by drugs.
Platelet derived growth factor Erythropoietin	Stimulation of wound healing
Interferon	Treatment of pathogenic viral infections, cancer
Interleukin	Enhancement of action of immune system
Vaccines	Prevention of infectious diseases such as hepatitis B, herpes, influenza, pertussis, meningitis, etc.

Application of Genetically Engineered Microbes

Microbes	Applications
Escherichia coli (gut bacterium)	Production of human insulin, human growth factor interferons, interleukin and so on.
Bacillus thuringiensis (soil bacterium)	Productions of endotoxin (Bt toxin), highly potent, safe and biodegradable insecticide for plant protection.
Rhizobium meliloti (bacterium)	Nitrogen fixation by incorporating "nif" gene in cereal crops.
	Scavenging of oil spills by digesting hydrocarbons of crude oil.
Pseudomonas putida (bacterium)	Bioremediation (cleaning of pollutants in the environment).
Bacterial strains capable of accumulating heavy metal Trichoderma (fungus)	Production of enzyme chitinases for bio-control of fungal diseases in plants.

Transgenics & their Potential Application

Transgenic	Useful appications
Bt Cotton	Pest resistance, herbicide tolerance, and high yield.
Flavr Savr Tomato	Increased shelf-life (delayed ripening) and better nutrient quality
Golden Rice	Vitamin A and Fe - rich
Cattle (cow, sheep, goat)	Therapeutic human proteins in their milk
Pig	Organ transplantation without risk of rejection

Polymerase Chain Reaction Technology (PCR - Technology)

- This technique was invented by Kary Mullis (1983).
- In 1993 Karry Mullis got a Nobel prize for PCR(for chemistry)
- PCR is a method for amplifying a specific region of DNA molecule without the requirement for time-consuming cloning procedures. – PCR reaction takes place in the Eppendorf tube.
- Using PCR-technique very low content of DNA available from samples of blood or semen or any other tissue or hair cell can be amplified many times and analysed. In this technique, Taq-Polymerase is used.
- Taq polymerase enzyme is used in PCR which is a special type of DNA polymerase enzyme which is resistant to high temperature. Taq Polymerase is isolated from the Thermus aquaticus bacterium.
- Some other examples of polymerase which are used in PCR are
- Pfu Polymerase - Isolated from Pyrococus furiosus bacterium.
- Vent Polymerase- Isolated from Thermococcus litoralis bacterium.

Three Main Steps in PCR

1. Denaturation
2. Annealing
3. Extension

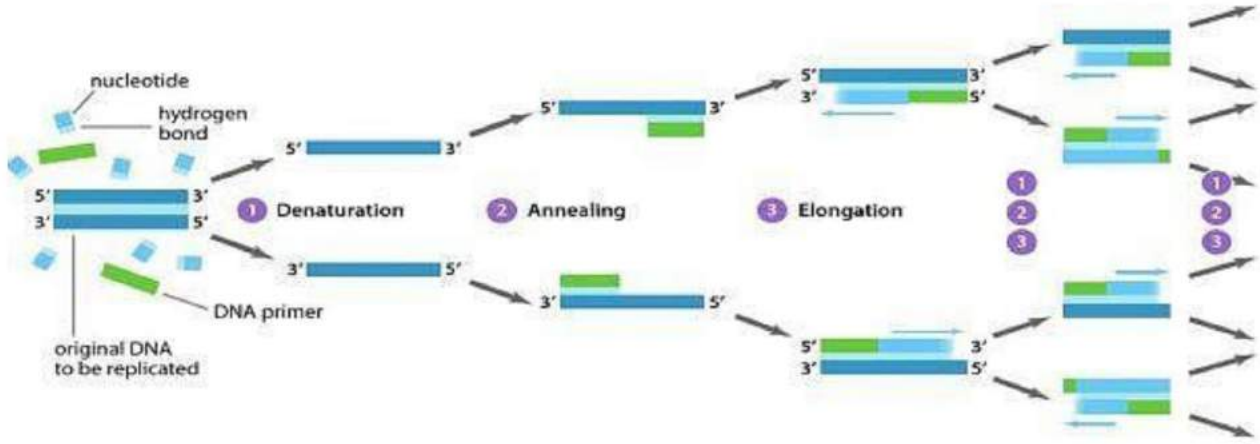


Fig: Polymerase chain reaction cycle

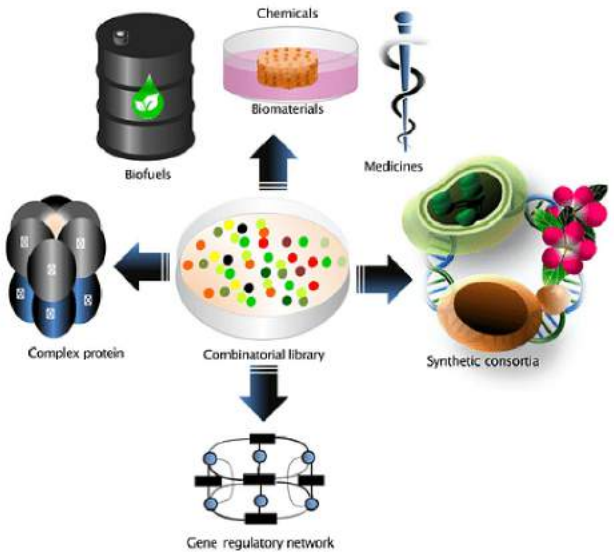
1. **Denaturation:** In this step, a double-stranded DNA molecule is placed at 94°C. So double-stranded DNA becomes single-stranded & each single-stranded DNA functions as a template.
2. **Annealing:** In this step, two primer DNA are attached at the 3' end of single-stranded DNA.
3. **Extension:** In this process, the Taq polymerase enzyme synthesizes DNA strain over the template. PCR is an automatic process because taq. polymerase enzyme is heat resistant.

Principles of Biotechnology: Genetic Engineering

GENETIC ENGINEERING

INTRODUCTION :

– **Genetic engineering** also referred as **'recombinant DNA technology '** or **'gene splicing '** is one kind of biotechnology involving manipulation of DNA.



Products of Genetic Engineering

It deals with the isolation of useful genes from a variety of sources and the formation of new combinations of DNA (recombinant DNA) for repair, improvement, perfection and matching of a genotype.

– Thus, genetic engineering may be defined *'as a technique for artificial and deliberately modifying DNA (gene) to suit human needs'.*

– In genetic engineering breakage of DNA molecule at two desired places is done with the help of restriction endonuclease to isolate a specific DNA segment and then insert it in another DNA molecule at a desired position.

– The new DNA molecule is recombinant DNA and the technique is called genetic engineering. Genetic engineering aims at adding, removing or repairing of a part of genetic material. Genetic engineering can be used to improve the quality of human life.

Paul Bergh (Father of genetic engineering). He transferred gene of SV-40 virus (simian virus) into E.coli with the help of l – phage. (Nobel prize - 1980) The concept of genetic engineering was the outcome of two very significant discoveries made in bacterial research. These were–

(i) presence of extra chromosomal DNA fragments called **plasmids** in the bacterial cell,which replicate along with chromosomal DNA of the bacterium.

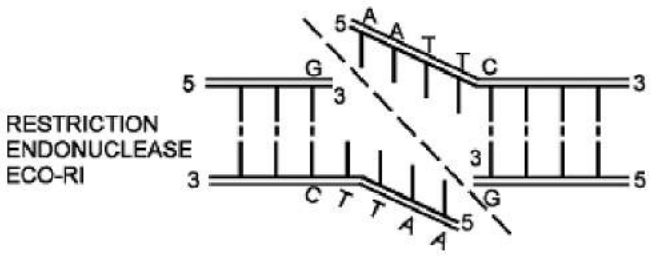
(i i) presence of enzymes **restriction endonucleases** which cut DNA at specific sites. These enzymes are, therefore, called **molecular scissors'.**

TOOLS AND TECHNIQUES OF GENETIC ENGINEERING

Tools

Genetic engineering involves cutting of desired segments of DNA and pasting of this D.N.A in a vector to produce a recombinant DNA (rDNA). The 'biological tools' used in the synthesis of recombinant DNA include enzymes, vehicle or vector DNA, passenger DNA and alkaline phosphatases.

1. **Enzymes.** A number of specific kinds of enzymes are employed in genetic engineering.
These include lysing enzymes, cleaving enzymes, synthesising enzymes and joining enzymes.
(i) **Lysing enzymes.** These enzymes are used for opening the cells to get DNA for genetic experiment.
Bacterial cell wall is commonly dissolved with the help of lysozyme.
(ii) **Cleaving enzymes.** These enzymes are used for DNA molecules. Cleaving enzymes are of three types; exonucleases, endonucleases and restriction endonucleases.



a) **Exonucleases** cut off nucleotides from 5' or 3' ends of DNA molecule.

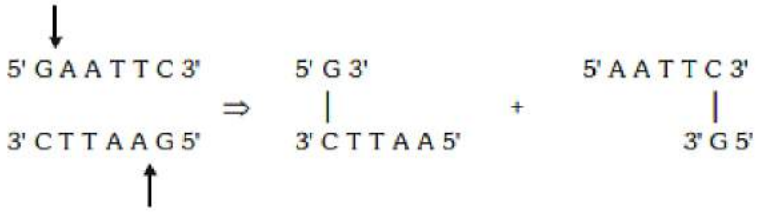
(b) **Endonucleases** break DNA duplex at any point except the end.

(c) **Restriction endonucleases** cleave DNA duplex at specific points in such away that they come to possess short single stranded free ends. For example, a restriction endonuclease ECOR-l (from *Escherichia coli)* recognize s the base sequence GA ATTC/CTTA AG in DNA duplex and cleaves it's strands between G and A.
Restriction enzymes are obtained from bacteria. They are useful to bacteria because the enzyme bring about fragmentation of viral DNA without affecting the bacterial genome. This is an adaptation against bacteriophages.
Restriction enzyme (Eco R-I) was discovered by Arber, Smith & Nathans (1978 Nobel prize). These enzymes exist in many bacteria beside cleavage some restriction endonuclease, also have the capability of modification.
Modification in the form of methylation, by methylation the bacterial DNA modifies and therefore protects it's own chromosomal DNA from cleavage by these restriction enzymes.

Restriction enzymes are used in recombinant DNA technology because they can be used in vitro to recognize and cleave within specific DNA sequence typically consisting of 4 to 8 nucleotides. This specific 4 to 8 nucleotide sequence is called **restriction site** and is usually **palin dromic ,** this means that the DNA sequence is the same when read in a 5'-3' direction on both DNA strand

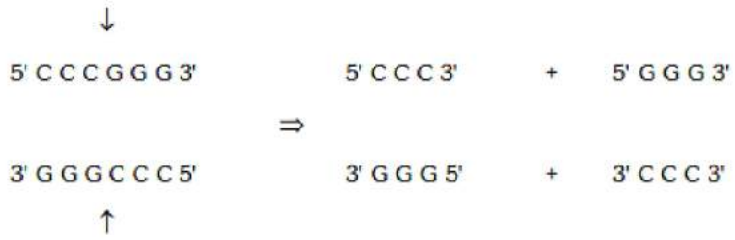
AND MADAM DNA

As a result the DNA fragments produced by cleavage with these enzymes have short single stranded overhang at each end these kinds of ends are called sticky or cohesive ends because base pairing between them can stick the DNA molecule back together again.



Therefore by cutting two different DNA samples with the same restriction enzyme and mixing the fragments together a recombinant DNA molecule can be generated. Exceptionally, some enzymes cleave both strand of DNA at exactly the same nucleotide position, typically in the center of the recognition sequence resulting in blunt end or flush end.

Sma I (Serratia marcescens)



Tools of Recombinant DNA Technology: Restriction Enzymes

Restriction Enzymes

"Restriction enzymes are the enzymes produced by certain bacteria that have the property of cleaving DNA molecule at or near specific base sequences."

What are Restriction Enzymes?

The restriction enzyme is a protein produced by bacteria that cleaves the DNA at specific sites. This site is known as the restriction site.
The restriction enzymes protect the live bacteria from bacteriophages. They recognize and cleave at the restriction sites of the bacteriophage and destroy its DNA.
Restriction enzymes are important tools for genetic engineering. They can be isolated from the bacteria and used in the laboratories.
The restriction enzymes recognize short and specific nucleotide sequences in the DNA known as the recognition sequences. When the restriction enzyme recognizes a DNA sequence, it hydrolyzes the bond between adjacent nucleotide and cuts through the DNA molecule.
The bacteria prevents its own DNA sequences from degradation by the addition of the methyl group at the adenine or cytosine bases within the recognition sequence with the help of enzyme methylases.

Types of Restriction Enzymes

Type I

These restriction enzymes cut the DNA far from the recognition sequences. However, they do not produce discrete restriction fragments, hence, are of not much practical value.
These are complex, multi-subunit restriction and modification enzymes. They were initially thought to be rare, but through genomic analysis, they are found to be common and are of considerable biochemical interest.

Type II

These enzymes cut at specific positions closer to or within the restriction sites. Discrete restriction fragments and gel banding patterns are observed. They are exclusively used for DNA analysis and gene cloning in the laboratories. These are a family of unrelated proteins. They are named after the bacterial species from which they are isolated. For eg., EcoRI is isolated from bacterial species *E.coli*. The restriction enzymes generate two different types of cuts. Blunt ends are produced when they cut the DNA at the centre of the recognition sequence, and sticky ends produce an overhang.

Type III

These are multi-functional proteins with two subunits- Res and Mod. It is a modification methyltransferase. The DNA sequence specific for the system is recognized by the Mod subunit.

Applications of Restriction Enzymes

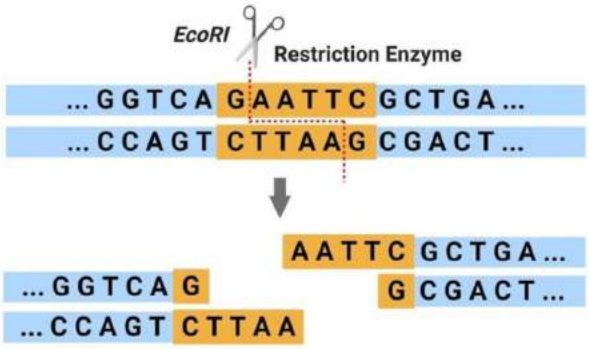
They are used in RFLP techniques to cut the DNA into smaller fragments to study the fragment length differences among the individuals.

In Gene Cloning

During cloning, a gene is inserted into a plasmid. Restriction enzymes cut the plasmid producing single-stranded overhangs. The two DNA molecules are ligated with the help of DNA ligase to form a single DNA molecule.

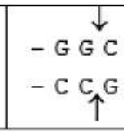
Nomenclature of enzyme – The first letter used for the enzyme is the first letter of the bacterium genus name (in Italics) then comes the first two letter of it's species (In Italics), next is the strain of the organism, last is Roman numerical signifying the order in which the enzymes were isolated from that strain of bacteria.

EXAMPLES OF RESTRICTION ENZYME



Recognition sequences of some restriction endonucleases:

Name	Recognition sequence	End after cleavage		Source
Eco RI	↓ – G A A T T C – – C T T A A G – ↑	– G – C T T A A	A A T T C – G –	Escherichia coli - containing drug resistant plasmid RI.
Hind III	↓ – A A G C T T – – T T C G A A – ↑	– A – T T C G A	A G C T T – A –	Haemophilus influenzae
Bam I	↓ – G G A T C C – – C C T A G G – ↑	– G – C C T A G	G A T C C – G –	Bacillus amyloliquefaciens

Hae III		– G G – C C	C C – G G –	Haemophilus aegyptius

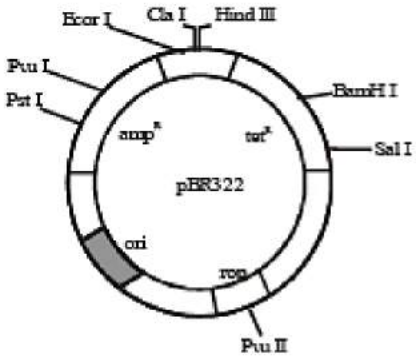
(iii) Synthesizing enzymes: These enzymes are used to synthesize new strands of DNA, complementary to existing DNA or RNA template. They are of two types; reverse transcriptases and DNA polymerases.
(a) Reverse transcriptases help in the synthesis of complementary DNA strands on RNA templates;
(b) DNA polymerases help in the synthesis of complementary DNA strands on DNA templates.
(iv) Joining enzymes: These enzymes help in joining the DNA fragments. For example DNA ligase from Escherichia colitis used to join DNA fragments. Joining enzymes are, therefore, called molecular glues.
(iv) Alkaline phosphatases: These enzymes cut off phosphate group from the 5' end of linearised circular DNA and prevent its re-circularisation.

Tools of Recombinant DNA Technology: Cloning Vectors

Vehicle DNA or Vector DNA: The DNA used as a carrier for transferring a fragment of foreign DNA into a suitable host is called vehicle or vector DNA.
You know that plasmids and bacteriophages have the ability to replicate within bacterial cells independent of the control of chromosomal DNA.
The following are the features that are required to facilitate cloning into a vector.

(i) Origin of replication (ori) : This is a sequence from where replication starts and any piece of DNA when linked to this sequence can be made to replicate within the host cells. This sequence is also responsible for controlling the copy number of the linked DNA. So, if one wants to recover many copies of the target DNA it should be cloned in a vector whose origin support high copy number.

(ii) Selectable marker : In addition to 'ori', the vector requires a selectable marker. Normally, the genes encoding resistance to antibiotics such as ampicilin, chloramphenicol, tetracycline or kanamycin, etc., are considered useful selectable markers for E. coli.



cloning sites : In order to link the alien DNA, the vector needs, recognition sites for the commonly used restriction enzymes. The ligation of alien DNA is carried out at a restriction site present in one of the two antibiotic resistance genes. For example, you can ligate a foreign DNA at the Bam H I site of tetracycline resistance gene in the vector pBR322. The recombinant plasmids will loss tetracycline resistance due to insertion of foreign DNA (insertional inactivation) now, it can be selected out from non-recombinant ones by plating the transformants on ampicillin containing medium. The transformants (plasmid transfer) growing on ampicillin containing medium are then transferred on a medium containing tetracycline. The recombinants will grow in ampicillin containing medium but not on that containing tetracycline. But, non-recombinants will grow on the medium containing both the antibiotics. In this case, one antibiotic resistance gene helps in selecting the transformants.

Selection of recombinants due to inactivation of antibiotics is a cumbersome (troublesome) procedure because it requires simultaneous plating on two plated having different antibiotics. Therefore, alternative selectable markers have been developed which differentiate recombinants from non-recombinants on the basis of their ability to produce colour in the presence of a chromogenic substrate. In this, a recombinant DNA is inserted within the coding sequence of an enzyme, which is referred to as insertional inactivation.

The presence of a chromogenic substrate X–gal (5–bromo–4 chloro– β-D galacto pyranoside) gives blue coloured colonies if the plasmid in the bacteria does not have an insert. Presence of insert results into insertional inactivation of the β-galactosidase (reporter enzyme) and the colonies do not produce any colour, these are identified as recombinant colonies.

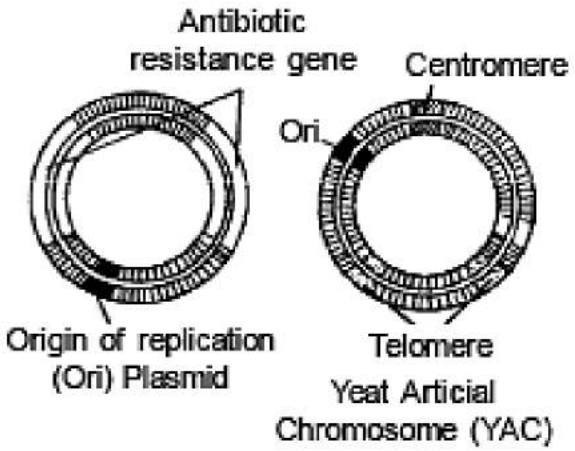
(iv) Vectors for cloning genes in plants and animals : Agrobacterium tumifaciens, a pathogen of several dicot plants deliver a piece of DNA known as 'T-DNA' to transform normal plant cells into a tumour .

Similarly, retroviruses in animals have the ability to transform normal cells intocancerous cells. A better understanding of the art of delivering genes by

pathogens in their vivo hosts has generated knowledge to transform these tools of pathogens into useful vectors for delivering genes of interest to humans. The tumour inducing (Ti) plasmid of Agrobacterium tumifaciens has now been modified into a cloning vector which is no more pathogenic to the plants but is still able to use the mechanisms to deliver genes (disarmed) and are now used to deliver desirable genes into animal cells. So, once a gene or a DNA fragment has been ligated into a suitable vector it is transferred into a bacterial, plant or animal host (where it multiplies).

(i) Plasmids. They are extra chromosomal DNA segments found in bacteria which can replicate independently. Plasmids can be taken out of bacteria and made to combine with desired DNA segments by means of restriction enzymes and DNA ligase. A plasmid carrying DNA of another organism integrated with it, is known as recombinant plasmid or hybrid plasmid or Chimeric plasmid.

(ii) Viruses. The DNA of certain viruses is also suitable for use as a vehicle DNA. Bacteriophage (bacterial virus) has been used to transfer gene for b galactosidase from Escherichia coli to human cells. Lambda phage (l phage) has been used for transferring lac genes ofE. coliinto haploid callus of tomato.
Vector type Insert size kb Plasmid 0.5 – 8 Bacterophage lambda 9 – 23 Cosmid 30 – 45 B AC 50 – 300
YAC 1000 – 2500



3. Passenger DNA. It is the DNA which is transferred from one organism into another by combining it with the vehicle DNA. The passenger DNA can be complementary, synthetic or random.

(i) Complementary DNA (cDNA)- It is synthesized on mRNA template with the help of reverse transcriptase and necessary nucleotides. The DNA strand is then separated from the hybrid RNA-DNA complex by using alkali. Complementary DNA

strand is then synthesized over the template of cDNA with the help of DNA polymerase. cDNA formed through reverse transcription is shorter than the actual or in vivo gene because of the absence of introns or non-coding regions.

(ii) Synthetic DNA (sDNA)- It is synthesized with the help of DNA polymerase on DNA template.
Kornberg (1961) synthesized first synthetic DNA from a mixture of deoxyribonucleotide triphosphates, DNA polymerase enzyme, metal ions and a segment of viral DNA.
Khorana (1968) synthesized first artificial gene (DNA) without a template. They synthesized the gene coding for yeast alaninet-RNA,which contained only 77 base pairs. However, it did not function in the living system. In 1979, Khorana was able to synthesize a functional tyrosine t-RNA gene of E. coli with 207 nucleotide pairs. Since then a number of genes have been synthesized artificially.

(iii) Random DNA - It refers to small fragments formed by breaking a chromosome with the help of restriction endonucleases.

Technique of Recombinant DNA

- The DNAs which are to be used as passenger DNA and the vehicle DNA are extracted out of their cells by lysing the cells with the suitable enzyme.
- The extracted DNAs are isolated from other cell contents by ultra centrifugation and purified.
- Both the passenger and vehicle DNAs are then, cleaved by using the same restriction endonuclease so that they have complementary sticky ends.
- The complementary sticky ends of the passenger and vehicle DNAs are joined with ligase enzyme. This gives rise to a recombinant DNA.
- The recombinant DNA is now inserted into a host cell such as Escherichia coli. The bacteria to be used as hosts should be without plasmids.
- The host cells are treated with calcium chloride or lysozyme. It creates transient (temporary) pores in their wall and makes the latter permeable to recombinant DNA.
- The recombinant DNA is added to the culture in which such bacteria are growing. The recombinant DNA is taken up by the bacteria. It replicates when the host bacteria divide and give rise to multiple copies of recombinant DNA.

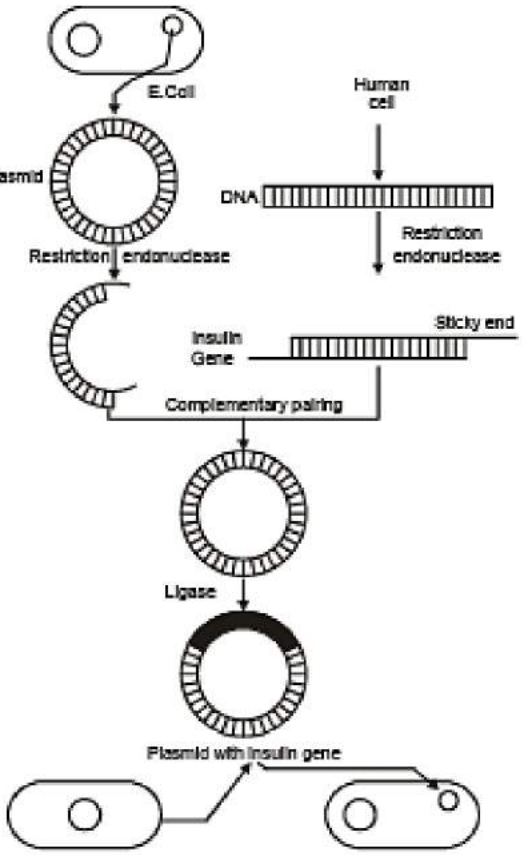


Fig.: Mechanism of transferring insulin gene from human DNA to E.coli with a plasmid

DNA Finger Printing, DNA Test & Cloning

DNA FINGER PRINTING / DNA TYPING / DNA PROFILING/ DNA TEST

- It is technique to identify a person on the basis of his/her DNA specificity.
- This technique was invented by sir **Alec. Jeffery** (1984).
- In India DNA Finger printing has been started by **Dr. V. K. Kashyap** & **Dr. Lal Ji Singh.**
- DNA of human is almost the same for all individuals but very small amount that differs from person to person that forensic scientists analyze to identify people.

These differences are called Polymorphism (many forms) and are the key of DNA typing. Polymorphism are most useful to forensic scientist. It consists of variation in the length of DNA at specific loci is called Restricted fragment. It is most important segment for DNA test made up of short repetitive nucleotide sequences, these are called VNTRs (variable number of tandem repeat).

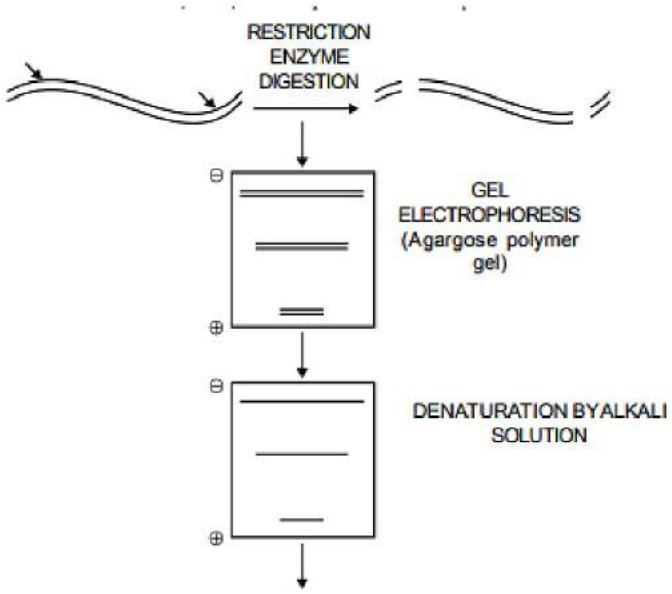
VNTR's also called mini satellites were discovered by Alec Jeffery. Restricted fragment consist of hyper variable repeat region of DNA having a basic repeat sequence of 11-60 bp and flanked on both sites by restriction site.

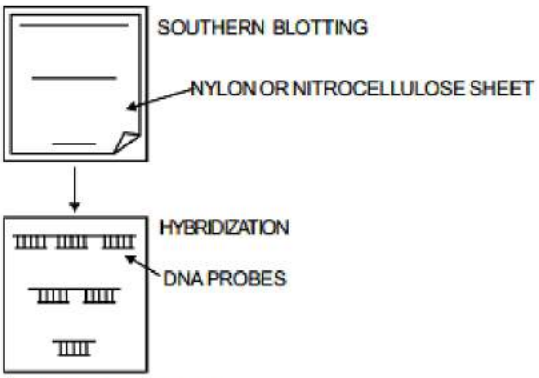
- The number and position of mini satellites or VNTR in restriction fragment is different for each DNA and length of restricted fragment is dependent on number of VNTR.
- Therefore, when the genome of two people are cut using the same restriction enzyme, the length of fragments obtained is different for both the people.
- These variations in length of restricted fragment is called RFLP or Restriction fragment length polymorphism.
- Restriction Fragment Length Polymorphism distributed throughout human genomes are useful for DNA Finger printing.
- DNA Fingerprint can be prepared from extremely minute amount of blood, semen, hair bulb or any other cell of the body.

DNA content of 1 - Microgram is sufficient.

Technique of DNA Finger printing involves the following major steps .

1. Extraction – DNA extracted from the cell by cell lysis . If the content of DNA is limited then DNA can be amplified by Polymerase chain reaction (PCR). This process is amplification.





Steps of DNA Fingerprinting

2 . Restriction Enzyme Digestion: Restriction enzyme cuts DNA at specific 4 or 6 base pair sequences called restriction site.
Hae III (Haemophilus aegyptius) is most commonly used enzyme. It cuts the DNA, every where the bases are arranged in the sequence GGCC. Theserestricted fragment transferred to Agarose Polymer gel.

3. Gel Electrophoresis :

- Gel electrophoresis is a method that separates macro-molecules-either nucleic acid or proteins-on the basis of size, electric charge.
- A gel is a colloid in a solid form. The term electrophoresis describes the migration of charged particles under the influence of an electric field. Electro refers to the energy of electricity. Phoresis, from the Greek verb phoros, means "to carry across." Thus, gel electrophoresis refers to the technique in which molecules are forced across a span of gel, motivated by an electrical current. Activated electrodes at either end of the gel provides the driving force. A molecule's properties determines, how rapidly an electric field can move the molecule through a gelatinous medium.
- Many important biological molecules such as amino acids , peptides, proteins, nucleotides, and nucleic acids posses ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cation (+) or anions (–). Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode.
- By the gel electrophoresis these restricted fragments move towards the positive electrode (anode) because DNA has –ve electric charge (PO_4^{3-}).
- Smaller Fragment more move towards the positive pole due to less molecular weight. So after the gel, electrophoresis DNA fragment arranged according to molecular weight.

- These separated fragments can be visualized by staining them with a dye that fluoresces ultraviolet radiation.
- This appears the specific restricted fragment length pattern. This length pattern is different in different individual.
- This is called Restricted Fragment length Polymorphism (RFLP).

4 . Southern transfer / Southern blotting : The gel is fragile. It is necessary to remove the DNA from the gel and permanently attaches it to a solid support. This is accomplished by the process of Southern blotting. The first step is to denature the DNA in the gel which means that the double-stranded restriction fragments are chemically separated into the single stranded form.
The DNA then is transferred by the process of blotting to a sheet of nylon. The nylon acts like an ink blotter and "blots" up the separated DNA fragments, the restriction fragments, invisible at this stage are irreversibly attached to the nylon membrane the "blot".
This process is called Southern blot by the name of **Edward Southern (1970).**

5. Hybridization: To detect VNTR locus on re stricted fragment, we use single stranded **Radioactive (P 32) DNA** probe which have the base pair sequences complimentary to the DNA sequences at the VNTR locus. Commonly we use a combination of at least 4 to 6 separate DNA probes.
Labelled Probes are attached with the VNTR loci of restricted DNA Fragments, this process is called Hybridization.

6. Autoradiography: Nylon membrane containing radio active probe exposed to X-ray. Specific bands appear on X-ray film. These bands are the areas where the radioactive probe bind with the VNTR.
These allow analyzer to identify a particular person DNA, the occurrence and frequency of a particular genetic pattern contained in this X-ray film. These x-ray film called DNA signature of a person, is specific for each individual.
The probability of two unrelated individuals having same pattern of location and repeat number of mini satellite (VNTR) is one in ten billion (world population 6.1 billion) In India, the centre for DNA finger printing and diagnosis (CDFD - center for DNA finger printing & diagnosis) located at Hyderabad.

Application of DNA Finger printing:

1 . Paternity tests. The major application of DNA finger printing is in determining family relationships. For identifying the true (biological) father, DNA samples of Child, mother and possible fathers are taken and their DNA finger prints are obtained. The prints of child DNA match to the prints of biological parents.

2 . Identification of the criminal. DNA finger printing has now become useful technique in forensic (crime detecting) science, specially when serious crimes such as murders and rapes are involved. For identifying a criminal, the DNA fingerprints of the suspects from blood or hair or semen picked up from the scene of crime are prepared and compared. The DNA fingerprint of the person matching the one obtained from sample collected from scene of crime can give a clue to the actual criminal.

CLONING

Clone is the exact carbon copy or copies produced by a single parent (mother or father) by non-sexual methods and are identical to their parent genetically and morphologically. Clone is a Greek word which means twig (Klon=twig). As all the branches of a tree are similar in morphology and genetical characteristics, in the same way clones are also similar to one another.

Cloning is the process of producing many identical organisms (clone), generally used to produce new plants with similar characteristics. Microbes produce clones through asexual reproduction. In higher animals, clones are produced by nuclear transplantation technique in which the nucleus from a somatic cell is transferred into an unfertilized enucleated egg. The world's most famous sheep 'Dolly' was a clone produced by this method.

Many plant species show vegetative reproduction. In these plants, the clones produced by a twig (detached shoot) are similar in their genotype as well as in phenotype (except environmental variations). Scientists have been much curious to apply this characteristics of plants on animals also to conserve the desired genotypes of some rare animals by making their clones. In higher animal, showing sexual reproduction, a zygote is formed after fertilization of the egg by spermatozoa. Zygote differs from its parents in genotype. It was revealed by the scientists through several experiments that only the egg and/or zygote has the potential to produce a whole individual from a single cell. J.B. Gurdon (1969) of Oxford University applied this fact while performing an experiment on frog. He destroyed the nucleus of an unfertilized egg of frog by treating with U.V rays and transferred the nucleus of intestinal epithelial cell of tadpole into the egg cell . In this experiment a few of the many transplanted eggs could develop into tadpoles. These developed tadpoles were identical in genotype and phenotype to their parents. This nuclear transplantation technique devised by Gurdon is still being used in cloning practice in some modified manner. A brief introductory history of cloning is given in table. In addition to the fact depicted in table attempts are continuously in progress in this field. In December, 2001 (report published in February, 2002) scientists at University, Texas, successfully produced the first cloned domestic pet named as copy cat (C.C). Further, in Aug. 2005 Woo-Sukhwang of South Korea produced the clone of an Afganian hound (Domestic dog used for hunting).

An introductory story of cloning			
Year	**Name of the Scientist**	**Brief account of the experiment (s)**	**Result**
1950's	Briggs & King	Nuclear transplantation from embryo to egg in frog.	Tadpoles produced but died before adulthood.
1960's	John B. Gurdon	Nuclear transplantation from cells of skin, liver, kidney into the egg in frog.	Tadpoles produced but died before adulthood.
1970's	Illmensee	Nuclear transplantation from embryo to egg in Drosophila	Larvae produced but died before adulthood
1984	Mcgrath & Solter	Nuclear transplantation from embryo to egg in mouse	A few mice born but none lived to adulthood.
1993	Hall & Stillman	Artificial splitting of an embryo of human into two identical twins	First artificially twinned embryos developed but abnormal
March, 1995	Roslin Institute team,Scotland	Nuclear transplantation from embryo to egg in sheep	Megan and Moragn sheep born normally
Feb, 1997	Roslin Institute team, Scotland	Nuclear transplantation udder cell to egg in sheep	"Dolly" sheep born normally
March, 1997	Don Wolf and Coworkers, Oregon	Nuclear transplantation from	Two monkeys "Neti"and

		embryo to egg in monkey	"Ditto" born normally
Dec. 1997	Roslin Institute team, Scotland	Nuclear transplantation from embryo to egg in sheep	Molly and Polly sheep born normally
1998	University of Hawai	Nuclear transplantation from adult cell to egg in mice	50 mice born normally
1999	Kato and Coworkers	Nuclear transplantation from adult cell to egg in cow	"George and Charlie" cows born normally
2000	Well and his Associated	Nuclear transplantation from skin cell to egg in cow	Many cows born normally
2001	Kabota	Nuclear transplantation from skin fibroblast culture to egg in cow	Six calves born

An Italian scientist Dr. S. Antenori is trying hard to produce human clone. It has triggered serious discussions and debates at global level, focussing on the medical and ethical issues.

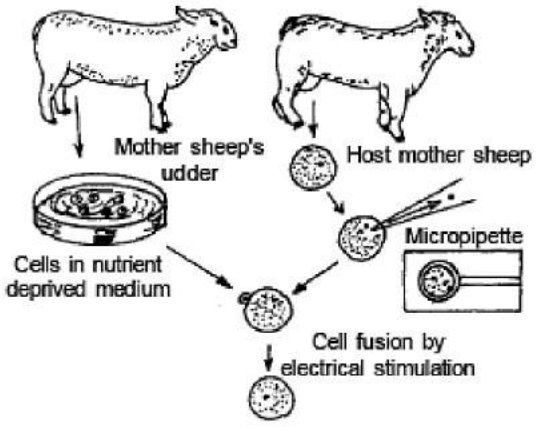
Types of Cloning Cloning is an extensive technique, which is divided into following types, on the basis of the experimental material used -(1) Gene cloning (2) Microbial cloning (3) Cell cloning (4) Plant cloning and (5) Animal cloning

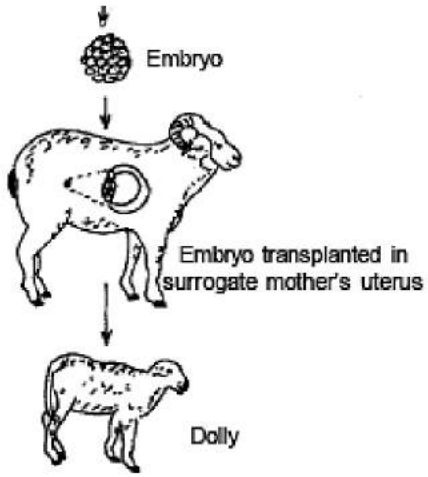
Animal cloning Embryonic cell in animals, are deprived of their totipotency by the time they enter into gastrula stage. So animal cloning is some what more difficult than plant cloning. On the basis of aims and end products animal cloning is of two types (i) Reproductive and (ii) Therapeutic.

A clone of the whole animal is prepared in reproductive cloning. The techniques which are used in such cloning include blastomere separation, nuclear transplantation and the Honolulu technique.

This technique can be used to conserve and increase the number of those animals

which are threatened to be extinct in the near future.
In nuclear transplantation technique, nucleus is removed from the egg obtained from the female. After then the nucleus of the desired cell (2n) is transferred into the enucleated egg cell which is then allowed to develop into an embryo in suitable conditions. The developing embryo is the clone of the donor cell from which nucleus was obtained for transplantation.
In Honolulu technique (1998-Teruhiko Wakayama - cloned mice) there is no fusion of the donor and recipient cells or its nucleus. Instead of ,nucleus from the donor cell which is in G0or G1, stage is substituted into enucleated egg cell (culture medium or chemical both is used in place of electric shock to stimulate development).
Therapeutic cloning technique may prove to be very useful in the field of medical science, particularly when there is a need for the transplantation to replace some damaged and diseased organ or tissue by a healthy organ from a suitable donor. In such condition, if the cells from the patient himself are taken and cultured to form desired organ. The organs developed in such manner(organ cloning) will be easily acceptable by the patient, and there will be no possibility of its rejection as often occurs otherwise.
A number of diseases like will be no possibility of its rejection as often occurs otherwise . A number of diseases like parkinsonia, alzheimer, diabetes and diseases related with kidneys will possibly be cured in future by the application of cloning technique. "Dolly" sheep was produced by using nuclear transfer technique by Dr. Ian Wilmut and his colleagues at Roslin Institute of Scotland in 1997.
They used somatic cells from udder (mammary glands) for forming this clone. One udder cell with its nucleus intact was selected because this nucleus carried the mother's genetic information.





Meanwhile, and unfertilized egg cell was taken from a different sheep. Its nucleus was sucked out and an enucleated egg cell was obtained. After then the udder cell nucleus was fused with the enucleated egg cell under electrical stimulation. Now this egg cell had the mother's nucleus. At last the fused egg was implanted into the uterus of surrogate mother, other than the egg donor where it grew into a lamb. Thus the Dolly was born, as a genetically identical copy of its mother.

Advantages of Cloning

1. This technique can be used to improve the breeds of live-stock used in agriculture.
2. Cloning is helpful for providing many useful substances and chemicals required for human body, and also in the cloning of such animals which can be used as a source of organs for transplantation purpose in medical practices.
3. British scientists have been successful in obtaining alpha anti-trypsin from sheep for curing an incurable disease emphysema and clotting factor- ix for curing hemophilia. It has been possible by the use of genetic engineering.
4. Many incurable diseases which are not curable so far, may be cured effectively in near future by applying the processes relating with cloning and genetic engineering.
5. Cloning is useful to increase the number of individuals of those species which are at the edge of extinction, thus helpful in conservation of biodiversity.

Disputes Related to Cloning

At present, the issue of cloning has been a matter of discussion and disputes among the scientists and the sociologists. Many questions are being raised with regards to the ethical, moral, and social aspects of cloning.
Doubts have also been there about the health and ageing of clones and the misuse of cloning.

Cloning has important role in treatment of serious and non-curable diseases, a view that is favoured by most scientists, however there is no agreement on the issue of human cloning.

Processes of Recombinant DNA Technology

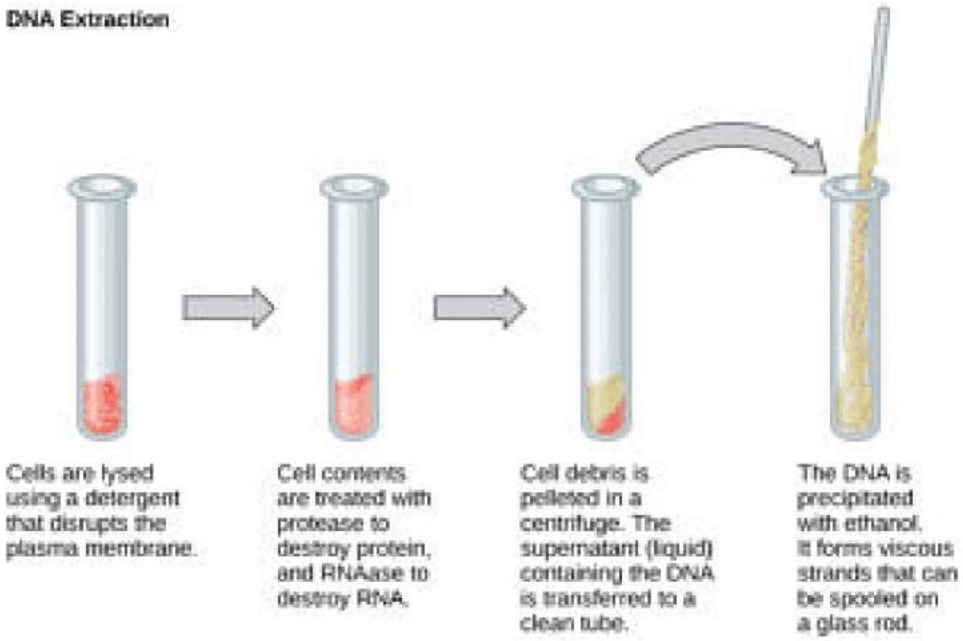
Recombinant DNA (rDNA) technology refers to the process of joining DNA molecules from two different sources and inserting them into a host organism, to generate products for human use. Can you put the DNA molecules in the host organism first and then cut and join them? No! This process involves multiple steps that have to proceed in a specific sequence to generate the desired product. Let's understand each stepin detail.

1. Isolation of Genetic Material

We already know that the genetic material of all living organisms is 'nucleic acid'. In most organisms, it is DNA, whereas in some it is RNA. The first step in rDNA technology is to isolate the desired DNA in its pure form i.e. free from other macromolecules.
However, in a normal cell, the DNA not only exists within the cell membrane, but is also present along with other macromolecules such as RNA, polysaccharides, proteins, and lipids. So, how do we break open the cell and obtain DNA that is free from other macromolecules? We can use the following enzymes for specific purposes:

- **Lysozyme** – to break bacterial cell wall.
- **Cellulase** – to break plant cell wall.
- **Chitinase** – to break fungal cell wall.
- **Ribonuclease** – removes RNA.
- **Protease** – removes proteins (such as histones that are associated with DNA).

Other macromolecules are removable with other enzymes or treatments. Ultimately, the addition of ethanol causes the DNA to precipitate out as fine threads. This is then spooled out to give purified DNA.

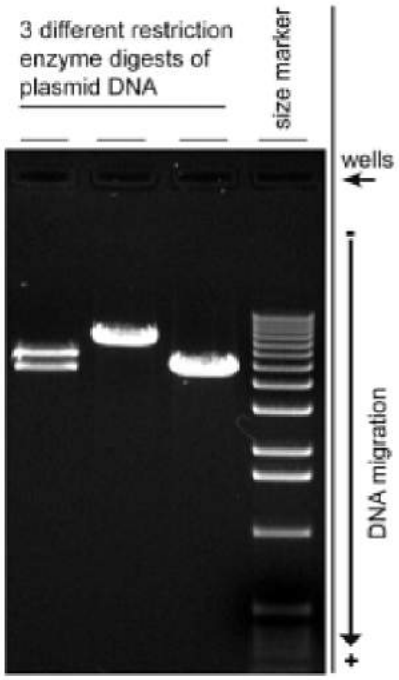


Steps in DNA isolation

2. Restriction Enzyme Digestion

Restriction enzymes act as molecular scissors that cut DNA at specific locations. These reactions are called 'restriction enzyme digestions'. They involve the incubation of the purified DNA with the selected restriction enzyme, at conditions optimal for that specific enzyme.

The technique – 'Agarose Gel Electrophoresis' reveals the progress of the restriction enzyme digestion. This technique involves running out the DNA on an agarose gel. On the application of current, the negatively charged DNA travels to the positive electrode and is separated out based on size. This allows us to separate and cut out the digested DNA fragments. The vector DNA is also processed using the same procedure.



digested DNA after agarose gel electrophoresis

3. Amplification Using PCR

Polymerase Chain Reaction or PCR is a method of making multiple copies of a DNA sequence using the enzyme – DNA polymerase. It helps to amplify a single copy or a few copies of DNA into thousands to millions of copies. PCR reactions are run on 'thermal cyclers' using the following components:

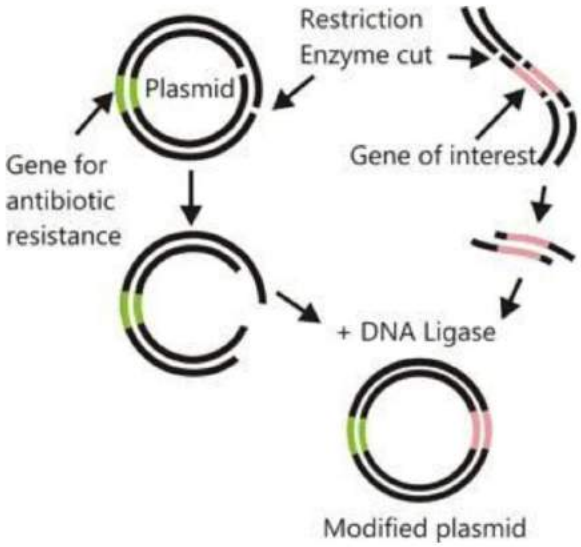
- **Template** – DNA to be amplified
- **Primers** – small, chemically synthesized oligonucleotides that are complementary to a region of the DNA.
- **Enzyme** – DNA polymerase
- **Nucleotides** – needed to extend the primers by the enzyme.

Thermal Cycler

The cut fragments of DNA can be amplified using PCR and then ligated with the cut vector as explained below.

4. Ligation of DNA Molecules

The purified DNA and the vector of interest are cut with the same restriction enzyme. This gives us the cut fragment of DNA and the cut vector, that is now open. The process of joining these two pieces together using the enzyme 'DNA ligase' is 'ligation'. The resulting DNA is 'recombinant DNA'.



Restriction enzyme digestion followed by ligation

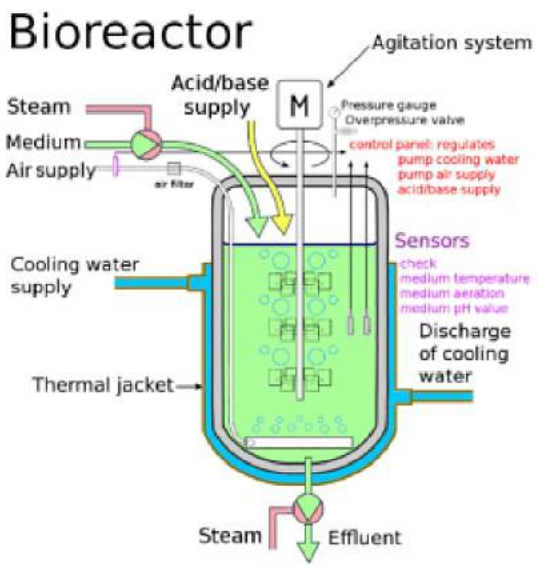
5. Insertion of Recombinant DNA Into Host.

In this step, the recombinant DNA is introduced into a recipient host cell. This process is 'Transformation'. Bacterial cells do not accept foreign DNA easily. Therefore, they are treated to make them 'competent' to accept new DNA. (The topic – Tools of Biotechnology explains a few ways to make cells competent).
During transformation, if a recombinant DNA bearing a gene for ampicillin resistance is transferred into recipient E. coli cells, then the E. coli cells also become ampicillin-resistant. This aspect is useful in differentiating transformed cells from non-transformed cells.
For example, if we spread the transformed cells on agar plates containing ampicillin, only the transformed, ampicillin-resistant cells will grow while the untransformed cells will die. Therefore, in this case, the ampicillin resistance gene acts as the 'selectable marker'.

6. Obtaining Foreign Gene Product

The recombinant DNA multiplies in the host and is expressed as a protein, under optimal conditions. This is now a recombinant protein. Small volumes of cell cultures will not yield a large amount of recombinant protein. Therefore, large-scale production is necessary to generate products that benefit humans. For this purpose, vessels called bioreactors are used.
Bioreactors are large containers with a continuous culture system, where the fresh medium is added from one side and used medium is taken out from another side. Bioreactors can process about 100-1000 litres of cell cultures. A bioreactor provides optimum conditions (temperature, oxygen, pH, vitamins etc.) to biologically convert raw materials into specific proteins, enzymes etc.
'Stirred-tank bioreactor' is the most common type of bioreactor. It is usually cylindrical and has the following parts:

- Agitator system – to stir the contents evenly
- Oxygen delivery system – to introduce air into the system
- Foam control system
- Temperature control system
- pH control system
- Sampling ports – to take out small amounts of culture



Bioreactor

7. Downstream Processing

Before the protein is marketed as a final product, it is subjected to downstream processing which includes:

- Separation and purification.
- Formulation with suitable preservatives.
- Clinical trials to test the efficacy and safety of the product.
- Quality control tests.

Competent Host

What are Competent Cells?

The bacterial cells that can take up the foreign DNA from the surroundings by a process called transformation are known as competent cells. Griffith first reported it in Streptococcus pneumoniae. E.coli cells are more likely to uptake the DNA if their cell walls are altered. The cells can be made competent by calcium chloride and heat shock treatment. The cells growing rapidly can be made competent more easily than those in other stages of growth.
The cells might express the acquired genetic information after transformation. The process is largely used to introduce recombinant plasmid DNA into competent bacterial cells. This process does not require a donor cell, but only a DNA in the surrounding environment.

Principle of Competent Cells

Competent cells have altered cell walls that allow the DNA to easily pass through it. Some cells need to be exposed to some chemical or electrical treatments to make them competent. Treatment with calcium ions is the standard method for the preparation of these cells. Bacterial cells can also take up DNA through a process called electroporation.

Methods of Preparation of Competent Cells

Competence is achieved in two ways:

- Natural Competence
- Artificial Competence

Natural Competence

Bacteria take up DNA from the surroundings by transformation, conjugation, and transduction. The foreign DNA directly enters into the bacterial cell through transformation. For this, the cells need to be in a competent state.
Frederich Griffith was the first one to discover natural competence. He injected the smooth strain of pneumococcus in mice, and the mice died. This strain is therefore known as virulent strain. But the mice did not die when injected with the rough strain (non-virulent strain). Heat-killing abolishes the virulent nature of the smooth strain. The heat-killed smooth strain and the rough strain were mixed. The rough strain adopted the smooth phenotype and became virulent. This suggests that a heat-stable, non-living material obtained from the smooth strain facilitated transformation.

Artificial Competence

In this, the cells are permeable to DNA in the laboratory. The competent cells can be prepared artificially in two ways, namely:

- **Calcium Chloride:** This method was proposed by Higa and Mandel. The bacterial cells were treated with calcium chloride and then suddenly exposed to high temperatures. This is known as heat shock treatment method.
- **Electroporation:** In this technique, cells are subjected to an electric field to increase their permeability. It is also known as electropermeabilization.