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SCHOOL OF BIO AND CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

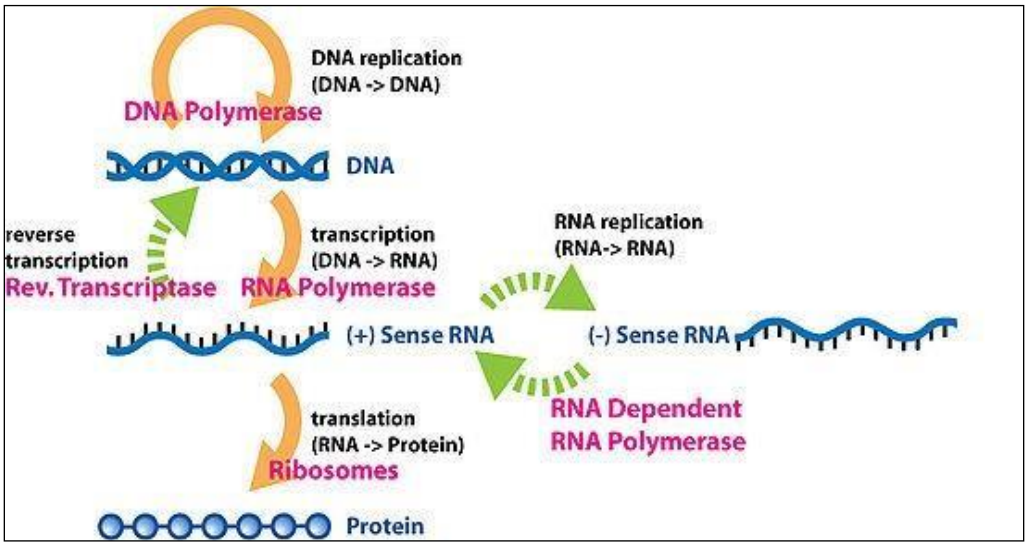
UNIT – I – Advanced Molecular Biology and Genetic Engineering – SBTA5201

Scope and milestones of rDNA technology

Genes and Genetic Engineering

A **gene** is a basic unit of heredity in a living organism. It is "a locatable region of genomic sequence, corresponding to a unit of inheritance, which is associated with regulatory regions, transcribed regions, and or other functional sequence regions ".

Central Dogma of Molecular Biology: The flow of genetic information in the cell starts at DNA, which replicates to form more DNA. Information is then 'transcribed" into RNA, and then it is "translated" into protein.



Genetic Engineering: The technology entailing all processes of altering the genetic material of acell to make it capable of performing the desired functions, such as producing novel substances. In other words, Genetic engineering is the deliberate controlled manipulation of genes in an organism in order to upgrade that organism.

Although there are many diverse and complex techniques involved, the basic principles of genetic manipulation are reasonably simple. The premise on which the technology is based is that genetic information, encoded by DNA and arranged in the form of genes, is a resource that

can be manipulated in various ways to achieve certain goals in both pure and applied science and medicine.

There are many areas in which genetic manipulation is of value, including the following:

- Basic research on gene structure and function
- Production of useful proteins by novel methods
- Generation of transgenic plants and animals
- Medical diagnosis and treatment
- Genome analysis by DNA sequencing

A Brief History of Genetic Engineering

Some major steps in the development of Genetic Engineering

1869	**Miescher** first isolates DNA from white blood cells harvested from pus-soaked bandages obtained from a nearby hospital.
1944	**Avery** provides evidence that DNA, rather than protein, carries the genetic information during bacterial transformation.
1953	**Watson and Crick** propose the double-helix model for DNA structure based on x-ray results of **Franklin and Wilkins**.
1955	**Kornberg** discovers DNA polymerase, the enzyme now used to produce labeled DNA probes.
1961	**Marmur and Doty** discover DNA renaturation, establishing the specificity and feasibility of nucleic acid hydridization reactions.
1962	**Arber** provides the first evidence for the existence of DNA restriction nucleases, leading to their purification and use in DNA sequence characterization by **Nathans and H. Smith**.
1966	**Nirenberg**, **Ochoa**, and **Khorana** elucidate the genetic code.
1967	**Gellert** discovers DNA ligase, the enzyme used to join DNA fragments together.
1972–1973	DNA cloning techniques are developed by the laboratories of **Boyer**, **Cohen**, **Berg**, and their colleagues at Stanford University and the University of California at San Francisco.
1975	**Southern** develops gel-transfer hybridization for the detection of specific DNA sequences.
1975–1977	**Sanger and Barrell** and **Maxam and Gilbert** develop rapid DNA-sequencing methods.

1981–1982	**Palmiter and Brinster** produce transgenic mice; **Spradling and Rubin** produce transgenic fruit flies.
1982	**GenBank**, NIH's public genetic sequence database, is established at Los Alamos National Laboratory.
1985	**Mullis** and co-workers invent the polymerase chain reaction (PCR).
1987	**Capecchi** and **Smithies** introduce methods for performing targeted gene replacement in mouse embryonic stem cells.
1989	**Fields and Song** develop the yeast two-hybrid system for identifying and studying protein interactions
1989	**Olson** and colleagues describe sequence-tagged sites, unique stretches of DNA that are used to make physical maps of human chromosomes.
1990	**Lipman** and colleagues release BLAST, an algorithm used to search for homology between DNA and protein sequences.
1990	**Simon** and colleagues study how to efficiently use bacterial artificial chromosomes, BACs, to carry large pieces of cloned human DNA for sequencing.
1991	**Hood and Hunkapillar** introduce new automated DNA sequence technology.
1995	**Venter** and colleagues sequence the first complete genome, that of the bacterium *Haemophilus influenzae.*
1996	**Goffeau** and an international consortium of researchers announce the completion of the first genome sequence of a eucaryote, the yeast *Saccharomyces cerevisiae.*
1996–1997	**Lockhart** and colleagues and **Brown and DeRisi** produce DNA microarrays, which allow the simultaneous monitoring of thousands of genes.
1998	**Sulston and Waterston** and colleagues produce the first complete sequence of a multicellular organism, the nematode worm *Caenorhabditis elegans.*
2001	Consortia of researchers announce the completion of the draft human genome sequence.

Isolation and Purification of Genomic DNA

Every gene manipulation procedure requires genetic material like DNA and RNA. Nucleic acids occur naturally in association with proteins and lipoprotein organelles. The dissociation of a nucleoprotein into nucleic acid and protein moieties and their subsequent separation, are the essential steps in the isolation of all species of nucleic acids. Isolation of nucleic acids is followed by quantization of nucleic acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture. Isolating the genetic material (DNA) from cells (bacterial, viral, plant or animal) involves three basic steps-

- Rupturing of cell membrane to release the cellular components and DNA
- Separation of the nucleic acids from other cellular components
- Purification of nucleic acids

Genomic DNA is found in the nucleus of all living cells with the structure of double-stranded DNA remaining unchanged (helical ribbon). The isolation of genomic DNA differs in animals and plant cells. DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells. The amount and purity of extracted DNA depends on the nature of the cell.

The method of isolation of genomic DNA from a bacterium comprises following steps

1. Bacterial culture growth and harvest.
2. Cell wall rupture and cell extract preparation.
3. DNA Purification from the cell extract.
4. Concentration of DNA solution.

Growth and harvest of bacterial culture

Bacterial cell culture is more convenient than any other microbe, as it requires only liquid medium (broth) containing essential nutrients at optimal concentrations, for the growth and division of bacterial cells. The bacterial cells are usually grown on a complex medium like Luria-Bertani (LB), in which the medium composition is difficult to decipher. Later, the cells are separated by centrifugation and resuspended in 1% or less of the initial culture volume.

Preparation of cell extract

Bacterial cell is surrounded by an additional layer called cell wall, apart from plasma membrane with some species of *E. coli* comprising multilayered cell wall. The lysis of cell wall to release the genetic material i.e. DNA can be achieved by following ways-

- Physical method by mechanical forces.
- Chemical method by metal chelating agents i.e. EDTA and surfactant i.e. SDS or enzyme (e.g. lysozyme).

Lysozyme

- present in egg-white, salivary secretion and tears.
- catalyzes the breakdown of cell wall i.e. the peptidoglycan layer.

EDTA (Ethylene diamine tetra-acetic acid)

- a chelating agent necessary for destabilizing the integrity of cell wall.
- Inhibits the cellular enzymes that degrade DNA.

SDS (Sodium dodecyl sulphate)

- helps in removal of lipid molecules and denaturation of membrane proteins.

Generally, a mixture of EDTA and lysozyme is used. Cell lysis is followed by centrifugationto pellet down the cell wall fractions leaving a clear supernatant containing cell extract.

Purification of DNA

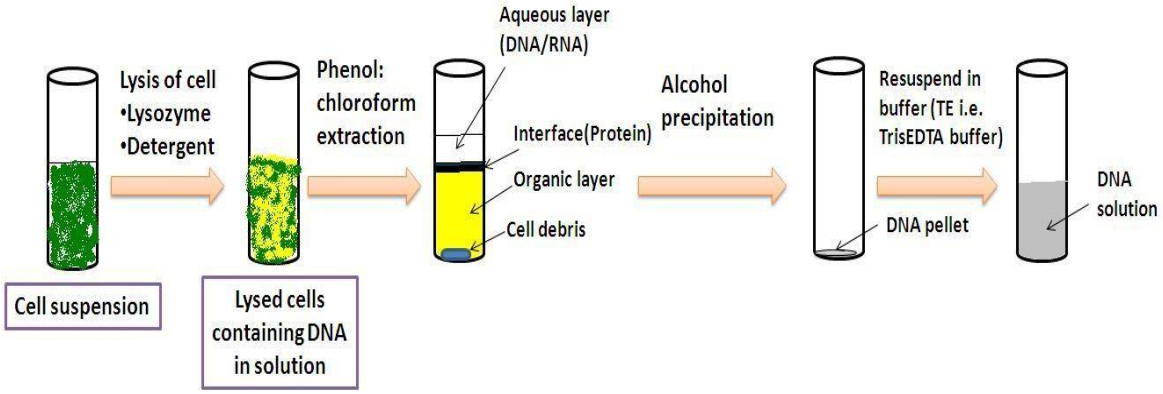
In addition to DNA, a cell extract contains significant quantities of protein and RNA which can be further purified by following methods-

Organic extraction and enzymatic digestion for the removal of contaminants

It involves the addition of a mixture of phenol and chloroform (1:1) to the cell lysate for protein separation. The proteins aggregate as a white mass in between the aqueous phase containing DNA and RNA, and the organic layer. Treatment of lysate with pronase or protease, in addition to phenol/chloroform, ensures complete removal of proteins from the extract. The RNA can be effectively removed by using Ribonuclease, an enzyme which rapidly degrades RNA into its ribonucleotide subunits. Repeated phenol extraction is not desirable, as it damages the DNA.

Using ion-exchange chromatography

This involves the separation of ions and polar molecules (proteins, small nucleotides and amino acids) based on their charge. DNA carrying negative charge binds to the cationic resin or matrix which can be eluted from the column by salt gradient. Gradual increase in salt concentration detaches molecules from the resin one after another.



Concentration of DNA samples

Concentration of DNA can be done using ethanol along with salts such as sodium acetate, potassium acetate etc. These salts provide metal ions like sodium ions (Na+), potassium ions (K+) which help in aggregation and hence precipitation of DNA molecules.

Advantage

It leaves short-chain and monomeric nucleic acid components in solution. Ribonucleotides produced by the ribonuclease treatment are separated from DNA.

Isolation and Purification of Plasmid DNA

Plasmids are circular, double stranded extra cellular DNA molecules of bacterium and most commonly used in recombinant DNA technology. The isolation of plasmid DNA involves three major steps-

1. Growth of the bacterial cell.
2. Harvesting and lysis of the bacteria.
3. Purification of the plasmid DNA.

Growth of the bacterial cell

It involves growth of the bacterial cells in a media containing essential nutrients.

Harvest and lysis of bacteria

Lysis of bacteria results in the precipitation of DNA and cellular proteins. Addition of acetate- containing neutralization buffer results in the precipitation of large and less supercoiled chromosomal DNA and proteins leaving the small bacterial DNA plasmids in solution.

Purification of Plasmid DNA

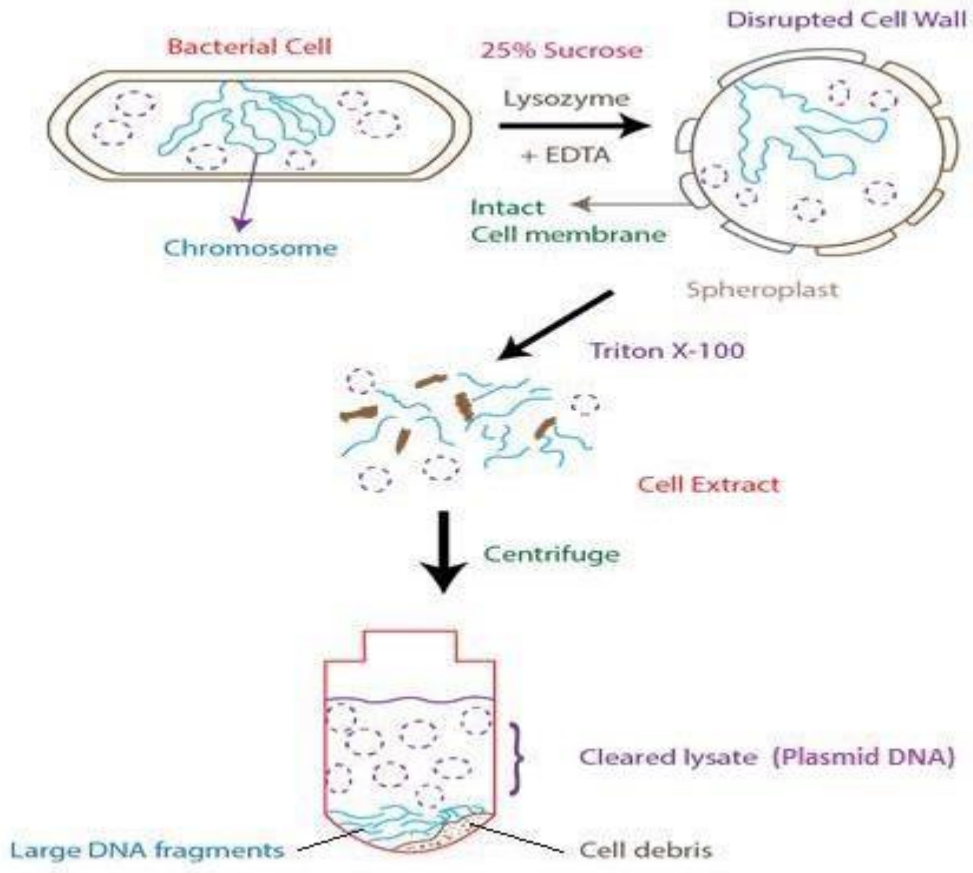
This step is same for both plasmid and genomic but former involves an additional step i.e. the separation of plasmid DNA from the large bacterial chromosomal DNA.

Methods for separation of plasmid DNA

Separation of plasmid DNA is based on the several features like size and conformation of plasmid DNA and bacterial DNA. Plasmids are much smaller than the bacterial main chromosomes, the largest plasmids being only 8% of the size of the *E. coli* chromosome. The separation of small molecules (i.e. plasmids) from larger ones (i.e. bacterial chromosome) is based on the fact that plasmids and the bacterial chromosomes are circular but bacterial chromosomes break into linear fragments during the preparation of the cell extract resulting in separation of pure plasmids. The methods of separation of plasmid DNA are described as below-

Separation based on size difference

- It involves lysis of cells with lysozyme and EDTA in the presence of sucrose (preventsthe immediate bursting of cell).
- Cells with partially degraded cell walls are formed that retain an intact cytoplasmicmembrane called as sphaeroplasts.
- Cell lysis is then induced by the addition of a non-ionic detergent (e.g. Triton X-100) or ionic detergents (e.g. SDS) causing chromosomal breakage.
- Bacterial chromosome attached to cell membrane, upon lysis gets removed with the celldebris.
- A cleared lysate consisting almost entirely of plasmid DNA is formed with very littlebreakage of the bacterial DNA.



Separation based on conformation

Plasmids are supercoiled molecules formed by partial unwinding of double helix of the plasmid DNA during the plasmid replication process by enzymes called topoisomerases. The supercoiled conformation can be maintained when both polynucleotide strands are intact, hence calledcovalently closed-circular (ccc) DNA. If one of the polynucleotide strands is broken, the double helix reverts to its normal relaxed state taking an alternative conformation, called open-circular (oc). Super coiling is important in plasmid preparation due to the easy separation of supercoiled molecules from non-supercoiled ones.

The commonly used methods of separation based on conformation are as follows-

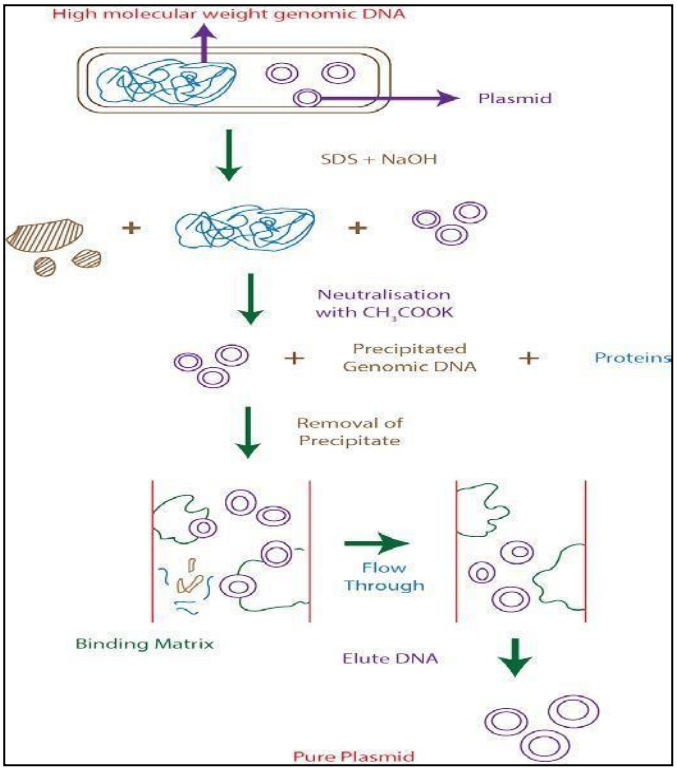
Alkaline denaturation method

- This method is based on maintaining a very narrow pH range for the denaturation of non-supercoiled DNA but not the supercoiled plasmid.
- Addition of sodium hydroxide to cell extracts or cleared lysate (pH12.0-12.5) results indisruption of the hydrogen bonds of non-supercoiled DNA molecules.

- As a result, the double helix unwinds and two polynucleotide chains separate.
- Further addition of acid causes the aggregation of these denatured bacterial DNA strands into a tangled mass which can be pelleted by centrifugation, leaving plasmid DNA in the supernatant.

Advantage

- Most of the RNA and protein under defined conditions (specifically cell lysis by SDS andneutralization with sodium acetate) can be removed by the centrifugation.
- No requirement of organic extraction.



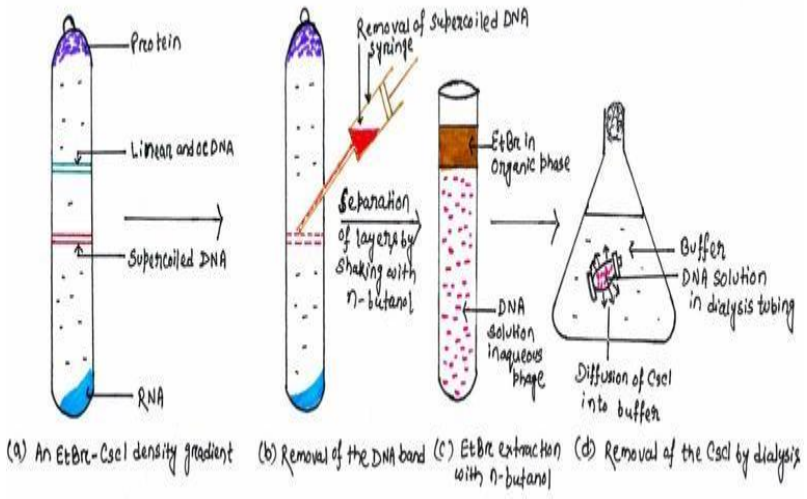
Ethidium bromide-cesium chloride density gradient centrifugation

- Density gradient centrifugation can separate DNA, RNA and protein. It is a very efficientmethod for obtaining pure plasmid DNA.
- A density gradient is produced by centrifuging a solution of cesium chloride at a very high speed which pulls the CsCl ions towards the bottom. This process is referred as isopycnic centrifugation.

- The DNA migrates to the point at which it has density similar to that of CsCl i.e.1.7 g/cm^3 in the gradient.
- In contrast, protein molecules having lower buoyant densities float at the top of the tube whereas RNA gets pelleted at the bottom.

Density gradient centrifugation in the presence of ethidium bromide (EtBr) can be used to separate supercoiled DNA from non-super coiled molecules. Ethidium bromide is an intercalating dye that binds to DNA molecules causing partial unwinding of the double helix.

Supercoiled DNA have very little freedom to unwind due to absence of free ends and bind to a limited amount of EtBr resulting in very less decrease in buoyant density (0.085 g/cm^3) than that of linear DNA (0.125 g/cm^3). As a result, they form a distinct band separated from the linear bacterial DNA. The EtBr bound to DNA is then extracted by n-butanol and the CsCl is removed by dialysis.



Bacteriophage DNA preparation

In bacteriophage DNA preparation, a cell extract is not the starting material, because bacteriophage particles can be obtained in large numbers from the extracellular medium of an infected bacterial culture. When such a culture is centrifuged, the bacteria are pelleted, leaving the phage particles in suspension. The phage particles are then collected from the suspension andtheir DNA extracted by a single deproteinization step to remove the phage capsid.

Isolation and Purification of RNA

RNA (Ribonucleic acid) is a polymeric substance consisting of a long single-stranded chain of phosphate and ribose units with the nitrogen bases adenine, guanine, cytosine and uracil bonded to the ribose sugar present in living cells and many viruses. The steps for preparation of RNA involve homogenization, phase separation, RNA precipitation, washing and re-dissolving RNA.

The method for isolation and purification of RNA are as follows-

1) Organic extraction method
2) Filter-based, spin basket formats
3) Magnetic particle methods
4) Direct lysis method.

Organic extraction method

This method involves phase separation by addition and centrifugation of a mixture of a solution containing phenol, chloroform and a chaotropic agent (guanidinium thiocyanate) and aqueous sample. Guanidium thiocyanate results in the denaturation of proteins and RNases, separating rRNA from ribosomes. Addition of chloroform forms a colorless upper aqueous phase containing RNA, an interphase containing DNA and a lower phenol-chloroform phase containingprotein. RNA is collected from the upper aqueous phase by alcohol (2-propanol or ethanol) precipitation followed by rehydration.

One of the advantages of this method is the stabilization of RNA and rapid denaturation of nucleases. Besides advantages, it has several drawbacks such as it is difficult to automate, needs labor and manual intensive processing, and use of chlorinated organic reagents

Direct lysis methods

This method involves use of lysis buffer under specified conditions for the disruption of sample and stabilization of nucleic acids. If desired, samples can also be purified from stabilized lysates. This method eliminates the need of binding and elution from solid surfaces and thus avoids bias and recovery efficiency effects.

Advantages

- Extremely fast and easy.
- Highest ability for precise RNA representation.
- Easy to work on very small samples.
- Amenable to simple automation.

Drawbacks

- Unable to perform traditional analytical methods (e.g. spectrophotometric method).
- Dilution-based (most useful with concentrated samples).
- Potential for suboptimal performance unless developed/optimized with downstreamanalysis.
- Potential for residual RNase activity if lysates are not handled properly.

Modifications of Cut Ends in DNA

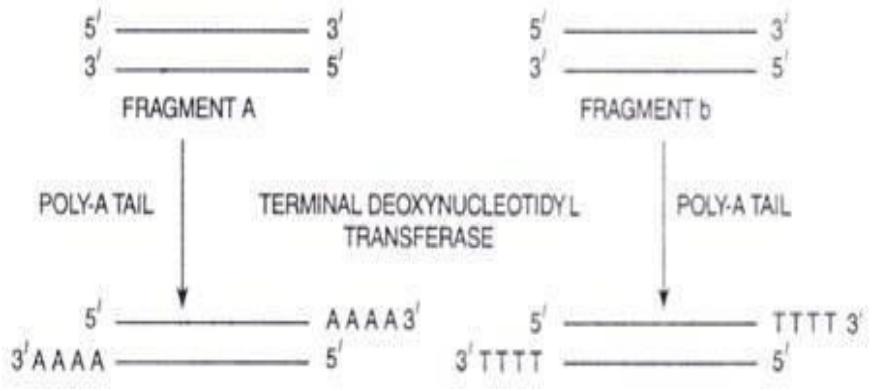
- The 3′-ends of DNA strands generated by cleavage always carry a free hydroxyl (—OH) group, while their 5′-ends always bear a phosphate group. Often the ends produced by restriction enzymes have to be modified for further manipulation of the fragments; some of the modifications are summarised below.

1. Removal of the 5'phosphate group of vector DNA by alkaline phosphatase treatment inorder to prevent vector circularization during DNA inserts integration.
2. Addition of a phosphate group to a free 5'hydroxyl group by T4 polynucleotide kinase.
3. Removal of the protruding ends by digestion with, say, S1 nuclease; this enzyme digests both 3′- and 5′- protruding ends.
4. Making the single-stranded protruding ends double-stranded by extending the recessed(shorter) strand with, say, Klenow fragment of E. coli DNA polymerase I
5. Removal of nucleotides from the 5′-ends using A exonuclease.
6. Removal of nucleotides from the 3′-ends using E. coli exonuclease III.

(Both the strategies 5 and 6 convert blunt ends into protruding single-stranded ends ofundefined base sequence).

7. Treatment of double-stranded DNAs with exonuclease Ball 1, which simultaneously digests both the strands (from both the ends) of a DNA molecule; this treatment produces shortened DNA fragments with blunt ends.

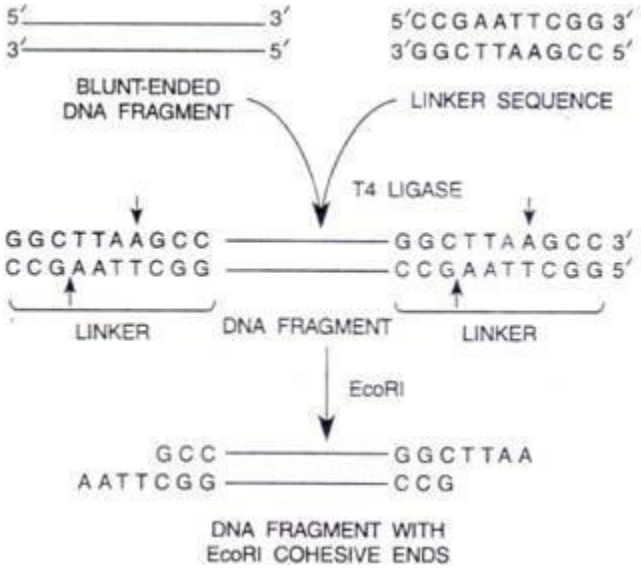
8. Synthesis of single-stranded tails (protruding ends) at the 3′-ends of blunt-ended fragmentsby the enzyme terminal deoxynucleotidy1 transferase; this is called tailing. In practice, the tails on vector and DNA inserts differ in length. As a result, short gaps remain when the ends of DNA insert pair with those of vector. Therefore, Klenow fragment is first used to fill in this gap before DNA ligase is used to ligate them together.



Tailing of blunt-ended DNA fragments A and B using terminal nucleotidyl transferase. Poly-A tail is added to the 3′ ends of fragment A, while poly-T tail is added to the 3′ ends of fragment B so that the protruding ends of fragment A arecomplementary to those of B.

9. Linker and/or adapter molecules can be joined to the cut ends. Linkers are short, chemically synthesized, double-stranded oligonucleotides, which contain within them oneor more restriction endonuclease sites, e.g., linker 5'CCGAATTCGG (only one strand of the linker is shown here) contains one EcoRl site. Linkers are fused with blunt-ended DNA fragments; cleavage of the linker with the appropriate restriction enzyme creates suitable cohesive protruding ends .

Linkers have the following two applications: creation of cohesive ends (1) on blunt-endedDNA fragments, and (2) on fragments having unmatched or undefined sequences in their protruding ends. In the latter situation, the DNA fragments are first made blunt-ended using either strategy 3 or 4, following which the selected linkers are ligated to them by T4 ligase.

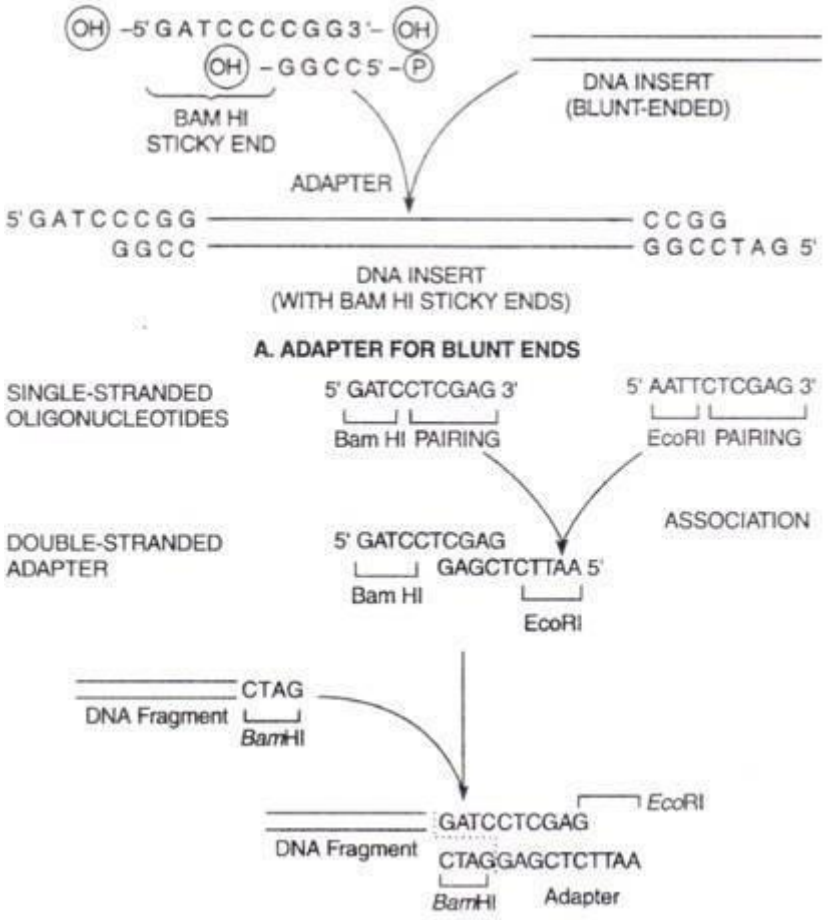


Creation of cohesive ends on blunt-ended DNA fragments. Suitable linkers areligated to the blunt ends by T4 DNA ligase. The linker is then cleaved with theappropriate restriction enzyme to generate sticky ends.

A potential drawback of linkers is as follows. The linkers must be cleared by the concerned restriction enzyme to generate the sticky ends. In case, one or more recognition sites for this enzyme are present within the DNA insert, it will also be cleaved into two or more pieces. In such a case, adapters are employed.

Adapters are short, chemically synthesized DNA double strands, which already have one or both sticky ends . When a blunt end is converted into a sticky end, the adapter hasone blunt end and one sticky end corresponding to the concerned restriction enzyme. The blunt end of the adapter is ligated to the blunt ends of the DNA insert, which are now converted into sticky ends.In order to prevent ligation of a further adapter molecule to a sticky end so produced, the 5′-terminus at the sticky end of the adapter molecules is occupied by a —OH rather than the normal

phosphate group. After the adapters have been attached to the DNA insert, their 5′-ends are phosphorylated by polynucleotide kinase so that the DNA insert can be ligated to the vector.



Examples of adapters. A. An adapter used to create BamHl sticky ends at the bluntends of a DNA insert. B. A conversion adapter produced by associating two oligonucleotides, each having a different recognition sequence at its 5′-end but a complementary sequence for base pairing at the 3′-end.

GENE TRANSFER METHODS

Chemical methods

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials amongthe different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compoundsare introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell. An ideal chemical used for DNA transfer should have the ability to-

- Protect DNA against nuclease degradation.
- Transport DNA to the target cells.
- Facilitate transport of DNA across the plasma membrane.
- Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

- Calcium phosphate
- DEAE dextran
- Cationic Lipid
- Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction.Inthis method,the precipitates of calcium phosphate and DNAbeing small and insolublecan be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis and the DNA gets integrated into the cell genome resulting in stable

or permanent transfection.

Uses

- This method is mainly used in the production of recombinant viral vectors.
- It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell
- lines. As the precipitate so formed must coat the cells, this method is suitable only forcells
- growing in monolayer and not for suspension cultures.

Advantages

- Simple and inexpensive
- Applicability to generate stably transfected cell lines
- Highly efficient (cell type dependent) and can be applied to a wide range of cell types.
- Can be used for stable ortransient transfection

Disadvantages

- Toxic especially to primary cells
- Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
- Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
- Limited by the composition and size of the precipitate.
- Random integration into host cell.

DEAE-Dextran (Diethylaminoethyl Dextran)mediated DNA transfer

This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer. Diethylaminoethyldextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactionsbetween DNA and endocytotic machinery of the cell. In this

method, the negatively chargedDNA and positively charged DEAE – dextran form aggregates through electrostatic interactionand form apolyplex. A slight excess of DEAE –dextran in mixture results in net positive chargein the DEAE – dextran/ DNA complex formed.These complexes, when added to the cells, bindto the negatively charged plasma membrane and get internalized through endocytosis.

Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol. Several parameters such as number of cells, polymer concentration, transfected DNAconcentration and duration of transfection should be optimized for a given cell line.

Advantages

- Simple and inexpensive
- More sensitive
- Can be applied to a wide range of cell types
- Can be used for transient transfection.

Disadvantages

- Toxic to cells at high concentrations
- Transfection efficiency varies with cell type
- Can only be used for transient transfectionbut not forstable transfection
- Typically produces less than 10% delivery in primary cells.

Lipofection

Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes. Liposomes areartificial phospholipid vesicles used for the deliveryof a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of 0.1 to 10 micrometer or 20-25 nanometers respectively. They can bepreloaded with DNA by two common methods- membrane-membrane fusion and endocytosisthus forming DNA- liposome complex. This complexfuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are

susceptible to lipofection method. Liposomes can be classified as either cationic liposome or pH-sensitive.

Cationic liposomes

Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

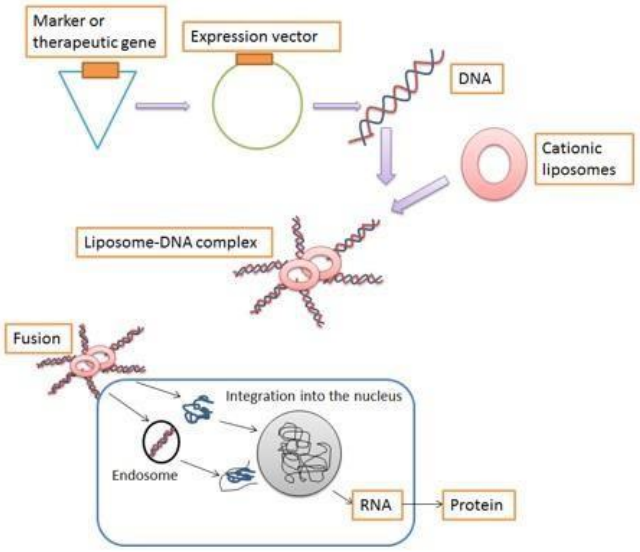
Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum.A positively charged lipid is often mixed with aneutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity. The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus. The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

Negatively charged liposomes

Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes. However, formation oflipoplex, a complex between DNA and anionic lipids can occur by using divalent cations (e.g.Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+}) which can neutralize the mutual electrostatic repulsion. Theseanionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which arephysiologically safe components. They are termed as **pH sensitive** due to destabilization at lowpH. The efficiency of both *in vivo* and *in vitro*gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene

delivery vehicles than the cationic liposomes.



Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.
- Delivery of the nucleic acids with minimal toxicity.
- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

Polyethylenimine

- Polyethylenimine (PEI) is a non-degradable, high molecular weight polymer which may accumulate in the body.
- PEI, due to its polycationic nature, condenses with the DNA molecule resulting in the formation of PEI-DNA complex which enters the cell by endocytosis, thus mediating gene transfer.

- PEI exhibit cytotoxicity due to its ability to permeabilize and disrupt cell membranes leading to necrotic cell death.
- The cytotoxicity may be reduced using various methods e.g. PEGylation and conjugation of low molecular weight polyethylenimine with cleavable cross-links such as disulfide bondsin the reducing environment of the cytoplasm.

Chitosan

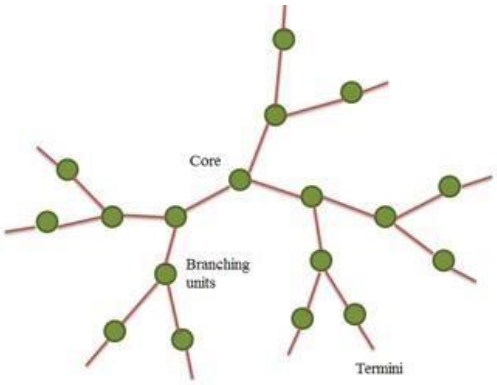
- Chitosan, a biodegradable polysaccharide is composed of D-glucosamine repeating units and canbe used as a non-viral gene carrier.
- It can efficiently bind and protect DNA from nuclease degradation.
- The biocompatibility and low toxicity profile makes it a safe biomedical material for clinical applications.
- Chitosan DNA nanoparticles can transfect several different cell types with relatively low transfection efficiency.
- Modified chitosans such as trimethylated chitosan and chitosan conjugated with deoxycholic acid have been developed to increase the solubility of chitosan at neutral pH which can efficiently transfect COS-1 cells.
- Chitosans with different molecular weights exhibit different DNA binding affinities. The efficiency of transfection is determined by the particle stability which is one of the rate-limiting steps in the overall transfection process.

Dendrimers

- Dendrimers are a new class of polymeric materials that are highly branched and monodisperse macromolecules. Due to their unique behaviour, they are suitable for a wide range of biomedical applications.
- They have positively charged amino groups (termini) on their surface which interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.
- This DNA-dendrimer complex has an overall net positive charge and interacts with negatively charged surface molecules of the cell membrane thus allowing the entry of complex into the cell through non-specific endocytosis.
- Once inside the cell, these complexes are then transported to the endosomes where these

are protected from nuclease degradation by being highly condensed within the DNA-dendrimer complex.

- The unprotonated amino groups on the dendrimers at neutral pH can become protonated in the acidic environment of the endosome leading to buffering of the endosome and thus inhibiting pH-dependent endosomal nucleases.



Various physical or mechanical methods are employed to overcome this and aid in gene transfer as listed below-

1. Electroporation
2. Microinjection
3. Particle Bombardment
4. Sonoporation
5. Laser induced
6. Bead transfection

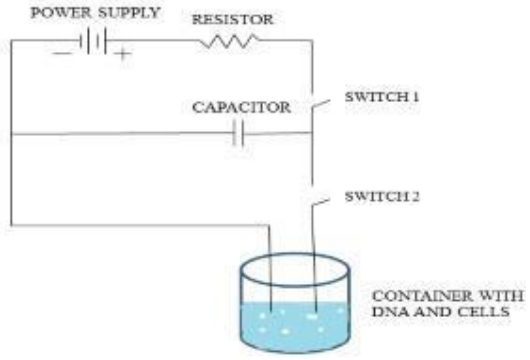
Electroporation

- Electroporation is a mechanical method used for the introduction of polar molecules into a hostcell through the cell membrane.
- This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer inmouse cells.
- It is now a widely used method for the introduction of transgene either stably or transiently intobacterial, fungal, plant and animal cells.
- It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer,

allowing the passage of molecules such as DNA.

The basis of electroporation is the relatively weak hydrophobic/hydrophilic interaction of the phospholipids bilayer and ability to spontaneously reassemble after disturbance. A quick voltage shock may cause the temporary disruption of areas of the membrane and allow the passage of polar molecules. The membrane reseals leaving the cell intact soon afterwards.

The host cells and the DNA molecules to be transported into the cells are suspended in a solution. The basic process inside an electroporation apparatus is represented in a schematic diagram



When the first switch is closed, the capacitor charges up and stores a high voltage which gets discharged on closing the second switch.

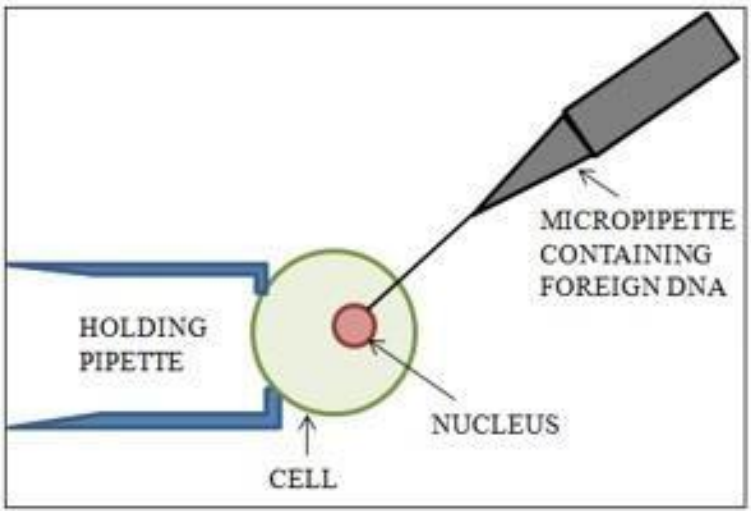
- Typically, 10,000-100,000 V/cm in a pulse lasting a few microseconds to a millisecond is essential for electroporation which varies with the cell size.
- This electric pulse disrupts the phospholipid bilayer of the membrane causing the formation of temporary aqueous pores.
- When the electric potential across the cell membrane is increased by about 0.5-1.0 V, the charged molecules e.g. DNA migrate across the membrane through the pores in a similar mannerto electrophoresis.
- The initiation of electroporation generally occurs when the transmembrane voltage reaches at 0.5-1.5 V. The cell membrane discharges with the subsequent flow of the charged ions and molecules and the pores of the membrane quickly close reassembling the phospholipid bilayer.

Microinjection

- DNA microinjection was first proposed by Dr. Marshall A. Barber in the early of nineteenth century.
- This method is widely used for gene transfection in mammals.
- It involves delivery of foreign DNA into a living cell (e.g. a cell, egg, oocyte, embryos of animals) through a fine glass micropipette. The introduced DNA may lead to the over or under expression of certain genes.
- It is used to identify the characteristic function of dominant genes.

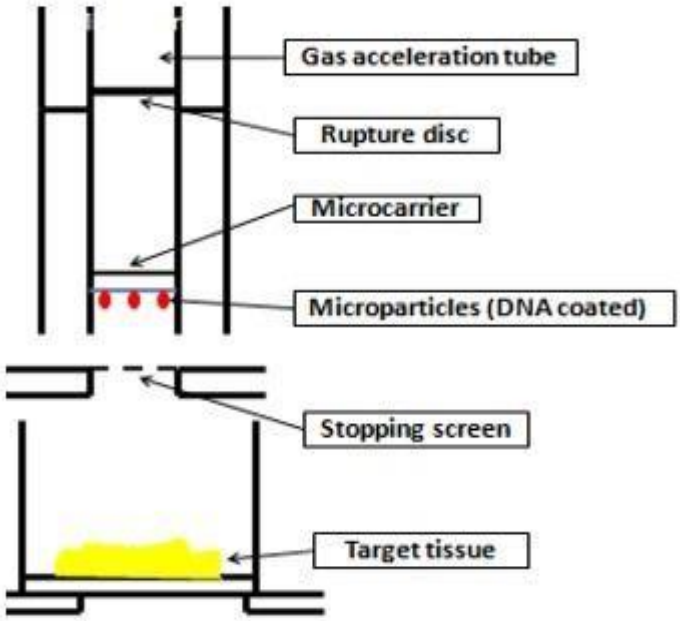
Procedure

- The delivery of foreign DNA is done under a powerful microscope using a glass micropipette tipof 0.5 mm diameter.
- Cells to be microinjected are placed in a container. A holding pipette is placed in the field of view of the microscope thatsucks and holds a target cell at the tip. The tip of micropipette is injected through the membrane of the cell to deliver the contents of the needle into the cytoplasmand then the empty needle is taken out.



Particle bombardment

This method is commonly employed for genetic transformation of plants and many organisms.



- This method is applicable for the plants having less regeneration capacity and those which fail to show sufficient response to *Agrobacterium*- mediated gene transfer in rice, corn,wheat,chickpea,sorghum and pigeon-pea.

  The biolistic gun employs the principle of conservation of momentumand uses the passage of helium gas through the cylinder with arrange of velocitiesrequired for optimal transformation of various cell types. It consists of a bombardment chamber which is connected to an outlet for vacuum creation. The bombardment chamber consists of a plastic rupture disk below which macro carrier is loaded with micro carriers. These micro carriers consist of gold or tungsten micro pellets coated with DNA for transformation.

Sonoporation

- Sonoporation involves the use of ultrasound for temporary permeabilization of the cell membrane allowing the uptake of DNA, drugs or other therapeutic compounds from the extracellular environment.
- This method leaves the compound trapped inside the cell after ultrasound exposure.
- It employs the acoustic cavitation of micro bubbles for enhancing the delivery of large moleculeslike DNA.The micro bubbles form complex with DNA followed by injection and ultrasound treatment to deliver DNA into the target cells.

- Unlike other methods of transfection, sonoporation combines the capability to enhance gene and drug transfer.

Laser induced transfection

- It involves the use of a brief pulse of focused laser beam.
- In this method, DNA is mixed with the cells present in the culture and then a fine focus of laser beam is passed on the cell surface that forms a small pore sufficient for DNA uptake into the cells. The pore thus formed is transitory and repairs soon.

Bead transfection

- Bead transfection combines the principle of physically producing breaks in the cellular membrane using beads.
- In this method, the adherent cells are incubated for a brief period with glass beads in a solution containing the DNA.

Immunoporation is a recently developed transfection process involving the use of new type ofbeads, Immunofect^{TM} beads, which can be targeted to make holes in a specific type of cells.

Biological methods

The main gene transfer methods using biological means are as follows:

- Bacterial gene delivery i.e. bactofection.
- Delivery using a viral vector i.e. transduction

Bactofection

It is a method of direct gene transfer using bacteria into the target tissue, organ or organism. The genes located on the plasmids of the transformed bacterial strains are delivered and expressed into the cells. The gene delivery may be intracellular or extracellular. It has a potential to express various plasmid-encoded heterologous proteins (antigens, toxins, hormones, enzymes etc.) in different cell types.

Uses

- Bactofection can be used for DNA vaccination against various microbial agents such as viruses,fungi, protozoans and other bacteria.
- It can be used in the treatment of several tumours like melanoma, lung carcinoma and coloncarcinoma in mice.

Advantages

- Simple, selective and efficient transfection.
- Low synthesis cost and can be administered easily.

Disadvantages

Unwanted side effects associated with host-bacteria interaction. This can be reduced by using genetically modified bacteria which contain suicide genes that ease the bacterial destruction and thus reduces the risk of clinical infections.

Transduction

This method involves the introduction of genes into host cell's genome using viruses as carriers.The viruses are used in gene transfer due to following features-

- Efficiency of viruses to deliver their nucleic acid into cells
- High level of replication and gene expression.

The foreign gene is packaged into the virus particles to enter the host cell. The entry of virus particle containing the candidate gene sequences into the cell and then to the nuclear genome is a receptor- mediated process. The vector genome undergoes complex processes ending up with ds-DNA depending on the vector that can persist as an episome or integrate into the host genome followed by the expression of the candidate gene .

Viral vectors

Vector	Host cells	Entry pathway	Vector genome forms	Transgene expression	Uses
Retrovirus	Actively dividingcells	Receptor-binding, membrane fusion	Integrated	Long term (years)	SCID, Hyperlipedemia, solid tumors
Lentivirus	Dividing and non-dividingcells	Receptor-binding, membrane fusion	Episomal	Stable	Hematopoetic cells, muscles, neuron, hepatocytes
Adeno virus	Most cells	CAR (Coxsackie and Adenovirus Receptor)-mediated endocytosis endosomal escape	Episomal	Transient (short term for weeks)	CNS, hepatocytes, pancreas
Adeno-associated virus	Most cells	Receptor-mediated endocytosis endosomal escape	Episomal (90%) Integrated (10%)	Medium to long term (year)	lung , muscle, heart, CNS
Herpes virus	Most cells	Endocytotic or membrane fusion	Episomal	Transient	Suitable particularly for nervoussystem

Adenoviruses

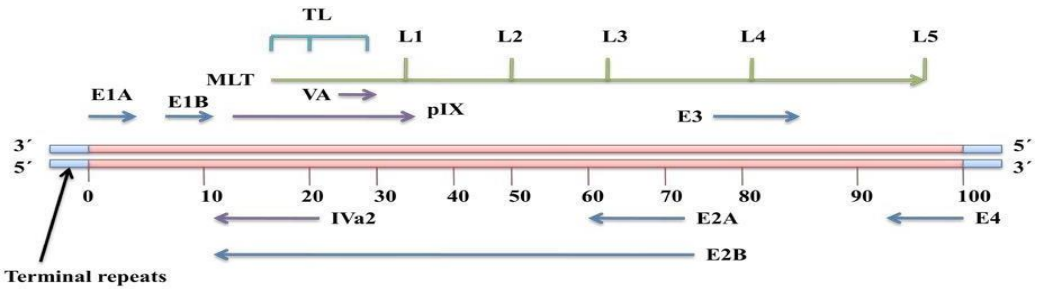
Adenoviruses are medium-sized (90-100 nm), non-enveloped, icosahedral viruses containing linear, double-stranded DNA of approximately 36 kb. 57 immunologically distinct types (7 subgenera) of adenoviruses cause human infections. They are unusually stable to physical or chemical agents and adverse pH conditions for long-term survival outside the body.

There are six early-transcription units, most of which are essential for viral replication, anda major late transcript that encodes capsid. They result in transient expression in dividing cells as they do not integrate efficiently into the genome, but prolonged expression can be achieved in post-mitotic cells, like neurons.

Adenoviruses are mostly attractive as gene therapy vectors, because the virions are takenup efficiently by cells *in vivo.* Adenovirus-derived vaccines have been used in humans withno reported side-effects.

The adenovirus infection cycle comprises two phases-early and late phase, separated by viral DNA replication. The first or "early" phase involves the entry of the virus into the host cell and virus genome to the nucleus. The late genes are transcribed from the major late promoter. The “late” phase involves the formation of gene products related to production and assembly of capsid proteins.

Wild Type Adenovirus Genome



wild type adenovirus genome. (E1A, E1B, E2A, E2B, E3, E4- early genes; L1 to 5- late genes; MLT- major late transcript; TL-tripartite leader; other genes are represented by pIX, IVa2, VA)

Adenoviral genes	Function
Early genes: E1A, E1B, E2, E3, E4	Transcription, replication, host immune suppression, inhibition of host cell apoptosis.
Delayed early genes: pIX, IVa2	Packaging
Major late gene (L)	Assembly

Construction of Adenoviral vectors

First generation adenoviral vectors were *replication deficient,* lacking the essential E1A and E1B genes and often the non-essential gene E3 and were called **'E1 replacement vectors'.** They had a maximum capacity of about 7 kb and were propagated in the cell lines transfected with DNA containing E1 genes e.g. human embryonic kidney line 293 (HEK 293).

Drawback

- These vectors caused **cytotoxic effect** due to low-level expression of the viral gene products, and chances of recombination between the vector and the integrated portion ofthe genome, resulting in the recovery of replication-competent viruses.
- Higher-capacity vectors lacking the E2 or E4 regions in addition to E1 and E3 provide a maximum cloning capacity of about 10 kb but still allow low level of transgene expression. These must be propagated on complementary cell lines providing multiple functions. The use of E1/E4 deletions is a sound strategy as the E4 gene is responsible for many of the immunological effects of the virus.

To overcome the above limitations, an alternative strategy employs insertion of 'stuffer DNA' into the nonessential E3 gene as part of the vector backbone so to maintain optimum vector size. Helper dependent adenoviral vectors (HDAd) are favoured for *in vivo* gene transfer due to deletion of all viral coding sequences.

Advantages of HDAd

- Large cloning capacity (up to 37 kb)
- High transduction efficiency

- Long term transgene expression
- Lack of immune response and cytotoxicity.

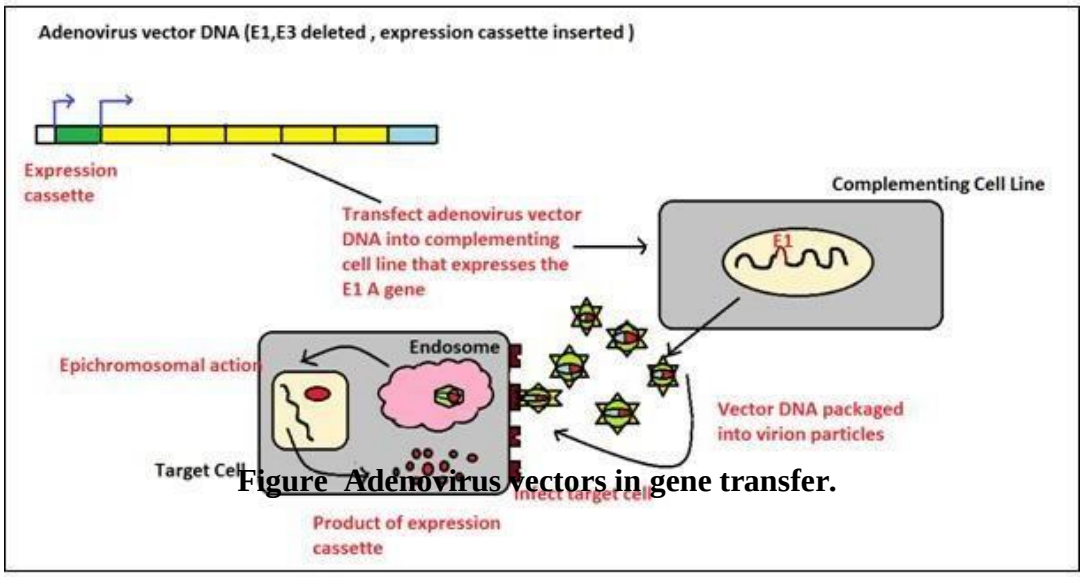


Figure Adenovirus vectors in gene transfer.

Advantages of Adenoviral vectors

- High transduction efficiency
- Insert size up to 8 kilobases
- Generation of high virus titres
- High level of expression in a wide variety of cell types
- No mutagenic effects due to lack of random integration into the host genome.

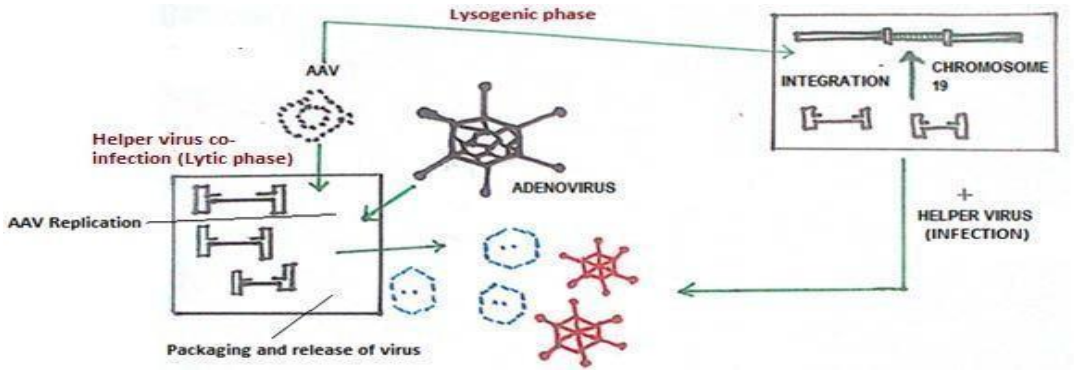
Disadvantages of Adenoviral vectors

- Transient expression due to lack of integration into the host.
- Pathogenic to humans.

Adeno-associated virus

- It was first discovered as a contaminant in an adenoviral isolate in 1965.
- It is a small, non-enveloped virus packaging a linear single stranded DNA belonging to Parvovirus family.
- It is naturally replication defective thus requiring a helper virus (usually adenovirus or herpes virus) for productive infection.

- In human cells, the provirus integrates predominantly into a 4-kb region (AAVS1) on chromosome19. Subsequent infection by adenovirus or herpes virus can 'rescue' the provirus and induce lytic infection.



AAV life cycle.

- AAV life cycle comprises two phases-lytic and lysogeny.
- In the presence of helper virus, AAV undergoes lytic phase comprising genome replication, expression of viral genes and production of virions (refer Figure)
- In the absence of helper virus, it undergoes lysogenic phase and integrates into the host cell's genome as a latent provirus. This latent genome undergoes replication by subsequent infection with helper virus.
- Both the stages of life cycle of AAV are controlled by complex interactions between the AAV genome and helper virus, AAV and host proteins.

Adeno-associated viral genome

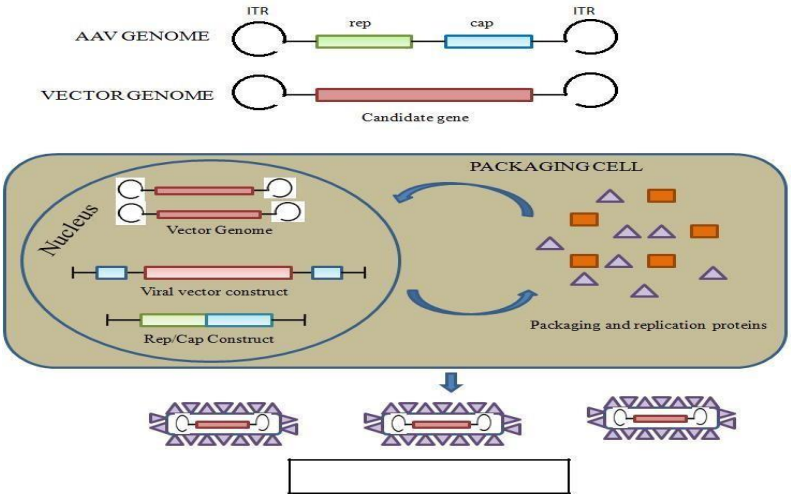
The AAV genome is small (about 5 kb) and comprises a central region containing rep (replicase)and cap (capsid) genes flanked by 145 base inverted terminal repeats (ITRs). The rep gene is involved in viral replication and integration whereas cap gene encodes viral capsid proteins. ITRs are required for replication, transcription, proviral integration and rescue.

In earlier AAV vectors, foreign DNA replaced the cap region and was expressed under the control of an endogenous AAV promoter. The transgene expression was

inefficient using heterologous promoters due to inhibition of their activity by Rep protein.

Rep interference with endogenous promoters resulted in cytotoxic effects of the virus. Toovercome the above limitations, such vectors in which both genes were deleted and the transgenewas expressed from either an endogenous or a heterologous promoter, were developed.

In vitro manipulation of AAV is facilitated by cloning the inverted terminal repeats in a plasmid vector and inserting the transgene between them. Transfection of this construct into cellsalong with a helper plasmid produced recombinant viral particles.



Recombinant AAV (rAAV) is used as an expression cassette containing a reporter or candidate gene of interest. The foreign gene replaces all of the viral genes present in a wild type virus. Only the inverted terminal repeats are left to function as the essential replication/packagingsignal.

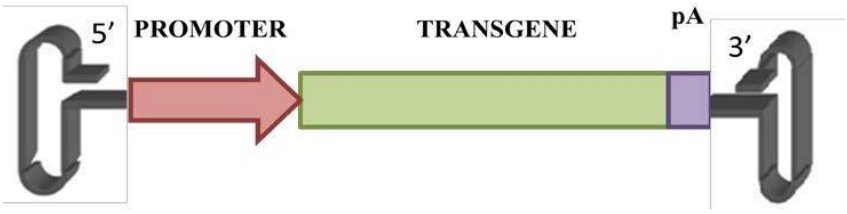


Figure Organization of a typical recombinant AAV (rAAV) genome. pA representsPoly A tail.

Advantages

- Stable and have a wide host range
- Lack of initiating an immune response
- The dependence of AAV on a heterologous helper virus provides higher control overvector replication, making AAV vectors safer for use in gene therapy
- Potential of targeted/site-specific integration
- Non-pathogenic

Disadvantages

- AAV uses concatemeric replication intermediates
- They must be closely screened as they are often contaminated with adenovirus or HerpesVirus.
- Insert size is limited (4Kb)
- Difficult generation of high virus titres

Herpes virus vectors

- The herpes viruses are linear ds-DNA viruses of approximately 150 kb size *e.g.* EBV (Epstein–Barr virus) and the HSVs (Hepatitis B virus, e.g. HSV-I, varicella zoster).
- Most HSVs are transmitted without symptoms (varicella zoster virus is exceptional) and cause prolonged infections.
- With the help of two viral glycoproteins, gB and gD, the virus binds to cells through an interaction with heparan sulphate moieties on the cell surface.
- Unlike EBV as a replicon vector (contains both *cis* and *trans* acting genetic elements required for replication), HSV-I have been developed as a transduction vector for purposeof gene transfer and can efficiently transduce a wide range of cell types.
- HSV virus is remarkably neurotropic and thus HSV vectors are particularly suitable for gene therapy in the nervous system. HSV can also be transmitted across neuronal synapses during lytic infections which can be used to trace axon pathways.
- Generation of recombinants in transfected cells takes place by homologous

recombination. These viral vectors may be replication competent or helper dependent.

- The plasmid based amplicon vectors carrying only the *cis*-acting elements required for replication and packaging can be constructed. These vectors require packaging systems toprovide the missing functions in *trans*.

Role in gene therapy

Most promised use of HSV vectors involves gene transfer to neural cells where it can cause a latent infection (e.g. spinal cord, brain, and peripheral nervous system).

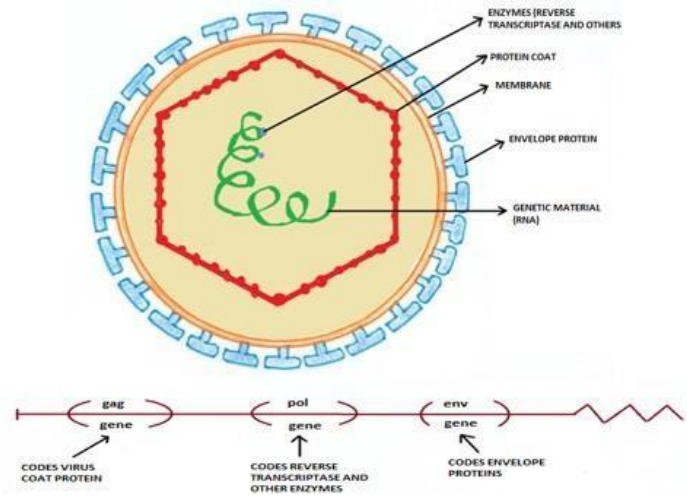
Advantages

- Infects a wide range of cell types
- Insert size up to 50 kb due to large viral genome size
- Natural tropism to neuronal cells
- Stable viral particles allow generation of high virus titres (10^{12}pfu/ml)

Disadvantages

- No viral integration into host genome and transient transgene expression
- High level of pre-existing immunity
- Cytotoxicity effects
- Risk of recombination with latently HSV-infected cells

Retroviruses are RNA viruses that replicate via a ds-DNA intermediate. The infection cycle begins with the interaction between viral envelope and the host cell's plasma membrane, delivering the particle into the cell. The capsid contains two copies of the RNA genome, as well as reverse transcriptase/integrase. After infection, the RNA genome is reverse transcribed to produce a cDNA copy, a DNA intermediate, which integrates into the genome randomly.



Life cycle of retroviruses

A retrovirus, on binding to a cell surface receptor, enters the cell where it reverse transcribes the RNA into double-stranded DNA. Viral DNA gets integrated into the cell chromosome to form a provirus. Cellular machinery transcribes, processes the RNA and undergoes translation into viral proteins. The viral RNA and proteins are then assembled to form new viruses which are released from the cell by budding

Retroviral genome

The integrated provirus comprises three genes (*gag, pol* and *env*). The *gag* gene encodes a viral structural protein, *pol* encodes the reverse transcriptase and integrase and *env* gene encodes viral envelope proteins. Retrovirus can be classified as oncoviruses, lentiviruses, and spuma-viruses. Oncoviruses are simple whereas lentiviruses and spuma-viruses are complex retroviruses.

Viral genomic RNA is synthesized by transcription from a single promoter located in the left LTR and ends at a poly-A site in the right LTR. Thus, the full-length genomic RNA is shorter than the integrated DNA copy and lacks the duplicated LTR structure. The genomic RNA is capped and polyadenylated, allowing the *gag* gene to be translated. The *pol* gene is also translated by read through, producing a Gag–Pol fusion protein, which is further processed into several distinct polypeptides. Some of the full-length RNA also undergoes splicing, eliminating the *gag* and *pol* genes and allowing the downstream *env*

gene to be translated. Two copies of the full-length RNA genome are incorporated into each capsid requiring a specific *cis* -acting packaging site termed ψ. The reverse transcriptase/ integrase are also packaged.

Construction of a retroviral vector and propagation in helper cell

The retroviral construct involved in gene delivery comprises two constructs-

- A vector consisting of all *cis* -acting elements required for gene expression and replication
- A helper cell expressing all the viral proteins (*gag*, *pol*, *env*) lacking in vector and support thereplication of vector. Helper cell lacks RNA containing packaging signal which is required for formation and release of infectious particles but not for non-infectious viral particles.

When the vector DNA is introduced into a helper cell, helper cell produces the viral proteins which help in the assembly of viral particles containing RNA transcribed from the viral vector. These viral particles on infecting the target cell, reverse transcribe the vector RNA into ds-DNA which gets integrated into the host genome forming a provirus which encodes the gene of interest. Target cells do not express viral proteins and cannot generate infectious viral particles containing the vector RNA and thus cannot infect other target cells .

Advantages

- Insert size up to 8 kb
- Integration into host genome resulting in sustained expression of the vector
- Vector proteins are not expressed in host

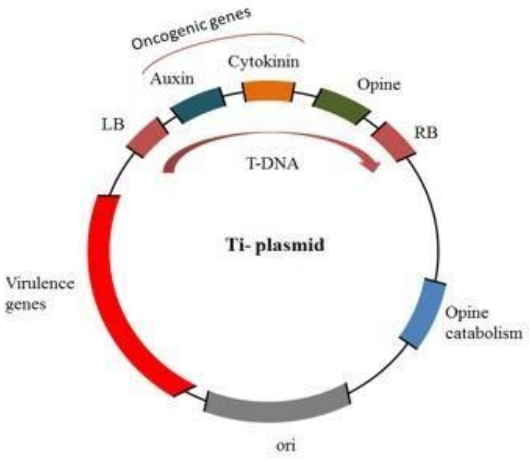
Disadvantages

- Infection by retrovirus requires dividing cells
- Low titres (10^6 -10^7)
- Random integration
- Poor *in vivo* delivery

***Agrobacterium*-mediated transformation**

Ti-plasmid

The virulent strains of *A. tumefaciens* harbor large plasmids (140–235 kbp) known as tumor- inducing (Ti) plasmid involving elements like T-DNA, vir region, origin of replication, region enabling conjugative transfer and o-cat region (required for catabolism of opines).



T-DNA

It is a small, specific segment of the plasmid, about 24kb in size and found integrated in the plantnuclear DNA at random site. This DNA segment is flanked by right and left borders.

The functions of T-DNA genes are listed

Gene	**Product**	**Function**
Ocs	Octopine synthase	Opine synthesis
Nos	Nopaline synthase	Opine synthesis
trns1 (iaaH, auxA)	Tryptophan-2-mono-oxygenase	Auxin synthesis
trns2 (iaaM, auxB)	Indoleacetamide hydrolase	Auxin synthesis
trnr (ipt, cyt)	Isopentyltransferase	Cytokinin synthesis
Frs	Fructopine synthase	Opine synthesis
Mas	Mannopine synthase	Opine synthesis
Ags	Agropine synthase	Opine synthesis

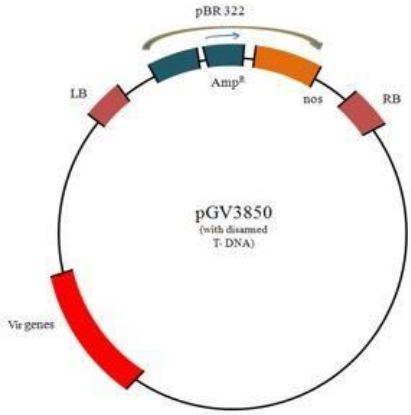
T- DNA:Border Sequences

- T-regions are defined by direct repeats known as T-DNA border sequences (Right and LeftBorder i.e. RB and LB of 25 bp each).

- These are not transferred intact to the plant genome, but are involved in the transfer process.
- The RB is rather precise, but the LB can vary by about 100 nucleotides.
- Deletion of the RB repeat abolishes T-DNA transfer, but the LB seems to be non-essential. TheLB repeat has little transfer activity alone.

Disarmed Ti-plasmid derivatives as plant vectors

Ti plasmid is a natural vector for genetically engineering plant cells due to its ability to transfer T-DNA from the bacterium to the plant genome. But wild-type Ti plasmids are not suitable as vectors due to the presence of oncogenes in T-DNA that cause tumor growth in the recipient plant cells. For efficient plant regeneration, vectors with disarmedT-DNA are used by making it non-oncogenic by deleting all of its oncogenes. The foreign DNA is inserted between the RB and LB and then integrated into the plantgenome without causing tumors.



Structure of the Ti-plasmid pGV3850 with disarmed T-DNA.

The creation of disarmed T-DNA is an important step forward, but the absence of tumor formation makes it necessary to use an alternative method for the identification of transformed plant cells. Opine production using *pGV3850* was exploited as a screenable phenotype, and the *ocs* and *nos*genes are now widely used as screenable markers.

Drawbacks

Several drawbacks are associated with disarmed Ti- vector systems as discussed below;

- Necessity to carry out enzymatic assays on all potential transformants.

- Not convenient as experimental gene vectors due to large size.
- Difficulty in *in vitro* manipulation and
- Absence of unique restriction sites in the T-DNA.

Co- integrate vectors

Co-integrate vectors are the deletion derivatives of Ti-plasmids. The DNA to be introduced intothe plant transformation vector is sub cloned in a conventional *Escherichia coli* plasmid vector for easy manipulation, producing a so-called *intermediate vector*. These vectors are incapable of replication in*A. tumefaciens* and also lack conjugation functions. Transfer is achieved using a 'triparental mating' in which three bacterial strains are mixed together:

(i) An *E. coli* strain carrying a helper plasmid able to mobilize the intermediate vector in *trans*;

(ii) *The E.coli* strain carrying the recombinant intermediate vector;

(iii) *A.tumefaciens* carrying the Ti plasmid. Conjugation between the two *E. coli* strains transfers the helper plasmid to the carrier of theintermediate vector,which in turn is

mobilized and transferred to the recipient *Agrobacterium.* Homologous recombination between the T-DNA sequences of the Tiplasmid and intermediate vector forms a large co- integrate plasmid resulting in the transfer ofrecombinant T-DNA to the plant genome.

Binary vector

- Binary vector was developed by Hoekma *et al* (1983) and Bevan in (1984).
- It utilizes the trans- acting functions of the vir genes of the Ti-plasmid and can act on any T-DNA sequence present in the same cell.
- Binary vector contains transfer apparatus(the vir genes) and the disarmed T-DNA containing thetransgene on separate plasm

Advantages of Binary vector

- Small size due to the absence of border sequences needed to define T-DNA region and virregion.
- Ease of manipulation

Molecular characterization of transgenes

• **PCR**- Simplest and fastest method. Prone to false positives.

• **Southern Blot**- Confirms insertion of the tDNA into the genomic DNA of the targetorganism, as well as provides insertion copy number.

• **Northern Blot**- Confirms the presence of RNA transcript accumulation from thetransgene of interest.

• **Western Blot**- Confirms presence of the PROTEIN produced from the inserted transgeneof interest.

• **qRT-PCR**- Provides a relative expression level for the gene of interest—transcript—likeNorthern blot.

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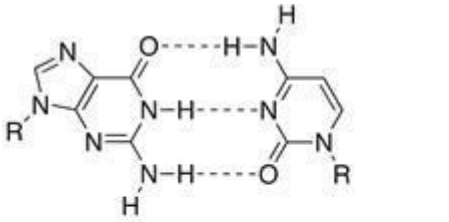
DEPARTMENT OF BIOTECHNOLOGY

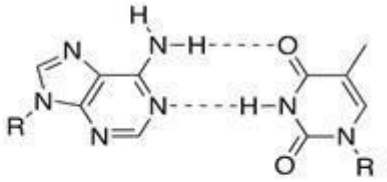
UNIT – II – Advanced Molecular Biology and Genetic Engineering – SBTA5201

Hybridization techniques

Nucleic acid hybridization is a basic technique in molecular biology which takes advantage of the ability of individual single-stranded nucleic acid molecules to form double-stranded molecules. According to Watson-Crick base pairing, adenine binds with thymine and guanine binds with cytosine by hydrogen bonding.



A GC base pair demonstrating three intermolecular hydrogen bonds



An AT base pair demonstrating two intermolecular hydrogen bonds

Southern hybridization

The basic principle behind the southern hybridization is the nucleic acid hybridization. Southern hybridization commonly known as southern blot is a technique employed for detection of a specific DNA sequence in DNA samples that are complementary to a given RNA or DNA sequence. It was the first blotting technique to be devised, named after its pioneer E.M Southern, a British biologist. Southern blotting involves separation of restricted DNA fragments by electrophoresis and then transferred to a nitrocellulose or a nylon membrane, followed by detection of the fragment using probe hybridization.

Separated by electrophoresis is transferred from gel to a membrane which in turn is used as a substrate for hybridization analysis employing labeled DNA or RNA probes specific to target fragments in the blotted DNA. Southern hybridization helps to detect specific fragment against a

background of many other restriction fragments. Southern blotting is a technique which is used to confirm the identity of a cloned fragment or for recognition of a sub-fragment of interest from within the cloned DNA, or a genomic DNA. Southern blotting is a prerequisite to techniques such as restriction fragment length polymorphism (RFLP) analysis.

Procedure:

1. The high-molecular-weight DNA strands are fractioned using restriction enzymes.

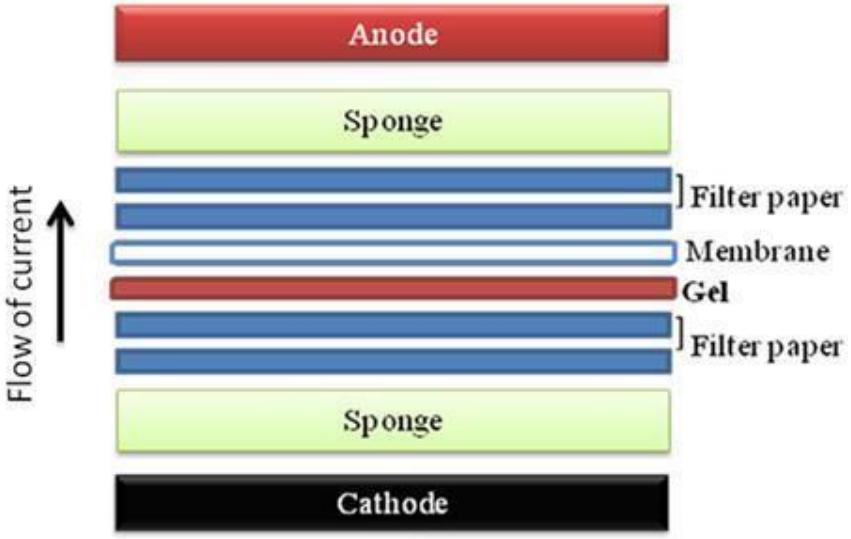
2. The DNA fragments are separated based on size by agarose gel electrophoresis.

3. The gel with the restricted fragments is then laid on a filter paper wick which serves as aconnection between the membrane and the high salt buffer.

4. The nitrocellulose membrane is placed on top of the gel and a tower of filter papers is used to cover it and these are kept in place with a weight. The capillary action drives the buffer soaking through the filter paper wick, through the gel and the membrane and into the paper towels. Alongwith the buffer passing through the gel the DNA fragments are also carried with it into the membrane and they bind to the membrane.

For DNA fragments larger than 15 kb, before blotting an acid such as diluted HCl is used to treatthe gel that depurinates the DNA fragments causing breakage of DNA into smaller pieces, resulting in more efficient transfer from the gel to membrane.

Now a day's blotting is also done by applying electric field. This **electro blotting** technique depends upon current and transfer buffer solution to nucleic acids onto a membrane. Following electrophoresis, a standard tank or semi-dry blotting transfer system is set up. A stack is put together in the following order from cathode to anode: sponge, three sheets of filter paper soakedin transfer buffer gel, PVDF or nitrocellulose membrane, three sheets of filter paper soaked in transfer buffer and then again sponge. Importantly the membrane should be located between the gel and the positively-charged anode, as the current and sample will be moving in that direction. Once the stack is prepared, it is placed in the transfer system, and suitable current is applied for aspecific period of time according to the materials being used.



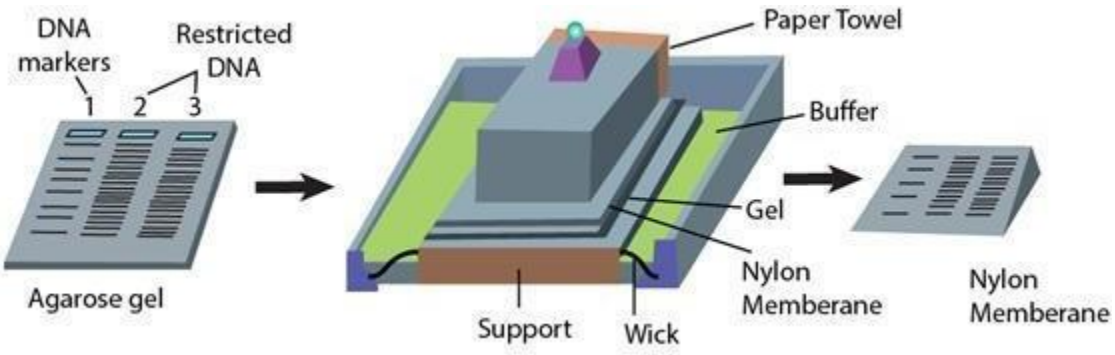
For using alkaline transfer methods, the DNA gel is placed into an alkaline solution (like that of sodium hydroxide) causing denaturation of the double-stranded DNA. Denaturation in an alkaline environment enhances the binding between the negatively charged DNA and the positively charged membrane, causing separation to single DNA strands for further hybridizationto the probe, alongside destroying any residual RNA that may persist in DNA. The membrane is washed with buffer to remove unbound DNA fragments.

The membrane which contains the transferred fragments is heated in presence or absence of vacuum at 80°C for 2 hours or exposed to ultraviolet radiation (nylon membrane) for permanent attachment of the transferred DNA to the membrane.

The obtained membrane is then hybridized with a probe (a DNA fragment with a specific sequence whose presence in the target DNA is to be determined).

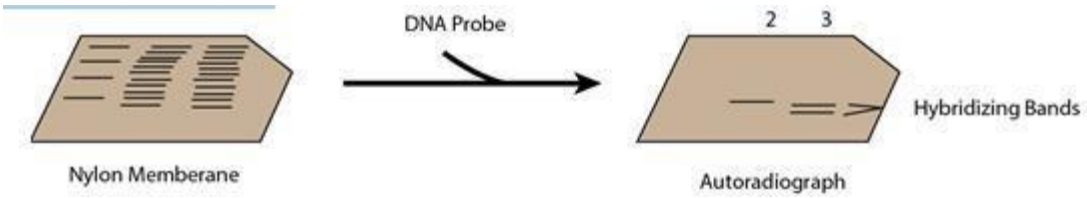
Labeling of the probe DNA is done for easy detection, usually radioactivity is incorporated or themolecule is tagged with a fluorescent or chromogenic dye. The hybridization probe may be madeof RNA, instead of DNA in some cases where the target is RNA specific.

Washing of the excess probe from the membrane is done by SSC buffer after the hybridization step and the hybridization pattern is studied on an X-ray film by autoradiography or via color development on membrane if a chromogenic detection method is employed



Analysis of Southern Blot:

Hybridization of the probe to a specific DNA fragment on the membrane indicates the presence of a complementary fragment in the DNA sequence. Southern hybridization performed by digestion of genomic DNA using a restriction enzyme digestion, helps in determining the number of sequences (or gene copies) in the genome. For a probe hybridizing to a single DNA segment that has not been cut by the restriction enzyme, a single band is observed and on the other hand multiple bands will likely be observed when the hybridization occurs between the probe and several highly similar target sequence (Due to sequence duplication). Alterations in the hybridization conditions like enhancing the hybridization temperature or decreasing salt concentration, helps in altering specificity and hybridization of the probe to sequences that are less than 100% similar.



Applications:

a) Clone identification: One of the most common applications of Southern blotting is identification and cloning of a specific gene of interest. Southern blotting is carried out for identification of one or more restriction fragments that contain the gene of interest in genomic DNA.. After cloning and tentative identification of the desired recombinant by employing colonyor plaque hybridization, southern blotting is further is used to confirm the clone identificationand possibly to locate a shorter restriction fragment, containing the sequence of interest.

b) Restriction fragment length polymorphism Analysis: Another major application of Southern hybridization is restriction fragment length polymorphism (RFLP) mapping, which is crucial in construction of genome maps.

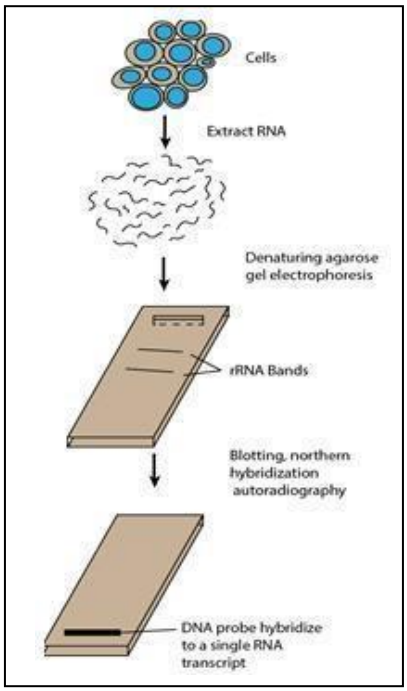
Northern hybridization:

Northern blotting was developed by James Alwine, George Stark and David Kemp (1977). Northern blotting drives its name because of its similarity to the first blotting technique, which isSouthern blotting, named after the biologist Edwin Southern. The major difference is that RNA being analyzed rather than DNA in the northern blot.

Expression of a particular gene can be detected by estimating the corresponding mRNA by Northern blotting. Northern blotting is a technique where RNA fragments are separated by electrophoresis and immobilized on a paper sheet. Identification of a specific RNA is then done by hybridization using a labeled nucleic acid probe. It helps to study gene expression by detection of RNA (or isolated mRNA) in a sample.

In Northern blotting, probes formed of nucleic acids with a sequence which is complementary to the sequence or to a part of the RNA of interest. The probe can be DNA, RNA or chemically synthesized oligonucleotides of minimum 25 complementary bases to

the target sequence.



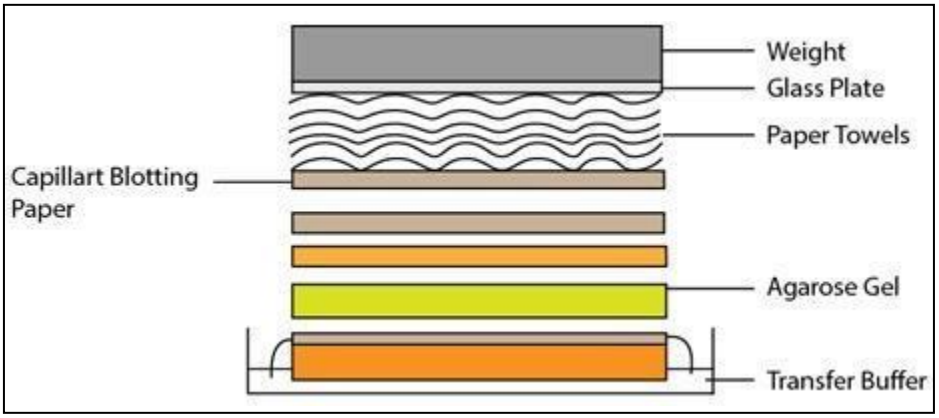
Procedure:

The northern blotting involves the following steps:

• Total RNA is extracted from a homogenized tissue sample or cells. Further eukaryotic mRNA can then be isolated by using of oligo (dT) cellulose chromatography to isolate only those RNAs by making use of a poly A tail.

• The isolated RNA is then separated by gel electrophoresis.

• The RNA samples separated on the basis of size are transferred to a nylon membraneemploying a capillary or vacuum based system for blotting.



Similar to Southern blotting, the membrane filter is revealed to a labeled DNA probe

that iscomplementary to the gene of interest and binds.
The labeled filter is then subjected to autoradiography for detection.

The net amount of a specific RNA in a sample can be estimated by using Northern blot. This technique is widely used for comparing the amounts of a particular mRNA in cells under different conditions. The separation of RNA samples is often done on agarose gels containing formaldehyde as a denaturing agent as it limits the RNA to form secondary structure.

Analysis of Northern Blot:

RNA extract is electrophoresed in an agarose gel, using a denaturing electrophoresis buffer (containing formaldehyde) to ensure that the RNAs do not form inter- or intra-molecular base pairs, as base pairing would affect the rate at which the molecules migrate through the gel. After electrophoresis, the gel is blotted onto a nylon or nitrocellulose membrane, and hybridized with alabeled probe. If the probe is a cloned gene, the band that appears in the autoradiograph is the transcript of that gene. The size of the transcript can be determined from its position within the gel, and if RNA from different tissues is run in different lanes of the gel, then the possibility of differentially expressed gene can be examined.

Applications:

Northern blotting helps in studying gene expression pattern of various tissues, organs, developmental stages, pathogen infection, and also over the course of treatment. It has been employed to study overexpression of oncogenes and down-regulation of tumor-suppressor genes in cancerous cells on comparison with healthy tissue, and also for gene expression of immune- rejection of transplanted organ.

The examination of the patterns of gene expressions obtained under given conditions can help determine the function of that gene. Northern blotting is also used for the analysis of alternate spliced products of same gene or repetitive sequence motif by investigating the various sized RNA of the gene. This is done when only probe type with variation in one location is used to bind to the target RNA molecule.

Variations in size of a gene product may also help to identify deletions or errors in transcript processing, by altering the probe target that can be used along the known sequence and

make it possible to determine the missing region of the RNA.

Western Blotting

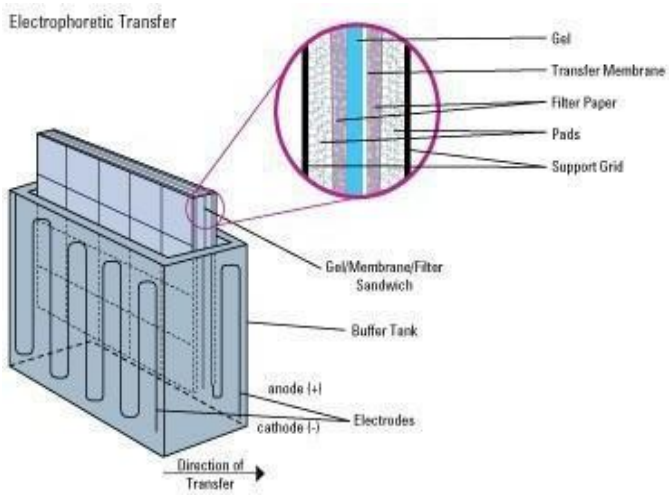
The first step in a Western blotting procedure is to separate the macromolecules using gel electrophoresis. After electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. Most commonly, the transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic precipitate on the membrane for colorimetric detection. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a byproduct. The light output can be captured using film, a CCD camera or a phosphorimager that is designed for chemiluminescent detection. Alternatively, fluorescently tagged antibodies can be used, which are directly detected with the aid of a fluorescence imaging system. Whatever system is used, the intensity of the signal should correlate with the abundance of the antigen on the membrane.

Electrophoresis of Proteins

Proteins are commonly separated using polyacrylamide gel electrophoresis (PAGE) to characterize individual proteins in a complex sample or to examine multiple proteins within a single sample. When combined with Western blotting, PAGE is a powerful analytical tool providing information on the mass, charge, purity or presence of a protein. Several forms of PAGE exist and can provide different types of information about the protein(s)

Following electrophoresis, the protein must be transferred from the electrophoresis gel to a membrane. There are a variety of methods that have been used for this process, including diffusion transfer, capillary transfer, heat-accelerated convectional transfer, vacuum blotting transfer and electroelution. The transfer method that is most commonly used for proteins is electroelution or electrophoretic transfer because of its speed and transfer efficiency. This method uses the electrophoretic mobility of proteins to transfer them from the gel to the membrane. Electrophoretic transfer of proteins involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose or other

suitable, protein- binding support and "sandwiching" this between two electrodes submerged in a conducting solution. When an electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane, where the proteins become tightly attached. The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel.



Transfer efficiency can vary dramatically among proteins, based upon the ability of a protein to migrate out of the gel and its propensity to bind to the membrane under a particular set of conditions. The efficiency of transfer depends on factors such as the composition of the gel, complete contact of the gel with the membrane, the position of the electrodes, the transfer time, size and composition of proteins, field strength and the presence of detergents and alcohol in the buffer.

After transfer and before proceeding with the Western blot, total protein on the membrane is often stained with a dye, such as Ponceau S or amido black 10B, to check the transfer efficiency; the gel may also be stained to confirm that protein has been moved out of the gel, but this does not ensure efficient binding of protein to the membrane. Because dyes may interfere with antibody binding and detection, a protein stain that is easily removable is ideal. Ponceau S stainis the most widely used reagent for reversibly staining proteins on a membrane, although it has limited sensitivity, does not photograph well and fades quickly, which makes documentation difficult. Superior alternatives for staining protein on nitrocellulose or PVDF membranes are available, which allow the detection of low-

nanogram levels of protein, are easily photographed and do not fade until removed.

Blocking Nonspecific Sites

The membrane supports used in Western blotting have a high affinity for proteins. Therefore, after the transfer of the proteins from the gel, it is important to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. A variety of blocking buffers ranging from milk or normal serum to highly purified proteins have been used to block free sites on a membrane.

Primary and Secondary Antibodies

Although other methods are used, Western blotting is typically performed by probing the blocked membrane with a primary antibody that recognizes a specific protein or epitope on a group of proteins (e.g., SH2 domain or phosphorylated tyrosine).

In general, the primary antibody which recognizes the target protein in a Western blot is not directly detectable. Therefore, tagged secondary antibodies or other detection reagents are usedas the means of ultimately detecting the target antigen (indirect detection). A wide variety of labeled secondary detection reagents can be used for Western blot detection. The choice of which depends upon either the species of animal in which the primary antibody was raised (the host species) or any tag on that antibody (i.e., biotin or DIG). For example, if the primary antibody is an unmodified mouse monoclonal antibody then the secondary antibody must be an anti-mouse IgG secondary antibody obtained from a non-mouse host.

Detection Methods

Enzymatic labels are most commonly used for Western blotting and, although they require extra steps, can be extremely sensitive when optimized with an appropriate substrate. Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensivelyas labels for protein detection. An array of chromogenic, fluorogenic and chemiluminescent substrates are available for use with either enzyme. Alkaline phosphatase offers a distinct advantage over other enzymes in that its reaction rate remains linear allowing sensitivity to be improved by simply allowing a reaction to proceed for a longer time period. Unfortunately, the increased reaction time often leads to high

background signal resulting in low signal:noise ratios.

Horseradish peroxidase (HRP) conjugated antibodies are considered superior to antibody-AP conjugates with respect to the specific activities of both the enzyme and antibody due the smallersize of HRP enzyme and compatibility with conjugation reactions. In addition, the high activity rates, good stability, low cost and wide availability of substrates make HRP the enzyme of choice for most applications.

PCR (Polymerase Chain Reaction)

PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

Components of PCR

Template

The sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strandsfrom each other.

DNA polymerase

type of enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used of these enzymes is *Taq* DNA polymerase (from *Thermus aquaticus*), whereas *Pfu*DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA. Although these enzymes are subtly different, they both have two capabilities that make them suitable for PCR: 1) they can generate new strands of DNAusing a DNA template and primers, and 2) they are heat resistant.

Primers

short pieces of single-stranded DNA that are complementary to the target sequence.

Thepolymerase begins synthesizing new DNA from the end of the primer.

Nucleotides (dNTPs or deoxynucleotide triphosphates)

• single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNAstrands.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of thereaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (anenzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps.

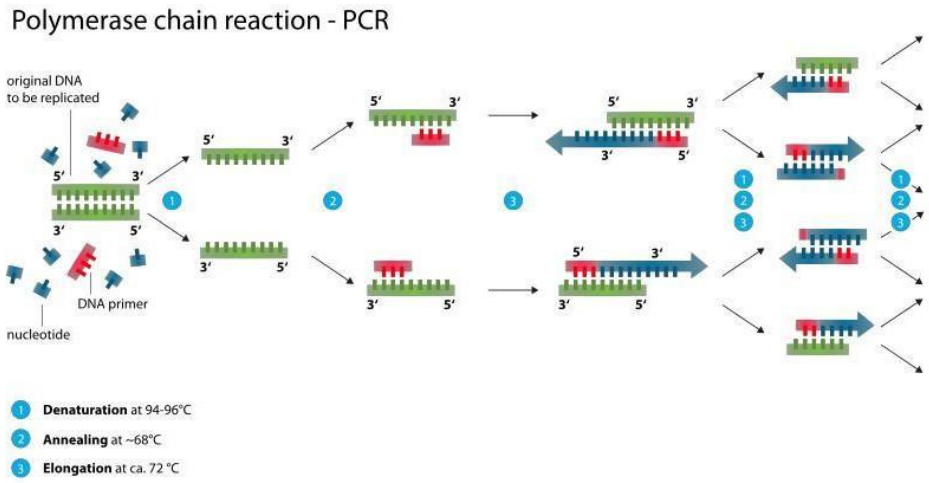
Typically, PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2–3 discrete temperature steps, usually three (Figure below). The cycling is often preceded by a single temperature step at a high temperature (>90 °C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPsin the reaction, and the melting temperature (Tm) of the primers.

- *Denaturation step*: *This* step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding

single-stranded DNAmolecules.

- *Annealing step*: *The* reaction temperature is lowered to 50–65 °C for 20–40 secondsallowing annealing of the primers to the single-stranded DNA template. This temperaturemust be low enough to allow for hybridization of the primer to the strand, but high enoughfor the hybridization to be specific, i.e., the primer should only bind to a perfectlycomplementary part of the template. If the temperature is too low, the primer could bindimperfectly. If it is too high, the primer might not bind. Typically the annealing temperature is about 3–5 °C below the Tm of the primers used. Stable DNA–DNA hydrogen bonds areonly formed when the primer sequence very closely matches the template sequence. Thepolymerase binds to the primer-template hybrid and begins DNA formation. It is very vital todetermine the annealing temperature in PCR. This is because in PCR, efficiency andspecificity are affected by the annealing temperature. An incorrect annealing temperaturewill cause an error in the test.

- *Extension/elongation step*: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to amplify. As a rule-of-thumb, at its optimum temperature, the DNA polymerase polymerizes a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.



Multiplex PCR

Multiplex polymerase chain reaction refers to the use of polymerase chain reaction to amplify several different DNA sequences simultaneously (as if performing many separate PCR reactions all together in one reaction). This process amplifies DNA in samples using multiple primers and

• temperature-mediated DNA polymerase in a thermal cycler. Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple sequences at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. Alternatively, if amplicon sizes overlap, the different amplicons may be differentiated andvisualised using primers that have been dyed with different colour fluorescent dyes.

Nested PCR

Nested polymerase chain reaction is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites

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Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product.

1. The target DNA undergoes the first run of polymerase chain reaction with the first set of primers, shown in green. The selection of alternative and similar primer binding sites gives a selection of products, only one containing the intended sequence.
2. The product from the first reaction undergoes a second run with the second set of primers, shown in red. It is very unlikely that any of the unwanted PCR products containbinding sites for both the new primers, ensuring the product from the second PCR has little contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences.

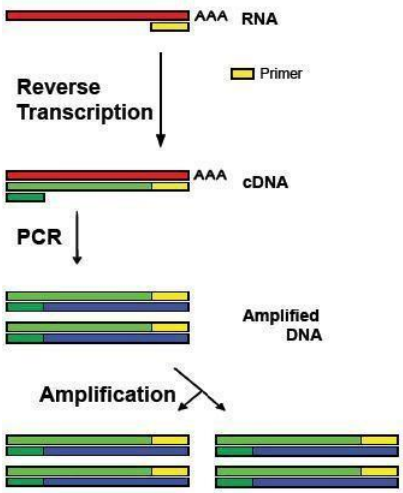
RT-PCR

RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA(cDNA) transcripts from RNA.

Although RT-PCR and the traditional PCR both produce multiple copies of particular DNA isolates through amplification, the applications of the two techniques are fundamentally different. Traditional PCR is used to exponentially amplify target DNA sequences. RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA

complement through the use of reverse transcriptase. Subsequently, the newly synthesized cDNAis amplified using traditional PCR.

In addition to the qualitative study of gene expression, quantitative PCR can be utilized for quantification of RNA, in both relative and absolute terms, by incorporating qPCR into the technique. The combined technique, described as quantitative RT-PCR or real-time RT-PCR is often abbreviated as qRT-PCR, RT-qPCR, or RRT-PCR. Compared to other RNA quantification methods, such as northern blot, qRT-PCR is considered to be the most powerful, sensitive, and quantitative assay for the detection of RNA levels. It is frequently used in the expression analysisof single or multiple genes, and expression patterns for identifying infections and diseases.



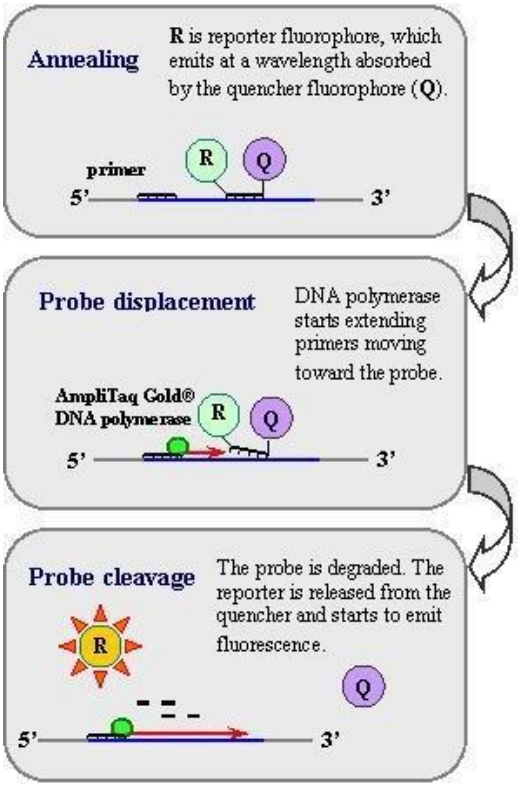
Real time PCR

A real-time polymerase chain reaction is a laboratory technique ofmolecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (Quantitative real-time PCR), semi-quantitatively, i.e. above/below a

certain amount of DNA molecules (Semi quantitative real-time PCR) or qualitatively (Qualitative real-time PCR).

TaqMan assay (named after *Taq* DNA polymerase) **was** one of the earliest methods introduced for real time PCR reaction monitoring and has been widely adopted for both the quantification of mRNAs and for detecting variation. The method exploits the 5' endonuclease activity of *Taq* DNA polymerase to cleave an oligonucleotide probe during PCR, thereby generating a

detectable signal. The probes are fluorescently labeled at their 5' end and are non-extendable at their 3' end by chemical modification. Specificity is conferred at three levels: via two PCR primers and the probe.



A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in

fluorescence intensity measured at each cycle. However, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as Primer dimer). This can potentially interfere with, or prevent, accurate monitoring of the intended target sequence.

In real-time PCR with dsDNA dyes the reaction is prepared as usual, with the addition of fluorescent dsDNA dye. Then the reaction is run in a real-time PCR instrument, and after each cycle, the intensity of fluorescence is measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). This method has the advantage of only needing a pair of primers to carry out the amplification, which keeps costs down; however, only one target sequence can be monitored in a tube.

DNA fingerprinting

In the 1980's, the use of hypervariable regions of DNA called minisatellites was introduced. The molecular techniques associated with these regions, typically referred to as "DNA fingerprinting," have been a useful tool for pedigree analysis and in estimating the genetic variation in populations.

The chemical structure of everyone's DNA is the same. The only difference between people (or any animal) is the order of the base pairs. There are so many millions of base pairs in each person's DNA that every person has a different sequence.Using these sequences, every person could be identified solely by the sequence of their base pairs. However, because there are so many millions of base pairs, the task would be very time-consuming. Instead, scientists are able to use a shorter method, because of repeating patterns in DNA. These patterns do not, however, give an individual "fingerprint," but they are able to determine whether two

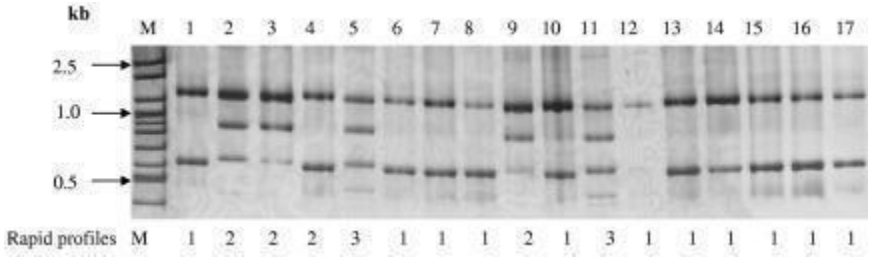
DNA samples are from the same person, related people, or non-related people. Scientists use a small number of sequences of DNA that are known to vary among individuals a great deal, and analyze those to get a certain probability of a match.

Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence.

Unlike traditional PCR analysis, RAPD (pronounced "rapid") does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or willnot amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced,resulting in a different pattern of amplified DNA segments on the gel.

Example



RAPD is an inexpensive yet powerful typing method for many bacterial species.

Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual strains.

Limitations of RAPD

- Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.

- PCR is an enzymatic reaction, therefore the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible.
- Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret.

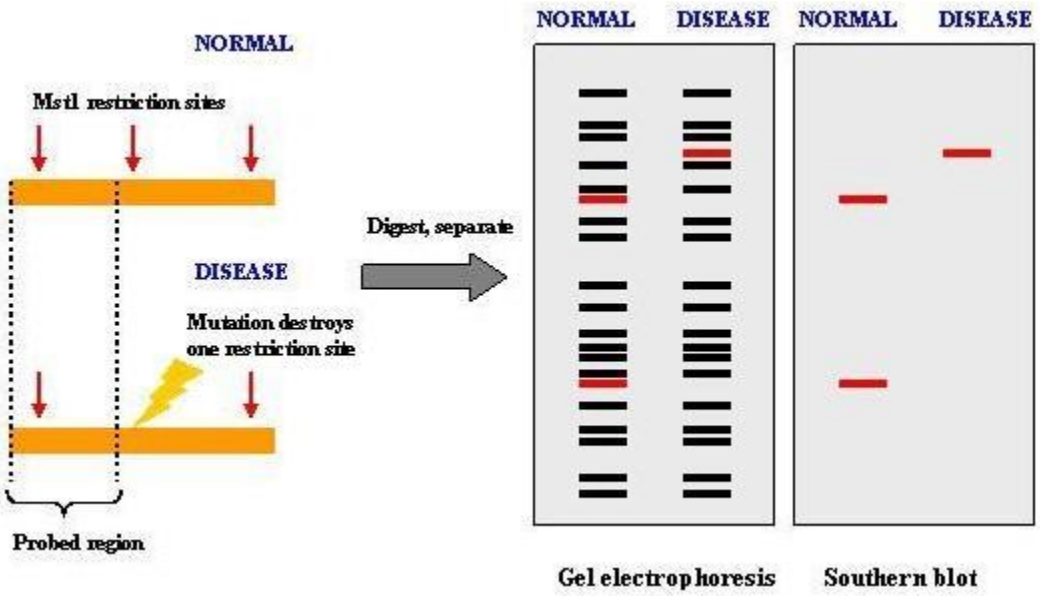
Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination.

Most RFLP markers are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific.

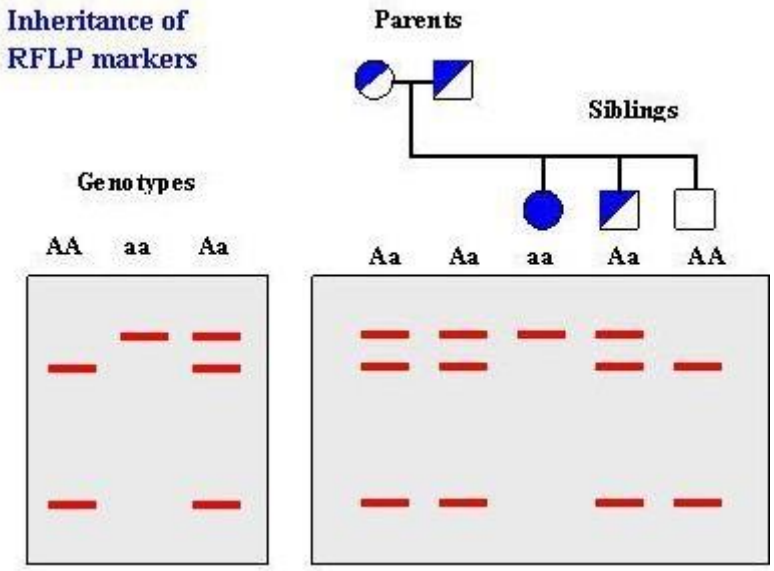
An RFLP probe is a labeled DNA sequence that hybridizes with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Short, single- or low- copy genomic DNA or cDNA clones are typically used as RFLP probes.

The RFLP probes are frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, hereditary disease diagnostics, etc.).



SNPs or INDELs can create or abolish restriction endonuclease (RE) recognition sites, thus affecting quantities and length of DNA fragments resulting from RE digestion.

Genotyping



Developing RFLP probes

- Total DNA is digested with a methylation-sensitive enzyme (for example, PstI), thereby enriching the library for single- or low-copy expressed sequences (PstI clones are based on the suggestion that expressed genes are not methylated).
- The digested DNA is size-fractionated on a preparative agarose gel, and fragments

ranging from 500 to 2000 bp are excised, eluted and cloned into a plasmid vector (for example, pUC18).

- Digests of the plasmids are screened to check for inserts.
- Southern blots of the inserts can be probed with total sheared DNA to select clones thathybridize to single- and low-copy sequences.
- The probes are screened for RFLPs using genomic DNA of different genotypes digestedwith restriction endonucleases. Typically, in species with moderate to high polymorphism rates, two to four restriction endonucleases are used such as EcoRI, EcoRV, and HindIII. In species with low polymorphism rates, additional restriction endonucleases can be tested to increase the chance of finding polymorphism.

Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphisms (AFLPs) are differences in restriction fragment lengths caused by SNPs or INDELsthat create or abolish restriction endonuclease recognition sites.

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA.

After final amplification, selectively amplified fragments are separated by gel electrophoresis and visualized autoradiographically. MseI-MseI fragments are excluded from the autorad because only EcoRI-directed primers are normally labeled. Typically, the autorad has 100-300 fingerprints with sizes ranging from 80 to 500 nucleotides. Only a subset (10-40) of these total bands is polymorphic between two related individuals, such as Arabidopsis thaliana Columbia and Landsberg erecta ecotypes.

Using 3-bp selective primer extensions gives 128 possible linker combinations. Therefore, 128 subsets of genomic DNA can be readily amplified. Thus, thousands of markers can be generated quite rapidly.

Weaknesses of AFLP

- Proprietary technology is needed to score heterozygotes and ++ homozygotes. Otherwise,AFLP must be dominantly scored.

- Developing locus-specific markers from individual fragments can be difficult.
- Need to use different kits adapted to the size of the genome being analyzed.

Sequence-Tagged Sites (STS)

Sequence-Tagged Site (STS)is a relatively short, easily PCR-amplified sequence (200 to 500 bp) which can be specifically amplified by PCR and detected in the presence ofall other genomic sequences and whose location in the genome is mapped.

The STS concept was introduced by Olson et al (1989). In assessing the likely impact of the Polymerase Chain Reaction (PCR) on human genome research, they recognized that single-copy DNA sequences of known map location could serve as markers for genetic and physical mapping of genes along the chromosome. The advantage of STSs over other mapping landmarks is that the means of testing for the presence of a particular STS can be completely described as information in a database: anyone who wishes to make copies of the marker would simply look up the STS in the database, synthesize the specified primers, and run the PCR under specified conditions to amplify the STS from genomic DNA.

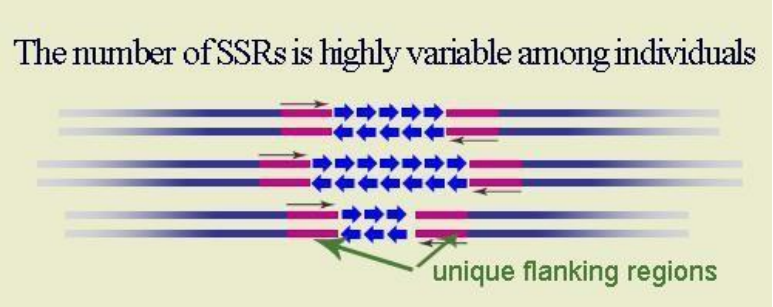
STS-based PCR produces a simple and reproducible pattern on agarose or polyacrylamide gel. In most cases STS markers are co-dominant, i.e., allow heterorozygotes to be distinguished from the two homozygotes.

The DNA sequence of an STS may contain repetitive elements, sequences that appear elsewhere in the genome, but as long as the sequences at both ends of the site are unique and conserved, researches can uniquely identify this portion of genome using tools usually present in any laboratory. Thus, in broad sense, STS include such markers as microsatellites (SSRs, STMS or SSRPs), SCARs, CAPs, and ISSRs.

Microsatellites

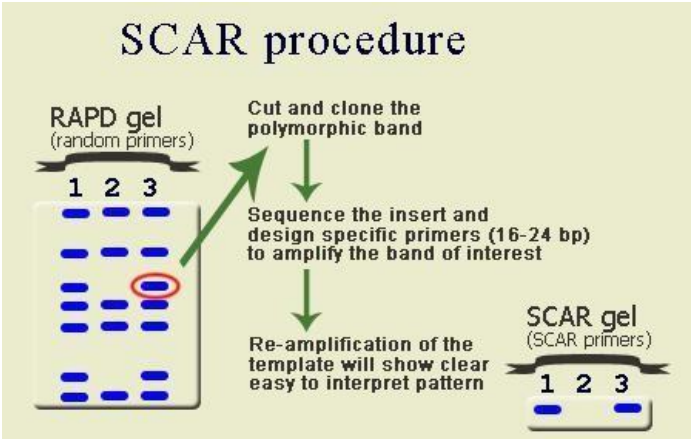
Polymorphic loci present in nuclear DNA and organellar DNA that consist of repeating units of 1-10 base pairs, most typically, 2-3 bp in length, also called Simple Sequence Repeats (SSR), Sequence-Tagged Microsatellite Sites (STMS) or Simple Sequence

Repeats Polymorphisms (SSRP). SSRs are highly variable and evenly distributed throughout the genome. This type of repeated DNA is common in eukaryotes. These polymorphisms are identified by constructing PCR primers for the DNA flanking the microsatellite region. The flanking regions tend to be conserved within the species, although sometimes they may also be conserved in higher taxonomic levels.



Sequence Characterized Amplified Region (SCAR)

DNA fragments amplified by the Polymerase Chain Reaction (PCR) using specific 15-30 bp primers, designed from nucleotide sequences established in cloned RAPD (Random Amplified Polymorphic DNA) fragments linked to a trait of interest. By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs. Obtaining a co-dominant marker may be an additional advantage of converting RAPDs into SCARs.

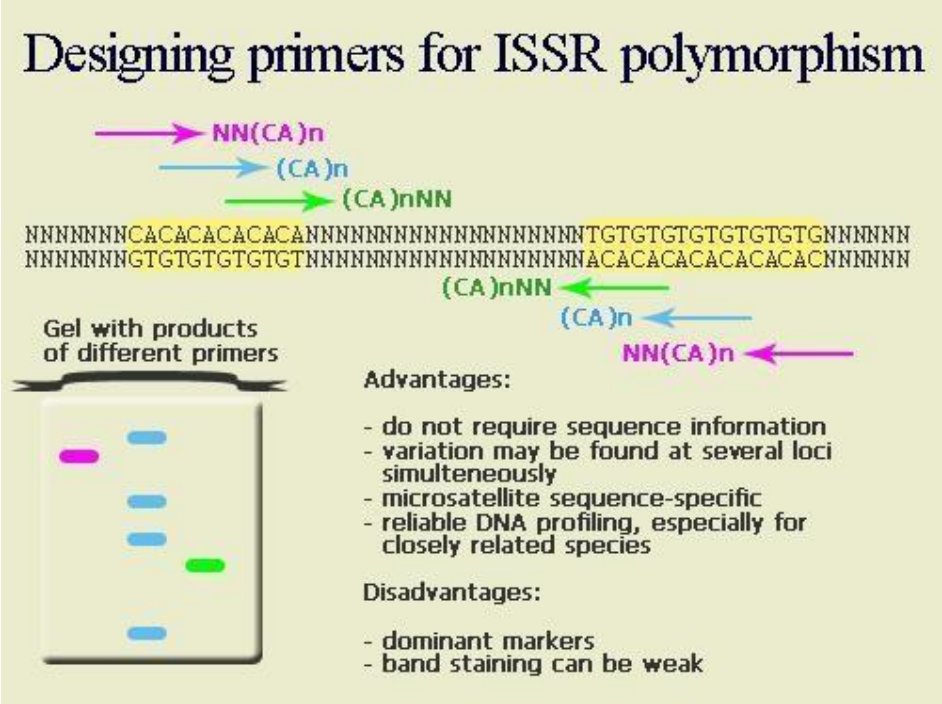


Cleaved Amplified Polymorphic Sequences (CAPS)

STS polymorphisms that can be detected by differences in restriction fragment lengths caused by SNPs or INDELs that create or abolish restriction endonuclease recognition sites in PCR amplicons produced by locus-specific oligonucleotide primers. In other words this technique aims to convert and amplified band that does not show variation by length of PCR product into a polymorphic one. More about CAPS in Overview of CAPS technology.

Inter-simple Sequence Repeats (ISSRs)

STS polymorphisms that are found between microsatellite repeats. Primers can be designed based on a microsatellite repeats exclusively, in which case this technique will target multiple loci due to known abundance of repeat sequences in the genome. Alternatively, primers can be extended outside or inside the ISSR in which case a unique region most likely will be amplified.



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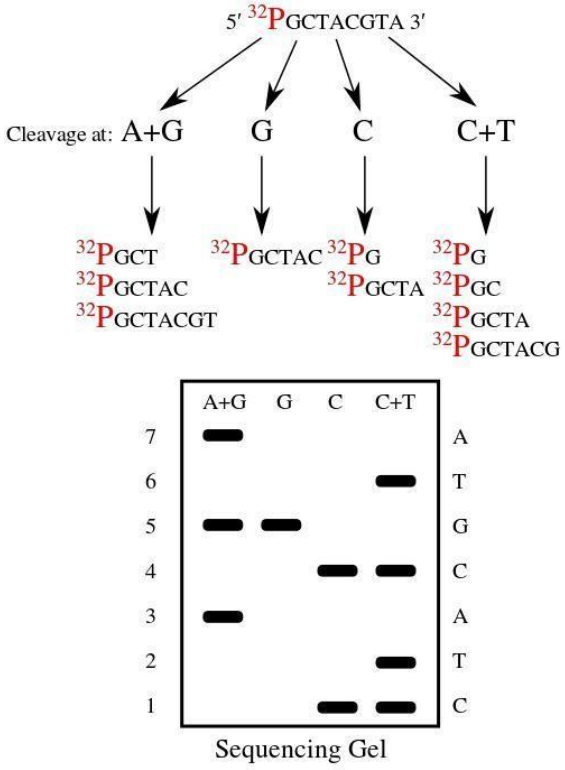
DEPARTMENT OF BIOTECHNOLOGY

UNIT – III – Advanced Molecular Biology and Genetic Engineering – SBTA5201

DNA sequencing is the process of determining the precise order ofnucleotides within a DNA molecule.Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as medical diagnosis,biotechnology, forensic biology, virology and biological systematics.

Maxam–Gilbert sequencing is a method of DNA sequencing developed by Allan Maxam and Walter 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



Maxam–Gilbert sequencing requires radioactive labeling at one 5′ end of the DNA fragment to be sequenced (typically by akinase reaction using gamma-^{32}P ATP) and purification of the DNA. Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, C+T). For example, the purines(A+G) are depurinated using formic acid, the guanines (and to some extent the adenines) are methylated by dimethyl sulfate, and the pyrimidines (C+T) are hydrolysed using hydrazine. The addition of salt (sodium chloride) to the hydrazine reaction inhibits the reaction of thymine for the C-only reaction. The modified DNAs may then be cleaved by hot piperidine;(CH2)5NH at the position of the modified base. The concentration of the modifying chemicals is controlled to introduce on average one modification per DNA molecule. Thus a series of labeled fragments is generated, from the radiolabeled end to the first "cut" site in each molecule.

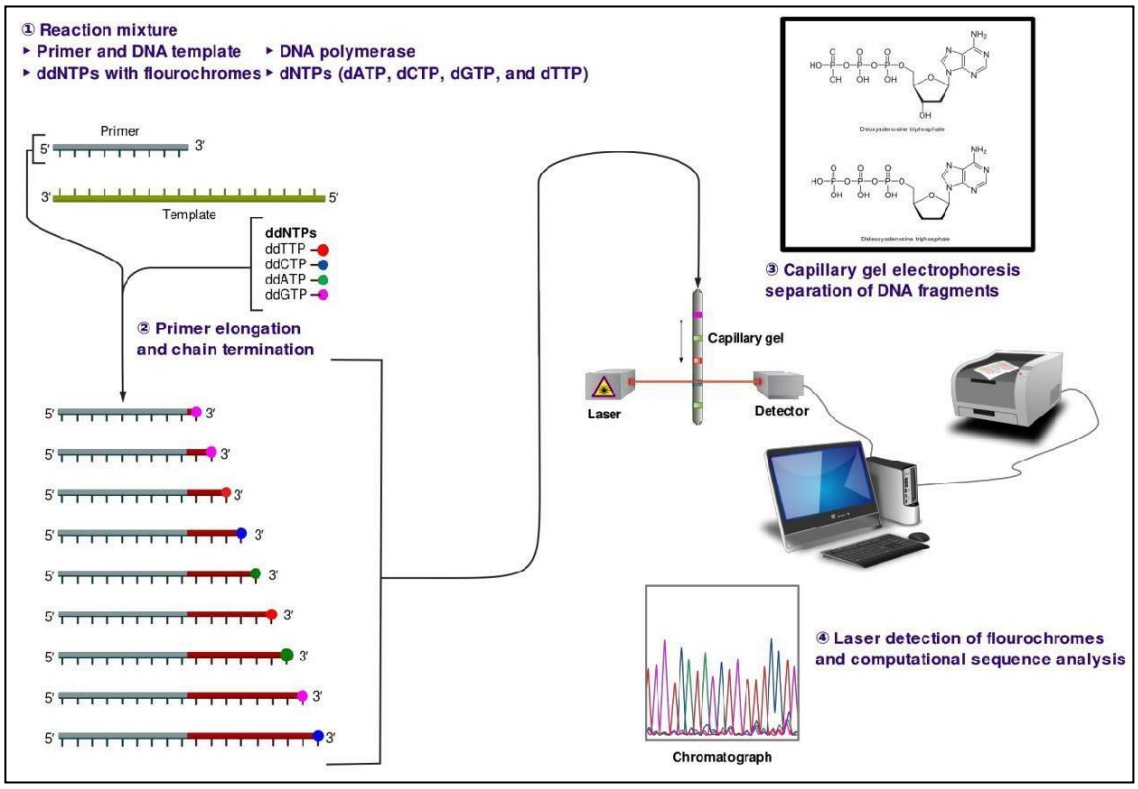
The fragments in the four reactions are electrophoresed side by side in denaturing acrylamide gels for size separation. To visualize the fragments, the gel is exposed to X-ray film for autoradiography, yielding a series of dark bands each showing the location of identical radiolabeled DNA molecules. From presence and absence of certain fragments the sequence maybe inferred.

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminatingdideoxynucleotides by DNA polymerase during in vitro DNA replication.

Developed by Frederick Sanger and colleagues in 1977, it was the most widely used sequencingmethod for approximately 25 years. More recently, Sanger sequencing has been supplanted by "Next-Gen" sequencing methods, especially for large-scale, automated genome analyses.However, the Sanger method remains in wide use, for smaller-scale projects, validation of Next- Gen results and for obtaining especially long contiguous DNA sequence reads (>500 nucleotides).

The classical chain-termination method requires a single-stranded DNA template, a DNA primer,a DNA polymerase, normal deoxynucleosidetriphosphates (dNTPs), and modified di- deoxynucleosidetriphosphates (ddNTPs), the latter of which terminate DNA strand elongation. These chain-terminating nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labeled for detection in automated sequencing machines.

The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides(dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the fourdideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP), while the other added nucleotides are ordinary ones. Following rounds of template DNA extension from the bound primer, the resulting DNA fragments are heat denatured and separated by size using gel electrophoresis. In the original publication of 1977,[2] the formation of base-paired loops of ssDNA was a cause of serious difficulty in resolving bands at some locations. This is frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C). The DNA bands may then be visualized by autoradiography or UV light and the DNA sequence can be directly read off the X- ray film or gel image.

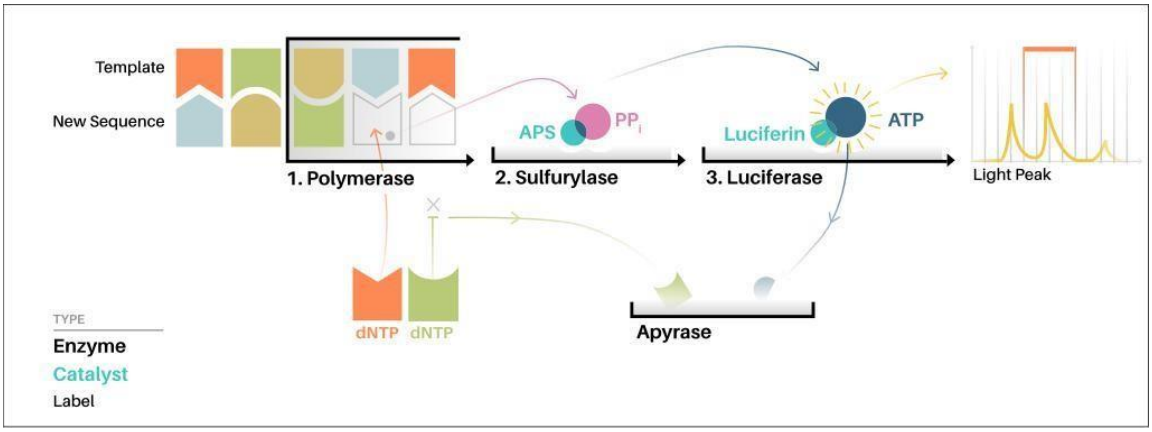


Pyrosequencing is a method of DNA sequencing (determining the order ofnucleotides in DNA) based on the "sequencing by synthesis" principle.

"Sequencing by synthesis" involves taking a single strand of the DNA to be sequenced and then synthesizing its complementary strand enzymatically. The pyrosequencing method is based on detecting the activity of DNA polymerase (a DNA synthesizing enzyme) with another chemoluminescent enzyme. Essentially, the method allows sequencing of a single strand of DNA by synthesizing the complementary strand along it, one base pair at a time, and detecting which base was actually added at each step. The template DNA is immobile, and solutions of A, C, G, andT nucleotides are sequentially added and removed from the reaction. Light is produced only when the nucleotide solution complements the first unpaired base of the template. The sequence of solutions which produce chemiluminescent signals allows the determination of the sequence of the template.

The single-strand DNA (ssDNA) template is hybridized to a sequencing primer and incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase, and with the substrates adenosine 5´ phosphosulfate (APS) andluciferin.

- The addition of one of the four deoxynucleoside triphosphates (dNTPs) (dATPαS, which is not a substrate for a luciferase, is added instead of dATP to avoid noise) initiates the secondstep. DNA polymerase incorporates the correct, complementary dNTPs onto the template. This incorporation releases pyrophosphate (PPi).
- ATP sulfurylase converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP acts as a substrate for the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a camera and analyzed in a pyrogram.
- Unincorporated nucleotides and ATP are degraded by the apyrase, and the reaction canrestart with another nucleotide.

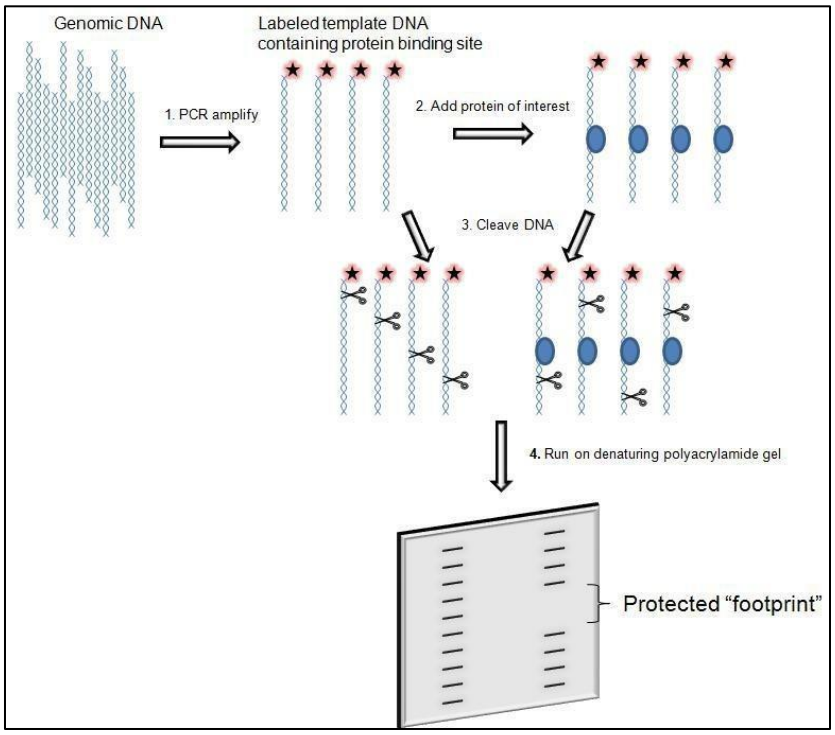


DNA footprinting

DNA footprinting is a method of investigating the sequence specificity of DNA-binding proteins in vitro. This technique can be used to study protein-DNA interactions both outside and within cells.

The regulation of transcription has been studied extensively, and yet there is still much that is notknown. Transcription factors and associated proteins that bind promoters,

enhancers, or silencers to drive or repress transcription are fundamental to understanding the unique regulation of individual genes within the genome. Techniques like DNA footprinting help elucidate which proteins bind to these associated regions of DNA and unravel the complexities oftranscriptional control.



In 1978, David Galas and Albert Schmitz developed the DNA footprinting technique to study thebinding specificity of the lac repressor protein. It was originally a modification of the Maxam- Gilbert chemical sequencing technique.

The simplest application of this technique is to assess whether a given protein binds to a regionof interest within a DNA molecule. Polymerase chain reaction (PCR) amplify and label region ofinterest that contains a potential protein-binding site, ideally amplicon is between 50 to 200 base pairs in length. Add protein of interest to a portion of the labeled template DNA; a portion shouldremain separate without protein, for later comparison. Add a cleavage agent to both portions of DNA template. The cleavage agent is a chemical or enzyme that will cut at random locations in asequence independent manner. The reaction should occur just long enough to cut each DNA molecule in only one location. A protein

that specifically binds a region within the DNA templatewill protect the DNA it is bound to from the cleavage agent. Run both samples side by side ona polyacrylamide gel electrophoresis. The portion of DNA template without protein will be cut at random locations, and thus when it is run on a gel, will produce a ladder-like distribution. The DNA template with the protein will result in ladder distribution with a break in it, the "footprint", where the DNA has been protected from the cleavage agent. Note: Maxam-Gilbert chemical DNA sequencing can be run alongside the samples on the polyacrylamide gel to allow the prediction of the exact location of ligand binding site.

A variety of cleavage agents can be chosen.

DNase I is a large protein that functions as a double-strand endonuclease. It binds the minor groove of DNA and cleaves the phosphodiester backbone. It is a good cleavage agent for footprinting because its size makes it easily physically hindered. Thus is more likely to have its action blocked by a bound protein on a DNA sequence. In addition, the DNase I enzyme is easilycontrolled by adding EDTA to stop the reaction. There are however some limitations in using DNase I. The enzyme does not cut DNA randomly; its activity is affected by local DNA structure and sequence and therefore results in an uneven ladder. This can limit the precision of predictinga protein's binding site on the DNA molecule.

Hydroxyl radicals are created from the Fenton reaction, which involves reducing Fe^{2+} with $H2O2$ to form free hydroxyl molecules. These hydroxyl molecules react with the DNA backbone, resulting in a break. Due to their small size, the resulting DNA footprint has high resolution. Unlike DNase I they have no sequence dependence and result in a much more evenly distributed ladder. The negative aspect of using hydroxyl radicals is that they are more time consuming to use, due to a slower reaction and digestion time.

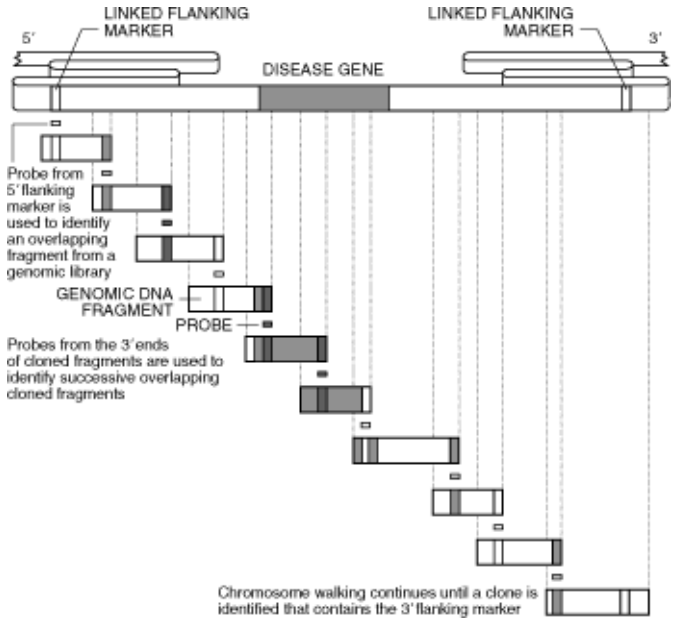
Ultraviolet irradiation can be used to excite nucleic acids and create photoreactions, which results in damaged bases in the DNA strand. Photoreactions can include: single strand breaks, interactions between or within DNA strands, reactions with solvents, or crosslinks with proteins. The workflow for this method has an additional step, once both your protected and unprotected DNA have been treated, there is subsequent primer extension of the cleaved products. The extension will terminate upon reaching a damaged

base, and thus when the PCR products are runside-by-side on a gel; the protected sample will show an additional band where the DNA was crosslinked with a bound protein. Advantages of using UV are that it reacts very quickly and can therefore capture interactions that are only momentary. Additionally it can be applied to *in vivo* experiments, because UV can penetrate cell membranes

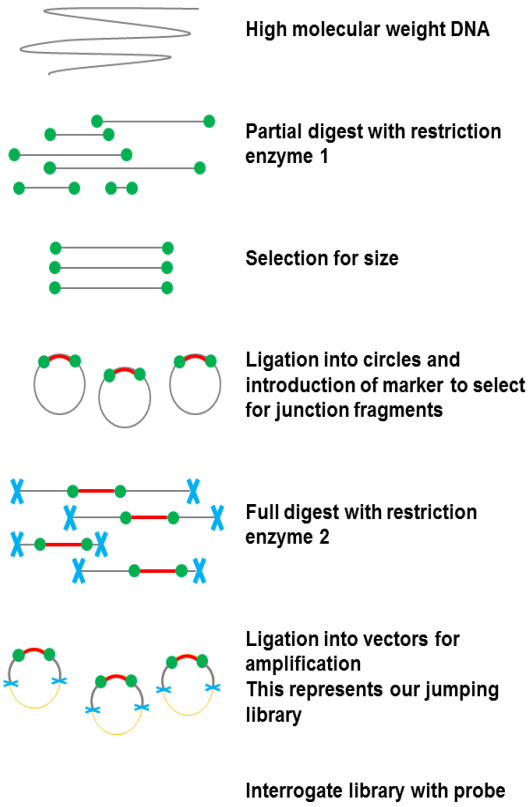
Chromosome walking

Chromosome walking is a method of positional cloning used to find, isolate, and clone a particular allele in a gene library. An allele is a gene for a particular genetic trait passed on from adults to their offspring, such as the allele for brown eyes in a gene for eye color. Sometimes, theapproximate location of a single allele in a string of deoxyribonucleic acid (DNA) may be known. To isolate a particular allele for a genetically transmitted disease, chromosome walking may need to explore for the desired specimen in an unmapped DNA sequence outside ofpreviously mapped sequences.

To locate a particular disease gene, the walking starts at the closest gene that has already been identified, known as a marker gene. Each successive gene in the sequence is tested repeatedly forwhat are known as overlap restrictions and mapped for their precise location in the sequence. Eventually, walking through the genes reaches the mutant gene in an unmapped sequence that binds to a fragment of a gene of that particular disease. Once the gene is cloned, its function can be fully identified. Throughout this process, tests are done to fully identify the properties of each


LINKED FLANKING MARKER
5′
LINKED FLANKING MARKER
3′
DISEASE GENE
Probe from 5′ flanking marker is used to identify an overlapping fragment from a genomic library
GENOMIC DNA FRAGMENT
PROBE
Probes from the 3′ ends of cloned fragments are used to identify successive overlapping cloned fragments
Chromosome walking continues until a clone is identified that contains the 3′ flanking marker

successive clone, to map their locations for future use.



Chromosome jumping

Chromosome jumping is a tool of molecular biology that is used in the physical mapping of genomes. It is related to several other tools used for the same purpose, including chromosome walking.

Chromosome jumping is used to bypass regions difficult to clone, such as those containing repetitive DNA, that cannot be easily mapped by chromosome walking, and is useful in moving along a chromosome rapidly in search of a particular gene.

In chromosome jumping, the DNA of interest is identified, cut into fragments with restriction enzymes, and circularised (the beginning and end of each fragment are joined together to form a circular loop). From a known sequence, a primer is designed to sequence across the circularised junction. This primer is used to jump 100 kb-300 kb intervals: a sequence 100 kb away would have come near the known sequence on circularisation. Thus,

sequences not reachable by chromosome walking can be sequenced. Chromosome walking can be used from the new jump position (in either direction) to look for gene-like sequences, or additional jumps can be used to progress further along the chromosome.

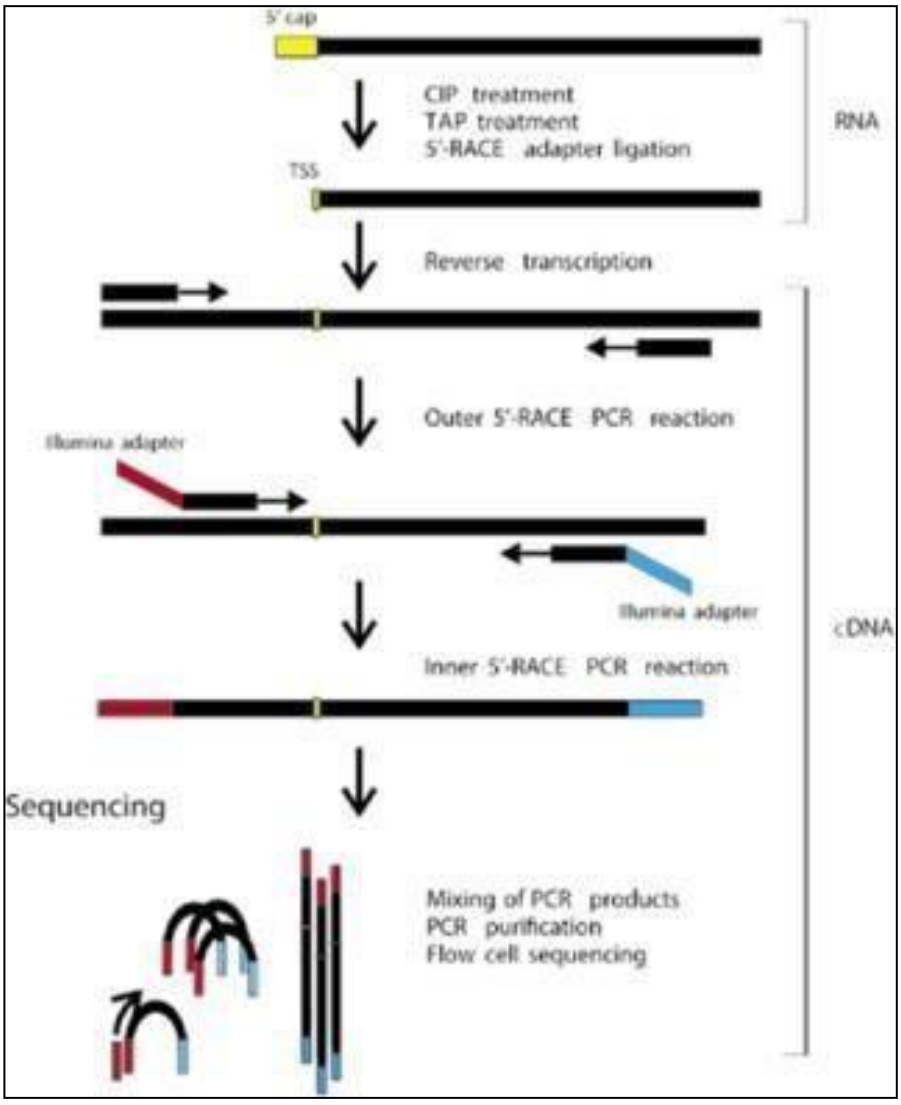
Rapid amplification of cDNA ends (**RACE**) is a technique used to obtain the full length sequence of an RNA transcript found within a cell. RACE results in the production of a cDNA copy of the RNA sequence of interest, produced through reverse transcription, followed by PCR amplification of the cDNA copies (see RT-PCR). The amplified cDNA copies are then sequenced and, if long enough, should map to a unique genomic region. RACE can provide the sequence of an RNA transcript from a small known sequence within the transcript to the 5' end (5' RACE-PCR) or 3' end (3' RACE-PCR) of the RNA. This technique is sometimes called *one-sided PCR* or *anchored PCR*.

The first step in RACE is to use reverse transcription to produce a cDNA copy of a region of the RNA transcript. In this process, an unknown end portion of a transcript is copied using a known sequence from the center of the transcript. The copied region is bounded by the known sequence,at either the 5' or 3' end.

The protocols for 5' or 3' RACES differ slightly. 5' RACE-PCR begins using mRNA as a template for a first round of cDNA synthesis (or reverse transcription) reaction using an *anti- sense* (reverse) oligonucleotide primer that recognizes a known sequence in the middle of the gene of interest; the primer is called a *gene specific primer* (GSP). The primer binds to the mRNA, and the enzyme reverse transcriptase adds base pairs to the 3' end of the primer to generate a specific single-stranded cDNA product; this is the reverse complement of the mRNA. Following cDNA synthesis, the enzyme terminal deoxynucleotidyl transferase (TdT) is used to add a string of identical nucleotides, known as a homopolymeric tail, to the 3' end of the cDNA. (There are some other ways to add the 3'-terminal sequence for the first strand of the de novo cDNA synthesis which are much more efficient than homopolymeric tailing, but the sense of the method remains the same). APCR reaction is then carried out, which uses a second anti-sense gene specific primer (GSP2) that binds to the known sequence, and a sense (forward) universal primer (UP) that binds the homopolymeric tail added to the 3' ends of the cDNAs to amplify a cDNA product

from the 5' end.

3' RACE-PCR uses the natural polyA tail that exists at the 3' end of all eukaryotic mRNAs for priming during reverse transcription, so this method does not require the addition of nucleotides by TdT. cDNAs are generated using an Oligo-dT-adaptor primer (a primer with a short sequence of deoxy-thymine nucleotides) that complements the polyA stretch and adds a special adaptor sequence to the 5' end of each cDNA. PCR is then used to amplify 3' cDNA from a known regionusing a sense GSP, and an anti-sense primer complementary to the adaptor sequence.



Gene silencing is a general term used to describe the regulation of gene expression. In particular, this term refers to the ability of a cell to prevent the expression of a certain gene. Gene silencing can occur during either transcription ortranslation and is often used in research. In particular, methods used to silence genes are being increasingly used to produce therapeutics to combat cancer and diseases, such as infectious diseases and neurodegenerative disorders.

Gene silencing is often considered the same as gene knockout. When genes are silenced, their expression is reduced. In contrast, when genes are knocked out, they are completely erased from the organism's genome and, thus, have no expression. Gene silencing is considered a gene knockdown mechanism since the methods used to silence genes, such as RNAi, CRISPR, or siRNA, generally reduce the expression of a gene by at least 70% but do not completely eliminate it. Methods using gene silencing are often considered better than gene knockouts since they allow researchers to study essential genes that are required for the animal models to survive and cannot be removed. In addition, they provide a more complete view on the development of diseases since diseases are generally associated with genes that have a reduced expression.

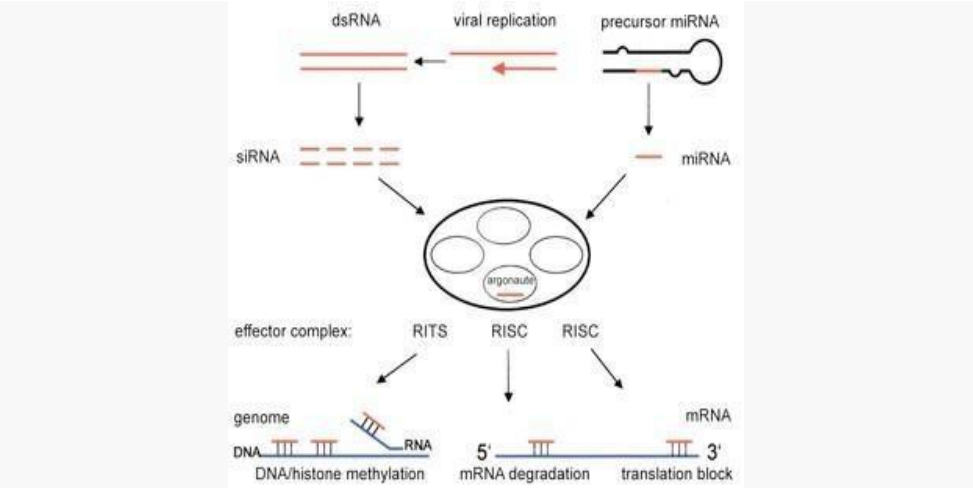
Antisense oligonucleotides

Antisense oligonucleotides were discovered in 1978 by Paul Zamecnik and Mary Stephenson. Oligonucleotides, which are short nucleic acid fragments, bind to complementary target mRNA molecules when added to the cell. These molecules can be composed of single- stranded DNA or RNA and are generally 13-25 nucleotides long. The antisense oligonucleotides can affect gene expression in two ways: by using an RNase H-dependent mechanism or by using a steric blocking mechanism. RNase H-dependent oligonucleotides cause the target mRNA molecules to be degraded, while steric-blocker oligonucleotides prevent translationof the mRNA molecule. The majority of antisense drugs function through the RNase H- dependent mechanism, in which RNase H hydrolyzes the RNA strand of the DNA/RNAheteroduplex. This mechanism is thought to be more efficient, resulting in an approximately 80% to 95% decrease in the protein and mRNA expression.

Ribozymes are catalytic RNA molecules used to inhibit gene expression. These molecules work by cleaving mRNA molecules, essentially silencing the genes that produced them. Sidney Altman and Thomas Cech first discovered catalytic RNA molecules, RNase P and group II intronribozymes, in 1989 and won the Nobel Prize for their discovery. Several types of ribozyme motifs exist, includinghammerhead, hairpin, hepatitis delta virus, group I, group II, and RNase Pribozymes. Hammerhead, hairpin, and hepatitis delta virus (HDV) ribozyme motifs are generally found in viruses or viroid RNAs. These motifs are able to self-cleave a specific phosphodiester bond on an mRNA molecule. Lower eukaryotes and a few bacteria contain groupI and group II ribozymes. These motifs can self-splice by cleaving and joining together phosphodiester bonds. The last ribozyme motif, the RNase P ribozyme, is found in *Escherichia coli* and is known for its ability to cleave the phosphodiester bonds of several tRNA precursors when joined to a protein cofactor.

The general catalytic mechanism used by ribozymes is similar to the mechanism used by protein ribonucleases. These catalytic RNA molecules bind to a specific site and attack the neighboring phosphate in the RNA backbone with their 2' oxygen, which acts as a nucleophile, resulting in the formation of cleaved products with a 2'3'-cyclic phosphate and a 5' hydroxyl terminal end. This catalytic mechanism has been increasingly used by scientists to perform sequence-specific cleavage of target mRNA molecules. In addition, attempts are being made to use ribozymes to produce gene silencing therapeutics, which would silence genes that areresponsible for causing diseases.

RNA interference



RNA interference (RNAi) is a natural process used by cells to regulate gene expression. It was discovered in 1998 by Andrew Fire and Craig Mello, who won the Nobel Prize for their discovery in 2006. The process to silence genes first begins with the entrance of a double- stranded RNA (dsRNA) molecule into the cell, which triggers the RNAi pathway The double- stranded molecule is then cut into small double-stranded fragments by an enzyme called Dicer. These small fragments, which includesmall interfering RNAs (siRNA) and microRNA (miRNA),are approximately 21-23 nucleotides in length. The fragments integrate into a multi-subunit protein called the RNA-induced silencing complex, which contains Argonaute proteins that are essential components of the RNAi pathway. One strand of the molecule, called the "guide" strand, binds to RISC, while the other strand, known as the "passenger" strand is degraded. The guide or antisense strand of the fragment that remains bound to RISC directs the sequence- specific silencing of the target mRNA molecule. The genes can be silenced by siRNA molecules that cause the endonucleatic cleavage of the target mRNA molecules or by miRNA molecules that suppress translation of the mRNA molecule. With the cleavage or translational repression of the mRNA molecules, the genes that form them are essentially inactive. RNAi is thought to have evolved as a cellular defense mechanism against invaders, such as RNA viruses, or to combat the proliferation of transposons within a cell's DNA. Both RNA viruses and transposons can exist as double-stranded RNA and lead to the activation of RNAi. Currently, siRNAs are being widely used to suppress specific gene expression and to assess the function of genes

Construction of siRNA vectors

There are several methods for preparing siRNA, such as chemical synthesis, in vitro transcription, siRNA expression vectors, and PCR expression cassettes. Irrespective of which method one uses, the first step in designing a siRNA is to choose the siRNA target site.

General Design Guidelines

If you prefer to design your own siRNAs, you can choose siRNA target sites in a variety of different organisms based on the following guidelines. Corresponding siRNAs can then be chemically synthesized, created by in vitro transcription, or expressed from a vector or

PCR product.

Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. that siRNAs with 3'overhanging UU dinucleotides are the most effective. This is also compatible with using RNA pol III to transcribe hairpin siRNAs because RNA pol III terminates transcription at 4-6 nucleotide poly(T) tracts creating RNA molecules with a short poly(U) tail.

In Elbashir's and subsequent publications, siRNAs with other 3' terminal dinucleotide overhangs have been shown to effectively induce RNAi. If desired, you may modify this target siteselection strategy to design siRNAs with other dinucleotide overhangs, but it is recommended that you avoid G residues in the overhang because of the potential for the siRNA to be cleavedby RNase at single-stranded G residues.

1. Select 2-4 target sequences.

Research has found that typically more than half of randomly designed siRNAs provide at leasta 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide a 75-95% reduction. Choose target sites from among the sequences identified in Step 1 based on the following guidelines:

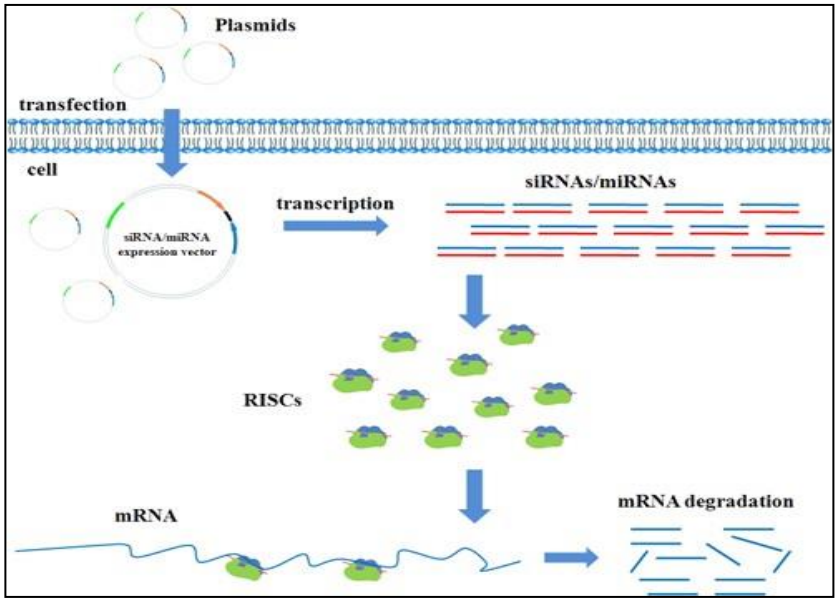
- Researchers find that siRNAs with 30-50% GC content are more active than those with ahigher G/C content.
- Since a 4-6 nucleotide poly(T) tract acts as a termination signal for RNA pol III, avoid stretches of > 4 T's or A's in the target sequence when designing sequences to be expressed from an RNA pol III promoter.
- Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.
- Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more

than 16-17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST,which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST.

2. Design appropriate controls.

A complete siRNA experiment should include a number of controls to ensure the validity of the data. The editors of Nature Cell Biology have recommended several controls (2). Two of these controls are:

- **A negative control siRNA with the same nucleotide composition as your siRNA but which lacks significant sequence homology to the genome.** To design a negative control siRNA, scramble the nucleotide sequence of the gene-specific siRNA and conduct a search to make sure it lacks homology to any other gene.
- **Additional siRNA sequences targeting the same mRNA.** Perhaps the best way to ensure confidence in RNAi data is to perform experiments, using a single siRNA at a time, with two or more different siRNAs targeting the same gene. Prior to these experiments, each siRNAshould be tested to ensure that it reduces target gene expression by comparable levels.



Site-directed mutagenesis

Site-directed mutagenesis is one of the most important techniques in laboratory for introducing mutation into a DNA sequence. However, with decreasing costs of oligonucleotide synthesis, artificial gene synthesis is now occasionally used as an alternative to site-directed mutagenesis.

The basic procedure requires the synthesis of a short DNA primer. This synthetic primer containsthe desired mutation and is complementary to the template DNA around the mutation site so it can hybridize with the DNA in the gene of interest. The mutation may be a single base change(a point mutation), multiple base changes, deletion, or insertion. The single-strand primer is then extended using a DNA polymerase, which copies the rest of the gene. The gene thus copied contains the mutated site, and is then introduced into a host cell as a vector and cloned. Finally, mutants are selected by DNA sequencing to check that they contain the desired mutation.

The original method using single-primer extension was inefficient due to a low yield of mutants. This resulting mixture contains both the original unmutated template as well as the mutant strand, producing a mixed population of mutant and non-mutant progenies. Furthermore the template used is methylated while the mutant strand is unmethylated, and the mutants may be counter-selected due to presence of mismatch repair system that favors the methylated template DNA, resulting in fewer mutants. Many approaches have since been developed to improve the efficiency of mutagenesis.

A large number of methods are available to effect site-directed mutagenesis, although most of them are now rarely used in laboratories since the early 2000s, as newer techniques allow for simpler and easier ways of introducing site-specific mutation into genes.

Kunkel's method

In 1987, Thomas Kunkel introduced a technique that reduces the need to select for the mutants.The DNA fragment to be mutated is inserted into a phagemid such as M13mp18/19 and is then transformed into an *E. coli* strain deficient in two enzymes, dUTPase (*dut*) and uracil

deglycosidase (*ung*). Both enzymes are part of a DNA repair pathway that protects the

bacterial chromosome from mutations by the spontaneous deamination of dCTP to dUTP. The dUTPase deficiency prevents the breakdown of dUTP, resulting in a high level of dUTP in the cell. The uracil deglycosidase deficiency prevents the removal of uracil from newly synthesized DNA. As the double-mutant *E. coli* replicates the phage DNA, its enzymatic machinery may, therefore, misincorporate dUTP instead of dTTP, resulting in single-strand DNA that contains some uracils(ssUDNA). The ssUDNA is extracted from the bacteriophage that is released into the medium, and then used as template for mutagenesis. An oligonucleotide containing the desired mutation is used for primer extension. The heteroduplex DNA, that forms, consists of one parental non- mutated strand containing dUTP and a mutated strand containing dTTP. The DNA is then transformed into an E. coli strain carrying the wildtype *dut* and *ung* genes. Here, the uracil- containing parental DNA strand is degraded, so that nearly all of the resulting DNA consists of the mutated strand.

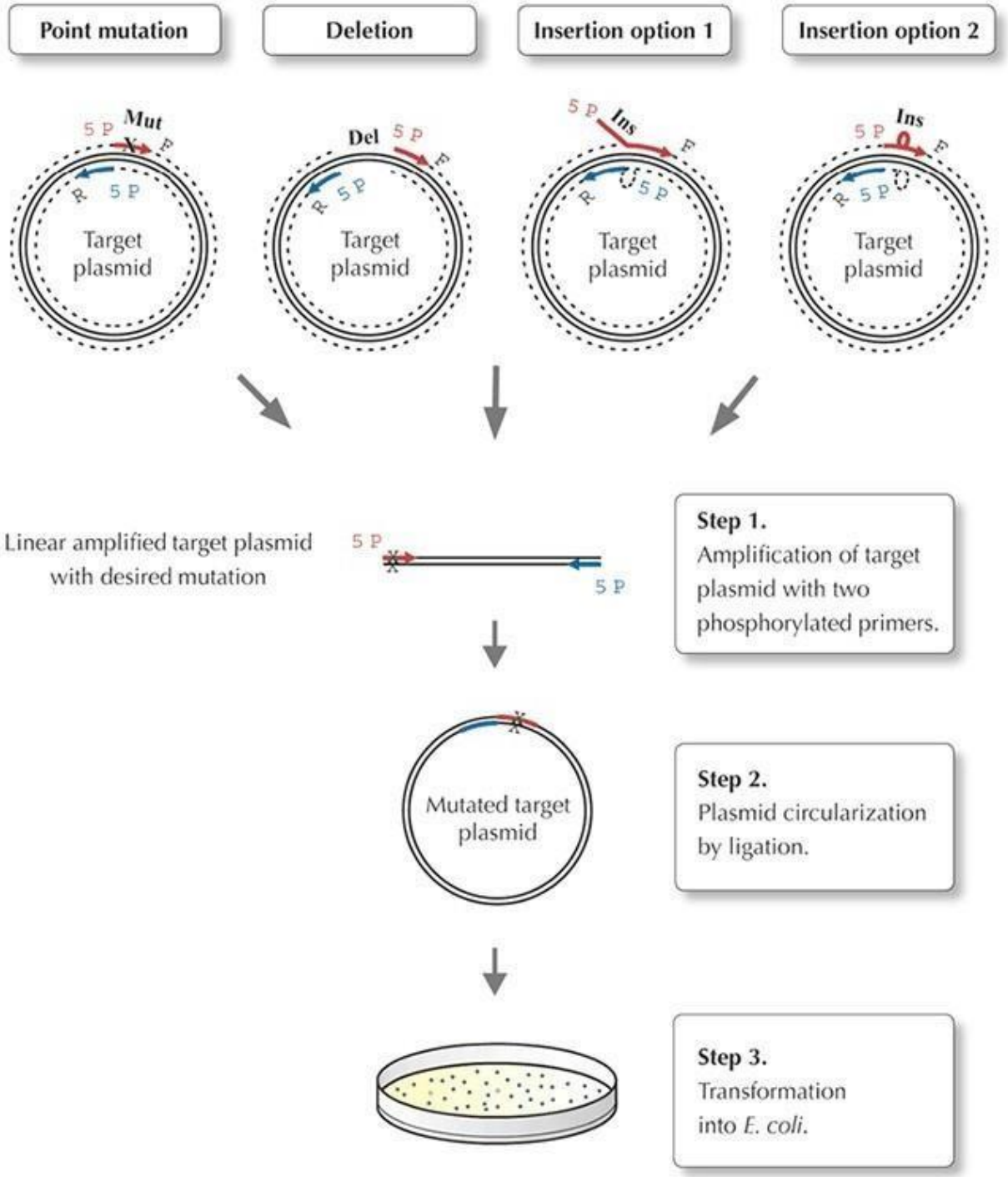
Cassette mutagenesis

Unlike other methods, cassette mutagenesis need not involve primer extension using DNA polymerase. In this method, a fragment of DNA is synthesized, and then inserted into a plasmid. It involves the cleavage by a restriction enzyme at a site in the plasmid and subsequent ligation of a pair of complementary oligonucleotides containing the mutation in the gene of interest to the plasmid. Usually, the restriction enzymes that cut at the plasmid and the oligonucleotide are the same, permitting sticky ends of the plasmid and insert to ligate to one another. This method can generate mutants at close to 100% efficiency, but is limited by the availability of suitable restriction sites flanking the site that is to be mutated.

PCR site-directed mutagenesis

The limitation of restriction sites in cassette mutagenesis may be overcome using polymerase chain reaction witholigonucleotide "primers", such that a larger fragment may be generated, covering two convenient restriction sites. The exponential amplification in PCR produces a fragment containing the desired mutation in sufficient quantity to be separated from the original, unmutated plasmid by gel electrophoresis, which may then be inserted in the original context using standard recombinant molecular biology techniques. There are many variations of the sametechnique. The simplest method places the mutation site toward one of the ends of the fragment

whereby one of two oligonucleotides used for generating the fragment contains the mutation. This involves a single step of PCR, but still has the inherent problem of requiring a suitable restriction site near the mutation site unless a very long primer is used. Other variations, therefore, employ three or four oligonucleotides, two of which may be non-mutagenic oligonucleotides that cover two convenient restriction sites and generate a fragment that can be digested and ligated into a plasmid, whereas the mutagenic oligonucleotide may be complementary to a location within that fragment well away from any convenient restriction site.These methods require multiple steps of PCR so that the final fragment to be ligated can contain the desired mutation.


Point mutation
Deletion
Insertion option 1
Insertion option 2
Mut
Del
Ins
Ins
5 P
F
R
Target plasmid
Linear amplified target plasmid with desired mutation
Step 1.
Amplification of target plasmid with two phosphorylated primers.
Mutated target plasmid
Step 2.
Plasmid circularization by ligation.
Step 3.
Transformation into E. coli.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – IV – Advanced Molecular Biology and Genetic Engineering – SBTA5201

Genetically modified crops (**GMCs**, **GM crops**, or **biotech crops**) are plants used in agriculture, the DNA of which has been modified using genetic engineering techniques. In most cases, the aim is to introduce a new trait to the plant which does not occur naturally in the species. Examples in food crops include resistance to certain pests, diseases, or environmental conditions, reduction of spoilage, or resistance to chemical treatments (e.g. resistance to a herbicide), or improving the nutrient profile of the crop. Examples in non- food crops include production of pharmaceutical agents,biofuels, and other industrially useful goods, as well as for bioremediation.

A number of techniques exist for the production of GM plants. The two most commonly employedare the bacterium *Agrobacterium tumefaciens*, which is naturally able to transfer DNA to plants, and the 'gene gun', which shoots microscopic particles coated with DNA into the plant cell. Generally, individual plant cells are targeted and these are regenerated into whole GM plants usingtissue culture techniques. Three aspects of this procedure have raised debate with regard to human health.

- The use of selectable markers to identify transformed cells
- Transfer of extraneous DNA into the plant genome (i.e. genes other than those being studied)
- The possibility of increased mutations in GM plants compared to non-GM counterparts due to tissue culture processes used in their production and the rearrangement of DNA around the insertion site of foreign genes.

To facilitate the transformation process, a selectable marker gene conferring, for example, resistance to an antibiotic (e.g. kanamycin, which will kill a normal non-GM plant cell), is often co-transferred with the gene of interest to allow discrimination of GM tissue and regeneration of GM plants. Critics of the technology have stated that there is a risk of the spread of antibiotic resistance to the bacterial population either in the soil or in the human gut after ingestion of GM food. However, these antibiotic resistance genes were initially isolated from bacteria and are already widespread in the bacterial population. In addition, kanamycin itself has GRAS status (Generally Regarded As Safe) and has been used for over 13 years without any known problems. Studies have concluded that the probability of transmission of antibiotic resistance from plants to bacteria is extremely low and that the hazard occurring from any such transfer is, at worst, slight. Nevertheless, other selection

strategies that do not rely on antibiotic resistance have been developed, and procedures to eliminate the selectable marker from the plant genome once its purpose has been fulfilled have also been designed.

The second aspect of the plant transformation procedure that has been criticized is that unnecessary DNA is transferred into the plant genome as a consequence of the engineering and transfer process. Of course, there is no reason that DNA *per se* should be harmful, as it is consumed by humans in all foods, but again plant technologists have responded to the criticism bydesigning 'minimal cassettes' in which only the gene of interest is transferred into the plant.

Finally, it has been claimed that GM plants carry more mutations than their untransformed counterparts as a result of the production method. Genome-wide mutations may be produced by the tissue culture process, generating so called somaclonal variation, and endogenous DNA rearrangements may occur around the integrated transgene. Theoretically, this may mean that plants may be produced with, for example, reduced levels of nutrients or increased levels of allergens or toxins (although the alternative must also hold true, that positive traits may be expressed).

Transgenic plants have genes inserted into them that are derived from another species. The inserted genes can come from species within the same kingdom (plant to plant) or between kingdoms (for example, bacteria to plant). In many cases the inserted DNA has to be modified slightly in order to correctly and efficiently expressin the host organism. Transgenic plants are used to express proteins like the cry toxins from *B. thuringiensis*, herbicide resistant genes, antibodies and antigensfor vaccinations. A study led by the European Food Safety Authority (EFSA) found also viral genes in transgenic plants.

Transgenic carrots have been used to produce the drug Taliglucerase alfa which is used to treat Gaucher's disease. In the laboratory, transgenic plants have been modified to increase photosynthesis (currently about 2% at most plants versus the theoretic potential of 9–10%).[48] This is possible by changing the rubisco enzyme (i.e. changing C3 plants into C4 plants[49]), by placing the rubisco in acarboxysome, by adding CO_2 pumps in the cell wall, by changing the leaf form/size. Plants have been engineered to exhibit bioluminescence that

may become a sustainable alternative to electric lighting.

Cisgenic

Cisgenic plants are made using genes found within the same species or a closely related one, where conventional plant breeding can occur. Some breeders and scientists argue that cisgenic modification is useful for plants that are difficult tocrossbreed by conventional means (such as potatoes), and that plants in the cisgenic category should not require the same regulatory scrutiny as transgenics.

Subgenic

In 2014, Chinese researcher Gao Caixia filed patents on the creation of a strain of wheat that is resistant to powdery mildew. The strain lacks genes that encode proteins that repress defenses against the mildew. The researchers deleted all three copies of the genes from wheat's hexaploid genome. The strain promises to reduce or eliminate the heavy use offungicides to control the disease. Gao used the TALENs and CRISPR gene editing tools without adding or changing any other genes. No field trials were immediately planned.

GENETICALLY MODIFIED CROPS

The first genetically modified crop approved for sale in the U.S. was the *FlavrSavr* tomato, which had a longer shelf life. It is no longer on the market.In November 2014, the USDA approved a GM potato that prevents bruising.

In February 2015 Arctic Apples were approved by the USDA,] becoming the first genetically modified apple approved for US sale. Gene silencing was used to reduce the expression of polyphenol oxidase (PPO), thus preventing enzymatic browning of the fruit after it has been slicedopen. The trait was added to Granny Smith and Golden Delicious varieties.[86][88]The trait includes a bacterial antibiotic resistance gene that provides resistance to the antibiotic kanamycin. The genetic engineering involved cultivation in the presence of kanamycin, which allowed only resistant cultivars to survive. Humans consuming apples do not acquire kanamycin resistance. The FDA approved the apples in March 2015.

Nutrition

Edible oils

Some GM soybeans offer improved oil profiles for processing or healthier eating. Camelina sativa has been modified to produce plants that accumulate high levels of oils similar to fish oils.

Vitamin enrichment

Golden rice, developed by the International Rice Research Institute (IRRI), provides greater amounts of Vitamin A targeted at reducing Vitamin A deficiency.

Researchers vitamin-enriched corn derived from South African white corn variety M37W, producing a 169-fold increase in Vitamin A, 6-fold increase in Vitamin C and doubled concentrations of folate. Modified Cavendish bananas express 10-fold the amount of Vitamin A asunmodified varieties.

Toxin reduction

A genetically modified cassava under development offerslower cyanogen glucosides and enhanced protein and other nutrients (called BioCassava).

In November 2014, the USDA approved a potato, developed by J.R. Simplot Company, that prevents bruising and produces less acrylamide when fried. The modifications prevent natural, harmful proteins from being made via RNA interference. They do not employ genes from non- potato species. The trait was added to the Russet Burbank, Ranger Russet and Atlantic varieties.

Stress resistance

Plants engineered to tolerate non-biological stressors such as droughtfrost,[102][103] high soil salinityand nitrogen starvationwere in development. In 2011, Monsanto's Drought Gard maize became the first drought-resistant GM crop to receive US marketing approval.

Herbicides

Glyphosate

As of 1999 the most prevalent GM trait was glyphosate-resistance. Glyphosate, (the active ingredient in Roundup and other herbicide products) kills plants by interfering with the shikimate pathway in plants, which is essential for the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. The shikimate pathway is not present in animals, which instead obtain aromatic amino acids from their diet. More specifically, glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3- phosphate synthase (EPSPS).

This trait was developed because the herbicides used on grain and grass crops at the time were highly toxic and not effective against narrow-leaved weeds. Thus, developing crops that could withstand spraying with glyphosate would both reduce environmental and health risks, and gives an agricultural edge to the farmer

Some micro-organisms have a version of EPSPS that is resistant to glyphosate inhibition. One of these was isolated from an *Agrobacterium* strain CP4 (CP4 EPSPS) that was resistant to glyphosate. The CP4 EPSPS gene was engineered for plant expression by fusing the 5' end ofthe gene to a chloroplast transit peptide derived from the petunia EPSPS. This transit peptide was used because it had shown previously an ability to deliver bacterial EPSPS to the chloroplasts of other plants. This CP4 EPSPS gene was cloned and transfected into soybeans.

The plasmid used to move the gene into soybeans was PV-GMGTO4. It contained three bacterial genes, two CP4 EPSPS genes, and a gene encoding beta- glucuronidase (GUS) from *Escherichia coli* as a marker. The DNA was injected into the soybeans using the particle acceleration method. Soybean cultivar A54O3 was used for the transformation.

Pest resistance

Insects

Tobacco, corn, rice and many other crops have been engineered to express genes encoding for insecticidal proteins from Bacillus thuringiensis (Bt). Papaya, potatoes, and squash have been engineered to resist viral pathogens such ascucumber mosaic virus which, despite its name, infects a wide variety of plants. The introduction of Bt crops during the period between

1996 and 2005 has been estimated to have reduced the total volume of insecticide active ingredient use in the United States by over 100 thousand tons. This represents a 19.4% reduction in insecticide use.

In the late 1990s, a genetically modified potato that was resistant to the Colorado potato beetle was withdrawn because major buyers rejected it, fearing consumer opposition.

Viruses

Virus resistant papaya were developed In response to a papaya ringspot virus (PRV) outbreak in Hawaii in the late 1990s. . They incorporate PRV DNA. By 2010, 80% of Hawaiian papaya plants were genetically modified.

Potatoes were engineered for resistance to potato leaf roll virus and Potato virus Y in 1998. Poor sales led to their market withdrawal after three years.

Yellow squash that were resistant to at first two, then three viruses were developed, beginning in the 1990s. The viruses are watermelon, cucumber and zucchini/courgette yellow mosaic. Squash was the second GM crop to be approved by US regulators. The trait was later added to zucchini.

Many strains of corn have been developed in recent years to combat the spread of Maize dwarf mosaic virus, a costly virus that causes stunted growth which is carried in Johnson grass andspread by aphid insect vectors. These strands are commercially available although the resistance isnot standard among GM corn variants.

By-products

Drugs

In 2012, the FDA approved the first plant-produced pharmaceutical, a treatment forGaucher's Disease. Tobacco plants have been modified to produce therapeutic antibodies

Biofuel

Algae is under development for use in biofuels. Modified jatropha offers improved qualities for fuel. Syngenta has USDA approval to market a maize trademarked Enogen that has been genetically modified to convert its starch to sugar for ethanol. In 2013, the Flemish Institute

for Biotechnology was investigating poplar trees genetically engineered to contain less lignin to ease conversion into ethanol. Lignin is the critical limiting factor when using wood to make bio-ethanol because lignin limits the accessibility of cellulose microfibrils to depolymerization by enzymes.

Bioremediation

Scientists at the University of York developed a weed (*Arabidopsis thaliana*) that contains genesfrom bacteria that can clean TNT and RDX-explosive soil contaminants. 16 million hectares in the USA (1.5% of the total surface) are estimated to be contaminated with TNT and RDX. However *A. thaliana* was not tough enough for use on military test grounds.

Genetically modified plants have been used for bioremediation of contaminated soils. Mercury, selenium and organic pollutants such as polychlorinated biphenyls (PCBs).

Marine environments are especially vulnerable since pollution such as oil spills are not containable. In addition to anthropogenic pollution, millions of tons of petroleum annually enter the marine environment from natural seepages. Despite its toxicity, a considerable fraction of petroleum oil entering marine systems is eliminated by the hydrocarbon- degrading activities of microbial communities. Particularly successful is a recently discovered group of specialists, the so- called hydrocarbonoclastic bacteria (HCCB) that may offer useful genes.

Resistance

Bt resistance

Constant exposure to a toxin creates evolutionary pressure for pests resistant to that toxin. Overreliance on glyphosate and a reduction in the diversity of weed management practices allowed the spread of glyphosate resistance in 14 weed species/biotypes in the US

One method of reducing resistance is the creation of refuges to allow nonresistant organisms to survive and maintain a susceptible population.

To reduce resistance to Bt crops, the 1996 commercialization of transgenic cotton and maize came with a management strategy to prevent insects from becoming resistant. Insect resistance management plans are mandatory for Bt crops. The aim is to encourage a large population of pestsso that any (recessive) resistance genes are diluted within the population.

Resistance lowers evolutionary fitness in the absence of the stressor (Bt). In refuges, non-resistant strains outcompete resistant ones.

With sufficiently high levels of transgene expression, nearly all of the heterozygotes (S/s), i.e., the largest segment of the pest population carrying a resistance allele, will be killed before maturation,thus preventing transmission of the resistance gene to their progeny.[167] Refuges (i. e., fields of nontransgenic plants) adjacent to transgenic fields increases the likelihood that homozygous resistant (s/s) individuals and any surviving heterozygotes will mate with susceptible (S/S) individuals from the refuge, instead of with other individuals carrying the resistance allele. As a result, the resistance gene frequency in the population remains lower.

Complicating factors can affect the success of the high-dose/refuge strategy. For example, if the temperature is not ideal, thermal stress can lower Bt toxin production and leave the plant more susceptible. More importantly, reduced late-season expression has been documented, possibly resulting from DNA methylation of the promoter. The success of the high-dose/refuge strategy has successfully maintained the value of Bt crops. This success has depended on factors independent of management strategy, including low initial resistance allele frequencies, fitness costs associated with resistance, and the abundance of non-Bt host plants outside the refuges.

Companies that produce Bt seed are introducing strains with multiple Bt proteins. Monsanto did this with Bt cotton in India, where the product was rapidly adopted. Monsanto has also; in an attempt to simplify the process of implementing refuges in fields to comply with Insect Resistance Management(IRM) policies and prevent irresponsible planting practices; begun marketing seed bags with a set proportion of refuge (non-transgenic) seeds mixed in with the Bt seeds being sold. Coined "Refuge- In-a-Bag" (RIB), this practice is intended to increase farmer compliance with refuge requirements and reduce additional labor needed at planting from having separate Bt and refuge seed bags on hand. This strategy is likely to reduce the likelihood of Bt- resistance occurring for corn rootworm, but may increase the risk of resistance for lepidopteran corn pests, such as European corn borer. Increased concerns for resistance with seed mixtures include partially resistant larvae on a Bt plant being able to move to a susceptible plant to survive or cross pollination of refuge pollen on to Bt

plants that can lower the amount of Bt expressed in kernels for ear feeding insects.

Herbicide resistance

Best management practices (BMPs) to control weeds may help delay resistance. BMPs include applying multiple herbicides with different modes of action, rotating crops, planting weed-free seed, scouting fields routinely, cleaning equipment to reduce the transmission of weeds to other fields, and maintaining field borders. The most widely- planted GMOs are designed to tolerate herbicides. By 2006 some weed populations had evolved to tolerate some of the same herbicides.Palmer amaranth is a weed that competes with cotton. A native of the southwestern US,it traveled east and was first found resistant to glyphosate in 2006, less than 10 years after GM cotton was introduced.

Plant protection

Farmers generally use less insecticide when they plant Bt-resistant crops. Insecticide use on corn farms declined from 0.21 pound per planted acre in 1995 to 0.02 pound in 2010. This is consistent with the decline in European corn borerpopulations as a direct result of Bt corn and cotton. The establishment of minimum refuge requirements helped delay the evolution of Bt resistance. However resistance appears to be developing to some Bt traits in some areas.

Transgenic animals

Genetically modified mammals are an important category of genetically modified organisms. Ralph L. Brinster and Richard Palmiter developed the techniques responsible for transgenic mice, rats, rabbits, sheep, and pigs in the early 1980s, and established many of the first transgenic models of human disease, including the first carcinoma caused by a transgene. The process of genetically engineering animals is a slow, tedious, and expensive process. However, new technologies are making genetic modifications easier and more precise.

The first transgenic (genetically modified) animal was produced by injecting DNA into mouseembryos then implanting the embryos in female mice.

Genetically modified animals currently being developed can be placed into six different broad classes based on the intended purpose of the genetic modification:

1. to research human diseases (for example, to develop animal models for these diseases);
2. to produce industrial or consumer products (fibres for multiple uses);
3. to produce products intended for human therapeutic use (pharmaceutical products ortissue for implantation);
4. to enrich or enhance the animals' interactions with humans (hypo-allergenic pets);
5. to enhance production or food quality traits (faster growing fish, pigs that digest foodmore efficiently);
6. to improve animal health (disease resistance)

Research use

Transgenic animals are used as experimental models to perform phenotypic and fortesting in biomedical research.

Genetically modified (genetically engineered) animals are becoming more vital to the discovery and development of cures and treatments for many serious diseases. By altering the DNA or transferring DNA to an animal, we can develop certain proteins that may be used in medical treatment. Stable expressions of human proteins have been developed in many animals, including sheep, pigs, and rats. Human-alpha-1- antitrypsin, which has been tested in sheep and is used in treating humans with this deficiency and transgenic pigs with human-histo-compatibility have been studied in the hopes that the organs will be suitable for transplant with less chances of rejection.

Producing human therapeutics

Within the field known as pharming, intensive research has been conducted to develop transgenic animals that produce biotherapeutics. On 6 February 2009, the U.S. Food and Drug Administration approved the first human biological drug produced from such an animal, a goat. The drug, ATryn, is an anticoagulant which reduces the probability of blood clots during surgery or childbirth. It is extracted from the goat's milk

Production or food quality traits

In 2006, a pig was engineered to produce omega-3 fatty acids through the expression of a roundworm gene.

Enviropig was a genetically enhanced line of Yorkshire pigs in Canada created with the capability of digesting plant phosphorus more efficiently than conventional Yorkshire pigs. The project ended in 2012. These pigs produced the enzymephytase, which breaks down the indigestible phosphorus, in their saliva. The enzyme was introduced into the pig chromosome by pronuclear microinjection. With this enzyme, the animal is able to digest cereal grain phosphorus. The use of these pigs would reduce the potential of water pollution since they excrete from 30 to 70.7% less phosphorus in manure depending upon the age and diet.[85][87] The lower concentrations of phosphorus in surface runoff reduces algal growth, because phosphorus is the limiting nutrient for algae. Because algae consume large amounts of oxygen, it can result in dead zones for fish.

In 2011, Chinese scientists generated dairy cows genetically engineered with genes from human beings to produce milk that would be the same as human breast milk.] This could potentially benefit mothers who cannot produce breast milk but want their children to have breast milk rather than formula. Aside from milk production, the researchers claim these transgenic cows to be identical to regular cows. Two months later scientists from Argentina presented Rosita, a transgeniccow incorporating two human genes, to produce milk with similar properties as human

breast milk. In 2012, researchers from New Zealand also developed a genetically engineered cowthat produced allergy-free milk.

Goats have been genetically engineered to produce milk with strong spiderweb-like silk proteins intheir milk.

Human gene therapy

Gene therapy, uses genetically modified viruses to deliver genes that can cure disease in humans. Although gene therapy is still relatively new, it has had some successes. It has been used to treat genetic disorders such as severe combined immunodeficiency, and Leber's congenital amaurosis. Treatments are also being developed for a range of other currently incurable diseases, such as cystic fibrosis, sickle cell anemia, Parkinson's disease, cancer diabetes, heart disease and muscular dystrophy.

Fish

Genetically modified fish are used for scientific research and as pets, and are being considered for use as food and as aquatic pollution sensors.

GM fish are widely used in basic research in genetics and development. Two species of fish, zebrafish and medaka, are most commonly modified because they have optically clear chorions (membranes in the egg), rapidly develop, and the 1-cell embryo is easy to see and microinject withtransgenic DNA.

The GloFish is a patented brand of genetically modified (GM) fluorescent zebrafish with bright red, green, and orange fluorescent color. Although not originally developed for the ornamental fishtrade, it became the first genetically modified animal to become publicly available as a pet when itwas introduced for sale in 2003. They were quickly banned for sale in California.

GM fish have been developed with promoters driving an over-production of "all fish" growth hormone for use in theaquaculture industry to increase the speed of development and potentially reduce fishing pressure on wild stocks. This has resulted in dramatic growth

enhancement in several species, including salmon, trout[] and tilapia. AquaBounty Technologies, a biotechnology company working on bringing a GM salmon to market, claims that their GM AquAdvantage salmon can mature in half the time as wild salmon. AquaBounty applied for regulatory approval tomarket their GM salmon in the US, and was approved in November 2015. On 25 November 2013 Canada approved commercial scale production and export of GM Salmon eggs but they are not approved for human consumption in Canada.

Several academic groups have been developing GM zebrafish to detect aquatic pollution. The lab that originated the GloFish discussed above originally developed them to change color in the presence of pollutants, to be used as environmental sensors. A lab at University of Cincinnati has been developing GM zebrafish for the same purpose, as has a lab at Tulane University.

Recent research on pain in fish has resulted in concerns being raised that genetic-modifications induced for scientific research may have detrimental effects on the welfare of fish.

Production of Vaccine Antigens in Plants

The aim of vaccination is to prevent infectious diseases. It can be considered as one of the most successful breakthrough of this century in the medical field. The principle of vaccination involves mimicking an infection in such a way that the specific natural defence mechanism of the host against the pathogen gets activated but the host remains free of the disease that normally results from such infection. Vaccination is also referred to as 'active immunization', since host immune system is 'activated' to respond to infection through humoral and cellular responses, resulting in acquired immunity against the particular pathogen. Further, most of the human pathogens that cause respiratory and intestinal diseases initiate infection at mucosal surfaces, where they encounter the mucosal immune system (MIS). Mucosal response is achieved more efficiently by oral instead of parenteral delivery of the antigens. Immunity *via* mucosal tissues can be induced bydelivering the antigens with live, replicating agents, such as attenuated or mutant strains of recombinant bacteria or viruses. Many of these attempts have had limited success and the use of live vaccines raises obvious safety issues. Most soluble, non-viable antigens are subject to

degradation in the gastrointestinal tract due to acidic and proteolytic environment of the gut. Thus, expressing the antigen in plant cell can be considered as a means of bioencapsulating protein for oral delivery. Plant cell wall consisting of cellulose, proteins and pectin will serve as barrier to enzymatic degradation, and cell membrane and internal organelle membranes provide further protection.

Escherichia coli Labile Toxin

Escherichia coli labile toxin (LT), which is responsible for causing diarrhoea, is composed of a 27 kDa A subunit and five 11.6 kDa B subunits that pentamerise. The B pentamer (LT-B) has been used as a vaccine component, as antibodies against this would block toxin activity. LT-B wasexpressed in transgenic potato. However, the maximum expression level achieved represented 0.01% of total soluble protein. Mice fed with such transgenic tuber developed an oral immune response. Further, a synthetic gene was created for LT-B that contained plant -preferred codons. As a result, the expression level increased to 0.19% of the total soluble protein. In a clinical trial, transgenic tubers expressing LT-B, when fed to human volunteers, showed the development of serum and mucosal immune response to LT-B. In another study, the recombinant LT-B, produced in potato tubers, was found immunogenic and, on oral administration, elicited a systemic and local IgA response in parenterally primed but not in naïve animals. A synthetic gene encoding, a variant of LT-B, was also expressed in transgenic corn and oral administration of transgenic corn elicited serum and mucosal immune responses in mice.

Development of Tolerance to Edible Antigens

'Oral tolerance' is generally characterised by the fact that an animal, fed an antigen at a certain level, becomes refractory or has diminished capacity to develop an immune response, which it normally develops when exposed to the same antigen by a systemic route. This could have a negative consequence, as it would lead to increased likelihood of disease rather than prevention. However, the concept of 'oral tolerance' has been used to advantage in suppressing insulitis in NOD (non-obese diabetic) mice. Insulin- dependent diabetes mellitus is an autoimmune disease in humans. This involves lymphocytic infiltration of Islets of Langerhans, which is followed by destruction of insulin producing β cells. Several antigens,

including insulin have been predicted as potential targets. Transgenic potato plants, synthesizing human insulin and its CTB conjugate, suppressed diabetes in NOD mice. This orally administered form of insulin did not have any metabolic effect on blood glucose as it got degraded but it might facilitate orally induced tolerance by creating smaller protein fragments that were better able to interact with GALT (gut associated lymphoid tissue). T cells that adoptively transfer suppression of experimental autoimmune diabetes following oral administration of antigen were triggered in an antigen-specific manner. These cells mediated their effect by release of the cytokine, transforming growth factor β (TGFβ), an antigen non-specific suppressor, in close proximity to effector cells that in turn down regulated the local inflammatory processes in pancreas.

Antibody Production in Plants (Plantibody)

Hiatt *et al* were the first to demonstrat the production of antibodies in plants. Since then, a number of groups have expressed plantibodies, either to modify plant performance, such as pest resistance, or with a view to exploiting plants as bioreactors for the large scale production of antibodies for the following reasons:

1. Plants can assemble heavy and light chains into complete antibodies.
2. Plants permit appropriate post-translational modification for the production of antibodies.
3. Several groups have expressed complete antibody by targeting the antibody *via* endoplasmic reticulum to apoplast, the extracellular aqueous region in which hydrolytic degradation is minimal and antibodies secreted into it can accumulate in a relatively stable environment. The extraction of antibody is also simpler and can be achieved by conditions milder than those required for proteins located elsewhere.
4. Genetically stable seed stocks of antibody-producing plants can be produced and stored indefinitely at low cost; the seed stock can be converted into a harvest of large quantity of antibody within one growing season.

Production of a fully functional antibody in plants is not a very straightforward task because of the multi-subunit structure of antibody molecule. Moreover, expression of a complete antibody may not be required for many applications. For example, production of antigen binding domain as present in single chain Fv or Fab molecules may be enough to

block binding of a pathogen or a virulence factor secreted by the pathogen and thus may limit the spread of the infection. However, a monovalent antibody fragment may have reduced affinity for binding to antigens as compared to a bivalent F(ab)2 fragment or a complete antibody. Further, divalent nature may be required for aggregation of cells or bacteria in some cases. Production of these fragments without complete constant region might be sufficient for the conditions where attenuation of the function of the antigen is required. Different antibody fragments have been expressed in plants for blocking actions of phytochrome A , abscisic acid as well as for sequestration of organic pollutants. However, antigen-antibody binding alone is not enough in some cases and secondary effector functions, attributed to constant region, are required. These functions include activation of complement and binding to phagocytes. Other features, which reside in constant region, are sites for glycosylation, placental transfer, association with J chain and secretary component.

The first human trial of a monoclonal secretory antibody produced in transgenic plants was conducted by Planet Biotechnology, Inc (Mountain View, CA). Their product CaroRx^{TM} was recombinant sIgA/G, purified from mature tobacco plants by ammonium sulphate precipitation and protein G immunoaffinity chromatography. The clinical efficacy of both plant sIgA/G and murine IgG monoclonal antibodies was tested by their application directly to teeth for three weeks with two applications per week.

Transgenic Plants with Enhanced Nutritive Value

The plastids of higher plants synthesize numerous useful compounds of nutritional value like β- carotene (provitamin A) and tocopherols (vitamin E), in addition to performing normal functions of photosynthesis. Dietary β-carotene is converted into vitamin A, which plays an important role in the normal development of humans, quenches free radicals, prevents cellular damage and supports human immune system. On the other hand, insufficient dietary vitamin A leads to eye disease, xerophthalmia. For this reason, it would be desirable to meet the daily requirements of vitamin A and this goal can be achieved by raising carotenoid levels

Production of Enzymes in Plants

α-Amylase

This enzyme from bacteria and fungi finds use in starch processing, alcohol production,

increasingbread volume, clarifying wines and juices and in detergent manufacture. The enzyme α-amylase from *Bacillus licheniformis* is most commonly used for starch liquefaction. The enzyme is suited because of its heat stability and its activity over a wide *p*H range. Tobacco has been transformed with the gene for α-amylase from *B. licheniformis*. The enzyme expressed at 0.3% of the total soluble protein. The biological activity did not differ from the bacterial counterpart of the enzyme.The nature and the quality of the products obtained from corn and potato starch with the help of plant-based α-amylase indicated that the enzyme produced in tobacco is well suited for the liquefaction of starch. Direct fructose production was engineered in transgenic potato tubers created by using a fusion gene encoding two thermostable enzymes, viz. α-amylase (from *Bacillusstearothermophilus*) and glucose isomerase (from *Thermus thermophilus*), which were placed under the control of granule-bound-starch synthase promoter. More than 100 independent transgenic lines were generated. This enzyme complex was active only at high temperature. When crushed transgenic tubers were heated for 45 min at 65°C, starch was degraded to produce glucose and fructose.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – V – Advanced Molecular Biology and Genetic Engineering – SBTA5201

GENETICALLY MODIFIED ORGANISMS

For centuries, humans have altered plants and animals by selective reproduction (breeding, hybridizing). As a result, we have a wide range of domestic animals and plants grown for food andfor a variety of non-food use (such as for fibers and decorative purposes and as a source of fuel).

These efforts to adjust the characteristics of organisms in nature do not involve direct genetic modification by humans, but involve human actions working with existing natural processes forselection of traits. These traits are in the genes, so there are some differences in the genes of theoriginal and modified versions of the plants and animals.

Direct genetic modification is a relatively new process based on a set of technologies that alter the genetic makeup of living organisms, including animals, plants, bacteria, or fungi by inserting genesrather than using cross-breeding and selection techniques. The purpose of the modification of the genes is to derive certain benefits. Genetic modification is accomplished by inserting one or more genes from one organism into a different organism (for example, from bacteria into a plant or fromone species of plant into another). Combining genes from different organisms is known as recombinant DNA technology ("gene splicing"), and the resulting organism is said to be "genetically modified," "genetically engineered," or "transgenic." The end product we use may be part of the genetically modified organism itself (e.g., the beans of the soy plant) or something produced by the modified organism (for example, a drug produced by fermentation using modifiedbacteria or fungi).

SOME QUESTIONS; ETHICAL AND PRACTICAL MATTERS

The rapid introduction of these genetic engineering technologies has posed the serious question of whether we are rushing into an area of potential danger without giving it adequate thought. One canraise the "Jurassic Park" specter of messing with DNA and having the results come back to "bite you" just when you thought everything was going so well and that the few initial problems were

resolved. Some of the fears are generated by and kept active among groups who discuss the matter without having any significant training in biology and who may be relying on misconceptions and incorrect information. Science fiction writers and movie makers may help play into these fears as will authors of "non-fiction" works who sensationalize and misrepresent certain scientific concerns. Real problems with genetic engineering can then blend into the imagined scenarios, to make peoplebecome very agitated.

There have also been a number of technology scares that lead to a general concern about moving into new areas like genetic engineering. One can think of nuclear weapons and the threat of global destruction; nuclear power and the problems of Chernobyl and Three Mile Island; the excessive use of the pesticide DDT and the potentially devastating outcomes for animals and humans that was averted by banning it; the contamination of water supplies with mercury and contamination of the soil with lead; the possibility that continued global warming and serious adverse consequences due to human activities, and so on. In these instances, drastic actions have been considered-and sometimes utilized-in order to prevent catastrophes, sometimes after a wake-up call from a limited disaster. In addition, there have been worries about human activities even from the non- technological end, such as decimating forests. So, some people simply want humans to back off from altering nature and leave things alone to the extent possible; that includes not altering the DNA of organisms.

On the other hand, most people like the numerous benefits of technology and simply want it to be used sensibly. Instead of protesting against technological innovations, they want to be assured thatreasonable safeguards are in place. Some of the genetic modifications have raised virtually no objections, such as the use of bacteria to make insulin and the use of yeasts to make enzymes for cheese. These technologies are unlikely to stir up much controversy because the genetically modified organisms stay inside the factory and no problems have yet been detected. Some of the fears about genetically modified foods are not consistent with our knowledge of biology and

toxicology. For example, eating a food that includes a protein (such as the one serving as a natural insecticide or protecting against herbicides) doesn't appear to pose any threat to humans (that protein already existed in nature and was present in small quantities in some foods). The protein is not toxic to humans and is broken down, like other proteins, into amino acids that nourish the body.The gene is not going to change human genes. Nor is the gene within the food we eat going to mutate into a virus or other pathogen. Eating genetically modified soybeans will not have a direct adverse effect on the person eating them.

However, the underlying issue of worrying about eating genetically modified foods is not entirely without certain merits, in that certain genetic modifications might affect humans. A process was developed to make soybeans a richer source of nutrition by adding a gene from Brazil nuts. The purpose was to make the balance of amino acids in soybeans better for nourishing humans (something that was really not necessary). Soybean nutrition is compromised slightly by a relative deficiency in its methionine content; the Brazil nut gene for producing a methionine rich protein was introduced into the soybean genes. The problem is that some people are allergic to the protein produced by the gene from the Brazil nut (they are allergic to these nuts and this protein happens tobe one of the allergenic substances). So, that GMO crop idea had to be abandoned (it had not been commercially introduced). Still, the fact that scientists went down this particular path of potential product development shows that the technology can get into areas of trouble.

Another question that is raised is the extent to which the genetic modification actually provides a benefit. As an example, studies have suggested that some of the pest-resistant GM crops do not actually result in a significantly lower amount of pesticides being used on them. Thus, any potentialrisks of using the technology might not be balanced by sufficient benefits. Also, nature can find a way around the genetic modifications. For example, with increasing sowing of the Roundup ready crops, this particular herbicide is being very extensively used; so weeds resistant to that pesticide are turning up.

Perhaps the biggest ethical problem is the one of the "slippery slope." Genetic engineering has definitely provided some benefits and also appears to have many more benefits to offer as the technology progresses. Companies and governments may rush into production one or more productsof the new technologies that will turn out to be harmful, either to the environment or to humans directly. Consider, for example, a country where a large part of the population is starving (example:North Korea) and where researchers might find a way to vastly increase the yield of a crop or the nutritional benefits of a food. There would be a lot of pressure to move quickly to put this GM crop into commercial use, and to downplay any objections raised (as well as to consider that any problems that might arise could be resolved later). These genetically modified organisms are not always confined to the country where they are being used (particularly in the case where pollen is spread by the wind). Who knows what kind of ecological disaster might arise from failure to consider the unintended consequences. Similarly, when bacteria are used in batch cultures to produce proteins (as in the case of producing insulin), often the bacteria is one that is commonly found in nature (e.g., *E. coli*). If it escapes into the environment, could it then cause problems?

Might these organisms be inadequately safe-guarded in some countries?

Objections that do not involve the biology of genetic alterations might still be mentioned here in passing, such as the consideration of economics and society. The leading technologies and the ability to make use of them on a large scale is often dominated by countries with the greatest wealth or companies with patent protections. There is some concern that the utilization of the technology and the economic benefits may not be equitably shared. This is not unique to genetic modification, but because of the diversity of genetic work that can be accomplished with the state of the art technology and production facilities, there is definitely a concentration of power in certain areas of the world.

The genetic engineering of animals has increased significantly in recent years, and the use of thistechnology brings with it ethical issues, some of which relate to animal welfare — defined by the World Organisation for Animal Health as “the state of the animal…how an animal is coping withthe conditions in which it lives”.

Several terms are used to describe genetically engineered animals: genetically modified, geneticallyaltered, genetically manipulated, transgenic, and biotechnology derived, amongst others. In the early stages of genetic engineering, the primary technology used was transgenesis, literally meaningthe transfer of genetic material from one organism to another. However, with advances in the field, new technology emerged that did not necessarily require transgenesis: recent applications allow for the creation of genetically engineered animals via the deletion of genes, or the manipulation of genes already present. To reflect this progress and to include those animals that are not strictly transgenic, the umbrella term "genetically engineered" has been adopted into the guidelines developed by the Canadian Council on Animal Care (CCAC). For clarity, in the new *CCAC guidelines on: genetically-engineered animals used in science* (currently in preparation) the CCAC offers the following definition of a genetically engineered animal: "an animal that has had a change in its nuclear or mitochondrial DNA (addition, deletion, or substitution of some part of the animal'sgenetic material or insertion of foreign DNA) achieved through a deliberate human technological intervention." Those animals that have undergone induced mutations (for example, by chemicals orradiation — as distinct from spontaneous mutations that naturally occur in populations) and cloned animals are also considered to be genetically engineered due to the direct intervention and planninginvolved in creation of these animals.

Cloning is the replication of certain cell types from a "parent" cell, or the replication of a certain part of the cell or DNA to propagate a particular desirable genetic trait. There are 3 types of cloning: DNA cloning, therapeutic cloning, and reproductive cloning . For the purposes of this paper, the term "cloning" is used to refer to reproductive cloning, as this is the most likely to lead toanimal welfare issues. Reproductive cloning is used if the intention is to generate an animal that has the same nuclear DNA as another currently, or previously existing animal. The process used togenerate this type of cloned animal is called somatic cell nuclear transfer (SCNT).

During the development of the *CCAC guidelines on: genetically- engineered animals used inscience,* some key ethical issues, including animal welfare concerns, were identified: 1) invasiveness of procedures; 2) large numbers of animals required; 3) unanticipated welfare concerns; and 4) how to establish ethical limits to genetic

engineering

Genetic engineering technology has numerous applications involving companion, wild, and farm animals, and animal models used in scientific research. The majority of genetically engineered animals are still in the research phase, rather than actually in use for their intended applications, orcommercially available.

Companion animals

By inserting genes from sea anemone and jellyfish, zebrafish have been genetically engineered to express fluorescent proteins — hence the commonly termed "GloFish." GloFish began to be marketed in the United States in 2003 as ornamental pet fish; however, their sale sparked controversial ethical debates in California — the only US state to prohibit the sale of GloFish as pets (5). In addition to the insertion of foreign genes, gene knock-out techniques are also being usedto create designer companion animals. For example, in the creation of hypoallergenic cats some companies use genetic engineering techniques to remove the gene that codes for the major cat allergen Companion species have also been derived by cloning. The first cloned cat, "CC," was created in 2002. At the time, the ability to clone mammals was a coveted prize, and after just a few years scientists created the first cloned dog, "Snuppy".

With the exception of a couple of isolated cases, the genetically engineered pet industry is yet to move forward. However, it remains feasible that genetically engineered pets could become part of day-to-day life for practicing veterinarians, and there is evidence that clients have started to enquireabout genetic engineering services, in particular the cloning of deceased pets .

Wild animals

The primary application of genetic engineering to wild species involves cloning. This technology could be applied to either extinct or endangered species; for example, there have been plans to clonethe extinct thylacine and the woolly mammoth (5). Holt et al (8) point out that, "As many conservationists are still suspicious of reproductive technologies, it is unlikely that cloning techniques would be easily accepted. Individuals involved in field conservation often harbour suspicions that hi-tech approaches, backed by high profile publicity would divert funding away from their own efforts." However, cloning may prove to be an important tool to be used alongside other forms of assisted reproduction to help retain genetic diversity

in small populations of endangered species.

Farm animals

Productivity of farm animal species can be increased using genetic engineering. Examples includetransgenic pigs and sheep that have been genetically altered to express higher levels of growth hormone.

Genetically engineered farm animals can be created to enhance food quality . For example, pigs have been genetically engineered to express the Δ12 fatty acid desaturase gene (from spinach) forhigher levels of omega-3, and goats have been genetically engineered to express human lysozymein their milk. Such advances may add to the nutritional value of animal-based products.

Farm species may be genetically engineered to create disease-resistant animals . Specific examplesinclude conferring immunity to offspring via antibody expression in the milk of the mother; disruption of the virus entry mechanism (which is applicable to diseases such as pseudorabies); resistance to prion diseases; parasite control (especially in sheep); and mastitis resistance (particularly in cattle).

Genetic engineering has also been applied with the aim of reducing agricultural pollution. The best-known example is the EnviropigTM; a pig that is genetically engineered to produce an enzyme that breaks down dietary phosphorus (phytase), thus limiting the amount of phosphorus released in its manure.

Despite resistance to the commercialization of genetically engineered animals for food production,primarily due to lack of support from the public ,a recent debate over genetically engineered AquAdvantageTM Atlantic salmon may result in these animals being introduced into commercial production . Effort has also been made to generate genetically engineered farm species such as cows, goats, and sheep that express medically important proteins in their milk. According to Dycket al , “transgenic animal bioreactors represent a powerful tool to address the growing need for therapeutic recombinant proteins.” In 2006, ATryn® became the first therapeutic protein producedby genetically engineered animals to be approved by the Food and Drug Administration (FDA) of the United States. This product is used as a prophylactic treatment for patients that have hereditary antithrombin deficiency and are undergoing surgical procedures.

Research animals

Biomedical applications of genetically engineered animals are numerous, and include understanding of gene function, modeling of human disease to either understand disease mechanisms or to aid drug development, and xenotransplantation.

Through the addition, removal, or alteration of genes, scientists can pinpoint what a gene does by observing the biological systems that are affected. While some genetic alterations have no obvious effect, others may produce different phenotypes that can be used by researchers to understand the function of the affected genes. Genetic engineering has enabled the creation of human disease models that were previously unavailable. Animal models of human disease are valuable resources for understanding how and why a particular disease develops, and what can be done to halt or reverse the process. As a result, efforts have focused on developing new genetically engineered animal models of conditions such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and cancer. However, as Wells points out: "these [genetically engineered animal] models do not always accurately reflect the human condition, and care must be taken tounderstand the limitation of such models."

The use of genetically engineered animals has also become routine within the pharmaceutical industry, for drug discovery, drug development, and risk assessment. As discussed by Rudmann andDurham : "Transgenic and knock out mouse models are extremely useful in drug discovery, especially when defining potential therapeutic targets for modifying immune and inflammatory responses…Specific areas for which genetically engineered animal models may be useful are in screening for drug induced immunotoxicity, genotoxicity, and carcinogenicity, and in understandingtoxicity related drug metabolizing enzyme systems."

Perhaps the most controversial use of genetically engineered animals in science is to develop the basic research on xenotrans-plantation — that is, the transplant of cells, tissues, or whole organs from animal donors into human recipients. In relation to organ transplants, scientists have developed a genetically engineered pig with the aim of reducing rejection of pig organs

by human recipients. This particular application of genetic engineering is currently at the basic research stage,but it shows great promise in alleviating the long waiting lists for organ transplants, as the number of people needing transplants currently far outweighs the number of donated organs. However, as a direct result of public consultation, a moratorium is currently in place preventing pig organ transplantation from entering a clinical trial phase until the public is assured that the potential disease transfer from pigs to humans can be satisfactorily managed.

Ethical issues of genetic engineering

Ethical issues, including concerns for animal welfare, can arise at all stages in the generation and life span of an individual genetically engineered animal. The following sections detail some of the issues that have arisen during the peer-driven guidelines development process and associated impactanalysis consultations carried out by the CCAC. The CCAC works to an accepted ethic of animal use in science, which includes the principles of the Three Rs (Reduction of animal numbers, Refinement of practices and husbandry to minimize pain and distress, and Replacement of animals with non-animal alternatives wherever possible). Together the Three Rs aim to minimize any pain and distress experienced by the animals used, and as such, they are considered the principles of humane experimental technique. However, despite the steps taken to minimize pain and distress, there is evidence of public concerns that go beyond the Three Rs and animal welfare regarding the creation and use of genetically engineered animals.

Concerns

The generation of a new genetically engineered line of animals often involves the sacrifice of someanimals and surgical procedures (for example, vasectomy, surgical embryo transfer) on others.

These procedures are not unique to genetically engineered animals, but they are typically requiredfor their production.

During the creation of new genetically engineered animals (particularly mammalian species) oocyteand blastocyst donor females may be induced to superovulate via intraperitoneal or subcutaneous injection of hormones; genetically engineered embryos may be surgically implanted to female recipients; males may be surgically vasectomized under general

anesthesia and then used to induce pseudopregnancy in female embryo recipients; and all offspring need to be genotyped, which is typically performed by taking tissue samples, sometimes using tail biopsies or ear notching.

However, progress is being made to refine the genetic engineering techniques that are applied to mammals (mice in particular) so that less invasive methods are feasible. For example, typical genetic engineering procedures require surgery on the recipient female so that genetically engineered embryos can be implanted and can grow to full term; however, a technique called non-surgical embryo transfer (NSET) acts in a similar way to artificial insemination, and removes the need for invasive surgery. Other refinements include a method referred to as "deathless transgenesis," which involves the introduction of DNA into the sperm cells of live males and removes the need to euthanize females in order to obtain germ line transmission of a genetic alteration; and the use of polymerase chain reaction (PCR) for genotyping, which requires lesstissue than Southern Blot Analysis .

Large numbers of animals required

Many of the embryos that undergo genetic engineering procedures do not survive, and of those thatdo survive only a small proportion (between 1% to 30%) carry the genetic alteration of interest .

This means that large numbers of animals are produced to obtain genetically engineered animals that are of scientific value, and this contradicts efforts to minimize animal use. In addition, the advancement of genetic engineering technologies in recent years has lead to a rapid increase in the number and varieties of genetically engineered animals, particularly mice . Although the technologyis continually being refined, current genetic engineering techniques remain relatively inefficient, with many surplus animals being exposed to harmful procedures. One key refinement and reductioneffort is the preservation of genetically engineered animal lines through the freezing of embryos or sperm (cryopreservation), which is particularly important for those lines with the potential to experience pain and distress .

As mentioned, the number of research projects creating and/or using genetically engineered animalsworldwide has increased in the past decade . In Canada, the CCAC's annual data on the numbers of animals used in science show an increase in Category D procedures (procedures with the potential to cause moderate to severe pain and distress) — at present the

creation of a new genetically engineered animal line is a Category D procedure . The data also show an increase in the use of mice , which are currently the most commonly used species for genetic engineering, making up over 90% of the genetically engineered animals used in research and testing. This rise in animal usechallenges the Three Rs principle of Reduction . It has been reasoned that once created, the use of genetically engineered animals will reduce the total number of animals used in any given experiment by providing novel and more accurate animal models, especially in applications such astoxicity testing . However, the greater variety of available applications, and the large numbers of animals required for the creation and maintenance of new genetically engineered strains indicate that there is still progress to be made in implementation of the Three Rs principle of Reduction in relation to the creation and use of genetically engineered animals.

Unanticipated welfare concerns

Little data has been collected on the net welfare impacts to genetically engineered animals or to those animals required for their creation, and genetic engineering techniques have been described asboth unpredictable and inefficient . The latter is due, in part, to the limitations in controlling the integration site of foreign DNA, which is inherent in some genetic engineering techniques (such as pro-nuclear microinjection). In such cases, scientists may generate several independent lines of genetically engineered animals that differ only in the integration site, thereby further increasing the numbers of animals involved. This conflicts with efforts to adhere to the principles of the Three Rs,specifically Reduction. With other, more refined techniques that allow greater control of DNA integration (for example, gene targeting), unexpected outcomes are attributed to the unpredictable interaction of the introduced DNA with host genes. These interactions also vary with the genetic background of the animal, as has frequently been observed in genetically engineered mice.

Interfering with the genome by inserting or removing fragments of DNA may result in alteration ofthe animal's normal genetic homeostasis, which can be manifested in the behavior and well-being of the animals in unpredictable ways. For example, many of the early transgenic livestock studies produced animals with a range of unexpected side effects including lameness, susceptibility to stress, and reduced fertility.

A significant limitation of current cloning technology is the prospect that cloned offspring

may suffer some degree of abnormality. Studies have revealed that cloned mammals may suffer from developmental abnormalities, including extended gestation; large birth weight; inadequate placentalformation; and histological effects in organs and tissues (for example, kidneys, brain, cardiovascular system, and muscle). One annotated review highlights 11 different original research articles that documented the production of cloned animals with abnormalities occurring in the developing embryo, and suffering for the newborn animal and the surrogate mother.

Genetically engineered animals, even those with the same gene manipulation, can exhibit a variety of phenotypes; some causing no welfare issues, and some causing negative welfare impacts. It is often difficult to predict the effects a particular genetic modification can have on an individual animal, so genetically engineered animals must be monitored closely to mitigate any unanticipated welfare concerns as they arise. For newly created genetically engineered animals, the level of monitoring needs to be greater than that for regular animals due to the lack of predictability. Once agenetically engineered animal line is established and the welfare concerns are known, it may be possible to reduce the levels of monitoring if the animals are not exhibiting a phenotype that has negative welfare impacts. To aid this monitoring process, some authors have called for the implementation of a genetically engineered animal passport that accompanies an individual animal and alerts animal care staff to the particular welfare needs of that animal. This passport document isalso important if the intention is to breed from the genetically engineered animal in question, so theappropriate care and husbandry can be in place for the offspring.

With progress in genetic engineering techniques, new methods may substantially reduce the unpredictability of the location of gene insertion. As a result, genetic engineering procedures maybecome less of a welfare concern over time.

Beyond animal welfare

As pointed out by Lassen et al, “Until recently the main limits [to genetic engineering] were technical: what it is *possible* to do. Now scientists are faced with ethical limits as well: what it is*acceptable* to do” (emphasis theirs). Questions regarding whether it is acceptable to make new

transgenic animals go beyond consideration of the Three Rs, animal health, and animal welfare, andprompt the discussion of concepts such as intrinsic value, integrity, and naturalness.

When discussing the "nature" of an animal, it may be useful to consider the Aristotelian concept of *telos,* which describes the "essence and purpose of a creature". Philosopher Bernard Rollin applied this concept to animal ethics as follows: "Though [*telos*] is partially metaphysical (in defining a way of looking at the world), and partially empirical (in that it can and will be deepened and refinedby increasing empirical knowledge), it is at root a moral notion, both because it is morally motivated and because it contains the notion of what about an animal we *ought* to at least try to respect and accommodate" (emphasis Rollin's). Rollin has also argued that as long as we are carefulto accommodate the animal's interests when we alter an animal's *telos,* it is morally permissible. Hewrites, "…given a *telos,* we should respect the interests which flow from it. This principle does not logically entail that we cannot modify the *telos* and thereby generate different or alternative interests" .Views such as those put forward by Rollin have been argued against on the grounds that health and welfare (or animal interests) may not be the only things to consider when establishing ethical limits. Some authors have made the case that genetic engineering requires us to expand our existing notions of animal ethics to include concepts of the intrinsic value of animals , or of animal "integrity" or "dignity" . Veerhoog argues that, "we misuse the word *telos* when we say that human beings can 'change' the *telos* of an animal or create a new *telos*" — that is to say animals have intrinsic value, which is separate from their value to humans. It is often on these grounds that people will argue that genetic engineering of animals is morally wrong. For example, in a case study of public opinion on issues related to genetic engineering, participants raised concerns about the "nature" of animals and how this is affected (negatively) by genetic engineering .

An alternative view put forward by Schicktanz argues that it is the human-animal relationship thatmay be damaged by genetic engineering due to the increasingly imbalanced distribution of power between humans and animals. This imbalance is termed "asymmetry" and it is raised alongside "ambivalence" as a concern regarding modern human-animal relationships. By using genetically engineered animals as a case study, Schicktanz argues that genetic

engineering presents “a troublingshift for all human-animal relationships.”

Opinions regarding whether limits can, or should, be placed on genetic engineering are often dependent on people’s broader worldview. For some, the genetic engineering of animals may not put their moral principles at risk. For example, this could perhaps be because genetic engineering isseen as a logical continuation of selective breeding, a practice that humans have been carrying out for years; or because human life is deemed more important than animal life. So if genetic engineering creates animals that help us to develop new human medicine then, ethically speaking, we may actually have a moral obligation to create and use them; or because of an expectation that genetic engineering of animals can help reduce experimental animal numbers, thus implementing the accepted Three Rs framework. For others, the genetic engineering of animals may put their moral principles at risk. For example costs may always be seen to outweigh benefits because the ultimate cost is the violation of species integrity and disregard for the inherent value of animals.
Some may view *telos* as something that cannot or should not be altered, and therefore altering the*telos* of an animal would be morally wrong. Some may see genetic engineering as exaggeratingthe imbalance of power between humans and animals, whilst others may fear that the release of genetically engineered animals will upset the natural balance of the ecosystem. In addition, there may be those who feel strongly opposed to certain applications of genetic engineering, but more accepting of others. For example, recent evidence suggests that people may be more accepting of biomedical applications than those relating to food production .

Such underlying complexity of views regarding genetic engineering makes the setting of ethical limits difficult to achieve, or indeed, even discuss. However, progress needs to be made on this important issue, especially for those genetically engineered species that are intended for life outsidethe research laboratory, where there may be less careful oversight of animal welfare. Consequently,limits to genetic engineering need to be established using the full breadth of public and expert

opinion. This highlights the importance for veterinarians, as animal health experts, to be involved inthe discussion.

Other ethical issues

Genetic engineering also brings with it concerns over intellectual property, and patenting of createdanimals and/or the techniques used to create them. Preserving intellectual property can breed a culture of confidentiality within the scientific community, which in turn limits data and animal sharing. Such limits to data and animal sharing may create situations in which there is unnecessary duplication of genetically engineered animal lines, thereby challenging the principle of Reduction. Indeed, this was a concern that was identified in a recent workshop on the creation and use of genetically engineered animals in science .

It should be noted that no matter what the application of genetically engineered animals, there are restrictions on the methods of their disposal once they have been euthanized. The reason for this isto restrict the entry of genetically engineered animal carcasses into the natural ecosystem until thelong-term effects and risks are better understood.

Implications for veterinarians

As genetically engineered animals begin to enter the commercial realm, it will become increasinglyimportant for veterinarians to inform themselves about any special care and management required by these animals. As animal health professionals, veterinarians can also make important contributions to policy discussions related to the oversight of genetic engineering as it is applied to animals, and to regulatory proceedings for the commercial use of genetically engineered animals.

It is likely that public acceptance of genetically engineered animal products will be an important step in determining when and what types of genetically engineered animals will appear on the commercial market, especially those animals used for food production. Veterinarians may also becalled on to inform the public about genetic engineering techniques and any potential impacts to animal welfare and food safety. Consequently, for the discussion regarding genetically engineered

animals to progress effectively, veterinarians need to be aware of the current context in which genetically engineered animals are created and used, and to be aware of the manner in which genetic engineering technology and the animals derived from it may be used in the future. Genetic engineering techniques can be applied to a range of animal species, and although many genetically engineered animals are still in the research phase, there are a variety of intended applications for their use. Although genetic engineering may provide substantial benefits in areas such as biomedical science and food production, the creation and use of genetically engineered animals not only challenge the Three Rs principles, but may also raise ethical issues that go beyondconsiderations of animal health, animal welfare, and the Three Rs, opening up issues relating to animal integrity and/or dignity. Consequently, even if animal welfare can be satisfactorily safeguarded, intrinsic ethical concerns about the genetic engineering of animals may be cause enough to restrict certain types of genetically engineered animals from reaching their intended commercial application. Given the complexity of views regarding genetic engineering, it is valuableto involve all stakeholders in discussions about the applications of this technology.

There are a number of ethical concerns over genetically modified (GM) foods and these have all affected public support of the products. The issues have also triggered controversy and regulations around GM foods and any company that produces these crops or products. Concerns range from theenvironment to risks to our food web or issues concerning disease, allergies and contamination.

Allergies And Disease

A key ethical concern about GM foods is their potential to trigger allergies or disease in humans. Given that a gene could be extracted from an allergenic organism and placed into another one that typically does not cause allergies, a person may unknowingly be exposed to an allergen. In turn, thiscould lead to an allergic reaction. There is also the fear that new allergies could occur from the mixing of genes from two organisms.

Disease is a major health worry with regards to GM foods. Given that some of the crops modified are done so with DNA from a bacterium or virus, there is concern that a new disease may occur in humans who consume the GM food. With some GM crops having antibiotic-resistant marker genes,there is also the worry that these genes could be passed on to microbes that cause disease and healthproblems in humans. With widespread antibiotic resistance currently already occurring, any new resistance could prove disastrous.

Damage To The Environment

Damage to the environment is another ethical fear with regards to GM crops. Unfortunately, the technology is still new enough that there is much we do not know about the effect of GM crop production on the environment. Long-term studies take decades to complete and most studies of GM crop production involve short-term effects of the technology. Another ethical issue around GMcrops is our ability to contain them in a specific area. There are fears that if these crops do negatively impact the environment, they will spread in an out-of-control fashion and we will not beable to stop their damaging effects. For instance, one type of sugar beet that had been engineered tobe resistant to a specific herbicide ended up unintentionally having the genes to resist a different herbicide. When farmers went to eliminate the crop, they still found that a small percentage had survived.

Cross-Pollination

Cross-pollination is a challenge for any crop growth but it can typically be managed if care is takento use good growing practices. There is the possibility of genes from GM foods spreading to other plants and crops, which could create overzealous weeds that can't be contained at all.

Food Web And Risks

Risks to the food web are a very real ethical concern around GM technology. Any pesticide or herbicide from the crop could harm animals and other organisms in the environment. For example,GM sugar beets that were produced to be resistant to herbicides did successfully reduce weeds.

However, Skylark birds that consume the seeds from this particular weed would now be

required tofind a new food source, thereby endangering their existence.

An animal could also consume the GM crop itself, which means that if the crop has been engineeredto produce a pesticide, the animal may become ill and die. In one North American study, caterpillars of the monarch butterfly were killed when they fed on pollen from GM corn crops.

Addressing Ethical Concerns For GM Foods

Unfortunately, the controversy and fears around GM foods and any company that produces these products still continue to persevere, although this could be viewed as a positive movement because it will challenge GM technology and help to make it safer and more regulated. In one public opinionpoll, it was found that the more people read about GM foods, the more concerned they became about the technology. Studies are ongoing into the many ethical concerns around GM foods but these are not conclusive and have thus far shown very mixed results. It is also very difficult to assess the long-term impact, thereby leaving many of the public fearing for the long-term safety of humans and the environment.

For now, it is hoped that people will become more educated on the ethical concerns about GMfoods, which will ideally fuel further research and accountability in the field.