ARPUTHA COLLEGE OF ARTS AND SCIENCE FOR WOMEN

(Affiliated to Bharathidasan University, Thiruchirappalli)

ARPUTHA NAGAR, VAMBAN

PUDUKKOTTAI(D.t), TAMILNADU, INDIA 622 303

III B.Sc., MICROBIOLOGY

SEMESTER VI

Major Based Elective II

RECOMBINANT DNA TECH NOLOGY

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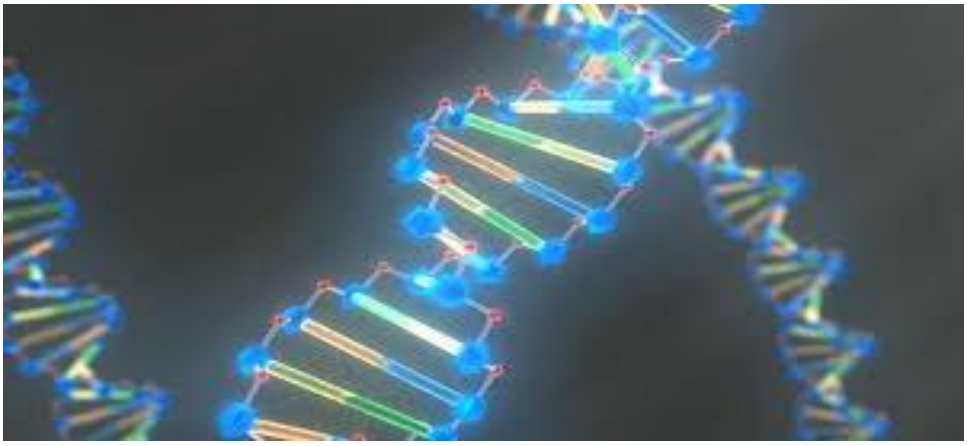
UNIT IV & UNIT V



K.S.POUNYA

Assistant professor in Microbiology

Arputha college of arts and science for women

Arputha nagar, Vamban

UNIT IV

Gene/ DNA transfer techniques: Physical – Biolistic Method (Gene gun), Chemical – Calcium chloride & DEAE methods, Biological invitro packaging method in viruses – Selection & screening of Recombinants: Direct method: Selection by Complementation, Marker inactivation methods – Indirect methods: Immunological & Genetic methods.

Definition of Gene transfer

Gene transfer: The insertion of unrelated genetic information in the form of DNA into cells. ... There are also different ways to transfer genes. Some of these methods involve the use of a vector such as a virus that has been specifically modified so it can take the gene along with it when it enters the cell.

What is meant by gene transfer?

The insertion of copies of a **gene** into living cells in order to induce synthesis of the **gene's** product: the desired **gene** may be microinjected directly into the cell or it may be inserted into the core of a virus by **gene** splicing and the virus allowed to infect the cell for replication of the **gene** in the cell's DNA.

There are different methods of gene transfer.They are as follows

1. Physical method of gene transfer
2. Chemical method of gene transfer
3. Biological invitro packaging method

Physical Methods of Gene Transfer

The following points highlight the ten main physical methods of gene transfer. The methods are:

1. Biolistic or Particle Bombardment 2. Electroporation 3. Microinjection 4. Pollen Transformation 5. Liposome Mediated Transfer 6. Microlaser 7. Macro-Injection 8. Silicon Carbide Fiber (SCF) Mediated Transfer 9. Poly Ethylene Glycol (PEG) Mediated Transformation 10. Ultrasound Mediated Transfer.

BIOLISTIC METHOD OF GENE TRANSFER

One of the most spectacular successes in transformation of broad range of plants devoid of discrimination is the biolystic or gene gun method. This method, undoubtedly, is in driver seat among several other proposed methods.

This is the combination of biological and ballistic method. Klein (1987) has emphatically described effective and versatile particle gum method for delivering nucleic acids into intact plant cells and eventually result in transient expression of foreign gene.

In particle bombardment mediated process, DNA coated micro-projectile is used to transform plant tissue. After being accelerated, micro projectile is propelled to pierce cell wall and membrane and enter intact plant cells. The micro projectile is small to penetrate the cell with limited damage and successfully introduce DNA or RNA.

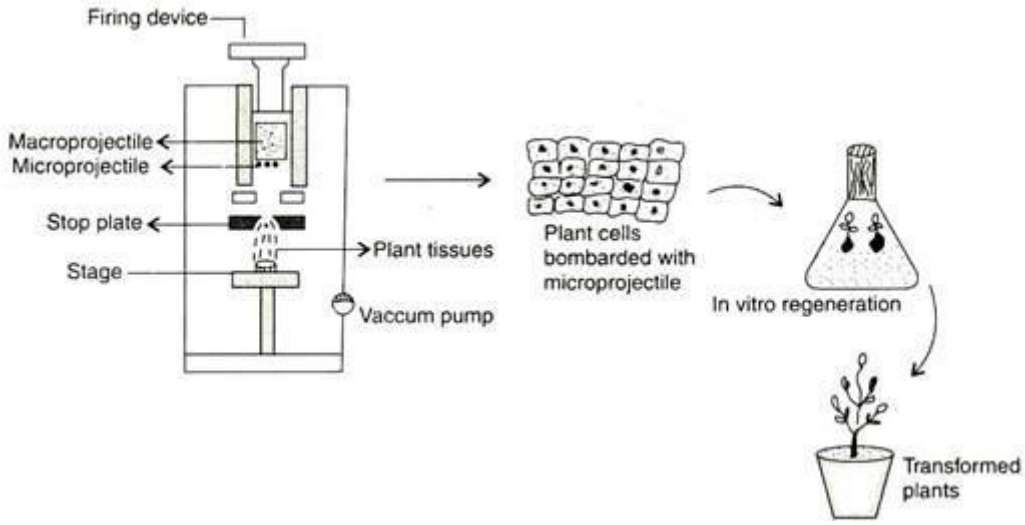
Biolystic process has been used to transform larger tissue and organs such as shoot tip, leaves, callus, cotyledon, zygotic and somatic embryos. This technique was first developed in 1987, intended to transform cereals. Infact, the first genetically modified (GM) crop like maize contains Bt-toxin gene was produced by this method.

Gene Gun Design:

Particle bombardment is based on the development of gas flow system such as powder driven (PDS-1000) or helium driven (PDS-1000/Hc). Efficiency of the system depends on selection of target material, particle to be used as micro projectile and acceleration.

Transformation efficiency depends on the amount of DNA dosage delivered into the cell, for example, low amount of nucleic acid delivery results in low transformation frequency and similarly high amount of DNA delivered into the cell leads to high copy number transformation efficiency.

In order to accomplish higher transformation rate at lower DNA concentration, the choice of chemical to coat particle have been modified, in which calcium chloride and polyamines are replaced by aminosiloxanes.



Diagrammatic representation of Particle bombardment method

Nature and Preparation of Micro-carriers:

In the basic design of particle gun, coating of DNA onto small dense particles known as micro-projectiles is required. Several chemically inert metal particles such as gold, tungsten, palladium and platinum are employed. The size of the particles may vary between 1 and 1.6 pm in diameter.

The size of gold and tungsten particles is generally between 1 and 1.5 pm and 1.2 and 4 pm, respectively. Micro-metals are initially subjected to ethanol and sterile water washing process. Micro carrier suspension is then stored at 4°C for tungsten and – 20°C for gold particles.

Once preliminary treatment is done, micro-particles are mixed with plasmid DNA. Fixing of DNA onto the particles is carried out by either using ethanol or $CaCl_2$ precipitation method. After precipitation, the particles are washed, resuspended and either dried or stored on ice as an aqueous suspension.

Bombardment Process:

- This was originally proposed by Klein (1987) to transform epidermal cells of Allium cepa (Onion). In this method, tungsten particles of 4 pm in diameter is coated with genomic RNA of tobacco and placed on the front surface of a cylindrical nylon projectile (macro projectile) of diameter 5 mm and 8 mm in length.

- The whole projectile is prepared as a suspension in 1-2 pi of water. A gunpowder charge, detonated with a firing device is used to propel (accelerate) the nylon projectile down the apparatus. The tungsten particles move towards the steel plate, designed to stop the movement of nylon projectile.

- The steel plate allows the micro projectile to pass through 1 mm aperture of stop plate. Tungsten projectile leaves particle gun with an initial velocity of 430 ms^{-1}. This high velocity can be estimated by chronograph. The target cells/tissues are placed 15-20 cm from the end of the device. Many cells are bombardment simultaneously and about 90% of the cells typically contain bombardment micro-projectiles.

Merits of Biolystic Device:

i.It is efficient and easy to handle.

ii. It can transfer genes into many cells due to multiple sites.

iii. Technique can be widely used to transform different plate material types such as culture cells, pollen, meristem, embryos, and somatic embryo. Hence, in vitro regeneration is feasible.

iv. Only cells present in the line of micro-projectile movement are killed.

v. Utility of technique can be extended to a wide group of plants including dicots and monocots.

Demerits of Biolystic Device:

i. Integration of high copy number DNA sequence into the chromosome.

ii. Equipment costly.

iii. Cell/tissue damage due to bombardment by uncontrolled velocity of micro-projectiles.

ELECTROPORATION

Electroporation is well suited for the transformation of plant cells and protoplast. Extensive work has been carried out regarding transformation of cereals using protoplast. Both linear and circular DNA can be transformed into the plant tissue. Intact plant cells of monocots have been transformed by electroporation. During electroporation, protoplast or intact plant cells are taken in electroporation chamber fitted with parallel steel electrodes.

The chamber is initially filled with buffer containing DNA of interest and high initial field strength of 1000-1500 volts with a short decay time in microseconds in applied. Pulse is applied by discharge of the capacitor across the cell. Alternatively, successful transformation is also carried out, by passing low voltage strength with larger decay of time.

Once protoplast is pulsed with low or high voltage DNA then migrated through pores into the plasma membrane induced by high voltage, eventually integrated into the genome. Most of the cereals, particularly rice and wheat have been successfully transformed by electroporation. Even other tissues such as callus and immature embryos are suggested.

Several methods have been suggested to increase transformation efficiency. Utility of osmotic buffer was well documented. Incubation of target material in high osmotic buffer before or after electroporation may increase efficiency of the technique. Addition of spermidine induces condensation of DNA, which results in enhanced efficiency of electroporation.

Advantages:

i. Efficient transformation.

ii. Large number of transformed cells can be obtained.

iii. Production of transformants with low transgene copy number.

iv. Electroporated cells exhibit same physiological status after transformation.

v. Least number of cells deaths.

vi. Electroporation of tissue can reduce in vitro regeneration problem.

vii. Low equipment cost.

viii. Does not require experties individual.

Disadvantages:

i. Requirement of protoplast for cumbersome in vitro regeneration of plants from protoplast.

ii. Difficulties associated with regeneration of plants from protoplast.

iii.Rise of obtaining genetic variation in protoplast mediated regenerated plants.

Microinjection:

Transformation of higher plant cells by intranuclear microinjection has been emerged as an attractive approach in recent days. Genetic transformation of animals and insects using microinjection of embryos has been well established.

In plant system, however, protoplast is selected as favourable choice for microinjection. This technique has advanced into diverse applications in key areas like cell biology, genetics and transgenic field. Recently, microinjection is widely employed in cereal transformation.

Microinjection is a precise way of delivering genetic material into the target cells. Several workers have demonstrated the feasibility of microinjecting substances into specific cells. In order to understand intercellular transport, fluorescent dyes were microinjected. The mode of virus infection was elucidated by microinjection of viral particles into intact plant cells.

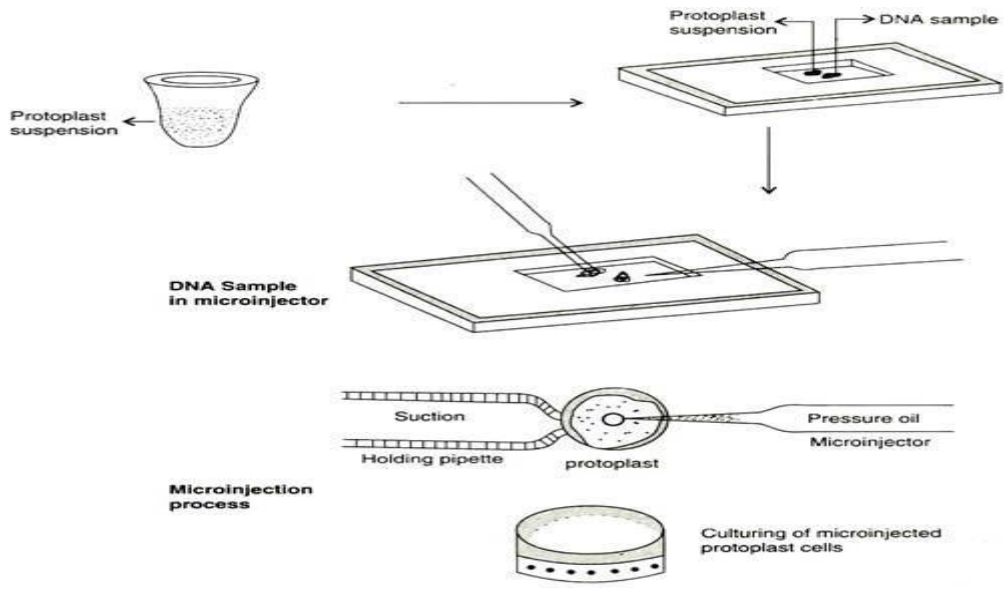
Microinjection involves direct physical approach in depositing DNA into specific target cells. Generally, microinjection requires micro-capillaries and microscopic devices to deliver DNA into cells in such a way that the injected cells survive the treatment and is able to proliferate in the cultural conditions.

During microinjection, plant protoplast or partially synthesized cells are fixed to glass coverslips with the help of poly L. lysine. Further process requires holding pipette and micromanipulator or micro-injector. If any cell type is reluctant to attach to cover slips by binding agent, holding pipette can be an essential factor in microinjection.

These cell types are firmly retained on fixed place by blunt holding pipette. The exogenous DNA of 1 pm is taken in micro-injector and the cells or protoplasts are firmly immobilized by holding

pipette by exerting suction pressure. Microinjection containing approximate dosage of DNA is then directly delivered inside the cells.

In microinjection, it is possible to microinject 200-350 protoplasts intra nuclearly and transformation frequency has been demonstrated with 20-60% success.



By means of reference marking on the coverslip, it is possible to locate microinjected cells/protoplast by recording with a video camera, which enables to work more freely from one microinjected cell to next one without interception.

Advantages:

i. The amount of DNA delivered can be optimized.

ii. Precise delivery of DNA. DNA delivery is predictable even into the cell nucleus.

iii. Small cell structures like microspores, callus and proembyros can be precisely targetted.

iv. Micro-culture is accomplished.

Disadvantages:

i. Only one cell receives DNA per injection.

ii. Handling of protoplast for microinjection requires skilled persons.

iii.Sophisticated equipment.

iv.Requirement of regeneration process from microinjected cells.

CHEMICAL METHOD OF GENE TRANSFER

Chemical. **Chemical** based **methods of gene** delivery can use natural or synthetic compounds to form particles that facilitate the **transfer** of **genes** into cells. These synthetic vectors have the ability to electrostatically bind DNA or RNA and compact the **genetic** information to accommodate larger **genetic** transfers.

CALCIUM CHLORIDE METHOD OF GENE TRANSFER

Calcium chloride transformation. **Calcium chloride** ($CaCl_2$) transformation is a laboratory **technique** in prokaryotic (bacterial) cell biology. ... The plasmid DNA can then pass into the cell upon heat shock, where chilled cells (+4 degrees Celsius) are heated to a higher temperature (+42 degrees Celsius) for a short time.

Competent cells are bacterial cells that can accept extra-chromosomal DNA or plasmids (naked DNA) from the environment. The generation of competent cells may occur by two methods: natural competence and artificial competence. Natural competence is the genetic ability of a bacterium to receive environmental DNA under natural or in vitro conditions. Bacteria can also be made competent artificially by chemical treatment and heat shock to make them transiently permeable to DNA.

When the foreign DNA enters inside the cells, it may be degraded by the cellular nucleases or may recombine with the cellular chromosome. However, natural competence and transformation are efficient for linear molecules such as chromosomal DNA but not for circular plasmid molecules. Artificial competence is not coded by the genes of the bacterial cells. It is a laboratory procedure in which cells are passively made permeable to DNA using unnatural conditions. The procedure of artificial competence is relatively simple and easy and can be used to engineer a bacterium genetically. However, transformation efficiency is very low as only a portion of the cells become competent to successfully take up DNA.

Reagents Required and Their Role Luria-Bertani Broth

Luria-Bertani (LB) broth is a rich medium that permits fast growth and good growth yields for many species including E. coli. It is the most commonly used medium in microbiology and molecular biology studies for E. coli cell cultures. Easy preparation, fast growth of most E. coli strains, ready availability and simple compositions contribute to the popularity of LB broth. LB can support E. coli growth (OD600 = 2–3) under normal shaking incubation conditions.

Calcium Chloride

Calcium chloride transformation technique is the most efficient technique among the competent cell preparation protocols. It increases the bacterial cell's ability to incorporate plasmid DNA, facilitating genetic transformation. Addition of calcium chloride to the cell suspension allows the binding of plasmid DNA to LPS. Thus, both the negatively charged DNA backbone and LPS come together and when heat shock is provided, plasmid DNA passes into the bacterial cell. Prepare 2000 ml of 50 mM Calcium
chloride stock solution by adding 14.701 g of CaCl2.2H2O in 2 l of milli-Q water, autoclave, and store at 4 °C.

Materials Required

1. LB broth: Yeast extract 0.5%, NaCl l%, tryptone 1%.
   2 LB agar: As above, plus 2% agar prior to autoclaving.
3. 0.1M CaCl2.
   Antibiotics are added to the above media after autoclaving. Tetracycline to a final concentration of 15 pg/mL and ampicillin to 50 kg/mL Solutions of these antibiotics are prepared with ampicillin at 50 mg/mL m slightly alkaline distilled water and tetracycline at 15 mg/mL in ethanol.

Method

1. Prepare a small, overnight culture of the bacteria in LB broth. Grow at 37°C without shaking.
2. About 2 h before you are ready to begin the main procedure, use 1.0 mL of the overnight culture to inoculate 100 mL of fresh LB broth. This culture is grown with rapid shaking at 37°C until it reaches roughly 5 x 107 cells/ml. Thus corresponds to an OD650 for our cultures, but you should calibrate this for each of your own strains
3. Take a 5 mL aliquot of each transformation reaction and transfer to sterile plastic centrifuge tubes. Cool on ice for 10 mm.
4. Pellet the cells by spinning for 5 mm at 5000g. It is necessary for the centrifugation to be performed at 4°C. We have found a refrigerated bench centrifuge ideal for this.
5. Pour off the supernatant and resuspend cells in 25 mL of cold 0.1M CaCl2. Leave on ice for at least 20 min.

6. Centrifuge as in Step 3. You should observe a more diffuse pellet than previously. This is an indication of competent cells.
7. Resuspend the cells in 0.2 mL of cold 0.1M CaCl2.
8. Transfer the suspensions to sterile, thin-walled glass bottles or tubes. The use of glass makes the subsequent heat shocks more effective.
9. To each tube add up to 0.1 mg of DNA, made up in a standard DNA storage buffer such as TE to a volume of 100 mL. Leave on ice for 30 min.
10. Transfer to a 42°C water bath for 2 min and return briefly to ice.
11. Transfer the contents of each tube to 2 mL of LB broth in a small flask. Incubate with shaking at 37°C for 60-90 min.
12. Plate 0.1 mL aliquots of undiluted, 10-1 and 10-2 dilutions onto LB plates to which the antibiotics to be used for selection have been added.
13. Incubate overnight at 37°C.

Advantages

This method generally gives 104-106 transformants/mg of closed circle plasmid DNA. Do note that the relationship between amounts of DNA added and yield is not totally linear

DEAE-Dextran Transfection

Diethylaminoethyl (DEAE)-dextran is a polycationic derivative of the carbohydrate polymer dextran, and it is one of the first chemical reagents used to transfer nucleic acids into cultured mammalian cells (Vaheri and Pagano, 1965). The cationic DEAE-dextran molecule tightly associates with the negatively charged backbone of the nucleic acid, and the net positive charge of the resulting nucleic acid-DEAE-dextran complex allows it to adhere to the cell membrane and enter into the cytoplasm via endocytosis or osmotic shock induced by DMSO or glycerol.

The advantages of DEAE-dextran method are its relative simplicity, reproducibility, and low cost, while its disadvantages include cytotoxicity and low transfection efficiency for a range of cell types (typically less than 10% in primary cells), as well as the requirement for reduced serum media during the transfection procedure. In addition, this method is limited to transient transfections, and is not suitable for generating stable cell lines.

METHODOLOGY

1. Mix nucleic acid with DEAE-dextran solution in transfection medium or phosphate-buffered saline solution. Nucleic acid-DEAE-dextran complexes are formed via electrostatic interactions between the polymer and phosphate backbone of the nucleic acid.

2. Add the nucleic acid-DEAE-dextran complexes to the cells, which adhere to the cell surface via electrostactic interactions.

3. Induce the uptake of the nucleic acid-DEAE-dextran complexes by osmotic shock using DMSO or glycerol.

4. Wash cells to remove the complexes and incubate to allow gene expression.

5. Assay cells for transient gene expression.

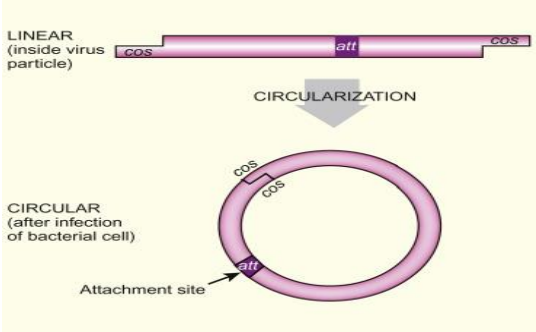
BIOLOGICAL INVITRO PACKAGING METHOD IN VIRUSES

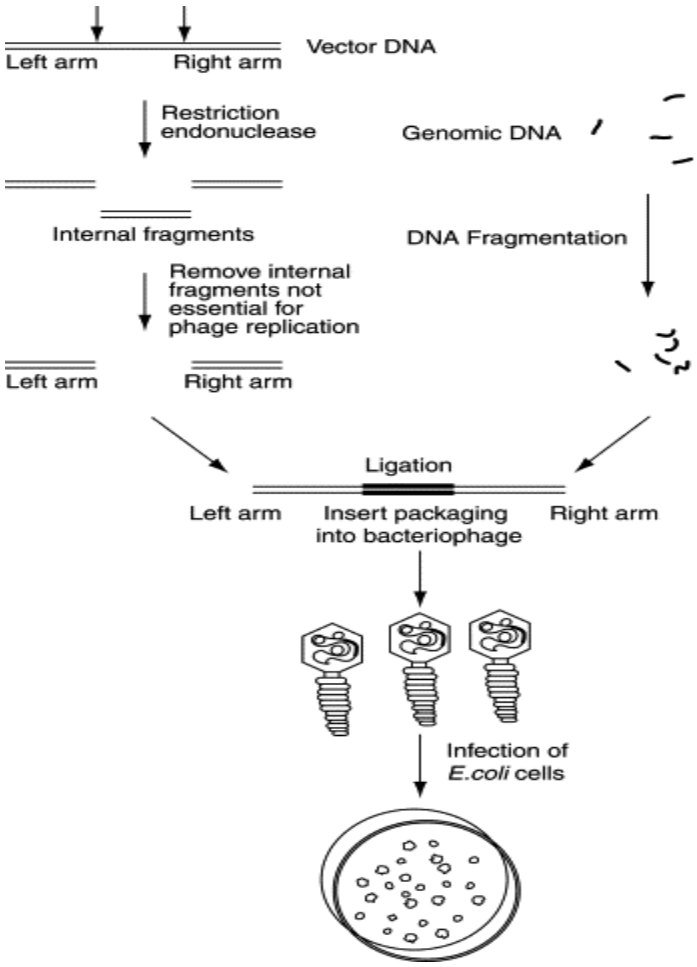
Genome **packaging** is a fundamental process in a **viral** life cycle. Many **viruses** assemble preformed capsids into which the genomic material is subsequently **packaged**. These **viruses** use a **packaging** motor protein that is driven by the hydrolysis of ATP to condense the nucleic acids into a confined space.

Bacteriophage Lambda mediated gene transfer

Bacteriophage lambda, a bacterial virus that infects *E. coli*, has been widely used as a cloning vector. Lambda is a well-characterized virus with both lytic and lysogenic alternatives to its life cycle. Although lambda DNA circularizes for replication and insertion into the *E.coli* chromosome, the DNA inside the phage particle is linear . At each end are complementary 12 bp long overhangs known as ***cos* sequences (cohesive ends)**. Once inside the *E. coli* host cell, these pair up and the cohesive ends are ligated together by host enzymes forming the circular version of the lambda genome.

If foreign DNA is inserted into the middle of lambda, the result is a linear DNA molecule with two cohesive ends. To get such constructs into an *E. coli* host cell efficiently requires ***in vitro* packaging** . In this technique, a mixture of lambda proteins is mixed with the recombinant lambda DNA *in vitro* to form phage particles





Infecting two separate *E. coli* cultures with two different defective lambda mutants generates the necessary lambda proteins. Each of the two mutants lacks an essential head protein and cannot form particles containing its own DNA. A mixture of the two lysates gives a full set of lambda proteins and when mixed with lambda DNA can -generate infectious phage particles.

SELECTION AND SCREENING OF RECOMBINANTS

SELECTION – After the introduction of **recombinant** DNA into the host cells, it is essential to identify those cells which received rDNA molecule - **screening** (or) **selection**. ... The ligated mix will comprise many copies of 13 different **recombinant** DNA molecules, one set of which carries the gene for kanamycin resistance.

There are two different methods of selection of recombinants. They are,

1.Direct method of selection of recombinants

2.Indirect method of selection of recombinants

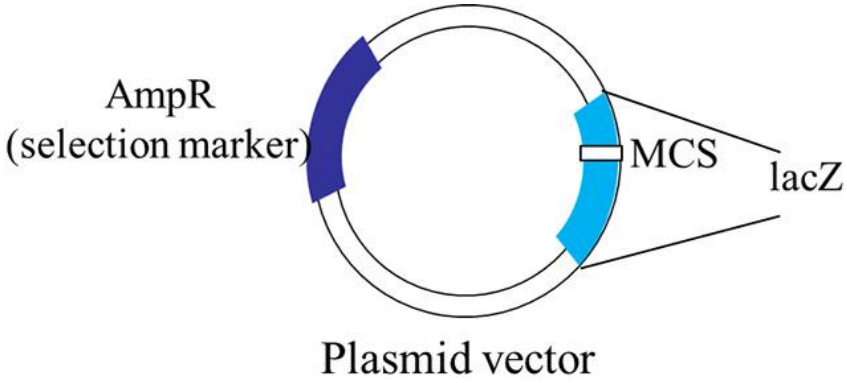
A number of **selection** systems have been developed for direct **selection of recombinant** plasmids in cloning experiments (positive **selection**). In this study, the commonly used LacZ-based α-**complementation** plasmid vectors have been used for designing a positive **selection** system for the **selection of recombinants**.

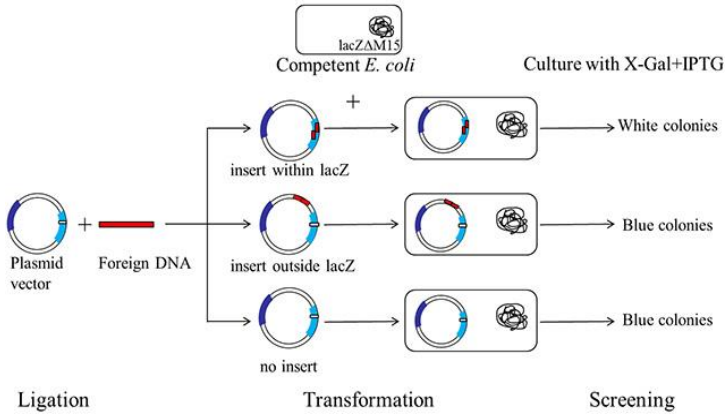
SELECTION OF RECOMBINANTS BY COMPLEMENTATION (Direct method)

Identification of Recombinant Bacteria

Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria. It relies on the activity of β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.

Preparation of Bacterial culture for transformation of RDNA through Viruses





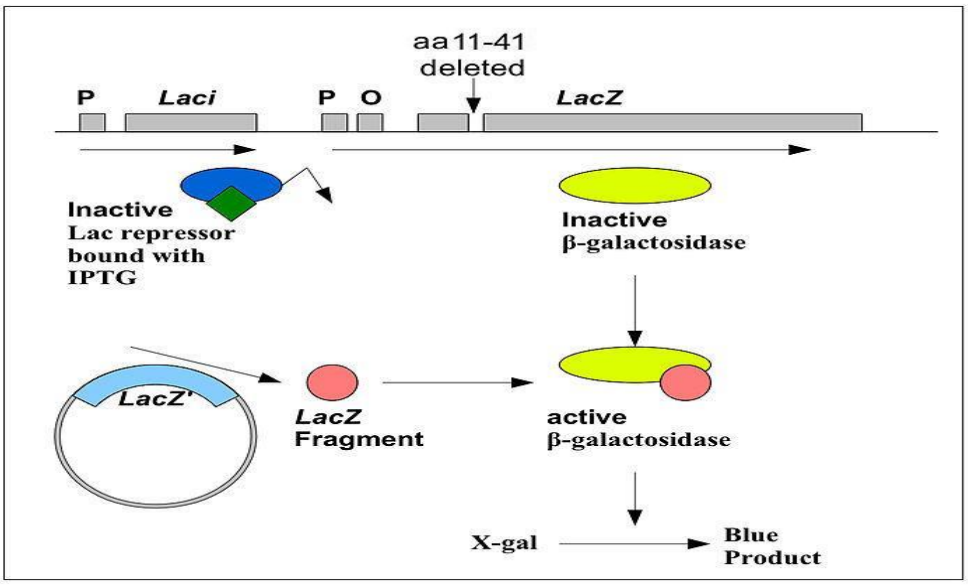
The **blue–white screen** is a screening technique that allows for the rapid and convenient detection of recombinant bacteria in vector-based molecular cloning experiments. DNA of interest is ligated into a vector.

The vector is then inserted into a competent host cell viable for transformation, which are then grown in the presence of X-gal. Cells transformed with vectors containing recombinant DNA will produce white colonies; cells transformed with non-recombinant plasmids (i.e. only the vector) grow into blue colonies.

This method of screening is usually performed using a suitable bacterial strain, but other organisms such as yeast may also be used.

Disrupting the LacZ Gene

The presence of lactose in the surrounding environment triggers the lacZ operon in *E. coli*. The operon activity results in the production of β-galactoisdase enzyme that metabolizes the lactose.

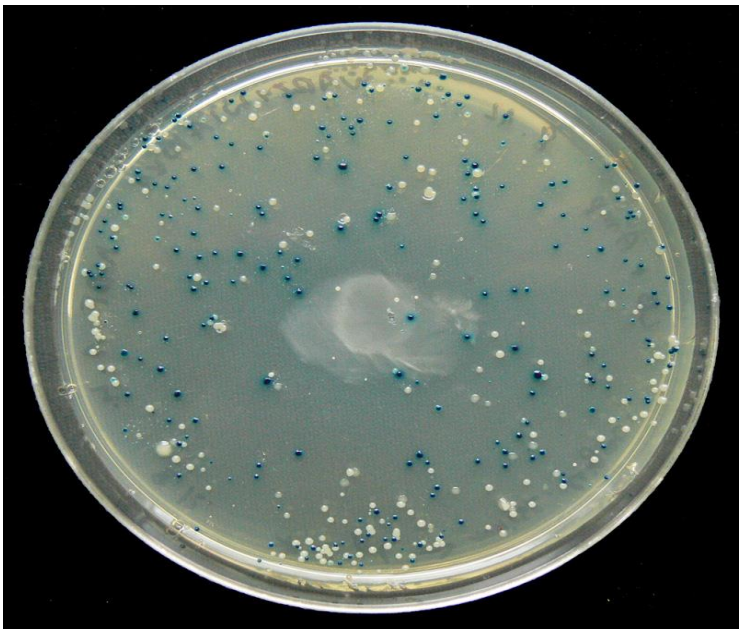


The plasmid vectors used in cloning are manipulated in such a way that this α-complementation process serves as a marker for recombination. A multiple cloning site (MCS) is present within the lacZ sequence in the plasmid vector.

This sequence can be nicked by restriction enzymes to insert the foreign DNA. When a plasmid vector containing foreign DNA is taken up by the host *E. coli*, the α-complementation does not occur, therefore, a functional β-galactosidase enzyme is not produced.

If the foreign DNA is not inserted into the vector or if it is inserted at a location other than MCS, the lacZ gene in the plasmid vector complements the lacZ deletion mutation in the host *E. coli* producing a functional enzyme.

Blue White Screening of Recombinants



For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.

Insertional Inactivation/ Marker inactivation method

- Insertional inactivation is a technique used in recombinant DNA technology. In this procedure, a bacteria carrying recombinant plasmids or a fragment of foreign DNA is made to insert into a restriction site inside a gene to resist antibiotics, hence causing the gene to turn non-functional or in an inactivated state.

- An example of this is a pUC19 plasmid vector in which the lacZ gene that encodes for beta-galactosidase can no longer be produced upon the insertion of a foreign gene. Selection or screening is a fundamental process in which once the recombinant DNA is inserted into a particular host cell, it becomes necessary to detect the cells that have received the recombinant or foreign DNA molecules.
- This process is referred to as screening or selection. It is based on non-expression or expression of certain traits or characteristics. Insertional inactivation is an effective method of screening. In this procedure, one of the genetic characteristics is disturbed by the introduction of foreign DNA. One of the most influential selection methods of recombinant plasmid for the insertional inactivation procedure is a method known as 'Blue-white' selection method.
- In this procedure, the lacZ gene which is a reporter gene is inserted in the vector. The enzyme β-galactosidase encoded by the lacZ gene comprises of a few recognition sites for restriction enzymes. The β-galactosidase enzyme splits a synthetic substrate X-gal, which is an organic compound abbreviated as BCIG (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) into an insoluble product that is blue in colour.
- If a foreign gene is introduced into lacZ, the gene will be deactivated. Hence no blue colour will develop as β-galactosidase is not produced due to deactivation of the lacZ gene. Consequently, the host cell comprising the rDNA will create white coloured colonies on the medium containing X-gal, whereas other cells bearing non-recombinant DNA will tend to develop blue coloured colonies. The recombinants are thus selected on the basis of the colour of the colony.

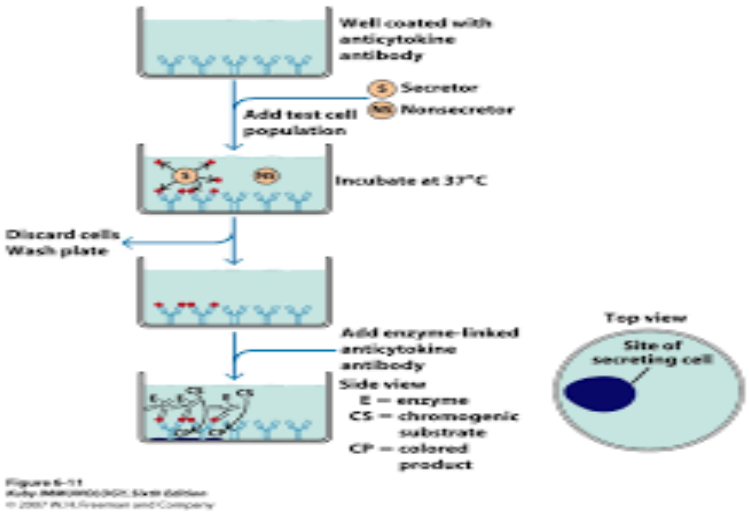
INDIRECT SELECTION METHOD OF RECOMBINANTS

An **immunological approach** to **screen recombinant** clones is possible if the gene of interest encodes a polypeptide for which specific antibodies can be prepared. In one **approach**, DNA complementary to mRNA is inserted in frame with the coding regions of genes present in E. coli plasmids.

Immunological method of screening

Immunoscreening is a method of to detect a polypeptide produced from a cloned gene. The term encompasses several different techniques designed for protein identification, for example Western blotting.

Clones are screened for the presence of the gene product (a protein). This strategy requires first that a gene library is implemented in an expression vector, and that antiserum to the protein is available. Radioactivity or an enzyme is coupled generally with the secondary antibody.

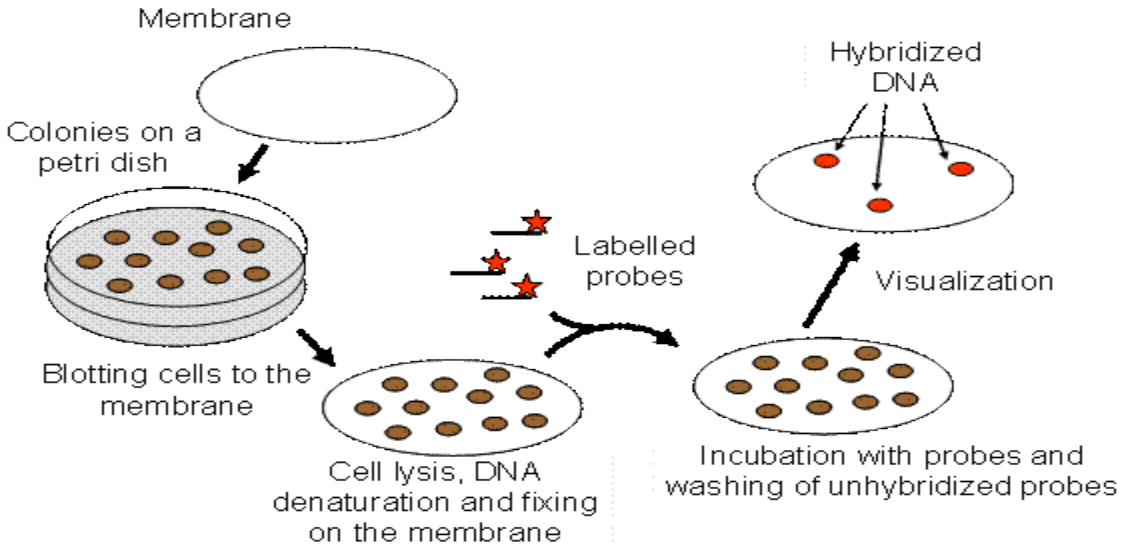


- The recombinant clones were filtered onto a hydrophobic grid membrane and grown up into individual colonies, and a replica was made onto nitrocellulose paper.
- The bacterial cells were then lysed with chloroform and the proteins were immobilized onto the nitrocellulose paper.
- The nitrocellulose paper is then reacted with a rabbit antibody preparation made against the particular antigenic product to detect the recombinant clone which carries the corresponding gene.
- The bound antibodies can be detected easily by a colorimetric assay using goat anti-rabbit antibodies conjugated to horseradish peroxidase.
- Positively reacting clones can be recovered from the master hydrophobic grid membrane filter for further characterization.

COLONY HYBRIDIZATION METHOD

Colony hybridization is a method of selecting bacterial colonies with desired genes. This method was discovered by Michael Grunstein and David S. Hogness. Colony hybridization is the "Blot analysis technique" where the bacterial cells are transferred from the solid nutrient medium to the absorbent material. Colony hybridization can define as the method for the isolation of the specific DNA sequences or genes from the bacterial cells containing hybrid DNA, by the means of a nitrocellulose membrane filter. The transferring medium then goes through several chemical and physical treatment.

- Colony hybridization begins with culturing sparsely populated bacterial colonies on a nutrient agar plate.
- These colonies are symmetrically replicated on a nitrocellulose filter by direct contact, after which the cells on the filter membrane are lysed and their DNA is denatured, allowing it to bind to the filter.
- These DNA clusters are then hybridized to a desired radioactively-labelled RNA or DNA probe (chosen specifically beforehand) and screened by autoradiography.
- DNA clusters that exhibit a desired gene are then matched up to the corresponding (living) bacterial colonies, which can be isolated for further growth and experimentation.



Transferring medium of Colony Hybridization:- The nitrocellulose filter paper is the transferring medium of the colony hybridization which forms replicas of the master plate. The

nitrocellulose acts as a membrane which contains the exact copies of the gene to that of the master plate. Nitrocellulose filter paper acts as the “Blotting pad”.

Colony hybridization involves the following steps:

- **Preparation of Master plate:-** First, inoculate the bacterial cell suspension on the solid agar medium to prepare the master plate. After the inoculation, the number of bacterial colonies will develop with different plasmids which refer as “Master or Reference plate”.
- **Formation of replicas over a nitrocellulose filter**:- Then transfer the bacterial cells from the master plate on to the membrane or filter by the means of “Nitrocellulose filter”. Press the nitrocellulose filter paper over the surface of the master plate. This compression of the filter membrane will form replicas or copies of the bacterial cells as that of the master plate.
- **Treatment of filter medium with SDS**:- After that treat the nitrocellulose filter paper with the detergent like SDS (Sodium dodecyl sulfate) to lyse the bacterial cells.
- **Treatment of filter medium with alkali**:- Treat the filter medium with the alkali like sodium hydroxide in order to separate the DNA into single strands.
- **Fixation of DNA onto the filter medium**:- To fix the DNA onto the nitrocellulose filter paper, either bake the filter paper at 80 degrees Celsius or expose it to the UV light.
- **Addition of radioactive probe**:- Hybridize the nitrocellulose filter paper containing imprints of the plasmid DNA by the addition of radioactive RNA probe. This radioactive RNA probe will code the desired gene of sequence from the bacterial cells.
- **Washing and Autoradiography**:- Wash the filter paper to remove unbound probe particles. After that, expose the nitrocellulose filter paper to the X-ray film by the method refer as “Autoradiography”. The colony which will appear after autoradiography will refer as “Autoradiogram” which carry the genes of interest.
- **Identification of the desired gene**:- Then compare the developed autoradiogram with the master plate to identify the colonies containing a gene of interest.
- The cells which contain the desired gene can grow in the liquid medium and can further process for the isolation of recombinant plasmid DNA.

UNIT V

Blotting (Southern, Western, Northern &North- eastern) techniques-PCR-basic steps in DNA amplification, RAPD, RFLP and their applications – DNA fingerprinting – DNA microarray analysis – Applications of recombinant DNA technology.

Blotting is used in molecular biology for the identification of proteins and nucleic acids and is widely used for diagnostic purposes. This technique immobilizes the molecule of interest on a support, which is a nitrocellulosic membrane or nylon. It uses hybridization techniques for the identification of the specific nucleic acids and genes.

The blotting technique is a tool used in the identification of biomolecules such ad DNA, mRNA and protein during different stages of gene expression. Protein synthesis involves expression of a DNA segment which gets converted to mRNA to produce the respective protein.

SOUTHERN BLOTTING TECHNIQUE

Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975).

Principle:

- This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/ Steps

1. Restriction digest: by RE enzyme and amplification by PCR
2. Gel electrophoresis: SDS gel electrophoresis
3. Denaturation: Treating with HCl and NaOH
4. Blotting
5. Baking and Blocking with casein in BSA
6. Hybridization using labelled probes
7. Visualization by autoradiogram

Step I: Restriction digest

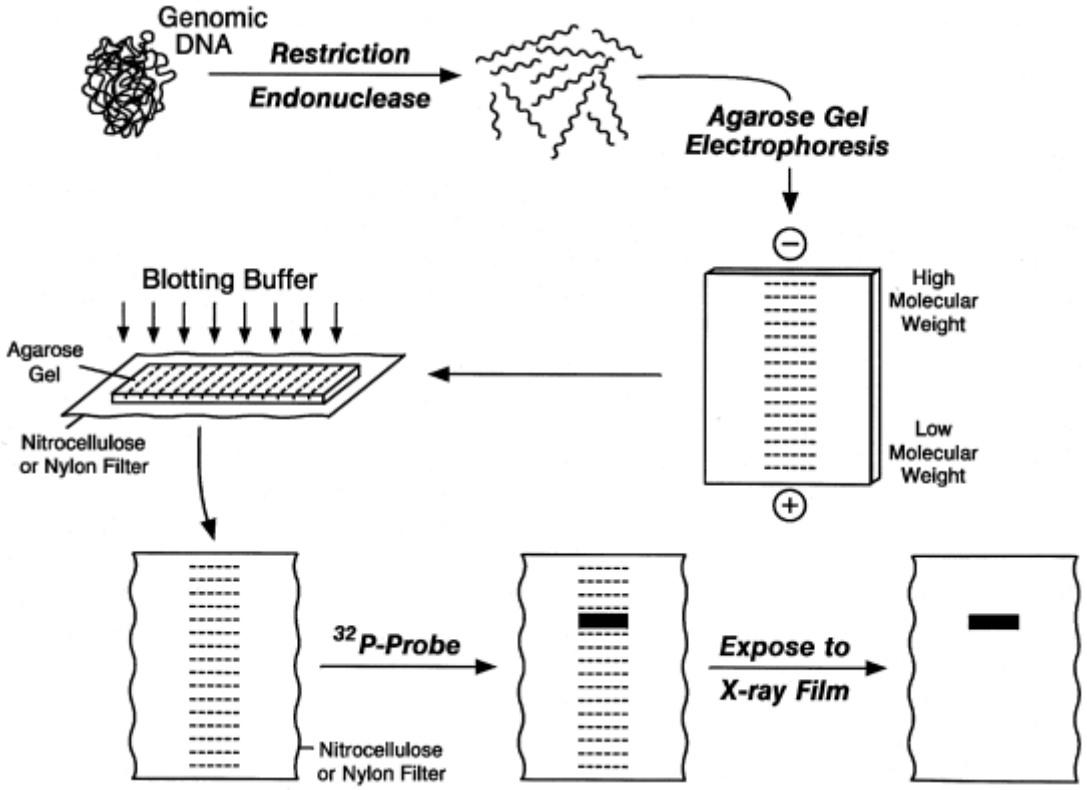
- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments
- The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

- The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.
- DNA strands get separated.



Step IV: Blotting

- The separated strands of DNA is then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

- The DNA bound to membrane is then treated with labelled probe
- The labelled probe contains the complementary sequences to the gene of interest
- The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Application of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting
3. Used for paternity testing, criminal identification, victim identification
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism
6. To identify mutation or gene rearrangement in the sequence of DNA
7. Used in diagnosis of disease caused by genetic defects
8. Used to identify infectious agents

NORTHERN BLOTTING GTECHNIQUE

Northern Blotting is a technique used for the study of gene expression. It is done by detection of particular RNA (or isolated mRNA). mRNA is generally represented as 5% of the overall RNA sequence.

The northern blot technique was developed in 1977 by James Alwine, David Kemp and George Stank at Stanford University. The technique got its name due to the similarity of the process with Southern blotting. The primary difference between these two techniques is that northern blotting concerns only about RNA.

Principle

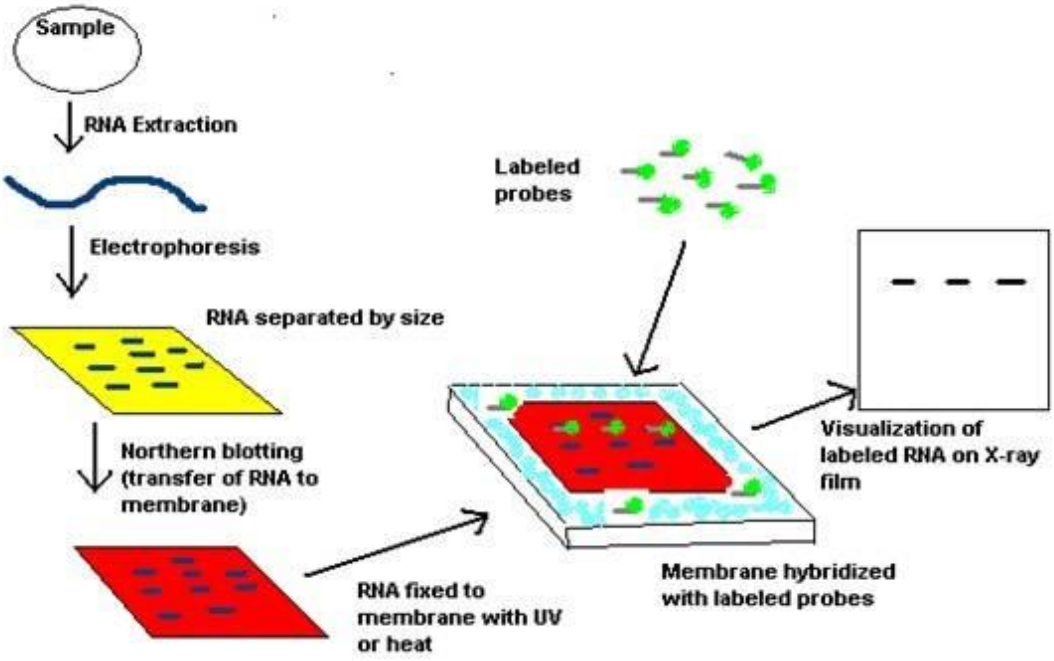
As all normal blotting technique, northern blotting starts with the electrophoresis to separate RNA samples by size. Electrophoresis separates the RNA molecules based on the charge of the nucleic acids. The charge in the nucleic acids is proportional to the size of the nucleic acid sequence. Thus the electrophoresis membrane separates the Nucleic acid sequence according to the size of the RNA sequence.

The selection of nylon membrane is contributed to the factor that nucleic acids are negatively charged in nature. Once the RNA molecules are transferred it is immobilized by covalent linkage.

The probe is then added, the probe can be complementary an ss DNA sequence. Formamide is generally used as a blotting buffer as it reduces the annealing temperature.

Procedure

1. The tissue or culture sample collected is first homogenized. The samples may be representative of different types of culture for comparison or it can be for the study of different stages of growth inside the culture.
2. The RNA sequence is separated in the electrophoresis unit an agarose gel is used for the purpose of the nucleic acid separation.



3. Now the separated RNA sequence is transferred to the nylon membrane. This is done by two mechanisms capillary action and the ionic interaction.

4. The transfer operation is done by keeping the gel in the following order. First, the agarose gel is placed on the bottom of the stack, followed by the blotting membrane. On top of these paper towels a mild weight (glass plate) is placed. The entire setup is kept in a beaker containing transfer buffer.
5. RNA transferred to the nylon membrane is then fixed using UV radiation.
6. The fixed nylon membrane is then mixed with probes. The probes are specifically designed for the gene of interest, so that they will hybridize with RNA sequences on the blot corresponding to the sequence of interest.
7. The blot membrane is washed to remove unwanted probe
8. Labeled probe is detected by chemiluminescence or autoradiography. The result will be dark bands in x ray film.

APPLICATIONS

- Southern blots are used in gene discovery , mapping, evolution and development studies, diagnostics and forensics (It is used for DNA fingerprinting, preparation of RFLP maps)
- identification of the transferred genes in transgenic individuals, etc.
- It is an invaluable method in gene analysis.
- Important for the conformation of DNA cloning results.
- Highly useful for the determination of restriction fragment length polymorphism (RFLP) associated with pathological conditions.

WESTERN BLOTTING TECHNIQUE

Western blot is often used in research to separate and identify proteins. In this **technique** a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein.

Western blot is the analytical **technique** used in molecular biology, immunogenetics and other molecular biology to detect specific proteins in a sample of tissue homogenate or extract. **Western blotting** is called so as the procedure is similar to Southern **blotting.**

Principle

It works on the principle of gel electrophoresis (SDS-PAGE). Proteins are separated based on their size on polyacrylamide gel.

Steps in western blotting:

- Sample prepration
- Gel electrophoresis of protein sample
- Protein transfer
- Protein staining
- Blocking non-specific antibody
- Antibody probing
- Washing
- Protein detection
- Digital imaging

1.Sample preparation

- Detergent for the lysis of tissue culture.
- Ultrasonication for cell suspension.
- Mechanical homogenization for plant animal tissue.
- Enzymatic digestion for bacterial, yeast, and fungal cells.

2.Gel electrophoresis

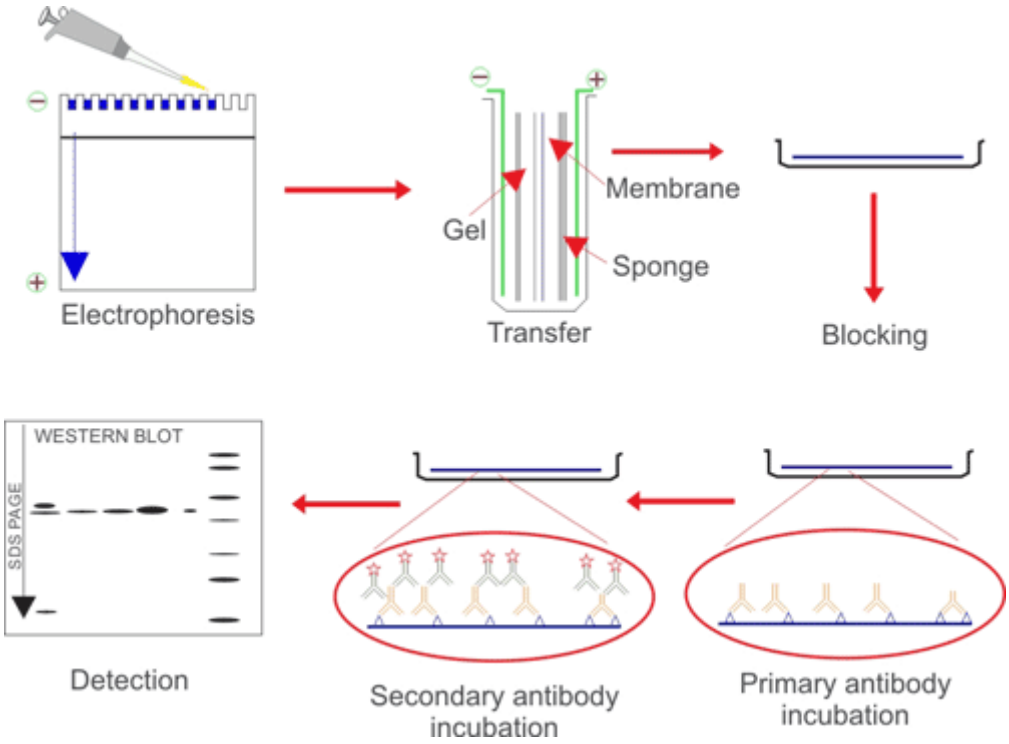
Gel electropgoresis is commonly used methos for separating proteins on the basis of size, shape or charge. In this SDS PAGE electrophoresis is used by which protein sample are separated according to there molecular weight.

PAGE protocol

- Load the sample into the wells of electrophoresis equipment.
- Load the sample into empty wells.
- Place the safety lid on the unit.
- Plug the color coded leads to the jacks in the power supply.
- Run the gel under the appropriate conditions.
- When tracking gel reached the bottom of the gel switched off the power supply.
- Disconnect the leads and removes safety.
- Remove the gel from electrophoresis appratus.
- Then proceed to protein transfer.

Western blotting procedure

- Load and separate protein sample on SDS-PAGE.
- Electrophoretically transfer fractionated proteins onto membrane.
- Block the membrane with neutral proteins (BSA or milk casein).
- Incubate the membrane with primary antibody specific to target protein.
- Incubate the membrane with HRP (Horse Radish Peroxidase) labeled secondary antibody specific to primary antibody.
- Incubate the blot with chemiluminescent HRP substrate and expose to X-ray film.



Blocking

For meaningful result the antibodies must bind only to the protein of interest and are not to the membrane. non-specific binding of antibodies can be reduced by blocking the unoccupied sites of membrane with an inert protein or non-ionic detergent.

Blocking agents possess a greater affinity towards membrane than the antibodies. example of blocking agents are:- **bovine serum albumin(BSA)**, **dry milk**, **gelatine**, **dilute solution of tween20**.

Antibody probing

- After blocking the blot is incubated with many antibodies.
- This uses a specific antibody to detect a localize the protein blotted to a membrane. The specificity of antigen of antigen-antibody binding permits the identification of a single protein in a complex sample.
- The non labeled primary antibody directed against the target protein, and specific labeled secondary antibody binds to the primary antibody.
- The secondary antibody is conjugated to an enzyme that is used to indicate the location of the protein.The secondary antibodies not only serves as a carrier of the label, but it is also helps to amplify the emitted signals.

Washing

Unbound antibodies can make high background and poor detection. hence washing the blot removes unbound antibodies from the membrane.

A diluted solution of tween-20 in TBS or PBS buffer is mainly used for washing.

Protein detection

After the unbound probes washed away, the western blotting is now ready for detection of the probes that are labeled and bound to the protein of interest.

Enzyme such as alkaline phosphatase & horse radish peroxidase are widely used in detection of proteins.

Analysis & Imaging

This is the last and major step of western blotting technique. Detection of signals using either x-ray film, scanners results in one or more visible proteins bands on the membrane image.

The molecular weight of the protein can be estimated by comparision with marker proteins and the amount of protein can be determined as this is related to band intensity.

Applications of Western blotting

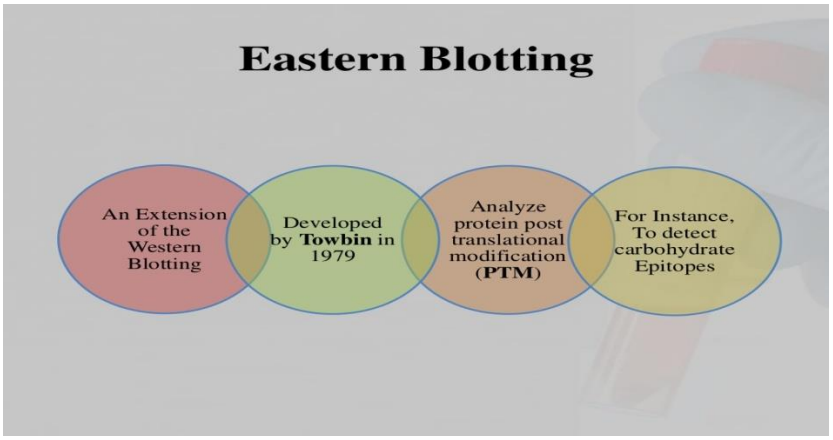
- Diagnosis of HIV by **ELISA**, includes the western blotting technique.
- Western blotting technique is also used to detect different forms of lyme disease.
- Western blotting technique is used in defence test for BSE, which is commonly known as mad cow disease.
- Confirmatory tests for hepatitis-B is done by western blotting technique.
- This technique is also employed in gene expression studies.

Limitation of western blotting

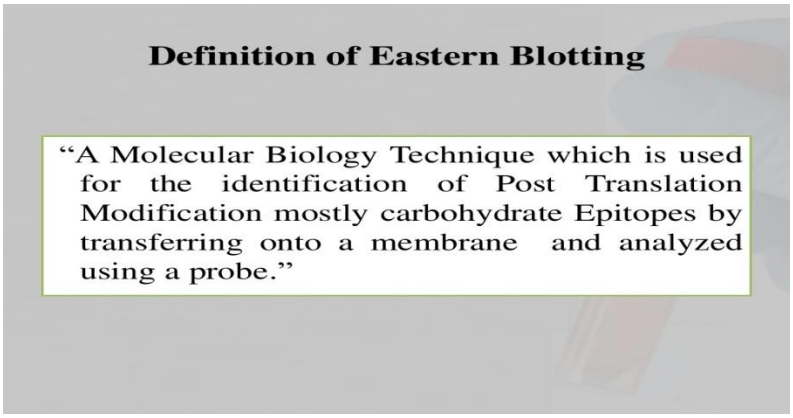
- Very delicate and time consuming process.
- Incorrect labeling of protein can happen due to the reaction of secondary antibody.

NORTH - EASTERN BLOTTING TECHNIQUE

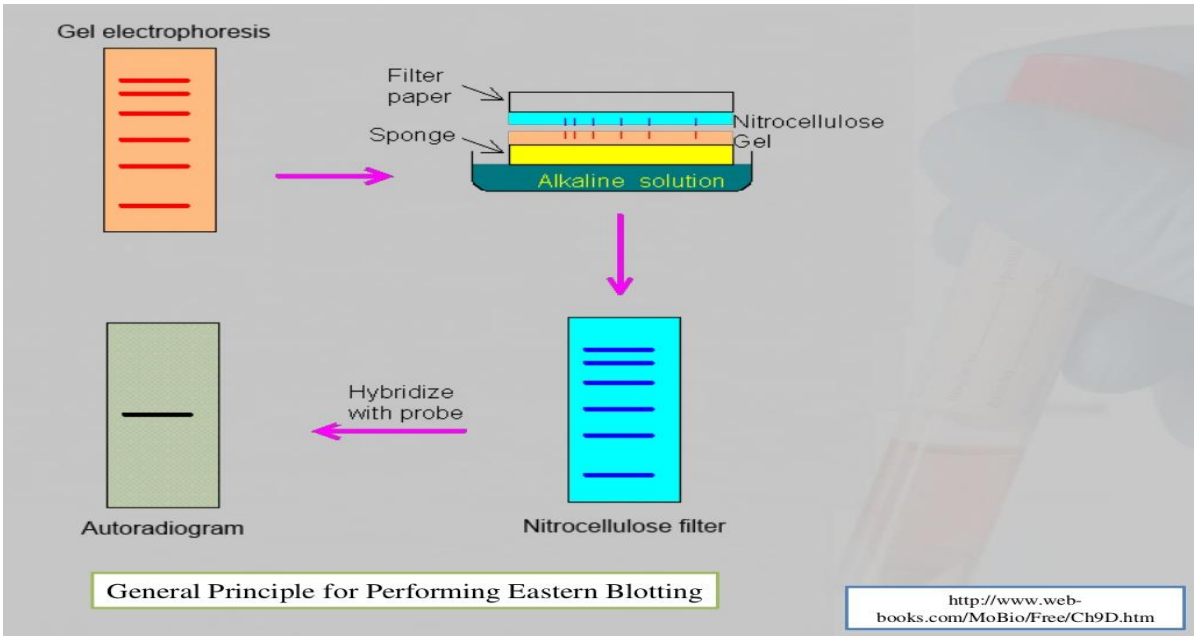
Introduction



Definition



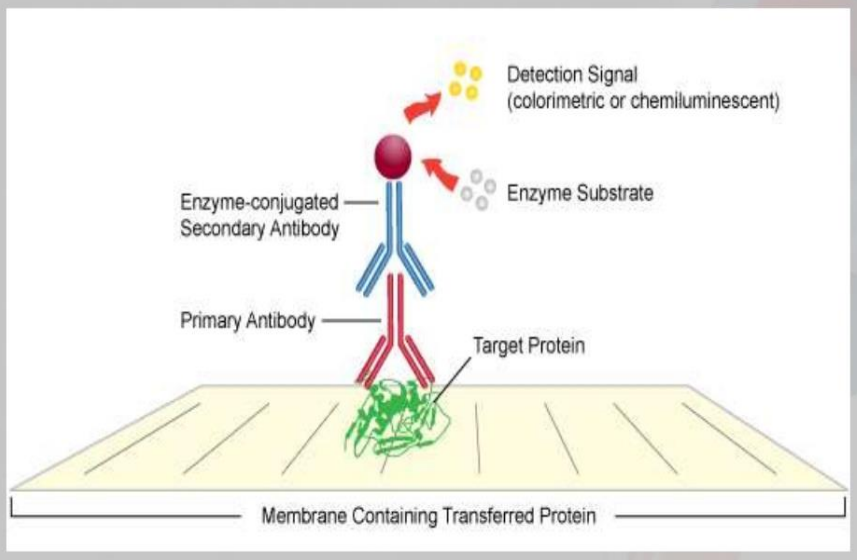
Principle



Procedure

Method

1. **Separation**: targeted molecules are vertically separated by using gel electrophoresis
2. **Transfer:** Separated molecules are transferred horizontally on the nitrocellulose membrane
3. **Addition of Primary Antibody**: primary antibody is added to the solution.
4. **Washing:** Then wash it to remove unbound primary antibody
5. **Secondary Antibody Addition**: Add labeled secondary antibody with an enzyme which will emit the fluorescence when product is formed.
6. **Confirmation**: These labeled probes confirm the molecule of interest by autoradiography.



Attachment of Antibodies with target molecule and luminescence Production

Example

Fingerprint by Eastern Blotting:

- Solasodine glycosides were developed on a TLC plate.
- The TLC plate was covered with the PVDF membrane and blotted by short heating.
- The blotted PVDF membrane was dipped in water containing NaIO4under stirring at room temperature for1 hr.
- After washing with water, a carbonate buffer containingBSA was added and stirred for 3 hr.
- ThePVDF membrane was washed twice with phosphate bufferfor 5 min
- The PVDF membrane was immersed in anti-solamargine MAb, stirred at room temperature for 1 hr. After washing the PVDF membrane twice with phosphate buffer and water, a 1000-fold dilution of peroxidase-labeled goat anti-mouse IgG in phosphate buffer (pH 7.2) was added and stirred at room temperature for 1 hr.
- The PVDF membrane was washed twice with phosphate buffer and water, then exposed to 4-choloro-1-naphthol (1 mg/mL)—H_2O_2(0.03%) in phosphate buffer(pH 7.2), and eastern blotting was stopped by washing with water.
- The immuno-stained PVDF membrane was allowed to dry.

Applications

Detection of protein modification.

Used for binding studies by using various ligands

Used to purify various phospholipids

Help in translocation studies across biological membranes.

Expression of post-translated proteins in several diseases

To study Protein modifications in bacterial species

Comparing the protein modification of two bacterial species

Antigenic proteins of the non-virulent E. muris is more post-translationally modified than the highly virulent IOE

POLYMERASE CHAIN REACTI0N

- PCR is a technique that results in exponential amplification of a selected region of a DNA molecule.
- PCR is widely held as one of the most important inventions of the 20th century in molecular biology.
- The idea of PCR is credited to *Kary Mullis*, a research scientist at California Biotech Company, Cetus, in 1983.
- For this work, Mullis received the Nobel Prize in Chemistry jointly with *Michael Smith* in 1993.

Principle:

- Polymerase chain reaction is method for amplifying particular segments of DNA. It is an enzymatic method and carried out invitro.
- PCR is very simple, inexpensive technique for characterization, analysis and synthesis of specific fragments of DNA or RNA from virtually any living organisms.

Procedure

PCR consists of three basic steps.

1. Denaturation:

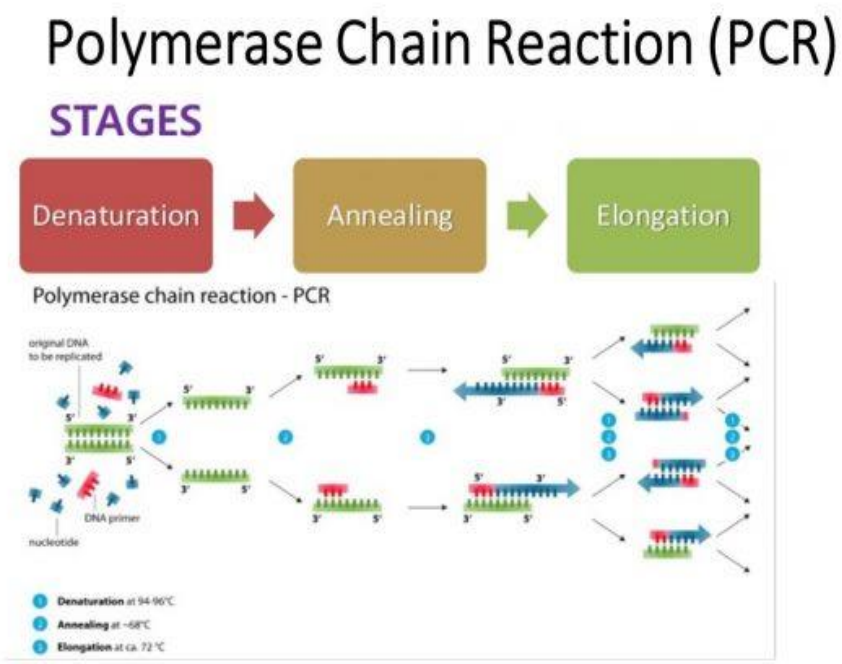
- Two strand of DNA separates (melt down) to form single stranded DNA
- This step is generaly carried out at 92C-96C for 2 minutes.

2. Annealing:

- Annealing of primer to each strand is carried out at 45C-55C

3. Extension:

- DNA polymerase adds dNTPs complementary to templates strands at 3'end of primer.
- It is carried out at temperature of 72C.
  -
  -



- These three steps are repeated 20-30 times in an automated thermocycler that can heat and cool the reaction mixture in tube within very short time. This results in exponential accumulation of specific DNA fragments.
- The doubling of number of DNA strands corresponding to target sequences can be estimated by amplification number associated with each cycle using the formula.
- Amplification=2n, where n=no. of PCR cycle.
- PCR can amplify a desired DNA sequences of any origin hundred or millions time in a matter of hour, which is very short in comparison to recombinant DNA technology.
- PCR is especially valuable because the reaction is highly specific, easily automated and very sensitive.
- It is widely used in the fields like- clinical medicine for medical diagnosis, diagnosis of genetic diseases, forensic science; DNA finger printing, evolutional biology

Factors affecting PCR

i. Primer

- PCR reaction needs two primer, a forward and a reverse primer
- Primer are synthesized oligonucleotide usually ranging from 15-30 bases long

- Primers are designed such that at 3'end they donot have more than two bases complementary to each other as this results in PRIMER-DIMER formation.
- The G+C contents is in the range of 40-60%
- The melting temperature (Tm) of both forward and reverse primer is usually the same.
- Low concentration of primer results in poor yield while high concentration may results in non specific amplification. Hence optimal concentration of primer is needed ie 0.1-1µ

ii. Amount of Template DNA

- Optimal amount of template DNA usually in nano gram. Higher concentration inhibit or results in non specific amplification.
- Taq DNA polymerase:
- Taq DNA polymerase is 94 KD thermostable DNA polymerase isolated from Thermus aquaticus.
- Optimal temperature for activity of Taq polymerase is 72° but it can tolerate high temperature and donot affects by denaturating temperature of 94°C.
- Taq DNA polymerase have both 5'-3' polymerase activity and 5'-3' exonuclease activity. But it lacks 3'-5' exonuclease activity (proof reading activity).

Types of PCR

1. Restriction fragment length polymorphism (RFLP)
2. Amplified fragment length polymorphism (AFLP)
3. Random amplified polymorphic DNA
4. Reverse transcriptase Polymerase chain reaction (RT-PCT): for RNA
5. One step RT-PCR
6. Two step RT-PCR
7. Real time PCR: for DNA or RNA
8. Dye binding to ds DNA
9. Fluorescent probes

Application:

1. Forensic science: DNA finger printing, paternity testing and criminal identification
2. Diagnosis: Molecular identification of microorganisms
3. Evolution study: evolutionary biology
4. Fossil study: paleontology
5. Gene cloning and expression
6. Gene sequencing

7. Vaccine production by recombinant DNA technology
8. Drug discovery
9. Mutation study
10. Human genome project.

Limitations

- One major limitation of PCR is that prior information about the target sequence is necessary in order to generate the primers that will allow its selective amplification.
- Like all enzymes, DNA polymerases are also prone to error, which in turn causes mutations in the PCR fragments that are generated.
- Another limitation of PCR is that even the smallest amount of contaminating DNA can be amplified, resulting in misleading or ambiguous results.

Random Amplified Polymorphic DNA (RAPD)

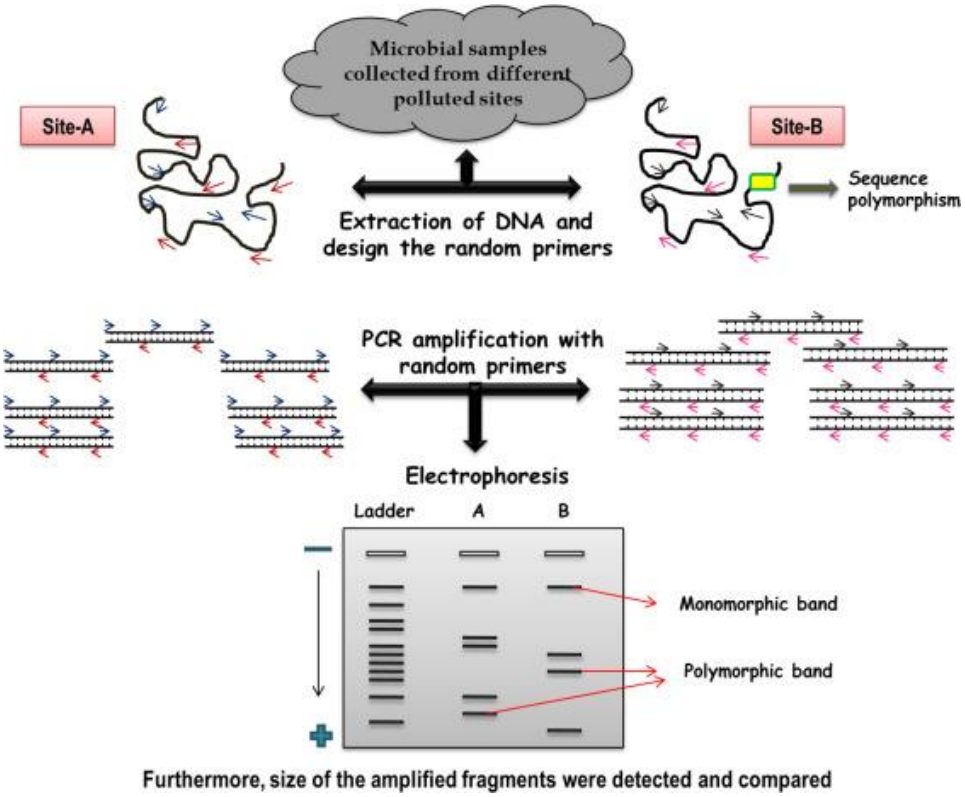
Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is a PCR based technique for identifying genetic variation. It involves the use of a single arbitrary primer in a PCR reaction, resulting in the amplification of many discrete DNA products. The technique was developed independently by two different laboratories.

Principle

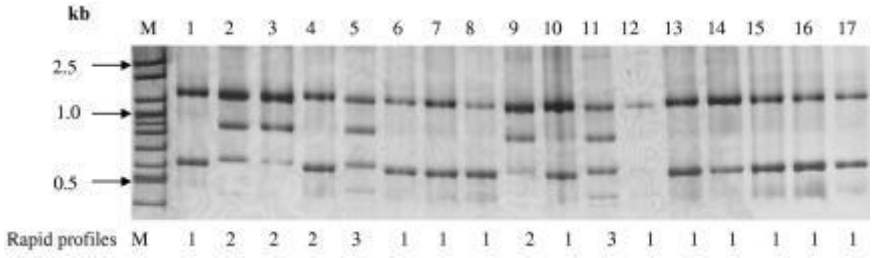
Unlike traditional PCR analysis, RAPD (pronounced "rapid") does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no 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.

Procedure



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 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.

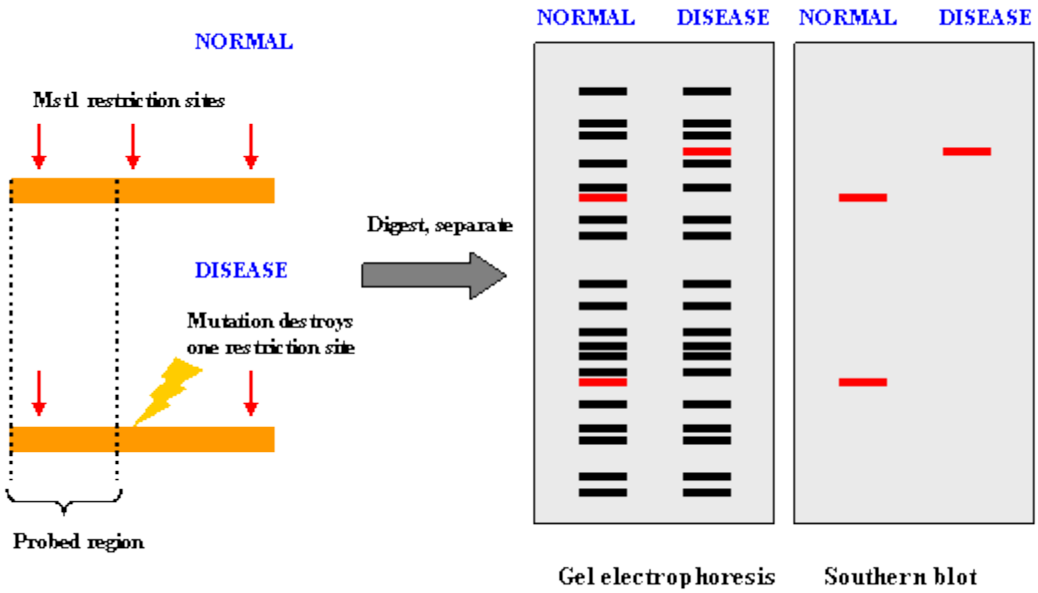
Restriction Fragment Length Polymorphism (RFLP)

Introduction

Restriction Fragment Length Polymorphism (RFLP)

- It 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 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.).

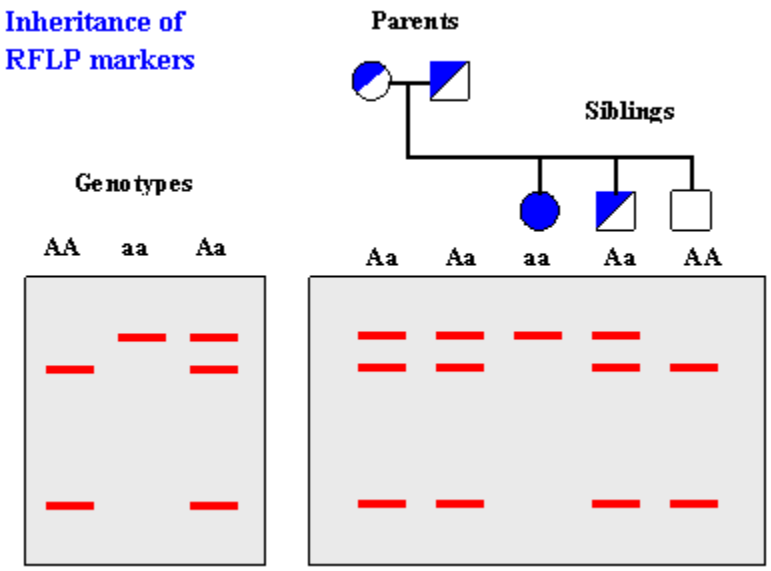
How It Works



Principle of RFLP:

RFLP is an enzymatic procedure for separation and identification of desired fragments of DNA. Using restriction endonuclease enzymes fragments of DNA is obtained and the desired fragment is detected by using restriction probes. Southern hybridization using restriction endonuclease enzymes for isolation of desired length of DNA fragments is an example of RFLP.

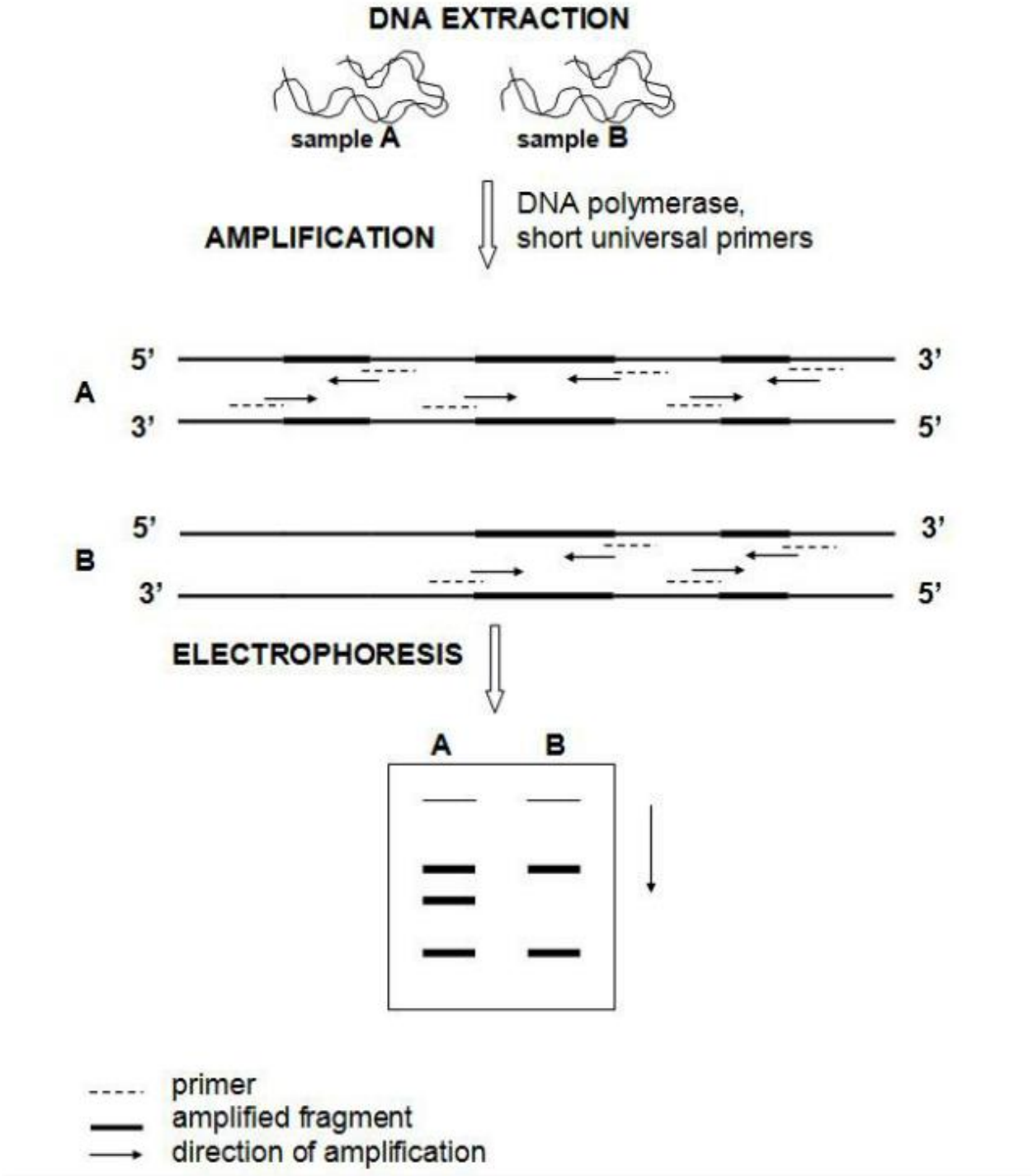
Genotyping



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 that hybridize to single- and low-copy sequences.
- The probes are screened for RFLPs using genomic DNA of different genotypes digested with restriction endonucleases. Typically, in species with moderate to high polymorphism rates, two to four restriction endonucleases are used such as EcoRI.

Procedure


DNA EXTRACTION
sample A
sample B
AMPLIFICATION
DNA polymerase,
short universal primers
A
5'
3'
3'
5'
B
5'
3'
3'
5'
ELECTROPHORESIS
A
B
primer
amplified fragment
direction of amplification

Step I: Restriction digest

- Extraction of desired fragments of DNA using restriction endonuclease (RE).
- The enzyme RE has specific restriction site on the DNA, so it cut DNA into fragments. Different size of fragments are generated along with the specific desired fragments.

Step II: Gel electrophoresis

- The digested fragment are run in polyacrylamide gel electrophoresis or Agarose gel electrophoresis to separate the fragments on the basis of length or size or molecular weight.
- Different size of fragments form different bands.

Step III: Denaturation

- The gel is placed in sodium hydroxide (NaOH) solution for denaturation so that single stranded DNA are formed.

Step IV: Blotting

- The single stranded DNA obtained are transferred into charge membrane ie. Nitrocellulose paper by the process called capillary blotting or electro-blotting.

Step V: Baking and blocking

- The nitrocellulose paper transferred with DNA is fixed by autoclaving.
- Then the membrane is blocked by using bovine serum albumin or casein to prevent binding of labelled probe nonspecifically to the charged membrane.

Step VI: Hybridization and visualization

- The labelled RFLP probe is hybridized with DNA on the nitrocellulose paper.
- The RFLP probes are complimentary as well as labelled with radioactive isotopes so they form color band under visualization by autoradiography.

Application of RFLP test:

- **Genome mapping:** helps in analysis of unique pattern in genome for organism identification and differentiation. It also helps in determining recombination rate in the loci between restriction sites.
- **Genetic disease analysis:** After identification of gene for particular genetic or hereditary disease, that gene can be analyzed among other family members.
- To detect mutated gene.
- **DNA finger printing (forensic test):** It is the basis of DNA finger printing for paternity test, criminal identification etc.

DNA MICROARRAY ANALYSIS

Introduction of DNA Microarray technique:

- Also termed as DNA chips, gene chips, DNA arrays, gene arrays and biochips.
- Biochips are latest generation of biosensors developed by use of DNA probes.
- DNA microarray is one of the molecular detection techniques which is a collection of microscopic characteristics (commonly DNA) affixed to a solid surface.
- DNA microarrays are solid supports usually made up of glass or silicon upon which DNA is attached in an organized pre-arranged grid design.
- Each spot of DNA, termed as probe, signifies a single gene.
- DNA microarrays can examine the expression of tens of thousands of genes concurrently.
- There are 2 types of DNA microarray i.e. cDNA based microarray and oligonucleotide based microarray.

Principle of DNA microarray:

- DNA microarray technology was originated from Southern blotting, in which fragmented DNA is attached to a substrate and then probed with a known DNA sequence.
- DNA microarray is based on principle of hybridization between the nucleic acid strands.
- Unknown sample of DNA sequence is termed as sample or target and the known sequence of DNA molecule is called as probe.
- Fluorescent dyes are used for labelling the samples and at least 2 samples are hybridized to the chip.
- Thus, the fluorescent labeled target sequences that pairs to the probe releases a signal that relies on the strength of the hybridization detected by the number of paired bases, hybridization conditions, and washing after hybridization.
- DNA microarrays employs relative quantization in which the comparison of same character is done under two different conditions and the identification of that character is known by its position.
- After completion of the hybridization, the surface of chip can be examined both qualitatively and quantitatively by use of autoradiography, laser scanning, fluorescence detection device, enzyme detection system.
- The presence of one genomic or cDNA sequence in 1,00,000 or more can be screened in a single hybridization by using DNA microarray.

Types of DNA microarray:

1. cDNA based microarrays
2. Oligonucleotide based microarrays

cDNA based microarrays:

- cDNA is used for the preparation of chips.
- cDNAs are amplified by PCR.
- It is a high throughput technique.
- It is highly parallel RNA expression assay technique that allows quantitative analysis of RNAs transcribed from both known and unknown genes.

Oligonucleotide based microarrays:

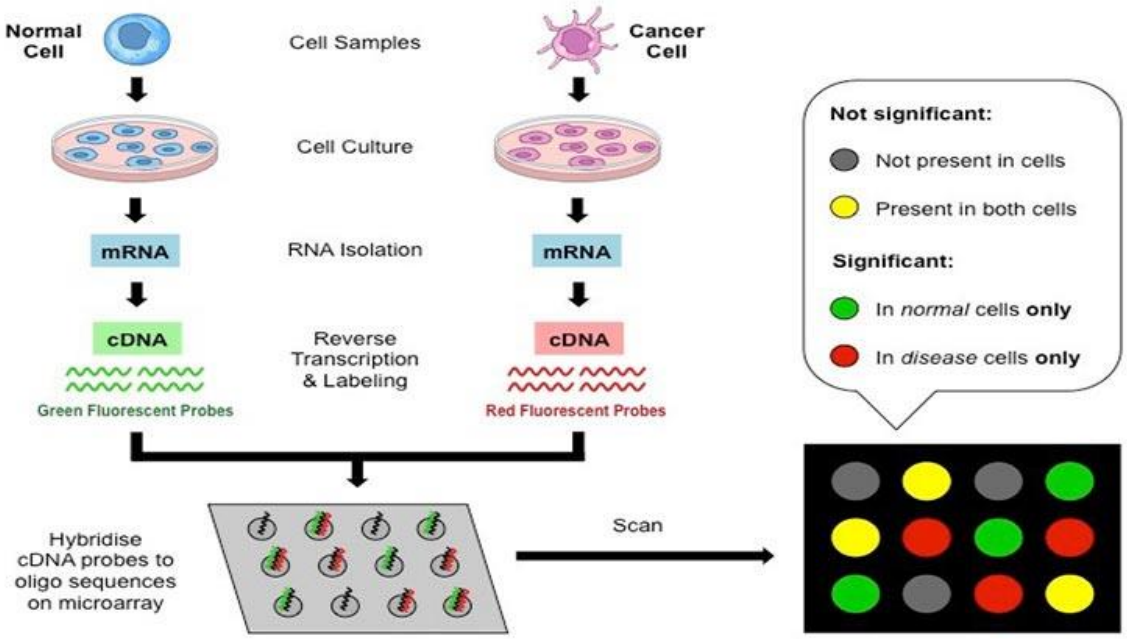
- In this type, the spotted probes contains of short, chemically synthesized sequences, 20-25 mers/gene.
- Shorter probe lengths allows less errors during probe synthesis and enables the interrogation of small genomic regions, plus polymorphisms
- Despite being easier to produce than dsDNA probes, oligonucleotide probes need to be carefully designed so that all probes acquire similar melting temperatures (within 5^0 c) and eliminate palindromic sequences.
- The probe's attachment to the glass slides takes place by the covalent linkage as electrostatic immobilization and cross-linking can result in significant loss of probes during wash steps due to their small size.
- The coupling of probes to the microarray surface takes place via modified 5′ to 3′ ends on coated slides that provide functional groups (epoxy or aldehyde)

Requirements of DNA microarray:

- DNA chip
- Fluorescent dyes
- Fluorescent labelled target/sample
- Probes
- Scanner

Steps involved in cDNA based microarray:

1. Sample collection
2. Isolation of mRNA
3. Creation of labeled cDNA
4. Hybridization
5. Collection and analysis



Sample collection:

- A sample can be any cell/tissue that we desire to conduct our study on.
- Generally, 2 types of samples are collected, i.e. healthy and infected cells, for comparing and obtaining the results.

Isolation of mRNA:

- The extraction of RNA from a sample is performed by using a column or solvent like phenol-chloroform.
- mRNA is isolated from the extracted RNA leaving behind rRNA and tRNA.
- As mRNA has a poly-A tail, column beads with poly-T tails are employed to bind mRNA.
- Following the extraction, buffer is used to rinse the column inorder to isolate mRNA from the beads.

Creation of labeled cDNA:

- Reverse transcription of mRNA yields cDNA.
- Both the samples are then integrated with different fluorescent dyes for the production of fluorescent cDNA strands which allows to differentiate the sample category of the cDNAs.

Hybridization:

- The labeled cDNAs from both the samples are placed on the DNA microarray which permits the hybridization of each cDNA to its complementary strand.
- Then they are thoroughly washed to remove unpaired sequences.

Collection and analysis:

- Microarray scanner is used to collect the data.

- The scanner contains a laser, a computer and a camera. The laser is responsible for exciting the fluorescence of the cDNA, generating signals.
- The camera records the images produced at the time laser scans the array.
- Then computer stores the data and yields results instantly. The data are now analyzed.
- The distinct intensity of the colors for each spot determines the character of the gene in that particular spot.

Applications of DNA microarray technique:

- Drug discovery
- Study of functional genomics
- DNA sequencing
- Gene expression profiling
- Study of proteomics
- Diagnostics and genetic engineering
- Toxicological researches
- Pharmacogenomics and theranostics.

APPLICATIONS OF RECOMBINANT DNA TECHNOLOGY

- Recombinant DNA technology has a wide range of application in industries, medical science, and agriculture as "well as molecular biology.
- Molecular diagnosis of diseases
- Gene therapy
- DNA finger printing
- Production of vaccines
- Commercial and pharmaceutical products

- **Agricultural Applications**
  - Improvements in nutritional value and yield
    - Tomatoes allowed to ripen on vine and shelf life increased
      - Gene for enzyme that breaks down pectin suppressed
    - BGH allows cattle to gain weight more rapidly
      - Produce meat with lower fat content and produce 10% more milk
    - Gene for β-carotene (vitamin A precursor) inserted into rice
    - Scientists considering transplanting genes coding for entire metabolic pathways

ALL THE BEST