GENETIC ENGINEERING ITS TECHNIQUES AND APPLICATIONS

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Introduction:

Genetic engineering refers to the direct manipulation of DNA to alter an organism's characteristics (phenotype) in a particular way. It is also called Genetic Modification or Genetic Manipulation. It is a part of Biotechnology.

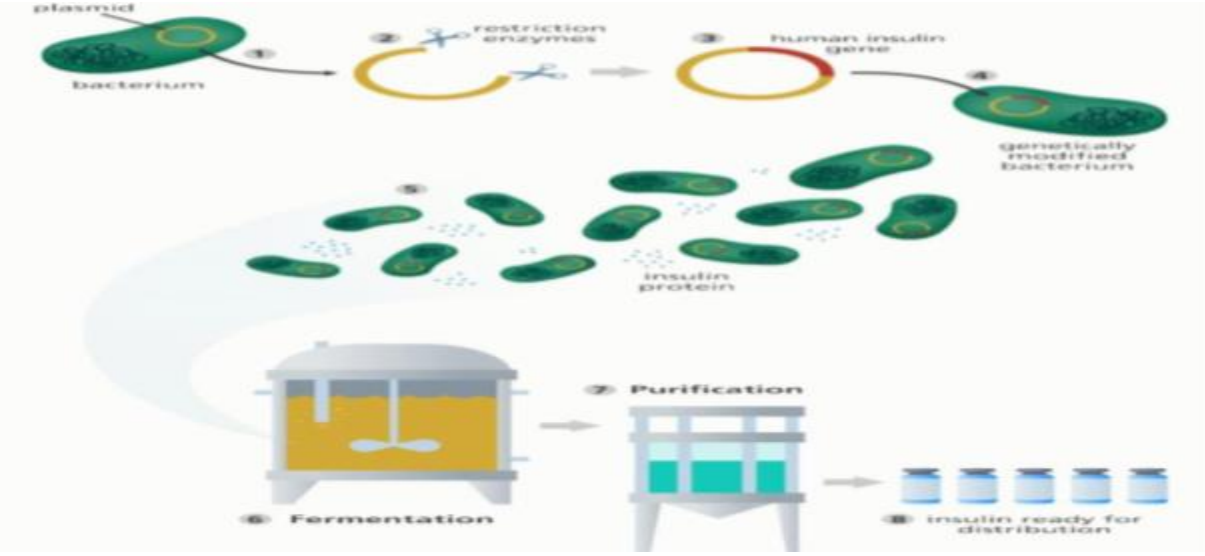
Biotechnology:

Definition:

It is the Industrial use of organisms for human welfare is called biotechnology. e.g. use of organisms for the manufacture of beer and milk products.

Genetic engineering:

It is the artificial manipulation Modification and Recombination of DNA or other Nucleic acid in order to modify an organism or population of organisms.



Genetically Modified Organism:

According to WHO, Genetically Modified Organism (GMO) can be defined as organisms (plants, animals, or microorganisms) in which the genetic material has been altered in a way that does not occur naturally by mating and or natural recombination. The technology is often called Modern Biotechnology or Gene technology. Sometimes also called Recombinant DNA or genetic engineering

Recombinant DNA:

Recombinant DNA is a combination of DNA from different organisms or different location in a given genome that would no normally be found in nature.

<u>General Process of Genetic Engineering:</u>

Main focus of genetic engineering is:

1.Gene isolation,

2.Gene modifications

3. Gene removal

4.Evaluating the success of resultant gene combinations.

So the process starts with the step is to isolate the candidate gene. The cell containing the gene is opened and the DNA is purified. The gene is separated by using the restriction enzymes thes are enzymes that are used to cut the DNA into fragments or polymerase chain reaction (PCR) ton amplify up the gene segment. These enzymes can be extracted by Gel Electrophoresis. So once isolated the gene is ligated into plasmid that is then inserted into a bacterium. For ligation, ligase enzymes are used to join the fragment with plasmid. The plasmid is replicated when the bacteria divide, ensuring unlimited copies of the gene are available. Before the gene is inserted into the target organism it must be combined with the other genetic elements. These include a promoter and terminator region, which initiate and end transcription. A selectable marker gene is added, which in most cases confers antibiotic resistance, so it can be easily determine which cells have been successfully transformed. The gene can also be modifiedat this stage for better expression or effectiveness. These manipulations are carried out using the recombinant DNA techniques.

DNA Cloning with Cloning Vectors

- The two molecules that are required for cloning are the DNA to be cloned and a cloning vector.
- **Cloning vector** is a DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial or yeast cell and produces many copies of itself and the foreign DNA. When a single recombinant DNA molecule, composed of a vector plus an inserted DNA fragment, is introduced into a host cell, the inserted DNA is reproduced along with the vector, producing large numbers of recombinant DNA molecules that include the fragment of DNA originally linked to the vector.

  Three features of all cloning vectors:

  1. Small in size.
  2. Sequences that permit the propagation of itself in bacteria or in yeast(The replication origin).
  3. A cloning site to insert foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes.

Multiple Cloning Site (MCS)

A method of selecting for bacteria or yeast containing a vector with foreign DNA; usually accomplished by selectable markers for drug resistance or/and Reporter genes.

Types of Cloning Vectors

- **Plasmid –**
  anextrachromosomal circular DNA molecule that autonomously replicates inside the bacterial cell; cloning limit: 100 to 10,000 base pairs or 0.1-10 kilobases (kb)

- **Phage -** derivatives of bacteriophage lambda; linear DNA molecules, whose region can be replaced with foreign DNA without disrupting its life cycle; cloning limit: 8-20 kb
- **Cosmids** - an extrachromosomal circular DNA molecule that combines features of plasmids and phage; cloning limit - 35-50 kb
- **Bacterial Artificial Chromosomes (BAC**).
- **Yeast Artificial Chromosomes (YAC**) an artificial chromosome that contains telomeres, origin of replication, a yeast centromere, and a selectable marker for identification in yeast cells; cloning limit: 100-1000 kb.
- **Human Artificial Chromosomes (HAC).**

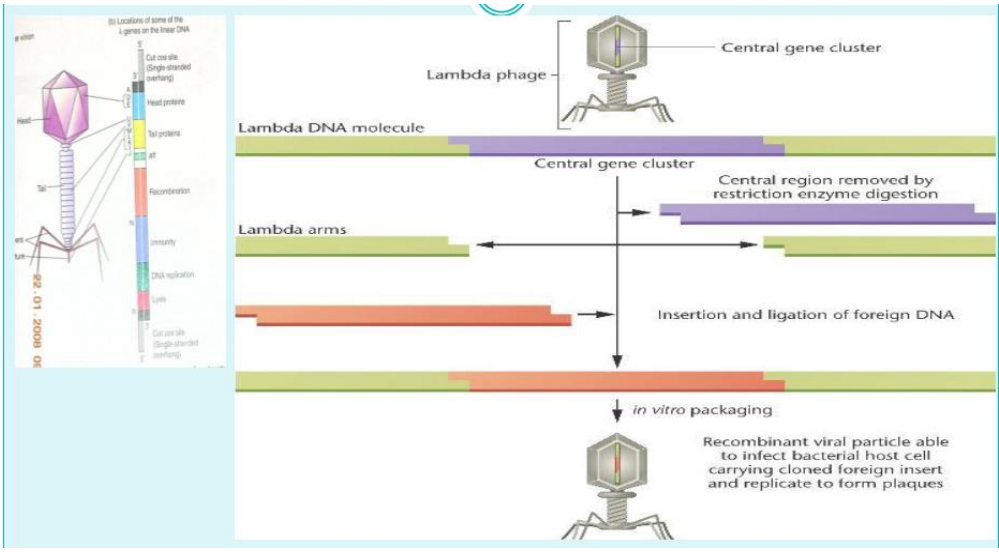
Plasmids

Plasmids are circular, double stranded DNA (dsDNA) molecules that are separate from a cell's chromosomal DNA. Plasmid is an autonomously replicating circular extra-chromosomal DNA. Plasmids range in size from a few thousand base pairs to more than 100 kilobases (kb). During cell division, at least one copy of the plasmid DNA is segregated to each daughter cell, assuring continued propagation of the plasmid through successive generations of the host cell.

- **For example**, some bacterial plasmids encode enzymes that inactivate antibiotics.
- Such drug-resistance plasmids have become a major problem in the treatment of a number of common bacterial pathogens.
- As antibiotic use became widespread, plasmids containing several drug-resistance genes evolved, making their host cells resistant to a variety of different antibiotics simultaneously.
- The plasmids most commonly used in recombinant DNA technology replicate in E. coli. Generally, these plasmids have been engineered to optimize their use as vectors in DNA cloning.

Bacteriophages

- The bacteriophages used for cloning are the phage λ and M13 phage.
- There is an upper limit on the amount of DNA that can be packed into a phage (a maximum of 53 kb).
- To allow foreign DNA to be inserted into phage, phage cloning vectors need to have some nonessential genes deleted.
- There are two kinds of λ phage vectors :insertion vector and replacement vector**.**
- **Insertion vectors** contain a unique cleavage site whereby foreign DNA with size of 5–11 kb may be inserted.
- **In replacement vectors**, the cleavage sites flank a region containing genes not essential for the lytic cycle, and this region may be deleted and replaced by the DNA insert in the cloning process, and a larger sized DNA of 8–24 kb may be inserted.



Difference between plasmid and bacteriophage

- Plasmid vectors replicate along with their host cells.
- λ vectors replicate as lytic viruses, killing the host cell and packaging the DNA into virions.
- **Cosmids** are plasmids that incorporate a segment of bacteriophage λ DNA that has the cohesive end site (cos) which contains elements required for packaging DNA into λ head particles.
- It is normally used to clone large DNA fragments between 28 to 45 Kb.
- **Bacterial artificial chromosome:** Insert size of up to 350 kb can be cloned in bacterial artificial chromosome (BAC). BACs are maintained in E. coli with a copy number of only 1 per cell.
- **Yeast artificial chromosome Human artificial chromosome**
- Yeast artificial chromosome (YAC): Insert of up to 3,000 kb may be carried by yeast artificial chromosome.
- **Human artificial chromosome (HAC):** may be potentially useful as a gene transfer vectors for gene delivery into human cells, and a tool for expression studies and determining human chromosome function.
- It can carry very large DNA fragment (there is no upper limit on size for practical purposes).
- It also avoids possible insertional mutagenesis caused by integration into host chromosomes by viral vector.

General Steps of Cloning with Any Vector

- Prepare the vector and DNA to be cloned by digestion with restriction enzymes to generate complementary ends.
- Ligate the foreign DNA into the vector with the enzyme DNA ligase.

- Introduce the DNA into bacterial cells (or yeast cells for YACs) by transformation.
- Select cells containing foreign DNA by screening for selectable markers (usually drug resistance).

APPLICATIONS OF GENETIC ENGINEERING

PHARMACEUTICAL & MEDICAL APPLICATIONS

The production of pharmaceutical products from transgenic animals is considered as "Pharming" (taken from "farming" and "pharmaceutical") Pharming involves the use of genetic engineering technique to insert genes into host animals or plants resulting in expression of useful pharmaceuticals products. Since 1980s pharming has resulted in a plethora of recombinant products made in E. coli. In this case donor is mostly human. Term Biopharmaceuticals was given to class of proteins created by genetic engineering.

Insulin	Treatment of diabetes
Haemoglobin	Used as a blood substitute
human protein C	Used as an anticoagulant
alpha-1 antitrypsin (AAT)	Treatment of AAT deficiency
Vaccines	Used as antigens
Growth hormones	Treatment of deficiencies
Factor VIII blood clotting factor	Used as blood clotting factor
Factor IX blood clotting factor	Used as blood clotting factor
Fibrinogen blood clotting factor	Used as blood clotting factor
Lactoferrin	Used as an infant formula additive
Tumor necrosis factor	Treatment of tumor cells
Interleukin-2 (IL-2)	Treatment of cancer, immune deficiency, and HIV infection
Taxol	Treatment for ovarian cancer
Interferon	Treatment for cancer ; viral infections

i. Human Insulin:

Importance: Insulin is a hormone produced in pancreas by the islets of Langerhans. It regulates the amount of glucose in the blood and malfunctioning of insulin production leads to a condition called diabetes. Earlier insulin from cows and pigs was used for treating diabetics but now a days it is obtained from humans through recombinant DNA technology. It was approved for human use in 1982.

Manufacture:

A and B chains are produced separately in different in separate bioreactors.

They are joined using lysosomes and cyanogen bromide.

- Purification by chromatography.

Brands: Some Common Brands are given in table below:

Human rh insulin	Eli Lilly
Novolinrh insulin	Novo Nordisk
Humalog (Insulin lispro)	Eli Lilly
Novolog (Insulin aspart)	Novo Nordisk
Actrapid	Novo Nordisk
Lantus (Insulin glargine)	Aventis
Optisulin	Aventis

ii. Human Growth Hormone: It is secreted by pituitary gland and responsible for normal body growth and development as well as development of muscles, bones, lengthening of height etc. are all regulated by HGH. Low production of HGH results in Dwarfism while High production of HGH results in giant stature or Gigantism. Brands: Genotropin by Pfizer

iii. Cytokines: Cytokines constitute the single most important group of biopharmaceutical substances. As coordinators of the immune and inflammatory response, can have a major influence on the body's response to a variety of medical conditions. Administration of certain cytokines can enhance the immune response against a wide range of infectious agents and cancer cells. Interferons (IFNs) were the first family of cytokines to be discovered. In 1957 researchers observed that if susceptible animal cells were exposed to a colonizing virus, these cells immediately become resistant to attack by other viruses. Brand: Betaferon by Bayer

iv. Vaccines: Importance: Vaccination intends to provide individuals with immunological protection before an infection actually takes place. Recombinant technology can serve as a gateway for the development of safe and effective vaccines that can be delivered effectively. New generation vaccines include highly purified synthetic or recombinant antigens that stimulate effective cell-mediated immune and mucosal immunity. Recombinant vaccine technology is rapid, compared to the traditional method of vaccine development and does not require the handling of live viruses. The use of recombinant proteins allows the targeting of immune responses focused against few protective antigens. There are a variety of expression systems with different advantages, allowing the production of large quantities of proteins depending on the required characteristics.

Manufacture: The gene for an antigenic determinant of a pathogenic organism is inserted into a plasmid. This genetically engineered plasmid comprises the DNA vaccine which is then injected into the host. Within the host cells, the foreign gene can be expressed (transcribed and translated) from the plasmid DNA, and if sufficient amounts of the foreign protein are produced, they will elicit an immune response.

v. Antibiotics: (Under Research) Antibiotics are small molecular weight compounds that inhibit or kill microorganisms at low concentrations. Antibiotics are produced by various bacteria, actinomycetes and fungi such as Bacillus, Streptomyces, and Penicillium. The significance of antibiotic production in microorganisms is still unclear which may be for ecological adaptation for the organism in nature. Example ecm gene (extra cellular matrix gene) cluster from Streptomyces lasaliensis was cloned and expressed in E. coli that directs the biosynthesis of the anti- tumor non- ribosomal peptide echinomycin.

vi. Gene Therapy: It involves correcting defective genes that are responsible for disease development. The most common form of gene therapy involves inserting a normal gene to replace an abnormal gene. Researchers are studying gene therapy for a number of diseases, such as Severe combined immuno-deficiencies (SCID) , Hemophilia, Parkinson's disease, Cancer, HIV, Bone problems. Research: Researchers may have just found a way to restore normal blood glucose levels in a mouse model of type 1 diabetes, which could prove to be a promising solution for people with type 1 or type 2 diabetes in the future. Intervertebral Disc Degeneration, Cancer, HIV, Parkinson's disease, Heart healing are also extensive fields of research.

vii. Metabolic engineering: It is a process to optimize the genetic and regulatory process. It induces capability of producing certain substance. Can be achieved through

- Over expressing a gene
- Blocking the competing metabolic pathways
- Heterologous gene expression
- Enzyme engineering Example engineering of a new fatty acid pathway (beta oxidation)

viii. Diagnostic purposes: Involves Diagnostic of parasitic disease through DNA probes Steps:

a) Isolate parasite from patient
b) Break DNA
c) Electrophoresis and smearing of DNA
d) Immobilize DNA on filter paper
e) Incubate with radiolabeled probe
f) Probe DNA complementary to parasite DNA will stick to it.
g) Wash the filter paper and observe in X-ray
h) Dark areas will show the positive infection.

Gene Therapy

Gene therapy is when DNA is introduced into a patient to treat a genetic disease. The new DNA usually contains a functioning gene to correct the effects of a disease-causing mutation.

Types

There are two different types of gene therapy depending on which types of cells are treated:

• **Somatic Gene Therapy**

If transfer of a section of DNA to any cell of the body that doesn‟t produce sperm or eggs. Effects of gene therapy will not be passed onto the patient‟s children.

• **Germline Gene Therapy**

If transfer of a section of DNA to cells that produce eggs or sperm. Effects of gene therapy will be passed onto the patient‟s children and subsequent generations

Gene Therapy Techniques

There are several techniques for carrying out gene therapy. These include:

• Gene Augmentation Therapy

This is used to treat diseases caused by a mutation that stops a gene from producing a functioning product, such as a protein. This therapy adds DNA containing a functional version of the lost gene back into the cell. The new gene produces a functioning product at sufficient levels to replace the protein that was originally missing. This is only successful if the effects of the disease are reversible or have not resulted in lasting damage to the body.

For example, this can be used to treat loss of function disorders such as cystic fibrosis by introducing a functional copy of the gene to correct the disease as below.

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• Gene inhibition therapy

Suitable for the treatment of infectious diseases, cancer and inherited disease caused by inappropriate gene activity.

The aim is to introduce a gene whose product either:

✓Inhibits the expression of another gene

✓Interferes with the activity of the product of another gene.

The basis of this therapy is to eliminate the activity of a gene that encourages the growth of diseaserelated cells. For example, cancer is sometimes the result of the over-activation of an oncogene (gene which stimulates cell growth). So, by eliminating the activity of that oncogene through gene inhibition therapy, it is possible to prevent further cell growth and stop the cancer in its tracks.

Killing of specific cells

Suitable for diseases such as cancer that can be treated by destroying certain groups of cells.

The aim is to insert DNA into a diseased cell that causes that cell to die. This can be achieved in one of two ways:

✓The inserted DNA contains a “suicide” gene that produces a highly toxic product which kills the diseased cell

✓The inserted DNA causes expression of a protein that marks the cells so that the diseased cells are attacked by the body‟s natural immune system.

It is essential with this method that the inserted DNA is targeted appropriately to avoid the death of cells that are functioning normally.

Transferring of DNA

A section of DNA/gene containing instructions for making a useful protein is packaged within a vector, usually a virus, bacterium or plasmid.
The vector acts as a vehicle to carry the new DNA into the cells of a patient with a genetic disease. Once inside the cells of the patient, the DNA/gene is expressed by the cell‟s normal machinery leading to production of the therapeutic protein and treatment of the patient‟s disease.

An illustration to show the transfer of a new gene into the nucleus of a cell via a viral vector. Image credit: Genome Research Limited

Challenges of Gene Therapy

Delivering the gene to the right place and switching it on:

✓it is crucial that the new gene reaches the right cell

✓ Delivering a gene into the wrong cell would be inefficient and could also cause health problems for the patient, even once the right cell has been targeted the gene has to be turned on cells sometimes obstruct this process by shutting down genes that are showing unusual activity.

Avoiding the immune response

The role of the immune system is to fight off intruders.

✓Sometimes new genes introduced by gene therapy are considered potentially-harmful intruders.

✓This can spark an immune response in the patient that could be harmful to them.

Scientists therefore have the challenge of finding a way to deliver genes without the immune system „noticing‟. This is usually by using vectors that are less likely to trigger an immune response.

The Cost of Gene Therapy

• Many genetic disorders that can be targeted with gene therapy are extremely rare.

• Gene therapy therefore often requires an individual, case-by-case approach. This may be effective, but may also be very expensive. Diseases Treated By **Gene Therapy**

Gene Therapy was initially meant to introduce genes straight into human cell, focusing on disease caused by single-gene defects such as cystic fibrosis, hemophilia, muscular dystrophy.

Three types of diseases for gene therapy can be distinguished:

• Monogenic disorders, single locus is defective and responsible for the diseases such as sickle cell anemia, severe combined immunodeficiency (ADA-SCID), cystic fibrosis, hypercholesterolemia

• Polygenic Disorders, multiple gene involved, diseases may be dependent on environmental factors and life style EXAMPLES: Heart disease, Cancer, Diabetes, Schizophrenia and Alzheimer’s disease

• Infectious diseases, Such as HIV

Severe Combined Immune Deficiency(ADA-SCID)

ADA-SCID is also known as the bubble boy disease. Affected children are born without an effective immune system and will succumb to infections outside of the bubble without bone marrow transplantation from matched donors. A landmark study representing a first case of gene therapy "cure," or at least a long-term correction, for patients with deadly genetic disorder was conducted by investigators in Italy. The therapeutic gene called ADA was introduced into the bone marrow cells of such patients in the laboratory, followed by transplantation of the genetically corrected cells back to the same patients.

Chronic Granulomatous Disorder (CGD)

CGD is a genetic disease in the immune system that leads to the patients' inability to fight off bacterial and fungal infections that can be fatal. Using similar technologies as in the ADASCID trial, investigators in Germany treated two patients with this disease, whose reconstituted immune systems have since been able to provide them with full protection against microbial infections for at least two years.

Hemophilia

Patients born with Hemophilia are not able to induce blood clots and suffer from external and internal bleeding that can be life threatening. In a clinical trial conducted in the United States, the therapeutic gene was introduced into the liver of patients, who then acquired the ability to have normal blood clotting time. The therapeutic effect however, was transient because the genetically corrected liver cells were recognized as foreign and rejected by the healthy immune system in the patients. This is the same problem faced by patients after organ transplantation, and curative outcome by gene therapy might be achievable with immune-suppression or alternative gene delivery strategies currently being tested in preclinical animal models of this disease.

Cancer

Multiple gene therapy strategies have been developed to treat a wide variety of cancers, including suicide gene therapy, oncolytic virotherapy, anti-angiogenesis and therapeutic gene vaccines. Two-thirds of all gene therapy trials are for cancer and many of these are entering the advanced stage, including a Phase III trial for head and neck cancer and two different Phase III gene vaccine trials for prostate cancer and pancreas cancer. Additionally, numerous Phase I and Phase II clinical trials for cancers in the brain, skin, liver, colon, breast and kidney among others, are being conducted in academic medical centers and biotechnology companies, using novel technologies and therapeutics developed on-site.

Neurodegenerative Diseases

Recent progress in gene therapy has allowed for novel treatments of neurodegenerative diseases such as Parkinson's Disease and Huntington's Disease, for which exciting treatment results have been obtained in appropriate animal models of the corresponding human diseases. Phase I clinical trials for these neurodegenerative disorders have been, or will soon be, launched.

Mesothelioma

Gene therapy research could result in effective treatments for mesothelioma patients. Although some types of gene therapy are aimed at specific cancers, early studies show promise for mesothelioma treatment. Suicide genes have also been used in clinical trials with

pleural mesothelioma patients. While early results are positive, more work is necessary to develop effective gene therapy treatments.

Adenosine deaminase (ADA) deficiency

ADA is another inherited immune disorder that has been successfully treated with gene therapy. In multiple small trials, patients' blood stem cells were removed, treated with a retroviral vector to deliver a functional copy of the ADA gene, and then returned to the patients. For the majority of patients in these trials, immune function improved to the point that they no longer needed injections of ADA enzyme. Importantly, none of them developed leukemia.

Hereditary blindness

Gene therapies are being developed to treat several different types of inherited blindness—especially degenerative forms, where patients gradually lose the light-sensing cells in their eyes. Encouraging results from animal models (especially mouse, rat, and dog) show that gene therapy has the potential to slow or even reverse vision loss.

The eye turns out to be a convenient compartment for gene therapy. The retina, on the inside of the eye, is both easy to access and partially protected from the immune system. And viruses can't move from the eye to other places in the body. Most gene-therapy vectors used in the eye are based on AAV (adeno-associated virus).

In one small trial of patients with a form of degenerative blindness called LCA (Leber congenital amaurosis), gene therapy greatly improved vision for at least a few years.

Future Prospects

More gene therapies are expected to reach the market in the next few years. Gene therapy to treat hemophilia approve soon. Much of the conversation in the gene therapy industry is focused on reducing manufacturing costs and determining the swiftest path through clinical trials. The FDA is doing its best to keep up with this rapidly changing landscape and adjust their framework to the particulars of a gene therapy clinical trial. As of 2020, the FDA has hired more than 50 clinical trial reviewers to adjust to the onslaught of gene therapy applicants.

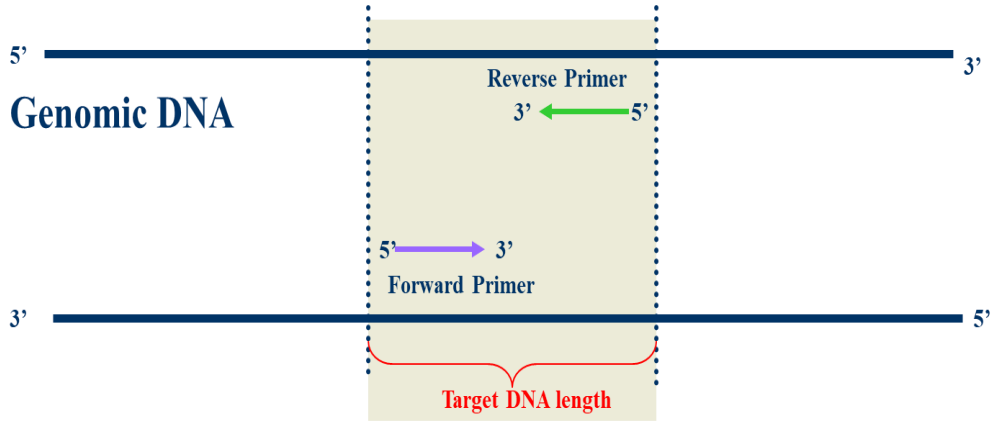
A number of clinical trials will soon be launched for various genetic disorders that include congenital blindness, lysosomal storage disease and muscular dystrophy, among others. Cancer Multiple gene therapy strategies have been developed to treat a wide variety of cancers, including suicide gene therapy, oncolyticvirotherapy, anti-angiogenesis and therapeutic gene vaccines.

What is PCR?

- An *invitro* process that detects, identifies, and copies (amplifies) a specific piece of DNA in a biological sample.
- Discovered by Dr. Kary Mullis in 1983.

PCR Requires the following:

- Template DNA to be amplified
- Pair of DNA primers
- Thermostable DNA polymerase
- dNTPs
- Buffer to maintain pH and to provide mg++Magnesium Ions
- Thermal cycler



How Does PCR Work?

A Three-Step Process:

Each step happens at a different temperature.

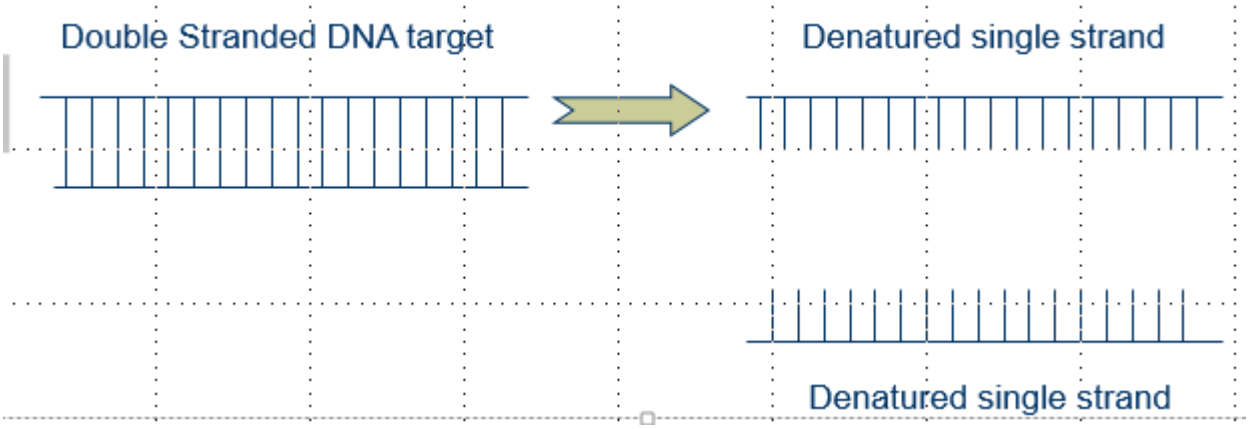
Step 1: Denaturation93 to 95°C 1min

Step 2: Annealing50 to 55°C 45sec

Step 3: Extension70 to 75°C 1-2min

Step 1: Denaturation

- Heat over 90ºC breaks the hydrogen bonds of DNA and separates double-stranded molecule into two single strands

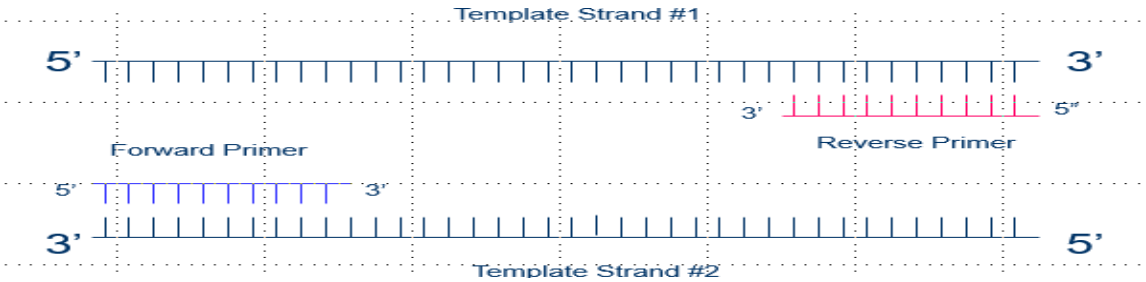


Step 2: Annealing

Primer Binding to Target also called Hybridization.

Temperature is reduced ≈ 50-65ºC

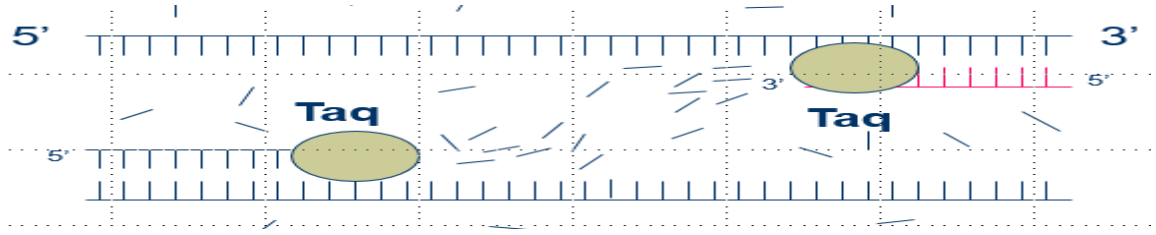
(Annealing temperature depends on primer lengthand G-C content



Step 3: Extension

Temperature is increased≈ 72ºC

(taq's ideal temperature)



<u>Equipment</u>

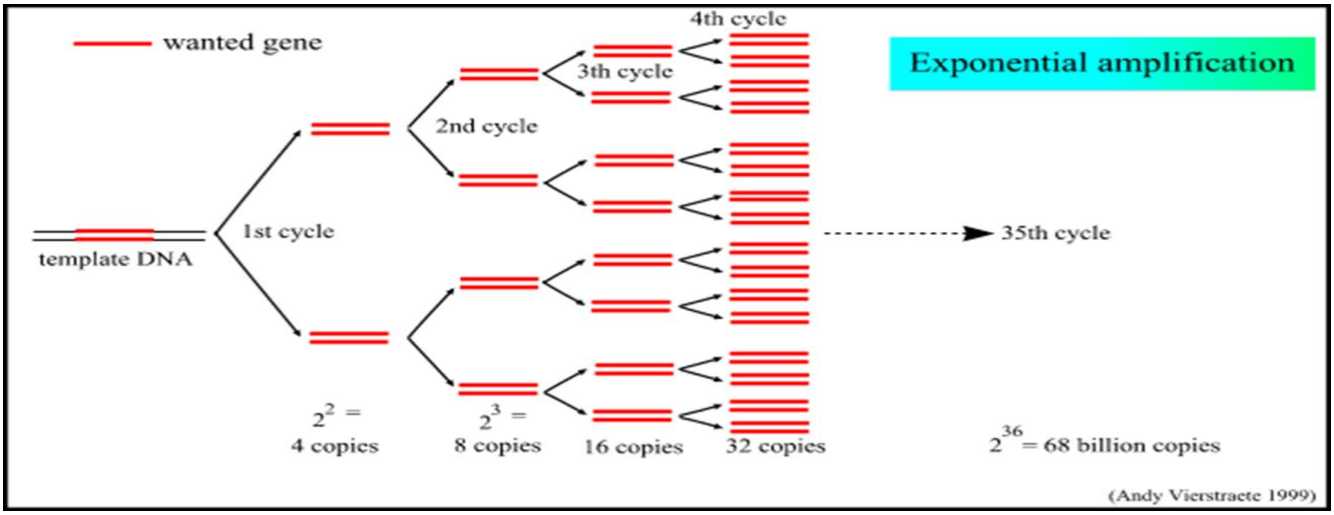
Thermal cycler:

- Thermal cyclers have metal heat blocks with holes where PCR reaction tubes can be inserted. The thermalcycler then raises and lowers the temperature of the block at each step (denaturation ~95℃, annealing ~55℃ and extension 72℃)

- At the end of the first PCR cycle, there are now two new DNA strands identical to the original target
- Multiple Cycles (30-40)
- Exponential Growth:
  # of Copies $=2^n$

(Where ^{n} is the number of cycles)

Exponential Amplification of the Target DNA Sequence



Cycles	Time	Temperature	Steps
1	5 min	95°C	Initialization
□30	30 sec	94°C	Denaturation
30 sec	55°C–65°C		Annealing
□ 1 min/kb	72°C		Elongation
1	10 min	72°C	Final elongation
1	∞	4°C	Final hold

VALIDATION:

- Do a negative control (no DNA) to validate that the PCR product is amplified from the intended DNA, not some other source of DNA.
- A positive control using DNA with good primers validates that the reaction conditions and thermal cycler work properly.

PCR: Analysis

At the end of a PCR reaction, there is aA LOT more of your target DNA than before the reaction started…billions of copies!

Now the sample is large enough to be seen on a gel (gel electrophoresis) and analyzed.

A powerful, versatile tool

APPLICATIONS OF POLYMERASE CHAIN REACTION (PCR)

Broadly, the applications of PCR in the biological sciences may be divided into:

a) Medical applications
b) Research applications

A. Medical Application of PCR

Polymerase chain reaction has helped in the realization of the potential of clinical DNA-based diagnoses by producing enough of the target sequence, so that simple, rapid and robust methods for identifying it could be employed. Specific applications of PCR in the medical sciences include:

- **Diagnosis of Monogenic Diseases**

Monogenic diseases are diseases resulting from single gene disorder e.g. sickle cell anaemia, β – thalassemia and some cases of hemophilia. Before now, the pre-natal diagnosis and carrier detection of these diseases relied upon the disorder using mixed DNA polymorphisms and family studies, and the procedures involved can take several weeks. However, with PCR, it is now possible to diagnose this disease directly and, indeed within a few days.

The possibility of testing for positive genetic traits in the pre-implanting embryo also arises because of the availability of PCR.

One or two cells could be removed from the embryo at the blastomere stage, and using PCR techniques, a genetic diagnosis could be made on the DNA of these cells. The embryo could undergo further growth in vitro and be later implanted into the uterus.

In some advanced countries/hospitals, the PCR technique is fast replacing the use of southern blotting in the diagnosis of monogenic diseases.

- **Diagnosis of Mutation Diseases**

Duchenne muscular dystrophy (DMD) and the Lesch-Nyhan (LN) mutation syndrome are genetic diseases arising from heterogeneous mutation at the loci for dystrophin and the Hypoxanthine phosphoribosyltransferase (HPRT) genes respectively.

The genes that are deficient in the two diseases are located on the X-chromosomes, but have many other different features.

Dystrophin is expressed in the muscle and the brain, and is not detectable in other tissues, while the HPRT gene product is ubiquitously expressed.

The polymerase chain reaction offers advantage for the diagnosis of dystrophin gene abnormalities due to its ease of application, speed and sensitivity, and has permitted the development of a rapid method of detecting 80-90% of all dystrophin gene deletions.

The general strategy for molecular diagnosis of newly arising human disease mutations is to use multiplex DNA amplification as a primary screen to detect DNA deletions. Cases not possible to diagnose by deletion studies can be further studied via DNA analysis, or if the target fragments are small enough, by direct sequence analysis of the mutations. The

mutant sequence information can then be used in simplified assays to diagnose future disease cases in the family or detect carrier state of the disease.

- **DNA Typing, Evolutionary Trends and Disease Susceptibility Studies.**

The capability of the PCR to amplify a specific segment of genomic DNA has made it an invaluable tool in the study of polymorphism and evolution, as well as in the analysis of genetic susceptibility to diseases. In all of these areas, a particular gene must be examined in a variety of individuals, either within a species, in different but closely related species, or in-patient and in healthy control populations.

Based on this principle, HLA class II polymorphism is valuable in the area of individual identification, tissue typing for transplantation and genetic susceptibility to specific autoimmune diseases.

Similarly, in molecular toxicology, PCR is used in the genotyping of carcinogen metabolism polymorphisms, since human genetic polymorphisms in metabolic activation and detoxification pathways appear to be important sources of inter-individual variation in susceptibility to cancer and diseases. Thus, individuals who inherit the "at risk" alleles of genes for enzymes such as glutathione S-transferases (GST) and N-acetyltransferases (NAT) may not be protected against carcinogens in cigarette smoke, diet industrial processes, and environmental pollution .

- **PCR and Forensic Science**

The ultimate goal of forensic DNA analysis is to obtain a positive identification of the donor of a biological evidence sample. The ability to detect DNA polymorphisms in biological evidence samples, have revolutionized forensic biology.

Whereas, methods like restriction fragment length polymorphism (RFLP) analysis and individual – specific DNA "fingerprints" requires 50-100ng of DNA, which is frequently not available from forensic evidence, the PCR technique requires much less quantity of DNA for a successful detection outcome.

Thus, it has been demonstrated that DNA typing results can be obtained from semen stains, bloodstains, single hair and epithelial cells . All of these had been used in the unequivocal settlement of criminal cases.

- **Detection of Human Infectious Diseases**

The PCR has revolutionized the detection of bacterial , fungal and viral pathogens . The targeted amplification of nucleic acid sequences provides not only dramatic increases in the number of copies to be detected, but concomitantly provides a nearly equivalent reduction in the complexity of the nucleic acid to be probed. Either DNA or RNA (following the production of complementary DNA using reverse transcriptase) can be used as a template for amplification.

Several laboratories have reported the detection of as few as three hepatitis B virus (HBV) genome (or about 300 virus particles per ml of serum) which represent a 10,000 fold increase in sensitivity over standard procedures .

Similarly, the use of PCR technique has brought great improvement in the clinical sensitivity and specificity of methods for detecting human immunodeficiency virus I and II , human papiloma viruses, nitrobacter population in soil, Clostridial organisms and a host of other organisms.

- **Detection of ras Oncogenes**

Cancer is thought to be mainly due to alteration in the cellular genome that affects the expression or function of genes controlling cell growth and differentiation. Present day cancer research aims at identifying the alterations responsible for the development oftumours, at characterizing the genes involved and at determining the consequences of the genes alterations for the control of cell growth and differentiation, and for the process of carcinogenesis using a variety of approaches.

- ras family consisting of three related genes, Hras, Kras and Nras, have been characterized as potential transforming genes by their presence in certain acutely transforming retrovirus, and in transformed NIH-3T3 cells transfected with DNA isolated from a variety of tumors. The PCR procedure has greatly simplified the analysis of ras mutations in human tumors, so that it has been possible to survey a large number of different tumours types at a sensitivity that could not be achieved by other techniques.

- **PCR and DNA Vaccine Production**

Genetic, DNA or nucleic acid-based immunization refers to the induction of an immune response to protein antigen, which is synthesized and expressed in vivo within the cells of the mammalian recipient of the DNA vaccine, subsequent to the administration of a DNA sequence or gene coding for an antigenic polypeptide. The sequence of the gene(s) inoculated contain a suitable eukaryotic transcription and translation control signals for the correct and efficient in vivo synthesis, conformation and post-translational processing of the proteins.

PCR plays a key role in the production of DNA vaccines. It is a much simpler, efficient and rapid procedure for the amplification (multiplication) of the desired sequences of the gene(s), once selected.

B. Research Applications of PCR

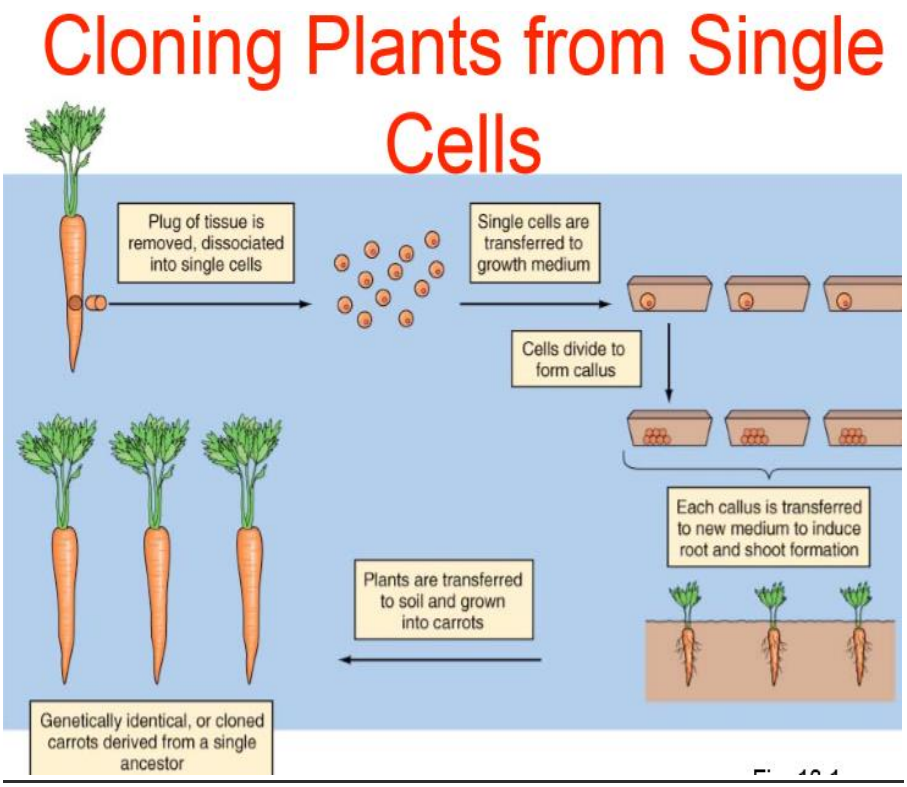
The ability to synthesize large amount of a specific DNA fragment from a complex template has significantly facilitated subsequent analysis. The nucleotide sequence of amplified DNA fragments can be determined directly without molecular cloning and preparation of template by growth of the host and biochemical purification of the vector. Most examples of applications of PCR in scientific research may be summarized as follows:

i. Direct sequencing of in vitro amplified DNA.
ii. Engineering DNA to meet specific needs.
iii. Detection of mutation
iv. Detection of gene expression.
v. Specific amplification of a DNA specie.
vi. Geometric amplification of unknown DNA sequence through inverse PCR.
vii. Analysis of DNA sequences in individual gametes.
viii. Evolutionary analysis.

WHAT IS CLONING?

The word “cloning” is referred as “making an identical copy” which has a Greek origin of “Asexual replication of an organism”. Cloning has been used in various fields of biology while the DNA molecule of cells with genetically identical structure is known as a clone.

Cloning plants from single cell:

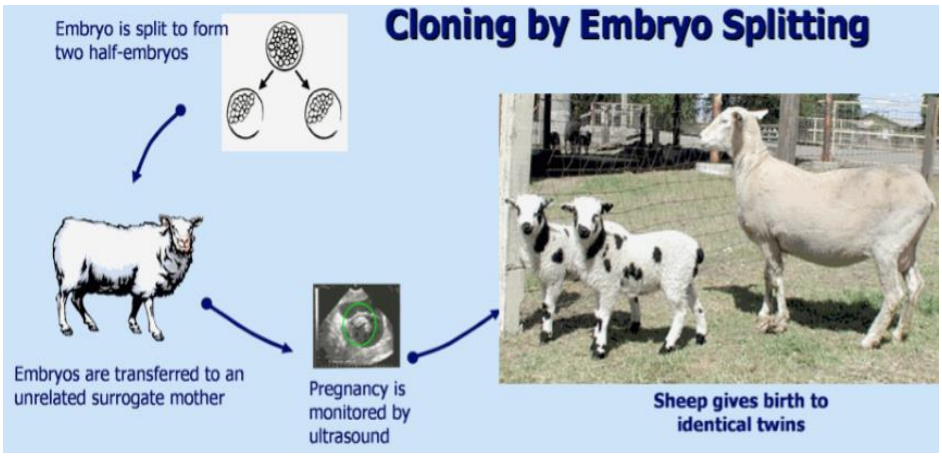


Cloning animals:

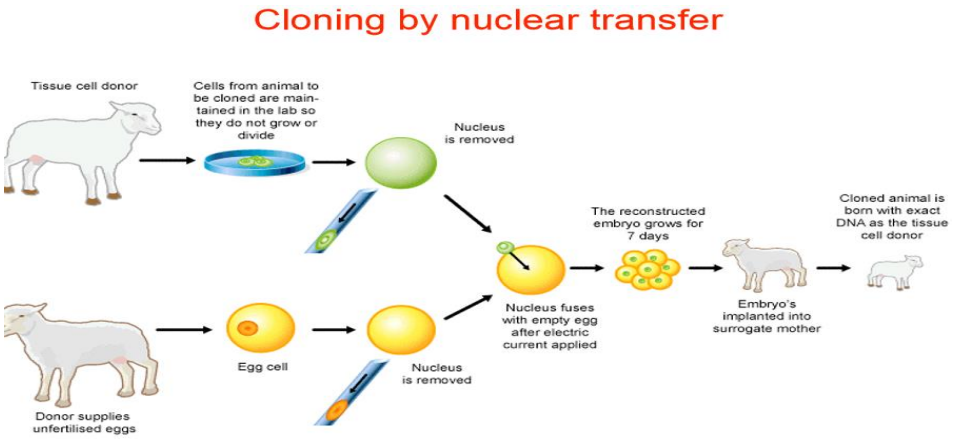
Two techniques

–Embryo splitting

–Nuclear transfer

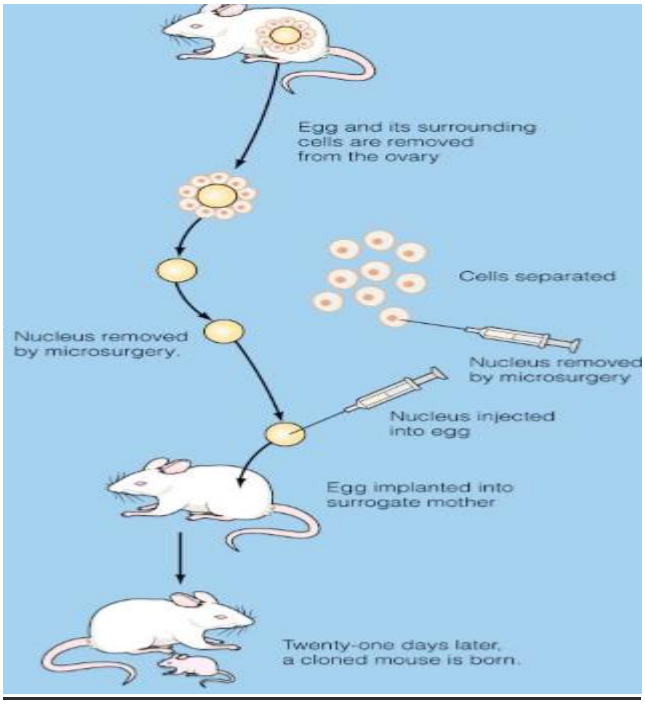


- Egg collected
- Fertilized by in vitro fertilization (IVF)
- Embryo is grown to 8–16 cells
- Cells are separated
- Separated cells grown into separate embryos
- Embryos transplanted into surrogate mothers
- May be used to clone any mammalian embryos, including humans



- More difficult
- Nucleus is removed from an egg
- Enucleated eggs are fused with other cells
- Embryos are transplanted into a surrogate mother
- In 1997, Dolly the sheep was the first mammalian clone from an adult donor cell

Cloning Mice by Injection of Nuclei from Adult Cells

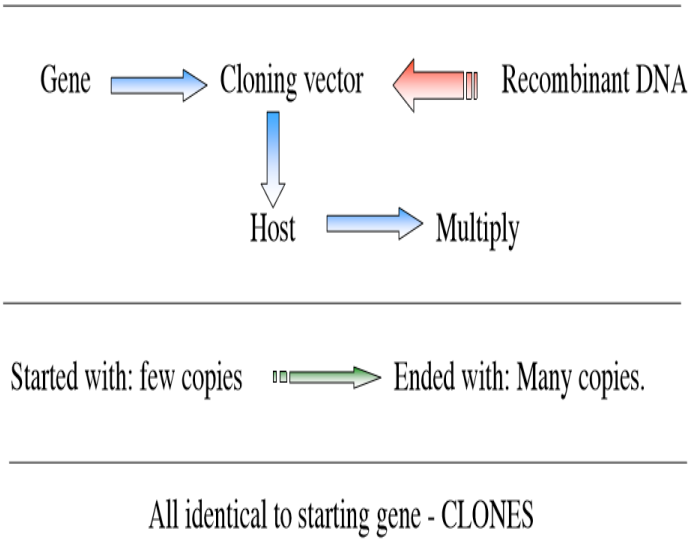


Problems -

- don't live as long
- not carbon copies/identical
- develop diseases early
- very low success rate - 0.1 - 3%
- Dedifferentiation/reprogramming may not be complete or accurate

Gene cloning

GOAL: To get enough copies of the gene to manipulate



APPLICATIONS OF CLONING

- Basic understanding on signal transduction together with genetic manipulation within the early human embryo has the potential to respond to many developmental diseases and defects requiring aesthetic and regenerative medicine to enter the field.
- Cells created by somatic cell nuclear transfer (SCNT) are beneficial in research of the causes of diseases, and as model systems for drug discovery. Cells produced with SCNT could eventually be used in cell transplantation, or for creation of organs in transplantation, called regenerative medicine.
- Stem cell therapy is cell transplantation in treatment or prevention of a disease or condition.
- Bone marrow transplantation is a widely used form of stem cell therapy. The potential use of stem cell therapy in treatment of several diseases is underway. Regenerative medicine would allow autologous transplantation of stem cells, and removes the risk of organ transplant rejection by the recipient.
- For instance, in liver diseases, a new liver may be grown using the same genetic material and transplanted to remove the damaged liver.
- Human pluripotent stem cells have been promised as a reliable source to generate human neurons, with the potential for regenerative medicine in brain and neural damages.

HUMAN CLONING

- **Reproductive Cloning**

Reproductive cloning is the process where the asexual cells are transferred to an egg while its DNA has been removed and after the development of an embryo, it is placed into the recipient uterus. This process can result in production of a human while the cloned individual would totally be identical to the genetic donor.

- **Therapeutic Cloning**

The therapeutic cloning also known as embryonic cloning is actually used to produce human embryos for research purposes. The objective of this type of cloning is not the production of a cloned human but the culture of cells is used in human researches and for treatment purposes in regenerative medicine. These cells are very important for biomechanics researchers because they can be used to produce any types of cells of human body. These cells are extracted from embryo after 4 days of cell division. The process of extraction ruins the embryo and this issue creates a lot of ethical concerns. The researchers hope to replace the cloned cells for the cells destroyed by diseases such as Alzheimer's, cancer, etc.

Advantages of Cloning

The cloning technology may have positive and negative effects with advantages as well as disadvantages and even can be with fatal effects. The most important advantages of cloning can be

(i) Replicating and propagating plants and animals,
(ii) Recreating and replicating extinct or going to extinct animals,
(iii) Propagating genes and saving newborns from hereditary diseases,
(iv) Helping to discover treatment methods of infertility,
(v) Dividing the developed embryo into several cloned embryos so that in case of probable incidents happening to one of them, the other clone can replace it,
(vi) Using it to reproduce the ambulated limbs and replicating them to culture and replace the destroyed organs such as liver, heart. One of the advantages can be that the cloned limbs have full genetic adaptation with the recipient individual who is the donor of the stem cells,
(vii) Helping to control population regarding shortages of male or female sex due to incidents such as war and earthquake, and
(viii) Helping to reduce sorrows and pains of people suffering from the death and absence of their loved ones by cloning them.32

Disadvantages of Cloning

(i) The cloned living organism may encounter genetic problems and complications in long term,
(ii) The more the cloned people are in the society, the more their extinction probability will be; because there are about one million four hundred thousand nucleotides in the body of every human and this remarkable variety is the origin of human generation survival; while the decrease in the genetic variety of individuals in a society, which is the result of cloning– highly increase the probability of their death by a special virus or a pathogen,
(iii) 99% of attempts to clone human may result in creation of monsters, (iv) Biological disorders such as cancer,
(iv) Premature aging: Dolly, the sheep, aged soon after cloning and the cloned baby will age at birth; because if the genetic donor is fifty-year-old, the new born will be a fifty-year-old one, thus, it will be suffering from premature aging like Dolly.

Bioethical Issues in Cloning

Bioethics as one of the new branches of "applied normative ethics" is a new field of research which reviews and analyzes challenges caused by using innovations and technologies in bioscience and biomedicine, and also regulates the does and does not in this area in the interdisciplinary space systematically.

Considering bioethics in cloning, it refers to different ethical issues especially from religious and secular points of views even human therapeutic and reproductive cloning are not presented commercially, but animals are currently cloned and the technique is used in livestock production.

In therapeutic cloning, generate tissue generation takes place to treat patients who cannot obtain transplants, resulting to avoidance of the need for immunosuppressive drugs,and to stave off aging effects.

In reproductive cloning, parents who cannot procreate are advised to have access to the cloning technology.The protest against therapeutic cloning is just on the use of embyronic stem cells, which is related to the abortion debate. Regarding reproductive cloning, there are concerns that cloning is not yet highly developed to confirm the safety of the technology, and could be prone to abuse and concerns about how cloned individuals could integrate with the society.In 2015, about 70 countries declared banning of human cloning.

Restriction Fragment Length Polymorphism Technique:

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA.

OR

A variation in the length of restriction fragments produced by a given restriction enzyme in a sample of DNA. Such variation is used in forensic investigations and to map hereditary disease.

Introduction:

>It is a technique that exploits variations in homologous DNA sequences.

>A restriction fragment length polymorphism is defined by the existence of alternative alleles associated with restriction fragments that differ in size from each other. Simply, the variations in the restriction DNA fragments lengthbetween individuals of a species is called RFLP.

>The basic technique of identifying such restriction fragment length polymorphisms involve fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest.

>The resulting DNA fragments are then separated by length through a processknown as agarose gel electrophoresis, and transferred to a membrane.

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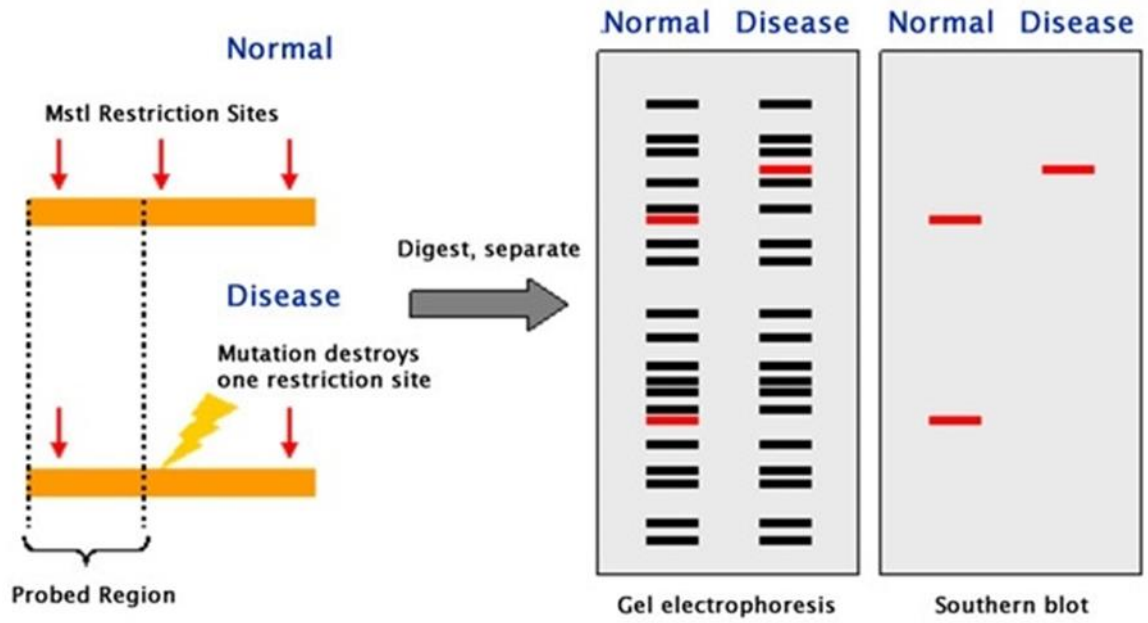
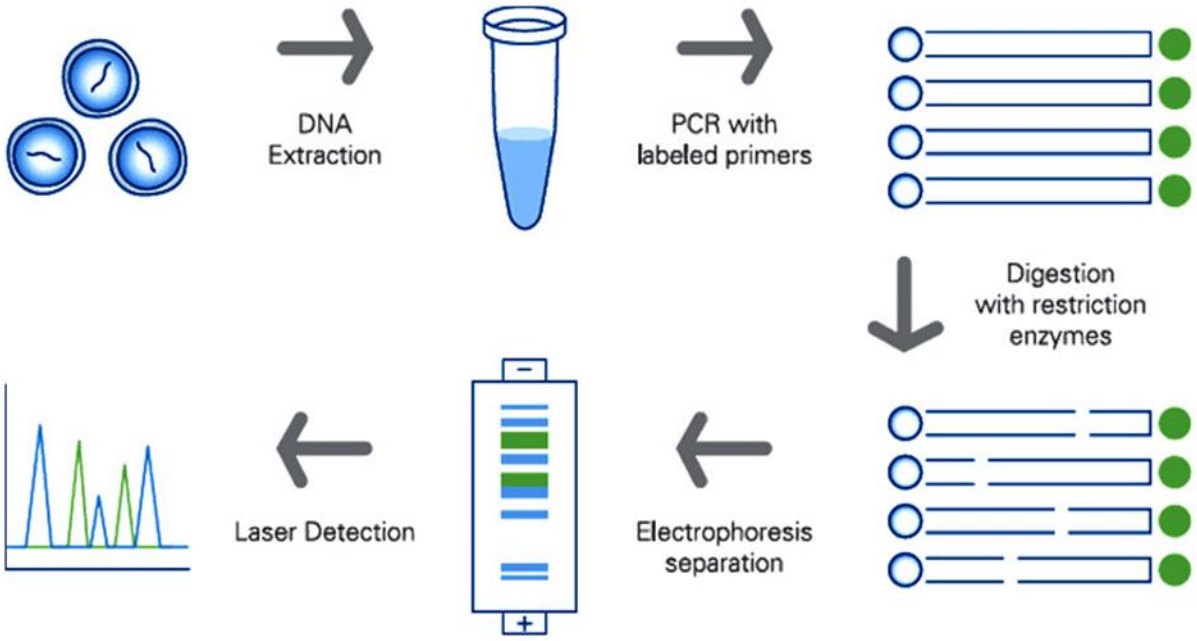


Principal:

If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity and differences of the patterns thus generated can be used to differentiate species (and even strains) from one another.

Steps Involved in Restriction Fragment Length Polymorphism (RFLP):

1. The first step in this process is to **isolate the DNA** from the target.
2. Once the DNA is isolated from the sample it is **subjected to restriction digestion** using restriction enzymes.
3. The digested DNA sample is then subjected to **gel electrophoresis**, in which the DNA is separated based on its size. Many DNA fragments with slight differences in length are produced.
4. The gel is then exposed to a chemical to denature double-stranded DNA to become single-stranded.
5. This is followed by southern blotting where DNA is transferred from gel to nylon membrane.
6. The nylon membrane is then exposed to solution with radioactive complementary nucleotide probes that hybridize to specifically chosen DNA sequences on nylon membrane.
7. The membrane is then placed against X- ray film, where hybridized radioactive probes cause exposure of X-ray film, producing an autoradiogram.
8. RFLP analysis is carried out to detect differences in pattern to confirm polymorphisms.



Applications:

RFLP can be used in many different settings to accomplish different objectives:

1: In paternity cases or criminal cases to determine the source of a DNA sample. (it has forensic applications).

2: Determining the disease status of an individual. (e.g. it can be used in the detection of mutations)

3: To measure recombination rates which can lead to a genetic map with the distance between RFLP loci.

4: In the characterization of genetic diversity or breeding patterns in animal populations.

5: RFLP has been developed for chromosomes mapping of humans, mice, maize, tomato, rice, etc.

Advantages:

1: The main advantage of RFLP analysis over PCR-based protocols is that no prior sequence information, nor oligonucleotide synthesis, is required.

2: Results are based on reliable genotypic characteristics' rather than on phenotypes.

3: RFLP based Genetic Marker

4: RFLP is the co-dominant marker thus can estimate heterozygosity.

5: RFLP & is very useful study in Genomic DNA Sequence**.**

Limitations:

>Slow

>Requires a large amount of sample DNA.

>Automation not possible

>Low levels of polymorphism in some species

>Few loci detected per assay

>Need a suitable probe library

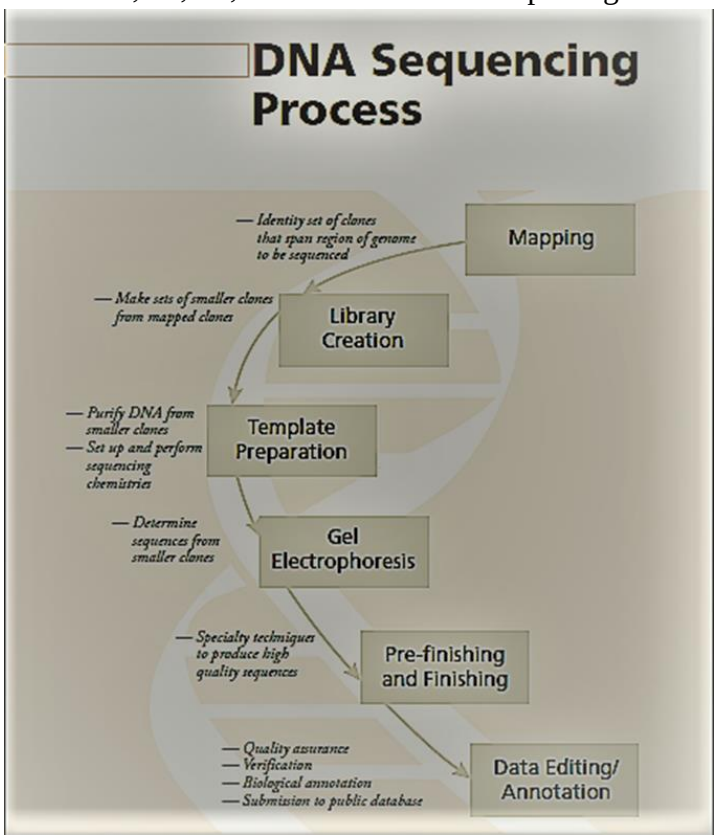
Human Genome Project (HGP):

The Genome: the complete set of genes or genetic material present in a cell or organism.
The Human Genome Project (HGP) was the international, collaborative research program whose goal was the complete mapping and understanding of all the genes of human beings. All our genes together are known as our "genome."

Human Genome Project Sequencing:

Chromosomes, which series in size from 50 million to 250 million bases, ought to first be broken down into a great deal shorter pieces. Every short piece is used as a template to produce a set of fragments that are different in extent from each other by a single base that will be recognized in a later step.

The wreckage in a set is divided by gel electrophoresis (separation step). New fluorescent dyes allow separation of all four fragments in a single lane on the gel.The last base at the conclusion of each fragment is recognized (base-calling step). This procedure recreates the original sequence of As, Ts, Cs, and Gs for each short piece generate in the first step.



<u>Goals and Aims of HGP:</u>

The mission of HGP is The quest to understand the human genome and the role it plays in both health and diseases.

According to Francis Collins (Director of HGP and NHGRI) **the true payoff from the HGP will be the ability to better diagnose, treat and prevent disease.**

Project goals were to

>Identify all the approximately 20,500 genes in human DNA.

>Determine the sequences of the 3 billion chemical base pairs that make up human DNA.
>Store this information in databases.
>Improve tools for data analysis.
> Transfer related technologies to the private sector.
>Address the ethical, legal, and social issues (ELSI) that may arise from the project.

Applications of the HGP:

Scientists estimate that chromosomes in the human population differ at about 0.1%. Understanding these differences could lead to discovery of heritable diseases, as well as diseases and other traits that are common to man.

Information gained from the HGP has many positive discoveries in health care. It include the cloning of genes responsible for Duchenne muscular dystrophy, retinoblastoma, cystic fibrosis, and neurofibromatosis.

Increasingly detailed genomic maps have also aided researchers seeking genes associated with fragile X syndrome, types of inherited colon cancer, Alzheimer's disease, and familial breast cancer.

In Diagnosis:

The tracing of genetic diseases to their molecular causes is rapidly expanding diagnostic and preventive options. The increased insights into molecular pathways, gained from high-throughput "functional genomics"
using DNA-chip and protein-chip approaches and specially designed animal model systems, will open great prospects for pharmacological and genetic therapies.

Current and potential applications of genome research will address national needs in molecular medicine, waste control and environmental cleanup, biotechnology, energy sources, and risk assessment.

Molecular Medicine:

1: Genetic screening will enable rapid and specific diagnostic tests making it possible to treat countless diseases.

2: DNA-based tests clarify diagnosis quickly and enable geneticists to detect carriers within families. Genomic information can indicate the future likelihood of some diseases.

3: Medical researchers will be able to create therapeutic products based on new classes of drugs, immunotherapy techniques, and possible augmentation or replacement of defective genes through gene therapy.

Waste Control and Environmental Cleanup:

In 1994, through advances gained by the HGP, the DOE formulated the Microbial Genome Initiative to sequence the genomes of bacteria useful in the areas of energy production, environmental remediation, toxic waste reduction, and industrial processing.

Resulting from human genome project, six microbes that live under extreme temperature and pressure conditions have been sequenced. By learning the unique protein structure of these microbes, researchers may be able to use the organisms and their enzymes for waste control and environmental cleanup.

Energy Sources:

Biotechnology, strengthened by the HGP, will be important in improving the use of fossil-based resources. Increased energy demands require strategies to circumvent the many problems with today's dominant energy technologies. Biotechnology will help address these needs by providing a cleaner means for the bioconversion of raw materials to refined products.

Ethical, legal, and social implications addressed by the Human Genome Project (ELSI):

The Ethical, Legal, and Social Implications (ELSI) program was founded in 1990 as an integral part of the Human Genome Project. The mission of the ELSI program was to identify and address issues raised by genomic research that would affect individuals, families, and society.

The ELSI program focused on the possible consequences of genomic research in four main areas:

>Privacy and fairness in the use of genetic information, including the potential for genetic discrimination in employment and insurance.

>The integration of new genetic technologies, such as genetic testing, into the practice of clinical medicine.

>Ethical issues surrounding the design and conduct of genetic research with people, including the process of informed consent.

>The education of healthcare professionals, policy makers, students, and the public about genetics and the complex issues that result from genomic research.

Risk Assessment:

Understanding the human genome will have an enormous impact on the ability to assess risks posed to individuals by environmental exposure to toxic agents.

Scientists know that genetic differences cause some people to be more susceptible than others to such agents. More work must be done to determine the genetic basis of such variability. There is need of long-term mission to understand the effects of low-level exposures to radiation and other energy-related agents, especially in terms of cancer risk.

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