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DNA Replication

DNA is capable of self reproduction. Parent DNA produces two daughter DNA molecules which are exact copies / replicas of parent DNA in N₂ base sequence. Hence DNA duplication is called as DNA replication.

Definition-- It is making exact copies of parent DNA. It is bidirectional.

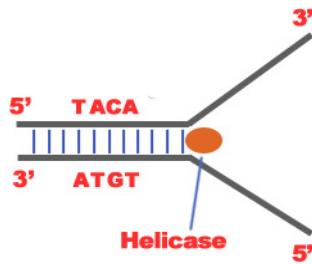
Steps of DNA Replication --

1)The first major step for the **DNA Replication** to take place is the breaking of hydrogen bonds between bases of the two antiparallel strands. The unwinding of the two strands is the starting point.

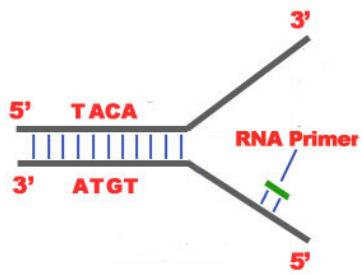
2)Activation of deoxyribonucleotides by energy & enzyme **Phosphorylase**.

3)Enzyme **Endonuclease** makes cut to one of the strands of DNA. The splitting happens in places of the chains which are rich in A-T. That is because there are only two bonds between Adenine and Thymine (there are three hydrogen bonds between Cytosine and Guanine). **Helicase** is the enzyme that splits the two strands. The initiation point where the splitting starts is called "origin of replication". The structure that is created is known as "**Replication Fork**".

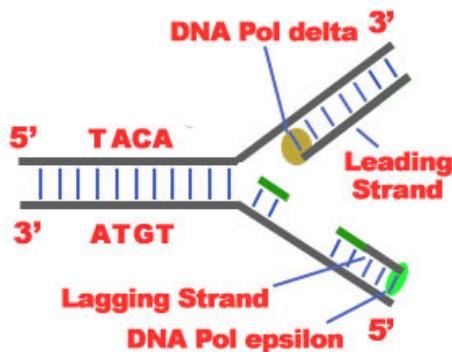
4)**Topoisomerase, helix destabilizing protein** stabilize replication fork.



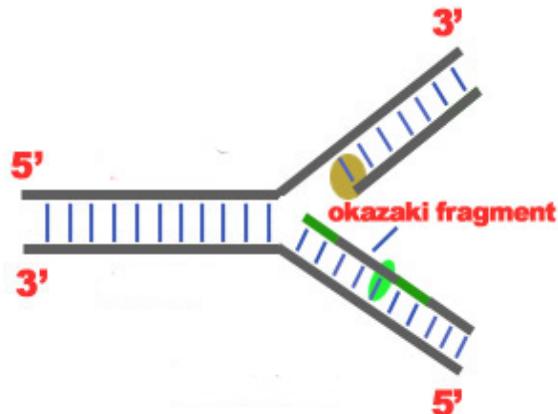
5) **Primase** synthesizes RNA primer that attaches at 3' end. RNA primer functions as 5' end of new strand.



- 6)** Two separated strands function as templates. Initiation of replication occurs at 3' end.
- 7)** DNA polymerase adds nucleotides in 5' → 3'direction continuously complementary to the nucleotides of the template, e.g. A—T. This strand is called **leading strand**.
- 8)** On the other strand DNA polymerase adds nucleotides in 5' → 3'direction in short fragments. Hence it is called as **lagging strand**. The newly synthesized DNA fragments are called as **Okazaki fragments**. These fragments are joined by **DNA ligase & become continuous**.



- 9)** Once replication is completed, RNA primer is removed& DNA nucleotides are synthesized by **DNA polymerase**.
- 10) Proof reading** – Mismatched N₂ bases are removed by **endonuclease** & appropriate N₂ bases are introduced by **DNA polymerase**.
- 11) In daughter DNA, 2 strands coil around each other to form helix.



12) Thus in daughter DNA one strand is old i.e. conserved & another strand is new. Hence it is known as "**semiconservative replication**".

Protein Synthesis:

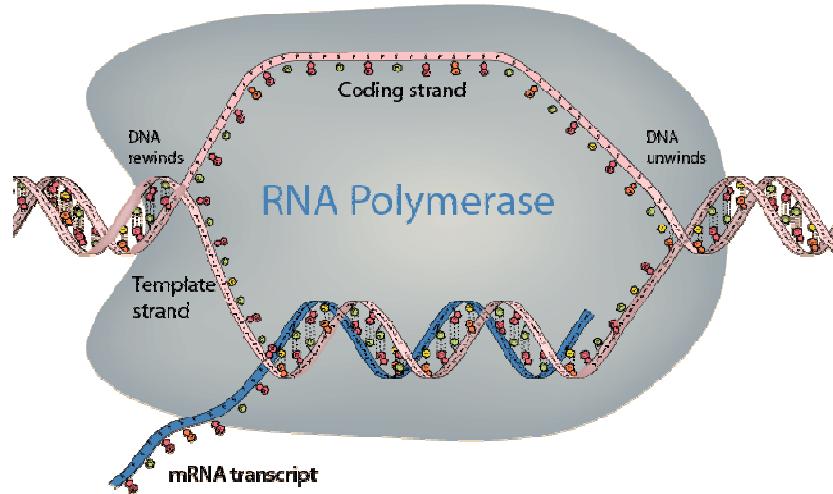
Protein synthesis is the main mechanism in body with respect to growth and changes in the cell. It results in the production of amino acid chains which are for proteins (important component in body). But for short term reactions importance of protein synthesis is the production of variety enzymes for different reactions as needed by the body for that moment. Since we cannot exist without enzymes, protein synthesis is needed for our existence. The process of protein synthesis translates the codons (nucleotide triplets) of the messenger RNA (mRNA) into the 20-symbol code of amino acids that build the polypeptide chain of the proteins.

DNA \longrightarrow mRNA \longrightarrow PROTEIN is known as CENTRAL DOGMA in the process of protein synthesis. It has 2 steps – transcription (DNA \longrightarrow mRNA) and translation (mRNA \longrightarrow PROTEIN).

TRANSCRIPTION

The first step in protein synthesis is the transcription of mRNA from a DNA gene in the nucleus. In this phase, one strand of DNA double helix acts as a template to synthesize its complimentary strand i.e. mRNA. Transcription starts with an enzyme called **polymerase** copying the DNA sequence to a similar molecule called messenger RNA (mRNA). This synthesis takes place in a specific portion of DNA which is known as transcription bubble. In the bubble, DNA strands are separated or unzipped

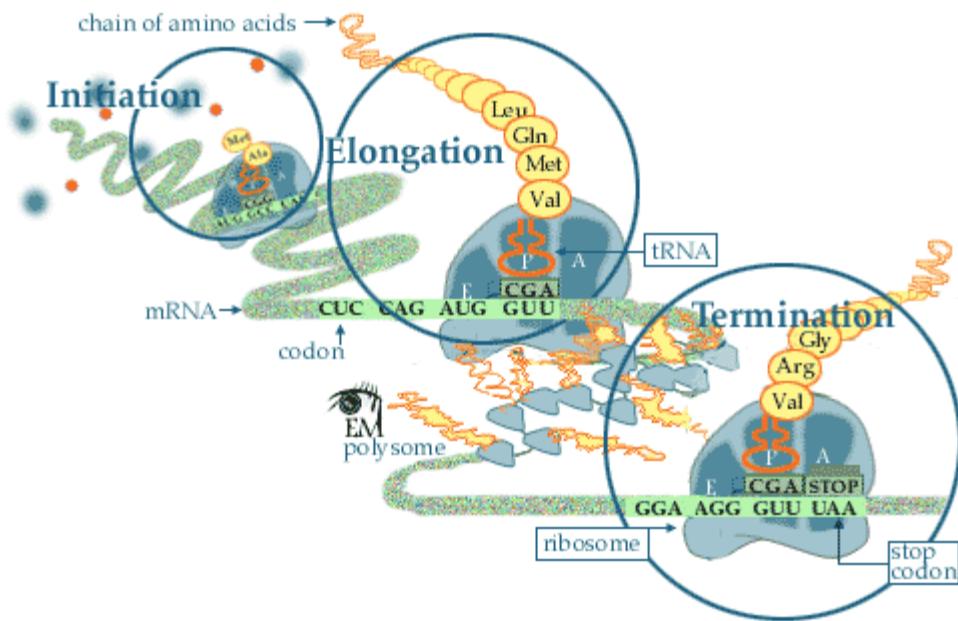
The process of mRNA translation begins from its 5'-end towards its 3'-end as the polypeptide chain is synthesized from its amino-terminal (N-end) to its carboxyl-terminal (C-end). It replaces T with U (Uracil), a helper base, making it clear that the mRNA is a copy. The bases (A, T, G, C) on one strand of the DNA specify the order of bases on the new strand of mRNA (A, U, G, C). At the end of transcription, DNA stays inside the nucleus while the RNAs migrate from the nucleus into the cytoplasm.



TRANSLATION

This is the second phase of protein synthesis where the ribosomes in the cytoplasm use transfer RNA (tRNA) to attach to the mRNA and translate the bases into amino acids. tRNA molecules bring the specified amino acids that the ribosome links together to make a protein. Translation has four steps viz. Activation and charging, Initiation, Elongation and Termination

Before the actual translation, **activation**, which is not part of translation in technical sense, occurs. In this step, the correct amino acid (AA) is joined to the correct tRNA. It is required for translation to proceed. When the activated tRNA has an amino acid linked to it, it is termed as "**charged**". The AA is joined by its carboxyl group to the 3' OH of the tRNA by an ester bond.



Initiation: In the cytoplasm, protein synthesis is actually initiated by the AUG codon on mRNA. The AUG codon signals both the interaction of the ribosome with m-RNA and also the tRNA with the anticodons (UAC). The tRNA which initiates the protein synthesis has N-formyl-methionine attached. The formyl group is really formic acid converted to an amide using the -NH₂ group on methionine (left most graphic)

The next step is for a second tRNA to approach the mRNA (codon - CCG). This is the code for proline. The anticodon of the proline tRNA which reads this is GGC. The final process is to start growing peptide chain by having amine of proline to bond to the carboxyl acid group of methionone (met) in order to elongate the peptide.

Elongation: Elongation of the peptide begins as various tRNA's read the next codon. In the example on the left the next tRNA to read the mRNA is tyrosine. When the correct match with the anticodons of a tRNA has been found, the tyrosine forms a peptide bond with the growing peptide chain. The proline is now hydrolyzed from the tRNA. The proline tRNA now moves away from the ribosome and back into the cytoplasm to reattach another proline amino acid.

Elongation and Termination: When the stop signal on mRNA is reached, the protein synthesis is terminated. The last amino acid is hydrolyzed from its t-RNA. The peptide chain leaves the ribosome. The N-formyl-methionine that was used to initiate the protein synthesis is also hydrolyzed from the completed peptide at this time.

The ribosome is now ready to repeat the synthesis several more times. The events following translation include post-translational modification and protein folding. During

and after synthesis, polypeptide chains often fold to attain secondary, tertiary and quaternary structures. This process is known as **maturation of protein** and it takes place in Golgi Complex.

GENETIC ENGINEERING

Recombinant DNA Technology, Genetic modification/manipulation (GM) and Gene splicing are terms that apply to the direct manipulation of an organism's genes. Genetic engineering is different from traditional breeding, where the organism's genes are manipulated indirectly.

Definition --Genetic engineering uses the techniques of molecular cloning and transformation to alter the structure and characteristics of genes directly.

Genetic engineering techniques have found some successes in numerous applications. Some examples are in improving crop technology, the manufacture of synthetic human insulin through the use of modified bacteria, the manufacture of erythropoietin in hamster ovary cells, and the production of new types of experimental mice such as the oncomouse (cancer mouse) for research.

Definition Clone: A clone is a group of identical copies. A clone of a cell is a group of cells of a single type isolated and allowed to reproduce to create a population of identical cells. A clone of a DNA molecule is an isolated DNA molecule which is multiplied number of times to produce a large amount of identical copies.

Definition Cloning : It is a method of producing identical copies of cells / molecules/organisms.

There are a number of ways through which genetic engineering and cloning together is accomplished. Essentially, the process has following steps :-

1. Isolation of the gene/ DNA fragment of interest (known function) from an organism (**A**) . It is known as an **insert**.
2. Enzymatic cleavage (**B**) and joining (**C**) of insert DNA to another DNA molecule (cloning vector) to form recombinant DNA (rDNA) i.e. vector + insert DNA (**D**)
3. Transformation of a host cell that includes transfer and maintenance of rDNA molecule in the host organism (**E**)
4. Identification of transformed cells (with rDNA) and their selection from non transformants
5. Amplification of rDNA to get multiple copies in a cell (**F**)
6. Cell multiplication (**G**) to get clones (population of genetically identical individuals carrying multiple copies of foreign DNA)

Isolation of gene of interest:

- Isolation is achieved by identifying the gene of interest that the user wishes to insert into the organism, usually on the basis of existing knowledge about various functions of genes. This segment of DNA is the molecule is used for the cloning process.
- Isolation is carried out by using variety of Restriction Endonucleases or Restriction Enzymes (e.g. *EcoR1*) that recognize the site of cleavage (cut) on DNA at specific palindromic sequences.

- Broadly these enzymes are categorized in three categories Type I, II and III. Recognition site and cleavage site is same in Type II while it is different in Type I and III. Thus, type II enzymes are widely used in genetic engineering especially for gene manipulation.

The details of these groups are as follows

Type I <i>EcoB</i> , <i>EcoK</i>	Recognition site for type I enzyme is 15 bp long and cleavage site 1000 bp away; the enzyme has restrictive subunit, modification subunit and specificity subunit; requires Mg ⁺⁺ , S-adenosyl methionine and ATP as cofactors
Type II <i>EcoR1/R2</i> <i>Hae III</i> <i>BamH1</i>	The length of recognition site for type II enzyme varies from 4/5/6/8 or more bp long and cleavage site 1000 bp away; the enzyme has restrictive subunit, modification subunit and specificity subunit; more stable and requires Mg ⁺⁺ as cofactors
Type III <i>MboII</i> <i>FokI</i>	Made up of 2 subunits for recognition and cleavage; requires ATP for energy and Mg as co factor; recognition site is non palindromic and cleavage site 25-27 bp away

- As a result of these enzymes, the DNA from donor cell is cut in such a way that it has sticky/staggered cuts or sometimes blunt ends.

Insertion of the above mentioned gene of interest into a cloning / transfer vector

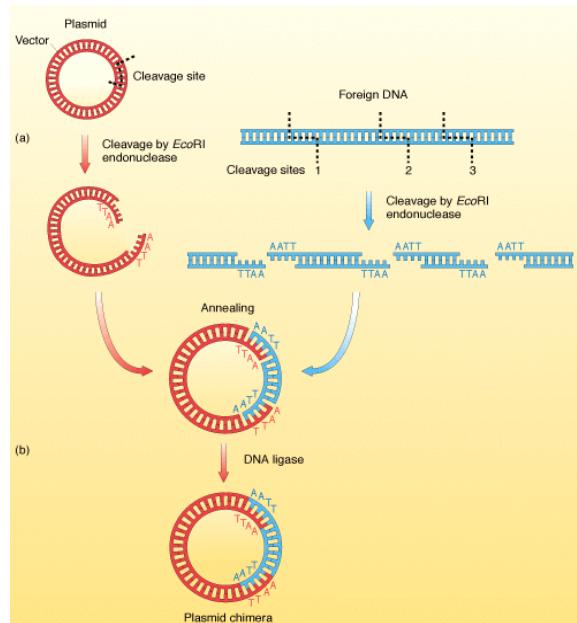
- This can be done with the opening of vector DNA molecule with a cut by the same restriction enzyme (i.e. producing similar kind of the sticky or blunt ends).
- Thus the foreign DNA and the vector DNA (plasmid) now share the complementarities at these ends.
- Then the addition of DNA ligase joins the two DNA molecules by ligating their nucleotides with each other. This prepares the recombinant DNA vector i.e. the recombinant plasmid.
- A vector is a cloning vehicle i.e. the agent used to multiply the isolated gene. It itself is a synthesized DNA molecule that carries the isolated gene into a host where it can replicate producing many of its copies.
- Hence the same number of the copies the gene of interest are generated. The product thus generated is called the **Recombinant DNA** (rDNA)and the technique is called gene cloning.
- A variety of vectors which are actually the cloning vehicles have been developed which allow the multiplication of the inserted gene.
- The most common vectors are plasmids. Other vectors can also be used, such as viral vectors, and non-prokaryotic ones such as liposomes, or even direct insertion using DNA guns.

Commonly used vectors:

- Plasmids:** Extra-chromosomal, closed, circular, double stranded DNA molecules present in bacterial cells. May present independently or integrated in the bacterial chromosome. Capable of independent replication and transmission. Many times

specify number of properties of the host. e.g. antibiotic resistance, heavy metal resistance, nitrogen fixation. Plasmids are used with *E. coli* cells. They are introduced in the bacterial cells by the process of transformation. The *E. coli* cells and plasmid DNA are incubated together at 0°C in CaCl₂ solution, then subjected to the heat shock by rapidly changing the temperature to 43°C. Few cells of the bacteria take up the plasmid DNA. Plasmid DNA can also be introduced in *E. coli* cells by electroporation during which the mixture of the cells and the plasmid is subjected to a high voltage pulse. The cell membrane becomes permeable and the plasmid can enter the cells.

- **Bacteriophages:** Also called phages which are the viruses that infect bacteria. They have the ability to attach the host (bacterium) and transfer their own DNA to the host. Most frequently used phage vectors are lambda (λ) phage and M13. The genome of λ phage is a linear double stranded with 12 bases long single stranded stretch at 5' end which can be used as cohesive/sticky ends. Also there is a large non-essential region in its DNA which can be replaced with the foreign DNA. Such λ vectors can accommodate large DNA inserts around 40,000 – 53,000 b p (base pair) long. These vectors can then be packaged into the infectious phage particles. The infectious phages bring about the lysis of the host cell.
- **BACs:** Bacterial artificial chromosomes, A **bacterial artificial chromosome (BAC)** is a DNA construct, from plasmids. They can contain very long DNA inserts (100,000-300,000) as a foreign DNA.
- **YACs:** **Yeast artificial chromosome** (short YAC) is a vector used to clone large DNA fragments (larger than 100 kb and up to 3000 kb). It is an artificially constructed chromosome and contains the sequences needed for replication and preservation in yeast cells.
- **Shuttle vectors:** these are the plasmid DNAs that can be propagated in the cells of 2 or more different species.



Transfer of the vector by transformation of the host cell is the next step. The commonest type of host organism used for introducing the vectors (plasmids and viral vectors) is *Escherichia coli*. Its DNA metabolism is well-understood and plasmids are the naturally occurring circular DNA molecules in the *E. coli*.

Transformation is the process by which the *E. coli* are made receptive by some heat shock or electric shock and the recombinant plasmid DNA is kept in the surrounding medium of such competent *E. coli* cells. The recombinant plasmid DNA enters the *E. coli* cells. Such organisms are called **GMO or Genetically Modified Organisms**.

Selection of the transformed organisms is required to find out only those organisms which have received the recombinant from those which have not. The selection is carried out by various ways –

a) Direct Selection:

- If the cloned DNA itself codes for resistance to the antibiotic ampicillin (amp^R), the recombinants can be allowed to grow on the minimal medium containing ampicillin.
- Thus only such recombinants will grow and develop colonies on medium, which contain amp^R gene on its plasmid vector.
- But with this method one can not segregate the transformed cells with re-ligated plasmid (i.e. cut by endonuclease & joined again)vector from the recombinant plasmid.

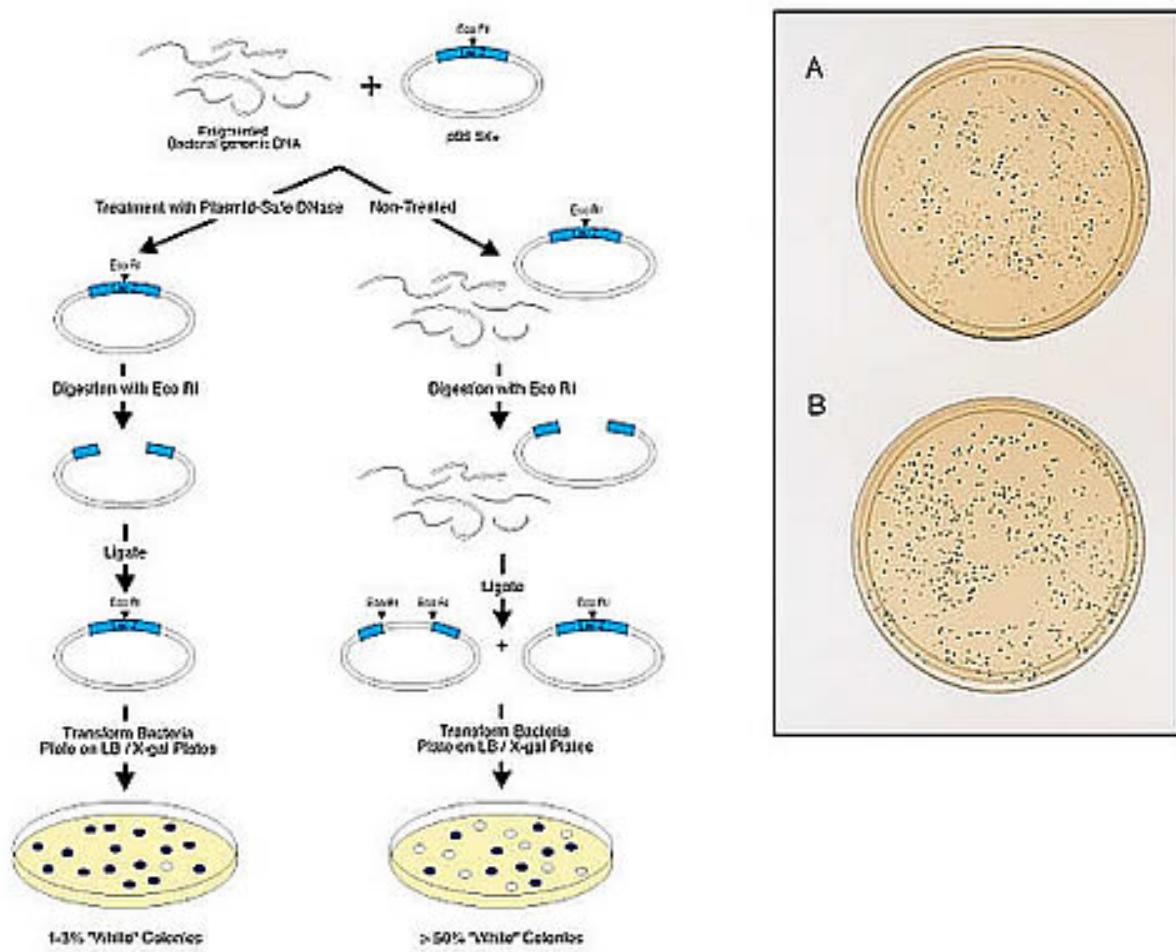
b) Insertional Selection Inactivation:

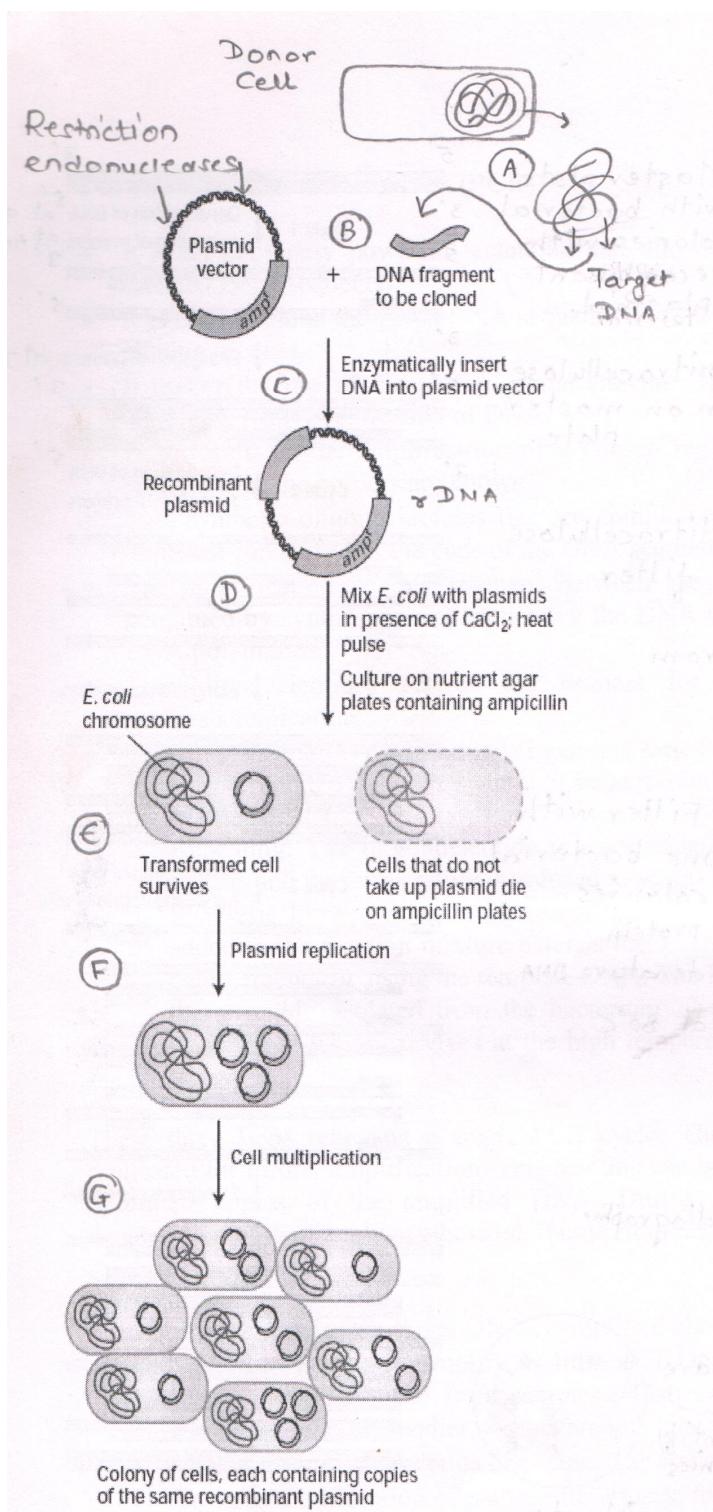
- In this method, as discussed earlier, the vector plasmid is constructed with some specific DNA sequences which confer the antibiotic resistance to the host cell to two different antibiotics (e.g. ampicillin and tetracyclin).
- It is designed in such a way that the foreign gene gets inserted in this part of the plasmid disrupting one of these genes. As a result the resistance for one antibiotic is lost by the recombinant plasmid whereas the plasmid DNA without the insert (foreign DNA) has the intact resistance for both the antibiotics.
- Thus the host cells that have not taken the plasmid cannot grow on the medium containing both the antibiotics.
- Those cells which get transformed by the non-recombinant DNA have intact resistance for both the antibiotics.
- The *E. coli* cells that get transformed by the recombinant plasmid lose resistance for one of the antibiotics and retain for the other.
- Hence the cells are first grown on medium with ampicillin and then on medium with tetracycline.
- This strategy can be exploited for identifying the host cells that have taken the foreign gene. These cells are then grown in cultures to produce multiple copies of the foreign gene (as explained in the diagrammatic representation).

c) Blue White Colony

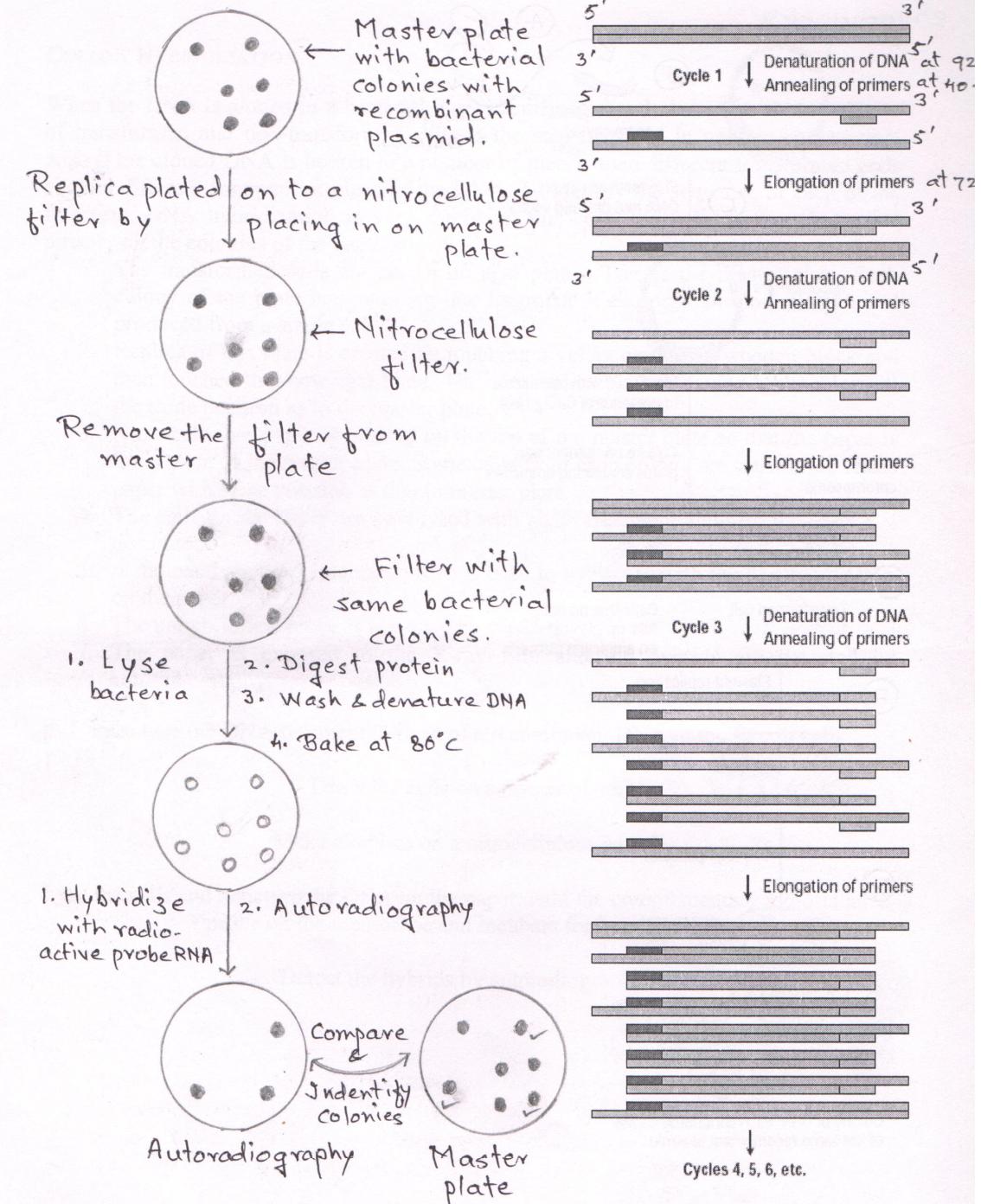
- In this method instead of antibiotic resistance gene, a lacZ gene is constructed which is responsible for the synthesis of beta-galactosidase enzyme.

- The enzyme reacts with chemical B galactose and develops blue colour.
- In the recombinant plasmid, the introduced gene disrupts(alter function) lacZ gene and thus prevents further reaction if grown on the medium containing B galactose.
- Against this, the non recombinant plasmid certainly develops blue colour on the growing plate and can easily be differentiated from white colonies of recombinant plasmids which use glucose from the medium.



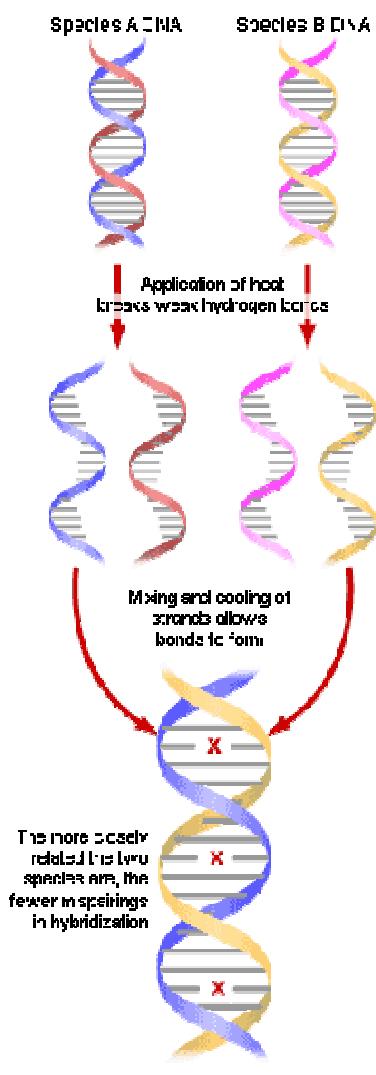


Gene Cloning



Colony Hybridization Polymerase Chain reaction

DNA HYBRIDIZATION:



- Hybridization technique makes use of the ability of DNA molecule to renature or reanneal when it is converted to single stranded structure.
- It is the method that detects DNA by its hydrogen bonding capability - we call this "probing" the DNA, and we say that the probe "hybridizes" to the target sequence.
- Also if any two DNA molecules share any complementarity, they can bind each other to form a double stranded DNA molecule.
- When any two different DNA samples are to be compared they are first denatured. They are then mixed and the mixture is cooled i.e. allowed to renature.
- During renaturation, the two DNA strands of the original molecule anneal and also the strands of the two different molecules anneal if they have significant similarity. Depending upon the extent of the similarity between them, they will form DNA duplexes of various lengths. Thus they form hybrids (if the renatured DNA has formed from the two different sources present in the mixture).
- These hybrids are the partial duplexes.
- This technique is highly useful in detecting similarity between two genes or DNA molecules from different organisms or species etc.
- Similarly, a small complementary probe also can hybridize with the small region of the DNA to be detected or studied. This probe is usually labeled with radioactive or fluorescent label.

The level of the radioactivity or fluorescence is detected after the two DNA solutions are mixed and allowed to renature. The intensity of the label is directly proportional to the complementarity of the two DNA molecules. This method can be implemented to detect a gene sequence.

Like DNA – DNA hybridization RNA –DNA hybridization can also be carried out.

The overall process can be summarize as follows,

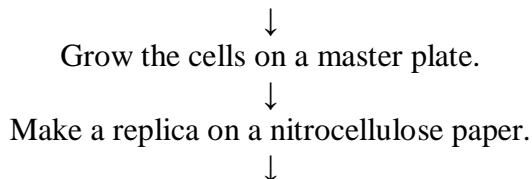
Denatured DNA sample 1 + Denatured DNA sample 2 → Cool for renaturation and/or hybridization → Hybrid DNA duplexes → Screen the label by its radioactivity or fluorescence.

COLONY HYBRIDIZATION

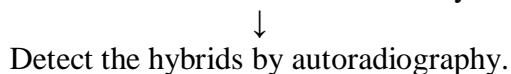
When the DNA is cloned in a bacterial host, identifying the cloned DNA in the mixture of transformed and non-transformed cells is the step involved in genetic engineering. Also if the cloned DNA is broken into number of pieces, then different transformed cells contain different fragment or piece of the DNA. To identify the sequence of each of the fragment, DNA hybridization is very useful. In this case, the hybridization is carried directly on the colonies of the bacterial cells.

1. The transformed cells are grown on agar plates. This is the master plate. Each colony of the plate is containing one fragment of the original cloned DNA and produced from a single cell.
2. Replica of this plate is created by touching a velvet cloth or a wooden block and then touched to a new agar plate. The new replica plate is having same colony at the same position as in the master plate.
3. Nitro-cellulose paper is pressed on the top of the master plate so that the paper is the replica of the master plate. Some cells from each colony are transferred to the paper with same position as that in master plate.
4. The cells on the paper are now lysed with alkali treatment and also their DNA is denatured.
5. A radioactive complementary probe is used to hybridize with the denatured DNA on the paper.
6. The unhybridized probe is removed by washing.
7. The paper is exposed to the X-ray film and the hybrids are detected by autoradiography.

Introduce the DNA fragment in form of a recombinant DNA in the E. coli cells



Lyse the cells and denature the DNA on the paper. Add the complementary radio-labeled probe on the membrane and incubate for hybridization



POLYMERASE CHAIN REACTION (PCR)

- It is an extremely powerful technique that allows a million fold amplification of selected DNA sequence.
- It is used to clone the given DNA sequence *in vitro* without using living cells during cloning process.
- It makes the use of the ability of the enzyme DNA polymerase to carry out the semi-conservative replication of DNA.
- PCR results in the amplification of a chosen region of DNA molecule when the sequences of the borders are known.
- Two synthetic oligonucleotides that are complementary to end parts of the chosen sequence can anneal to the ends of the DNA segment.
- The amplification of this segment between the two defined ends can be then continued by synthesizing or replicating the DNA with the help of a special enzyme DNA polymerase.
- The oligonucleotides act as the primers for the enzyme to complete the synthesis/replication.
- This amplification is achieved by a repetitive series of cycles involving three steps.
 1. Denaturation: The DNA sample to be amplified (template DNA) is denatured by heating at 92°C.
 2. Annealing: The oligonucleotide primers added to the separated template DNA strands and the temperature is reduced to 40-60 °C.
 3. Synthesis by extending the primers: At 72°C DNA polymerase that has been added in the reaction mixture extends the 3' ends of the oligonucleotide primers complementarily using the template DNA. The DNA polymerase used in PCR is a thermostable, isolated from the bacterium *Thermus aquaticus*. It is called Taq polymerase which survives at the high temperature required during denaturation step.

These three steps represent a single PCR cycle. The products of the first cycle are replicated for further amplification. This reaction can be performed many times to supply unlimited copies of the amplified DNA. During repetition of the cycle regular denaturation of the freshly synthesized double stranded DNA molecules is carried out.

Applications of PCR

- After 25 cycles the target DNA is amplified about 10^6 fold.
- PCR can detect and amplify as little as 1 DNA molecule. It allows successful cloning of DNA even from samples which are more than 40,000 years old or mummified human bodies, extinct animals like wooly mammoth or dinosaurs.
- Highly useful in new fields like molecular archaeology, molecular palaeontology
- To trace the evolution of pathogenic viruses, forensic medicine, detection of viral infections before causing the disease or showing the symptoms, prenatal diagnosis of genetic disorders
- It was used for Human Genome Project

TISSUE ENGINEERING

Tissue engineering is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions. In practice the term is closely associated with applications that repair or replace portions of or whole tissues (i.e., bone, cartilage, blood vessels, bladder, etc.). The term **regenerative medicine** is often used synonymously with tissue engineering, although those involved in regenerative medicine place more emphasis on the use of stem cells to produce tissues.

Stem cells are cells found in most, if not all, multi-cellular organism. They are characterized by the ability to renew themselves through mitotic cell division and differentiating into a diverse range of specialized cell types. The two broad types of mammalian stem cells are: **embryonic stem cells** that are isolated from the inner cell mass of blastocysts, and **adult stem cells** that are found in adult tissues.

Stem cells can now be grown and transformed into specialized cells with characteristics consistent with cells of various tissues such as muscles or nerves through cell culture. Highly plastic adult stem cells from a variety of sources, including umbilical cord blood and bone marrow, are routinely used in medical therapies. Embryonic cell lines and autologous embryonic stem cells generated through therapeutic cloning have also been proposed as promising candidates for future therapies.

Powerful developments in the multidisciplinary field of tissue engineering have yielded a novel set of tissue replacement parts and implementation strategies. Scientific advances in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created unique opportunities to fabricate tissues in the laboratory from combinations of engineered extracellular matrices ("scaffolds"), cells, and biologically active molecules. Among the major challenges now facing tissue engineering is the need for more complex functionality, as well as both functional and biomechanical stability in laboratory-grown tissues destined for transplantation. The continued success of tissue engineering, and the eventual development of true human replacement parts, will grow from the convergence of engineering and basic research advances in tissue, matrix, growth factor, stem cell, and developmental biology, as well as materials science and bioinformatics.

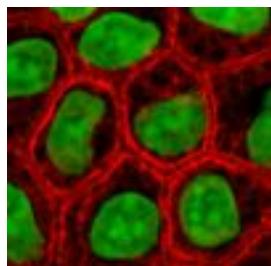
In 2003, a report entitled "The Emergence of Tissue Engineering as a Research Field" has been published, which gives a thorough description of the history of this field.

Examples

- Bioartificial liver device — several research efforts have produced hepatic assist devices utilizing living hepatocytes.
- Artificial pancreas — research involves using islet cells to produce and regulate insulin, particularly in cases of diabetes.
- Artificial bladders — Anthony Atala (Wake Forest University) has successfully implanted artificially grown bladders into seven out of approximately 20 human test subjects as part of a long-term experiment.

- Cartilage — lab-grown tissue was successfully used to repair knee cartilage.
- Doris Taylor's heart in a jar
- Tissue-engineered airway
- Artificial skin constructed from human skin cells embedded in collagen
- Artificial bone marrow

Cells as building blocks



Stained cells in culture

Tissue engineering utilizes living cells as engineering materials. Examples include using **living fibroblasts** in **skin** replacement or repair, **cartilage** repaired with living **chondrocytes**, or other types of cells used in other ways.

Cells became available as engineering materials when scientists discovered how to extend telomeres in 1998, producing immortalized cell lines. Before this, laboratory cultures of healthy, noncancerous mammalian cells would only divide a fixed number of times, up to the **Hayflick limit**.

Extraction

From fluid tissues such as blood, cells are extracted by bulk methods, usually centrifugation or apheresis. From solid tissues, extraction is more difficult. Usually the tissue is minced, and then digested with the enzymes trypsin or collagenase to remove the extracellular matrix that holds the cells. After that, the cells are free floating, and extracted using centrifugation or apheresis. Digestion with trypsin is very dependent on temperature. Higher temperatures digest the matrix faster, but create more damage. **Collagenase** is less temperature dependent, and damages fewer cells, but takes longer and is a more expensive reagent.

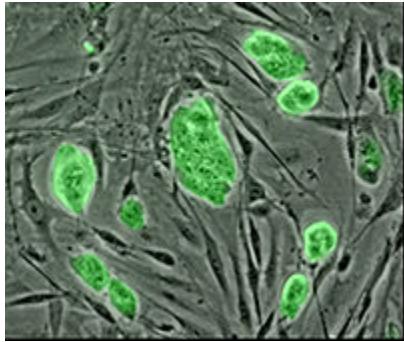
Types of cells

Cells are often categorized by their source:

- **Autologous** cells are obtained from the same individual to which they will be reimplanted. The distinguishing features of Autologous cells are:
 1. They have the very few problems with rejection and pathogen transmission however in some cases might not be available. For example in genetic disease suitable autologous cells are not available. Also very ill or elderly persons, as well as patients suffering from severe burns, may not have sufficient quantities of autologous cells to establish useful cell lines. Moreover since this category of cells needs to be harvested from the patient, there are also some concerns related to the necessity of performing such surgical operations that might lead to donor site infection or chronic pain. Autologous cells also must be cultured from samples before they can be used: this takes time, so

autologous solutions may not be very quick. Recently there has been a trend towards the use of mesenchymal stem cells from bone marrow and fat.

2. These cells can differentiate into a variety of tissue types, including bone, cartilage, fat, and nerve.
3. A large number of cells can be easily and quickly isolated from fat, thus opening the potential for large numbers of cells to be quickly and easily obtained. Several companies have been founded to capitalize on this technology, the most successful at this time being Cytori Therapeutics.



Mouse embryonic stem cells.

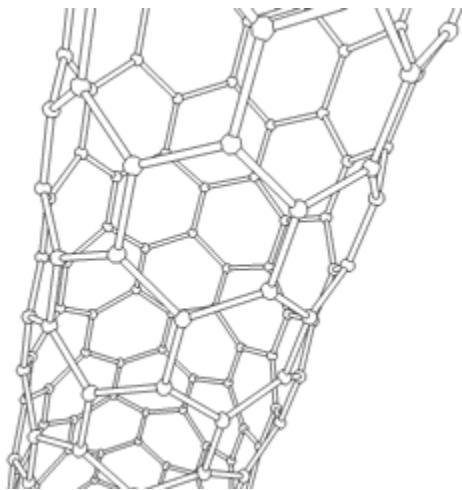
- **Allogenic** cells come from the body of a donor of the same species. While there are some ethical constraints to the use of human cells for *in vitro* studies, the employment of dermal fibroblasts from human foreskin has been demonstrated to be immunologically safe and thus a viable choice for tissue engineering of skin.
- **Xenogenic** cells are these isolated from individuals of another species. In particular animal cells have been used quite extensively in experiments aimed at the construction of cardiovascular implants.
- **Syngenic or isogenic** cells are isolated from genetically identical organisms, such as twins, clones, or highly inbred research animal models.

Primary cells are from an organism.

Secondary cells are from a cell bank.

Stem cells are undifferentiated cells with the ability to divide in culture and give rise to different forms of specialized cells. According to their source stem cells are divided into "adult" and "embryonic" stem cells, the first class being multipotent and the latter mostly pluripotent; some cells are totipotent, in the earliest stages of the embryo. While there is still a large ethical debate related with the use of embryonic stem cells, it is thought that stem cells may be useful for the repair of diseased or damaged tissues, or may be used to grow new organs.

Scaffolds/Extracellular matrices



Cells are often implanted or 'seeded' into an artificial structure capable of supporting three-dimensional tissue formation. These structures, typically called scaffolds, are often critical, both *ex vivo* as well as *in vivo*, to recapitulating the *in vivo* milieu and allowing cells to influence their own microenvironments. The purposes served by the scaffolds are:

- Allow cell attachment and migration
- Deliver and retain cells and biochemical factors
- Enable diffusion of vital cell nutrients and expressed products
- Exert certain mechanical and biological influences to modify the behaviour of the cell phase

This rotating Carbon nanotube shows its 3D structure. Carbon nanotubes are

1. Among the numerous candidates for tissue engineering scaffolds
2. They are biocompatible
3. Resistant to biodegradation
4. Can be functionalized with biomolecules.
5. However, the possibility of toxicity with non-biodegradable nano-materials is not fully understood.

Also the requirements of an ideal scaffold are:

- A high porosity and an adequate pore size are necessary to facilitate cell seeding and diffusion throughout the whole structure of both cells and nutrients.
- Biodegradability is often an essential factor since scaffolds should preferably be absorbed by the surrounding tissues without the necessity of a surgical removal.
- The rate at which degradation occurs has to coincide as much as possible with the rate of tissue formation: this means that while cells are fabricating their own natural matrix structure around themselves, the scaffold is able to provide structural integrity within the body and eventually it will break down leaving the neotissue, newly formed tissue which will take over the mechanical load.
- Injectability is also important for clinical uses.

Materials: They are usually functionally customized and the ideal properties are:

- | | |
|--------------------------|-----------------------------|
| a. injectability, | e. transparency |
| b. synthetic manufacture | f. nano-scale fibers |
| c. biocompatibility | g. low concentration |
| d. non-immunogenicity | h. desired resorption rates |

The different types of materials:

1. **Natural or constructed from natural materials** - different derivatives of the extracellular matrix. Proteic materials, such as collagen or fibrin, and polysaccharidic materials, like chitosan or glycosaminoglycans (GAGs), are all suitable in terms of cell compatibility, but some issues with potential immunogenicity still remains. Among GAGs hyaluronic acid, possibly in combination with cross linking agents (e.g. glutaraldehyde, water soluble carbodiimide, etc...), bioresorbable sutures like collagen
2. **Synthetic** – PuraMatrix, PLA - polylactic acid. This is a polyester which degrades within the human body to form lactic acid, a naturally occurring chemical which is easily removed from the body; polyglycolic acid (PGA) and polycaprolactone (PCL): their degradation mechanism is similar to that of PLA, but slightly slower.

The materials can be biodegradable or non-biodegradable.

Synthesis

A number of different methods has been described in literature for preparing porous structures to be employed as tissue engineering scaffolds. Each of these techniques presents its own advantages, but none is devoid of drawbacks.

- **Nanofiber Self-Assembly:** Molecular self-assembly is the method to create biomaterials with properties similar in scale and chemistry to that of the natural in vivo extracellular matrix (ECM). The polymers are immersed in the hydrogels and assemble on their own thus known as self assembly. These are hydrogel scaffolds, superior in vivo toxicology and biocompatibility.
- **Textile technologies:** these techniques include the preparation of non-woven meshes of different polymers. e.g. non-woven polyglycolide structures. Such fibrous structures are useful to grow different types of cells. The drawbacks -difficulties of obtaining high porosity and regular pore size.
- **Solvent Casting & Particulate Leaching (SCPL):** the preparation of porous structures with regular porosity, but with a limited thickness.
 - 1.the polymer is dissolved into a suitable organic solvent (e.g. polylactic acid could be dissolved into dichloromethane),
 - 2.the solution is cast into a mold filled with porogen particles of inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres. The size of the porogen particles and the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure.
 3. The solvent is allowed to fully evaporate,
 - 4.the composite structure in the mold is immersed in a bath of a liquid suitable for dissolving the porogen. Once the porogen has been fully dissolved a porous structure is obtained.

The drawback of SCPL - its use of organic solvents which must be fully removed to avoid any possible damage to the cells seeded on the scaffold.

- **Gas Foaming:** to overcome the necessity to use organic solvents and solid porogens a technique using gas as a porogen has been developed.
 1. disc shaped structures made of the desired polymer are prepared by means of compression molding using a heated mold.
 2. The discs are then placed in a chamber where are exposed to high pressure CO₂ for several days.
 3. The pressure inside the chamber is gradually restored to atmospheric levels. During this procedure the pores are formed by the carbon dioxide molecules that abandon the polymer, resulting in a sponge like structure.

The drawbacks: prohibits the incorporation of any temperature labile material into the polymer matrix; the pores do not form an interconnected structure.
- **Emulsification/Freeze-drying:** this technique does not require the use of a solid porogen like SCPL.
 1. a synthetic polymer is dissolved into a suitable solvent (e.g. polylactic acid in dichloromethane),
 2. water is added to the polymeric solution
 3. the two liquids are mixed in order to obtain an emulsion.
 4. the emulsion is cast into a mold
 5. quickly frozen by means of immersion into liquid nitrogen.
 6. The frozen emulsion is subsequently freeze-dried to remove the dispersed water and the solvent, thus leaving a solidified, porous polymeric structure.

Drawbacks -it still requires the use of solvents, pore size is relatively small and porosity is often irregular.)
- **Thermally Induced Phase Separation (TIPS):** similar to the previous technique, (this phase separation procedure requires the use of a solvent with a low melting point that is easy to sublime. For example dioxane could be used to dissolve polylactic acid, then phase separation is induced through the addition of a small quantity of water: a polymer-rich and a polymer-poor phase are formed. Following cooling below the solvent melting point and some days of vacuum-drying to sublime the solvent a porous scaffold is obtained.) Liquid-liquid phase separation presents the same drawbacks of emulsification/freeze-drying.
- **CAD/CAM Technologies:** since most of the above described approaches are limited when it comes to the control of porosity and pore size, computer assisted design and manufacturing techniques have been introduced to tissue engineering. First a three-dimensional structure is designed using CAD software, then the scaffold is realized by using ink-jet printing of polymer powders or through Fused Deposition Modeling of a polymer melt.

Assembly methods

One of the continuing, persistent problems with tissue engineering is mass transport limitations. Engineered tissues generally lack an initial blood supply, thus making it difficult for any implanted cells to obtain sufficient oxygen and nutrients to survive, and/or function properly.

Self-assembly may play an important role here, both from the perspective of encapsulating cells and proteins, as well as creating scaffolds on the right physical scale for engineered tissue constructs and cellular ingrowth.

It might be possible to print organs, or possibly entire organisms. A recent innovative method of construction uses an ink-jet mechanism to print precise layers of cells in a matrix of thermoreversible gel. Endothelial cells, the cells that line blood vessels, have been printed in a set of stacked rings. When incubated, these fused into a tube.

Tissue culture

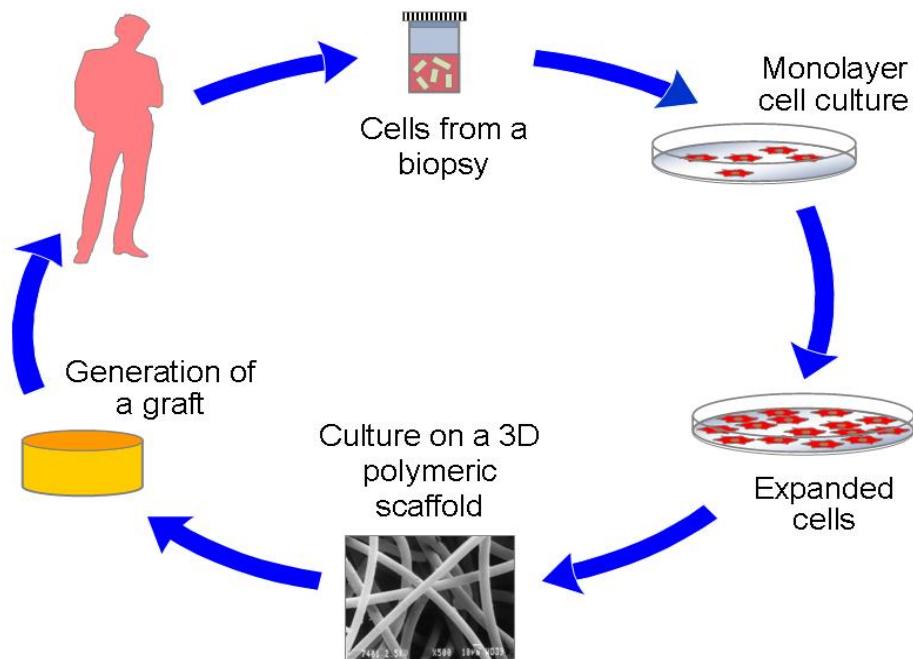
In many cases, creation of functional tissues and biological structures *in vitro* requires extensive culturing to promote survival, growth and induction of functionality. In general, the basic requirements of cells must be maintained in culture, which include oxygen, pH, humidity, temperature, nutrients and osmotic pressure maintenance.

Tissue engineered cultures also present additional problems in maintaining culture conditions. In standard cell culture, diffusion is often the sole means of nutrient and metabolite transport. However, as a culture becomes larger and more complex, such as the case with engineered organs and whole tissues, other mechanisms must be employed to maintain the culture.

Another issue with tissue culture is introducing the proper factors or stimuli required to induce functionality. In many cases, simple maintenance culture is not sufficient. Growth factors, hormones, specific metabolites or nutrients, chemical and physical stimuli are sometimes required. For example, certain cells respond to changes in oxygen tension as part of their normal development, such as chondrocytes, which must adapt to low oxygen conditions or hypoxia during skeletal development. Others, such as endothelial cells, respond to shear stress from fluid flow, which is encountered in blood vessels.

Basic principle of Tissue engineering is illustrated in the following figure. Cells can be isolated from the patient's body, and expanded in a petri dish in laboratory. Once we have enough number of cells, they can be seeded on a polymeric scaffold material, and cultured *in vitro* in a bioreactor or incubator. When the construct is matured enough, then it can be implanted in the area of defect in patient's body.

Basic principles of Tissue engineering



The application of the principles & methods of engineering & life sciences towards the fundamental understanding of structure, function & relationships in normal & pathological mammalian tissue & the development of biological substitutes to restore, maintain & improve tissue function.

... Symposium on Tissue Engineering (1988)

Defect	Organ	Function
Mechanical	Cartilage	Resist compression
Metabolites	Liver	Nitrogen metabolism
Synthetic	Pancreas	Insulin production
Communication	Nerve	Coordination
Combination	Skin	prevents water loss immunologic barrier