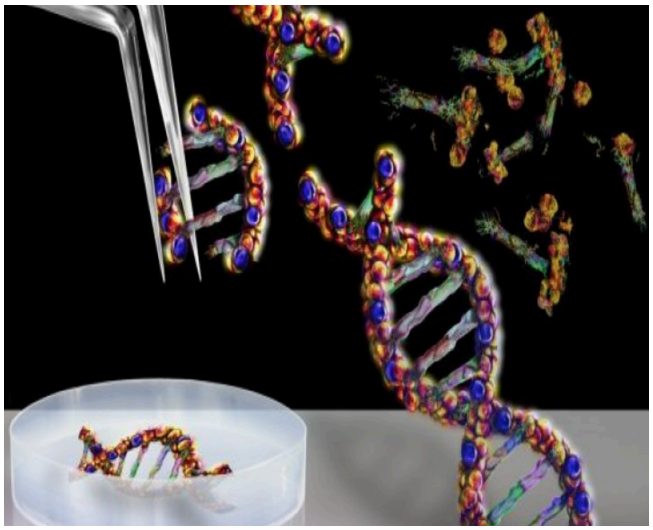

BIOTECHNOLOGY AND GENETIC ENGINEERING

ABSTRACT

This class discusses the fundamental aspects of biotechnology and genetic engineering its importance to mankind in a concise and lucid explanation of this newly founded science. The course emphasizes how cell structure and function is a vital starting point for knowledge of genetic engineering and gene technology. Lectures will underlie the principles and application of Recombinant DNA technology in industrial, agricultural, pharmaceutical, and biomedical fields.

Dr. Saad mutlk

Fourth stage

Biology Department

College of science

Origin of the term "Biotechnology"

According to Robert Bud1, this term (Biotechnology) was firstly used by the Hungarian **Károly Ereky** during 1919 to describe a technology based on converting raw materials into a more useful product in a book called "Biotechnology of Meat, Fat and Milk Production in an Agricultural Large-Scale Farm ». For Ereky, the term "biotechnology" indicated the process by which raw materials could be biologically upgraded into socially useful products. Since its inception, the concept of biotechnology has been variously defined.

Definitions used by governments and organizations

FDA's working definition of biotechnology is "the application of biological systems and organisms to technical and industrial processes". This definition is necessarily broad. It takes in both the "old" and "new" science: the age-old techniques for making beer or yogurt as well as the most advanced uses of recombinant DNA technology. It takes in many applications, from production of enzymes for laundry detergents, to selective breeding of plants and animals, to genetic engineering of bacteria to clean up oil spills.

Federal Republic of Germany: "Biotechnology deals with the introduction of biological methods within the framework of technical processes and industrial production. It involves the application of microbiology and biochemistry together with technical chemistry and process engineering"

Biotechnology a multidisciplinary field

Biotechnology is a clearly multidisciplinary field involving biochemistry, molecular biology, genetics, immunology, microbiology, pharmacology, fermentation, agriculture, to name just a few. Each of the contributing subject areas brings its own special vocabulary and nomenclature standards and considerable difficulties of communication is the result. It is therefore important to become familiar with terminology.

HISTORY & EVOLUTION OF BIOTECHNOLOGY

This section introduces to biotechnology concepts through the presentation of timeline showing the progression from the earliest domestication of crops and animals (before the Common Era) to modern methods of biotechnology in the 21st Century. The classification in ancient biotechnology (1st generation), classical biotechnology (2nd generation) and modern biotechnology (3rd generation) is presented. Dates are benchmarks of scientific, social responses and regulatory breakthroughs, and scientific evidence on the important of the role of biotechnology as tools to improve food production (crops, food, and animal's husbandry) is highlighted.

Biotechnology Timeline

The historical application of Biotechnology throughout is provided below since before the common era.

Before Common Era:

- 7000 BCE – Chinese discover fermentation through beer making.
- 6000 BCE – Yogurt and cheese made with lactic acid-producing bacteria by various people.
- 4000 BCE – Egyptians bake leavened bread using yeast.
- 500 BCE – Moldy soybean curds used as an antibiotic.
- 250 BCE – The Greeks practice crop rotation for maximum soil fertility.
- 100 BCE – Chinese use chrysanthemum as a natural insecticide.

Pre-20th Century:

- 1663 – First recorded description of dying cells by Robert Hooke.
- 1675 – Antonie van Leeuwenhoek discovers and describes vagina and protozoa.
- 1798 – Edward Jenner uses first viral vaccine to inoculate a child from smallpox.
- 1802 – The first recorded use of the word biology.
- 1824 – Henri Dutrochet discovers that tissues are composed of living cells.
- 1838 – Protein discovered, named and recorded by Gerardus Johannes Mulder and Jöns Jacob Berzelius.

- 1862 – Louis Pasteur discovers the bacterial origin of fermentation.
- 1863 – Gregor Mendel discovers the laws of inheritance.
- 1864 – Antonin Prandtl invents first centrifuge to separate cream from milk.
- 1869 – Friedrich Miescher identifies DNA in the sperm of a trout.
- 1871 – Ernst Hoppe-Seyler discovers invertase, which is still used for making artificial sweeteners.
- 1877 – Robert Koch develops a technique for staining bacteria for identification.
- 1878 – Walther Flemming discovers chromatin leading to the discovery of chromosomes.
- 1881 – Louis Pasteur develops vaccines against bacteria that cause cholera and anthrax in chickens.
- 1885 – Louis Pasteur and Emile Roux develop the first rabies vaccine and use it on Joseph Meister.

20th century:

- 1919 – Károly Ereky, a Hungarian agricultural engineer, first uses the word biotechnology.
- 1928 – Alexander Fleming notices that a certain mould could stop the duplication of bacteria, leading to the first antibiotic: penicillin.
- 1933 – Hybrid corn is commercialized.

- 1942 – Penicillin is mass-produced in microbes for the first time.
- 1950 – The first synthetic antibiotic is created.
- 1951 – Artificial insemination of livestock is accomplished using frozen semen.
- 1952 – L.V. Radushkevich and V.M. Lukyanovich publish clear images of 50 nanometre diameter tubes made of carbon, in the Soviet Journal of Physical Chemistry.
- 1953 – James D. Watson and Francis Crick describe the structure of DNA.
- 1958 – The term bionics is coined by Jack E. Steele.
- 1964 – The first commercial myoelectric arm is developed by the Central Prosthetic Research Institute of the USSR, and distributed by the Hangar Limb Factory of the UK.
- 1972 – The DNA composition of chimpanzees and gorillas is discovered to be 99% similar to that of humans.
- 1973 – Stanley Norman Cohen and Herbert Boyer perform the first successful recombinant DNA experiment, using bacterial genes.
- 1974 – Scientist invent the first biocement for industrial applications.
- 1975 – Method for producing monoclonal antibodies developed by Köhler and César Milstein.
- 1978 – North Carolina scientists Clyde Hutchison and Marshall Edgell show it is possible to introduce specific mutations at specific sites in a DNA molecule.
- 1980 – The U.S. patent for gene cloning is awarded to Cohen and Boyer.
- 1982 – Humulin, Genentech's human insulin drug produced by genetically engineered bacteria for the treatment of diabetes, is the first biotech drug to be approved by the Food and Drug Administration.
- 1983 – The Polymerase Chain Reaction (PCR) technique is conceived.
- 1990 – First federally approved gene therapy treatment is performed successfully on a young girl who suffered from an immune disorder.
- 1994 – The United States Food and Drug Administration approves the first GM food: the "Flavr Savr" tomato.
- 1997 – British scientists, led by Ian Wilmut from the Roslin Institute, report cloning Dolly the sheep using DNA from two adult sheep cells.
- 1999 – Discovery of the gene responsible for developing cystic fibrosis.
- 2000 – Completion of a "rough draft" of the human genome in the Human Genome Project.

21st Century:

- 2001 – Celera Genomics and the Human Genome Project create a draft of the human genome sequence. It is published by Science and Nature Magazine.
- 2002 – Rice becomes the first crop to have its genome decoded.
- 2003 – The Human Genome Project is completed, providing information on the locations and sequence of human genes on all 46 chromosomes.
- 2008 – Japanese astronomers launch the first Medical Experiment Module called "Kibo", to be used on the International Space Station.

- 2009 – Cedars-Sinai Heart Institute uses modified SAN heart genes to create the first viral pacemaker in guinea pigs, now known as iSANs.

Plant tissue culture

In general Tissue culture is referred to the culture and maintenance of plant cells or organs in sterile, nutritionally and environmentally supportive conditions (*in vitro*), in which all product cells (clones) have the same genotype (unless affected by mutation during culture). It has applications in **research** and **commerce**. In commercial settings, tissue culture is primarily used for plant propagation and is usually meant to as micropropagation.

⇒ 1920's known to be as a first commercial use of plant tissue culture on artificial media (germination and growth of orchid plants).

⇒ During 1950's and 60's there was a great deal of research specially when a reliable artificial medium was developed and leads to plant tissue culture really 'took off' commercially.

⇒ Its methods are used for **virus eradication**, **genetic manipulation**, **somatic hybridization** and other procedures that benefit propagation, plant improvement and basic research.

Several advantages were arisen from using different techniques in plant tissue culture which including:

◊ The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
◊ Ability to quickly produce mature plants.
◊ The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
◊ The regeneration of whole plants from plant cells that have been genetically modified.
◊ The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and *Nepenthes*.
◊ To clear particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

o It relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency).

o Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

Tissue culture has several critical requirements:

- Appropriate tissue (some tissues culture better than others).
- A suitable growth medium containing energy sources and inorganic salts to supply cell growth needs. This can be liquid or semisolid.
- Aseptic (sterile) conditions, as microorganisms grow much more quickly than plant and animal tissue and can overrun a culture.
- Growth regulators - in plants, both auxins & cytokinin's.
- Frequent subculturing to ensure adequate nutrition and to avoid the build-up of waste metabolites.

Appropriate tissue (Explant):

These explants include (Cell, tissue or organ of a plant) can be used for tissue culture, although axillary buds and meristems are most commonly used. It is important to remove microbial contaminants firstly. Which is usually performed by chemical surface sterilization of the explants with an agent such as bleach at a concentration and for a duration that will kill or remove pathogens without injuring the plant cells beyond recovery.

Nutrition medium:

When an explant is isolated, it is no longer able to receive nutrients or hormones from the plant, and these must be provided to allow growth *in vitro*. The composition of the nutrient medium is for the most part similar, although the exact components and quantities will vary for different species and purpose of culture. Types and amounts of hormones vary greatly. In addition, the culture must be provided with the ability to excrete the waste products of cell metabolism. This is accomplished by culturing on or in a defined culture medium which is periodically replenished.

- A nutrient medium is defined by its mineral salt composition, carbon source, vitamins, plant growth regulators and other organic supplements.
- pH determines many important aspects of the structure and activity of biological macromolecules. Optimum pH of 5.0-6.0 tends to fall during autoclaving and growth.

Mineral salt composition:

⇒ Macroelements: referred to the elements (N, K, P, Ca, S, Mg, Cl) required in concentration > 0.5 mmol/l

⇒ Microelements: referred to the elements (Fe, Mn, B, Cu, Zn, I, Mo, Co) required in conc. < 0.5 mmol/l

An optimum concentration → maximum growth rate

Carbon sources and vitamins:

- Sucrose or glucose (sometimes fructose), concentration 2-5%
- Most media contain myo-inositol, which improves cell growth.
- An absolute requirement for vitamin B1 (thiamine).
- Growth is also improved by the addition of nicotinic acid and vitamin B6 (pyridoxine).

In addition, some media contain pantothenic acid, biotin, folic acid, p-amino benzoic acid, choline chloride, riboflavin and ascorbic acid (C-vitamin).

Plant growth regulators (Body building Plants)

Auxins: (2,4-D, NAA, IAA, IBA, pCPA)

⇒ prompts cell division, cell elongation, swelling of tissues, formation of callus, formation of adventitious roots.
⇒ reduces adventitious and axillary shoot formation.

Cytokinins: (BAP, Kinetin, zeatin, 2iP)

shoot induction, cell division

Gibberellins: GA3

⇒ plant regeneration, elongation of internodes

Abscisic acid: ABA

⇒ induction of embryogenesis

Organic supplements:

⇒ **N** in the form of amino acids (glutamine, asparagine) and nucleotides (adenine).
⇒ **Organic acids:** TCA cycle acids (citrate, malate, succinate, fumarate), pyruvate.
⇒ **Complex substances**: yeast extract, malt extract, coconut milk, protein hydrolysate.
⇒ **Activated charcoal** is used where phenol-like compounds are a problem, absorbing toxic pigments and stabilizing pH. Also, to prevent oxidation of phenols PVP (polyvinylpyrrolidone), citric acid, ascorbic acid, thiourea and L-cysteine are used.

Applications:

Plant tissue culture is used widely in the plant sciences, forestry, and in horticulture and its applications include:

- ⇒ The commercial production of plants used as potting, landscape, and florist subjects, which uses meristem and shoot culture to produce large numbers of identical individuals.
- ⇒ To conserve rare or endangered plant species.
- ⇒ A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g., herbicide resistance/tolerance.
- ⇒ Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals.
- ⇒ To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
- ⇒ To rapidly study the molecular basis for physiological, biochemical, and reproductive mechanisms in plants, for example *in vitro* selection for stress tolerant plants.
- ⇒ To cross-pollinate distantly related species and then tissue culture the resulting embryo which would otherwise normally die (Embryo Rescue).
- ⇒ For chromosome doubling and induction of polyploidy, for example doubled haploids, tetraploids, and other forms of polyploids. This is usually achieved by application of antimitotic agents such as colchicine or oryzalin.
- ⇒ As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.
- ⇒ Certain techniques such as meristem tip culture can be used to produce clean plant material from virus stock, such as sugarcane, potatoes and many species of soft fruit.
- ⇒ Large scale production of artificial seeds through somatic embryogenesis.

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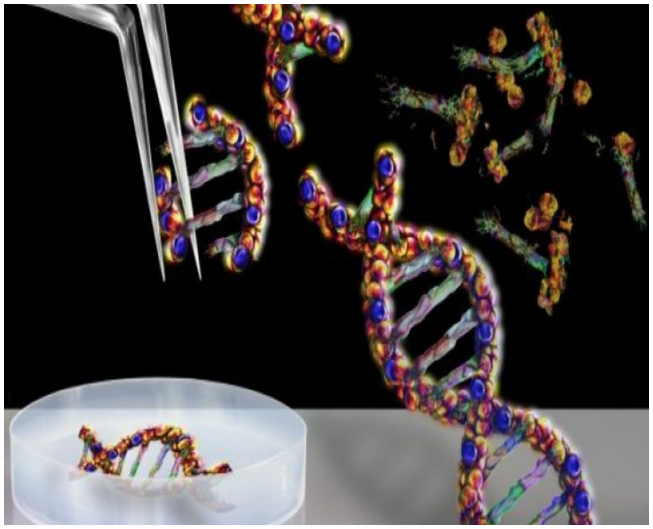


BIOTECHNOLOGY AND GENETIC ENGINEERING

ABSTRACT

Animal cell culture technology in today's scenario has become crucial in the field of life sciences, which provides a basis to study regulation, proliferation, and differentiation and to perform genetic manipulation. It requires specific technical skills to carry out successfully. This lecture describes the essential techniques of animal cell culture as well as its applications. describes the basics of animal cell culture along with the most recent applications.

Dr. Saad mutlk

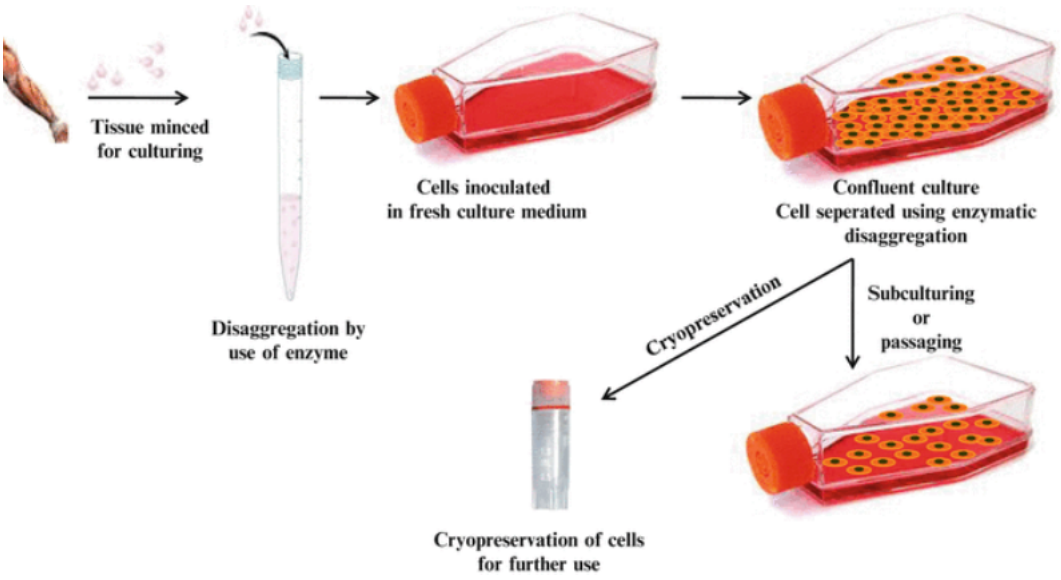
Fourth stage

Biology Department

College of science

What is Cell and Tissue Culture?

Tissue Culture is the general term for the removal of cells, tissues, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid or semi-solid medium that supplies the nutrients essential for survival and growth. The culture of whole organs or intact organ fragments with the intent of studying their continued function or development is called **Organ Culture**. When the cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relation- ships with neighbouring cells, it is called **Cell Culture**. Although animal cell culture was first successfully under taken by Ross Harrison in 1907, it was not until the late 1940's to early 1950's that several developments occurred that made cell culture widely available as a tool for scientists. For example, there was the development of antibiotics that made it easier to avoid many of the contamination problems that plagued earlier cell culture attempts.



Types of cell culture:

1- Primary cell culture:

These cells are obtained directly from tissues and organs by mechanical or chemical disintegration or by enzymatic digestion. These cells are induced to grow in suitable glass or plastic containers with complex media. These cultures usually have a low growth rate and are heterogeneous; however, they are still preferred over cell lines as these are more representative of the cell types in the tissues from which they are derived.

2- Secondary cell culture:

When primary cell cultures are passaged or subcultured and grown for a long period of time in fresh medium, they form secondary cultures and are long-lasting (unlike cells of primary cell cultures) due to the availability of fresh nutrients at regular intervals. The passaging or subculturing is carried out by enzymatic digestion of adherent cells. This is followed by washing and re-suspending of the required amount of cells in appropriate volumes of growth media. Secondary cell cultures are preferred as these are easy to grow and are readily available; they have been useful in virological, immunological, and toxicological research.

According to the life span of culture, the cell lines are categorized into two types:

- ## Finite cell lines

These cell lines are known to have limited number of cell division during their life span. The cells passage several times and then lose their ability to proliferate, which is a genetically determined event known as senescence. Cell lines derived from primary cultures of normal cells are finite cell lines.

- ## Continuous cell lines

When a finite cell line undergoes transformation and gains the ability to divide indefinitely, it becomes a continuous cell line. Such transformation/mutation can occur spontaneously or can be chemically or virally induced or from the establishment of cell cultures from malignant tissue.

Quantitation:

Quantitation is carried out to characterize cell growth and to establish reproducible culture conditions.

Hemocytometer

Cell counts are important for monitoring growth rates as well as for setting up new cultures with known cell numbers. The most widely used type of counting chamber is called a hemocytometer. It is used to estimate cell number. The concentration of cells in suspension is determined by placing the cells in an optically clear chamber under a microscope. The cell number within a defined area of known depth is counted, and the cell concentration is determined from the count.

Electronic counting

For high-throughput work, electronic cell counters are used to determine the concentration of each sample.

Other quantitation

In some cases, the DNA content or the protein concentration needs to be determined instead of the number of cells.

Growth Requirements

The culture media used for cell cultures are generally quite complex, and culture condition widely varies for each cell type. However, media generally include amino acids (source of nitrogen), vitamins (cofactors), salts (maintain osmotic pressure), glucose (source of energy, carbon,) a bicarbonate buffer system (maintains a pH between 7.2 and 7.4), growth factors and hormones (growth stimulators), O2 and CO2. To obtain best growth, addition of a small amount of blood serum is usually necessary, and several antibiotics, like penicillin and streptomycin are added to prevent bacterial contamination. Temperature varies on the type of host cell. Most mammalian cells are maintained at 37^{o}C for optimal growth, while cells derived from cold- blooded animals tolerate a wider temperature range (i.e., 15^{o}C to 26^{o}C).

Advantages of animal cell culture

1- Physiochemical and physiological condition: Role and effect of pH, temperature, O2/CO2 concentration, and osmotic pressure of the culture media can be altered to study their effects on the cell culture.
2- Metabolism of cell: To study cell metabolism and investigate the physiology and biochemistry of cells.
3- Cytotoxic assay: Effect of various compounds or drugs on specific cell types such as liver cells can be studied.
4- Homogenous cultures: These cultures help study the biology and origin of the cells.
5- Valuable biological data from large-scale cell cultures: Specific proteins can be synthesized in large quantities from genetically modified cells in large-scale cultures.
6- Consistency of results: Reproducibility of the results that can be obtained by the use of a single type/clonal population.
7- Identification of cell type: Specific cell types can be detected by the presence of markers such as molecules or by karyotyping.
8- Vaccines production, Virus cultivation and study, Gene therapy, Cancer research, Cellular and molecular study.

Disadvantages of animal cell culture

1- Expenditure and expertise: This is a specialized technique that requires aseptic conditions, trained personnel, and costly equipment.
2- Dedifferentiation: Cell characteristics can change after a period of continuous growth of cells in cultures, leading to differentiated properties compared to the original strain.
3- Low amount of product: The miniscule amount of mAB and recombinant protein produced followed by downstream processing for extracting pure products increases expenses tremendously.
4- Contamination: Mycoplasma and viral infection are difficult to detect and are highly contagious.
5- Instability: Aneuploidy chromosomal constitution in continuous cell lines leads to instability. In addition, this system cannot replace the complex live animal for testing the response of chemicals or the impact of vaccines or toxins.

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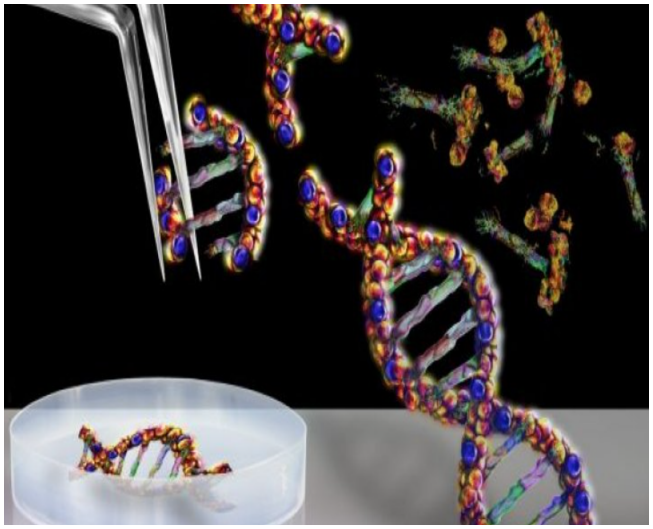

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Dr. Saad mutlk

Fourth stage

Biology Department

College of science

What are Biosensors?

A biosensor is a diagnostic device used to investigate the concentration and presence of a specific substance in a biological sample. It is an analytical device that convert a biological response into an electrical signal. Essentially biosensors must be highly specific, independent of physical parameters such as pH and temperature and should be reusable[1].

Construction of biosensors, its materials, transducing devices, and control methods requires multidisciplinary research in chemistry, biology, and engineering. The materials used in biosensors are categorized into three groups based on their mechanisms: biocatalytic group comprising enzymes, bioaffinity group including antibodies and nucleic acids, and microbe based containing microorganisms.

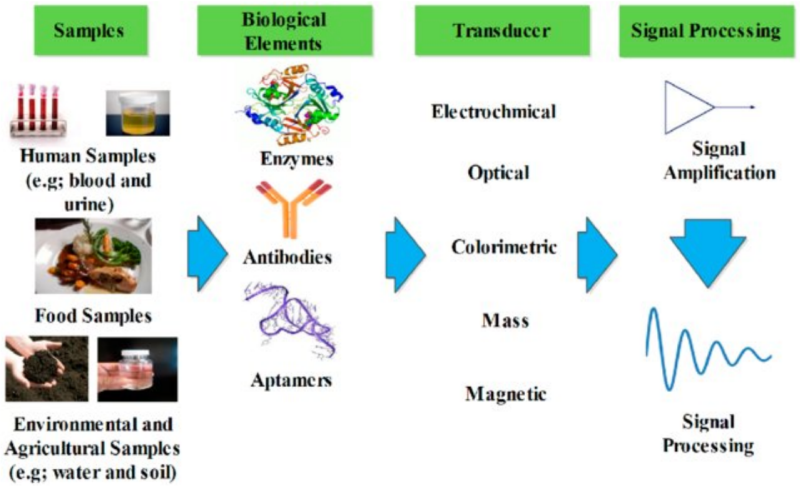


Figure.1 Schematic of different parts of a biosensor including biological recognition elements, transducers, and detectors.

Types of biosensors

Biosensors started in the 1960s by the pioneers **Clark** and **Lyons**. Various types of biosensors being used are enzyme-based, tissue-based, immunosensors, DNA biosensors, and thermal and piezoelectric biosensors.

Enzyme-based biosensors have been devised on restriction methods such a adsorption of enzymes by van der Waals forces, ionic bonding or covalent bonding. The commonly used enzymes for this purpose are oxidoreductases, polyphenol oxidases, peroxidases, and aminooxidases.[2]

Tissue-based sensors arise from plant and animal sources. The analyte of interest can be an inhibitor or a substrate of these processes. Organelle-based sensors were

made using membranes, chloroplasts, mitochondria, and microsomes. However, for this type of biosensor, the stability was high, but the detection time was longer, and the specificity was reduced.

Immunosensors were established on the fact that antibodies have high similarity towards their respective antigens such as antibodies specifically bind to pathogens or toxins, or interact with components of the host's immune system.

The DNA biosensors were devised on the property that single-strand nucleic acid molecule is able to recognize and bind to its complementary strand in a sample. The interaction is due to the formation of stable hydrogen bonds between the two nucleic acid strands.

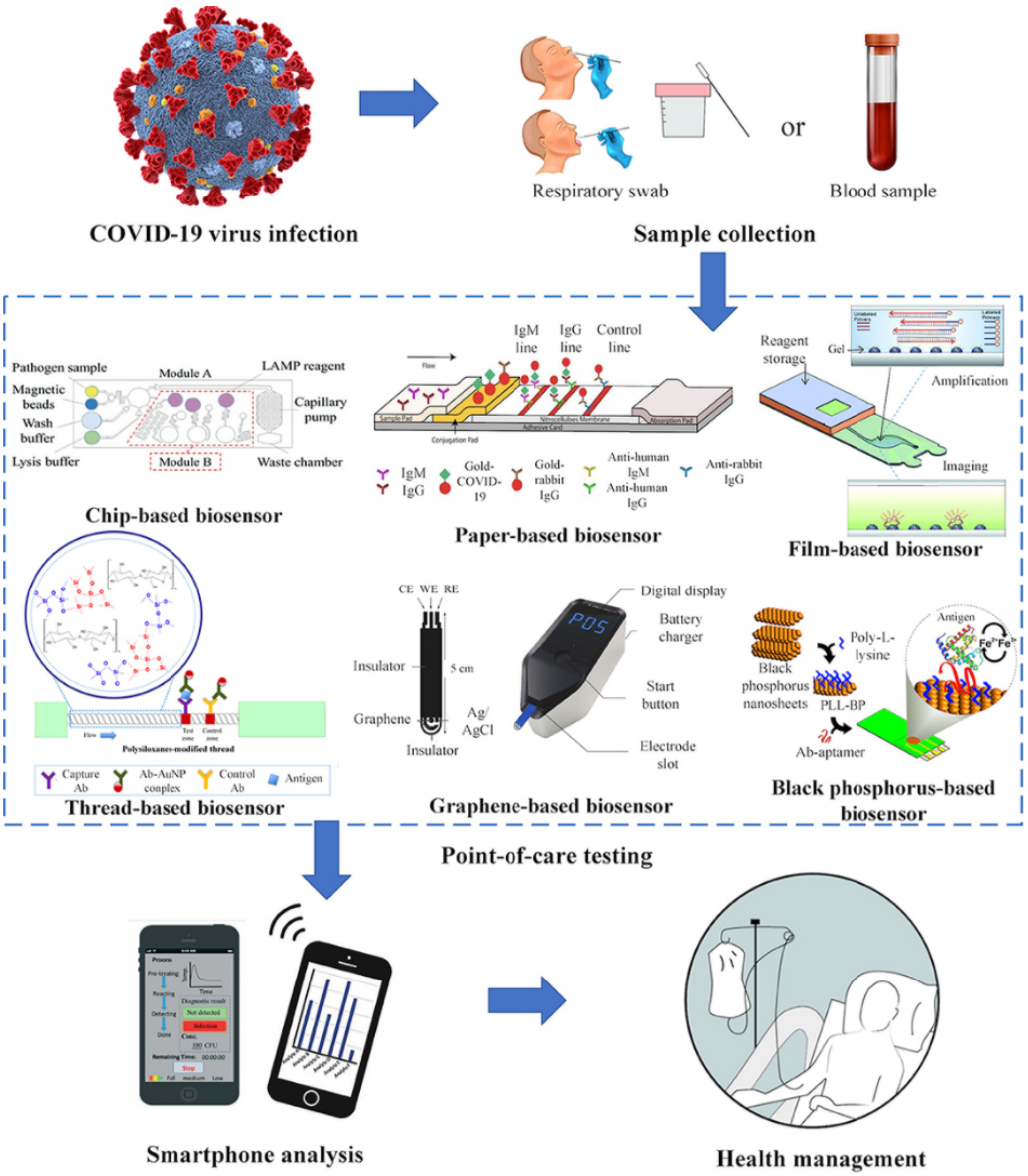
Magnetic biosensors: miniaturized biosensors detecting magnetic micro- and nanoparticles in microfluidic channels using the magnetoresistance effect have great potential in terms of sensitivity and size.

Thermal biosensors or calorimetric biosensors are developed by assimilating biosensor materials as mentioned before into a physical transducer.

Piezoelectric biosensors are of two types: the quartz crystal microbalance and the surface acoustic wave device. They are based on the measurement of changes in resonance frequency of a piezoelectric crystal due to mass changes on the crystal structure.

Optical biosensors consist of a light source, as well as numerous optical components to generate a light beam with specific characteristics and to beeline this light to a modulating agent, a modified sensing head along with a photodetector.[3]

Green fluorescent protein and the subsequent auto fluorescent protein (AFP) variants and genetic fusion reporters have aided the development of genetically-encoded biosensors[4]. This type of biosensor is user-friendly, easy to engineer, manipulate and transfer into cells. Peptide and protein biosensors are easily manufactured through synthetic chemistry followed by enzymatic labelling with synthetic fluorophores.



Applications of biosensors:

Biosensors have been applied in many fields namely food industry, medical field, marine sector etc., and they provide better stability and sensitivity as compared with the traditional methods.

1. In food processing, monitoring, food authenticity, quality and safety):

An arduous quandary in food processing industry is of quality and safety, maintenance of food products and processing. The development of biosensors in response to the demand for simple, real-time, selective and inexpensive techniques is seemingly favourable.

Biosensors are used for the detection of pathogens in food in which presence of Escherichia coli in vegetables, is a bioindicator of faecal contamination in food[5].

One of the popular food additives extensively used today are **sweeteners**, which are adversely causing undesirable diseases including dental caries, cardiovascular diseases, obesity and type-2 diabetes.

2. In fermentation processes:

In fermentation industries, process safety and product quality are crucial. Thus effective monitoring of the fermentation process is imperative to develop, optimize and maintain biological reactors at maximum efficacy. Biosensors precisely control the fermentation industry and produce reproducible results due to their simple instrumentation, formidable selectivity, low prices and easy automation. Nowadays, several kinds of commercial biosensors are accessible; capable of detecting biochemical parameters (glucose, lactate, lysine, ethanol etc.) and are widely used in China, occupying about 90% of its market.

3. Biosensing technology for sustainable food safety:

The term food quality refers to the appearance, taste, smell, nutritional value, freshness, flavour, texture and chemicals. Smart monitoring of nutrients and fast screening of biological and chemical contaminants are of paramount importance, when it comes to food quality and safety. Material science, nanotechnology, electromechanical and microfluidic systems are striding in to make sensing technology imminent for use in market. Efforts are being made for developing control systems ensuring food quality and safety and, as a consequence, human health. **Glucose** monitoring becomes indispensable as during storage the food content and composition may get altered[6].

4. In medical field:

Glucose biosensors are widely used in clinical applications for diagnosis of diabetes mellitus, which requires precise control over blood-glucose levels[7]. Blood-glucose biosensors usage at home accounts for 85% of the gigantic world market[8] .

5. Fluorescent biosensors:

Fluorescent biosensors are imaging agents, for use in cancer and drug discovery. These are small scaffolds onto which one or several fluorescent probes are mounted (enzymatically, chemically or genetically) through a receptor. Fluorescent biosensors are used in drug discovery programmes for early detection of biomarkers in molecular and clinical diagnostics, for monitoring disease progression and response to treatment/therapeutics, for intravital imaging and image guided surgery[9]. A genetically-

encoded FRET biosensor developed for detection of Bcr-Abl kinase activity was used on cancer patient cells to assess Bcr-Abl kinase activity and to establish an interrelation with the disease status in chronic myeloid leukaemia.

6. Biodefense biosensing applications:

Biosensors can be used for military purposes at times of biological attacks. The main motive of such biosensors is be to sensitively and selectively identify organisms posing threat in virtually real time called biowarfare agents (BWAs) namely, bacteria (vegetative and spores), toxins and viruses.

7. In metabolic engineering:

Environmental concerns and lack of sustainability of petroleum-derived products are gradually exhorting need for development of microbial cell factories for synthesis of chemicals. They have also envisioned that a substantial fraction of fuels, commodity chemicals and pharmaceuticals will be produced from renewable feedstocks by exploiting microorganisms rather than relying on petroleum refining or extraction from plants.

8. Biosensors in plant biology:

Revolutionary new technologies in the areas of DNA sequencing and molecular imaging, have led to advancements in plant science. However, this information can be easily successfully tapped using biosensors. Roger Tsien's lab was the first to develop protein prototype sensors to measure caspase activity and control levels of calcium in live cells[10]. Biosensors can be utilized to identify missing components pertinent to metabolism, regulation, or transport of the analyte.

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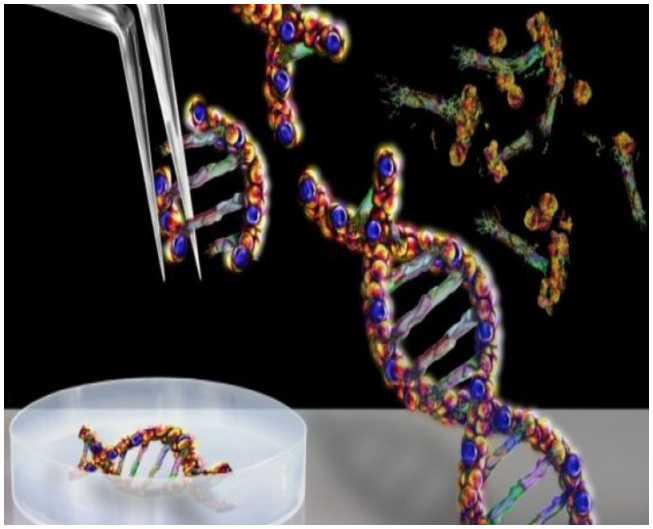


BIOTECHNOLOGY AND GENETIC ENGINEERING

ABSTRACT

Animal cell culture technology in today's scenario has become crucial in the field of life sciences, which provides a basis to study regulation, proliferation, and differentiation and to perform genetic manipulation. It requires specific technical skills to carry out successfully. This lecture describes the essential techniques of animal cell culture as well as its applications. describes the basics of animal cell culture along with the most recent applications.

Dr. Saad mutlk

Fourth stage

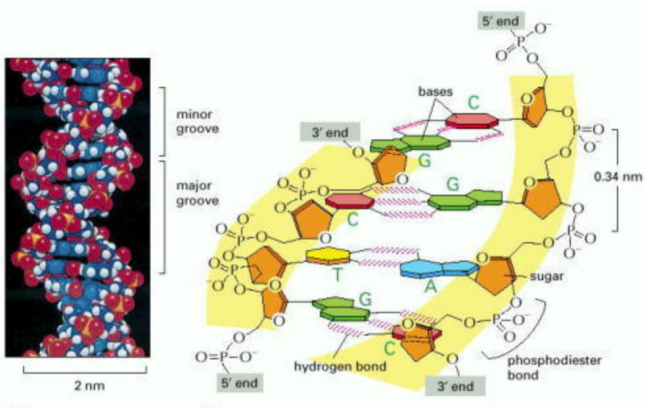
Biology Department

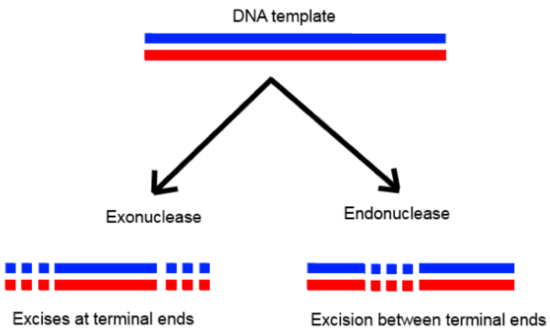
College of science

Endonucleases

What is endonuclease?

Are enzymes that cleave the phosphodiester bond within a polynucleotide chain. Some, such as deoxyribonuclease I, cut DNA relatively non-specifically (without regard to sequence), while many, typically called restriction endonucleases or restriction enzymes, cleave only at very specific nucleotide sequences. Endonucleases differ from exonucleases, which cleave the ends of recognition sequences instead of the middle (endo) portion. They found naturally in a wide variety of prokaryotes (Eubacteria and Archaea) and have an important tool for manipulating DNA.





Biological Roles:

- Most bacteria use them as a defence against bacteriophages.
- Restriction enzymes prevent the replication of the phage by cleaving its DNA at specific sites.
- The host DNA is protected by Methylases which add methyl groups to adenine or cytosine bases within the recognition site thereby modifying the site and protecting the DNA.

History of Restriction Enzyme:

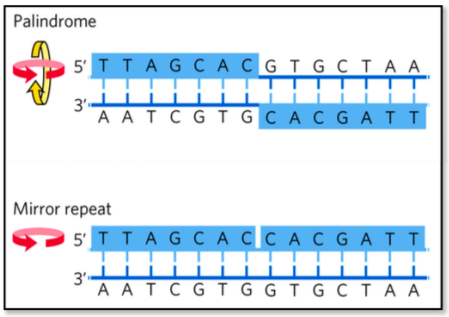
- Restriction enzymes was firstly isolated in 1970 by Hamilton Smith (*Hin*dII).
- He also done the subsequent discovery and characterization of numerous restriction endonucleases.
- From then more than 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially and are routinely used for DNA modification and manipulation in laboratories.

Mechanism of Action:

These Restriction Endonuclease scan the length of the DNA, binds to the DNA molecule when it recognizes a specific sequence and makes one cut in each of the sugar phosphate backbones of the double helix – by hydrolyzing the phosphodiester bond. Specifically, the bond between the 3' O atom and the P atom is broken. The 3'OH and 5' P are produced and Mg^{2+} is required as a cofactor for the catalytic activity of the enzyme.

Palindrome Sequences:

- A **palindrome** is a word, phrase, or sentence that is spelled identically read either forward or backward.
- This term is applied to regions of DNA with **inverted repeats** of base sequence having twofold symmetry over two strands of DNA.
- The mirror like palindrome in which the same forward and backwards are on a single strand of DNA strand, as in GTAATG.
- Inverted repeat palindromes are more common and have greater biological importance than mirror- like palindromes.



Ends of Restriction Fragments

Blunt ends

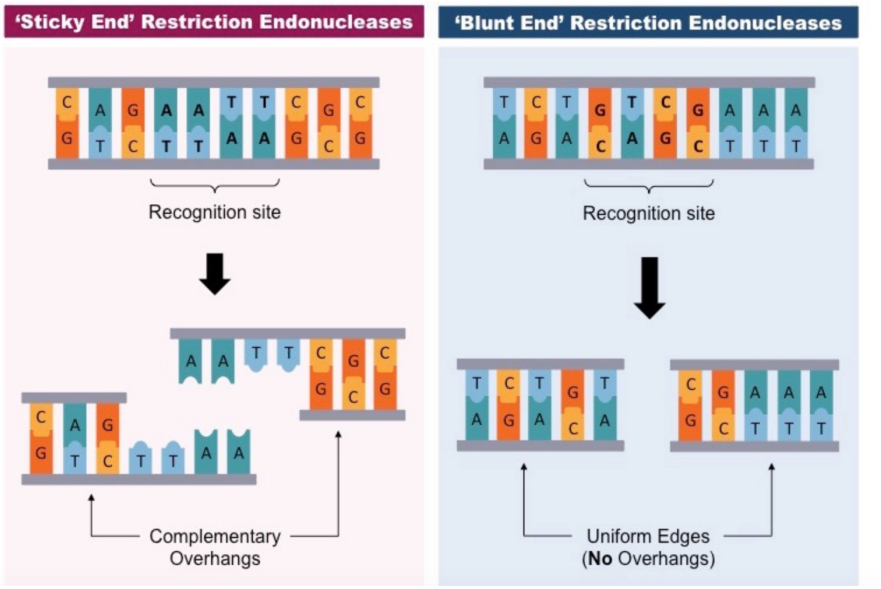
Some restriction enzymes cut DNA at opposite base

- They leave blunt ended DNA fragments which can be joined to any other blunt ends' DNA fragment. These Enzymes are useful tools for certain types of DNA cloning experiments.

Sticky ends

- Most restriction enzymes make staggered cuts on the two DNA strands, leaving two to four nucleotides of one strand unpaired at each resulting end.
- Staggered cuts produce overhanging piece of single-stranded DNA, these products are called sticky ends or cohesive ends.

"Sticky Ends" Are Useful DNA fragments with complimentary sticky ends can be combined to create new molecules which allows the creation and manipulation of DNA sequences from different sources.



Isoschizomers & Neoschizomers

Isoschizomers are restriction enzymes that have the same recognition sequence as well as the same cleavage site.

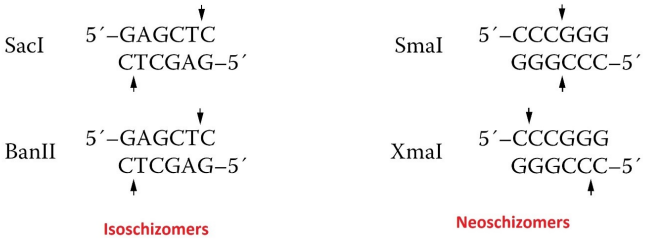
Neoschizomers are Restriction enzymes that have the same recognition sequence but cleave the DNA at a different site within that sequence.

Nomenclature of restriction enzyme

Each enzyme is named after the bacterium from which it was isolated using a naming system based on bacterial genus, species and strain.

For e.g. *Eco*RI

E	*Escherichia*	Genus
co	*coli*	Species
R	Ry 13	Strain
I	First identified	Order ID in bacterium



Types of restriction enzymes

There are four general groups of restriction endonucleases (I- IV), in which their classification according to:

1) Their composition.
2) Enzyme co-factor requirement.
3) the nature of their target sequence.
4) position of their DNA cleavage site relative to the target sequence.

Type I

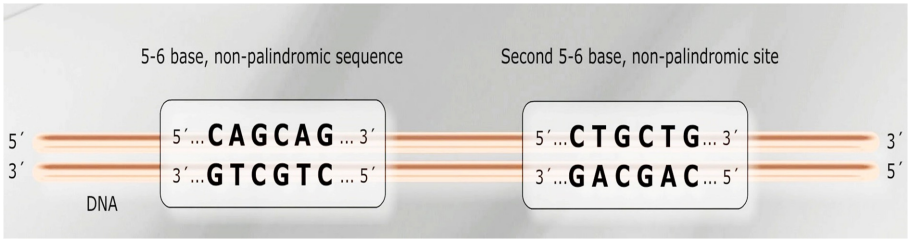
- Capable of both restriction and modification activities
- The cofactors S-Adenosylmethionine (AdoMet), ATP, and Mg^{+} are required for their full activity
- Contain:
    - two R (restriction) subunits
    - two M (methylation) subunits
    - one S (specificity) subunits
- Cleave DNA at random length from recognition sites

Type II

- These are the most commonly available and used restriction enzymes.
- They are usually composed of only one subunit.
- Their recognition sites are usually undivided and palindromic and 4-8 nucleotides in length.
- They generally recognize and cleave DNA at the same site.
- They do not use ATP for their activity.
- They usually require only Mg^{2+} as a cofactor.

Type III

- recognize two separate non-palindromic sequences that are inversely oriented.
- They cut DNA about 20-30 base pairs after the recognition site.
- These enzymes contain more than one subunit.
- And require AdoMet and ATP cofactors for their roles in DNA methylation and restriction.



Type IV

- Cleave modified DNA (methylated, hydroxymethylated and glucosylhydroxymethylated bases).
- Recognition sequences have not been well defined.
- Cleavage takes place ~30 bp away from one of the sites

Application of restriction enzymes

- They are used in gene cloning and protein expression experiments.
- Used in biotechnology to cut DNA into smaller strands in order to study fragment length differences among individuals (Restriction Fragment Length Polymorphism – RFLP).
- Each of these methods depends on the use of agarose gel electrophoresis for separation of the DNA fragments.

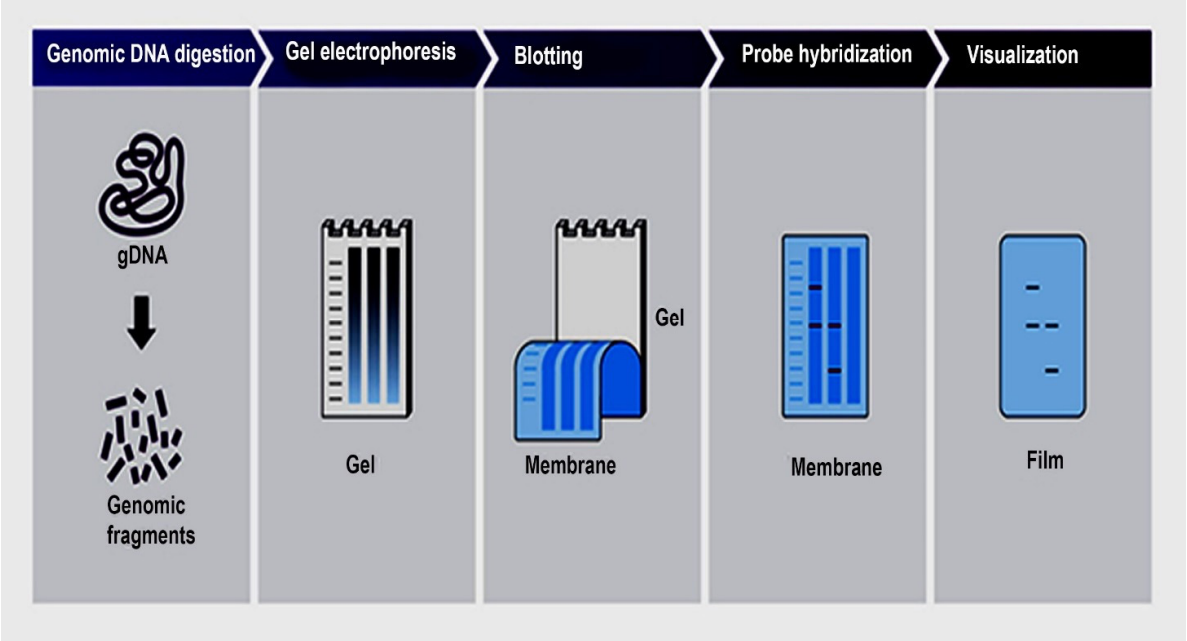
What is RFLP?

Restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples.

This method involves the following steps:

1. In the first step fragmentation of a sample of DNA is done by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest

2. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis.
3. Then transferred to a membrane via the Southern blot procedure.
4. Hybridization of the membrane to a labelled DNA probe will done and then determines the length of the fragments which are complementary to the probe.
5. Then we will observe the fragments of different length. An RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analysis



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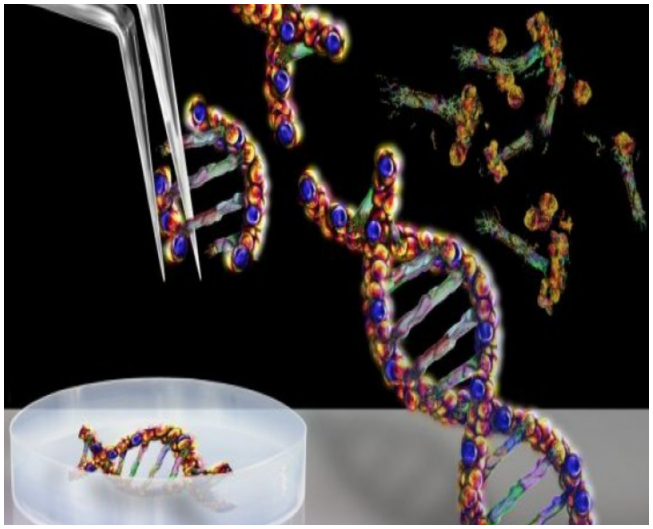

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ABSTRACT

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Fourth stage

Biology Department

College of science

Molecular cloning & Cloning vectors

What is Molecular cloning?

Molecular cloning is an essential technique in molecular biology and biotechnology laboratories. It is a useful method to study a gene, modify the gene, reintroduce the modified gene into the natural host or another host, or to produce protein. This can be achieved by combining a piece of DNA into a plasmid (a recombinant DNA) to make more identical recombinant DNA in a living host.

Materials for cloning:

To start with, it is important to prepare some important materials for cloning:

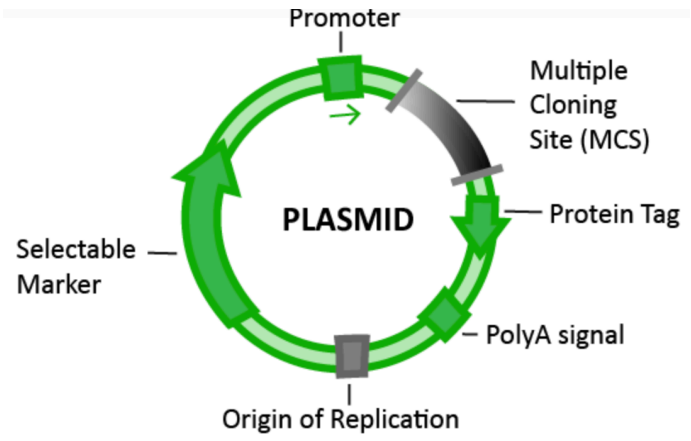
1- DNA Fragment

The source for a DNA fragment or "DNA insert" can be genomic DNA, complementary DNA, plasmid DNA, PCR product, or synthetic DNA. The DNA insert must contain particular sequences at the end of the fragments compatible with the prepared vector. You can add these particular sequences onto your DNA insert by PCR.

2- Vector

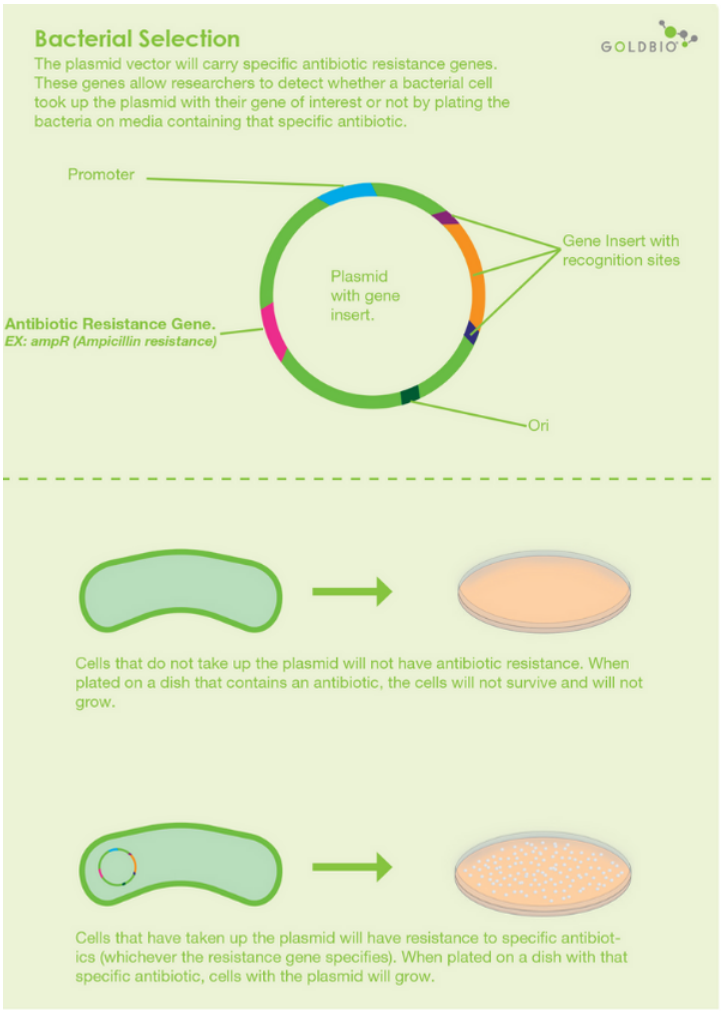
A vector is a DNA molecule which can carry a DNA insert to generate a recombinant DNA and replicate in a particular host. Examples of vectors are:

- **Cosmid**: a large DNA vector containing λ phage DNA sequence. It can carry large DNA fragment up to 45 kilobases into the host.
- **Artificial Chromosomes**: a large DNA vector which can perform the functions of a chromosome.
- **Plasmid**: a small extrachromosomal circular DNA which can replicate in a cell, commonly used in cloning.



Elements in a plasmid vector:

- **Origin of Replication (ORI)**: a required sequence or element on the plasmid for its replication inside the host.
- **Selectable Marker**: a required element for cloning to select a host, which carries the DNA construct. The only host cell growing in the growth medium containing a particular selective agent has the DNA construct with a selectable marker inside the cell. For an example, selectable markers in the DNA construct often contain antibiotic resistance genes. A transformed host has the antibiotic resistance gene on the DNA construct; therefore, it can grow in the medium with that particular antibiotic. On the other hand, the host cell without the DNA construct can't survive in the selective medium.
- **Multiple Cloning Site (MCS)**: an element on the plasmid fragment which contains restriction enzyme sites to allow DNA insertion. Compatible restriction enzymes cut on the MCS of plasmid and a DNA insert during preparation step of cloning.
- **Promoter Region**: a region which drives the protein expression of the cloned DNA.
- **Protein Tag**: a particular sequence which produces a protein with specific function, and it is usually attached to the recombinant protein. An example of a protein tag is luciferase or GFP, to monitor or quantify the protein.
- **Poly-adenylation signal**: an element containing poly-A which is important to produce a protein.



Competent Cells

After a DNA fragment is incorporated into the plasmid vector, the next cloning step is to perform a transformation step. In this transformation step, the recombinant DNA is introduced into the competent cell by chemical reaction or electroporation. Competent cells are cells which are temporarily permeable to extracellular DNA. The host organisms which are commonly used in the laboratories are *Escherichia coli* and *Saccharomyces cerevisiae.*

Selective Medium

It is a growth medium containing a selective agent to grow the transformed host. When you choose antibiotic selection for cloning, your growth medium must contain antibiotics. The most common antibiotics used for selection are Ampicillin, Kanamycin, and Chloramphenicol.

Cloning methods

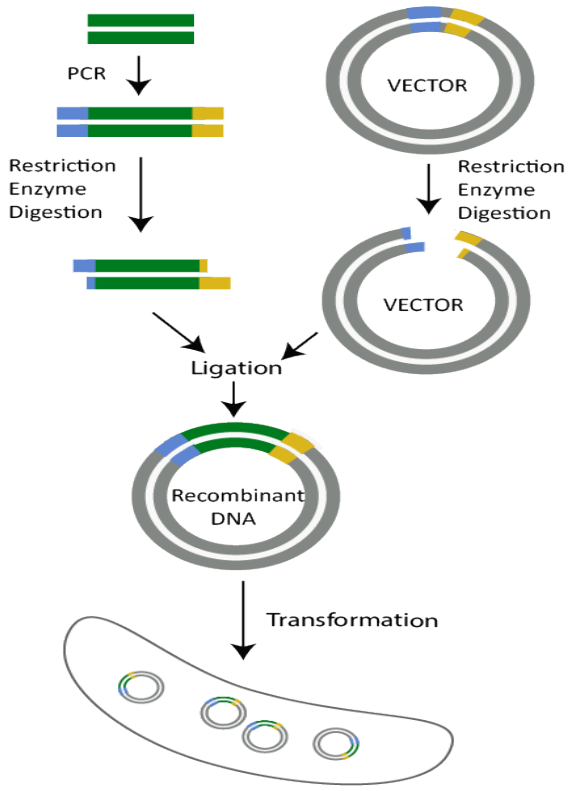
There are several different approaches to clone and you will need to find the right approach for your research. Below are some examples of popular cloning methods to generate a recombinant DNA construct:

Restriction Enzyme Based Cloning

As known before restriction enzymes are enzymes which cut DNA near at a specific short nucleotide sequence called a restriction site. The restriction enzyme based cloning method depends on the activity of restriction enzymes to ‘cut’ both a vector and a DNA insert and the method also depends on a DNA ligase to ‘paste’ the DNA fragment into the vector. This method is useful when have one DNA insert to incorporate into the plasmid.

- ◊By using PCR, you can add restriction enzyme sites on your DNA insert to accommodate this method. Your DNA insert must not contain an internal restriction site similar to the restriction site on your plasmid. Your restriction enzyme can cut your DNA insert at this internal restriction site and produce unwanted smaller pieces of DNA fragments.
- ◊You can choose to use one restriction enzyme or two enzymes to cut your DNA fragment and vector. When using two enzymes, both enzymes must be compatible or work well in the same restriction enzyme buffer.

Restriction Enzyme Based Cloning. 1. Short sequences containing restriction sites are added into the 5' ends of primers for DNA amplification by PCR. 2. Both the vector and DNA fragment are digested with restriction enzymes to create cohesive ends. 3. The vector and DNA fragment are ligated. 4. The recombinant DNA enters the host cell during transformation.



PCR Cloning

PCR cloning relies on a process called ligation, which is a method of inserting a DNA fragment into a vector using DNA ligase. The reason ligation is important for this step is because it is responsible for inserting the PCR product into a 'T-tailed' plasmid.

PCR amplified inserts contain an adenine residue at the 3' end of the DNA fragments ('A-tailed' ends). A 'T-tailed' plasmid vector has a single 3' deoxythymidine (T) at each end of the arms of a linearized plasmid. Therefore, these PCR products can be ligated into 'T-tailed' vectors by using DNA ligase, and this step is followed by transformation.

You can choose this method when your restriction enzymes are not compatible or you find an internal restriction enzyme site in your DNA insert.

One disadvantage of this method is you will need a specific 'T-tailed' vector to perform PCR cloning. But 'T-tailed' vectors may not have supportive elements for your protein research, such as promoter region or protein tag.

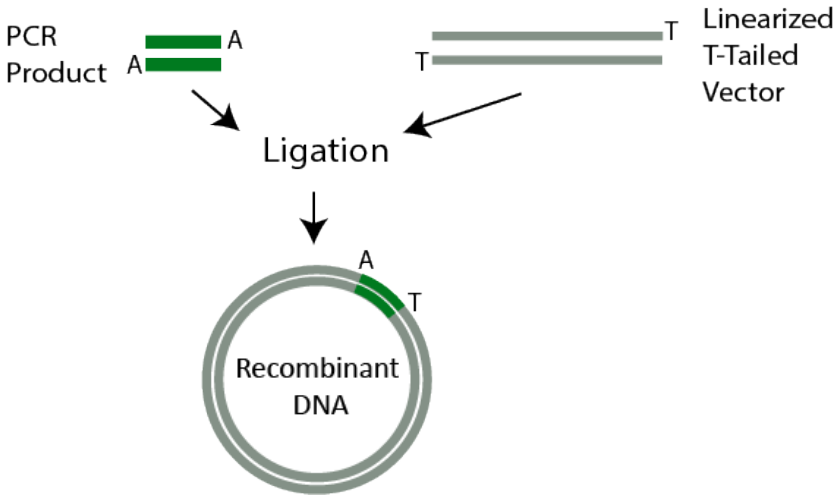


Figure1. PCR cloning.1. PCR Product with A-tailed ends is combined with T-tailed vector. 2. During ligation, PCR product is inserted into the vector.

Ligation Independent Cloning (LIC)

Ligation independent cloning (LIC) is performed by generating short sequences at the end of a DNA insert that match to the short sequences of a plasmid vector. Enzymes with 3' to 5' exonuclease activity chew 3' ends and generate cohesive ends between the DNA fragment and the linearized vector. The two materials are then combined for annealing step. During transformation, the host organism repairs the nicks on the recombinant DNA. The advantage of this method is it won't create any new restriction sites or unwanted sequences in the final DNA construct.

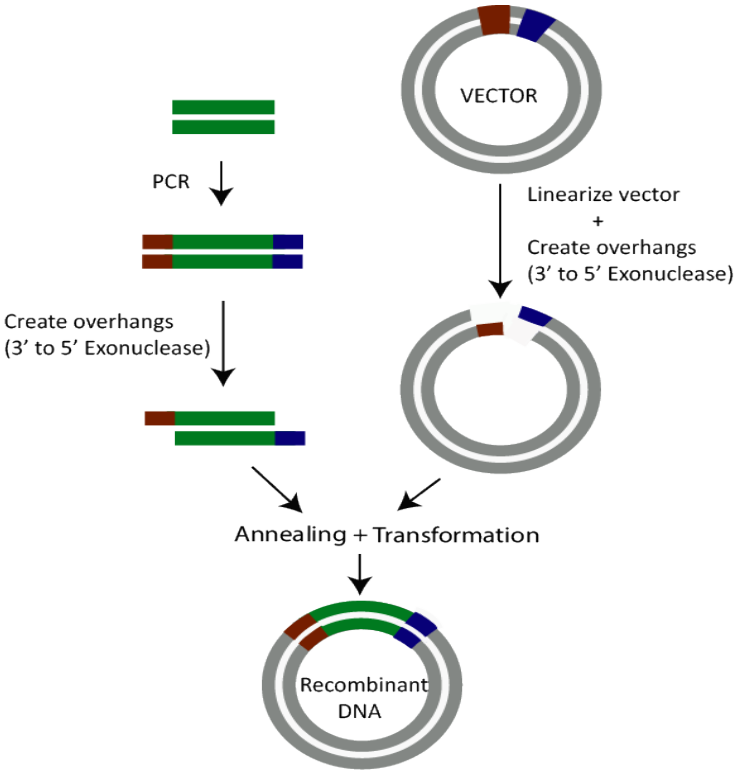


Figure2. LIC Cloning. 1. Short sequences which matches with sequences on the plasmid are added into the 5' ends of primers for DNA amplification by PCR. 2. Plasmid is linearized by using restriction enzyme. 3. Both DNA insert and vector are treated with 3' to 5' exonuclease to create cohesive overhangs. 4. Both DNA and vector are annealed. 5. After transformation, the host cell repairs the nicks on the recombinant DNA.

Seamless Cloning (SC)

The seamless cloning (SC) technique (similar to LIC) depends on matching short sequences at the ends of a DNA fragment to the short sequences on a plasmid vector. SC method requires an enzyme with 5' to 3' exonuclease activity to create 3' overhangs, a DNA polymerase to fill in gaps, and a DNA ligase to seal the nicks. The advantage of LIC and SC over the restriction enzyme-based cloning is it allows insertion of more than one DNA fragment into a vector. In addition, when you find an internal restriction enzyme site on your DNA fragment, you can use LIC or SC as an optional cloning method.

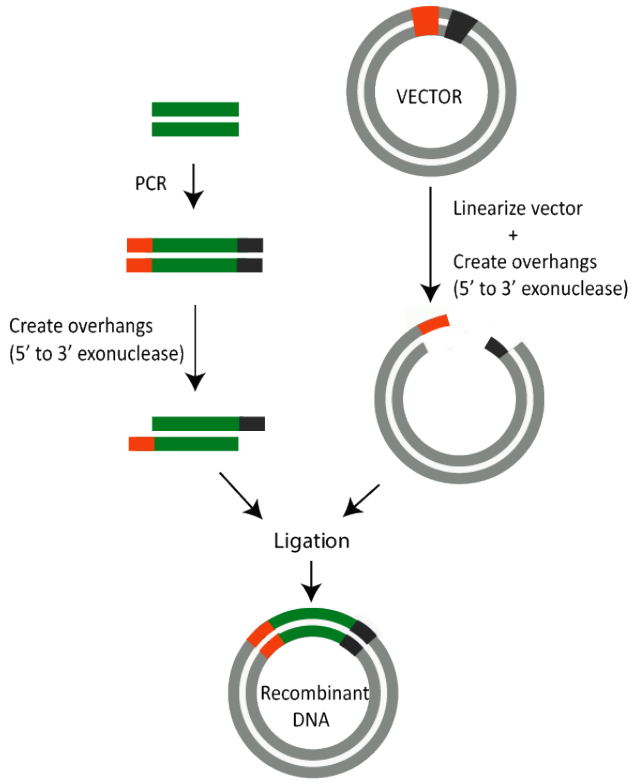


Figure 3. Seamless Cloning. 1. Short sequences are added into the 5' ends of primers for DNA amplification by PCR. 2. Vector is digested by a restriction enzyme. 3. Both DNA fragment and vector are treated with an enzyme with 5' to 3' exonuclease activity to create cohesive overhangs. 4. During ligation, the DNA fragment is inserted into the vector.

Recombinational Cloning

This method requires site-specific DNA recombinase enzymes, which exchange and recombine DNA pieces with particular recombination sites. The first step in this method is to insert a DNA fragment into an entry vector generating an entry clone. Another way to create an entry clone is by swapping and recombining a donor vector into an entry clone. After creating an entry clone, the next step is to swap and recombine the entry clone into a destination clone. The benefit of this approach is it can be used to place more than five elements into a single vector. It is commonly used to identify protein-binding interactions or to optimize protein expression, purification and solubility. To perform this method, you will need a particular plasmid which has recombination sites.

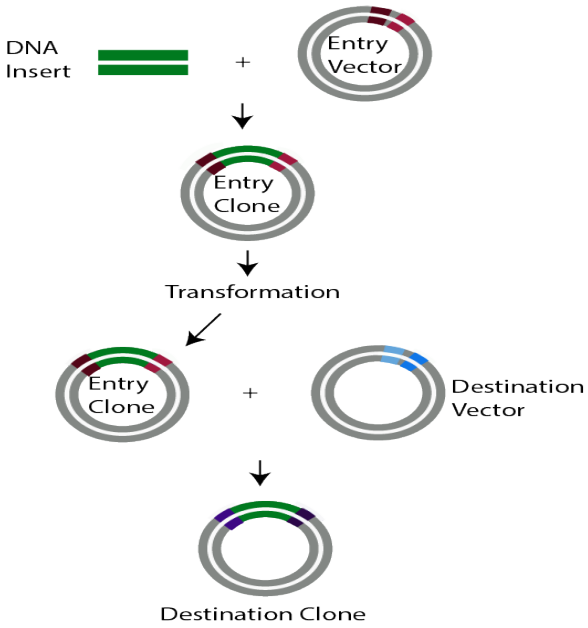


Figure 4. Recombinational Cloning. 1. DNA fragment is inserted into an entry vector to create an entry clone. 2. Entry clone and destination vector are combined by a recombinase enzyme to create a destination clone.

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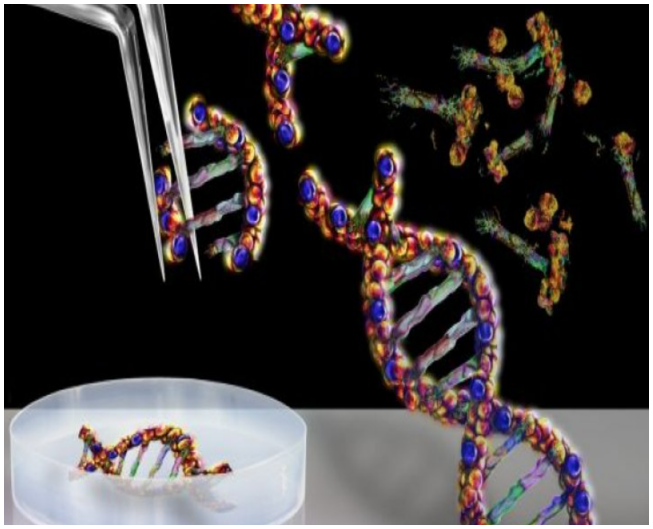

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Fourth stage

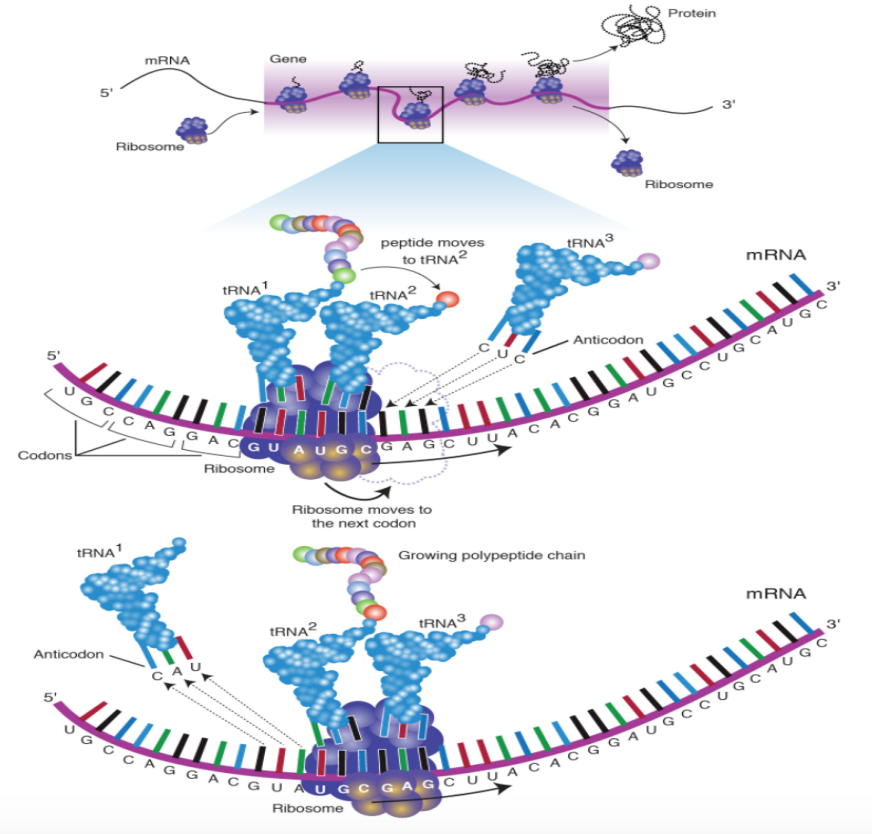
Biology Department

College of science

Gene expression in cloned cells

What is Gene expression?

The **central dogma** of biology describes the way by which information is taken from genes and used to create proteins. To do so DNA transcription result in RNA (mRNA), then RNA translation constructs proteins. This procedure is known as **gene expression** and all life forms use it to create the building blocks of life from genetic information. this requires reading the genetic code (3 letters) written in the DNA (template). So that the cell translates the genetic code, as it is each group of three letters it adds one of the 20 different amino acids that are the basic units needed to build proteins (final product) as shown in the figure below.



This is a controlled process in which genes are expressed enables the cell to control its size, shape and functions. The expressed gene affects the organism's phenotype such as eye and hair color.

Gene expression profiling:

After gene being transformed into cloned cell, it's important to measure which genes are being expressed in a cell at any given moment. This method can measure thousands of genes at a time; some experiments can measure the entire genome at once. Gene expression profiling measures mRNA levels, showing the pattern of genes expressed by a cell at the transcription level. This frequently means measuring relative mRNA amounts in two or more experimental conditions, then considering which conditions resulted in specific genes being expressed.

Gene expression profiling is used by a variety of biomedical researchers, from molecular biologists to environmental toxicologists. This technology can provide accurate information on gene expression, towards numerous experimental objectives.

It tells us how a cell is functioning at a specific time. This is due to cell gene expression is influenced by external and internal stimuli, including whether the cell is dividing, what factors are present in the cell's environment, the signals it is receiving from other cells, and even the time of day.

Several techniques are used to determine gene expression. These include DNA microarrays and sequencing technologies.

Uses of Gene expression profiling

1- It enables us to investigate the effects of different conditions on gene expression by altering the environment to which the cell is exposed, and determining which genes are expressed. Alternatively, if we previously know a gene is involved in a certain cell behaviour, this helps us to determine whether a cell is carrying out this function. For example, certain genes are known to be involved in cell division; if these genes are active in a cell, we can say the cell is undergoing division, or whether a cell is differentiated.

2- It's used in hypothesis generation. If very little is known about when and why a gene will be expressed, expression profiling under different conditions can help design a hypothesis to test in future experiments. For example, if gene A is expressed only when the cell is exposed to other cells, this gene may be involved in intercellular communication.

3- It is also investigated the effect of drug-like molecules on cellular response. We could identify the gene markers of drug metabolism, or determine

whether cells express genes known to be involved in response to toxic environments when exposed to the drug.

4- It can be used as a diagnostic tool. If cancerous cells express higher levels of certain genes, and these genes code for a protein receptor, this receptor may be involved in the cancer, and targeting it with a drug might treat the disease. So that gene expression profiling might then be a key diagnostic tool for people with this cancer.

Types of gene expression profiling:

1- RNA sequencing.
2- real-time quantitative PCR.
3- quantification by digital PCR

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