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Fundamentals and Advances in Medical Biotechnology

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"Dedicated to those from whom we learnt"

Preface

The applications of the field of Biotechnology have been around for many decades, though, earlier, people would benefit from the processes integral to the field without having knowledge of the science behind these processes like the technology of fermentation. The science of Biotechnology deals with deciphering the use of biological organisms and products, and its applications have traversed through all the scientific disciplines during the past few decades like agricultural biotechnology, marine biotechnology, industrial biotechnology, and unarguably the most noteworthy "Medical Biotechnology."

Medical Biotechnology encompasses the use of the principles and products of biotechnology for medical outcomes. Today, this field has expanded to become more and more interdisciplinary with the introduction of stem cell technology, stem cell banking and retrieval, nanotechnology, robotics, biopharmaceuticals, and so on. The introduction and widespread use of the "omics approach" in Medical Biotechnology, like other fields of Biotechnology, has made it possible to make more accurate prognosis and diagnosis of various previously uncharacterized diseases and disorders. It has also been highly advantageous in prediction of risk factors at genetic level for some of the most common chronic ailments and therefore plays a key role in preventive medicine. During the years to come, Biotechnology, aided with latest technological advancements and research modalities, is expected to penetrate deeper into the field of medicine not only for general prevention and treatment of diseases but also for customized treatment approaches for various diseases, depending upon the genetic background, environmental influences, lifestyle, and so on. These advancements will revolutionize the field of medicine and also pave the way for still greater advancements in the field.

The realization of the increasing influence of Biotechnology in Medicine makes it pertinent that we understand the basis of the subject matter and learn about some of the most commonly used techniques, scientific breakthroughs, and research-based advancements made in the field. This book has been written with the view to provide this critical information to readers in a comprehensible and reader-friendly manner. Reading this book will be likely to equip readers to understand and foresee the role that Medical Biotechnology will play in the field of medicine in future by

understanding its existing roles and contributions to medicine. At the outset, this book also aims to provide a tribute to some of the pioneers of the field who have done not only tremendous but *ahead of their time* work in the field and it is because of the efforts of these people that the field has attained its current status.

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Sincerely,
Mumtaz Anwar
Corresponding Editor

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Chapter 1
The Advent of Medical Biotechnology

Zeenat Farooq, Samia Rashid, Safrun Mahmood, Akhtar Mahmood, and Mumtaz Anwar

Abstract In the modern world, medical biotechnology plays an essential role in the interpretation of the molecular causes behind disease, improvement of diagnostic methods, and targeted drugs. This technology allows treatment of diseases with fascinating new module of drugs targeting a milieu of causes. It also allows quality care for individual patients in the form of personalized medicine. The field, however, took decades to reach where it stands today, with enormous contributions made by some of the greatest scientists of this century. In this chapter, we shall talk at length about the advent of the field of medical biotechnology, describe in detail some of the biggest advancements of the field, and discuss the legacy of the most noteworthy and celebrated scientists of the field whose contributions have made it possible for the field to reach where it stands today.

Keywords Biotechnology history and applications · Technology · Stem cells · CRISPR · Genetic engineering

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1.1 Definition and Historical Resume

1.1.1 Definition of Medical Biotechnology and its Evolution

Definition of Medical Biotechnology and its evolution is a discipline of medical sciences that use living organisms/cells/cell products or materials to produce products of diagnostic and therapeutic value. This field makes dedicated use of biotechnology towards the production of medically important products. One classical example to understand the concept and definition of medical biotechnology is production of recombinant human insulin [1]. Back in the day, type I (and sometimes type II) diabetes was treated by prescribing insulin to the patients that was obtained from bovine sources after sacrificing the animals and extracting insulin from the pancreas [2]. Although this method improved the condition of many patients, especially those with uncontrolled diabetes and chronic complications, it was ethically questionable. Also, the amount of insulin obtained from each animal was very low that a large number of animals were sacrificed for obtaining sufficient insulin for patient care, which endangered their lives. Therefore, few years later, insulin was produced from the recombinant bacteria which carried the human insulin gene and produced recombinant human insulin inside the bacteria. This development followed the ground-breaking invention of producing transgenic bacteria by introducing a gene of foreign origin into them. Biotechnology typically makes extensive use of technology to take it from "bench to bed-side" and today, with applications like molecular diagnosis, laser surgeries, PCR and antibody based diagnosis, use of nano-technology in improved drug delivery, use of robotics based surgeries in the battle fields, personalised medication for cancer treatment and many more. Medical biotechnology is not just advancing but sky-rocketing towards achieving healthcare standards that were unimaginable for human society, only two or three decades ago.

The recent invention of an "RNA based vaccine" for COVID-19 and the global use of polymerase chain reaction-based detection for COVID-19 infection also exemplify the widespread applications and merits of medical biotechnology.

1.1.2 History of Medical Biotechnology

Biotechnology has been applied in the medical science for hundreds of years with mankind's revelation that diseases can be cured from living organisms by using their products. The earliest known use of antibiotics can be traced back to 2500 years ago, when the mouldy curds made from soybeans were used by ancient Chinese to fight infection.

Louis Pasteur is considered one of the pioneers in the improvement of modern antibiotics. In 1870s, he discovered that saprophytic bacillus can check the growth of anthrax bacteria (*Bacillus anthracis*). Alexander Fleming discovered penicillin in 1928.

In 1973, the medical age of biotechnology was started by Herb Boyer and Stanley Cohen when they could develop a technique of introducing DNA into an *E. coli* bacterium and created a transgenic bacterium. Later, this recombinant DNA technology was used to successfully introduce the human insulin gene into *E. coli*. The genetically engineered *E. coli* was able to synthesize human insulin. Based upon Boyer and Cohen's recombinant DNA technique, Werner Aber, Daniel Nathans, and Hamilton Smith discovered restriction endonuclease enzymes and received Nobel Prize for Medicine in 1978.

1.1.3 Earliest Applications in Disease Detection

1.1.3.1 Gram Staining

The method of Gram staining was developed in 1884 by the Danish Hans Christian Gram, while working in the city hospital of Berlin [3]. This technique works by staining bacterial cells by using crystal violet. Gram positive cells have a thick peptidoglycan layer in their cell walls and hence retain the stain and appear purple. However, gram negative cells have a thin layer of peptidoglycan and hence are not able to retain the stain upon addition of ethanol. These cells are hence stained pink upon the addition of counter stain. Gram staining is capable of distinguishing between the two types of bacteria and is used till date for preliminary identification of bacterial infection on clinical samples like biopsies or cerebrospinal fluid because it offers an easy and quick way of detecting bacteria than cell culture [4]. However, this technique was basically developed to make bacteria visible and apparent upon observation of clinical lung tissue [5].

1.1.3.2 Haematological Staining and *Magic Bullet* by Paul Ehrlich

Paul Ehrlich (1854–1915) a German physician and scientist who was well known for cure of Syphilis (which he famously called as the magic bullet), which used to be a deadly disease in his times. He also developed a staining method for staining of different types of blood cells such as eosinophils, neutrophils, and mast cells. This led to the identification and also study of blood cells for diagnostic purposes. His staining studies had deep roots in chemistry and are said to be the precursor for Gram staining (Sect. 1.1.3.1).

1.1.3.3 Karyotyping

Karyotyping is the process by which photographs of chromosomes are aligned and the chromosomes are observed for abnormalities in number, shape, position of segments et cetera [6]. Each somatic human cell contains 46 (23 pairs) chromosomes. The whole process can be broken down into these steps:

1. *Isolation and culture of cells*. Cells are obtained from a desired tissue followed by culture of cells to allow the cells to propagate for the desired time. For the detection of genetic anomalies, karyotyping in humans usually involve white blood cells due to their ease of isolation and propagation in culture.
2. *Colchicine treatment*. Cells are treated with the drug called colchicine, also called as mitotic poison to arrest dividing cells. At metaphase, chromosomes are the most condensed and hence metaphasic chromosomes are generally utilized for karyotyping.
3. *Lysis of cells*. Cultured cells are treated with a hypotonic solution which makes the cells to swell and burst to release the chromosomes in solution.
4. *Staining of chromosomes and preparation of slides*. Each chromosome has a particular banding pattern and hence can be observed for analysis. Chromosomes are usually stained with dyes like Giemsa for karyotyping. However, other dyes and fluorescent probes are also used nowadays. Fluorescence In situ hyberdization (FISH). In fish, fluorescent probes are used against each chromosome so that each chromosome develops its fluorescent banding pattern upon observation. This technique is highly sensitive and specific, compared to conventional staining techniques.
5. *Alignment of chromosomes and analysis*. Finally, micrographs are aligned, and chromosomes are analysed. Chromosomes are distinguished from one another based on differences in shape, size, position of centromeres, number and position of satellite regions, distribution of heterochromatin.

Karyotyping is used for analysing genetic abnormalities in individuals which can arise due to:

Absence or excess of chromosomes. For example, Down's syndrome is characterized by lack of an entire chromosome (ch 21), whereas Klinefelter's syndrome is characterized by presence of an extra chromosome (second copy of X chromosome in XY genotype) within and organism.

Absence or excess of arms/fragments of arms of the chromosomes. For example, in Cri du chat syndrome, a fragment of the short arm of chromosome 4 is missing.

Karyotyping has also been used for determination of congenital abnormalities in an unborn child by analysing cells of the amniotic fluid which are amniotic in origin (come from the unborn child). This process is known as amniocentesis, and it has great potential for early detection and hence correction of various neonatal abnormalities. However, the technique can also be used for sex determination of unborn individuals and has greatly been mis-used for female foeticide.

1.2 Pioneers of the Field

1.2.1 "Re-visiting the History of Vaccination": The Story of Edward Jenner and His Predecessors

1.2.1.1 Origin of Smallpox and Introduction of Variolation

Smallpox is believed to have appeared in North-eastern Africa around 10,000 BC and afterwards spread to India, China, and Europe. It remained a deadly disease for centuries, claiming 400,000 lives in the eighteenth century in Europe alone and blinding an even larger number [7]. The term Variola meaning "stained" or "mark on the skin" was used for smallpox, based on its symptom presentation and the word "small" was chosen to distinguish it from another utterly devastating disease syphilis which was even more deadly [8]. During earlier attempts to cure and control smallpox, people began to realize that survivors of smallpox somehow resisted future attacks from the disease. This knowledge soon paved the way for the earliest attempts of "inoculation" which involved "subcutaneous instillation of material from a smallpox pustule of a patient to a healthy individual with the aid of a lancet". This practice started in many countries to combat the disease. The practice of variolation, which was similar to inoculation, was introduced in the sixteenth century in Turkey and involved subcutaneous instillation into children, so that they become, in today's language, immune for later infections against smallpox. This is a lot like the "vaccination" practices adopted today for babies and young kids and perhaps marks the first organized and systematic way of early life immunization.

Edward Jenner

Edward Jenner was born in England on 17th of May 1749 in the house of the Vicar of Berkeley, Gloucestershire. Right from his schooling years, Jenner had a great interest in nature and studies on nature. He did an apprenticeship at the age of 13 years and during this time he coincidently heard a maid saying that she would not get smallpox because she has survived cowpox. This struck him hard and during his training in surgical and medical practice, he started to work more closely on this observation and years later, he concluded that cowpox not only makes a person who contracts it immune to smallpox, but this effect can also be transmitted from one person to another. In May 1796, he inoculated a small boy James Phipps with material from cowpox pustule of a dairymaid. Two months later, he inoculated the same boy with a smallpox pustule and showed that he did not contract the disease. He called this process "vaccination" meaning "from cow in Latin" to the method of "variolation and inoculation" as described previously since he used less virulent material (in this case, cowpox pustule) to raise immunity against a deadly disease without the fear of serious illness that may occur with inoculation of virulent material itself. This event also made possible transportation of cowpox pustule materials,

the first vaccines in history, over long distances for vaccination of people. Besides vaccination, Edward Jenner is also known for his well-known discovery of foster-parent egg eviction by cuckoo hatchlings, building, and launching of hydrogen balloons [9, 10]. Jenner also had interest in the studies of geology and human blood [9].

Louis Pasteur

Louis Pasteur was a French biologist, chemist, and microbiologist. He was born to a poor tanner on December 27,1822. His studied philosophy in his college and earned a bachelor's degree in 1840. He later earned degrees in mathematics and science and was appointed as a Professor at the college of Tournon. Afterwards, he started his research in crystallography and submitted his thesis in both chemistry and physics in the same year 1847. Louis Pasteur's studies provided great support to germ theory of disease [11], according to which "germs grew from pre-existing germs and not spontaneously". He used sterilized and closed culture flasks to prove no germs grow therein and open sterile flasks to observe the growth of germs [11]. Among his famous medical discoveries are discovery of vaccines for Anthrax and Rabies. His most notable discovery in chemistry is asymmetry of certain crystals and the process of racemization. His early career investigations resulted in the first resolution of optical isomers of tartaric acid and therefore laid the foundation for the understanding of one of the greatest principles of organic chemistry. In the field of what is now called as "Food Technology". He discovered that milk and wine can be prevented from spoiling by heating them to an elevated temperature for a specified time. This process is famously named after him as "pasteurization" and is still applied in preservation of a large number of food products. His discovery of micro-organisms entering human body and making it ill also encouraged and laid the foundations of adoption of sterile methods in surgery.

Chicken Cholera Vaccine

While he was working on chicken cholera, Pasteur inoculated cultures of the infecting bacteria in chicken broth. While working, a given culture broth had spoiled and failed to induce the disease in chicken. Interestingly, these chickens failed to get infected upon subsequent attack by healthy bacterial cultures. This made Pasteur to conclude that "weakened bacteria made the chicken immune to infections by virulent bacteria" and laid the foundation of "immunization" [12].

Anthrax Vaccine

Later in 1870s, Pasteur extended his "immunization" theory to Anthrax which was killing thousands of cattle. In 1881, Pasteur discovered that growing Anthrax bacteria at 42 °C made them "inactive" and unable to produce disease. Later on, this heat

inactivated anthrax bacteria containing vaccine was used in a trial on sheep, goats, and cows with immense success. Although the concept of "vaccination" was introduced by Edward Jenner after the discovery of smallpox vaccine, Pasteur's contribution to the field of vaccination and immunization is immense since he used artificially weakened germs, which culminated the need of obtaining naturally weakened germs. This discovery greatly boosted and encouraged widespread vaccination.

He also isolated rabies vaccine from the saliva of rabid dogs, inoculated it into rabbits and used dried nerve tissues for obtaining weakened virus which was used for vaccination. In this study, Rabies vaccine was used in trial on a large number of dogs, before beginning human trials and yielded immense success.

Paul Ehrlich: A Biochemist Ahead of His Time

Paul Ehrlich was born on March 14 in 1854 in Poland in the house of an innkeeper. At a very early age, he became inspired by his cousin Karl Weigert (a pathologist who stained bacteria for the first time ever) to study the process of staining microscopic substances. Following his passion, he studied monophenylrosanilin (a red dye) during his dissertation programme at his university which led to his first publication [13, 14] and in 1978, he obtained his doctorate on the study entitled "Contributions to the Theory and Practice of Histological Staining". He discovered that an unknown blood cell type with the help of an alkaline dye and named it "mast cell". After obtaining his doctorate, he focused on studying histology, haematology, and chemistry behind stains (dyes). He worked extensively on the chemistry of pigments involved in staining dyes. He not only studied various acidic and alkaline dyes but also created neutral dyes by applying "principles of chemistry". He performed elaborate studies on the different types of white blood cells on the basis of differences in granular structures present within them. He also discovered nucleated red blood cells, and hence laid the foundation for the study of red blood cell precursors. He created the first systematic classification of distinguishing red cell anaemias and also laid the foundation of studying leukaemia on the basis of staining techniques. His work was so deep-rooted in chemistry that many biologists and haematologists of that time did not understand his work and discredited it.

In 1891, he joined Berlin Institute of Infectious Diseases where he soon became the funding director of Institute of Serum Research and Testing. He also served at various other places in several capacities afterwards. In 1909, Ehrlich discovered Salvarsan, a drug against Syphilis, which was considered a deadly disease at that time. This was the first time in history that a drug was used for fighting a microbial infection and he introduced the term "Chemotherapy" that is, "therapy with the aid of chemicals" for the first time.

1.3 Importance of the Field

1.3.1 Most Recent Advances and Their Importance (Fig. 1.1)

1.3.1.1 Robotics-Operated Surgeries

In such forms of surgeries, doctors can operate on patients remotely from their computer screens with the help of robotic arms. This form of surgeries was originally launched for military soldiers requiring surgeries at remote locations. However, because of the precision of such surgeries due to robotics, these have been extrapolated to cater to the needs of wider populations. It offers the benefits of being operated by a specialist surgeon who is an expert in the field on a patient sitting in another corner of the world. In addition, the use of robotics has been advanced over the years and have made surgical interventions with minimum possible invasions a reality. The biggest examples of routine utilization of this technology are the

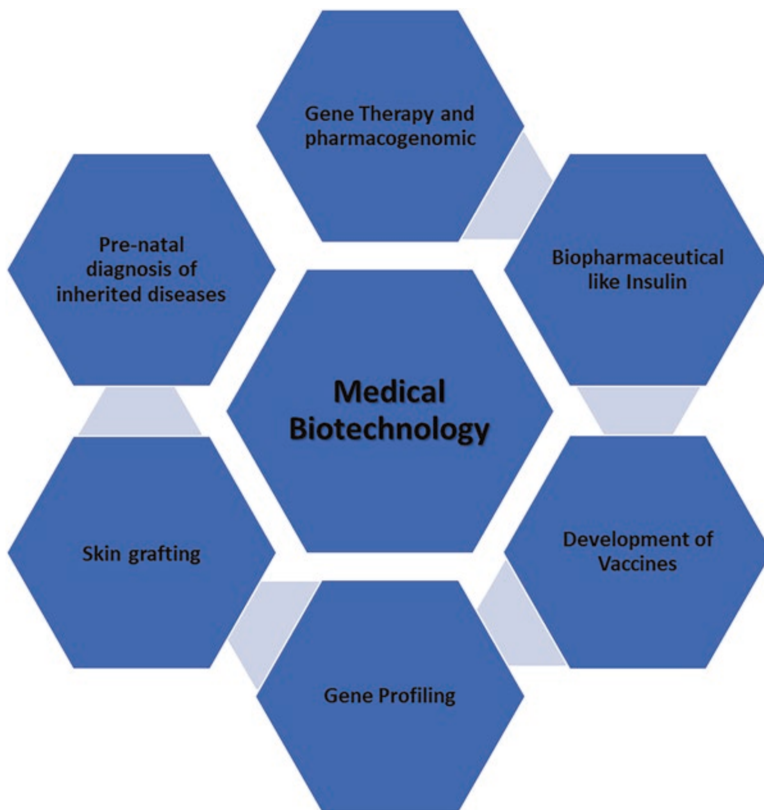


Fig. 1.1 Schematic representation of some of the best-known applications of Medical Biotechnology

laparoscopic surgeries. Also, surgeries of brain promising minimal damage to nerve tissue holds great promise for many patients worldwide requiring cranial surgeries.

1.3.1.2 Stem Cell Research

Stem cells are the cells which have the ability to divide into different types of cells. Earlier it was thought that compared to normal cells, the embryonic stem cells have enough stemness and plasticity to grow into different cell types, which made stem cell research ethically challenging and practically impossible to take the research from "bench to bedside". However, more advanced research performed in the area suggests that every organ has its own "stem cells" which renews the cells being lost due to injury or after completing their lifetime. There are stem cells that have the ability to develop into many but not all cell types. These are known as the pluripotent stem cells.

Research has shown that stem cells take differentiation decisions and follow specific paths upon differentiation into particular cell types. In fact, many factors have been identified which promote differentiation of stem cells into different lineages. These observations prompted researchers to try and differentiate stem cells in the labs (in vitro) to help replace damaged or worn-out cells in patients. Imagine a patient of neurodegeneration disorder getting benefitted by replacement of newly synthesized neurons. We can think of it as receiving blood after an injury or accident to make up for the loss in our body. The field of stem cells have made tremendous progress over the years. However, there is a dire need for research to have more research-based evidence into this field because this field is expected to see massive growth and even commercialization in clinical practices over the next decade.

1.3.1.3 Human Genome Project

Often lauded as the one of greatest feats of exploration in human history, the Human Genome Project (HGP) was an international scientific research project coordinated by the National Institutes of Health and the U.S. Department of Energy. It was officially launched in 1990 with the goal of determining the sequence of nucleotide base pairs that make up human DNA. In April 2003, the researchers announced that they had completed a preliminary sequencing of the entire human genome. This work of the HGP has allowed researchers to begin to understand the blueprint for building a person. As researchers learn more about the functions of genes and proteins, it has aided them in identifying genes that cause diseases.

1.3.1.4 Targeted Cancer Therapies

Currently, established standard chemotherapies are toxic for healthy cells. Targeted cancer therapies are drugs that work either by interfering with the function of specific molecules or by only targeting known cancerous cells, in order to minimize

damage to healthy cells. According to the National Cancer Institute, “Eventually, treatments may be individualized based on the unique set of molecular targets produced by the patient’s tumour”.

1.3.1.5 Three-Dimensional (3D) Visualization and Augmented Reality for Surgery

Surgery is brutal on a human body, and medical breakthroughs that make the surgical and healing process more efficient is always welcomed. Biotechnology has now made it possible for doctors to view an entire 3D image of the inside of a patient’s body through the use of MRI and CT scans. This allows each organ to be precisely projected so that the surgeon can make small, targeted incisions to minimize bodily trauma to the patient. Furthermore, augmented reality would allow pertinent information to be displayed directly overlaid over the relevant body parts.

1.3.1.6 HPV Vaccine

Human papilloma virus (HPV) is one of the causative agents of cervical cancer. It is the second most lethal cancer in women, second only to breast cancer, killing 275,000 women worldwide every year. Therefore, a successful HPV vaccination is considered a major medical accomplishment. The U.S. Food and Drug Administration (FDA) has approved HPV vaccines such as Gardasil and Cervix for use among females between 9 and 26 years of age.

1.3.1.7 Face Transplants

A face transplant is a process of using skin grafts to replace all or a part of the patient’s face with a donor’s face. The first partial face transplant was performed in Amiens, France, in 2005. The next successful transplant was performed 5 years later in Spain; this was also the first-ever full-face transplant. The transplant patient, whose face was severely damaged in an accident, received a new nose, lips, teeth, and cheekbones during the 24-h long surgery.

1.3.1.8 CRISPR

Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) is a relatively new gene-editing system that has been hailed as a ground-breaking tool in medical research. Of its many uses, HIV research is one of them. Researchers can now keep up with the constant genetic mutations by actively testing newly found mutations and constantly editing them to tweak targeted therapies.

1.3.1.9 Three-Dimensional (3D) Printed Organs

Artificial limbs have been in use for centuries, and there has been a steady improvement in the mobility and versatility of bionic limbs. Currently, new advances in bionic technology and 3D printing have taken it even further. It has made it possible to artificially construct internal organs such as heart, kidney, and liver. Doctors have been able to implant these into individuals that need them successfully.

1.3.1.10 Nerve Regeneration

Nerve damage from neurodegenerative disease and spinal cord injury has largely been considered irreversible. However, researchers have made significant progress in synthesizing rare enzymes that promote regeneration and growth of injured nerve cells. Neurotrophins are proteins that promote the development of neurons. It is a sequence of small molecular chains that possess potent neurotrophic properties. Although these neurotrophins have some of the shortcomings of protein-based agents, researchers are pursuing this as a possible avenue for nerve regeneration.

1.3.1.11 Brain Signals to Audible Speech

Scientists are working on creating a device that can translate brain signals to audible speech using a voice synthesizer. This would serve as an incredible tool in communicating with individuals paralysed with the disease or traumatic injuries. Furthermore, scientists have found that they can use these devices on epileptic patients to isolate the source of their seizures.

1.3.2 Invention of PCR

There are a few milestone inventions and discoveries in the history of biotechnology in general and medical biotechnology in particular that have changed the face of the field for ever. One of the greatest inventions of all times is invention of polymerase chain reaction (PCR). PCR was invented in 1983 by Kary Mullis. The technique is so powerful that many other techniques and/or landmarks that followed it would never have been possible without its invention like gene cloning, genotyping, and Sanger sequencing. In fact, the entire field of modern molecular biology/biotechnology is based on amplification of nucleic acids (DNA and RNA) like bioinformatics, computational biology, high throughput sequencing et cetera and therefore rests on the technique of PCR or its variants [15]. The significance of PCR has been put together very nicely by Stephen Scharf, a former colleague of Kary Mullis as “One of PCR’s distinctive characteristics is unquestionably its extraordinary versatility. That versatility is more than its “applicability” to many different situations. PCR is a tool that has the power to create new situations for its use and those required to use it”.

PCR was invented by Kary Mullis while he was working on the detection of point mutations using Sanger sequencing for Cetus Corporation at California. While working on this project, Mullis realized that detection of point mutations by Sanger sequencing with the aid of oligonucleotide primers and ddNTPs was impossible because the primers would bind at many places throughout the length of DNA by finding sequence complementarity. Mullis began thinking about finding a way to increase the concentration of the fragment of DNA under investigation. While driving in San Francisco California, Mullis had a vision and reasoned that "if we could use a pair of oligonucleotide primers complementary to the DNA (one complementary to upper strand and the other complementary to lower strand)", technically we should be able to exponentially amplify the DNA that lies between the primer binding sequences. Following his idea, the workers at Cetus developed the first PCR thermocycler of history known as "Mr. Cycle" that could alter the temperature appropriately to carry out PCR reaction optimally. The *E. coli* DNA polymerase used initially would be denatured in each following cycle due to denaturation (95 °C temperature) and therefore was required to be replenished after each cycle. This requirement not only made the overall reaction cost intensive but also hampered the automation of PCR because of manual interference to change DNA Pol after each cycle. However, in 1985, Mullis came up with the idea of using DNA polymerase from extremophilic bacteria *Thermophilus aquaticus* (called *Taq* polymerase), the reaction became both cost effective and fully automatic soon after. Ever since, a large number of other heat stable as well as high fidelity thermostable DNA polymerases are being used, so are the variants of the original techniques like RT-qPCR. PCR is unarguably the most commonly used technique in molecular biology. Kary Mullis was awarded the Noble prize in Physiology or Medicine for this ground-breaking invention (Fig. 1.2).

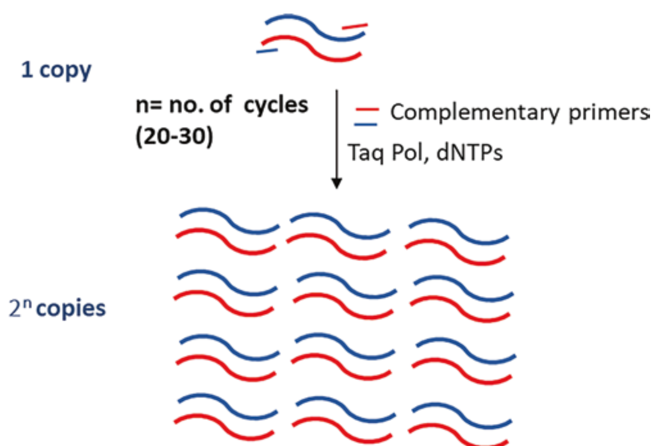


Fig. 1.2 Schematic showing the principle behind amplification potential of PCR in a geometric progression, utilizing key components such as enzyme (*Taq* Pol), dNTPs, and complementary primers. In practice, this reaction requires many more components like a buffer, divalent magnesium ions ($MgCl_2$), molecular biology grade water, and dimethyl sulfoxide (DMSO) on certain occasions

The technique has applications in all the domains of research in molecular biology as follows:

Detection of SNPs Single nucleotide polymorphisms are variations at a single nucleotide position in a particular gene among different individuals. In humans, each gene contains a particular sequence which is important for its expression and function. In some individuals at certain positions within the gene, a different nucleotide might be present instead of the original one. This condition is called as SNP and its outcome can sometime be highly deleterious for the carriers. For example, sickle cell anaemia in humans is caused due to a point mutation (SNP) by which glutamic acid is replaced by valine at position 6 in the β-chain of haemoglobin. As a result, RBCs become sickle shaped and often burst inside the blood vessels. The average life span of these RBCs is about 10–20 days as against 120 days for normal RBCs, which causes anaemia. For detecting this condition, a small blood sample is drawn from the patient, subjected to DNA extraction followed by PCR. PCR of the sequence of interest amplifies it manifold to enable sequencing of the fragment to detect the SNP. Also, PCR amplified normal and mutated versions of the gene settle at slightly different positions after migrating through an agarose gel. Therefore, these can also be identified by simply running on an agarose gel after PCR amplification, even without sequencing. This technique is called "genotyping".

Disease Prediction SNP and genotyping can also be used and is routinely being used for "disease prediction" by looking at point mutations in different target genes responsible for pathological conditions on a large number of individuals in a population. Not only SNP of protein coding genes, but other sequencing-based techniques used for disease prediction like mutations in introns, promoter regions, non-coding RNAs et cetera are also being very widely used.

Forensic Sciences and Agriculture PCR has also got immense application potential in forensic science. A small trace of any material like a nail, hair, blood drop, drop of sputum et cetera can be amplified by PCR to obtain "detectable levels" which can later be matched with DNA sequence of the suspect to identify the criminals. On the other hand, the identity of deceased unrecognizable victims can also be established by performing PCR on any sample that can yield DNA like hair strands and later "matched" with a sample of DNA from the person that is suspected to be the deceased victim (if available) or parents of suspects to "match the DNA". There are certain "molecular markers" within the genome (DNA) of living organisms, including plants, animals, and humans which can be amplified, and the sequences compared with those from the parents to establish identity. The markers used for establishing identity in such a manner are usually co-dominant. This application of PCR is used to resolve parental disputes and to establish purity of a breeding line in "Agricultural Biotechnology".

1.3.3 Production of Synthetic Insulin

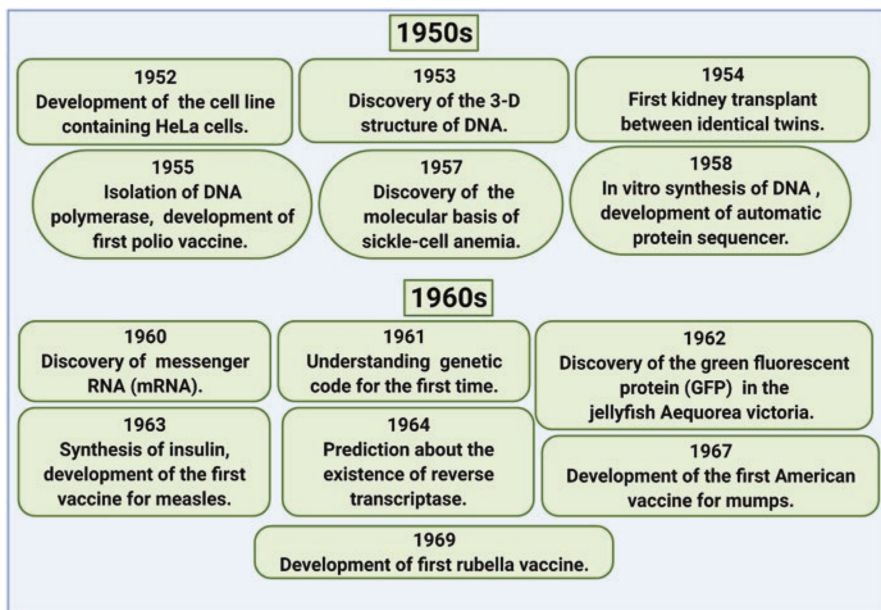
Insulin was discovered in 1921 at the University of Toronto, Canada by Sir Frederick G. Banting, Charles H. Best and J. J. R. Macleod and subsequently purified by James B. Collip. Before this discovery, it was not uncommon for people with diabetes to live for a year or couple of years after the onset of disease. There was not much that doctors at that time could do to help these patients. The most effective way of treating or managing such patients was to put them on strict low-calorie diets which eventually led to death of many patients due to undernutrition and starvation. In the year 1889, two German researchers Oskar Minkowski and Joseph von Mering found out that dogs in which pancreas was removed developed diabetes-like symptoms and died. This observation led to the idea that pancreas contained a substance that was involved in diabetes and in 1910, Sir Edward Albert Sharpey-Shafer suggested that a single chemical from pancreas was responsible for diabetes [16]. This substance was called insulin meaning Island L. In 1921, Frederick Banting and his assistant Charles Best succeeded in removing insulin from the pancreas of dogs and used it to keep dogs with severe diabetes alive for up to 70 days. Later on, Banting and Best, along with J.B. Collip and John Macleod purified insulin from the pancreas of cattle. In 1922, a 14-year-old boy named Leonard Thompson became the first person to an injection of insulin at a hospital in Toronto, Canada. In 1924, this group of researchers received the Nobel Prize in Medicine. The pioneering work of Stanley Cohen and Herbert Boyer through invention of technique of DNA cloning proved to be the one of the biggest landmarks in the history of biotechnology [17] since it paved the way for transfer of genes with economical, agricultural, and therapeutic benefits from one species to another for large scale production of the proteins of interest. With the help of cloning, genes encoding human insulin and human growth hormone were cloned and expressed in *E. coli* in 1978 and 1979, respectively. The first biosynthetic insulin was sold commercially by the firm Eli Lilly in 1982 and was named as Humulin. The administration of human insulin or any therapeutic substance of human origin to patients has two major benefits: (1) it can be produced on a massive scale without harming any animals or humans and (2) it offers the advantage of eliminating the risk of allergic reactions which was a huge concern in many patients receiving bovine insulin. Ever since, many versions of the insulin like slower-acting insulins have been developed. Today, human insulin is being synthesized mainly in *E. coli* and *Saccharomyces cerevisiae* for therapeutic purposes and there are more than 300 FDA approved biopharmaceutical products in the market with sales exceeding USD100 billion [18, 19].

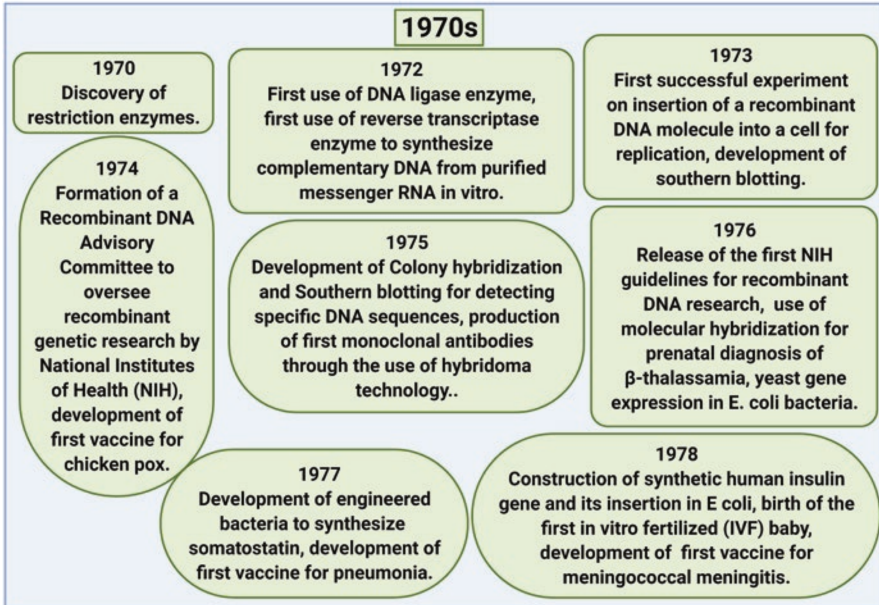
Box 1.1 Stem Cell Banking

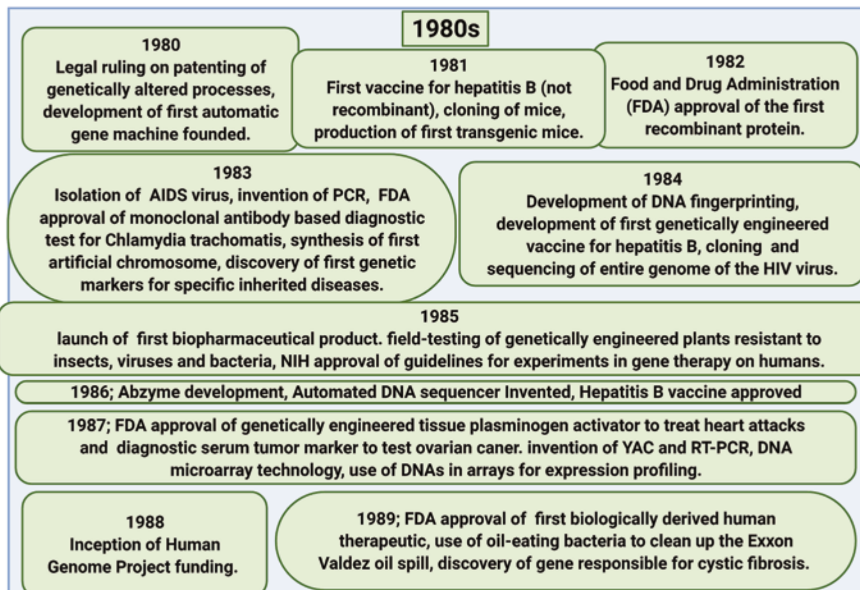
One of the major fallacies of any biotechnological technique, process or initiative with great scope for clinical application is that often times, there is exploitation of the technique and misrepresentation of facts in order to make money. One great example is “stem cell banking”. The theme of stem cell banking sounds very attractive but the following informational facts should be kept in mind.

Stem cell banking is the systematic storage of stem cells taken from the umbilical cord of an infant at birth and preserved (banked) under appropriate conditions, with the consent and fees taken from the parents for maintaining the stem cells in long term storage. The objective of stem cell banking is to preserve cells of an individual at birth, which have full potential of differentiating into any cell type. These cells could be used later in the life of that individual, should he require them. These could be differentiated into any cell type, as required, and transplanted into the recipient individual. The best flexibility with these cells is that these are "*self-cells*" and hence do not pose any problem of rejection by immune system activation. This can greatly help to evade use of immunosuppressants. However, there are many factors, ethical and otherwise, that one should consider before embracing and endorsing such advancements.

1. Maintaining the genetic integrity upon long-term storage is critical since random mutations can occur in these cells and can find their way into the recipient and cause serious conditions like cancer, if not screened appropriately. Mutations could also arise due to culture conditions which also need careful monitoring.
2. The stem cell banking facilities are generally commercial and hence there is always a risk of your stem cells being used unethically for research and/or donation without information.
3. Most of the times, the commercial banking systems do not offer enough information to their clients and charge a large amount of money, both for banking and for requesting re-use.




1970s
1970
Discovery of restriction enzymes.
1972
First use of DNA ligase enzyme, first use of reverse transcriptase enzyme to synthesize complementary DNA from purified messenger RNA in vitro.
1973
First successful experiment on insertion of a recombinant DNA molecule into a cell for replication, development of southern blotting.
1974
Formation of a Recombinant DNA Advisory Committee to oversee recombinant genetic research by National Institutes of Health (NIH), development of first vaccine for chicken pox.
1975
Development of Colony hybridization and Southern blotting for detecting specific DNA sequences, production of first monoclonal antibodies through the use of hybridoma technology..
1976
Release of the first NIH guidelines for recombinant DNA research, use of molecular hybridization for prenatal diagnosis of β-thalassamia, yeast gene expression in E. coli bacteria.
1977
Development of engineered bacteria to synthesize somatostatin, development of first vaccine for pneumonia.
1978
Construction of synthetic human insulin gene and its insertion in E coli, birth of the first in vitro fertilized (IVF) baby, development of first vaccine for meningococcal meningitis.


1980s
1980
Legal ruling on patenting of genetically altered processes, development of first automatic gene machine founded.
1981
First vaccine for hepatitis B (not recombinant), cloning of mice, production of first transgenic mice.
1982
Food and Drug Administration (FDA) approval of the first recombinant protein.
1983
Isolation of AIDS virus, invention of PCR, FDA approval of monoclonal antibody based diagnostic test for Chlamydia trachomatis, synthesis of first artificial chromosome, discovery of first genetic markers for specific inherited diseases.
1984
Development of DNA fingerprinting, development of first genetically engineered vaccine for hepatitis B, cloning and sequencing of entire genome of the HIV virus.
1985
launch of first biopharmaceutical product. field-testing of genetically engineered plants resistant to insects, viruses and bacteria, NIH approval of guidelines for experiments in gene therapy on humans.
1986; Abzyme development, Automated DNA sequencer Invented, Hepatitis B vaccine approved
1987; FDA approval of genetically engineered tissue plasminogen activator to treat heart attacks and diagnostic serum tumor marker to test ovarian caner. invention of YAC and RT-PCR, DNA microarray technology, use of DNAs in arrays for expression profiling.
1988
Inception of Human Genome Project funding.
1989; FDA approval of first biologically derived human therapeutic, use of oil-eating bacteria to clean up the Exxon Valdez oil spill, discovery of gene responsible for cystic fibrosis.

1990s

1990
First successful gene therapy, Human Genome Project launched, first hepatitis C antibody test licensing, bioengineered form of interferon gamma, enzyme replacement therapy to treat SCI

1992
Launch of genetic dog tag program, approval of genetically engineered clotting factor to treat hemophilia A, launch of in vitro embryo testing for genetic abnormalities such as cystic fibrosis and hemophilia..

1993
Approval of recombinant protein to treat multiple sclerosis, launch of study of Human Polymorphisms.

1994; Approval of recombinant protein for growth hormone deficiency and human DNase, identification of BRCA1 (first breast cancer gene). Identification of genes associated with apoptosis, bipolar disorder, cerulean cataracts, melanoma, thyroid cancer, syndrome, prostate cancer, and dwarfism.

1995; First baboon-to-human bone marrow transplant on an AIDS patient, first vaccine for hepatitis A, first full gene sequencing of Haemophilus influenzae, identification of genetic cause of deafness.

1996; Development of the first GeneChip, sequencing of Saccharomyces cerevisiae, development of diagnostic biosensor test for instantaneous detection of a toxic strain of E coli responsible for food-poisoning.

1997; Creation of first human artificial chromosome (HAC) and genetic cassette for gene therapy, approval of recombinant follicle stimulating hormone to treat infertility and antibody to treat lymphoma., completion of Borrelia burgdorferi and Helicobacter pylori sequencing.

1998; In vitro synthesis of human skin, completion of Caenorhabditis elegans sequencing, first vaccine for Lyme disease, approval of novel monoclonal antibody for Crohn's disease, approval of the HER2 inhibitor for breast cancer.

1999
The complete genetic code of the human chromosome deciphered.

2000s

2000
Completion of Human Genome Project draft.

2001
Publication of the human genome sequence.

2002
shotgun sequencing of major genomes, including mouse, chimpanzee, dog and hundreds of other species completed.

2004
first anti-angiogenic monoclonal for cancer therapy approved, DNA microarray test system, aiding in selecting medications for a variety of conditions cleared.

2006
Approval of recombinant vaccine against human papillomavirus, determination of 3-D structure of the human immunodeficiency virus.

2007; Use of human skin cells to create stem cells.

2008
Creation of DNA molecule from synthetic parts, first step towards creating the world's first living artificial organism.

2009
First FDA approved clinical trial on embryonic stem cells.

1.4 Sub-Disciplines: Allied Fields, Inter-Relationships with Other Fields

The field of biotechnology and science at large is inter-connected in which each branch relies on other branches in some manner. In fact, any application in one of these fields relies on understanding and application of inter-related fields to achieve an outcome. The best example for the subject matter of this book would be use of biomedical engineering in medical biotechnology. Biomedical engineering is the discipline at play behind the gigantic instruments such as ventilators, ECG machines, NMR operators, PCR machines, blood pressure measuring devices, and literally every piece of appliance used in medical biotechnology. Biomedical engineering combines the principles of engineering, primarily grounded in mathematics and physics, and combines them into making devices required in the medical field. In a similar manner, there are so many examples of inter-relationship of the fields and here we mention some of the most observed and best-known inter-relationships (Fig. 1.3).

1. *Biotechnology, molecular biology, and genetic engineering*. These disciplines play a deeply embedded role in medical biotechnology for as long as the field of modern medical biotechnology exists. Detection of defects and disease using PCR, genotyping are the best examples to illustrate this relationship. The use of micro-organisms to produce bio-pharmaceuticals like production of human insulin in *E. coli* and *S. cerevisiae* are also noteworthy. For further reading, this book contains separate chapters on biotechnology and the principles behind its applications.
2. *Nanotechnology*. Nano, as the name suggests means "very small, on a nano-scale". In recent years, various encapsulated nano-drugs have been synthesized with the help of nanotechnology and have been delivered to patients in a tar-

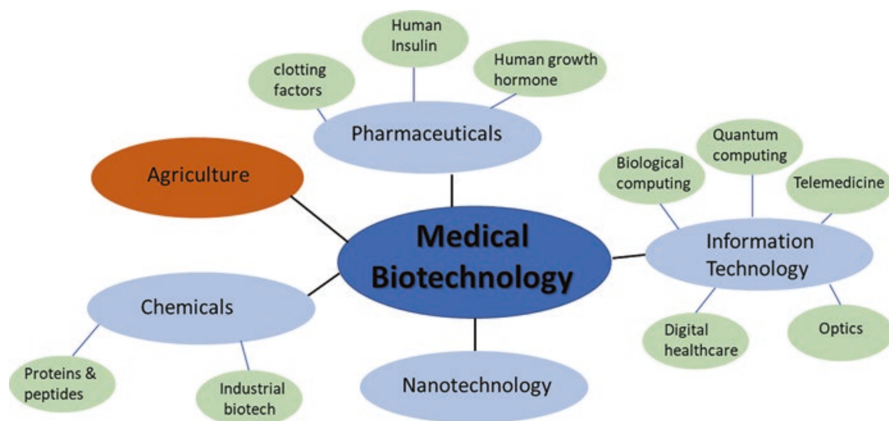


Fig. 1.3 Schematic representation of different sub-branches of medical biotechnology and their essential components

geted, tissue specific manner using a targeted drug delivery approach. For example, some drugs that have cholesterol lowering effect which need to be targeted to visceral fat in order to achieve best efficacy. Using the conventional drug delivery system (oral or IV), the effective amount that reaches the target tissue is very small. Consequently, higher doses are required to be given to the patients to achieve a certain level of benefit. This increases the amount of consumption which might not be suitable for some recipients. In the targeted drug delivery system, the drug only reaches its intended target which reduces overall drug load, improves efficacy and tolerance. These drugs are encapsulated by materials which increase their uptake by target tissue for better efficacy. For example, fat-lowering nano-drugs are encapsulated by lipid soluble nanoparticles. In some cases, these nano-capsules can also contain "ligand-like recognition molecules" which allows only target cells to uptake the drugs and hence drastically reduce off-target side effects. Currently, many anti-cancer drugs are being designed for animal and cell culture testing in labs using the same strategy with molecules on their surface which can be uniquely recognized by cancer cells. These applications, in turn, require a detailed understanding of chemistry, physiology, immunology, enzymology, and so on.

3. *Stem cell technology*. Stem cell technology has created a revolution in modern day medical biotechnology. The field makes extensive use of molecular biology and biomedical engineering to produce effective treatment options in the form of stem cell therapy. Tissues and 3D organoids have been created in labs with the help of stem cell technology for use in medical biotechnology. For further reading, this chapter contains a section on stem cell banking and stem cell technologies have been discussed in a separate chapter in this book.

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Chapter 2
Biotechnology in Medicine: Fundamentals

Sajad Ahmad Bhat, Zarka Sarwar, Asiya Batool, and Sameer Ahmed Bhat

Abstract Biotechnology is a diverse field with important implications in everyday life. It finds its use in research, medicine, agriculture, and chemical industry. Various biotechnology techniques are utilized for different purposes. Polymerase chain reaction (PCR) is used in molecular cloning, genotyping, and medical diagnostics. Molecular cloning in particular is a multiple step process involving PCR amplification of the gene of interest, restriction digestion of the vector, and the insert, ligation, screening, and sequencing. Vectors like plasmids are utilized to express the gene of interest. Amplification of plasmids involves transformation into bacteria and the expression of the gene of interest is achieved by transfection into the host cells. Real-time PCR measures gene expression at the RNA level. It is currently being used for COVID-19 screening. Site-directed mutagenesis is a technique to introduce specific mutations in DNA. It is utilized to perturb the function of a protein. Crispr-Cas9 system is a recent addition to the DNA manipulation tool kit. It finds its use in knock-out studies where guide RNA directs the Cas9 nuclease to the target gene. The system can also be utilized for knock-in studies and mutagenesis by providing a homology directed repair (HDR) template, along with Cas9 and guide, to introduce specific changes.

Keywords Applications of PCR in molecular biology · Mutagenesis · Cloning · Gene screening

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Sajad Ahmad Bhat, Zarka Sarwar and Asiya Batool contributed equally

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2.1 Introduction, Importance of Biotechnology in Everyday Life, Utilization of Biotechnology for Human Welfare

2.1.1 Introduction

Karl Ereky, a Hungarian engineer, first used the term "Biotechnology" to refer to the science of employing living organisms for the production of products from raw materials. With the advent of novel techniques for studying and manipulating living systems, biotechnology keeps on stretching its boundaries. It has evolved into a broad and multidisciplinary field of biology. Modern biotechnology has extensively harnessed the potentials of molecular biology and genetic engineering. This perhaps differentiates it from conventional biotechnology, where technologies like fermentation of products and selective plant breeding were more prominent. Biotechnology has made unprecedented progress across different fields raising the need to define it in the contemporary contexts. Biotechnology applies living systems, organisms, or biologically derived materials for production of products and services. At the fundamental level, it is grounded in molecular and cell biology. The Organization for Economic Co-operation and Development (OECD) defines biotechnology in a broader context that encompasses modern biotechnology as well as many traditional and borderline activities. It defines biotechnology as "the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services". Below we will discuss how biotechnology affects our daily lives and human welfare in general.

2.1.2 Importance of Biotechnology in Everyday Life

The use of biotechnology traces back to ancient times when humans harnessed the potential of living organisms for the production of bread, cheese, and herbal remedies for treatment of ailments. They were based on observations without understanding the underlying scientific principles. This phase was followed by the classical or traditional biotechnology with widespread use of fermentation technology at the industrial level to produce antibiotics, enzymes, and organic acids and selective breeding of plants [1]. The elucidation of DNA structure and development of recombinant DNA technology ushered the era of modern biotechnology. Today, with its versatility and potential, biotechnology has pervaded

all aspects of our lives to make it convenient and comfortable. From the food we eat, safety of water we drink, medications for healthcare, improvement of environment we live in, to fuels we put in vehicles, we rely on this "technology of hope" for our sustenance and development.

2.1.3 Utilization of Biotechnology for Human Welfare

Biotechnology has the potential to address the major world problems like shortage of food, replacement of fossil fuels, treatment of diseases, safety of environment, and drinking water. There are four broad areas where biotechnology has made important contributions for human welfare. These include medical applications also called as red biotechnology, in the field of agriculture called as green biotechnology, industrial applications called as white biotechnology, and blue biotechnology that involves the aquatic environment.

Medical biotechnology has improved the diagnosis, treatment, and control of common as well as rare diseases. Recombinant DNA technology has revolutionized the production of therapeutic proteins, enzymes, and antibodies. Molecular tests and advanced diagnostics offer great resolution to provide treatment that is more accurate. Now personalized treatments are explored based on individual genetic profiles. Novel technologies are being developed for early detection of debilitating diseases like cancer, AIDS, and neurodegeneration. Biotechnology has also been at the forefront of dealing with pandemic situations that can halt economic growth. This can be observed in recent COVID-19 pandemic where the processes have been fast-tracked to provide vaccinations and bring the life back to normal.

With a surge in population, the demand for food increases. Many factors preclude us from realizing the full potential of our agriculture system. These include environmental stress, diseases, and pests. Agricultural biotechnology aims to produce fertile plants that are more resistant and introduce friendly fertilizers and biopesticides for sustainable environment. For example, insect-resistant gene from the bacterium *Bacillus thuringiensis* was introduced into crop plants such as cotton, corn, and soybean to protect them from certain insect pests and improve the yield. Similarly, resistance gene was transferred to susceptible cultivated variety of rice from wild-type species, which imparted the resistance against bacterium *Xanthomonas oryzae* [2]. Recombinant DNA technology has also accelerated the process of introducing the gene of interest in the desired plant species by skipping the time-consuming conventional breeding methods. Introduction of high yielding varieties is increasing the crop yield. In addition, the crops are engineered to introduce desirable nutrients to prevent some chronic diseases and deficiencies.

Biotechnology is applied on industrial scale to produce useful products utilizing the microorganisms or biologically derived materials like enzymes. The products range from food, chemicals, textiles, paper, and much more. The aim of modern applications is to come up with products that are more economical and environment friendly. We have excessively utilized the non-renewable resources causing their

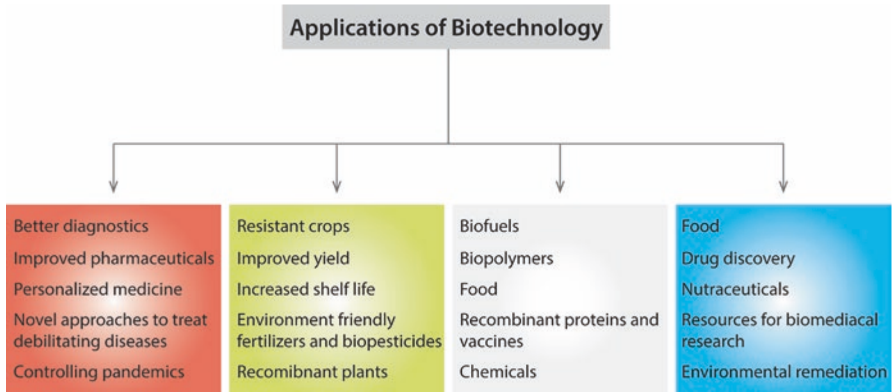

Fig. 2.1 Biotech applications in the four broad areas of red, green, white, and blue biotechnology

depletion and in the process of the extraction, refining, and use of these resources, the environment has been polluted. We are in desperate need for sustainable and clean energy. Biotechnology aims to provide better alternatives in the form of biofuels and biopolymers. Biofuels such as bioethanol and biodiesel can be obtained from renewable organic sources. These products are claimed to replace the conventional sources of energy.

Biotechnology also explores to harness the marine bioresources as a target or source of biotechnological applications. It helps to secure food supply by providing nutraceuticals such as amino acids, fish proteins, microalgae, and much more. It has also led to the identification of useful resources like Green Fluorescent Protein (GFP) from the jellyfish, Luciferase enzyme from Vibrio fischeri, Shrimp alkaline phosphatase and Taq polymerase from hot spring Archaea. These resources have significant applications in molecular biology and biomedical research. Given its vast share in biosphere and rich biodiversity, blue biotechnology has a great potential in applications related but not limited to food supply, drug discovery, biofuels, and environmental remediation (Fig. 2.1).

2.2 Polymerase Chain Reaction, Essential Components of Polymerase Chain Reaction, Modifications of PCR, Applications of PCR

2.2.1 Polymerase Chain Reaction

Polymerase Chain Reaction forms the backbone of modern molecular cloning. With some basic information about the nucleotide sequence, PCR can be employed to selectively and rapidly amplify the target sequences to millions of copies. It is a fairly simple technique which has found extensive use in molecular cloning

approaches like cDNA and genomic cloning, DNA sequencing, and mutagenesis. Some of the attributes that have made PCR so common in labs are its sensitivity, rapidity, accuracy, and cost-effectiveness.

The PCR works on the premise of thermal or temperature cycling which enables (1) DNA melting, (2) annealing of oligonucleotide primers to complimentary DNA targets, and (3) the bursts of enzyme-catalyzed DNA synthesis on the target sequences. Hence, a typical PCR reaction comprises of three stages with three different temperatures for denaturation, annealing, and extension in each cycle [3].

Denaturation. During this step, the temperatures rises to approximately 95 °C and melts the hydrogen bonds. This causes the unwinding of the DNA double-stranded helix into single-stranded form. In standard PCR reactions, it lasts for around 15–30 s.

Annealing. In this step, the temperature plummets to around 45–65 °C for approximately 20 s allowing the synthetic oligonucleotide primers to anneal to the complimentary nucleotide sequences. The temperature used depends on the length and the guanine-cytosine content of the primers used in the reaction.

Extension/elongation. During this step, the DNA polymerase initiates the synthesis of complementary DNA at the 3′ end of the oligonucleotide primers using the pool of nucleotides. The temperature used depends on the optimum activity temperature of the thermostable DNA polymerase employed (~72 °C for Taq polymerase). The extension time is directly proportional to target DNA length.

The three steps are cycled for about 25–35 times with the number of DNA copies increasing exponentially with each cycle such that 2^n copies are accumulated at the end of n number of cycles. However, it is only at the end of third cycle that the double-stranded DNA molecules corresponding to the exact size of the target molecules are synthesized (Fig. 2.2). Subsequently, these desired target molecules outnumber the other molecules with inaccurate sizes and sufficiently dilute them. The efficiency of PCR reaction in practice is less than 100%. The PCR reactions often conclude with a final extension step at ~72 °C for 5 min and serves to complete the extension of amplicons that are not fully extended. In addition, the first PCR cycle often begins with denaturation for 5 min to allow the long DNA molecules to fully denature. This step is, however, unnecessary for linear DNA molecules and may not be required for DNA templates with low GC content. The step can in fact be deleterious sometimes [4] and denaturation for 45 s suffice for routine linear DNA amplification [5].

2.2.2 *Essential Components of Polymerase Chain Reaction*

Template DNA. It is the original DNA template containing the chosen region or target sequence to be amplified in either single or double-stranded form. Linear DNA is modestly more efficiently amplified than closed circular DNA. In principle, PCR is sensitive enough to amplify infinitesimally small traces of DNA. In practice,

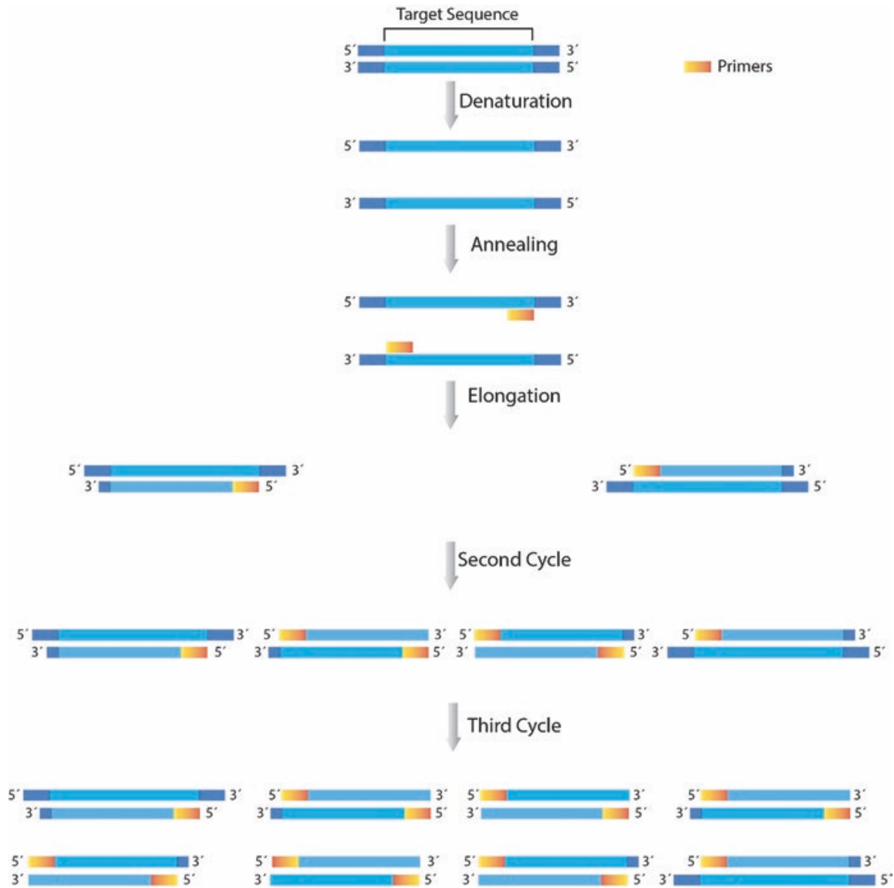

Fig. 2.2 Schematic representation of a Polymerase Chain reaction

however, about 1 μg (~3 × 10^5 copies) for mammalian genomic DNA, 1 ng for bacterial genomic DNA, and 1 pg for plasmids are routinely used.

Deoxynucleoside triphosphates (dNTPs). These are supplied in the form of dATP, dGTP, dTTP, and dCTP and are required by the DNA polymerase for synthesizing new DNA copies. For standard PCR reactions involving Taq polymerase having 1.5 mM $MgCl_2$, 200–250 μM of each dNTP is endorsed while higher concentrations (>4 mM) tend to be inhibitory likely due to sequestration of Mg^{2+}.

Buffer. A buffer, Tris-Cl, having pH between 8.3 and 8.8 at room temperature to ensure optimal conditions is required. The pH of the buffer drops to an optimum of nearly pH 7.2 during extension step when temperature is ~72 °C.

Divalent cations. Free divalent cations, usually Mg^{2+}, are required by all thermostable DNA polymerases for activity. They serve two functions: form a complex by reacting with dNTPs to act as substrate for the polymerase and for the stability of primer-template complex [5]. Very low concentrations decrease the efficiency of primer annealing while as very high concentrations of the Mg^{2+} hamper denaturation of the DNA duplexes. Substantial concentrations of chelating agent like

ethylenediaminetetraacetic acid or negatively charged ions such as PO_4^{3-} can cause Mg^{2+} sequestration and lead to reaction failure.

Primers. Primers are synthetic oligonucleotides complementary to the terminal ends of the target DNA that are required to initiate DNA synthesis. Efficient design of primers is crucial for the successful amplification of the PCR products or amplicons. The essential considerations for primer designing are listed as follows:

1. The ideal length for the region complementary to the target sequence is 18–30 nucleotides. Shorter primers are vulnerable to non-specific binding while as longer ones tend to form secondary structures. The difference in the length of the primer pair should not exceed more than three bases.
2. The G + C content should ideally lie in the range of 40–60% with uniform distribution of A, T, G, and C along the primer length. This avoids secondary structure formation.
3. Presence of inverted repeats or self-complimentary sequences should be avoided as they form hairpin structures hampering primer annealing.
4. The complementarity between the primer pairs especially at the 3′ ends should be avoided, as they tend to anneal with each other forming primer dimers.
5. Having G at the 3′ end of the primers and the existence of G or C bases within last five bases help in the strong binding of the 3′ ends because of stronger hydrogen bonding of GC pairs. This is known as GC clamp.
6. The melting temperature (T_m) is the temperature at which 50% of oligonucleotide primers are single-stranded and other half are double-stranded or hydrogen bonded to their exact complements. Nowadays, this is calculated by free software tools and is affected by oligonucleotide sequence, primer concentration, and salt concentration. The difference in the melting temperatures of the primer pair should not ideally exceed by 2–3 °C. T_m of the primer-DNA hybrids are calculated using several equations. No single equation, however, is perfect and applicable to every reaction and is subject to personal preferences. An empirical equation commonly used for calculating the T_m for perfect duplexes of 15–30 nucleotides in high ionic strength solvents is known as the "Wallace Rule" [6, 7]. It is calculated as:

$$T_m = 2(A + T) + 4(G + C)$$

T_m = melting temperature in °C. (A + T) is the sum of A and T residues and (G + C) is the sum of G and C residues in the primer.

DNA polymerase. The mercury levels soar to high temperatures in the PCR and thus requires a thermostable polymerase to carry out the DNA synthesis. These polymerases are derived from bacteria living in extreme conditions up to 90 °C or higher. The most commonly used one is the *Taq* polymerase from *Thermus aquaticus* [8, 9]. It has the 5′ → 3′ exonuclease activity but lacks the 3′ → 5′ exonuclease activity hence devoid of proofreading function. This increases the probability of misincorporation of dNTPs (one nucleotide in nearly 9000 nucleotides) [10]. DNA polymerases have subsequently been isolated from other thermophilic bacteria and archaea that possess the proofreading function like *Pfu* polymerase from *Pyrococcus*

furiosus [11] and *Tli* polymerase from *Thermococcus litoralis*. They can be used for reactions requiring high fidelity. Nowadays, engineered DNA polymerases like phusion and Q5 with high fidelity are routinely used for PCR amplification during cloning of genes.

2.2.3 Modifications of PCR

The PCR is versatile and has been modified in numerous ways to cater various research purposes. Below is the list of some commonly used variants.

1. *Reverse transcription PCR (RT-PCR)*. It employs an enzyme reverse transcriptase to reverse transcribe RNA and form RNA:DNA hybrid. This hybrid is then denatured and PCR amplified to form the double-stranded complementary DNA (cDNA). It can be used for purposes like measuring gene expression and comparative expression analysis.
2. *Real-time quantitative PCR (qPCR)*. Again, reverse transcriptase is used but employs fluorescent probes or DNA-binding dyes to detect and quantitate the amount of amplification in real time. This is made possible by measuring the light emitted by fluorescent probes, which is in proportion to the amount of amplification.
3. *Hot-start PCR*. In this modification, the DNA polymerase is supplied to the reaction mixture after the DNA has attained the melting temperature. This hampers the formation of primer dimers and inhibits non-specific hybridization.
4. *Nested PCR*. This method involves two sequential rounds of PCR where the amplicon or the product of the first PCR serves as a template for the second round. The second set of primers are nested downstream of the first set within the first target sequence. It is unlikely that any unwanted PCR products may bind to the second primer set. This reduces the non-specific PCR amplifications.
5. *Touchdown PCR*. It is a PCR modification where the initial annealing temperature is higher than the projected T_m of the primers in use, and decreases progressively over the course of reaction to the optimum melting temperature. At higher temperatures, non-specific primer binding reduces while specific primers are more likely to generate desired products. At the optimum $T_{m,}$ the amount of specific PCR products outcompetes undesired amplicons due to the exponential nature of the PCR [12].
6. *Multiplex PCR*. It employs several primer sets to simultaneously amplify different target sequences. The annealing temperatures of various primer pairs have to be compatible to work in the same reaction and the various amplicon sizes have to be discrete enough to be distinguishable. It is employed for various purposes like detection of mutations, genomic rearrangements, and polymorphism.
7. *Inverse PCR*. When a known DNA sequence is flanked by unknown sequences, inverse PCR variant is used. This can be used to identify the insertion sites of various transposons and retroviruses. A low-moderate frequency restriction enzyme, whose site is absent in the known sequence, is employed to digest the

DNA. The result would be that known sequence is flanked by unknown sequences with sticky ends that are ligated to make circular DNA product. Subsequently, two primers facing outwards form the known sequence are used for PCR amplification. Finally, the PCR amplicons can be sequenced and the location determined by running comparisons in databases.

2.2.4 Applications of PCR

PCR is a robust and versatile technique that has a wide range of applications in basic research, medical diagnostics, microbiology, forensics, and host of other fields. Here are some examples:

1. Comparing gene expression among different tissues or cells at a given time point. It is also used to identify the effect of various drugs or mutations on gene expression. This is usually achieved by employing RT-PCR.
2. For genotyping, to identify and characterize normal as well as mutant alleles.
3. It is widely used in molecular biology for cloning and mutagenesis to introduce desired mutations in the gene of interest.
4. One of the most important applications of PCR is its use in medical diagnostics to detect genetic as well as infectious diseases.
5. DNA fingerprinting uses a PCR based method to determine individual's DNA characteristics. It relies on short tandem repeats (STRs) and variable number of tandem repeats (VNTRs), which are polymorphic repetitive DNA sequences used as markers in DNA profiling.
6. Traces of DNA from precious samples like fossils can be amplified and subsequently sequenced to derive valuable molecular information.
7. It is employed in plant research and agriculture for genotyping and breeding.

2.3 Restriction Enzymes: Types and Mechanism of Action

2.3.1 Introduction

The restriction enzymes, also known as molecular scissors, originated from the studies of lambda phage. They derive their name by the ability of certain bacteria to restrict the activity of the lambda phage by the enzymatic cleavage of the phage DNA. The enzymes that allow these host bacteria to restrict the growth by DNA cleavage were termed as restriction enzymes. These enzymes selectively target foreign DNA in a process called restriction digestion while the host DNA is protected by the modification enzyme, methyltransferase. Together, these constitute the restriction-modification system. The discovery and characterization of restriction enzymes paved the avenues for DNA manipulation and led to the development of recombinant DNA technology. For their contribution in the field Werner Arber,

Daniel Nathans and Hamilton O. smith were awarded the Nobel Prize for Physiology or Medicine in 1978 [13–15].

2.3.2 Nomenclature

The restriction enzymes derive their name from the bacterium from which it was identified. The first three letters are italicized with the first letter adapted from the genus and the next two letters form the bacterial species. These may be followed by the numbers or letters denoting strain or serotype. Finally, a Roman numeral follows a space to designate chronology of identification. For example, *Eco*R I derives its name from *Escherichia coli*, strain RY13 and I indicates its order of identification. Similarly, *Hin*d III from *Haemophilus influenza* serotype d and was third (III) of the four enzymes identified in the bacterium. However, the use of italics for the first three-letter acronym and space before the Roman numeral is not consistently employed and may be omitted [16].

2.3.3 Types of Restriction Endonucleases

Restriction enzymes identify and cleave DNA at or near a specific sequence of nucleotides known as recognition sequences. They make a double-stranded cut, one incision in each strand, and are also known as restriction endonucleases. The restriction enzymes are classified into four main types, Type I, II, III, and IV based on the

Table 2.1 Classification of restriction endonucleases

Type	Features	Co-factors	Example
Type I	• Cleaves variably, often remote from the recognition site • Multifunctional protein possessing both restriction and methyltransferase activity	Mg^{2+}, ATP, *S*-Adenosyl-L-methionine	EcoKI
Type II	• Cleave within or at particular positions close to recognition site	Mg^{2+}	EcoRI
Type III	• Cleave at sites that are at fixed position from the recognition site	Mg^{2+}, ATP	EcoPI
Type IV	• Cleave at variable distance from the recognition site • Targets modified DNA e.g., methylated, hydroxymethylated, and glucosyl-hydroxymethylated DNA	Mg^{2+}	No typical example

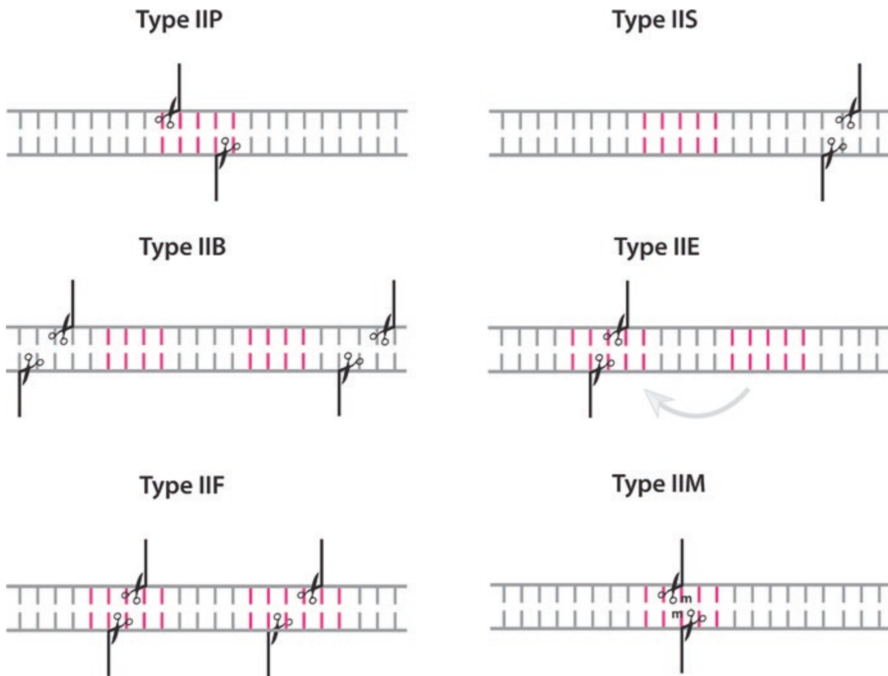

Fig. 2.3 Type II restriction enzymes subtypes based on cleavage properties

Table 2.2 Sub-classification of type II restriction endonucleases

Subtype	Characteristic features	Example
Type IIP	Palindromic recognition sequence, cleaves symmetrically usually within the recognition site	EcoRI
Type IIA	Asymmetric recognition site	FokI
Type IIB	Cleaves on both sides of recognition site	BcgI
Type IIC	Enzymes containing both cleavage and modification domains within a single polypeptide	HaeIV
Type IIE	Interact with two recognition sequences with one being the actual cleavage target and other serving as an allosteric effector	EcoRII
Type IIF	Interact with two recognition sequences and both required for cleavage. Concerted reaction by homotetramer	NgoMIV
Type IIG	Enzymes that have both restriction and modification domains in a single polypeptide and may be stimulated or inhibited by Adenosylmethionine	Eco571
Type IIH	Enzymes that have genetic features resembling type I systems but biochemically act as type II enzymes	BcgI
Type IIM	Recognizes specific methylated sites	DpnI
Type IIS	Asymmetric recognition sites and cleave usually outside of the recognition sequence at defined positions	FokI
Type IIT	Heterodimeric restriction enzymes having different subunits with restriction and modification activities	Bpu10I

composition, characteristics of the restriction site, and the enzyme cofactor requirements.

Type II restriction endonucleases do not require ATP for their activity and cleave faithfully within or at particular positions close to recognition sites. These enzymes are, therefore, most commonly used restriction endonucleases in gene analysis and recombinant DNA technology. They are further classified into various subtypes [16] (Fig. 2.3).

2.3.4 Isoschizomers and Neoschizomers

Isoschizomers are restriction endonucleases that have same recognition sequences and cleavage sites. For example, SphI (CGTAC↓G) and BbuI (CGTAC↓G). The first example discovered to recognize a given sequence is called as prototype while the subsequently identified enzymes that recognize the same site are isoschizomers of the prototype. Neoschizomers, on the other hand, are the restriction endonucleases with the same recognition sequences but cleave at different positions. For example, SmaI (CCC↓GGG) and XmaI (C↓CCGGG).

2.3.5 CRISPR-Cas9: A Novel Tool in the DNA Editing Toolbox

An additional defense mechanism identified recently in bacteria that has evoked great interest in the field of biomedical research is the CRISPR-Cas9 system [17, 18]. It is based on CRISPR (clustered regularly interspaced short palindromic repeats) and the CRISPR-associated (Cas) endonuclease. CRISPR are the DNA sequences in the prokaryote genomes such as bacteria adapted from the DNA fragments of bacteriophages that had previously infected the bacteria. This confers a form of acquired immunity to the prokaryote. The bacteria later transcribes these integrated sequences during subsequent infections and the small RNAs produced act as guide for the Cas endonucleases that degrades the phage DNA. This defense system has been harnessed into a versatile gene-editing tool that enables precise and efficient DNA manipulations. For their contribution in developing the technology, Jennifer Doudna and Emmanuelle Charpentier were awarded the Nobel Prize in chemistry in the year 2020.

Table 2.3 Carrying capacities of different cloning vectors

S. No	Vector	Carrying capacity (kb)
1	Plasmid	15
2	Phage lambda	25
3	Cosmid	45
4	Bacteriophage	70–100
5	Bacterial artificial chromosome (BAC)	120–300
6	Yeast artificial chromosome (YAC)	250–3000

2.4 Cloning: Introduction, Cloning Vectors, Cloning of PCR Products

2.4.1 Over-View of Cloning

Cloning is the process of producing DNA, either a portion of a regulatory region like promoter or a part of a functional gene. The basic steps of cloning a gene of interest in a suitable vector include the following [19, 20]:

(a) Gene isolation/amplification.
(b) Digestion of vector and gene using restriction endonucleases.
(c) Ligation of the digested vector and gene.
(d) Incorporation of recombinant DNA into host cells.
(e) Selection/screening of cells containing recombinant DNA.

2.4.1.1 Enzymes Involved in Cloning Procedure

(a) DNA polymerase: The initial requirement of molecular cloning is the isolation of the gene of interest. DNA polymerase is used to amplify the foreign DNA using site specific primers.
(b) Restriction Endonucleases: These are used for sequence-specific DNA cleavage to assist insertion of genes into suitable vectors.
(c) Ligase: It is required to seal the cohesive/blunt ends after restriction digestion. It uses ATP or NAD^+ as cofactor to form a phosphodiester linkage between 3′ and 5′ end.
(d) Alkaline phosphatase: It removes the 5′ terminal phosphate groups to prevent recircularization of the digested linear plasmid. Hence, ligation occurs only in the presence of the insert DNA. Use of alkaline phosphatase is essential in non-directional cloning where a single restriction enzyme is used and optional in directional cloning where two different restriction enzymes are utilized.

2.4.1.2 Molecular Accessories Required to Facilitate Cloning

(a) Linker Molecule: A linker molecule is an (8–10) nucleotide long dsDNA with a restriction site at the end. It is ligated with the foreign DNA (usually a genome product) to generate chimeric DNA containing the restriction site. Once digested, the chimeric DNA produces cohesive ends and facilitate cloning.
(b) Adaptor Molecule: An adaptor molecule is a short dsDNA with flanking DNA sequences. It promotes efficient ligation by providing free hydroxyl groups. Restriction digested vector is incubated with the chimeric DNA containing adapter DNA to get the circular clone [21, 22].

2.4.2 Cloning Vector

A cloning vector is a genetic vehicle that delivers foreign DNA into recipient cells where it can be amplified and/or expressed.

2.4.2.1 Criteria of a Good Cloning Vector

To be able to carry the foreign DNA and replicate in the host cells, the following properties are desirable in a cloning vector [21, 22]:

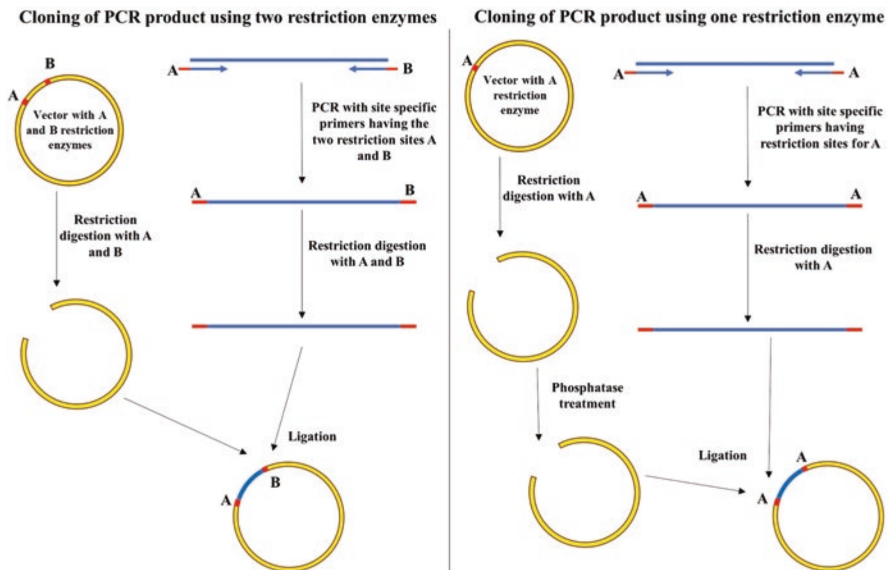

Fig. 2.4 Schematic representation of cloning of PCR products using one and two restriction enzymes

(a) It must be able to self-replicate inside host cells. For this, it must contain the origin of replication.
(b) It must possess a multiple cloning site (MCS) containing unique restriction endonuclease sites.
(c) The vector DNA should possess a marker gene to impart some additional phenotypic change in the host cells for identification of recombinant cells. An antibiotic resistance gene usually serves this purpose.
(d) The donor DNA fragment should not hinder the replication of the vector.
(e) A high copy number and small size of the vector is also desirable.

Different host-specific vectors are available for cloning (see Table 2.3) like plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and mammalian artificial chromosomes (MACs) [23].

2.4.2.2 Plasmids

Plasmids are circular dsDNA molecules present as extra chromosomal self-replicating elements in the bacterial cells. They exist in three different forms; open circular (nick in one strand), covalently closed circular (intact double strands), and supercoiled (extra twists in the double helix), supercoiled being the in vivo form. The following features make plasmids widely applicable for cloning [23]:

(a) Origin of replication: In order to start making its own replication copies, plasmids need an origin of replication. Origin of replication is specific to the host bacterial strain, for instance, plasmids with *ori* from *RSF1010* or *RP4* will be able to grow in both gram (+) and gram (−) bacteria while as plasmid with *ori* from *Col E1* are able to grow in E. coli species only.
(b) Selection marker: Plasmids often carry an antibiotic resistance gene, thereby imparting phenotypic changes to the recipient cell corresponding to the antibiotic supplied in the medium.
(c) Size: The size of plasmids ranging from 5.0 kb to 400 kb allows them to accommodate 10 kb of insert DNA.
(d) Promoter: It is required for the expression of the selectable marker and/or foreign DNA.

2.4.2.3 Bacteriophages

Bacteriophages are viruses that multiply as intracellular obligate parasites inside the bacterial cells. They have a specialized mechanism of delivering their genome into the bacterial cell. Most of their genome contains non-essential elements, which makes them suitable for cloning of larger DNA fragments (up to 20 kb) by replacement of the genome with foreign DNA [21, 23].

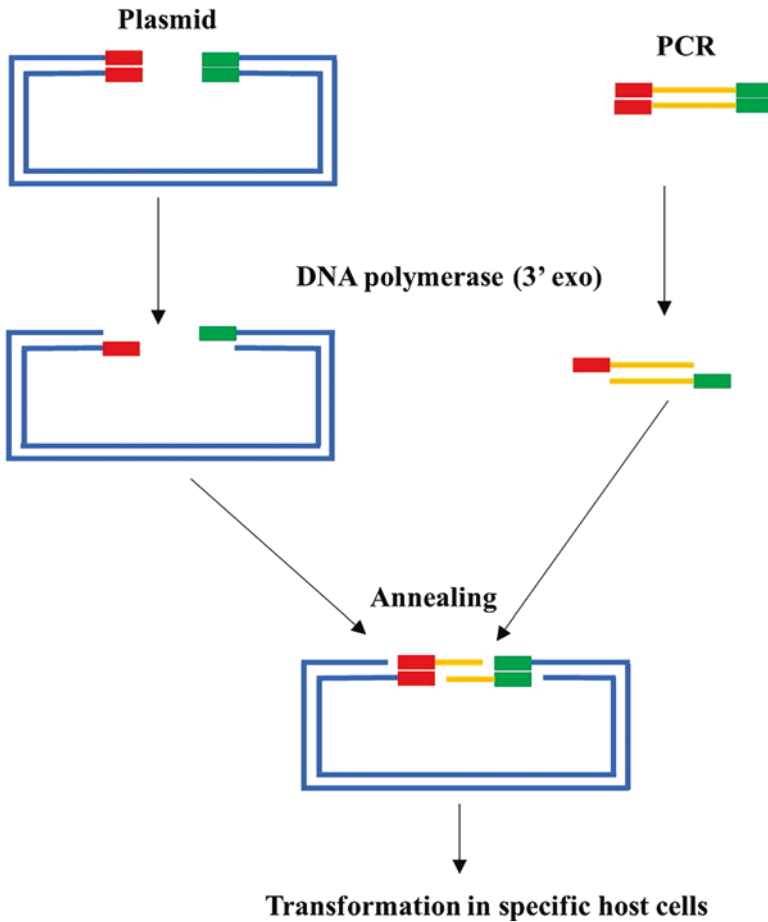

Fig. 2.5 Schematic representation of ligation-independent cloning

2.4.2.4 Cosmids

A Cosmid vector is a chimeric vector having origin of replication from bacteria and cos sites from bacteriophage λ. The flanking cos sites result in the circularization of the vector inside the host cells. Cosmids can accommodate 45 kb of DNA segments [23].

2.4.2.5 Bacterial Artificial Chromosomes (BAC)

Bacterial artificial chromosomes (BACs) are plasmids constructed with stable origin of replication (*F* factor) that maintains the plasmid as a single copy per cell. They can hold very large DNA fragments ranging in size from 75 to 300 kb. They

are commonly used for genome sequencing for instance human genome project [23].

2.4.2.6 Yeast Artificial Chromosomes (YAC)

YACs are yeast expression vectors that contain all the necessary elements required to maintain a eukaryotic chromosome in a yeast nucleus. They can accommodate very large DNA segments (up to 3000 kb). They are ideal for mapping of complex genomes [23].

2.4.3 Cloning of PCR Products

The cloning methods established for successful cloning of PCR products into suitable vectors are divided into following groups:

2.4.3.1 Ligation Dependent Cloning

1. *Blunt end cloning:* This method is used to ligate blunt end PCR products and dephosphorylated vectors with blunt cuts. Since blunt ends lack protruding ends, the ligation reaction is less efficient compared to sticky ends and depends solely on the random collisions between the blunt ends. For this reason, the concentration of ligase used in blunt end cloning is ten times more than sticky end ligation to compensate for the lower efficiency. PCR generally gives blunt-ended products, except when using *Taq* polymerase that may add an extra deoxynucleotide to the 3′ hydroxyl end of the PCR product. This may inhibit ligation reaction. To overcome this, PCR products are treated with Klenow fragment of DNA polymerase I [24], Pfu [25] or T4 [25, 26] polymerase to enhance the ligation efficiency by refining the ragged DNA end. The blunt-ended PCR products are also treated with T4 polynucleotide kinase prior to ligation as they lack a 5′-phosphate which is important particularly if the vector is dephosphorylated.
2. *Sticky end cloning:* Sticky end cloning is the most commonly used method of cloning PCR products using restriction endonucleases. For this, restriction sites are introduced at the 5′ termini of the PCR primers, that get integrated into PCR products after amplification (Fig. 2.4). This is followed by digestion with the respective restriction endonucleases to yield sticky ends at both ends of the PCR products. Digested vector and corresponding PCR products are then ligated using T4 DNA ligase with much higher efficiency.

   Problems routinely encountered during sticky-end cloning:

   - Restriction endonucleases being palindromes facilitate generation of primer dimers that may diminish the amplification efficiency.

- If the two restriction sites are too close to each other, the restriction endonucleases fail to effectively cleave the recognition sites.
- Restriction sites close to the 5′ ends of double-stranded PCR products result in unstable binding of the restriction endonuclease. To overcome this difficulty, extra nucleotides are added at the 5′ ends outside the restriction sites [27, 28].

3. *T-A cloning:* T-A cloning exploits the terminal transferase activity of *Taq* polymerase to create a 3′ dA overhang in the PCR amplified DNA duplex [29]. This duplex is then cloned in T-vector containing dT overhang. T-A cloning is a special kind of sticky end cloning with only one base at the sticky end.

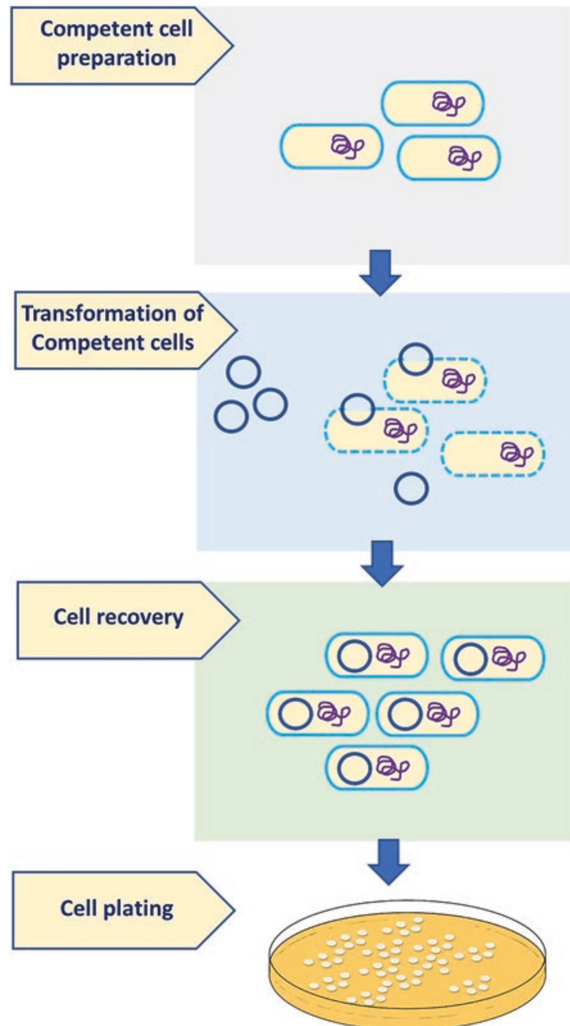

Fig. 2.6 Workflow of bacterial transformation

2.4.3.2 Ligation-Independent Cloning

Ligation-independent cloning (LIC), also known as ligase free cloning, does not require in vitro ligation but rather uses in vivo cloning. In this technique, the linearized vector and the PCR products having similar terminal sequences are co-transformed into host strain like JC8679 strain of E. Coli [30]. The method uses host homologous recombination system to clone the desired product in the vector (Fig. 2.5). This is a simple method but has limitations due to restricted range of host cells. Several LIC methods like Enzymatic Modification-Mediated LIC and PCR induced LIC are being adopted for different cloning strategies.

2.4.3.3 Gateway Cloning

This is a recombination based cloning procedure. For gateway cloning, primers are designed with flanking attB sites required for recombination. Gateway cloning involves two step cloning procedure: BP and LR reactions. Gene of interest is amplified using primers with flanking attB sites. BP reaction is then carried out, which results in the generation of donor vector containing the gene of interest. Donor vector is meant to transfer the gene to different destination vectors. BP reaction is followed by LR reaction, which transfers the insert to the destination vector. Destination vector is transformed using competent cells like NEB stable. These cells are selected with appropriate antibiotics for which the selection marker is present in the vector. Selected colonies are finally screened for the presence of the gene of interest using PCR/restriction digestion.

2.5 Introduction to Bacterial Transformation, Different Methods of Transformation, Animal Cell Transfection

2.5.1 Introduction

The process of transformation was discovered in 1928 by Frederick Griffith while working with Streptococcus pneumonia [31]. Bacterial transformation is the process of uptake of exogenous naked DNA by the competent bacterial cell. This results in the assimilation and expression of newly acquired traits in the recipient bacterium by the process of homologous recombination. Following are some of the basic features of bacterial transformation [32, 33]:

1. This process is based on the natural ability of the bacteria to spontaneously release DNA and requires only free DNA (transforming principle) in the extracellular environment without the need of any living donor cell.
2. The success of transformation rests on the ability of the recipient cell to incorporate exogenous DNA.

3. Heat shock or electroporation increases the permeability of the cell wall thereby increasing the competence of the bacterial cells.
4. Transformation has emerged as the most common method of transfer of artificially engineered DNA into recipient cells. It can transfer up to tens of kilobases of DNA.

2.5.2 Types of Bacterial Transformations

Transformation can be divided into two types; natural transformation and artificial transformation.

2.5.2.1 Natural Transformation

Bacteria that are able to naturally take DNA from extracellular environment are said to be naturally competent and the process is called natural transformation. This usually happens during decrease in growth rate, DNA damage, cell starvation or competition among bacteria, e.g. a small subset of Bacillus subtilis cells become competent during stationary phase or during increase in cell density to incorporate new genes for cell survival. Natural transformation can occur both in gram-positive and gram-negative bacteria with some differences. The basic difference lies in the structure of the cell envelope between gram-positive and gram-negative bacteria. In gram-positive bacteria, the DNA binds to the DNA receptor on the surface of the competent cell. One of the strands gets degraded by the nucleases and the other passes via the cytoplasmic membrane with the help of DNA translocase. The ssDNA then gets integrated into the genome by RecA mediated recombination. On the other hand, gram-negative cells form channel of secretins on the outer membrane to allow entry of specific DNA. Pilin may also be required for competence [32, 33].

2.5.2.2 Artificial Transformation

The bacteria that are incompetent of undergoing natural transformation can be induced to become competant artificially using different laboratory procedures [32, 33]. For this, the cells are made passively permeable to DNA using several treatments, to allow entry of foreign DNA. This includes the following:

1. Incubating cells in a solution of divalent cations like calcium chloride followed by heat shock.
2. Subjecting cells to chemicals like polyethylene glycol (PEG) or dimethyl sulfoxide (DMSO).
3. Electroporation—in which the cells are exposed to a short pulse of high voltage electric filed using an electroporator.

2.5.3 *Work Flow of Bacterial Transformation*

The four key steps in the process of bacterial transformation are as follows (Fig. 2.6):

2.5.3.1 Competent Cell Preparation

The most common bacterial species used in transformation procedures is E. coli. The starter culture of the desired strain of bacteria is carefully checked for active growth by monitoring the optical density at 600 nm (OD_{600}) continuously. OD_{600} between 0.4 and 0.9 is desirable for higher transformation efficiency, with optimal value depending upon culture strain, culture volume, and protocol in practice. Cells are harvested in mid-log phase as per the method of transformation. For heat shock method, cells are incubated in ice-cold calcium chloride ($CaCl_2$) to increase the permeability of the cell membrane [34, 35]. Cations like potassium (K^+), cobalt III hexamine ($[Co(NH_3)_6]^{3+}$), manganese (Mn^{2+}), dimethyl sulfoxide (DMSO), and/or dithiothreitol (DTT) may also be supplemented to increase competence [36]. Cells are then stored in 10% glycerol. In case electroporation is the method of choice for transformation, the harvested cells are washed several times with de-ionized ice-cold water and then stored at −80 °C by resuspension in 10% glycerol [37].

2.5.3.2 Transformation of Cells

The two most popular methods of bacterial transformation are as follows:

(a) *Chemical transformation*: In this method, 50–100 μL of chemically competent cells are incubated with 1–10 ng of DNA for 5–30 min on ice. This is followed by brief exposure to elevated temperature (37–42 °C) for 25–90 s as suitable for the particular bacterial strain. This is known as heat shock. Heat-shocked cells are incubated back on ice for ≥2 min.

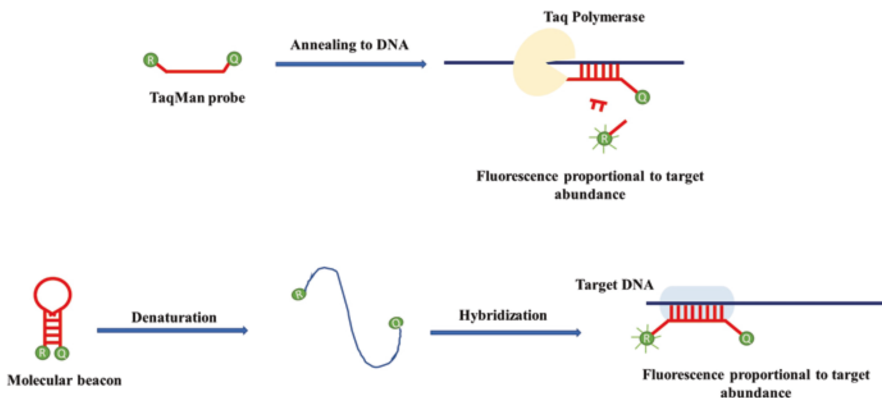

Fig. 2.7 Mechanism of quantification of TaqMan and Molecular beacon

(b) *Electroporation:* This procedure involves the exposure of competent cells to a short pulse of high voltage electric field (>15 kV/cm) in the presence of DNA. This induces transient pores for the entry of DNA through the cell membrane. Exponential decay is the most common type of electric pulse used for transformation wherein the applied voltage is allowed to decay for some milliseconds.

2.5.3.3 Cell Recovery

After transformation, cells are cultured in glucose and $MgCl_2$ rich, antibiotic-free liquid medium for about 1 h at 37 °C with shaking at 225 rpm. This increases the transformation efficiency and allows the expression of antibiotic resistance gene(s).

2.5.3.4 Cell Plating

After recovery, cells are plated on agar plates containing suitable antibiotic for identification of successful recombinants. The cells plated should be sufficient in amount to yield distinct colonies. Also, the cells should be evenly spread on the plate with the help of glass beads or cell spreader to avoid formation of bacterial lawn. Following this, plates are incubated overnight at 37 °C in an inverted position.

2.5.4 Animal Cell Transfection

Transfection commonly refers to the deliberate introduction of naked or purified nucleic acids into eukaryotic cells. It involves the transient opening of pores in the cell membrane and subsequent entry of nucleic acids. The incorporation of foreign nucleic acid results in some changes in the cell properties that allows the functional study of the gene. The introduced nucleic acid can persist in the cell for long time and get passed into progeny (stable transfection) or can exist a limited period of time and get lost during subsequent passaging of cells (transient transfection).

Various transfection technologies are available today but not all can be applied to every cell. The ideal approach is selected based on the cell type. Broadly, the techniques are divided into three categories:

2.5.4.1 Chemical

Chemical methods use carrier molecules either to neutralize the negatively charged nucleic acids or to impart a positive charge. Calcium phosphate transfection is one of the cheapest methods [38]. Others include cationic lipid transfection,

DEAE-dextran transfection or by other cationic polymers (e.g., polyethylenimine (PEI), polybrene, dendrimers, etc.)

2.5.4.2 Biological

Biological methods (also known as transduction) use genetically engineered viruses to transfer non-viral genes into cells and include adenoviruses, lentiviruses, etc.

2.5.4.3 Physical

Physical methods deliver nucleic acids directly into the nucleus or cytoplasm of the cell. These include Electroporation, Direct microinjection, Laser-mediated transfection (photo-transfection), Biolistic particle delivery (particle bombardment), etc. [39–42].

2.6 Real-Time PCR

2.6.1 Introduction

Real-time PCR or quantitative PCR (qPCR) is a robust molecular biology technique used for monitoring the amplification of the nucleic acid (DNA/RNA) present in a sample in real time, i.e. during the course of PCR reaction. Real-time PCR combines the detection and amplification into a single step by collecting data throughout the PCR reaction. This is achieved by the use of various fluorescent dyes that corelate fluorescence intensity with PCR product concentration [43].

This technique has emerged as the most common means of gene quantification and has the following advantages:

1. It is a highly sensitive technique.
2. It is very sequence-specific technique.
3. It has a large dynamic range.
4. It is cost-effective and time efficient.
5. It is amenable to high sample throughput.
6. It requires 1000 folds less template DNA or RNA than conventional PCR.
7. It needs no post-amplification processing.

2.6.2 Principle of Real-Time PCR

The principle of real-time PCR depends on the use of fluorescent dyes that fall under two categories (Fig. 2.7):

(a) DNA-binding dye (Intercalating dye-based method).
(b) Sequence-specific probe (Probe based detection method).

The choice of dye depends upon the type of tissue used and the cost of the sample.

2.6.2.1 DNA-Binding Dyes

These dyes have their own fluorescence. Their fluorescence increases by 100–1000 folds once they bind to dsDNA. Therefore, the fluorescence increases proportionally as more PCR product accumulates during cycling reaction [44]. While the method is rapid and cost-effective, it often yields false positives due to the lack of sequence-specific binding of the dye [45]. SYBR green and EvaGreen are two commonly used dyes in real-time PCR. A detailed protocol of SYBR Green I PCR master mix is illustrated in Ramos-Pay en et al. [46].

2.6.2.2 Probe Based Detection

This method relies on the use of single short sequence-specific probes. These are further divided into two types:

(a) *Linear probe*: Linear probes are either fluorescent or radiolabeled which bind DNA in a sequence-specific manner. TaqMan probe is a classical example of linear probe that works in conjunction with Taq DNA polymerase. It contains a fluorescent dye (reporter) at the 3′ end and a quencher at the 5′ end. When the probe is bound to the DNA, the quencher being in close proximity of the reporter molecule, quenches its fluorescence. During the extension step, the 5′ exonuclease activity of Taq polymerase degrades the probe. This separates the reporter from the quencher dye, resulting in augmented fluorescence emission [47].
(b) *Molecular beacons*: The molecular beacons are the simplest hairpin probes that operate in a thermodynamically stable structure. They comprise of two important regions–(1) the central region that is complementary to the target sequence and (2) the stems that contain the reporter and quencher dyes at their ends and are complimentary to each other [48]. When the beacon is in hairpin conformation, the reporter and quencher dyes are close to each other that results in reduction in fluorescence. When the probe binds the target DNA, the quencher is separated from the reporter, allowing reporter emission.

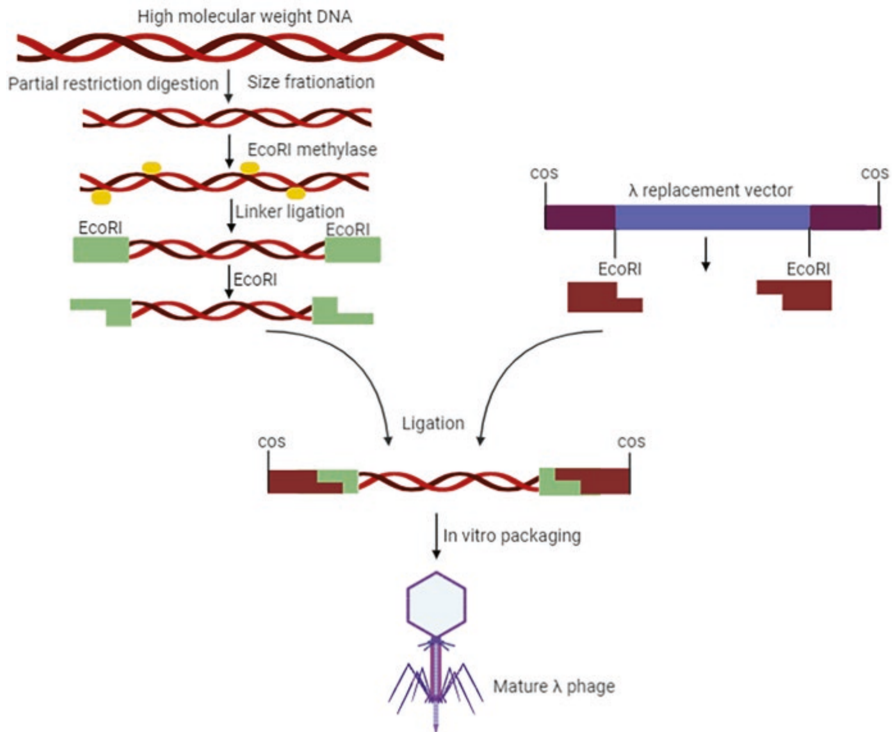

Fig. 2.8 Schematic representation of construction of genomic library

2.7 Genomic Libraries, cDNA Libraries, and PCR Based Libraries

A DNA library is an assembly of DNA fragments. Typical DNA cloning strategies involve four steps:

1. Generation of foreign DNA fragments.
2. Insertion of foreign DNA into a vector.
3. Transformation of the recombinant DNA molecule into a host cell in which it can replicate.
4. Selecting or screening clones to identify those that contain the particular recombinant we are interested in.

In this section we will discuss the types and construction of different types of libraries.

DNA fragment libraries are designated:

1. Genomic DNA library.
2. cDNA library,
3. PCR based library.

2.7.1 Genomic Libraries

Genomic libraries are organism specific and contain fragments of all DNA sequences present in the genome. Chromosomes contain an enormous amount of DNA. Even the simplest organism like E. Coli contains ~4.6 Mbp of DNA and it is impossible to clone this amount of DNA in any of the available vectors. It thus becomes imperative to fragment the DNA before cloning it in a vector.

Quality of the final library depends a lot on the method of fragmentation that is used. To ensure that the library contains representative copies of all DNA fragments present within the genome, an ideal approach is to cleave the DNA into random and overlapping fragments prior to cloning. There are two basic mechanisms that are employed for DNA fragmentation.

1. Mechanical shearing.
2. Restriction enzyme digestion.

Mechanical shearing: Genomic DNA is first purified and then passed through a narrow-gauge syringe needle (which gauge needles are generally used) many times. Alternatively, it can be subjected to sonication to generate DNA fragments that are suitable for cloning.

PROS: DNA fragmentation is random making it possible to incorporate every gene in the library.

CONS: The major limitation of this method is that large quantities of DNA (>1 μg)) are required, and also that the DNA fragment size is not uniform. Mechanical shearing does not produce DNA with cohesive ends. Therefore, further manipulation of DNA fragments is necessary to make them suitable for cloning in vectors.

Restriction enzyme digestion: In practice mechanical shearing is often considered undesirable, and fragmentation by *partial restriction enzyme digestion* is the preferred method for construction of libraries. Partial digestion of genomic DNA with restriction enzymes is the mostly used method for construction of genomic DNA libraries. Genomic DNA is treated with restriction enzymes in such a way that the digestion does not go to completion. Partial restriction digestion ensures that not all DNA recognition sequences are cut and, consequently, that the library produced should contain copies of genes that may possess multiple restriction enzyme recognition sequences. Practically, restriction digestion is normally performed using either a single restriction enzyme or often two, that recognize and cleave very commonly occurring sequences. Partial digestion is ensured by varying the enzyme concentration or the duration of digestion. Restriction digestion can lead to generation of either blunt end or sticky end DNA fragments. The blunt-ended DNA fragments can be difficult to clone whereas the ligation of sticky ended DNA is considerably more efficient. As such, it is preferable to generate genomic fragments that contain sticky ends in the cloning process. Sticky termini can be generated in two ways:

(a) Using Linkers and Adaptors:

Linkers:

Linkers are short pieces of double-stranded DNA of known nucleotide sequence that are synthesized in the test tube. A typical linker is blunt-ended but contains a restriction site.

Adaptors:

An *adaptor*, is also a short synthetic oligonucleotide but it is designed in such a way that it already has one sticky end. DNA fragments with blunt ends can be ligated to a series of oligonucleotides which can be either linkers or adaptors. The DNA fragments are first treated with a specific DNA methylase to protect them from restriction enzyme cleavage [49]. Treatment of the DNA fragments with the EcoRI methylase, in the presence of S adenosylmethoinine, will result in the methylation of the internal-most 'A' residue within the EcoRI recognition sequence (5'-GAATTC-3'). DNA modified in this fashion is unable to be cleaved by the restriction enzyme (see Fig. 2.8). The oligonucleotide linkers are then added to the methylated DNA in a large excess, in the presence of high concentration of DNA ligase. Subsequent treatment with the EcoRI restriction enzyme produces sticky ends.

(b) Using restriction enzymes that generate sticky ends:

The most prevalent method for generation of DNA fragments is the digestion of the genomic DNA with a commonly occurring restriction enzyme that generates sticky ends.

2.7.1.1 Cloning of DNA Fragments

Next step in the generation of genomic libraries is to clone the DNA fragments in a suitable vector. Although a variety of vectors are available for cloning large DNA fragments, λ based vectors are most commonly used. The recombinant vector and insert combinations are ligated and transformed into E. *coli*. A single bacterial colony or viral plaque arises from the ligation of a single genomic DNA fragment into the vector. *E. coli* cells infected with either a λ phage or transformed with a plasmid DNA are unable to take up additional DNA molecules of the same type. Thus one bacterial colony or λ plaque contains multiple copies of the same recombinant DNA molecule. These colonies or plaques are then pooled in such a way that sufficient colonies are present to ensure that each genomic DNA fragment is represented at least once. In this way a library of DNA fragments is generated. The main advantage of cloning large DNA fragments is that fewer individual clones can be pooled together to form a representative library. An obvious question to ask here is how many individual colonies or plaques must be pooled to ensure that a library is truly representative of the genomic DNA from which it was made. This depends on:

1. The size of the genome from which the library is made.
2. The average size of the cloned DNA fragments within the library.

For instance, if a library of the *E. coli* genome (4.6 Mbp) was constructed containing 5 kbp fragments, then the fraction of the genome size to the average individual cloned fragment size (f) would give the lowest possible number of clones (n) that the library must contain [50]

$$n = \frac{\text{genome} \quad \text{size}}{\text{fragment} \quad \text{size}} = \frac{4{,}600{,}000 \quad \text{bp}}{5000 \quad \text{bp}} = 920$$

Therefore, an *E. coli* genomic library of this size would require at least 920 independent clones. Going by the same formula, a human genomic library containing similar sized inserts would require at least 580,000 independent recombinants to construct a representative library.

Recombinant clones are washed off the growth plates and pooled together in a test-tube. The pool should contain a representative copy of each DNA molecule from which it was produced. This pooling together of either recombinant plaques or bacterial colonies generates a *primary library*. There is always a probability that some DNA molecules are not incorporated in the library.

A major disadvantage of a primary library is that it has low titer and is often quite unstable. This limitation is mitigated by an amplification step which increases both the stability and the titer. Bacterial colonies or plaques are plated out once more and the collection of resulting progeny leads to the formation of an *amplified library*. The advantage of an amplified library is that it has a much larger volume than the primary library, and thus can be subjected to multiple screenings.

2.7.2 cDNA Libraries

For organisms with relatively smaller genomes like bacteria, yeast, and fungi, the number of clones needed for a complete genomic library is manageable. For higher eukaryotic organisms like plants and animals, the genomes are quite big and therefore a complete library requires a large number of different clones that makes identification of a desired clone very difficult. Thus for multicellular organisms, a more feasible approach is the construction of cDNA library. Thus, the mRNA that is contained within a cell gives us a snapshot of the genes being expressed within that cell at any particular time. mRNA actually represents only a small fraction of the total RNA contained within a cell. With multicellular organisms a second type of library, specific not to the whole organism but rather to a particular cell type, might be more useful.

Although every cell in a multicellular organism contains the same set of genes, each cell is different because different sets of genes are switched on in different cells, while others are silent. Every individual cell is defined by the genes that are expressed and the proteins that they produce. Only those genes that are being expressed are transcribed into messenger RNA (mRNA). Using mRNA as a starting

material will therefore result in clones of only a selection of the total number of genes in the cell. Cloning of mRNA is particularly desirable in genes that are expressed at a high rate. Cloning of mRNA for highly expressed gene would obtain a large number of clones specific for that gene.

2.7.2.1 mRNA Can Be Cloned as Complementary DNA

The major difficulty with mRNA is that it cannot be maintained in stable vectors. A more feasible approach is to clone a DNA copy (called complementary DNA, or *cDNA*) of the mRNA for the construction of library. The conversion of RNA to DNA is dependent upon the action of *reverse transcriptase*, which is a RNA dependent DNA polymerase. Reverse transcriptase synthesizes a DNA polynucleotide complementary to an existing RNA strand. After the synthesis of cDNA strand, RNA is partially degraded by treating with ribonuclease HI. Fragmented RNAs then act as primers for DNA polymerase I, which synthesizes second cDNA strand. The resulting double-stranded DNA fragment can be ligated into a vector and cloned.

2.7.3 PCR Based Libraries

The main advantage of constructing a cDNA library is that these libraries are stable and of high quality given that the cDNA fragments are physically cloned and are screened multiple times. Although this makes cDNA libraries desirable for isolating cDNA clones, a major drawback is that the generation of cDNA library is cumbersome and time-consuming. Therefore, at times, the requirement of cloning for cDNA library can be circumvent by synthesis of PCR products from mRNA. The limitation with this approach is that it is feasible only when nucleic acid hybridization is used for screening and is not applicable when functional analysis of the encoded protein is required. Despite the screening constraints, PCR based libraries are both easy to construct and can be screened quickly. The key to PCR based libraries is to use reverse transcriptase enzyme in PCR, a technique known as *RT–PCR* [51]. Using RT-PCR we can:

1. Determine the presence or absence of a transcript.
2. Estimate expression levels.
3. Clone cDNA products without the necessity of constructing and screening a cDNA library.

In this approach, reverse transcriptase is first used to produce a cDNA strand [52]. Being a sensitive technique, even a single strand of cDNA suffices for the progression of the PCR stage, where second-strand cDNA synthesis and subsequent PCR amplification is performed using a thermostable DNA polymerase. The need for the use of two enzymes has now been bypassed by single-enzyme reverse transcription and PCR amplification protocols [53]. This has been made possible by using

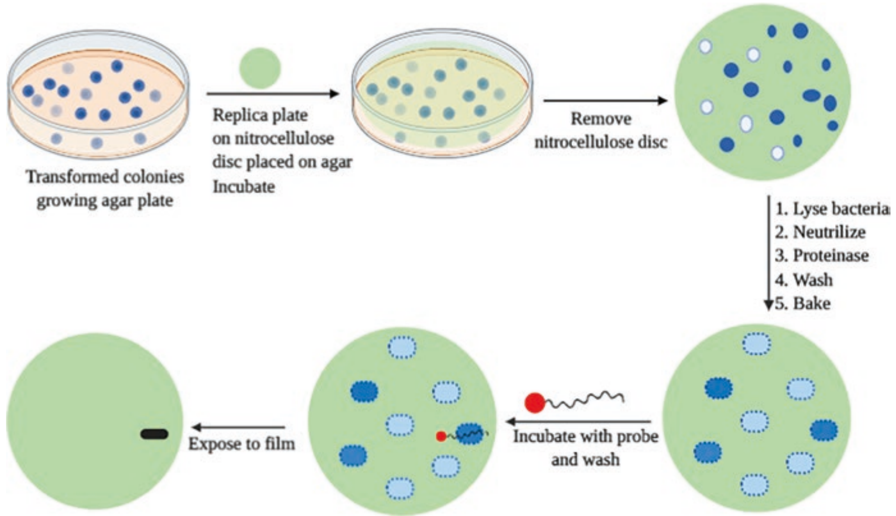

Fig. 2.9 Schematic representation of nucleic acid hybridization screening

enzymes which possess both DNA dependent DNA polymerase as well as reverse transcriptase activity. One such enzyme is *Thermus thermophilus* (*Tth*) DNA polymerase which possess a reverse transcriptase activity in the presence of manganese ions. Nowadays, to eliminate the likelihood of contaminating the reaction with the use of multiple buffers, systems have been devised where the reverse transcriptase reaction and PCR are performed in the same buffer [54]. A major check for this system is that it is most suited for the amplification of mRNA molecules whose sequence is already known. This ensures the use of highly specific primers. For the construction of an amplified representative library, it must be ensured that each mRNA molecule within the population is represented within the library. For this purpose, many methods have been utilized.

Using an oligo-dT primer, reverse transcriptase synthesizes the first cDNA strand following which additional, unique sequences are added at the 5′-end. Prior to the addition of multiple C residues at the 3′-end of the DNA molecule using terminal transferase, RNaseH is used to remove the mRNA strand of the RNA–DNA hybrid. An oligo-dG primer with unique sequences at its 5′-end is utilized to synthesize a second cDNA strand. Primers containing sequences complementary to the unique sequences at the 5′- and 3′-ends of the resulting double-stranded cDNA are then used to prime subsequent PCR reactions. This results in the production of large number of copies of each cDNA molecule produced in the RT reaction.

2.8 Gene Screening: Introduction, Nucleic Acid Hybridization Screening, Immune Screening, Functional Screening, Interaction-Based Screening

2.8.1 Introduction

Screening of clones for a single specific recombinant clone appears to be somewhat difficult. The selection process for a recombinant clone relies on one of the following criteria:

1. DNA sequence of the clone;
2. Protein sequence of the encoded polypeptide;
3. A biochemical function of the polypeptide; or.
4. The ability of the polypeptide encoded by the recombinant clone to interact with other polypeptides.

2.8.2 Screening by Nucleic Acid Hybridization

Nucleic acid hybridization screening is based on the complementary base pairing of nucleic acids. A probe sequence which is identical or complimentary to a specific DNA sequence of the recombinant clone can be used to identify the recombinant clones that contain the sequence of interest. A major requirement for this screening is the knowledge about the DNA sequence of interest so that a probe can be designed to search for that sequence. Nonetheless, hybridization has remained as a method of screening for many years now. One of the main advantages of this type of screening is that it does not depend on the expression of the cloned DNA fragments within the library and it can be applied to almost any vector system into which a library has been cloned.

A universal scheme for the identification of recombinant clones was originally described by Grunstein and Hogness, but was modified later on. The modified protocol utilizes a filter lift procedure and allows screening of a large number of bacterial colonies from a single plate [36, 55, 56]. The DNA contained within each clone is first immobilized on a nitrocellulose or a nylon membrane. For this bacterial colonies are first grown on agar plates containing selection marker (antibiotic). After that nylon is placed on top of the colonies and then replica plating is done. The nylon replica is then chemically treated with 0.5 M NaOH which causes bacterial lysis and DNA denaturation. This is followed by neutralization and proteinase K treatment in order to remove the protein. Denatured DNA remains bound to the membrane which is then baked at 80 °C, or treated with UV light, to firmly adhere the DNA to the membrane. Thus a nitrocellulose sheet containing single-stranded DNA copies of bacterial colonies present on the agar plate is obtained. A labeled single-stranded probe is allowed to hybridize to the nitrocellulose membrane to

reveal the location of colonies on the original dish that contain identical, or at least similar, DNA sequences. The probe can be any single-stranded nucleic acid sequence and does not need to match the target sequence precisely.

2.8.2.1 Properties of DNA Probe

Hybridization experiments require DNA probes that are homologous to the sequence that is to be detected. A gene isolated from one organism can also be used as a hybridization probe to detect a homologous gene in a DNA library generated from a different organism. An ideal size for the probe is approximately 14–20 nucleotides. The probe is chemically synthesized and degenerated but a larger number of degeneracies in a probe sequence will result in the loss of specificity. Specific binding of the probe to the sequence of interest is also determined by the stringency of washing the probe from the membrane.

Radioactively labeled probes are usually used for hybridization screening because of their easy detection. Oligonucleotide probes are treated with polynucleotide kinase in the presence of γ-^{32}P-ATP. This results in the transfer of radioactive phosphate group from the ATP molecule to the 5′-end of the oligonucleotide. Digoxigenin labeling is an example of non-radioactive alternative [57]. Non-radioactive approaches are useful for certain experimental procedures, but the sensitivity and detection power of radioactivity has been difficult to surpass.

Rather than screening a library with a single oligonucleotide to search for homologous sequences, a more feasible alternative is screening by PCR using two primers to amplify homologous portions of genes. The major advantage of this approach is speed. Since PCR reaction occurs directly with the naked DNA template this method mitigates the need for plating of the library prior to screening. Portions of homologous genes from the library can be amplified using degenerate primers [58]. The isolated PCR products usually represent only a small region of the gene. However, this isolated fragment can be used as a highly specific probe in a traditional hybridization screen, or as a starting point to amplify the 5′- and 3′-ends of the gene using various PCR methods [59] (Fig. 2.9).

2.8.3 Immunoscreening

If a DNA fragment library is cloned into an expression vector, each individual clone can be expressed to yield a polypeptide. Although all libraries can be screened using hybridization or PCR screening methods, these approaches become compromised when the DNA sequence of target clones in not known and thus there is no possibility of designing a probe or set of primers. In this case, identification of the clone can be done by identifying a sequence of expressed peptide. These are known as expression libraries. Expression libraries exploit some structural or functional properties

of the gene product and allow a range of alternative techniques to be employed for screening.

Antibodies are usually used for the detection of polypeptide sequences. If a protein is available, it is relatively straightforward to produce a purified or even partially purified antibody against that protein. Antibodies specifically recognize antigenic determinants on the polypeptide synthesized by a target clone. Screening of this type does not rely upon any particular function of the expressed foreign protein. Even if the protein is not fully functional, the sequence of the expressed peptide is likely to be unique within the host cell. It only requires the availability of antibody that binds to the protein of interest. Since this screening can be applied to any protein for which an antibody is available, it qualifies for one of the most versatile expression cloning strategies.

As already mentioned, there is no requirement for that protein to be functional. An antibody recognizes a short sequence of amino acids that folds into a particular three-dimensional conformation on the surface of the protein which is called the epitope. Epitopes form even if the polypeptide chain is incomplete or when expressed as a fusion with another protein because folding of epitopes is independent of the rest of the protein. Under denaturing conditions, when the overall conformation of the protein is abnormal, many new epitopes can be formed on the protein.

Immunological screening techniques were first developed in late 1970s, when plasmid vectors were widely used for construction of expression libraries. The method developed by Broome and Gilbert (1978) was routinely used at that time [60].

This method was based on two properties of antibodies.

1. Antibodies adsorb very strongly to certain types of plastic, such as polyvinyl.
2. IgG antibodies can be readily labeled with ^{125}I by iodination in vitro.

Cells transformed with the plasmid expression vector were allowed to form colonies on petri dish plates. The colonies were lysed using chloroform vapor or by spraying an aerosol of virulent phage. This results in the release of antigen from positive clones. A sheet of polyvinyl coated with the appropriate antibody was then applied to the surface of the plate, allowing the formation of antigen–antibody complexes. Following removal of the sheet, it was exposed to ^{125}I-labeled IgG, which was specific to a *different* epitope on the surface of the antigen (i.e. a determinant not involved in the initial binding of the antigen to the antibody-coated sheet). Ultimately, the sheet was washed and exposed to X-ray film. The clones identified by this procedure could then be isolated from the replica plate. A major limitation of this “sandwich” technique was the requirement of two antibodies recognizing different epitopes of the same protein.

While plasmid libraries have been useful for expression screening [61, 62], they have now been largely replaced by bacteriophage-λ insertion vectors. A generalized scheme for the immunoscreening of a DNA library is summarized here. cDNA is cloned into the expression vector *λ*ZAP in such a way that the foreign DNA is placed under the control of the bacterial *lac* promoter. Recombinant *λ* phages are

pooled and plated out onto a suitable bacterial host on agar plates followed by incubation of the plates until small plaques appear. A nitrocellulose sheet previously soaked in IPTG is placed on top of the plaques. IPTG acts as a gratuitous inducer of the *lac* promoter. The nitrocellulose sheet is left on top of the agar for 4 h. This induces the expression of the polypeptides encoded by the cDNA, and causes binding of the proteins that are produced when the *E. coli* cells lyse consequent to the phage infection. The nitrocellulose sheet is then removed from the plate. The sheet, now containing the proteins that were expressed in each individual plaque, is incubated with a specific antibody to the protein for which the gene is sought. This is followed by subsequent washings to remove any unbound antibody and non-specific bindings. After washing, the sheet is incubated with a labeled secondary antibody to detect the presence of the bound primary antibody.

Immunoscreening methods originally involved the use of radio-labeled primary antibodies to detect the antibody binding to the nitrocellulose sheet [60]. These methods have now been surpassed by antibody sandwich technique described above. The secondary antibody is conjugated to an enzyme like horseradish peroxidase or alkaline phosphatase with easily detectable activity [63, 64]. This conjugated secondary antibody recognizes the constant region of the primary antibody and can be assayed directly on the nitrocellulose sheet either calorimetrically or by using X-ray film. Multiple secondary antibodies are able to bind to each primary antibody; antibody sandwiches produce amplified signals. The use of polyclonal antibodies increases the sensitivity for immunological screening, but compromises on the specificity. Monoclonal antibodies and cloned antibody fragments can also be used, but since they recognize only a single epitope, they are less sensitive.

2.8.4 Screening by Function

Functional cloning relies on the screening methods that depend on the full biological activity of the protein. While immunoscreening depends only on the expression and not the function of the protein, functional screening essentially requires the protein to retain its function. Unlike positional cloning, described above, functional cloning does not require a prior knowledge of the gene in the genome, nor of the nucleotide sequence of the clone or the amino acid sequence of its product. As long as the expressed protein is functional, that function can be exploited to screen an expression library and the corresponding clone can be identified.

2.8.4.1 Screening by Functional Complementation

Functional complementation is the process by which a particular DNA sequence compensates for a missing function in a mutant cell, and thus restores the wild-type phenotype. This type of screening requires a host cell that either lacks a biochemical function that can be selected for or that is specifically disabled in some function that

can be compensated for by a protein produced from an expression library. *Functional complementation* is particularly useful for identifying genes from one organism that perform the same role as a defective gene in another organism.

Ratzkin and Carbon (1977) provide an early example of how certain eukaryotic genes can be cloned on the basis of their ability to complement auxotrophic mutations in *E. coli* [65]. *E. coli* cells having a defective copy of the *his*B gene are not able to grow on media lacking histidine. This gene codes for the enzyme imidazole glycerol phosphate dehydratase that is essential for the biosynthesis of histidine. Transformation of hisB defective *E. coli* cells with an expression library from yeast and plating of these cells onto media lacking histidine, will allow only growth of those cells that contain a functional copy of the yeast enzyme—encoded by the *HIS3* gene. Although the yeast *HIS3* and *E. coli his*B genes share little DNA sequence similarity (less than 20% overall identity at the amino acid level), their encoded proteins perform the same enzymatic function.

If the function of the gene is highly conserved, it is quite possible to carry out functional cloning of, for example, mammalian proteins in bacteria and yeast. Functional cloning has been particularly successful in the isolation of higher eukaryotic genes as functional homologues of genes found in more experimentally amenable lower-eukaryotic cells. For example, many higher eukaryotic genes have been isolated by their ability to complement defects in their yeast counterparts.

These include

- the genes coding for several human metabolic enzymes [66];
- the *Drosophila* topoisomerase II gene [67];
- a number of human RNA polymerase II transcription factors [68]; and.
- mouse cell cycle control genes [69].

A major limitation of this approach is that a detectable mutation within the host cell must be available that can be compensated by the gene expressed from the foreign DNA. Such assays are thus not available for many genes because:

1. The mutation may not be fully compensated by the foreign gene.
2. The foreign gene may only be partially functional within the host cell.
3. A foreign gene may not be expressed within the host cell.
4. Expressed proteins may not be subject to appropriate post-translational modifications to produce the active form of the protein.

Besides these shortcomings, complementation screening is not suitable if two or more different foreign gene products are required to produce the active protein.

If the expressed proteins are able to confer new phenotypes upon the host cell into which they are transformed, genes coding such proteins may be cloned as a consequence of function. An example of such proteins is oncoproteins. Cellular oncogenes can be isolated from human DNA libraries based on their ability to stimulate cell proliferation in culture [70]. This is known as "gain of function screening." Although it has limited uses, but this is an extremely powerful way to identify specific genes with important cellular functions.

2.8.5 Screening by Interaction

Proteins usually do not exist as single entities but interact with other cellular proteins. Most proteins interact with a range of other proteins that either regulate their function or assemble them into larger functional complexes [71]. Thus, once a gene that encodes a protein is cloned, we can look for its interacting partners.

2.9 Mutagenesis: Introduction, Primer Extension Mutagenesis, PCR Based Mutagenesis, Random Mutagenesis

2.9.1 Introduction

Site-directed mutagenesis (SDM) is a method that is used to alter DNA sequences in a specific and highly directed way. Introducing such changes to make specific DNA alterations is used to study:

1. Changes in the activity of protein as a result of mutation.
2. To select or screen for desirable mutations.
3. To introduce or remove restriction sites or tags.

These mutations can be insertions, deletions, or substitutions depending on the desired purpose of introducing the mutation. Binding of oligonucleotides to complementary DNA sequences can be used to create mutant mismatches. The mutant oligonucleotide is then used as a primer for DNA synthesis such that the new DNA contains mutations. Mechanisms to create mutations at highly specific regions of DNA have been developed. Advent of SDM has enabled a highly directed approach to the study of the relationship between genes and proteins. The use of oligonucleotides in creating site-directed mutations was devised in the laboratory of Michael Smith, who shared the 1993 Nobel Prize in Chemistry for his discovery. Smith and his colleagues used single-stranded M13 genomic DNA as a hybridization template for a synthetic oligonucleotide [72]. The oligonucleotide is designed in such a way that one or more mutations (non-complementary base pairings) occur when it binds to complementary sequence within the single-stranded DNA genome of M13. Complementary base pairing that occurs between nucleotides other than the mutant base, stabilizes the binding of oligonucleotide to the single-stranded DNA. An oligonucleotide can alter individual bases, introduce base insertions or deletions into a gene.

Three different methods of site-directed mutagenesis have been devised: [73].

1. Primer extension mutagenesis.
2. PCR based mutagenesis.
3. Random mutagenesis.

2.9.2 Primer Extension Mutagenesis (The Single-Primer Method)

The simplest method of site-directed mutagenesis is the single-primer method [72, 74]. An oligonucleotide (7–20 nucleotides long) that carries a base mismatch with the complementary sequence is chemically synthesized and used to prime in vitro DNA synthesis. Cloning the gene in M13-based vectors makes this process feasible because this method requires that the DNA to be mutated is available in single-stranded form. However, DNA cloned in a plasmid and obtained in duplex form can also be converted to a partially single-stranded molecule that is suitable [75].

The synthetic oligonucleotide primes DNA synthesis and is itself incorporated into the resulting heteroduplex molecule. The reaction mixture is then transformed into *E. coli*. The heteroduplex gives rise to homoduplexes whose sequences are either that of the original wild-type DNA or that containing the mutated base. The frequency of mutant clones compared with wild-type clones is low. Screening of the mutants can be done by nucleic acid hybridization with ^{32}P-labeled oligonucleotide as probe. Under proper conditions of stringency defined by the temperature and cation concentration, only a mutant clone will give a positive signal. In this way the desired mutant is readily selected [75–77]. To exclude the possibility of other extraneous changes, it is mandatory to check the sequence of the mutant directly by DNA sequencing. This was particularly required with the early versions of the technique which made use of *E. coli* DNA polymerase. With the advent of high fidelity DNA polymerases from phages T4 and T7, not only the possibility of adventitious changes has been minimized but the time for copying the second strand has also been reduced. Another advantage of using high fidelity polymerases is that these polymerases do not “strand-displace” the oligomer, a process which would eliminate the original mutant oligonucleotide.

In 1980s, the primer extension site-directed mutagenesis procedure became widely adopted. However, there are a number of deficiencies with this single-primer method.

1. The DNA that is to be mutated needs to be cloned into the M13 genome.
2. The efficiency with which the single-primer method yields mutants is quite low. The yield on mutants is dependent upon many factors like efficient oligonucleotide binding, DNA replication, and DNA ligation. Each of these procedures is likely to be less than 100% efficient and therefore wild-type DNA strands will predominate in the mixture that is transformed into bacteria.
3. The major reason for this low yield of mutant progeny is that the newly synthesized DNA will not be methylated as it is produced in vitro, while the wild-type M13 genome, isolated from bacterial cultures, will be methylated. Methyl directed mismatch repair system of *E. coli* favors the repair of non-methylated DNA. In the cell, newly synthesized DNA strands that have not yet been methylated are preferentially repaired at the position of the mismatch, thereby eliminating a mutation. This is important because the mismatch repair systems of the

E. coli favor the repair of non-methylated DNA. This will result in the mismatches between wild-type and the mutant DNA strands being repaired in favor of a return to the wild-type sequence.
4. Identification of mutant phages by differential screening is a time-consuming process and often results in the isolation of wild-type rather than mutant phage.

2.9.3 PCR Methods of Site-Directed Mutagenesis

PCR method of SDM is an immensely powerful tool for introducing DNA alteration into the ends of linear DNA fragments. In this method often referred to as "two step PCR mutagenesis," the mutations are introduced within the PCR primers themselves. PCR protocols have been developed to enable the creation of mutation at any point throughout the length of the PCR product [78]. Two primary PCR reactions produce two overlapping DNA fragments, both bearing the same mutation in the overlap region. The overlap in sequence allows the fragments to hybridize. One of the two possible hybrids is extended by DNA polymerase to produce a duplex fragment. The other hybrid has recessed 5′ ends and, since it is not a substrate for the polymerase, is effectively lost from the reaction mixture. As with conventional primer extension mutagenesis, deletions and insertions can also be created. The method of Higuchi et al. [78] requires four oligonucleotide primers and three separate PCR reactions (a pair of PCRs to amplify the overlapping segments and a third PCR to fuse the two segments). A simpler method, which utilizes three oligonucleotide primers to perform two rounds of PCR, has been described by Sarkar and Sommer [79]. In this method, the product of the first PCR serves as a *megaprimer* for the second PCR.

Efficiency of the PCR based mutagenic protocol makes it a desirable choice for SDM. Using this method, the target mutation is obtained with 100% efficiency. There are, however, some disadvantages to this method.

1. Since thermostable DNA polymerases have low fidelity, the entire PCR product needs to be sequenced to eliminate the possibility of any extraneous mutations. Alternatively, thermostable polymerases with improved fidelity can be used [80–82].
2. The PCR product usually needs to be ligated into a vector, although Sarkar and Sommer [79] have generated the mutant protein directly, using coupled in vitro transcription and translation.
3. It is difficult to amplify large DNA fragments using PCR limiting the use of this method.

2.9.4 Random Mutagenesis

Our understanding of protein function has been revolutionized by the creation of specific directed mutants within genes using oligonucleotides. The methods of SDM that we have discussed so far are limited to the alteration of specific bases within a gene to other defined bases. If the alterations are within a codon of a gene, it will result in a mutant protein with defined amino acid changes. However, it is not always possible to predefine the alterations that are required. Cunningham et al. devised a systematic approach to this problem by changing each amino acid coding triplet within a gene to an alanine codon [83]. This method known as *alanine scanning mutagenesis* does not alter the overall structure of the protein but only identifies amino acid side chains that are important for protein function and eliminates only amino acid side chain interactions. A prerequisite for this type of approach is that a functional screen is available for identifying protein. Owing to the number of individual mutations that must be constructed this method is mostly suitable for small proteins or protein domains. An alternative approach called *charged to alanine scanning mutagenesis* converts sets of charged amino acid residues that occur consecutively within a linear polypeptide sequence to alanine [84]. This method explores the fact that most proteins contain a hydrophobic core with charged residues on the outside surface of the protein. Consequently, the surface of the protein contains clusters of charged amino acids in a linear protein sequence and may therefore participate in, for example, protein–protein interactions. Creating mutations in these charged clusters are more likely to disrupt these protein–protein interactions than mutagenesis of other residues.

Introduction of random mutations in individual genes can be accomplished by one of the two methods.

1. *Doped cassette mutagenesis.*
2. *Error-prone PCR*.

Again, these methods require that a screening method to analyze mutants with an appropriate phenotype must be available. However, they do not suffer from limiting mutation types to individual residues or from the types of alteration that can be made.

2.9.4.1 Doped Cassette Mutagenesis

In this method the DNA between two restriction enzyme recognition sites is removed from a plasmid by restriction digestion and replaced using a pair of synthetic oligonucleotides. Here, however, the oligonucleotides do not encode a unique sequence. Libraries of oligonucleotides are produced that are based on the same sequence, but contain certain random changes. Such oligonucleotides are called *doped oligonucleotides* and are synthesized by using a mixture of bases. For example, if the next base to be added to an extending oligonucleotide was an 'A', then rather than chemically adding only the 'A' precursor to the growing oligonucleotide chain, a mixture

of 'A' and a small quantity of the other nucleotide precursors would be added. Though the probability is that such mixtures contain 95% of the wild-type nucleotide and only 1.7% of each of the other nucleotides, the level of 'doping' gives some control over the level of mutagenesis that will be obtained.

2.9.4.2 Error-Prone PCR

Certain DNA polymerases that are used in PCR lack proofreading activity and are thus error prone. In particular, Taq DNA polymerase lacks 3′–5′ exonuclease proofreading activity. This implies that significant mutations may be introduced into PCR products simply as a consequence of the PCR itself [85]. Thus this is a more straight forward method for introducing random mutations as only a PCR reaction needs to be carried out. The PCR product can then be cloned and screened functionally. By altering a variety of the PCR reaction conditions, the error rate of *Taq* DNA polymerase may be increased. Elevated error rate can be achieved by the following:

1. Adding manganese ions to the reaction.
2. Increasing the concentration of magnesium [86].
3. Changing the concentration of deoxynucleotides.
4. Changing the concentration of the polymerase itself.
5. Increasing the length of the extension step of the reaction.

Random mutagenesis is a popular choice for SDM owing to the ease at which PCR based random mutagenesis can be performed. The main drawback of the technique is the reliance on an enzyme to create random mutations. DNA polymerases have preferences in the mistakes they make. In the case of *Taq* DNA polymerase, transitions are favored over transversions [87], so some mutations are difficult to obtain.

2.10 Summary

Molecular biology techniques form the basis to characterize novel genes and to study mechanisms of various cellular pathways. Knowledge of PCR, restriction digestion, ligation, bacterial culture, and mammalian cell culture are essential for any basic molecular biology study. PCR in particular is a versatile technique which finds its use in cloning, genotyping, DNA fingerprinting and polymorphism analysis. Site-directed mutagenesis is used to study the function of important amino acids and motifs in a protein. qPCR has been utilized to evaluate gene expression at RNA level. In particular, regulation of oncogenes and tumor suppressors has been extensively studied in cancers using qPCR. Nowadays, qPCR is being used for COVID-19 screening. Comprehension of biotechnology as such is necessary for basic research and to diagnose and study various diseases.

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Chapter 3
Biotechnology in Medicine: Advances-I

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Abstract The recent advances in biologic medicine have paved a way to successful treatment of patients with serious diseases such as cancer, neurological cases like multiple sclerosis, autoimmune diseases such as rheumatoid arthritis (RA), and to fight against a very recent pandemic outbreak like COVID-19 disease. Biotechnology is an important field that is applied to the medical sector with the aim of improving the distinct targeted genes and customized medicines. Understanding of human genome via the human genome project, opened a new era of gene specific targeted therapy based on different disease conditions. There are numerous methods which facilitated for the advancement of medical biotechnology including microarray and next-generation sequencing. These advanced techniques of biotechnology are commonly used to improve medicines due to the advantages and pieces of knowledge it provides such as understanding the genetic composition of the different species using next-generation sequencing techniques. Understanding of genetic sequencing of an organism will provide a foundational structure of hereditary diseases manipulation and repairing of damaged genes to cure the diseases. In this chapter, we will discuss how medical biotechnology revolutionized the traditional medicine by understanding the in-depth of various diseases at a single nucleotide level using advanced biotechnological tools available till date. In addition, we will provide a

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brief overview on some of latest areas of medical biotechnological advancements include pioneering work in genetic testing, gene therapy and advanced drug treatments.

Keywords Sequencing techniques · Phylogeny · Microarrays · Genetic engineering · Gene therapy

3.1 Genome Sequencing

Genome sequencing refers to the entire genome sequencing of an organism, where genome refers to the entire set of genes present in an organism. There is a wealth of data obtained after the sequencing of the entire genome which helps us in studying it completely. Studying the gene functions one by one is time-consuming whereas genome sequencing is easy as it allows studying the whole genome functions in very less time. Prokaryotic whole genome sequencing is easy as their genome is small with no repetitive sequences. But eukaryotic genome sequencing is difficult due to its large set of sequence with more repetitive sequences and thus, sequencing of very large genomes like human genome requires computerized technology for the data handling. The initiation of the Human Genome Project led to the development and execution of several high throughput sequencing techniques [1].

A breakthrough that helped scientists to determine the human genetic code was using the sequencing technology, developed by Frederick Sanger and this technology named after him as Sanger sequencing [2–4]. The automated version of Sanger sequencing is still in use to sequence a short piece of DNA. It is believed to take years to sequence a human genome using Sanger sequencing method. However, the next-generation sequencing has sped up the process of sequencing as of now. Using next-generation sequencing, it is now achievable to sequence huge amounts of DNA, for example, all the portion of an individual's DNA that provide instructions for making proteins [5]. These portions are called as exons, and it is believed that this will make up 1% of an individual's genome. All the exons in a genome are known as the exome, and the method of sequencing them is known as whole exome sequencing. This method allows variations in the protein-coding region of any gene to be identified, rather than in only a select few genes [5]. Because most known mutations that cause disease occur in exons, whole exome sequencing is thought to be an efficient method to identify possible disease-causing mutations [6, 7]. However, recent studies state that DNA variations outside the exons can affect gene activity and protein production and lead to genetic disorders—variations that whole exome sequencing would miss. Another method, called whole genome sequencing, determines the order of all the nucleotides in an individual's DNA and can determine variations in any part of the genome [6–10].

While many more genetic changes can be identified with whole exome and whole genome sequencing, the significance of much of this information is unknown. Because not all genetic changes affect health, it is difficult to know whether identified variants are involved in the condition of interest. Sometimes, an identified variant is associated with a different genetic disorder that has not yet been diagnosed. In addition to being used in the clinic, whole exome and whole genome sequencing are valuable methods for researchers [6]. Continued study of exome and genome sequences can help determine whether new genetic variations are associated with health conditions, which will aid disease diagnosis in the future.

3.1.1 Human Genome Project (HGP)

HGP was a 15 year 3-billion-dollar program. This project was set up to determine the human genetic map, physical map of the human genome, and sequence map [11]. It produced an accurate sequence to be referenced for each human chromosome excluding the heterochromatic regions with a small number of gaps [11, 12]. Through this project, the technology was infused into biology resulting in small and big science research. Hence, the HGP has opened several avenues in technology, biology, computation, and medicine which need to be enormously exposed [13, 14].

The foundation of the human genome project orbited around two major principles. First, to make the information of whole human genome sequencing publicly available for the use of researchers and layman. Second, to understand the shared molecular heritage of human for the benefit of diverse approaches, this project embraced collaborators from through the world moving beyond borders. These founding principles ensured unrestricted access for the scientist in academia and in industry, which made very useful for disease-based discoveries by researchers of all types. Before going into the huge project, the collaborative team started with the two early goals. First, building genetic and physical maps of the human and mouse genomes. Second, performing the test run by sequencing the smaller genomes like yeast and worm. After getting a successful positive outcome with yeast and worm sequencing, the collaborative team proceeded for sequencing of human genome [3, 12].

The human genome project employed a two-phase approach to deal with the human genome sequence based on the understandings obtained from the yeast and worm studies. The first phase called as shotgun phase, in which the human chromosome has been divided into smaller segments of DNA, which were then sequenced based on overlapping DNA fragments. The shotgun phase generated massive amount of data, which were generated and analyzed using physical map of the human genome that established earlier as an important goal [11, 14]. The second phase of the project called as finishing phase. This phase involves the filling in gap experiments and resolving DNA sequences in abstruse areas which were not obtained during the shotgun phase [11]. Overall, the shotgun phase yielded 90% of the human genome sequence in conscription form. The three main steps involved in

shotgun phase are; (1) Acquiring a DNA clone to sequence. (2) Sequencing the DNA clone, and (3) Compiling sequence data from multiple clones to determine overlap and establish a contiguous sequence [11, 12, 15].

The second phase called, finishing phase of the human genome project has been hounded after the completion of the draft shotgun phase. The finishing phase yielded 99% of the human genome in final form. Throughout this phase, the investigators filled in gaps and resolved DNA sequences in ambiguous areas that were not solved during the shotgun phase. The final form of the human genome contained 2.85 billion nucleotides, with a projected error rate of 1 event per 100,000 bases sequenced. Though the earlier draft publications had anticipated as many as 40,000 protein-encoding genes, while the finishing phase lowered this estimate to between 20,000 and 25,000 protein-encoding genes [15].

One of the major findings of the human genome project is that the human nucleotide sequence is nearly identical between any two individuals, on the other hand, even a single nucleotide in a single gene can be responsible for causing disease. Molecular mechanisms underlying an array of genetic disorders have become understandable due to the contribution of human genome project [12, 15, 16]. Furthermore, a merging of cytogenetic approaches with the human genome sequence will continue to propel our understanding of human disease to an entirely new level. With the sequence of the human genome in hand, we have realized that it entails more than just wisdom of the order of the base pairs in our genome to cure human disease. Thus, the human genome project will undoubtedly be proclaimed as one of the truly essential scientific accomplishments twenty-first century [11, 17, 18]. Regrettably, the preliminary promise of hastening the breakthrough for new treatments for disease was not inevitably achieved by the human genome project [18].

3.1.2 Introduction to Manual and Automated Sequencing, Assembly

The prominent method for DNA sequencing is the Sanger sequencing technique which can be carried in both manually and automated methods [5]. The manual DNA sequencing technique uses four different ddNTPs individually and radioactive isotopes to label the DNA which is the reason to generate a lot of liquid and solid waste. Whereas, in automated DNA sequencing technique fluorescent dye is used to label the DNA and a mixed ddNTPs reaction is carried out producing a minimal amount of waste. Thus, automated DNA sequencing can eliminate the need for radioactive isotopes and is more reliable when compared to manual DNA sequencing [5].

3.1.2.1 Manual DNA Sequencing

Allan Maxam and Walter Gilbert first developed the radiolabeling method of DNA sequencing in 1977, later named as Maxam and Gilbert DNA sequencing. Further this method was refined by Frederick Sanger using chain termination method [4]. The Sanger method, is the technology which produced the first human genome in 2001, shepherding in the age of genomics [2, 8]. Since then, drastic new approaches and automation have been successful in making DNA sequencing faster, easier, and more cost effective. However, manual DNA sequencing techniques continue to be used by smaller scale laboratories [4, 7].

The manual DNA sequencing consists of two steps. In the first step the sample DNA is used, either directly or as a template, to generate sets of fragments. Each set contains multiple lengths of DNA, all of which end in one of the four nucleotide bases. These fragments are typically radiolabeled to enable detection. In the second step, the fragments are separated on a denaturing Polyacrylamide Gel Electrophoresis (PAGE). Each band on the gel represents a position in the DNA sequence, and each position appears only in the fragment set which terminates in the correct base(s). The bands are then visualized using Autoradiography [4].

Though the Maxam and Gilbert sequencing method was the first widely used technique, but no longer predominating and possess few advantages in certain applications. The four sets of reactions engaged in this method cleave the DNA at specific bases or base sets to produce four sets of fragments. No prior knowledge of the DNA sequence is required as the sample DNA is itself processed into fragments. Maxam and Gilbert sequencing is thus useful for sequencing fragments of completely unknown sequence and is essential for foot printing protocol [8].

Sanger dideoxy terminator sequencing is currently the most widely used chemistry. Although it requires prior knowledge of at least 15–20 bases of the sample sequence, it is far less laborious, and more reliable than Maxam and Gilbert sequencing, particularly for long substrate sequences [2]. In this system, the sample DNA is used as a template for a DNA polymerase, typically a bacteriophage polymerase. Four polymerase reactions are set up for each substrate, each containing enzyme, primer, and sample DNA, along with dNTP's. In addition, each reaction contains one of the four dideoxy NTP's. Dideoxy nucleotides do not have a 3′ OH. They are linked to the growing DNA chain through their 5′ OH, and the chain stops there for lack of a 3′ OH to link to the next base. Each reaction contains one of the four bases as a dideoxy NTP, thus each reaction will contain only fragments which terminate at that base. Proper balance of the levels of DNA, primer, enzyme, ddNTP, and dNTP allow reactions which can give readable sequence out to 1500 bases from the primer. In most dideoxy sequencing, ^{35}S labeled dNTP(s) are included in the polymerase reactions to label the fragments produced. Alternatively, ^{33}P dNTP's or ^{32}P labeled primers can be used [4].

3.1.2.2 Automated Sequencing

Automated DNA sequencing utilizes a fluorescent dye to label the nucleotides instead of a radioactive isotope. The fluorescent dye is not an environmentally hazardous chemical and has no special handling or disposal requirements. Instead of using X-ray film to read the sequence, a laser is used to stimulate the fluorescent dye. The fluorescent emissions are collected on a charge coupled device that can determine he wavelength. Typically, in an automated sequencer, all that is required is to load a sample tray with template DNA and the equipment performs the labeling and analysis. The other option is to perform the labeling reactions with fluorescent dyes, load the samples onto a gel, and place the gel into the DNA sequencer and equipment performs the separation and analysis. The system automatically identifies the nucleotide sequence and saves the information on the computer. The greatest impediment to researchers when converting from manual to automatic DNA sequencing is being required to learn the use of computer software necessary to interpret the results [19].

Automated DNA sequencing equipment can eliminate the need for radioactive isotopes to label DNA, thereby reducing the volume of low-level radioactive waste generated on campus. As a general approximation, one template of manual DNA sequencing will produce 83 mL of liquid waste and 0.167 gallon of solid waste. As a result, every 45 templates processed by automated DNA sequencing reduces the amount of manual DNA sequencing. The time saved is due to not having to perform autoradiography or associated tasks required for working with radioactive materials such as radiation surveys, inventory/disposal documentation, etc. Finally, automated DNA sequencing provides more reliable research results than manual DNA sequencing, thus maintaining the integrity of the research [10].

3.1.3 Next-Generation Sequencing (NGS)

NGS is a deep sequencing technology that has revolutionized genomic research. In contrast to the Sanger sequencing technique using NGS, an entire genome of humans can be sequenced within a day. NGS can be performed for small fragments of DNA using different sequencing techniques (Fig. 3.1). It can either be used to sequence a specific area or an individual gene or entire genomes [5]. NGS enables the interrogation of hundreds to thousands of genes at one time in multiple samples, as well as discovery and analysis of different types of genomic features in a single sequencing run, from single nucleotide variants (SNVs), to copy number and structural variants, and even RNA fusions. NGS provides the ideal throughput per run, and studies can be performed quickly and cost-effectively [9, 10]. Additional advantages of NGS include lower sample input requirements, higher accuracy, and ability to detect variants at lower allele frequencies than with Sanger sequencing [3, 7]. A typical NGS experiment shares related steps irrespective of the instrument technology used (Fig. 3.1).

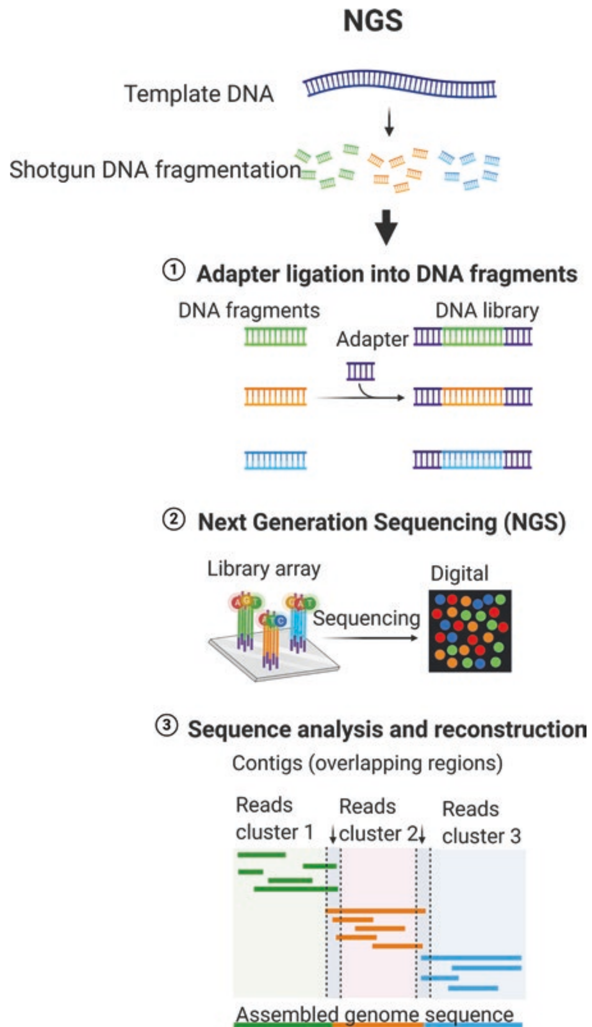

Fig. 3.1 Model illustration of Nextgen sequencing where is step 1 DNA is ligated into fragments, whereas in step 2 the NGS is carried out and finally in step 3 sequence analysis is done

Step 1: Template DNA and Shotgun DNA Fragmentation The template DNA sample is processed into short double-stranded fragments (100–800 bp). Depending on the specific application, DNA fragmentation can be performed in a variety of ways, including physical shearing, enzyme digestion, and PCR-based amplification of specific genetic regions.

Step 2: Adapter Ligation into DNA Fragments and Library Preparation Following to DNA fragmentation, the resulting DNA fragments are ligated to experiment specific adaptor sequences to form a fragment library. Typically, these adaptors contain a unique molecular "barcode" to tag each sample a unique DNA sequence. This helps if there are multiple samples which needs to be

mixed and sequenced at the same time. This approach, called “pooling” or “multiplexing,” which saves time and money during sequencing experiments and controls for workflow variation, as pooled samples are processed together.

Step 3: Sequencing The DNA in the library prepared from previous steps are sequenced using a sequencing instrument. Although each NGS technology is distinctive, they all employ a version of the “sequencing by synthesis” method, in which the instrument read individual bases as they grow along a polymerized strand. Most sequencing instruments use optical detection to determine nucleotide incorporation during DNA synthesis. Some instruments use electrical detection to sense the release of hydrogen ions, which naturally occurs when nucleotides are incorporated during DNA synthesis.

Step 4: Sequence Analysis and Reconstruction Depending on the NGS experiment, large quantity of complex data consisting of short DNA reads are generated. Although each technology platform has its own algorithms and data analysis tools, they share a similar analysis “pipeline” and use common metrics to evaluate the quality of NGS data sets. Overall, the analysis of the data can be divided into three steps. Primary analysis, in which the raw detector signals generates FASTQ files with sequencing reads, and quality scores. Secondary analysis, in which the FASTQ files were aligned to reference genome for genetic annotations. Tertiary analysis or downstream analysis, in which the results of the specific experiments were generated using NGS are interpreted using various computational platforms.

3.1.4 Sequence Comparison Techniques

Sequence comparison must be done for obtaining sequences from different sequencing techniques to identify the genetic disorders by comparing the sequences of unhealthy and healthy individuals and to detect similarity between them. Sequence alignment is a bioinformatics tool used to compare two or more sequences that include local and global alignment. Local alignment aligns only a portion of sequences but the whole sequence can be compared as well as their phylogenetic tree can be constructed using the global alignment technique [8].

3.1.5 Genome Annotation Techniques

As the number of genomic sequencing is finished, the sequencing of other organisms begins and their demand for genome annotation will also increase. The accuracy in genome annotations supports the current drug delivery systems to validate the drug targets against the genomic sequences. The bioinformatics approach is gaining importance to develop automatic annotation techniques [20].

Gene prediction algorithms search a genome for putative gene structures such as signals associated with transcription, protein-coding potential, and splicing. Although the manual gene prediction algorithms were used in the early analysis of the human genome, they are limited in both accuracy and coverage. The current automated gene annotation tools, such as Ensembl, provide fast computational annotation of eukaryotic genomes using evidence derived from known RNA-Seq data and protein sequence databases. The computational annotation system provided an overview of gene content in the newly sequenced genomes [12, 16, 20, 21].

A collaborative initiative called ENCODE project was established to investigate all functional elements in the human genome. Another team called Human and Vertebrate Analysis and Annotation (HAVANA) team is based at the Welcome Trust Sanger Institute, UK, conducted to assess the accuracy of computational gene annotation compared with a manually annotated test-set produced. To present several publicly available web-based interfaces for viewing annotations are being used by several research groups throughout the globe, for example, the Ensembl Genome and the UCSC browser. All transcripts are assigned to a unique stable identifier and this only changes if the structure of the transcripts are altered, making the temporal tracking of sequences easy [20].

3.2 Origin of Genomes

3.2.1 Acquisition of New Genes

Some 3.5 billion years ago, cellular life was established and was the late stage of biochemical evolution with the presence of self-replicating polynucleotides that were the progenitors of first genomes. Until photosynthesis evolved the oxygen content in the atmosphere was very low and later ammonia and methane became the most abundant gases [13]. Several experiments were attempted to recreate the ancient atmospheric conditions using the ammonia–methane mixture which resulted in the chemical synthesis of various amino acids found in the proteins like alanine, valine, glycine, formaldehyde, and hydrogen cyanide were also formed which led to the additional reactions synthesizing sugars, pyrimidines, and purines [22].

The acquisition of new genes is one of the most important processes that generate new genes and creates a genome evolution [23, 24]. The following are the different ways involved in the process of new gene formation:

1. Duplication of all the genes or groups of the genes in the genome.
2. Duplication of a part of a chromosome.
3. Acquiring the genes from other organisms/species.

3.2.2 Origins of Introns

In 1977, eukaryotic genes were analyzed to study their discontinuous structure and their splicing mechanism that puts the exons together that beyond doubt became the puzzling and unexpected discoveries of the twentieth century [25]. Immediately a question of why genes in pieces? Was aroused by Walter Gilbert thus, leading to the introns' early hypothesis and came into the notion that introns played a major role in the recombination of protein modules [25, 26]. Introns are absent in prokaryotes, certainly, a complication that is explained by "genome streamlining" postulate that explained the evolution of maximization of prokaryotic replication rate thus, eliminating all the non-essential parts from the genomes [25].

3.2.3 Genetics to Genomics and Genomics to Functional Genomics

Both genetics and genomics deal with the study of the gene which are the hereditary units of life and is often used incorrectly. Genetics deals with the study of single genes and their role in passing on conditions or traits from generation to generation. Genes hold the entire genetic information in DNA molecule which is chemically made up of strings of bases and their sequence or order determines the genetic message of each individual. The study of all the genes in an organism, including all the information and sequences is said to be genome. This study also involves how exactly the genes in the genome interact with each other [1, 16, 26].

When it comes to functional genomics, it describes the interactions and functions of both proteins and genes using the data obtained from genomic sequence analysis. It usually combines the data obtained from various techniques related to gene expression, DNA sequencing, and protein function techniques such as protein–protein, protein–DNA, protein–RNA interactions, coding and noncoding transcription, and protein translation [1].

3.2.4 Forward and Reverse Genetics

Forward genetics deals with identifying the genotypes that are responsible for a particular phenotype thus, finding the genetic base of a train or a phenotype. The discovery of the function of defective gene mutants is the main objective of forward genetics. Genetic mapping, gene approach, insertion mutagenesis, Exmore sequencing, and microarray expression analysis are the few approaches of forward genetics [27–29]. In contrast to forward genetics, reverse genetics identifies the phenotypes of the sequences of desired gene of interest. It uses transgenic technology to alter the gene and its function. Determining the function of gene activity through target modulation is the main goal of reverse genetics.

Forward genetics is a traditional method to study the specific function of a gene. The key goal of the forward genetics is to identify the respective gene sequence for a mutant phenotype, which generally occur in two conditions, like spontaneous mutation and induced mutation. Errors in the replication is responsible for the alterations occurred in the gene sequence are known as spontaneous mutations, whereas mutagens are responsible for induced mutations. Additionally, indiscriminate mutations can be induced by mutagens to generate the desired phenotype leading to the crossbreeding methods to genetically map corresponding genetic markers. Expression of the mutant phenotype can be identified by microarrays after identifying the mutant allele corresponding to the mutant phenotype.

Reverse genetics is the contrasting approach to the forward genetics in functional genomics. Consequently, it involves the study of the gene function starting from an already known gene sequence. To study the reverse genetics, alteration of the sequence of gene should be carried out to see effect on corresponding phenotype. Furthermore, in vitro mutagenesis and gene disruption are the two main tools of reverse genetics. In vitro mutagenesis is responsible for introducing mutations in the desired gene sequence and the methods that can introduce mutations in a gene sequence are random mutagenesis, site-directed mutagenesis, homologous recombination, and transposable element excision. Likewise, gene disruption is responsible for the inactivation of the gene or its product. Gene knockout or gene depletion or gene overexpression is another name for this process. In which, a cloned gene can be integrated into the genome of the wild-type organism, mutating the gene (gene overexpression). If not, the function of the gene can be silenced by RNA interference (gene knockout). Ultimately, the effect of the alteration is observable in the successive generations [27].

Some of the similarity can be found in comparing forward and reverse genetics. Like, both are two opposite approaches of functional genomics. Both are involved in the study of the relationship between the gene sequence and the corresponding phenotype. In other words, both approaches study the function of a gene. Also, both high throughput or large-scale experimental methods and statistical analysis are in the experimental procedure of both types of approaches. Whereas when come to the important difference, forward genetics refers to the approach of determining the genetic basis responsible for a phenotype while reverse genetics refers to the approach used to understand the function of a gene by analyzing the phenotypic effects of specific engineered gene sequences.

3.3 Phylogeny

The evolutionary history of the group of organisms in terms of their biological evolution is known as phylogeny. It represents how a particular taxon exists through several biological processes. Phylogeny involves the analytical and molecular approaches in knowing the development of species and their evolutionary history. The phylogeny of a living organism is represented in the form of a phylogenetic tree

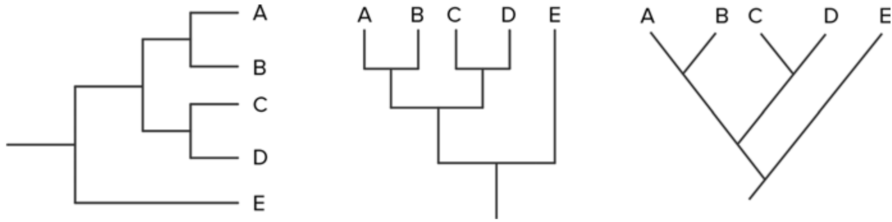

Fig. 3.2 Representing the evolutionary relationship in the form of phylogenetic tree among A, B, C, D and E species

or also called an evolutionary tree by analyzing their evolutionary occurrence. The organism's history or the evolutionary relatedness obtained through morphological data matrices and molecular sequencing data between various groups of living organisms is explained by phylogenetics [30].

The phylogenetic tree may be either rooted or unrooted, the closely related sequences with the common ancestor are said to have a rooted phylogenetic tree. While in contrast, there is no presence of a common ancestor in the case of an unrooted phylogenetic tree (Fig. 3.2). The tree diagrams help us in understanding the ecology, evolutions, genetics, and biodiversity, as well as their evolutionary relatedness, can be understood by just looking at the length and positioning of the "branches" [22, 30].

3.3.1 COGs (Cluster of Orthologues Genes)

COGs of proteins were produced as the result of a comparison between the protein sequences of complete genomes. Every cluster consists of a group of proteins obtained by the phylogenetic classification [21, 22]. The COGs database was constructed based on the criteria of genomic specific best hits of all the protein sequences in the genome [23]. This database uses the COGNITOR program that helps in fitting new proteins into the database and can also be applied for phylogenetic and functional annotation to obtain newly sequenced genomes [23]. The construction of COG is based on the notion that the proteins likely belong to the orthologous family only if the three groups of proteins obtained from distant genomes are most similar to each other when compared to any other proteins of the same genome [24]. Also, the sets of COGs are used to describe phylogenetic patterns [22, 23].

3.3.2 Paralogues Genes and Gene Displacement

The homologous genes after the speciation that is an evolution of different species give rise to orthologous genes. These genes maintain similar functions which were evolved from their ancestral gene. Wherein the paralogous genes are formed due to

the diversion within species and unlike orthologous genes these genes hold on a new function as they arise due to the gene displacement/gene duplication where the gene gets mutated thus, giving rise to a new gene with the new function [31].

3.4 Microarrays: Oligonucleotide Microarray Chips

A microarray is a tool used to detect the expression of thousands of genes at a time. Microarrays are of different types depending upon the type of question asked and the number of probes under examination. Oligonucleotide microarray uses the short sequences as probes that are designed to match the predicted open reading frame or parts of already known sequences. Single gene or gene families are used to design the oligonucleotide sequences that are further utilized to produce oligonucleotide arrays and these sequences might be either shorter or longer depending on their desired purpose, shorter probes are cheaper to manufacture and are used to spot the higher density across the array while the longer probes are specific to target genes [32].

3.4.1 SAGE (Serial Analysis of Gene Expression) Microarrays

The SAGE technique is used to produce the snapshot of the messenger RNA population in the sample provided. SAGE is very much similar to DNA microarrays and is based on mRNA output sequencing [33]. The gene expression quantification is exact in SAGE as it involves the direct count of transcripts. Several variants of SAGE have been developed such as RL-SAGE (Robust LongSAGE), SuperSAGE, and LongSAGE. The LongSAGE was developed in the year 2002 and has higher throughput capacity due to which it is the best version of SAGE [34]. Furthermore, depending on the mRNA insert size the LongSAGE protocol was improved to form RL-SAGE. SuperSAGE is another derivative of SAGE used to cut long sequence tags of 26 base pairs using III-endonuclease of phage P1 [33, 34].

SAGE Technology was initially developed by Velculescu et al. Two fundamental principles trigger the SAGE methodology: (1) a concise sequence tag (10 bp) inside the defined position holds adequate information to distinctively identify a transcript; (2) the chain of tags in a serial manner accepts for an increased effectiveness in a sequence-based analysis. The SAGE technology in brief, poly(A)$^+$ RNA is isolated by oligo-dT column chromatography. cDNA is synthesized from poly(A)$^+$ RNA using a primer of biotin-5′–T18-3′. The cDNA is cleaved with an anchoring enzyme (e.g., NlaIII) and the 3′-terminal cDNA fragments are bound to streptavidin-coated beads. An oligonucleotide linker comprising detection sites for a tagging enzyme is linked to the bound cDNA. The tagging enzyme is a class II restriction endonuclease that cleaves the DNA at a constant number of bases 3′ to the recognition site. This results in the release of a short tag plus the linker from the beads after digestion

with the tagging enzyme. The 3′-ends of the released tags plus linkers are then blunted and ligated to one another to form 102 bp linked ditags. After PCR amplification of the 102 bp ditags, the linkers and tags are released by digestion with the anchoring enzyme. The tags are then gel purified, concatenated, and cloned into a sequence vector. Sequencing the concatemers enables individual tags to be identified and the abundance of the transcripts for a given cell line or tissue determined [33–36].

3.4.2 cDNA Microarrays

The cDNA microarray is a gene expression technique and is one of the most powerful techniques among several microarray techniques as it is used in large-scale gene discovery, polymorphism screening, genomic DNA mapping for expressing thousands of genes [37]. This high throughput RNA expression permits the analysis of both unknown and known genes, also used to diagnose certain diseases by comparing pathogenic and normal cells. This novel technology provides a wide range of applications in different fields which provides new targets for disease therapies and drug development [1, 32].

cDNA microarrays are regarded as an enabling technology because they allow scientists to address previously intractable problems and to uncover potentially novel gene targets, perhaps those underlying genetic causes of many human diseases. They are also applicable to many other areas of biomedical science, including biological defense, and environmental monitoring. The utilization of microarray technology has significantly increased since 1995, with the largest increase in gene expression studies, and to a lesser extent, microbial identification. All microarray experiments rely upon probe specificity and probe sensitivity, and a unifying algorithm that interprets the multiple signals coming from array probes. Probe specificity is of particular importance because one has to be able to adequately distinguish between closely related nucleic acid targets, which may differ by only a few nucleotides. The oligonucleotide probes must possess adequate sensitivity to nucleic acid targets in order to provide a high signal-to-noise ratio, else the signal will be drowned by background noise. Also, an adequate relationship between target concentration and observed signal must exist in order to detect target fold changes upon different treatments. The image acquisition and array software is the integrative component of a microarray experiment because it not only interprets signal from multiple probes that vary in terms of their specificities and sensitivities, but it also plays a pivotal role in calling and/or quantifying a gene target, or several gene targets, within a pool of potentially similar targets [38].

3.4.3 Cancer and Genomic Microarrays

As already mentioned, the microarray technique is used to diagnose several human diseases like cancer. One of the major advantages of microarray is, it extracts and integrates a huge amount of molecular information which can further be used to compare thousands of genes concurrently; hence, this technique has revolutionized cancer diagnostics which classifies the normal and tumor samples and discovers new diagnostic and therapeutic markers [35]. The genomic expression studies carried out through microarray provides valuable insights for phenotypic differences observed between diseased and normal person. The high throughput whole genome sequencing is possible through microarray technology with increased specificity and sensitivity [39–43].

Microarray mediated analysis of tumor genome have employed arrays focused on a particular region of the genome, selected regions known to be commonly aberrant in tumors or genome-wide arrays. Application of genome-wide microarray in different tumors is revealing that they differ not only in the regions that are aberrant, but also the types of copy number aberrations that are present. Thus, for a specific type of tumor, the copy number profiles, or copy number phenotypes, arise due to both selection for changes affecting gene expression as well as distinct kinds of underlying genetic instability. For example, cytogenetic analyses have shown previously that colon tumors with defects in mismatch repair (MMR) have fewer copy number alterations than MMR-proficient colon tumors. These tumors also differ in their histology, in the genes that are inactivated and in their response to therapy. Thus, they can be readily distinguished based on the types of genetic instability displayed and on the selection of the genes that are altered. Analysis of the numbers and types of aberrations in the array CGH copy number profiles from MMR-proficient and -deficient cell lines confirmed the cytogenetic observations, but also found that MMR-deficient cell lines with alterations in *MSH2* had fewer aberrations than those with alterations involving *MLH1*. Further studies of a model system demonstrated the importance of the genetic background of cells in shaping the copy number profiles of tumor cell genomes [39–41, 43].

3.5 Application of Microarrays with Examples

Microarray technique assists with several pharmacological approaches such as cancer research, treating oral lesions, genomic studies, and much more. This technique can analyze a large number of sequences/samples.

3.5.1 In Cancer

In cancer, the formation of tumors involves variation in cells and genes. In this regard, microarray plays a major role as it can simultaneously test a large amount of genetic samples (Fig. 3.3). Cancer-causing specifications can be identified using microarrays such as mutations, drug discovery, target genes of tumor suppressors, tumor classification, cancer biomarkers, and chemo resistance-associated genes. A new molecular taxonomy can be developed by the data obtained from the above identification techniques and also helps in clustering the cancer gene expressions [44].

The gene expression profile of a cell determines its function, phenotype, and response to stimuli. Thus, gene expression profiles can help elucidate cellular functions, biochemical pathways, and regulatory mechanisms. Gene expression analysis using either oligonucleotide or cDNA microarrays is usually for "class comparison," "class prediction," and "class discovery." Although cancer classification has improved over the past 30 years, there has been no general approach for identifying new cancer classes (class discovery) or for assigning tumors to known classes [43, 45].

Many microarray analyses are aimed at finding significant biomarkers, many studies can be placed in this category. Some studies classified as belonging to one of the above three classifications can also belong to this category. Our group published gene expression profiling associated with multi-drug (5-FU, doxorubicin, and cisplatin) resistance in human gastric cancer cells using Affymetrix U133A microarrays [44–46].

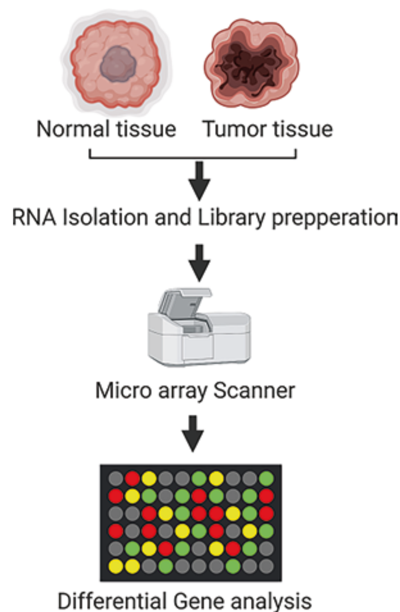

Fig. 3.3 Model illustration of application of microarray technique in differentiating cancerous tissue from normal tissue. In brief, the RNA isolated form the tissues will be subjected for library preparation and flowed by sequencing and computational methods included analysis

3.5.2 In Antibiotic Treatment

Usually, the antibiotics fail due to the resistance occurred by the bacteria towards particular antibiotics. The pathogenicity of bacteria affects the disease process. Anaerobic bacteria like actinomyces are not easily cultivable that infect and cause an oral cavity. In such a case, the microarray technique comes into the picture and helps in determining the bacterial genomic DNA where a small amount of DNA sample can be used to diagnose the specific bacterial disease [47].

3.5.3 Early Detection of Oral Lesions

The white lesions or leukoplakia formed in the oral cavity are the resultant of reversible myriad conditions. The lesions in the oral cavity progress into oral cancer and these small lesions fail in microscopic examinations [39]. The clinicians require genomic fingerprints or gene expression profiles to differentiate the white lesions from precancerous lesions or very early cancer. Thus, the gene expression studies of microarray technique have availed the early diagnosis of oral cancer leading to the effective identification and treatment of early cancerous oral lesions.

Microarray has emerged to be the most promising technique in several healthcare fields used to identify, classify, diagnose, monitor, prevent, and treat several diseases.

3.5.4 Microarray Data Analysis and Interpretation

The microarray data is primarily analyzed using image processing and it is the basis for further analysis. The image generated in the microarray technology is first analyzed to detect the spots that are orderly arranged making the spot detection easier. Secondly, the region corresponding to the spots must be selected to obtain the background intensity and spot signal. After getting the spot signal and background area details, the statistical summary such as pixel, mean and median for each spot is calculated and reported.

3.6 Genetic Engineering

Genetic engineering refers to the recombination, modification, or manipulation of DNA or other nucleic acids to modify the population of organisms. The genetically modified/altered organisms are obtained through the genetic engineering method as this method helps in altering the heredity constituent of an organism [15]. Several

biomedical techniques are involved in this procedure like cloning, artificial insemination, gene manipulation, and in vitro fertilization. Recombinant DNA technology is the more commonly used term where two or more DNA molecules are combined in vitro and then injected into the host organism to propagate. The organisms produced from this technique are referred to as transgenic animals.

The recombinant DNA technology was discovered when restriction enzymes were discovered by Swiss microbiologist in 1968. Following it, Hamilton O. Smith an American microbiologist purified the type II restriction enzyme which was the most essential tool used to cleave the specific site in the DNA to perform genetic engineering, further Daniel Nathans made contributed towards the advancement of this technique [48, 49].

3.6.1 Genetic Engineering in Animals: Pronuclear Injection, Somatic Cell Nuclear Transfer

Pronuclear microinjection is the first technique that helped in generating transgenic animals by inserting the DNA into the fertilized oocyte obtained from an organism [50, 51]. This technique was first employed on the mouse. The success rate of it in rats and mice is 3%, whereas only 1% in pigs, cattle, and sheep [50, 51]. Creating transgenic animals through the pronuclear injection approach leads to high variability in transgene expression in animals due to effects in chromosomal positioning that occurred during the integration of transgene. This method has successfully generated many transgenic cattle but has lacked in developing the bovine transgenic.

Somatic Cell Nuclear Transfer (SCNT) has been developed to overcome the disadvantage caused in developing the bovine transgenic using pronuclear injection technique. Identical copies of genetically modified organisms can be created using the nuclear transfer technique. The donor nucleus is placed or transferred into the cytoplasm of the enucleated oocyte. The sex of an organism that has to be created through genetic engineering can be predetermined using SCNT technique, this being an advantage over the pronuclear injection technique [51–53].

3.7 Gene Therapy: Introduction

Gene therapy is known to treat a serious illness by transferring DNA or RNA into cells. It can cure or decrease the abnormality of a medical condition by replacing the faulty gene with the normal gene in an organism [54]. Gene therapy is further divided into somatic and germ cell therapy, where somatic gene therapy introduces new genes into the somatic cells of an organism. Germ cell therapy helps in modifying the germ cells of the human body leading to the genetic alterations that are

Genetic Engineering Milestones

Understanding the milestones achieved in Genetic engineering is very crucial for the current state of the field. Major milestone of genetic engineering involves invention of double helical structure of DNA, recombinant DNA generation, invention of gene editing using CRISPR technology and more as detailed below:

1953: The twisted-ladder structure of deoxyribonucleic acid (DNA) familiar to us today as the "double helix" was pioneered in 1953 by James Watson and Francis Crick.

1958: Arthur Kornberg synthesized nucleotides in the lab for the first time.

1962: Osamu Shimomura isolated The green fluorescent protein (GFP) is naturally present in the Aequorea Victoria jellyfish and researchers Martin Chalfie and Roger Tsien further developed it into an indispensable biological tool.

1967: The discovery of DNA ligases as a combined effort by Gellert, Lehman, Richardson, and Hurwitz laboratories.

1968: Werner Arber, discovered and hypothesized about Restriction enzymes in bacterial strains fought off bacteriophage infection by chopping off its DNA.

1970: Hamilton Smith, a molecular biologist at Johns Hopkins University purified the type II restriction enzymes from bacterium *Haemophilus influenzae* Rd.

1971: Paul Berg became the first scientist to ever accomplish creating recombinant DNA from more than one species, which came to be known as the "cut-and-splice" method.

1972: First Recombinant DNA (rDNA) was created. The key to rDNA is that it can replicate naturally, despite being artificially introduced in another organism, and this was the achievement of Stanley N. Cohen & Herbert W. Boyer in 1972.

1975: Hybridoma technology using human B cell and myeloma cells by George Kohler & César Milstein producing identical antibodies known as "monoclonal antibodies," which help fight immune diseases.

1981: The research team led by Thomas Wagner at Ohio University transferred the gene of a rabbit into the mouse genome by using a method now standard in genetics known as "DNA microinjection" leading to the generation of first Transgenic animal.

1982: Synthesis of first genetically engineered human drug – insulin.

1983: Kary Mullis discovered the polymerase chain reaction (PCR) was integral and later DNA experiments and breakthroughs.

1985: Discovery of Zinc finger nucleases, which led to the pioneering in backword genetics in which scientists were able to identify the gene causing mutations at different disease conditions.

(continued)

1986: Pablo Valenzuela created the world's first recombinant vaccine using Saccharomyces cervisiae cells for Hepatitis B.

1988: The first Genetically modified crop appeared in United States, known as 'Bt corn' as it was genetically modified using the genes form bacterium Bacillus thuringiensis (Bt), to eradicate the pest impact and to increase the crop yield.

1996: The project led by Ian Wilmut of Roslin institute, cloned the first mammal from adult cell with the same genetic identity. This was one of the biggest mile stone that science has ever seen, as 'Dolly' the sheep.

1999: AS a part of the Human genome project, the scientists working, demonstrated that they had completely mapped out the sequence for Chromosome 22.

2001: Glivec (imatinib), is the first Gene-targeted drug therapy that got approved.

2003: The whole Human genome got mapped and new regulations are set.

2006: Gardasil, was the first preventive cancer recombinant vaccine to ever reach the market.

2006: Shinya Yamanaka from University of California San Fransisco, first introduced the induced pluripotent stem cell (iPSC) technology.

2010: World's first synthetic life form, Mycoplasma mycoides JCVI-syn 1.0 (1.08 mega base pair) was created by Craig Venter and his team.

2012: Jennifer Doudna, Emmanuelle Charpentier, and their teams elucidated the biochemical mechanism of CRISPR (Clustered regularly interspaced short palindromic repeats) technology.

2015: First genetically modified Salmon produced by the company AquaBounty were sold in Canadian markets.

2015: CRISPR technology was used to edit human embryos by Junjiu Huang at the Sun Yat-Sen University in Guangzhou.

2017: Cytotoxic T Lymphocyte therapy was first approved for lymphoblastic leukemia in children and advanced lymphoma in adults.

passed on to the offspring [55, 56]. Following are the procedures involved in gene therapy:

(a) Identification/selection of gene of interest.
(b) Acquiring a copy of the normal gene.
(c) Cloning of therapeutic gene into the vector, a vehicle used to carry and deliver the gene of interest.

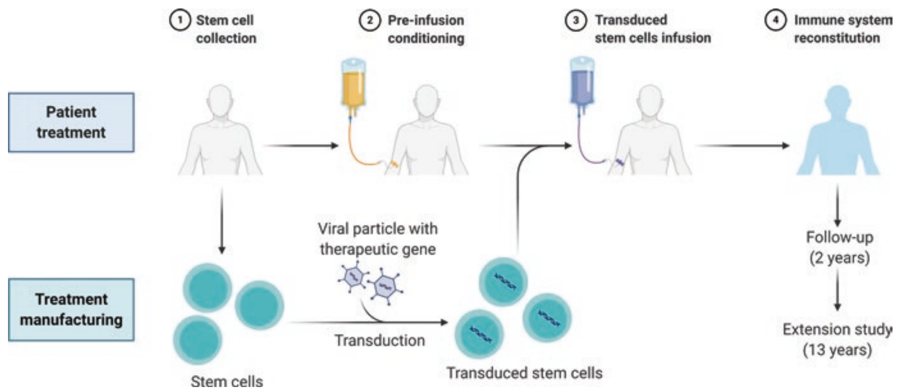

Fig. 3.4 Illustrative image showing of gene therapy at translational level using stem cells. Briefly, the stem cells collected form disease dependent patients, were transduced with indicated viral particle with therapeutic gene were infused into same patient and let to reconstitute the immune system

The concept behind gene therapy is to introduce the exogenous genes into the somatic cells selected from an organism to provide the desired therapeutic effects against a particular disease/disorder [55]. In recent days, gene therapy has grown into a vast subject as its application in various health care sectors has been used to treat autoimmune diseases, salivary gland diseases, bone repair, dermatological disorders, and DNA vaccination (Fig. 3.4).

3.7.1 Applications of Gene Therapy with Examples

The presence of defective genes or the absence of certain biological or cellular factors is responsible for causing various diseases/disorders. The exogenous gene used in gene therapy helps in delivering suitable therapeutics to the target cells that codes the biological factor. Neurological disorders, infectious diseases, cancer, cardiac diseases, and several inherited conditions can be treated using gene therapy [57].

3.7.1.1 Neurological Disorders

Several neurological disorders such as Alzheimer's disease, Parkinson's disease, and motor neuron diseases are treated using gene therapy. Parkinson's disease is caused due to the lack of dopamine in the brain; ProSavin is currently used to trail on Parkinson's disease patients [58].

3.7.1.2 Infectious Diseases

Gene therapy vaccines developed towards malaria, influenza, tuberculosis, and HIV have been trialed against the patients. The malaria vaccines containing its antigen have been taken from chimpanzee adenovirus. The tuberculosis vaccine contains genetically modified MVA and fowlpox virus which contains tuberculosis antigen [59].

3.7.1.3 Cancer

The gene products developed to treat cancer are targeted to genes that inhibit tumor angiogenesis, tumor suppressor genes, and immunotherapy are the primary target cells in cancer. Certain types of cancers are sensitive to immunotherapy and thus gene therapy is developed against such cancer (Fig. 3.5) [60].

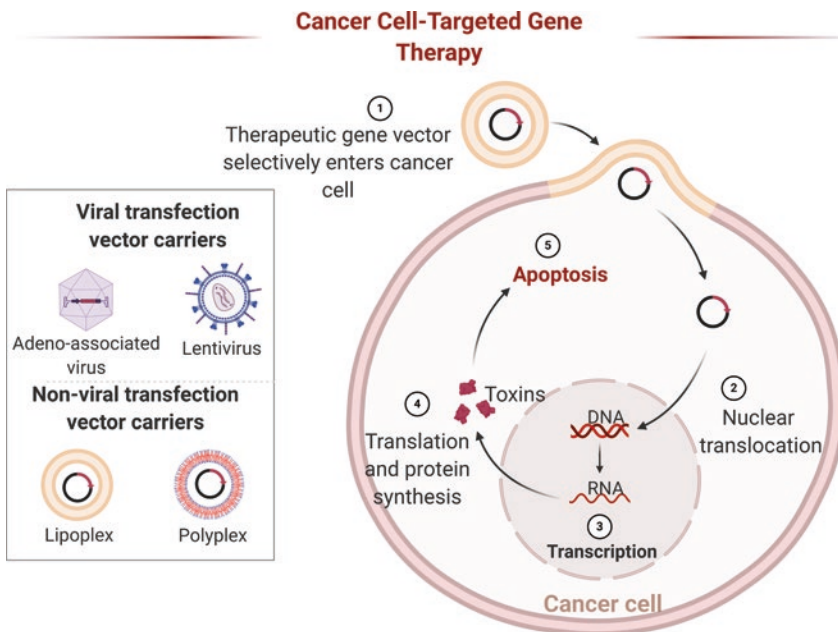

Fig. 3.5 Mechanistic illustration of gene therapy. Nontoxic gene carriers, such as viral and non-viral vectors, can be used to encapsulate and deliver genetic material to target cells. In particular, this approach can offer a targeted cancer treatment. Therapeutic genes that are encapsulated within these vectors are able to selectively enter cancer cells where they are transcribed and translated into cytotoxic proteins. The latter are capable of inactivating protein synthesis, ultimately leading to apoptosis of the cancer cell

3.7.1.4 Inherited Diseases

Inherited diseases such as *Hemophilia* and *Cystic fibrosis* are caused due to the deletion of genes and gene mutations, such type of diseases can be treated using gene therapy. Presently the lentivirus vectors and adenovirus are used against *Hemophilia,* whereas CFTR gene is used to conduct the gene expression studies to treat against *Cystic fibrosis* [61, 62].

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Chapter 4
Biotechnology in Medicine: Advances-II

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Abstract Therapeutically important proteins have been obtained from relevant organisms using biotechnological methods. The main caveat in procuring these proteins in such a way is their insufficient quantity in the natural sources. This difficulty has been circumvented by the recombinant protein technology, which entails the abundant expression and purification of the protein of interest in a heterologous system. Certain drawbacks in the *E. coli* (bacterial) expression system, the most widely used and economical expression system, have prompted the development of alternative expression systems like insect and mammalian ones for therapeutic and diagnostic products including human insulin, growth hormones, and antibody fragments. Major expression systems are described in this chapter along with a focus on a variety of protein purification tags. Brief information on expressed sequence tags is also provided. Later, the role of bioinformatics in handling huge amounts of genomics and proteomics data together with an application of various tools for protein structure analysis is explained. Finally, the significance of different protein detection arrays in biomarker discovery and diagnostics is reviewed followed by methods for data interpretation and analysis.

Keywords Protein expression system · Protein purification · Purification tags · Protein detection array · Protein domains · Homology modeling

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4.1 Protein Expression: Introduction to *E. coli* Expression System, Yeast Expression System, Insect Expression System. Higher-Eukaryotic Expression Systems

4.1.1 Protein Expression

The main objective of recombinant protein expression is to produce a target protein in commercial quantity in a cost-effective manner without compromising its biochemical and biological activity. Several protein expression systems have been used to produce large-scale proteins for therapeutic (biopharmaceuticals) and diagnostic purpose. Expression and purification of full-length protein is sometimes unnecessary, as the desired activity or property of the protein of interest can be achieved by a specific domain(s). Once the desired protein or domain(s) is finalized, next critical step is choosing the suitable expression system.

An expression system is defined as genetic constructs (expression vector with the desired gene sequence) specifically designed to produce protein of interest at high level, inside a host cell. The fundamental criteria for expression system selection include anticipated application of desired protein; resources availability; expenditure cost; and time. The four most well established expression systems in use for pharmaceutical purposes are bacteria, yeast, baculovirus, and mammalian, and will be discussed in details.

4.1.1.1 Bacterial Expression System

Bacterial expression system is most popular and first choice for rapid and economical production of pharmaceutically important recombinant proteins [1] *Escherichia coli* has emerged as most commonly used industrial microorganism, as about one-third of all the pharmaceutical relevant proteins are purified from it [2]. Other bacterial strains used for pharmaceutical purpose are *Lactococcus lactis* and *Bacillus subtilis*. The major advantages of using this system are: (1) Simple and scalable, with no sophisticated equipment requirement, (2) Quickest, (doubling time of *E. coli* is 20 min), (3) Easily manipulated due to availability of its genetics knowledge, and (4) Economical, as one can produce tones of recombinant protein in no time [3, 4].

An expression vector is required to insert the target gene in the bacterial cells. Numerous articles are available online that extensively discuss the desired properties of an expression vector. Inexpensive usage, a little or no leaky expression with a reliable and tunable induction promoter are some of the desirable qualities of an expression vector for large-scale protein production [5, 6]. Commonly used and commercially available vectors are the pET series, pQE series (Qiagen), pGEX, etc. for single protein expression and pACYC, pBAD, and pSC101 series or single plasmid system like the Duet vectors (Novagen) for more than one proteins expression at the same time.

Despite being the most efficient and cost-effective expression system, it has several shortcomings especially when expressing mammalian proteins. These problems include stability of mRNA and protein inside the host, codon bias [7], inclusion bodies formation [8], and protein folding and solubility [9], and absence of key post-translational modifications (glycosylation, carboxylation, and amidation). Increasing demand of the pharmaceutically important proteins in the last 5 years has led to the development of new codon optimized and genetically modified bacterial strains capable of performing desired post-translational modification, and improved expression vectors to overcome above mentioned problems [10–12]. A list of the commercially available modified strains with their key features is given in Table 4.1. The use of other strategies like growing cells at lower temperature to prevent protein aggregation 15–23 °C [15, 16], co-expression of molecular chaperones or the post-translational modifying enzyme [17–19], and translocating protein to periplasmic space has helped in increasing protein yield [20–22]. The protein expression in periplasmic space emerged as most successful strategy to produce several pharmaceutical proteins such as scFc, growth hormone, etc. owing to several advantages over others [23]. Protein isolation from inclusion bodies is also a viable and cost-effective option for pharmaceutical companies, as the purity of the protein in IBs is ~95% [24–26]. It is often better to change expression system to higher hierarchy as they are better equipped in folding and complex post-translational modifications to maintain the bioactivity of desired protein.

4.1.1.2 Yeast Expression System

Yeast being a eukaryotic organism and acts as a connecting link between *E. coli* and mammalian systems. It has properties similar to both the systems thus serves as an excellent expression system for pharmaceutically relevant protein production. The major advantages it has over other eukaryotic expression systems are: (1) Ability to grow in high densities in limited time, (2) Simple and cheap media requirements, (3) Easy genetic manipulation, and (4) Safe pathogen-free production [27]. Unlike bacterial expression system, yeast expression system has the ability to accomplish proper posttranslational modifications and extracellular expressions [28, 29]. *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe* [30] are few commonly used yeasts. *S. cerevisiae* and *P. pastoris* are used extensively for the production of several therapeutic important recombinant proteins such as human insulin, human serum albumin, alpha 2b, trypsin, collagen, hepatitis B vaccines, and human papillomavirus (HPV) vaccines [31, 32]. The expression vector is designed so that genomic integration of expression cassette could take place leading to generation of stable expression clones with multiple copies of target gene [33]. *P. pastoris* based vectors also have inducible promoters (P_{AOX1} (most widely used), GAP, FLD1, PEX8, and YPT1) and antibiotic selection markers, for the selection of multi-copy transformants [34]. Some commercially available plasmids have these features incorporated (such as the pYEDIS, pPIC9K, pPICZα vector).

Table 4.1 List of modified bacterial strains [10]

Strain	Key feature	Application	Company
Promoter inducible strains			
BL21(DE3)	• IPTG-inducible T7 RNAP(DE3) promoter • Protease deficiency	General protein expression	Multiple companies
BL21 star (DE3)	• IPTG-inducible T7 RNAP(DE3) promoter • Mutation in RNaseE gene, resulting in longer mRNA half-life	General protein expression from low-copy plasmid	Invitrogen™ (ThermoFisher)
BL21(DE3) pLysS	• Contains pLysS plasmid which express T7 lysozyme to suppress leaky expression	Toxic protein expression	Multiple companies
BL21-AI	• T7RNAP gene under control of the araBAD promoter • Tight regulation of protein expression	Toxic protein expression	Invitrogen™ (ThermoFisher)
BLR (DE3)	• RecA-deficient, improves plasmid monomer yield • Stabilizes plasmids with repetitive sequences	Expression of unstable proteins that might cause loss of DE prophage	Novagen (Merck)
Tuner(DE3) and derivatives	• Mutation in lac permease (*lacY*) allows uniform entry of IPTG • High regulation of IPTG induced protein expression	Expression of difficult protein: membrane proteins, toxic proteins, and proteins prone to insoluble expression	Novagen (Merck)
Lemo21(DE3)	• Modulated levels of T7 lysozyme (inhibitor of T7RNAP) by L-rhamnose addition • Tunable expression of protein	Expression of difficult protein: membrane proteins, toxic proteins, and proteins prone to insoluble expression	NEB
RiboTite	• Integration of orthogonal riboswitches upstream of the T7RNAP gene thereby permitting fine tuning of protein expression	For secretion of recombinant proteins in periplasm	Dixon laboratory
OverExpress™ C41(DE3) and C43(DE3)	• Derived from standard BL 21 (DE) strains • Contain genetic mutation in *t7rnap*, lowering the T7 RNAP accumulation • It phenotypically selected for conferring toxicity tolerance.	Expression of membrane toxic proteins from all classes of organism (yeast, plant, virus, and mammals)	Lucigen and Sigma [13]

(continued)

Table 4.1 (continued)

Strain	Key feature	Application	Company
Marionette	• Protein coexpression independent control of expression using 12 different inducers	Protein complexes	Addgene
(KRX) Competent Cells	• Stringent control provided by the rhamnose-driven T7 RNA polymerase	Expression of proteins	Promega
Codon biased strains			
BL21 (DE3) CodonPlus-RIL/RP	• Contains pRI(P)L plasmid provides extra copies of rare tRNAs genes • Codon bias correction	Enable efficient high-level expression of heterologous proteins in *E. coli*	Stratagene
Rosetta or Rosetta (DE3)	• Contains pRARE plasmid provides extra copies of rare tRNAs genes • Good for "Universal" translation	Enable efficient high-level expression of heterologous proteins in *E. coli*	Novagen(Merck)
Others			
Origami and derivatives	• Has mutation in trxB and gor reductase resulting in more oxidant conditions in the cytoplasm	Disulfide-bonded protein production	Novagen (Merck)
SHuffle® T7	• Contains the deletions of the genes for glutaredoxin reductase and thioredoxin reductase (Agor ΔtrxB) • Constitutively expresses a chromosomal copy of the disulfide bond isomerase DsbC, which promotes the correction of mis-oxidized proteins • DsbC is also a chaperone that can assist in the folding of proteins that do not require disulfide bonds	Expression potentially toxic protein	NEB
ArcticExpress (DE3)	• Production of aggregation-prone proteins/constitutive expression of chaperones Cpn10 and Cpn60 from the psychrophilic bacterium Oleispira antarctica, which show high refolding activities at 4–12 °C		Agilent
TatExpress BL21	• Strong inducible promoter, *ptac* upstream of *tatABCD* operon for increased levels of Tat secretion pathway	Enhanced production of industrially challenging proteins	Robinson laboratory [14]

In recent times, *P. pastoris* being a obligate aerobic yeast has gained more prestige as it can use methanol as a carbon source which lead to the development of an expression system based on the utilization of the inducible AOX1 promoter [35]. Other benefits include correct protein folding and secretion (by Kex2, PHO1, or α-MF as signal peptidase) outside the cell [36].

Despite being an efficient expression system, yeast has drawbacks like limited secretory expression of heterologous proteins resulting in low protein yield and limits its commercial use [37]. To address this issue, strategies involving optimization of cultivation parameters (induction temperature and time, pH, oxygen, and nutrient supply), protein-based host selection, gene copy number, co-expression of secretory proteins such as chaperones, engineering of secretory pathways, have been employed to improve the expression of proteins [38, 39]. The other major drawback is hyper *N*- and *O*-linked glycosylation of proteins unlike mammalian system, which affects the protein immunogenicity [40]. To overcome this shortcoming humanization of yeast by expressing mammalian specific glucose transferase and omitting yeast specific genes involved in glycosylation has been tried and found its application in producing humanized IgGs in yeast [41–45]. Latest technologies such as CRISPR/Cas9 [46] and GlycoSwitch [47] are now used for yeast genome engineering for this purpose. Many improvements are still required in the yeast expression system before it can be used for therapeutic commercial purposes; therefore, companies are shifting their focus to other more complex expression systems.

4.1.1.3 Insect Cell Expression System

Baculovirus-mediated insect cell expression systems are widely used these days to produce large quantities of proteins for pharmaceutical purpose. These proteins are difficult to express either in bacteria or yeast due to improper protein folding or posttranslational modifications. The major advantage of this system remains the presence of post-translational modifications similar to mammalian system, thus avoiding the problem of immune-reactivity [48]. The other advantages include: (1) Cheap protein production cost as compared to mammalian system, (2) High capacity of expressing multiple genes at the same time due to large and flexible viral genome (130 kb), (3) Safe, as they do not infect humans, (4) High protein yield driven by the strong promoters such as polyhedrin or p10, and (5) Easy downstream purification [49, 50].

Baculovirus vectors are used to insert desired gene and transfected into cultured insect cells. The most commonly used baculovirus system are Bac-to-Bac (Invitrogen), BacPAK (Takara), and BaculoGold (BD Biosciences), they are commercially available and has been widely used [51]. Recently, two rapid and simple baculovirus expression vector systems have been developed named as MultiBac [52] and Golden Gate-based system [53], which can be used to express multiple genes at the same time. The most common insect cells used for the production of protein are Sf9, Sf21 (*Spodoptera fugiperda),* High5 (*Trichopulsia ni*), and S2 (*Drosophila melanogaster* embryos) cell lines [54]. These cell lines have proven themselves for the application

of recombinant protein expression from a variety of expression platforms due to their ability to grow in suspension and serum-free medium [55]. Recent advancement in genetic engineering of baculovirus system, this system has been promoted from being used during pest control to production of several recombinant proteins (viral antigens) by biopharmaceutical companies for human use [56]. Several vaccines are now available for the commercial use, which include Cervarix™ (GSK, Rixensart, Belgium), FluBlok™ (Protein Sciences Corp., Connecticut, USA), Provenge (DendreonInc., Seattle, WA), and Chimigen (Virexx Medical Corp., Calgary, Canada), [56–58]. Several of the known subunit vaccines against Chandipura virus, hepatitis E virus, and West Nile virus are also synthesized in insect cells [59]. Since, baculovirus are known not to infect humans, they are being evaluated to be used as efficient delivery vehicles for gene and cell therapy [60].

Despite being a promising expression system, baculovirus has several drawbacks. For instance, time consuming cloning procedure to generate stable recombinant virus, expensive media requirements, and cell lysis by baculovirus infections resulting in suboptimal protein processing. The major drawback is differential glycosylation pattern in comparison to humans, thus limiting the therapeutics use of recombinant protein [50]. In recent years, efforts are directed to deal with these problems. The stable transformation of insect cell line with plasmid having early baculovirus constitutively active promoters (e.g., IE1) [61] or using pre-infected cells [baculovirus infected insect cells (BIIC)] to avoid making of stable system has significantly reduce the processing time along with improved protein production [62]. Engineering of insect cells to have mammalian glycosyltransferases enzymes is also tested [63]. Regardless of recent advancements there is a room for improvement to produce cost-effective, industrial scale, and therapeutics standard recombinant protein in insect cell lines [64]. Several good articles are available which covers the baculovirus system in more details [65–67].

4.1.1.4 Mammalian Expression System

Mammalian expression system is the ideal choice for production of therapeutically important proteins because they perform similar post-translational modifications along with proper protein folding, which are critical for bioactivity of the protein. Other advantages include secretion of proteins in the cell culture, preventing additional step of protein purification [68]. Some mammalian cell lines can grow in suspension culture and serum-free chemically defined media, enabling large-scale reproducible protein production [69]. Several different mammalian host cell lines, such as Chinese Hamster Ovarian (CHO) cells, baby hamster kidney (BHK21) cells, and *murine myeloma*-Sp2/0/NS0 cells, have been used for large-scale production of therapeutic proteins [70]. Recently, the use of human cell line (Human Embryonic Kidney (HEK 293) cells and fibro-sarcoma HT-1080 cells) has gained importance due to identical post-translational modifications of expressed proteins [71].

Like any other expression systems, plasmid based or viral based vectors (adenoviral vector, vaccinia vector, Semliki forest viral vectors) are used to transfect desired gene into cultured mammalian cells to form either transient or stable cell lines. However, efficient integration of transgenes into correct genomic loci still remains a major challenge during stable cell line production. Incorporation of chromosomal elements (nuclear scaffold/matrix attachment regions (S/MARs) and ubiquitous chromatin opening elements (UCOEs)) into plasmid vectors is found to have a positive effect on stable gene expression. Transposon based vectors and site-specific recombinase systems, such as Cre-Lox and Flp-FRT, are also found useful in targeted integration of the gene in host genome for the production of stable cell line expressing recombinant proteins [72].

In the year 1968, the production of FDA approved first recombinant glycoprotein, tissue plasminogen activator (tPA, Activase) in CHO made a revolution [69]. Subsequently, a number of vaccines like Herpes simplex virus (HSV) vaccine [73], Synagis® vaccine used against respiratory syncytial virus [74] etc. and several therapeutic proteins, Drotrecogin alfa (XIGRIS®; Eli Lilly Corporation, Indianapolis, IN), recombinant factor IX Fc and VIII Fc fusion protein (Biogen, Cambridge, MA), dulaglutide (TRULICITY®; Eli Lily, Indianapolis, IN) etc. [68] are produced in mammalian cell line. Some of them have already received FDA approval.

The major drawback of using mammalian expression system is high cost of protein production, due slow cell growth, expensive media, and culture conditions (continuous CO_2 supply, expensive transfection reagents). In recent years, mammalian cells have been further developed for the commercial production of broader range therapeutic proteins by selecting high protein-producing stable cell clones using methotrexate (MTX) amplification or glutamine synthetase (GS) system technology and high-throughput fluorescence-activated cell sorting (FACS)-based screening method [75–77]. Other advancements include genetic modification of mammalian cells by over-expression of anti-apoptotic proteins (bcl-2 family members and Bcl-x(L)) [78] or by inducing cell cycle arrest by adding anti-mitotic agents (such as hydroxyurea, nocodazole, colchicine, paclitaxel or vinblastine) [77] to increase cell viability along with high cell density which eventually lead to elevated protein productivity.

4.2 Protein Purification: Principle of Heterologous Protein Purification following Expression. Use of His Tag, GST-Tag, MBP-Tag, TAP-Tag, Myc-Tag

4.2.1 Protein Purification

Procuring pure and biologically active desired protein after expression is a daunting task. Separating desired protein from the rest of cellular protein pool is an essential prerequisite step for commercial production of therapeutic proteins. Several different chromatographic techniques like size exclusion, ion exchange, hydrophobic

interaction, affinity, and ammonium sulfate cut-off method, are widely used to isolate desired protein from other cellular impurity. These techniques rely on exploiting protein properties like, electric charge, solubility, size and hydrophobicity, therefore, optimization of the specific purification method is a time consuming and cumbersome task. Among the above mentioned techniques, affinity chromatography is the most time and cost-effective, and usually a single step purification method [79].

In affinity chromatography, protein is expressed in fusion to an affinity tag which significantly help in protein purification. Some of the known affinity tags also increase protein solubility without affecting biological activity of the protein, an additional advantage [80]. Polyhistidine tag (his-tag) is the most commonly used affinity tag. The purification is based on IMAC (immobilized metal-ion affinity chromatography), where adsorption of protein occurs due to coordination between an immobilized metal-ion (Ni^{2+} or Cu^{2+} ion) and an electron donor groups from the protein surface (stretch of 6–10 histidine (tryptophan and cysteine)). The protein is purified using imidazole gradient [81]. The other commonly used tags are polyarginine-tag, FLAG-tag, c-Myc-, S-, and Strep-tag; they all are around the same ~10 amino acid. Due to their small size, fusing these tags either at N- or C-terminus of desired protein usually does not affect its structure or biological activity. FLAG-tag is a hydrophilic octapeptide (DYKDDDDK) recognized by the M1 mAb resin. Due to non-reusability of antibody resin, the effective cost is high thereby restricting its widespread use. Recently, the development of anti-FLAG molecularly imprinted polymers (MIPs) approach using tetrapeptide DYKD as template has provides a cost-effective alternative solution for purifying FLAG-derived recombinant proteins [82]. Strep-tag (WRHPQFGG) binds to biotin and therefore, recombinant protein can eluted using biotin as competitor in buffer. Use of desthiobiotin facilitates regeneration and repeated use of these resins. Recent development of Strep-tag®II and Strep-Tactin has enhanced the use of Strep-tag owing to their higher affinity for the biotin [83]. Next comes the large molecular weight affinity tags (more than 200 amino acids), glutathione-*S*-transferase (GST), maltose-binding protein (MBP), N-utilization substance protein A (NusA), thioredoxin (Trx), ubiquitin, and SUMO [84]. Some of these affinity tags not only aid in purification but also increase the solubility of protein. MBP and GST act as both solubility enhancer and affinity tag, while NusA, SUMO, and Trx only increase solubility [85, 86]. MBP is a periplasmic *E. coli* protein with high solubility. MBP when fused with desired protein increases fused protein solubility due to its intrinsic chaperone activity [87]. NusA is a 55 kDa, elongation factor which regulates transcription in *E. coli.* As a fusion partner it improves the solubility of the protein due to its intrinsic high solubility [88]. SUMO (small ubiquitin-related modifier) protein is a reversible post-translational modification at ε-NH_2-group of lysine residues of target protein. It is known to increase the solubility and expression of the protein. SUMO-specific proteases removes the SUMO tag from the target proteins thereby reducing the erroneous cleavage within the target protein [80]. Thioredoxin (Trx) is a small and highly soluble *E. coli* protein. Like NusA tag, Trx itself does not act as an affinity tag and thus, requires fusion partners during purification step. List of affinity tags are given in Table 4.2. However, now-a-days larger soluble tags are being replaced

Table 4.2 Affinity and solubility tags for recombinant proteins [modified from refs [89, 90]

Tag	Size (kDa)	Sequence	Characteristics	
			Binding	Elution
His-tag	0.84	H_6 or H_{10}	Metal-ion based adsorption	Imidazole 20–250 mM or low pH
Arg-tag	0.80	RRRRR	Cation exchange resin	NaCl linear gradient from 0 to 400 mM at alkaline pH > 8.0
FLAG	1.01	DYKDDDDK	Use anti-FLAG monoclonal antibody for purification	pH 3.0 or 2–5 mM EDTA
Step-tag II	1.06	WSHPQFEK	Strep-Tactin (modified streptavidin)	2.5 mM desthiobiotin
HA-tag	0.99	YPYDVPDYA	HA-tag-specific antibody	High concentration of the HA-tag peptide or by low pH buffer
c-myc	1.20	EQKLISEEDL	Monoclonal antibody	Low pH
T7 tag	1.21	MASMTGGQQMG	Monoclonal antibody	Low pH
S-tag	1.75	KETAAAKFERQHMDS	S-fragment of RNaseA	3 M guanidine thiocyanate, 0.2 M citrate pH 2, 3 M magnesium chloride
Calmodulin binding domains	2.96	KRRWKKNFIAVSAANRFKKISSSGAL	Calmodulin	EGTA or EGTA with 1 M NaCl

Tag	Size (kDa)	Sequence	Characteristics	
			Binding	Elution
Chitin binding domain	5.99	TNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ	Chitin	Fused with intein: 30–50 mM dithiothreitol, b-mercaptoethanol, or cysteine
Thioredoxin (Trx)	12	Protein	Does not have intrinsic affinity properties (require other fusion tag)	Depends other affinity tag
Glutathione *S*-	26.0	Protein	Glutathione	5–10 mM reduced

by small soluble tags like SET tag [91] and Fh8 tag [87] to overcome the problem faced by size of large tags.

Tandem affinity purification (TAP) has also gained popularity in recent times. In TAP, desired protein is fused with at least two different affinity tags [92] or one affinity and another solubility enhancer fusion tag [93], depending on need. This helps in purifying desired protein using both the tags sequentially resulting in considerable reduction of nonspecific proteins. Overall it helps in increasing the purity of the desired protein making it useful for therapeutic purpose [80]. Numerous combinations of solubility-enhancing and affinity tags have been exploited in order to enhance both protein solubility and yield of the desired protein. Some of the commercially available TAPs are S3S-TAP-tag (is a recently developed system suitable for purification of mammalian protein complexes), FF-ZZ TAP-tag, Strep/FLAG-TAP (SF-TAP), GS-tag, PTP-tag, etc. [80].

Even though affinity tags are routinely used in laboratory protein purifications, they have a limited use in commercially therapeutic applications. Several times these tags cause structural and activity changes or immunogenicity problems. Sequence specific protease or chemical cleavage methods are developed for the removal of these tags. The tobacco etch virus (TEV) protease, thrombin, Enterokinase, and factor Xa are some of the most commonly used protease to remove tags [89]. Most of the commercially available vectors have a protease cleavage site designed between fusion tags and desired proteins. The solubility of a desired protein after tag removal cannot be predicted and enzymatic cleavage might cause negative effects, such as product heterogeneity due to cleavage at multiple sites, precipitation or poor recovery [94]. Chemical cleavage with CNBr-based method has advantages over enzymatic cleavage, as it is easy to remove from the reaction mixture and is cheap. Their use is largely restricted due to their harsh reactive nature and unwanted protein modifications making purified protein unsuitable for therapeutic use [85]. The use of the poly-ionic peptide tags (addition of 3–5 charge amino acid sequence) has shown to enhance solubility of the desired protein, regardless of their position at N- or C-terminus of the protein. The tags enhance the solubility of protein by increasing repulsive electrostatic interactions between protein molecules due to additional of charge from the tags. Due to their small size, the presence of poly-ionic peptide tags do not affect the structure or biological activity of the protein, an add-on advantage [94].

4.3 Proteomics: Introduction, Protein Detection Array, Protein Informatics, Domain Analysis, and Structure Prediction

Proteomics is defined as the study of proteome or a set of proteins found in a cell, tissue, or a whole organism. The importance of proteomics lies in the fact that unlike genome, the proteome is not constant and changes from cell to cell over time,

making an individual unique or different. The proteome provides a snapshot of the cell in action and the proteomics aims at understanding the proteome status at a large scale under certain physiological or diseased conditions [95]. The term "protein" was initially introduced in 1938 by the Swedish chemist JönsJakob Berzelius, working in electrochemistry while trying to describe a class of macromolecules made up of linear chains of amino acids [96]. Although proteomics research began in 1975 with the introduction of 2-Dimensional Gel Electrophoresis by O'Farrell and Klose, it was not until the early 1990's, when the term "proteomics" was coined by Mark Wilkins, a Ph.D. student at the Macquarie University, Australia [97].

Proteomics is a rapidly growing field with cutting-edge technologies used to investigate expression of proteins, post-translational modifications, and involvement of proteins in metabolic pathways and protein interactomics. The most commonly applied are mass spectrometry (MS)-based techniques such as Tandem-MS and gel-based techniques such as differential in-gel electrophoresis (DIGE). Another technique complementing to MS is protein microarray that has been widely applied as a promising proteomic technology with great potential for protein expression profiling, biomarker screen, drug discovery, drug target identification, and analysis of signaling pathways in health and disease [98]. The recording and analyses of the enormous amount of data generated by these high-throughput technologies are facilitated by the development of databases and online servers that are critical not only for recording and storing this data but also enable structure, function, and domain prediction of a protein [99]. For example, four major databases—UniProtKB, IntAct, Reactome, and PRIDE are responsible for storing all the up-to-date information generated for a protein [100–102]. In addition to that, several prediction software and servers such as Phyre2, FoldX, BisKit, etc. have facilitated protein structure prediction [103–105].

4.3.1 Protein Detection Array

Protein array analysis is a technique by which proteins spotted in defined locations on a solid support (a protein microarray, or protein chip) are probed for interactions with a probe molecule in a high-throughput, parallel manner [106–108]. Protein array analysis is used to screen protein function, drug discovery, biomarker discovery, expression profiling, and antibody analysis [109, 110]. Typically, a protein microarray is prepared by immobilizing proteins onto a microscope slide using a standard contact spotter or noncontact microarrayer [111]. The microscopic slide surface can be made of aldehyde and epoxy-derivatized glass that get attached to amines and nitrocellulose or the surface could be nickel-coated that relies on more specific affinity attachment of His6-tagged proteins which results in the generation of ten-fold better signals. After proteins are immobilized on the slides, they can be probed for a variety of functions/activities [112, 113]. Finally, the resulting signals are usually measured by detecting fluorescent or radioisotope labels. Protein microarrays are majorly categorized into two classes: analytical and functional [114,

115]. In addition, tissue or cell lysates can also be fractionated and spotted on a slide to form a reverse-phase protein microarray [116].

4.3.1.1 Analytical Microarrays

Analytical microarray is majorly represented by the antibody array which primarily employs the "analyte-labeled" assay format where an array is queried with: (1) a probe (labeled antibody or lig-and) or (2) an unknown biologic sample (e.g., cell lysate or serum sample) containing analytes of interest [117]. By tagging the query, molecules with a signal-generating moiety, a pattern of positive and negative spots is generated. For each spot, the intensity of the signal is proportional to the quantity of applied query molecules bound to the bait molecules. An image of the spot pattern is captured, analyzed, and interpreted [118, 119]. This format, successfully, found alterations in protein expression in cancer cell development, epithelial and stromal cells. However, one of the major limitations of the antibody array approach is the production of specific antibodies in a high-throughput manner. In addition, targeted protein labeling may lead to epitope destruction because of some chemical reactions [120].

This assay can also be explained as the original enzyme-linked immunosorbent assay (ELISA) in a multiplexed format, but can only detect dozens to hundreds of analytes simultaneously because cross-reactivity between antibodies can occur [120]. Recombinant antibodies have become a promising means of overcoming this problem; however, their fabrication issues such as cloning and protein expression add to complexities to their practical use [121]. To improvise on the sensitivity and specificity, analytical microarrays usually employ "sandwich" assay format [106]. This format employs two different antibodies to detect the targeted protein (1) the capture antibody that immobilizes the targeted protein on the solid phase and (2) the reporter or detection antibody that generates a signal for the detection system. This format was applied to successfully detect 75 cytokines with high specificity, femtomolar sensitivity, a 3-log quantitative range, and economy of sample consumption [106, 122, 123].

4.3.1.2 Functional Microarray

Functional protein microarrays are constructed using individually purified proteins that enable the study of various biochemical properties of proteins, such as binding activities, including protein–protein, protein–DNA, protein–lipid, protein–drug, and protein–peptide interactions, and enzyme–substrate relationships via various types of biochemical reactions [106, 120]. Functional protein microarrays are constructed by printing a large number of individually purified proteins, and in principle, it is feasible to print arrays comprised of virtually all annotated proteins of a given organism, effectively comprising a whole proteome microarray [124]. Functional protein microarrays have been successfully applied to identify

protein–protein, protein–lipid, protein–antibody, protein–small molecules, protein–DNA, protein–RNA, lectin–glycan, and lectin–cell interactions, and to identify substrates or enzymes in phosphorylation, ubiquitylation, acetylation, and nitrosylation, as well as to profile immune response [114]. The first use of functional protein microarrays was demonstrated by Zhu et al. [125] to determine the substrate specificity of protein kinases in yeast. Since then, reported applications of functional protein microarrays in basic research, as well as in clinical applications, have increased rapidly [126]. Significant achievements in providing the whole proteome of several organisms (i.e., human, yeast, *E. coli*, virus) on arrays have provided the tools for many important biological discoveries [126].

4.3.1.3 Reverse-Phase Protein Microarrays

This format immobilizes an individual complex test sample in each array spot such that an array is comprised of hundreds of different patient samples or cellular lysates. Each array is incubated with one detection protein (i.e. antibody), and a single analyte end point is measured and directly compared across multiple samples. This method allows for the analysis of many samples obtained at different states by directly spotting tissue, cell lysates, or even fractionated cell lysates on a glass slide. Many different probes can be tested to specifically identify certain proteins in lysate samples [120]. This type of microarray was first established by Paweletz and colleagues to monitor histological changes in prostate cancer patients [127]. Using this method, they successfully detected microscopic transition stages of pro-survival checkpoint protein in three different stages of prostate cancer: normal prostate epithelium, prostate intraepithelial neoplasia, and invasive prostate cancer. The high degree of sensitivity, precision, and linearity achieved by reverse-phase protein microarrays enabled this method to quantify the phosphorylation status of some proteins (such as Akts and ERKs) in these samples; phosphorylation was statistically correlated with prostate cancer progression.

4.3.2 Protein Informatics

With the advent of high-throughput technologies like Next-Generation Sequencing (NGS), a wealth of information about genomic sequences from a variety of organisms has been amassed. This has led to a rapid buildup of protein sequence data in the form of new protein databases and updation of the existing ones. The exponential increase in the protein related data has prompted computational biologists to develop an advanced infrastructure that facilitates better organization, structural and functional annotation, and evolutionary analyses [128]. Along with a myriad of protein analysis tools, numerous protein related databases have been created that can be categorized as sequence databases, family and domain databases, 3D structure databases, gene expression databases, enzyme and pathway databases, PTM databases,

protein-protein interaction databases, etc. [129]. More information can be accessed at http://www.oxfordjournals.org/our_journals/nar/database/cap/, https://proteininformationresource.org/staff/chenc/MiMB/dbSummary2015.html or Expasy, a Swiss Bioinformatics Resource Portal.

4.3.3 Domain Analysis

Protein domains are defined as basic units of structure, function, and evolution. Ranging from 30 to 600 amino acids in length, these units are able to fold independently into stable tertiary structures [130–132]. Compact structures with separate hydrophobic cores represent structural domains wherein contacts between residues within the domain are found to be more extensive than between domains [133, 134]. Identification of domains forms the basis of protein classification and annotation. This is exemplified by many protein sequence databases like Pfam, SMART and Interpro, and protein structure databases such as SCOP, CATH, and PALI, which consider domains as the basis for their classification of proteins. The domain-level approach has strongly influenced our understanding in the areas of evolutionary history, homology detection and modeling, protein fold recognition, etc. Interestingly, a relatively random domain shuffling process is thought to have led to domain linkages during the course of evolution, resulting in a few beneficial domain associations being selected and propagated in the interest of cell fitness [135–138].

Most sequence-based domain recognition methods rely on the conservation of contiguous homologous segments, which is complicated by domain shuffling and recombination in multidomain proteins, accessory domains, and evolutionary divergence of sequences. Therefore, to get an insight into the functional and structural interplay of domains in multidomain proteins, all domains in the full-length amino acid sequence need to be considered simultaneously for protein classification. Keeping this in view, an alignment-free tool, named CLAP (CLAssification of Proteins), was developed for effective classification of multidomain proteins, bypassing the need for identification of domains and their sequential order [135].

Proteins are generally composed of one or more domains arranged in a distinct way that largely dictates the protein function. This is referred to as domain architecture [139, 140].A limited fraction of domain combinations have been found in proteins ruling out the possibility of random combinations. In accordance with power law distribution, the covalent linkage between domains is such that most domains have few partners with a smaller fraction of abundant domains being highly connected [141–143]. Based on domain co-occurrence or context, a novel approach, dPUC (Domain Prediction Using Context) was developed for domain prediction and identification. The scores are assigned by analyzing whether two domain families frequently co-occur (positive context) or have never been found as a pair (negative context) [144].

As we move from prokaryotes to eukaryotes or from unicellular eukaryotes to animals, the number of unique domains and the fraction of multidomain proteins

increase. This organismal complexity-associated trend, called domain accretion, is thought to play a significant role in evolution. While researchers have likened the genomes to natural language texts, protein domains are considered analogous to words, with domain architectures and amino acids representing sentences and letters, respectively [145]. So *n*-gram analysis, a well-known probabilistic language-modeling (linguistic) technique helpful in the identification of meaningful word combinations by treating consecutive words in sentences as a unit, is utilized to probe the rules of domain association leading to distinct domain architectures. The set of rules, termed "proteome grammar" is employed to study genome complexity and domain evolution [146]. Some domains tend to be involved in many different domain architectures, a phenomenon called protein domain promiscuity. A bigram analysis has been employed to study the evolution of this promiscuity. Bigram refers to a pair of domains on a protein sequence [139, 147]. Domain rearrangements and domain accretion are two important aspects of evolution. It has been found that there is a nearly universal value of information gain (loss of entropy) associated with a transition to the observed domain architectures from random domain combinations. This highly conserved constant value corresponds to the minimum complexity required to maintain a functioning cell and is governed by "quasi-universal grammar." However, two major groups viz. a subset (extremely simplified cells) of Archaea and animals (extreme complexity) have deviations from this constant value [146].

4.3.3.1 Domain Parsing

The accurate prediction of domain boundaries (domain parsing) is crucial for the design of chimeric proteins with multi-functional domains and the experimental structure determination of proteins where crystallization is adversely affected by flexible regions. It also makes the multiple sequence alignments more reliable [148, 149]. The defining feature of structural domains underlies some effective algorithms that assign domain boundaries using 3D structures. Some protein features such as signal peptide, trans-membrane helices, low-complexity, and disordered regions and coiled coils, which are not found in globular domains, can be easily predicted using relevant tools. These analyses performed subsequent to a sequence search with BLAST constitute initial steps in domain prediction. The domain boundary prediction employing templates with known structure involves three steps [150]. Sequence search against protein structure databases like PDB comprises the first step and helps in retrieving alignments between target sequence and template structure. The more accurate alignments with a percentage identity of at least 30% and enough coverage (at least 100 residues) with few gaps can be used for 3D model generation. Many methods including a highly sensitive HHPred server have been developed for detecting remote structural homologs by performing a search against a wide range of databases such as PDB, SCOP, Pfam, and COGs [151]. The final step involves 3D model generation using a modeling program like MODELLER (incorporated in HHSearch server) [152–154]. Phyre2 [105, 155], I-TASSER [156,

157], and ROBETTA [158] are some of the methods that detect templates and develop the model automatically.

4.3.4 Structure Prediction

A few advanced techniques, viz. X-ray crystallography, nuclear magnetic resonance (NMR), and recently developed cryo-electron microscopy (cryo-EM), have been instrumental in solving the 3D structures of proteins. In spite of this, a rapid non-proportional progress in the field of genomics has widened the already existing gap between the number of protein sequences identified and the number of available protein 3D structures [159]. To address this issue, a variety of computational methods that are faster, easier, and economical have been developed. One of the methods involves sampling the conformational space (c-space) of a protein through deterministic or heuristic approaches. With deterministic methods like homology modeling, entire or part of the c-space is scanned and sub-spaces excluded based on a priori knowledge. In heuristic algorithms (ab initio modeling, Monte Carlo, and molecular dynamics simulations), only a fraction of the c-space is sampled without a priori knowledge generating a representative set of Boltzmann-weighted conformations [160, 161].

Homology modeling (also called comparative modeling) consists of predicting 3D structure from the primary sequence of the protein. It is useful in identifying therapeutic targets, studying structure and function of proteins, protein interaction networks and signaling pathways, and mutagenesis associated with certain diseases [159, 162]. It also has applications in molecular modeling of protein complexes and in the refinement of cryo-EM 3D structures [163–165]. It involves multiple steps starting with the identification and selection of suitable templates by searching PDB (Protein Data Bank), an online database of known crystal structures. The protein Basic Local Alignment Search Tool (BLASTp) is employed to look for templates with a sequence identity of more than 40%. There are other algorithms available including PSI-BLAST (Position-Specific Iterated BLAST), hidden Markov models (HMMs), and profile–profile alignments, for templates with low homology [166]. Subsequent to the optimization of the selected alignments, 3D model is built using rigid-body assembly method (as in 3D-JIGSAW and SWISS-MODEL programs), segmented matching method (used by SegMod/ENCAD), spatial restraint method (used by MODELLER and DRAGON), or the artificial evolution method (used by NEST). Next, loop modeling is performed either by scanning a structure database like PDB (a knowledge-based approach used by MODELLER, 3D-JIGSAW and SWISS-MODEL) or by optimizing a scoring function through Monte Carlo or molecular dynamics methods for randomly chosen conformations (an energy-based method using an ab initio fold prediction approach). Addition of side chains to the main backbone requires rotamer libraries, which contain statistical distributions of side chain and backbone orientations extracted from known crystal structures. These are tested sequentially and scored using energy functions. Some of the tools

used for side chain packing include SCWRL, FASPR, and SCAP [160–162]. To improve the quality of the model thus generated, optimization is done by energy minimization through molecular mechanics force fields. Other ways of model refinement employ molecular dynamics and Monte Carlo simulations. The relationship between the energy of a protein and its conformation is described by the potential energy hyper-surface (PEHS) modeled with quantum or molecular mechanical methods. The native conformation of a protein in the funnel shaped PEHS is ideally represented by a global energy minimum conformation (GEMC), although a canonical ensemble of structures is required to describe the system state completely. The top portion of the PEHS funnel contains high energy conformations resulting from steric and hydrophilic/hydrophobic clashes and unoptimized bond lengths and angles, etc. These conformations are eliminated as a protein folds and GEMC is reached with the narrowing of the funnel [160, 167].

Finally, the model is evaluated and validated by considering stereochemistry, physical parameters, statistical mechanics, etc. To perform this task, Distance-matrix ALIgnment (DALI; http://ekhidna2.biocenter.helsinki.fi/dali/) or Verify3D online servers are used. Many homology modeling programs and online servers like SWISS-MODEL [168–170] and Phyre2 [105] have been developed that perform most of the aforementioned steps in an automated fashion. Other homology modeling programs include MODELLER [153], I-TASSER [157], Rosetta [171], Raptor X [172, 173], GalaxyTBM [174], AlphaFold [175], etc.

4.4 Expression Sequence Tags (ESTs), Application of Protein Detection Microarray with Examples

4.4.1 Expression Sequence Tags (ESTs)

Expressed sequence tags (ESTs) are partial cDNA sequences, resulting from single pass sequencing of clones obtained from cDNA libraries. They are used for decoding genome organization and determining gene expression profiles in specific tissues under different conditions. The foremost utilization of ESTs in genome organization studies is to regulate the chromosomal localization of analogous genes employing somatic hybrid cell panel. Furthermore, ESTs contribute in comparative genetics of different species to decipher their gene function. Overall the ESTs lead to integrated genomic approach by the combination of sequence, functional, and localization data. To date, over 45 million ESTs have been generated from over 1400 different eukaryotic species. They have been proven very useful in gene identification and predictions because they are low-cost alternative to whole genome sequencing [176]. This is particularly important for eukaryotes which tend to have “less gene-dense genomes” [177].

For the generation and processing of ESTs mRNA is collected either from whole organisms or specific tissues depending on the size of the organism. This is followed

by the extraction of pooled mRNAs and purified typically on the basis of their polyadenylation. Subsequently, a cDNA library is constructed from this pool and clones are randomly picked for a single pass sequencing read. The raw data are then processed to derive the underlying sequence which is followed by further processing that removes low-quality and contaminating sequences associated with the vectors. The purified sequence data is the submitted to EST database such as dbEST [178–180]. The continued generation of ESTs for different species along with the technological advancement has led to its exploitation in range of applications. For example, tandem mass spectrometry matches peptide fragments to known protein sequences. However, limited number of sequences in protein databases leads to computational bias against poorly characterized proteins. ESTs are beginning to have a widespread appeal in identifying and characterizing alternative spliced isoforms [181].

4.4.2 Application of Protein Detection Microarray

Microarray technology was developed in 1989 by Roger Ekins, based on ambient analyte immunoassay [182, 183]. Later, it was transformed into DNA microarray for simultaneous detection of mRNA expression levels in multiple genes. However, the mRNA expression in a cell does not always correspond to exact protein levels [184]. Therefore, to overcome these limitations of DNA microarrays, protein microarray was developed for functional analysis of proteins as they are the major driving forces behind all cellular processes. Immunoassays are first protein microarray, based on specific antigen-antibody interactions, later expanded to antibody microarray which enabled parallel detection of multiple proteins in minute sample quantity with high sensitivity and reproducibility [184]. High-throughput protein array was developed by immobilization of purified proteins on chip glass slide/bead/nitrocellulose membrane or microtiter plate chip [106].

As discussed earlier, the arrays are divided into three main categories; (1) analytical protein microarrays (2) reverse phase protein microarrays, and (3) functional protein microarrays (Fig. 4.1). Analytical protein microarrays (APMs) or capture microarrays are composed of antibodies, aptamers, or affibody libraries attached to a solid support that binds to a specific protein in cell lysate. The APMs provide information regarding protein expression, their binding affinities and specificity; however, cross-hybridization of antibodies is still the major challenge associated with these microarrays. Analytical microarrays are generally used for identification and profiling of treated/non-treated cells and diseased/non-diseased tissues. The reverse phase protein microarray (RPPA) separates complex mixture of proteins in tissue lysate; detected by fluorescent or chemiluminescent assays, and are useful to identify altered proteins or post-translational modifications in diseased cell. Unlike APMs and RPPMs, functional protein microarray (FPM) is used to study biochemical activities in entire proteome. These microarrays are composed of full-length purified functional protein or protein domain arrays immobilized on protein chip. FPMs are used to identify protein–protein, protein–DNA, protein–RNA,

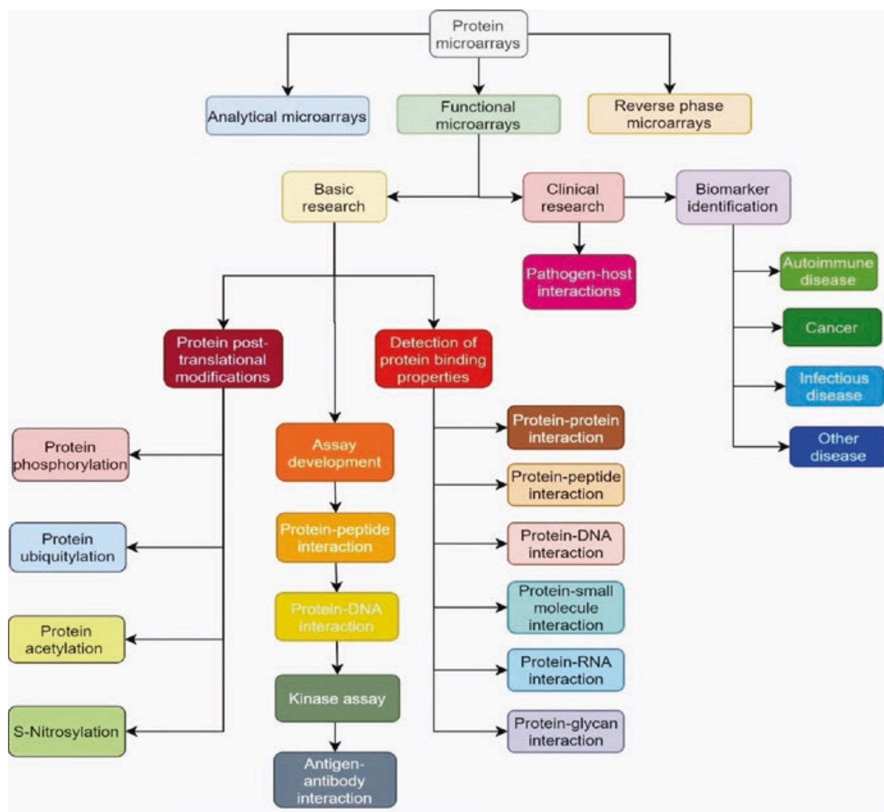

Fig. 4.1 Types of protein microarrays and their application in basic and clinical research

protein–phospholipids, and protein–small molecule interactions, detect antibodies, and determine enzyme activity and its specificity. Compared to other methods, FPMs are more capable in detecting low level of protein expression and weak interactions. On the basis of recent developments in FPMs, they are divided into four categories, namely, (1) Purified proteome microarray, (2) Purified protein family microarray, (3) Purified protein domain microarray and, (4) Cell-free protein/peptide microarray.

The purified proteome microarray, consisting of genome-wide expressed proteins immobilized on a microarray, is widely utilized in *E. coli*, *S. cerevisiae,* and human system to study their functional and biochemical properties. Two DNA repair proteins, namely YbaZ and YbcN, were identified by Chen et al. using *E. coli* proteome microarray consisting of 4256 unique proteins [185]. In another study, using similar microarray, Spr phase switch and DNA binding proteins were identified in type 1 fimbria [186]. Unique antimicrobial peptide and glycosaminoglycans protein targets were identified using *E. coli* proteome microarray [187, 188]. Additionally, *CobB* deacetylation enzyme was identified as a strong binder of cyclic di-GMP (bacterial second messenger) while Yojl was found to be involved in

bacterial cell invasion by probing human brain microvascular endothelial cells on *E. coli* proteome microarray [189, 190]. Besides this, *E. coli* proteome microarray has also been applied for identification of glycoproteins [191], tyrosine sulfation [192], and ClpYQ protease [193] to study bacterial physiology and host–microbial interactions.

A total of 33 novel calmodulin and more than 150 phospholipid binding proteins were identified with biotinylated calmodulin and fluorescently labeled liposomes, respectively, utilizing yeast proteome microarray [125]. The same microarray was used for the identification of SMIR3 and SMIR4 rapamycin inhibitors, Arg5,6 mitochondrial enzyme, and Pus4 and App1 brome mosaic virus antiviral proteins [194–196]. Lin et al. further demonstrated two signaling pathways, NuA4 complex-mediated protein acetylation reactions involved in yeast aging and substrates for HECT domain ubiquitin E3 ligase Rsp5 [197]. All these studies demonstrate the usefulness of bacterial and yeast proteome microarray in basic research. However, human proteome microarray is still the most widely used in clinical research, pharmaceutical industry, and translational research. HuProt composed of ~21,000 full-length purified human proteins, ProtoArray ~9000 purified human proteins from insect cells, and NAPPA with 10,000 human proteins are the three popular human proteome microarrays. Human proteome microarray is broadly applied in five major areas: (a) diagnostics, (b) proteomics, (c) protein functional analysis, (d) antibody characterization, and (e) treatment development. Diagnostics includes profiling of sera to discover new disease biomarkers and monitoring of disease states/responses to therapy in personalized medicine. In 2010, Song et al. identified and validated three highly specific anti-hepatitis biomarkers RPS20, Alba-like, and dUTPase with 47.5%, 45.5%, and 22.7% sensitivity, respectively, using human protein microarray consisting of 5011 non-redundant proteins [198]. In another study, a microarray with 1626 purified human recombinant proteins was utilized for validation of six highly specific biomarkers against autoimmune hepatitis with 82% sensitivity and 92% specificity [199]. Also, six highly specific biomarkers, namely PTPRN2, MLH1, MTIF3, PPIL2, NUP50, QRFPR associated with type 1 diabetes were recently validated using Nucleic Acid Programmable Protein Array (NAPPA) [200]. In addition to this, Protoarray was used for validation of transglutaminase 4 (TGM4) biomarker specific infertility causing autoimmune polyendocrine syndrome type 1 (APS1) in males [201]. Furthermore, validation of RNA Polymerase II subunit A C-terminal domain phosphatase (CTDP1) biomarker specific to Behcet disease was performed using HuProt array [202]. Three highly specific p53, PTPRA, and PTGFR biomarkers were validated against ovarian cancer using NAPPA 5177 tumor antigens microarray with 98.3% specificity [203]. In other study, four SNX1, PQBP1, IGHG1, and EYA1 biomarkers specific to glioma were identified by probing ~17,000 human protein microarray [204]. HuProt array was used for identification of COPS2, NT5E, TERF1, and CTSF biomarkers for diagnosis of gastric cancer and validation of p53, HRas, and ETHE1 for early detection of lung cancer [205, 206]. Moreover, three specific FGFR2, CALM1, and COL6A1 prostate cancer biomarkers were identified and validated using 123 purified antigens microarray platform [207]. Human proteome array has been utilized to validate IGHG4, STAT6,

CRYM, HDAC7A, EFCAB2, SELENBP1, and CCNB1 biomarkers against Meningiomas [208]. In a more recent study, a highly specific biomarker panel, identified and validated using protein array based approach, was employed to discriminate Zikavirus and Dengue virus infections [209]. High-Density Nucleic Acid Programmable Protein Array (HD-NAPPA) of the pathogen *Mycobacterium tuberculosis* (*Mtb*) was used in the identification of eight antibody targets, viz. Rv0054, Rv0831c, Rv2031c, Rv0222, Rv0948c, Rv2853, Rv3405c, Rv3544c, for tuberculosis serology [210].

Currently, there are two methods to detect protein signals (1) labeled and (2) label free. The ideal protein array detection method should produce low background noise and generate high signal frequency. Therefore, the most common and widely used detection method is fluorescence labeling which is highly sensitive, safe, and compatible with readily available microarray laser scanners. Other labels used are affinity, photochemical, or radioisotope tags. As these labels are attached to the probe itself which can interfere in the probe-target protein reaction; thus, a number of label free detection methods are developed such as surface Plasmon resonance (SPR), carbon nanotubes, carbon nanowire sensors, and microelectromechanical system cantilevers. Most of these methods are relatively new and not very suitable for detection of high-throughput protein interactions; however, they do offer much promise for the future.

4.5 Data Analysis and Interpretation of Protein Detection Arrays

Protein microarrays provide wealth of information regarding protein interactions, protein functions, and signaling pathways which could be used for clinical diagnosis. However, data translation requires automated data processing and interpretation for generation of meaningful information. Protein microarray data analysis mainly depends on the design of surfaces, content, detection method, data preprocessing, inference, classification, and validation (Fig. 4.2). The design of array is a crucial step as it significantly affects data analysis and its final interpretation. Inclusion of biological replicates is recommended as they provide higher statistical confidence; however, they also make results more complex to evaluate. Data preprocessing, which includes image analysis, normalization, and data transformation, also greatly affects data analysis and interpretation. Image-processing algorithms distinguish foreground and background intensities and inference based on data analysis variability [211]. Different data analysis strategies for different types of array generate variable results. These arrays provide variety of tools for disease analysis but lack standard analytical and data processing strategy which enhance complexity in data analysis.

The data analysis strategies like spot-finding on slide images, *Z*-score calculations, and significance analysis of microarrays (SAM) have their origin in DNA microarray analysis; however, concentration-dependent analysis (CDA) is specific for protein microarrays (Fig. 4.3) [212].

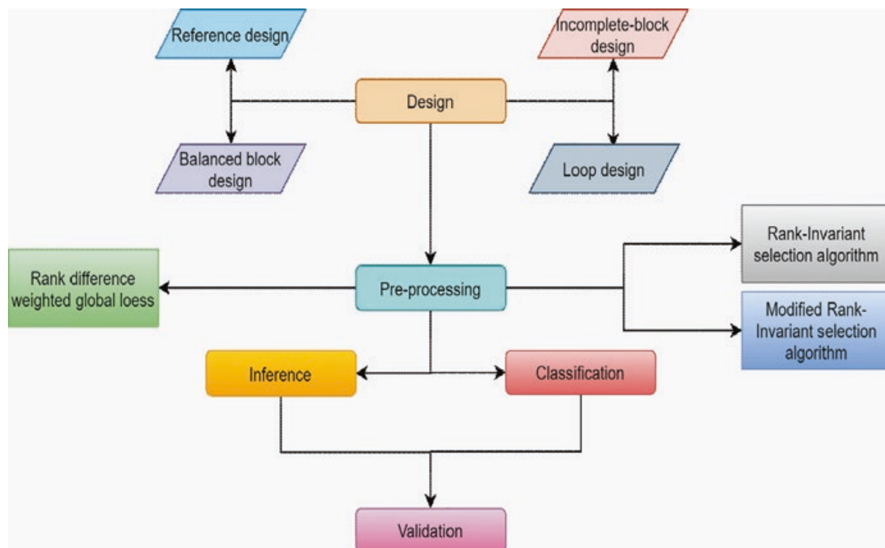

Fig. 4.2 Protein microarray strategy for data analysis

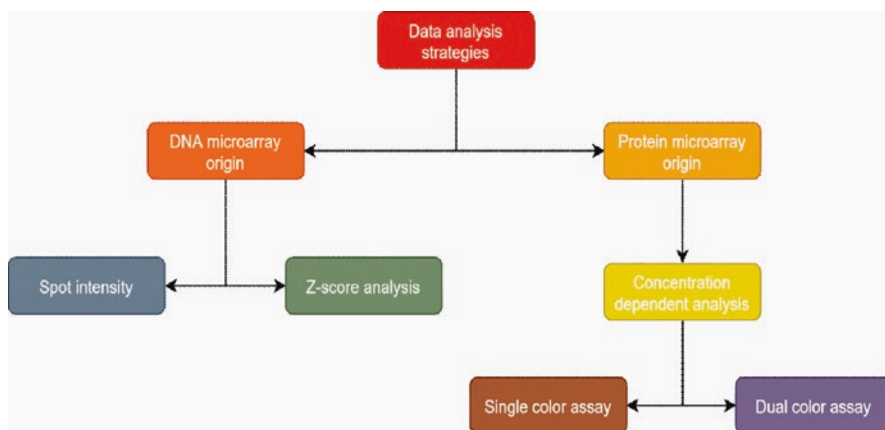

Fig. 4.3 Methods for data analysis of protein microarrays

In spot intensity determination method, microarray image analysis starts with the fixing of spot intensity. A grid of circles with adjusted position and size are placed over protein spots to get reliable intensity data. The output file is created by GenePix Pro software (Molecular Devices, CA). In *Z*-score analysis, *Z*-score equation ($Z_s = S_s - \mu/\sigma$) is analyzed to determine the significantly different from the expected values, where Z_s is the *Z*-score for the *s*th spot, S_s is the signal for that spot', μ is the mean signal across all spots, and σ is the standard deviation across all spots. In concentration-dependent analysis (CDA), absolute signals generated depend on protein concentration in the sample. To overcome this issue an iteration process is

used to calculate *Z*-score ($Z_s = S_s - \mu_w/\sigma_w$) and remove outliers. In the equation, Z_s represents *Z*-score for the *s*th spot, S_s spot signal, μ_w mean signal, and σ_w standard deviation (Fig. 4.3).

The signals produced are detected by fluorescent dyes or colorimetric assays. There are two types of assays for colorimetric detection of proteins: single color assay and dual color assay. Single color assay is a single antibody based microarray which uses internal control system based on two colors for quantification of antigen and antibody. In the dual color assay, each sample is labeled with different fluorescent dyes and their signal intensity is measured using fluorescence image scanners. Dual color assays have better reproducibility and discriminatory efficiency then single color assays [213]. To prevent undesired technical artifacts caused by electric charges, different protein sizes, hydrophobic protein interaction of proteins and antibody/antigen binding kinetics in dual color assays the data pre-processing protocols like filtering, background correction and data normalization are required [214]. Furthermore, it employs four different microarray designs: (a) Reference in which sample of interest and reference sample is labeled with different fluorescent fluorochromes. This design is generally used for comparative studies; (b) Balanced-block where two samples bearing two different fluorochromes are hybridized to make a single block; (c) Incomplete block, more than two samples bearing only two fluorescent fluorochromes are co-hybridized on microarray; (d) Loop design where samples are hybridized in different arrays using different fluorochromes which leads to duplication of arrays.

For data normalization, different algorithms, rank-invariant selection, modified rank-invariant selection and rank difference weighted global loess are used to define the set of probes. Rank-invariant selection algorithm is used in the absence of housekeeping controls. However, its major limitation is that it does not cover entire intensity range [215]. Modified Rank-Invariant Selection Algorithm corrects intensity values through extrapolation of curve to lower and upper intensity limits. Rank Difference Weighted Global Loess is applied to whole probes on array to get global normalization. Despite the differences among data processing methods in microarray analysis the general recommendations which need to be considered for data processing in microarray analysis are: Bayesian approaches to examine intersections, quality-control, validation methods, and standardized testing platforms.

4.6 Summary

Medical biotechnology has provided several products essential for research, therapeutics, and diagnostic purpose. Recombinant protein technology is the connecting link between medical biotechnology, and mass production of therapeutic and diagnostic products. For a long time, *E. coli* has served as the cost-effective and low-maintenance expression system for the production of pharmaceutically important recombinant proteins. The limitation of expressing several human proteins with specific post-translational modifications, essential for their biological activity, in

E. coli (which lacks post-translational modification machinery), has forced biochemists to look for alternative expression systems for large recombinant protein production. Using new and improved recombinant methods, humanization of *E. coli,* which has failed so far, could be tried and made successful. Lately, insect cell lines, non-human mammalian cell lines, and human cell lines with engineered genome are extensively used to produce therapeutic proteins such as growth factors, vaccines against infectious diseases, monoclonal antibodies, and IFNs to treat cancers and other diseases. The advantages and disadvantages of the different expression systems are reviewed in this chapter. We have also discussed the significance of protein tags used during protein purification following its expression. Some of these tags are also known to increase the solubility of the proteins, ultimately leading to high protein yields required for commercial purpose.

Ever since informative machineries started to evolve, proteomics technologies have been aimed at the comprehensive detection of the downstream proteins to evaluate complex disease diagnosis, allied mechanism and concerned therapy for effective management of the diseases. Moreover, to understand the complex biological organization, it is imperative to understand regulatory interconnections between DNA, RNA, and protein. For instance, microarrays, automated sequencing, and mass spectrometry have significantly contributed to systems biology approach by investigating protein–protein interactions, signal pathway analysis, studies of PTMs, or/and detection of toxins. It also has a wide array of opportunities in disease biomarker discovery to enable better disease management through improved diagnostics. More importantly, various forms of protein microarray have gradually evolved for proteomics research. With the progressive development, standardization of the experimental workflow and data interpretation, protein microarray holds promises in diagnostic applications. For protein microarray data analysis, various techniques including spot intensity determination method, *z*-score calculation, and concentration-dependent analysis have been used. Moreover, colorimetric assays involving fluorescent dyes are used for detecting signals.

Expressed sequence tags and cDNA provide direct evidence for all sample transcripts and are the most important resources for transcriptome exploration. ESTs have proven useful in different applications along with individual tools and pipelines for EST analysis.

Finally, an increasing number of bioinformatics methods have been developed to meet the needs of researchers for rigorous analysis of a vast amount of data generated through high-throughput genomic and proteomic techniques. From sequence-based analysis to protein structure prediction, analysis tools have been developed that focus on individual steps of the process or perform the whole process in an automated way. For instance, different protein databases along with certain analysis tools have been used for protein data analysis. Correct identification of domain boundaries for elucidating domain architecture and predicting protein structure from primary sequences using homology modeling have become possible with some of the finest tools developed recently. All these methods have facilitated the research on therapeutic agents that could be used in drug designing and other areas.

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Chapter 5
Analytical Techniques in Medical Biotechnology

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Abstract Biomedical research, diagnosis, and treatment thrive on the use of various analytical biotechniques. These analytical biotechniques include a vast array of tools and techniques aimed at examining biological specimens at the level of single molecule, cell, tissue, and whole organism. From the use of DNA fingerprinting yielding forensic evidence, fluorescence in situ hybridization detecting chromosomal abnormalities, ELISA detecting drugs in hair and urine specimens, microscopy in cancer diagnosis, chromatography leading to the world of infinite metabolites in biological samples and spectroscopy non-invasively analyzing soft tissues; core analytical biotechniques are invariably used in the biomedical field. In fact, technological progress goes hand in hand with basic science and medical discoveries. This chapter presents an overview of some such analytical techniques employed in

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medical biotechnology. Each technique description includes explanation of its core concepts followed by discussion of its applications to medicine.

Keywords Chromatography · Electrophoresis · ELISA · Microscopy · Spectroscopy · Radioisotopes

5.1 Introduction

The evolution of human T cell lymphotropic virus type III (HIV as known today) antibody assay from 1983 to 2016 is a simple example of how analytical biotechniques of ELISA, immunofluorescence, and Western blotting have aided early diagnosis of the deadly AIDS disease by reducing the antibody negative window from 8–10 to 2 weeks [1]. Another example is the quantitation of circadian phase biomarker, the pineal hormone melatonin, in biological fluids. Initial fluorometry based assay of 1960s has been replaced with highly sensitive ELISA and mass spectrometry-based assays [2, 3]. Most celebrated use of the analytical biotechniques in transforming clinical medicine is in the field of cancer. Liquid biopsy analyses using techniques like FISH, immunoblotting, and dielectrophoresis have aided early diagnosis, prognosis, treatment strategy, and patient monitoring [4]. Advances in life science research and biomedicine depend upon analytical techniques. This chapter is a collective attempt to summarize some of these techniques.

In both, basic research and medicine fields, seeing is believing. We begin this chapter with the art of viewing objects, the microscopy. We briefly touch upon different types of microscopy (light, fluorescence, and electron) and comment upon their applications in biomedicine. Another technique that is based on interaction of light and matter is the spectroscopy. Enumerating different radiations and resultant spectroscopic technique types, nuclear magnetic resonance is described in detail. Observing different components in a specimen through microscopy and spectroscopy is succeeded by separation using chromatography and electrophoresis with visualization using hybridization. Other technique that can precisely detect and quantify components in a specimen is ELISA when used in combination with fluorescence or visible spectroscopy. Lastly, we enumerate the applications of radioisotopes in biology.

5.1.1 Microscopy

Microscopy is a technique that microscopes to magnify and study objects that are too small to be visualized by the naked eye. From the first view of cork cells by Robert Hooke in 1665 to the visualization of the novel coronavirus, SARS-CoV-2 the technique of microscopy has undergone tremendous advancement [5]. The

technique, however, continues to work on simple laws of physics: (1) Snell's law of refraction (light traveling from one medium to another refracts or bends) and (2) Huygens' principle of diffraction (when light encounters an object it bends, and the bend depends upon wavelength of the light and the size of the opening. Besides, the scattering of the light wave generates secondary waves which can intersect. Constructive interference results with overlapping of secondary waves in the same phase while destructive interference occurs when secondary waves are in different phases) [6].

5.1.1.1 Background and Types

Medical science relies on magnification of minute yet distinct features of cells and cellular components. In microscopy this is defined as the resolution (the ability to distinguish between very small and closely spaced objects). Resolving power (RP) of a microscope is the minimum distance that it can distinguish. RP depends upon wavelength of light and numerical aperture of the objective lens. Illumination of the specimen with white or ultraviolet light is the most essential factor for visualization usually achieved using condenser lenses. Current microscopes utilize Kohler illumination where a series of condenser lenses and diaphragms are used to focus light on the specimen [7]. Microscopes are meant to magnify images (magnification is the ratio of limit of resolution of eye, ~150 μm and limit of resolution of microscope). Detail on microscope types is covered in Handbook of optics [8].

Microscopy can be broadly classified into (a) light and (b) fluorescence. Brightfield microscopy is the most widely used type of light microscopy. In this the illumination light is transmitted through the specimen. Differential absorption and differential refraction produce contrast. Contrast is crucial to differentiate the object (for example, a transparent cell) from the surrounding medium. The limitation of the brightfield microscopy is low contrast for weakly absorbing materials like bacterial cells. High contrast can be achieved using different dyes and stains (hematoxylin, eosin, methylene blue, Rose Bengal), by phase contrast and by differential interference contrast (DIC) [9]. Phase contrast is often used when high magnifications are required for a colorless specimen with fine details. Structures in a specimen have different densities and hence retard the light to different degrees creating a contrast between internal structures and the surrounding medium. In phase contrast, phase plate is placed in path of the light refracted from the specimen. Barely refracted light passes through the center of the plate and is not retarded while highly refracted light passes through the edges of the phase plate and is retarded, thus generating contrast. In DIC a pseudo-3-dimensional shape is provided to live specimens through manipulation of phases of light. Specifically, light entering into the specimen is split into beams, the sample beam that passes through the specimen emerges with a phase difference compared to the reference beam. When the two beams are combined interference is generated [10]. Dark field microscopy, also a type of light microscopy, produces dark images against a bright background. It uses

Table 5.1 Comparison of major forms of microscopy

Type	Description	Pros	Cons
Brightfield	Light transmitted through the specimens Contrast is enhanced by dyes	Simple and cost-effective	Most cells and tissues being transparent require dyes for visualization Fixation and sectioning of the specimen is required
Phase contrast	Contrast generated due to difference in refraction indices of cellular components due to varying densities	Live cells can be used	Chances of out of focus and higher noise-to-signal ratio
Darkfield	Scattered light is imaged emergent from obliquely illuminated sample	Sensitive High signal-to-noise ratio Can be used on live cells	Only structures that can scatter light are visible
Epifluorescent	Specimen illuminated at excitation wavelength and light is emitted by the excited fluorophores	Sensitive Specific Multiple structures can be probed at the same time Simple Fast High signal to noise ratio	Autofluorescence of cellular components can interfere Out of focus fluorescence can blurry images
Confocal	Out of focus illumination is blocked by pinhole apertures, allowing collection of only in-focus emitted light	Sharp in focus images are obtained	Longer image collection and processing times
Transmission electron microscopy (TEM)	Electron beams transmitted through ultrathin sections	High resolution	Skilled sample preparation, image interpretation and specialized equipment is required
Scanning electron microscopy (SEM)	Detects secondary electrons scattered off the surface of the sample	Provides 3D information	Skilled sample preparation, image interpretation, and specialized equipment is required

a central stop placed before the condenser allowing some but not all light to pass through the sample (see comparison of different microscopy types in Table 5.1).

As opposed to light microscopy, fluorescence microscopy uses ultraviolet (UV) light sources. Fluorescence microscopy is used in combination with fluorescent dyes (fluorophores), molecules that absorb light of one wavelength (the excitation wavelength that is shorter and has higher energy) and emit longer wavelength of light (the emission wavelength with lower energy). Generally, fluorescence microscopy uses epifluorescence in which the same objective is used for both illuminating

the sample with the excitation light and detecting emission light from the sample. A fluorescence filter cube separates both the lights based on wavelength and prevents interference. To allow visualization of the cellular molecules/structures with low intrinsic fluorescence, two methods are generally employed, (a) introduction of a fluorophore (like calcein AM, cyanine nucleic acid dyes) [11] or fluorescent tagged antibodies specific to proteins (immunofluorescence), into the cells [12] and (b) genetic introduction of a fluorescent protein like green fluorescent protein or red fluorescent protein into the genome of the cell under the control of an endogenous promoter [13]. The choice and use of fluorophores are restricted by their properties like Stokes shift. The distance between excitation and emission wavelengths (Stokes shift) is a key determinant in detection of emitted fluorescence in biological samples. Overlapping and indistinguishable emission wavelengths can be avoided by using tandem dyes that are composed of two covalently attached fluorescent molecules (one serving as the donor and the other as acceptor) and exhibit excitation properties of the donor and emission properties of the acceptor molecule. The principle behind the use of tandem dyes is Forster or fluorescence resonance energy transfer (FRET) that allows one fluorophore to pass its excitation energy to the acceptor fluorophore. Quantum dots are another type of fluorophores. These are nanocrystals which upon excitation emit wavelength proportional to the size of the particle. As size of the particle is highly controlled the emission can be controlled as well allowing advantage over conventional fluorophores. Common epifluorescence microscopes allow imaging four colors at a time: filter sets with 405-, 488-, 561-, and 640-nm excitation wavelengths are used. For immunofluorescence, typical dyes used are the nuclear stain 4′,6-diamidino-2-phenylindole, green dyes such as Alexa 488 or fluorescein, red dyes such as Cy3 or Alexa 568, and far-red dyes such as Cy5 or Alexa 647 [9] (see [14, 15], https://www.biolegend.com/en-us/multicolor-staining, and https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/fluorescent-probes.html).

Fluorescence in situ *hybridization* (FISH) is a variant technique of Southern blotting (see Sect. 5.6.1). In FISH, selected nucleotide probes coupled with reporter molecules are used to detect the target complementary DNA or RNA in interphase or metaphase chromosomes affixed to a microscope slide, bone marrow or peripheral blood smears or fixed and sectioned tissue [16]. These reporters can be radio labels, fluorophores (allowing direct detection) or a hapten (detected indirectly via enzymatic or immunologic reaction visualized via light or fluorescence microscopy) [17]. Reporter molecules generally used for direct detection include fluorescein isothiocyanate, rhodamine, Texas Red, while biotin, digoxigenin and dinitrophenol are used for indirect detection. Novel forms of FISH have been developed like single molecule (sm) FISH [18] and CASFISH [19]. In *sm*FISH multiple short single-stranded, fluorescent labeled DNA probes are used to hybridize to target RNA molecules in fixed cells. This way, both quantification and localization of cytoplasmic and nuclear RNA can be achieved at the single-cell level and single molecule resolution. In CASFISH (Cas9-mediated fluorescence in situ hybridization) fluorescent labeled in vitro constituted nuclease-deficient *c*lustered *r*egularly

*i*nterspaced *s*hort *p*alindromic *r*epeats (CRISPR)/CRISPR-associated caspase 9 (Cas9) complexes are used as probes to label sequence-specific genomic loci. As an important advantage over traditional FISH, CASFISH can be conducted without global DNA denaturation. Additionally, it is rapid and can be applied to primary tissue sections.

Confocal microscopy A major limitation of epifluorescence microscopy is that out of focus light is also detected by the camera resulting in blurry images with reduced contrast and resolution that is especially true for thicker sections and at higher magnification. Laser scanning confocal microscopy (LSCM) is useful in eliminating this out of focus light. In this technique the sample is illuminated (by laser beam) at single point in the sample focal plane and the emitted light is detected through a pinhole such that light emitted only from the sample focal plane is recorded by the detector and light from all out of focus planes is blocked by the pinhole. Thus, an optical section of the specimen at that focal plane is obtained. By changing the focal plane, several optical sections can be obtained [20]. Detectors used to create point by point image in LSCM are not as sensitive as cameras. Spinning disk confocal microscopy (SDCM) overcomes this limitation. In this, a disk of pinholes is employed that revolves over every point in the sample during single exposure. SDCM offers advantages of high speed and sensitivity and is a popular choice for live cell imaging and intracellular organelle dynamics [21].

In *total internal reflection fluorescence microscopy (TIRFM)* the excitation beam is entirely reflected at the coverslip/liquid interface generating an exponentially decaying fluorescence excitation wave that excites only the molecules near the glass coverslip [9]. TIRFM provides high signal-to noise-ratio and temporal resolution capability. TIRFM has been used to study membrane trafficking, cell cortex, and cytoskeletal dynamics [22–24].

Electron microscopy (EM) instead of light utilizes beam of electrons focused by coiled electromagnets to illuminate the specimen. Short wavelength and high focus ability of the electrons allow EM to achieve high spatial resolution compared to light microscopy. Two most common types of EM are transmission electron microscopy (TEM) and scanning electron microscopy (SEM). TEM generates image of the internal structure of a thin sample, SEM generates topological image of the sample [7]. In TEM image is formed by the electrons passing through extremely thin (<100 nm) specimen of fixed and preserved tissue. Specimens are pre-stained with electron dense heavy metals which show preferential binding to cellular structures like uranyl ions react strongly with phosphate and amino groups of nucleic acids and proteins, respectively. The stain absorbs or scatters part of the electrons of incident electron beam. Hence, this step allows enhancement of contrast among different cellular structures. In SEM, an image is formed as a high energy electron beam scans across the surface of a specimen producing different types of electron signals of different energies like X-rays, secondary electrons, etc. Like TEM, specimens for SEM are usually coated with a thin film of gold or platinum to improve contrast.

5.1.2 Applications of Microscopy in Medical Biotechnology

The main purpose of microscopy is visualization of real and unblurred biological structures. New super resolution microscopy techniques are being developed to look at smaller structures at very high resolution like the light sheet microscopy that allows imaging of whole organism at cellular level [10]. Microscopy, (1) allows better understanding of biological structures and processes; (2) aids diagnosis and treatment; and (3) assists in development of novel therapeutics [25]. Detailed discussion of microscopy applications to biomedicine is outside the scope of this chapter. Brief examples of the main applications are summarized. The study of the cellular degradation pathway of autophagy illustrates the use of microscopy in understanding biological processes [12, 26, 27]. Epifluorescence and LSCM are used to examine initiation of autophagy as well as its dynamics. TEM is a preferred method to visualize autophagic vacuoles enclosing organelles to be degraded. SDCM is used to monitor autophagic flux [28, 29]. Cellular localization of biomolecules, like RNA or protein localization, is also a frequently used application of microscopy. For example, binding of HKDC1 protein to mitochondria was visualized as colocalization signal of mitochondria specific dye and HKDC1 specific immunofluorescence [30]. Study of spatiotemporal interaction of two short chain fatty acid G protein coupled receptors on monocyte membranes and heterologous expression systems has been made possible with FRET [31]. Microscopic examination of tissues (biopsies by brightfield is classical example of use of microscopy) aids in clinical diagnosis of cancers, autoimmune diseases [32], and infectious diseases [33]. Chromosomal aberrations, aneuploidy, gene fusions for diagnosis of genetic diseases, and prognosis can be examined by FISH (see Box 5.1) [16].

Restriction mapping utilizes sequential DNA digestion by a combination of restriction enzymes (RE) with different target sequences. The resulting fragments are resolved by agarose gel electrophoresis allowing recognition of restriction sites across the DNA molecule. Subsequent combined digestion by both RE reveals a different number of sites. Lastly, partial restriction by any one of the enzymes proves diagnostic and allows complete identification of the map (Fig. 5.2). Restriction mapping, however, works well only for smaller DNA fragments (up to 50 kb) with fewer restriction sites. Second type of physical mapping method is FISH which allows direct visualization of marker DNA sequences due to

Box 5.1 Physical Maps and Techniques Utilized in Their Construction
Physical maps of DNA show the absolute location of landmarks like genes across a chromosome (Fig. 5.1) [34]. Three most important techniques to construct physical maps are restriction mapping, FISH and sequence tagged site (STS) mapping.

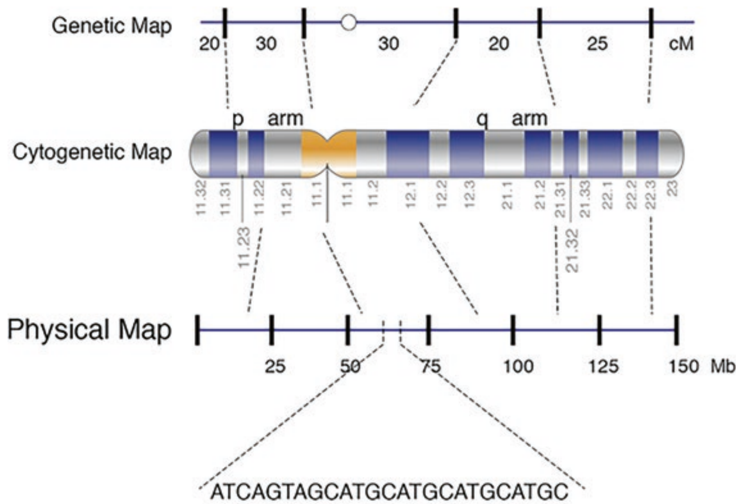


Fig. 5.1 Physical map of gene

hybridization with a fluorescent probe. FISH provides higher sensitivity and resolution over radioactive hybridization (basic concept of FISH has been covered earlier). Briefly, DNA is denatured to allow annealing of fluor labeled probe with specific sequence that can be visualized. Fluorolabels with different emissions are used to allow hybridization of multiple probes that allows simultaneous mapping of probe sequences.

Three different kinds of probes used by scientists are: *locus specific probes* that bind to a specific region of a chromosome allowing ascertaining location of an unknown gene in a chromosome or copies of a gene within a particular genome; *alphoid or centromeric repeat probes* bind to repetitive sequences found in the middle of each chromosome allowing scientists to determine if an individual possesses correct number of chromosomes. When used in combination of locus specific probes, alphoid repeat probes can also help determine loss of genes from a chromosome. Third kind of probes are *whole chromosome probes*. These are collection of smaller probes specific for different sequences along the length of a chromosome. When distinctly labeled *whole chromosome probes* set is used full color map of the chromosome, known as spectral karyotype is obtained. This is often used to identify chromosomal aberrations. FISH can also be used to compare evolutionary chromosomal arrangements of genes across species. FISH was originally used with highly condensed metaphase chromosomes allowing resolution of novel markers 1 Mb apart. Mechanically stretched metaphase chromosomes prepared by including centrifugation steps in isolation increases resolution of FISH to 200–300 kb apart markers. Use of uncondensed interphase chromosomes brings resolution down to 25 kb.

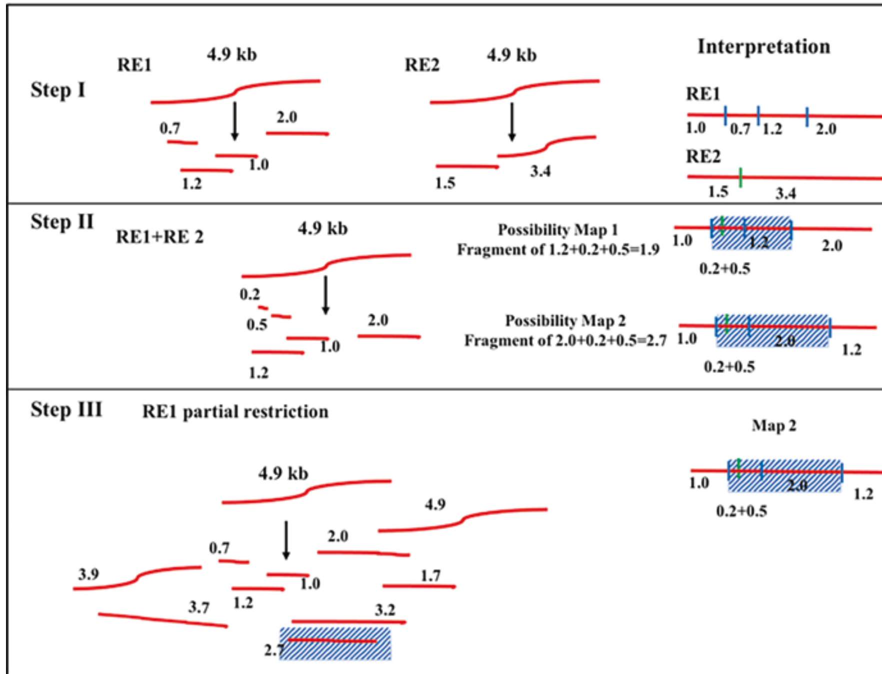


Fig. 5.2 Restriction mapping. Illustration demonstrating RE1 and RE2 sites in a linear 4.9 kb DNA molecule. Steps I and II depict single and double restrictions. Two possible maps that can be constructed post-double restriction are shown. Partial restriction using RE1 allows deduction of correct map shown in step III

Further resolution (to 10 kb) can be increased with use of purified DNA (in approach called as fiber FISH).

Most powerful technique for physical mapping of genome is STS mapping. The genomic DNA is fragmented using rare cutters (RE with rare target sites in the genome) into large overlapping fragments. These large fragments are cloned into high-capacity cloning vectors like the yeast artificial chromosome or in special rodent cells called radiation hybrids that lack their own genome but contain fragments of chromosomes from other eukaryotic cells. Two strategies can then be used to identify overlapping clones: clone fingerprinting and chromosome walking. In clone fingerprinting overlapping clones are identified based on common features in RE fragments or repeated content between the clones. In chromosome walking, hybridization of two clones to a particular STS marker is used for identification of the overlap. STS map is then generated by arranging the overlapping fragments to an STS marker using computational approaches. STS marker is a region, 200–300 bases long, that is found nowhere else in the genome. Two unique STS markers are Expression Sequence Tag (ESTs) obtained by partial sequencing of the cDNA clones and Simple Sequence Length Polymorphism (SSLPs) that are array of repeat

sequences that depict length variations. The STS are amplified by polymerase chain reaction (PCR) and used as probes or markers.

5.2 Spectroscopy

Spectroscopy is one of the most widely used analytical techniques for determination of identity or concentration of a substance. Spectroscopy can be defined as the study of the interaction of light and matter. This technique relies on absorption, emission or scattering of electromagnetic radiation by atoms or molecules. The absorption or emission of different forms of electromagnetic radiation is related to different types of transitions. Microwave radiation is associated with molecular rotational transitions, infrared radiation is associated with molecular vibrational transitions, and UV/visible radiation is associated with electronic transitions.

5.2.1 *Background and Types*

Properties of electromagnetic radiation The ranging of all types of electromagnetic radiation in order of their increasing wavelengths or decreasing frequencies is known as complete electromagnetic spectrum [35] (Fig. 5.3). The visible (from violet to red through rainbow colors) represent only little portion of the electromagnetic spectrum. The portion above the visible is called infrared while that below it is called ultraviolet. The boundaries between the regions are not sharp, though molecular processes associated with each region are quite different giving rise to different types of spectroscopic techniques (Table 5.2).

Two parameters used to describe the electromagnetic waves (Fig. 5.4) are frequency (v) and wavelength (λ). Frequency refers to the number of full wavelengths that pass by a given point in space every second; the SI unit for frequency is Hertz (Hz) where 1 Hz is 1 cycle per second. Wavelength and frequency are inversely proportional, thus, the shorter the wavelength, the higher the frequency, and vice versa. The relationship is given by the equation:

$$c = \lambda v \tag{5.1}$$

where c, the speed of light, is a constant. Hence, all electromagnetic radiations travel at the speed of light. A photon is an elementary unit of light and can be absorbed or emitted by the matter. When a photon is absorbed, its energy is transferred to the matter. Conversely, when matter loses energy, it emits a photon of an energy equal to the loss in energy of the matter. This change in energy is directly proportional to the frequency of photon emitted or absorbed. This relationship is described by Planck's equation:

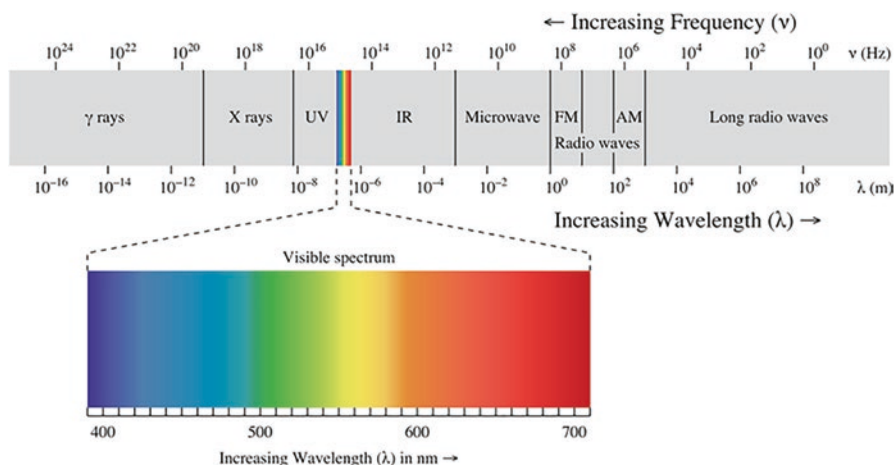


Fig. 5.3 The electromagnetic spectrum. Increasing wavelength regions are: X-ray region: energy changes involving the inner electrons of an atom or molecule, Visible and ultra violet: electronic spectroscopy (separations between the energies of valence electrons), Infrared region: Vibrational spectroscopy (change in molecular and vibrational states), Microwave: rotational spectroscopy (energy change involved is that of molecules arising from spin of electrons), Radio frequency: nuclear magnetic resonance (NMR) spectroscopy (energy change involved is that arising from spin of nucleus (nuclear spin))

Table 5.2 Spectroscopic techniques in different regions of electromagnetic radiation

Region of electromagnetic spectrum	Spectroscopic technique
Gamma ray	Mossbauer spectroscopy
X-ray	X-ray diffraction
Ultraviolet and visible	UV-visible spectroscopy Atomic absorption spectroscopy Fluorescence spectroscopy Phosphorescence spectroscopy
Infrared	Infrared spectroscopy Raman spectroscopy
Microwave	Microwave spectroscopy

$$E = h\nu \tag{5.2}$$

where E is the energy of the photon absorbed or emitted (in Joules, J), ν is the frequency of the photon (in Hz), and h is Planck's constant, 6.626×10^{-34} J·s. Using Eq. (5.1),

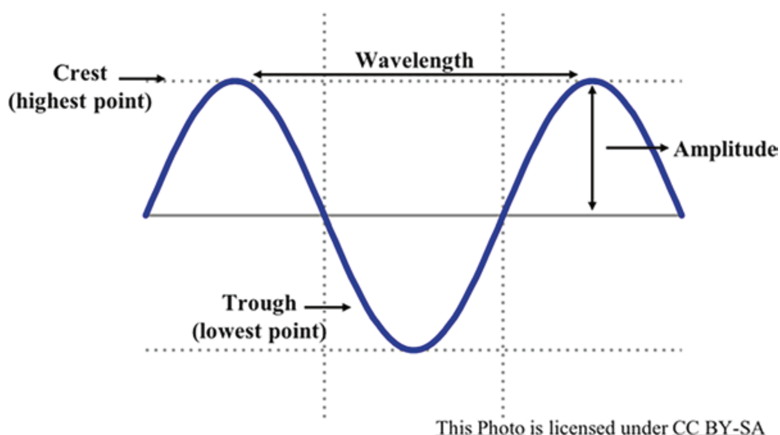


Fig. 5.4 Schematic representation of electromagnetic radiation: wavelength, horizontal distance between two consecutive crests or troughs; amplitude, vertical distance between the tip of the crest and central axis of the wave

$$E = h\nu = h\frac{c}{\lambda} \tag{5.3}$$

5.2.2 Nuclear Magnetic Resonance (NMR)

NMR is a robust and theoretically composite analytical tool. In NMR experiments are carried out on the nuclei of atom, and not on electron [36]. *Nuclear SPIN* Subatomic particles (electrons, protons, and neutrons) possess angular spin momentum. Only those nuclei which possess a finite value of spin quantum number $I > 0$ will process along the axis of rotation. The rules governing the net spin of nucleus are as follows:

Nuclei with both protons and neutrons even (charge mass even): Zero Spin.
Nuclei with proton and neutron odd (charge odd but mass = $p + n$, even): 1, 2, 3….
Nuclei with odd mass ($n + p$ = odd): 1/2, 3/2, 5/2.

A spinning nucleus can have $2I + 1$ possible orientations. For proton $I = ½$, therefore proton can have two possible orientations with respect to external magnetic field (Fig. 5.5). If a proton is placed in a magnetic field, then the energy splits into two orientations with respect to the axis of the external field: parallel with the field and antiparallel with the field. Each alignment is defined by magnetic quantum number, m. Preferred alignment is in parallel direction in which there is net excess of protons. From the high energy opposed orientation, a proton can come back to low energy (stabilized form) by losing energy. The transition from one energy state to other is called flipping of the proton. This transition between two energy states

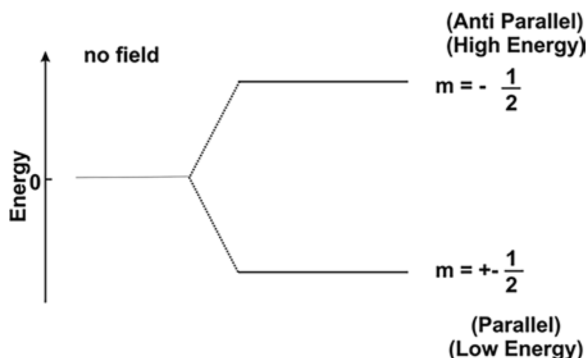


Fig. 5.5 Possible energy levels of a nucleus with spin quantum number ½

can be brought about by the absorption of a quantum of electromagnetic radiation in the radiowave region with energy $h\nu$. *Calculating transition energy* The energy required to flip the proton depends upon the strength of the external magnetic field. Stronger the field, greater will be the tendency of the nuclear magnet to remain lined up with it and higher the frequency of radiation needed to flip the proton to the higher energy state. Transition energy is calculated with the equation, $\nu = \gamma H \div 2\pi$, where ν is the frequency in cycles per second, H is the strength of magnetic field in gauss, and γ is the magnetogyric ratio that is a fundamental nuclear constant which has different values for every nucleus. Once any organic molecule is exposed to a powerful field, the protons in the substance will precess at different frequencies. These precessing protons are exposed to steadily changing frequencies (to promote or flip protons from the low energy state to high energy state) and measure the frequency at which absorptions occur. If the beam has the same frequency as that of precessing particle, it can interact coherently with these particles and energy can be exchanged. Absorption happens and signal is observed. This phenomenon is called resonance, thus for nuclei it is called nuclear magnetic resonance (NMR) [37].

Chemical Shift When a molecule is put in a magnetic field, its electrons give rise to secondary magnetic field, i.e. induced magnetic field. Spinning of electrons about the proton itself leads to generation of field in such a way that it opposes the applied field. Thus the field felt by the proton is reduced and the proton is said to be shielded. On the other hand, if the induced field reinforces the applied field, the proton feels a greater strength and as such is said to be deshielded. Shielding shifts the absorption upfield and deshielding shifts the absorption downfield to get effective field strength necessary for absorption. Such shifts in the position of NMR absorptions which arise due to shielding and deshielding of protons by electrons are called chemical shifts. For calculating chemical shifts of various protons in a molecule, the signal for tetramethyl silane (TMS) is taken as a reference. Instrument design of NMR spectrophotometer consists of: sample tube, magnet, radio frequency source, radio frequency detector and amplifier and recorder. The sample to be investigated is placed inside a glass tube and is placed between the poles of a magnet. A radiofrequency is made to fall on the sample under precess. A signal is generated if the nucleus in the sample resonates with the source. Δ E is energy needed to flip the

proton and is equal as that of the source. Energy is transferred from the source via nuclei to the detector coil. Samples in solutions are rotated frequently to eliminate discrepancies. This process helps in generating a uniform signal and different set of protons come to resonance and the signal from the detector produces a peak on the chart paper. Each peak in the NMR spectrum means a set of protons.

5.2.3 UV-Visible Spectroscopy

UV-visible spectroscopy is one of the oldest analytical techniques established on the interaction of light in UV and visible regions with the matter (an analyte) at a particular wavelength. The analyte absorbs light of specific wavelength yielding a characteristic spectrum. Therefore, a unique relationship exists between each analyte and its UV/visible spectrum. The spectrum can therefore be used to quantify or identify an analyte [38].

Light is absorbed by a molecule when the energy of the light is greater than or matches the energy difference between two electronic states. The electrons upon absorption of a photon of light get excited to higher energy, unstable level, and return to ground state again by emitting photon of light. This process of electronic transitions is unique to a molecule. Three different kinds of electronic transitions have been described:

σ–σ^* high energy requiring transition of electrons in strong single covalent bonds.
π–π^* relatively low energy requiring transition of electrons in double or triple bonds.
n–σ^* or n–π^* lowest energy requiring transition of lone pair of electrons to antibonding orbital.

Principle UV-visible spectroscopy works on the phenomenon of absorption of light by a substance and that absorption of light is directly proportional to the concentration of the substance. A typical absorption spectrophotometer measures the intensity of the incident light at each wavelength before (I_0) and of the emitted light (I) after it passes through the solution. The quantity of light absorbed is expressed as percent transmittance (I/I_0) or more frequently as absorbance or optical density (the negative log of transmittance). The amount of light absorbed is related to the concentration of the substance through Beer's and Lambert's law.

Beer's Law states that the log of the ratio of intensities of incident light and emergent light is directly proportional to the concentration of the chromophore in a solution provided the thickness of the solution through which the light passes is constant.

$$\log\frac{I_0}{I} = K1c \tag{5.4}$$

where I_0 = Intensity of the incident light, I = Intensity of the emergent light, c = concentration of the chromophore, $K1$ = constant depending upon the wavelength of the light, nature of the chromophore, and thickness of the solution.

Lambert's law states that the log of the ratio of the intensities of incident light and emergent light is directly proportional to the thickness of the solution through which light passes provided the concentration of chromophore is constant.

$$\log \frac{I_0}{I} = K2L \tag{5.5}$$

where, L = thickness of the solution traversed by light, $K2$ is a constant depending upon the wavelength of incident light, nature of the chromophore and its concentration.

Beers–Lambert's law which is a combination of Beer's law and Lambert's law is,

$$\log \frac{I_0}{I} = \varepsilon c L \tag{5.6}$$

where ε is the molar absorptivity or extinction coefficient that depends upon the wavelength of the incident light and the nature and concentration of the chromophore. Its units as deduced from the Eq. (5.6) are L mol^{-1} cm^{-1}.

Thus, Beer–Lambert's law states that the log of wavelength of the incident and the emergent light is directly proportional to the concentration of the chromophore and the thickness of the solution through which the light passes. The ratio between two intensities I/I_0 is defined as transmittance. The relation between absorbance and transmittance is expressed as

$$A = 2 - \log_{10} T\% \tag{5.7}$$

The main components of the UV/visible spectrophotometer are a light source (deuterium, hydrogen, tungsten, mercury, and xenon lamps are commonly used UV sources and tungsten, mercury vapor, and carbon lamps are used as visible sources), a sample, holder, a dispersive device to separate the different wavelengths of light (a monochromator), and a suitable detector.

5.2.3.1 Absorption and Intensity Shift in UV-Visible Spectroscopy of Biomolecules

The absorption spectra of nucleic acids in the 200–300 nm region is due to purine and pyrimidine bases and in region <200 nm is due to the backbone. Absorption spectrum of nucleic acids is sensitive to perturbations in the environment. This makes UV-visible spectroscopy a perfect qualitative and quantitative tool for nucleic acids. Nucleic acids give maximum absorbance at 260 nm while proteins absorb maximally at 280 nm. Thus, the ratio of absorbance at 260–280 nm of 1.8–2.0 denotes pure DNA. Concentration of nucleic acids can be extrapolated by the following absorbance at 260 nm (see Box 5.2). For peptides and proteins, amide bonds, amino acid side chains and prosthetic groups like hemes contribute to absorption

Box 5.2 Some Important Concentration Terms and Applications of UV-Visible Spectroscopy

- As a rule, for a 1-cm pathlength, the absorbance at 260 nm equals 1.0 for the following solutions of nucleic acids:
  - a 50 μg/mL solution of double-stranded DNA;
  - a 33 μg/mL solution of single-stranded DNA;
  - a 20–30 μg/mL solution of oligonucleotide;
  - a 40 μg/mL solution of RNA.
- Applications of UV-visible spectroscopy.

spectrum. Spectra in the far UV 170–250 nm is dominated by contributions of the peptide bonds and in the near UV region 250–300 nm originate from aromatic amino acids with contribution from disulphide bonds. Among the aromatic side chains, tryptophan residues give a peak at 279 nm with several transitions in 240–290 nm region, tyrosine gives a peak at 274 nm and phenylalanine at 258 nm.

This simple and quick technique finds multiple applications in biomedical/research field. Some are enumerated as follows:

1. Determination of molecular weight: this is usually achieved with formation of derivatives of an unknown compound.
2. Detection of impurities: This is one of the most important uses of UV-visible spectroscopy. Absorption spectra is characteristic of each compound and hence any additional peaks depict impurities present in the sample. Additionally, the nature and identity of the impurities can also be judged from the peaks.
3. Quantitative analysis: Using Beer and Lambert's law concentration of the compounds can be determined.
4. Qualitative analysis: Unknown compounds can be characterized based on identity of their absorption spectrum with that of reference compounds in spectral libraries.
5. Detection of functional groups: As different chromophores have distinct absorption peaks, their absence or presence can be detected with UV-visible spectroscopy
6. Chemical kinetics: Based on changes in absorption of the reaction intermediates and final product the progress or kinetics of an enzymatic reaction can be followed by UV-visible spectroscopy.
7. Denaturation or deviation from physiological conformation: As biomolecules exist in a preferred native physiological state, deviations arising in this state due to effect from external environment (change in pH or temperature, binding of ligands, aggregation) can be detected by UV-visible spectroscopy.

5.2.4 CD Spectroscopy

Circular dichroism (CD) spectroscopy is a crucial technique to investigate protein folding, form and function. It can give rapid and authentic information about the folded conformation and stability of a protein. This technique has two major advantages: it requires use of small amount of test material in physiological buffers and hence, can be used to monitor any structural alterations that might result from changes in environmental conditions, such as pH, temperature, and ionic strength. CD depends on chirality (asymmetric nature) of the molecule. Protein chromophores are generally achiral. It is the interaction between the chromophores and chiral protein field that leads to optical activity [39].

Circular Dichroism can be defined as unequal absorption of left-handed and right-handed circularly polarized light ($\Delta E = E_L - E_R$). As CD is an absorption phenomenon, chromophores contributing to CD spectrum are same as those contributing to absorption spectrum.

A beam of light has time-dependent electric and magnetic fields that oscillate perpendicularly in direction of light wave propagation (Fig. 5.6). If the light is polarized by passing through suitable prisms or filters, its electric field, *E*, rotates around the propagation axis maintaining a constant magnitude. When viewed from

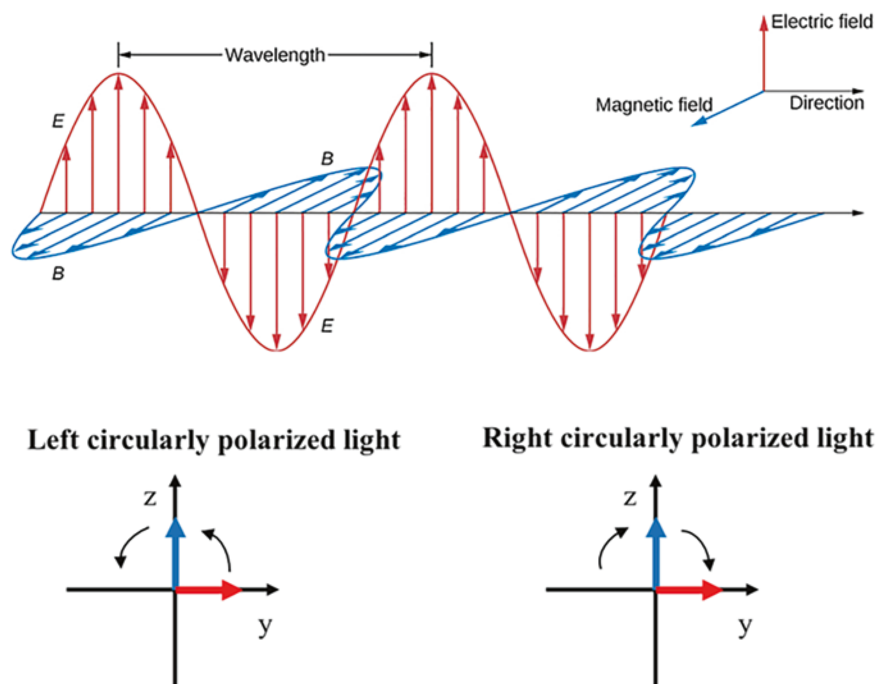


Fig. 5.6 Light polarization in the electric and magnetic fields (upper panel, vertically polarized light and bottom panel, left and right circularly polarized light)

the front, the axis of propagation of the electric field appears to trace to a circle consisting of two vectors of equal length, one that rotates clockwise (E_R) and the other that rotates counterclockwise (E_L).

These waves are at 90° phase difference to one another and can be separated using a variety of prisms or electronic devices. When asymmetric molecules interact with light, they may absorb right- and left-handed circularly polarized light to different extents (hence the term circular dichroism) and the resulting electric vector traces out as an ellipse with the light said to be elliptically polarized. CD is reported either in units of ΔE, the difference in absorbance of E_R and E_L by an asymmetric molecule, or in degrees ellipticity, which is defined as the angle whose tangent is the ratio of the minor to the major axis of the ellipse. $[\theta]$, the molar ellipticity in deg cm^2 dmol^{-1} = 3298ΔE [39].

In the CD spectrometer the sample is placed in a cuvette and a beam of linearly polarized light emerging from successive passage through a monochromator (that yields single wavelength light) and photoelastic modulator (that yields circular polarized light) is incident on the sample. The incident light on the sample switches direction of polarization resulting in absorption changes and the differential molar absorptivity is calculated.

5.2.5 Applications of Spectroscopy in Medical Biotechnology

X ray diffraction has been extensively used for the determination of the structure of proteins. The structures revealed are all stored in the protein data bank. X-ray fluorescence spectroscopy is a used to determine the elemental structure of almost all types of materials [40]. *Spectrophotometry* is routinely used for qualitative and quantitative analysis of substances in pure and biological mixtures, obtaining differential absorption spectra, monitoring the binding of monoclonal antibody with its antigen (second order differential spectroscopy) and for structural studies of protein and nucleic acids. *Atomic absorption spectroscopy* finds uses in the detection of trace elements in liquid and estimation of metal content of the sample. *Spectrofluorimetry* Fluorescence assay helps in understanding the physiochemical properties of proteins and other macromolecules. It also helps in tracing the interaction of protein with one another protein and with other smaller molecules. The fluorescence properties of DNA and RNA are of particular interest for the performance of molecular biological studies. Reaction mechanisms of analgesic, antibiotics, cardiovascular drugs, alkaloids, and muscular relaxants can also be done fluorometrically.

InfraRed FTIR is a highly sophisticated analytical technique and helps in correlating mixed sample profile to biological origin, metabolic status, and exposure to chemical or environmental stimuli. FTIR microspectroscopy is used for studying human arteries, cancers, tumors, and brain tissues. It is also used widely in toxicological studies.

Raman Spectroscopy is used for the identification of traces in semen, blood, saliva, sweat during forensic investigations [41]. In vivo Raman spectroscopy has been used to measure levels of the antioxidant molecules leutin and zeaxanthin in the retina of eyes. Manoharan et al. [42] and Lawson [43] have reviewed the application of Raman spectroscopy to study human arteries, tumors, and gall-stones. NMR is used in the determination of molecular structure and conformational changes, for example, high resolution protein structures of thioredoxin, plastocynanin, and interleukins are determined by NMR. It is also used to study in vitro enzyme kinetics of enzymes such as trypsin, chymotrypsin, pepsin, and papain. DNA-binding (drug-DNA and DNA-binding proteins) is studied by NMR. Phosphate concentration both in intracellular and extracellular spaces can be measured in living cells and tissues by NMR. Chief use of CD spectroscopy is in the study of biomolecules like protein and nucleic acids: determining secondary structure of proteins by far-UV CD, examining unfolding of proteins in presence of denaturing conditions by far- and near UV CD (see Box 5.3); studying protein–ligand, protein–nucleic acid, and protein–protein interactions.

Box 5.3 Use of CD in Determining Higher Order Structure of Biomolecules

CD is often used to determine higher order structures of *biosimilars* and compare them to original molecules. This is a criterion for approval of biosimilars by the Food and Drug Administration (USA). Biosimilars are a generic version of a biotherapeutic protein. Biocomparability and analytical comparability to the original biotherapeutic is required for the development of biosimilars [44]. CD bands of protein and peptides appear in two regions. The amide region (far UV 170–250 nm) is dominated by contributions of the peptide bonds. Two electronic transitions are responsible for this region: weak $n–\pi^*$ transition occurring as a negative band around 220 nm and strong $\pi–\pi^*$ occurring as a positive band around 192 nm and a negative band around 210 nm. CD bands in the near UV region (250–300 nm) originate from aromatic amino acids. Disulphide bonds give rise to several spectral bands and protein lacking chromophores other than amino acids do not give bands beyond 300 nm. Furthermore, each structural element of proteins have characteristic CD spectra: α-helical proteins have negative bands at 222 and 208 nm and a positive band at 193 nm; antiparallel β-pleated sheets (β-helices) have negative bands at 218 nm and positive bands at 195 nm, while disordered proteins have very low ellipticity above 210 nm and negative bands near 195 nm. These characteristics are utilized to compare a biosimilar to original biotherapeutic. For example, biosimilars are being developed for filgrastim, a recombinant human granulocyte-colony stimulating factor, widely used to treat congenital and acquired neutropenia [45]. Fig. 5.7 depicts CD comparison of filgrastim (black line) and biosimilars suggesting that they all share rich α-helical structure with positive band at 193 nm and negative bands at 208 and 222 nm.

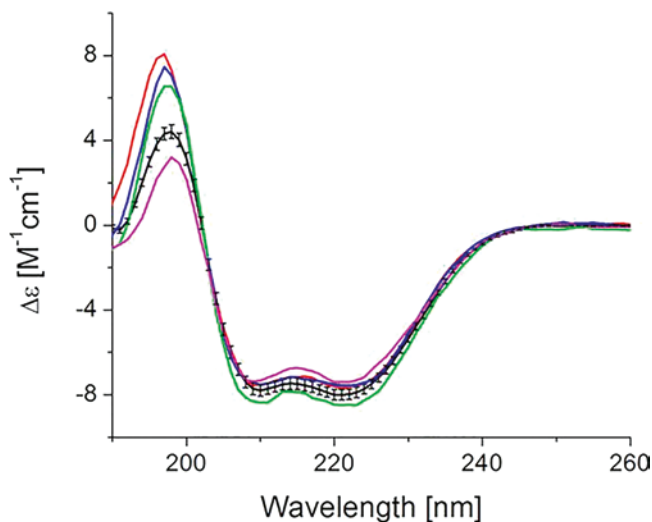


Image from Halim, L.A., Márquez, M., Maas-Bakker, R.F. *et al.* Quality Comparison of Biosimilar and Copy Filgrastim Products with the Innovator Product. *Pharm Res* **35,** 226 (2018), open access by **CC-BY 4.0.**

Fig. 5.7 CD spectra of filgrastim (black line and red line: two different batches) and different biosimilars (red, blue, green, and pink lines)

5.3 Chromatography

Chromatography is a technique by which two or more compounds in a mixture are physically separated by distributing between two phases: a stationary phase which can be a solid or liquid supported on a solid and a mobile phase either a gas or a liquid which flows continuously around the stationary phase. The separation of the components results from the relative difference in the affinity of individual components of the mixture for the stationary phase.

5.3.1 Principle

Chromatography is the most widely used separation technique for chemical analysis. The technique is based on the basic principle where molecules in form of a mixture are placed on a surface, a solid or fluid stationary phase. The components of the sample partition between the two phases, the stationary bed with a greater surface area and the other either liquid or gas known as the mobile phase, which penetrates and move through the stationary phase. Mobile phase carries the sample through the column. Samples equilibrate between the two phases based on the degree of solubilities or their tendencies to get absorbed in each of the respective

phases. The sample components called mainly as solutes or analytes partition from each other on the basis of their affinities for both the phases, which is known as elution chromatography.

The factors operative on this partitioning process include molecular characteristics like adsorption and partition in case of liquid–solid phases, molecular weight differences, and affinity, etc. [46]. Due to these differences, some of the mixture components stay longer in the stationary phase and move at a slower pace, while others move at a steady and rapid pace and leave the system earlier [47]. There are three major components of the technique: *Stationary phase:* The phase is generally a solid phase or a solid phase anchoring a layer of liquid adsorbed on the surface. *Mobile phase:* This phase generally comprises of liquid or gaseous components which move through the stationary phase. *Separated molecules:* The basic effective component on separation of molecules from each other is the type of interaction between stationary phase, mobile phase, and substances contained in the mixture.

Types of chromatic techniques and their applications in molecular analysis: The chromatographic methods are classified basically on the stationary and mobile phases.

Column chromatography This technique is used for the purification of biomolecules. On a column (stationary phase) firstly the sample to be separated and then wash buffer (mobile phase) are applied. Their flow inside the column material placed on a fiberglass support is ensured. The samples are accumulated at the bottom of the device in a time- and volume-dependent manner [48]. Since a column is used in this partitioning technique, hence the name column chromatography (Fig. 5.8). This column is filled or packed with a solid adsorbent, the stationary phase (e.g., silica gel or beads). Column chromatography is used to carry out all the major types of chromatographic techniques. These are: Adsorption, Ion exchange,

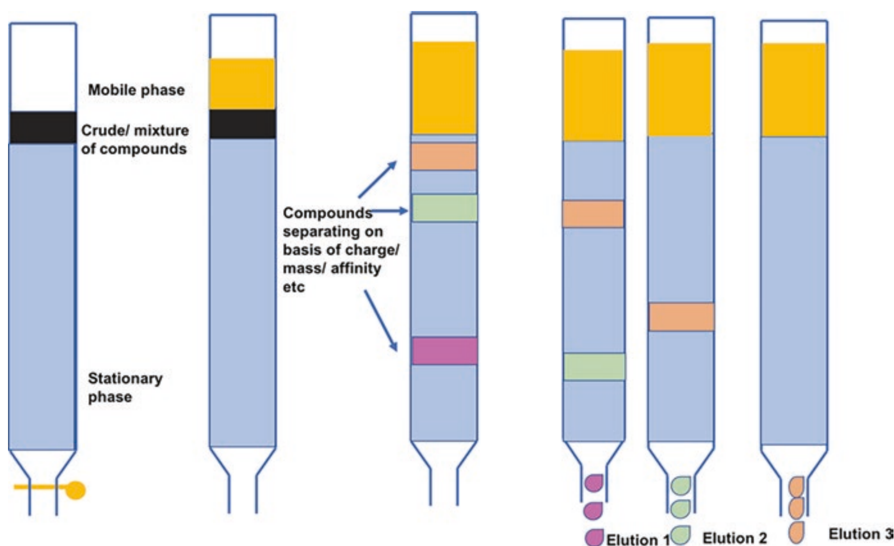


Fig. 5.8 Basic overview of column chromatography

Table 5.3 Protein properties forming basis of different chromatographic procedures

Protein property	Technique
Size	Size exclusion chromatography
Charge	Ion exchange chromatography
Biorecognition (ligand specificity)	Affinity chromatography

Gel filtration, Affinity, Gas, and High-performance liquid chromatography (Table 5.3). Major uses include separation of proteins and other molecules [49, 50], amino acids [51], and quantitative and qualitative analyses, etc.

Paper chromatography The support material here is thick filter paper (made of cellulose) which is saturated completely with water. Water droplets in the pores of the paper settle down acting as a stationary liquid phase. Mobile phase is kept in a tank, and hence, this method is a liquid–liquid chromatography technique. Paper chromatography finds major uses in the estimation of peptidase activity in white blood cells [52], desalting biological fluids [53], determination of urinary amines [54], detection of compounds such as imidazole [55], and identification of anaerobic bacteria [56].

Gas chromatography (GC) is used for analysis of mixtures containing inorganic or organic compounds that have vapor pressures greater than about 0.1 Torr (10 Pa) at instrument operating temperatures. GC can be used to analyze solids, liquids, and gases. Amounts ranging from 10^{-2} to 10^{-12} g can be introduced and analyzed. The instrument has five main components: Carrier Gas Supply, Sample Introduction, Columns, Temperature Programming (PTGC), and Detectors. GC is characterized by chromatographic distribution of either a gas mobile phase on a solid adsorbent (gas-solid chromatography) or a liquid on an inert support (gas-liquid chromatography). GC can be hyphenated with various detection techniques such as GC combined with mass spectrometry (GC-MS), GC combined with tandem mass spectrometry (GC-MS/MS) or GC combined with time-of-flight mass spectrometry (GC-TOF-MS), thus greatly increasing the versatility, sensitivity and accuracy of the method [57, 58]. GC has been extensively used in separation of natural compounds [59, 60], in identifying anaerobic bacteria [61, 62] and their metabolic products [63–65]. The head-space GC technique has been used mainly in dairy research, e.g., for the evaluation of microbial contamination of food [66–69]. It has also been used in analyses of fungi [70].

Thin-layer chromatography (TLC) is classified as a "solid-liquid adsorption" chromatography (Fig. 5.9).

The stationary phase in this method is a solid adsorbent material like alumina, silica gel, and cellulose coated on a glass plate. In this method stationary phase is used by the mobile phase to travel upwards, the solvents move up the plate soaked with the solvent in a capillary action. This upward traveling rate depends on the solid phase, polarity of the material and of the solvent [71]. If sample molecules are colorless, radioactivity can be used to produce a visible colored product so as to identify their positions on the chromatogram. Visible color formation can be observed under UV light. The location of each molecule in the mixture can be determined by calculating the ratio between the distances moved by the molecule and the

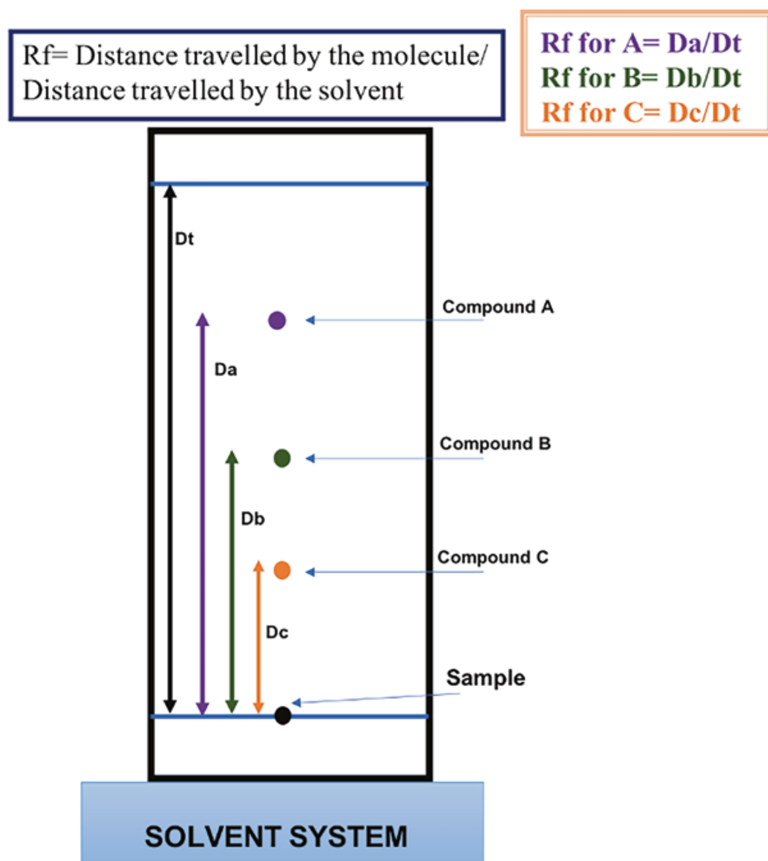
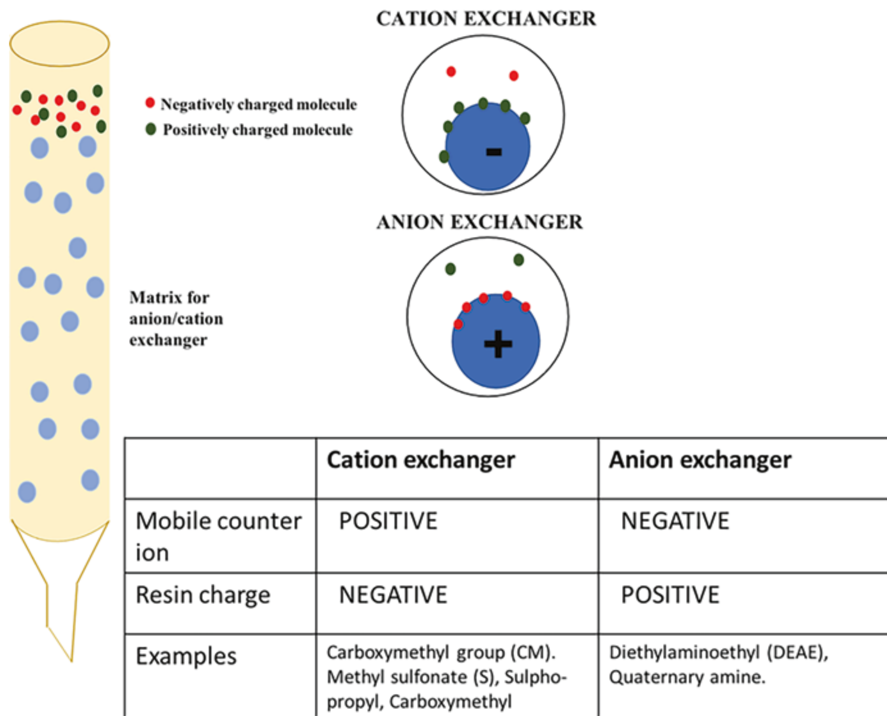


Fig. 5.9 Basic principle of thin layer chromatography (TLC)

solvent. This measurement value is called relative mobility (Rf). This value is used for qualitative description of the molecules [72].

Ion-exchange chromatography (IEC) is based on electrostatic interactions between solid support material (matrix) and charged protein groups. The matrix has an ion load inverse to that of the targeted protein for separation, and the affinity with which the protein binds to the column is achieved with ionic ties (Fig. 5.10).

Proteins are detached from the column either by changing the concentration of the ion salts, pH or the ionic strength of the buffer [73]. Anion exchange matrices carry positive charged ions and adsorb negatively charged proteins. While cation-exchange matrices which are bound with negatively charged groups adsorb positively charged proteins [74]. The protein holds surface charges depending on pH and pI (isoelectric point) of the environment. The ion exchanger is made of a base matrix generally in the form of porous beads to offer enough surface area for adsorption. Above its pI, protein has negative charge and attaches to anion exchanger, below pI, protein is positively charged and binds to cation exchangers. The ion



	Cation exchanger	Anion exchanger
Mobile counter ion	POSITIVE	NEGATIVE
Resin charge	NEGATIVE	POSITIVE
Examples	Carboxymethyl group (CM). Methyl sulfonate (S), Sulpho-propyl, Carboxymethyl	Diethylaminoethyl (DEAE), Quaternary amine.

Fig. 5.10 Ion exchange chromatography

exchanger behaves itself as an acid or base and the disproportionation of the charges rely on the pH. Major uses of IEC include, de novo purification, purification of a protein with known pI, size, and primary structure, preparative and industrial separation of proteins including high resolution separation of protein variants and/or isoforms [75].

Size exclusion chromatography (SEC) also known as molecular sieve chromatography works on the basic principle of separating macromolecules based on their variances in molecular sizes. This technique is basically used to determine proteins' molecular weights, and for concentrating protein solutions [76]. In a SE column stationary phase consists of inert molecules with small pores. A solution which contains molecules of different dimensions are continuously passed at a constant flow rate through the column. Molecules having larger size than the pore cannot pass through the gel particles and pass through the free spaces between the porous particles, moving rapidly out of the column. Whereas smaller molecules are diffused in the pores and they leave the column later, having longer retention time [77].

Sephadex G type is the most commonly used column material. Besides, agarose and polyacrylamide are also used [77]. SEC is the predominant technique used to monitor protein aggregation because of both its reproducibility and speed [78–81] (see Box 5.4). SEC is also a precise method if validated with an orthogonal method, such as sedimentation velocity analytical ultracentrifugation (SV-AUC) [82–84].

Affinity chromatography is based principally on the molecular recognition of a target molecule by a molecule previously bound to a column. It has been reported that over 60% of all purification techniques encompasses affinity chromatography [89]. Affinity purification has three major steps: incubation-crude sample is incubated

Box 5.4 Size Exclusion Chromatography (SEC)

SEC is a commanding tool for the separation of biotherapeutics such as antibody drug conjugates, monoclonal antibodies (mAb), biosimilars, and bispecific mAbs. Immunogenic responses are caused by heterogenic impurities in proteins. Greater than 99% purity is needed for the medicinal use. SEC is used to screen this purity level in the quality control process of the biopharmaceutical industry [85]. SEC is often implied as a tool to help in manufacturing process and formulation development. As a part of the manufacturing procedure development SEC can be important for guide cell-line selection. These data in not only allow choosing a cell-line that gives the lowest levels of aggregates, but can also differentiate amongst aggregate forms which may be difficult to eliminate during downstream purification steps [86]. Another important use for SEC during cell-line development is to guarantee that the specific activity of the purified protein is not underreported or in rare cases over-reported as a result of increased aggregation [87, 88].

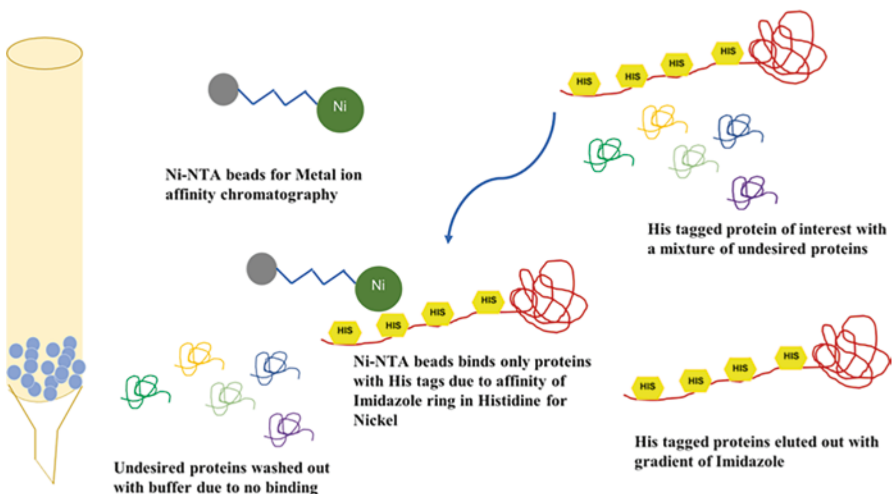


Fig. 5.11 Metal affinity chromatography

with the affinity support to let the target molecule in the sample bind to the immobilized ligand, washing-unbound sample components from the support are washed out with the buffer, elution-for the dissociation and recovery of the molecule targeted from the immobilized ligand by varying the buffer conditions so that the binding interactions are seized [90]. This chromatography technique is used for the purification of hormones, enzymes, nucleic acids, antibodies, and specific proteins [91]. Some of the most commonly used affinity chromatography techniques are Ni-NTA affinity and GST tagged chromatography. Ni-NTA Agarose is used majorly for purifying recombinant proteins carrying a His tag. Histidine residues (His tag) bind to the empty positions in the coordination sphere of the stationary nickel ions, immobilized on the matrix with high affinity and specificity (Fig. 5.11). His-tagged proteins are bound, and other proteins pass through the matrix. After washing, His-tagged proteins are eluted in buffer under native or denaturing conditions. Glutathione-*S*-transferase (GST) is 26 kDa, 211 amino acid long protein widely used as affinity tag that increases solubility of the desired protein. GST tag shows affinity for immobilized glutathione and is used for prokaryotic expression more commonly.

It can be bonded to either the N-terminus or C-terminus of a protein. Glutathione Affinity is an effectual technique for single-step purification of proteins fused to a GST tag. GST can be expressed in the *E. coli* cytoplasm as a soluble protein in high amounts and with full enzymatic activity.

5.4 Radioisotopes and Their Application in Biological System

Radioisotopes are elements that produce radioactivity. Radioactivity is a process by which an unstable nucleus undergoes conversion to an energetically stable form by emitting energy in the form of particles (alpha, beta or gamma) and photons. Alpha emitting radioisotopes are rarely used for biological work as their decay releases strongly ionizing alpha particles. In beta decay, a proton or neutron is lost by any of these reactions: negatron or beta emission ($n^0 \rightarrow p^+ + e^-$), positron emission ($p^+ \rightarrow n^0 + e^+$) and electron capture ($p^+ \rightarrow n^0 + e^-$) where n is the neutron, e^- is the electron, e^+ is the positron, and p is the proton. Beta emission is accompanied by the release of neutrinos and gamma rays (electromagnetic rays of short wavelength) and electron capture is accompanied with emission of gamma and X-rays. Most radioisotopes used in research and medicine decay by beta emission like tritium, beryllium-7, strontium-90, caseium-137. Iodine-125, chromium-51, cobalt-57 are some radionuclides used in research and medicine that decay by electron capture. For extended reading on radioisotopes see [92, 93].

Use of radioactive phosphorus in biological system was first propounded by Hevesy in 1935 [94] followed by use of carbon-14, fluorine-18, oxygen-15, thallium-201. This culminated in the use of iodine-131 for treatment of hypothyroidism

in humans [95]. Now several radioisotopes are used routinely for diagnostic applications like technetium-99m (^{99m}Tc) in single photon computer tomography (SPECT) and others have been approved for pharmaceutical use by US FDA like radium-223 (^{223}Ra) for bone metastases [96]. Though the use of certain radioisotopes accompanies undesirable health/environment effects, high accuracy, high sensitivity and ability to capture natural milieu makes their use inevitable in various biomedical techniques. Traditional uses of radioisotopes in biomedicine include tracer techniques (determining the fate of biomolecules, e.g., of pyruvate in citric acid cycle), metabolic studies (e.g., protein turnover, glucose uptake, nucleic acid metabolism), quantitative determination of analytes in biological fluids (e.g., determining concentration of hormones in plasma by radio-immunoassay) [97, 98]. Another important application of radioisotopes in biomedical research is for determination of subcellular localization of biological macromolecules. It is usually achieved by differential centrifugation (size and density dependent separation of organelles in an ultracentrifuge) that separates cell homogenates into distinct fractions. These fractions or tissue/cell homogenates can then be separated by electrophoresis (see Sect. 5.5) and radiolabeled antibodies or nucleic acid probes can be used for localization of specific protein (Western blotting, see Sect. 5.6) or nucleic acid (in situ hybridization). Through this technique, however, molecular location and spatiotemporal changes in macromolecules location cannot be determined.

5.4.1 Histochemistry, Immunohistochemistry

Radioisotopes are widely used for in vivo imaging, an aspect not discussed here. Histochemistry using nucleic acid probes (in situ hybridization, ISH) and immunocytochemistry (ICC) using antibodies are two other approaches used for detection of macromolecules in their specific spatial settings. With radioisotopes these techniques provide the advantage of quantitation, not available or difficult with non-radio isotopic techniques. In ICC, cells and tissues are processed in similar way as for regular microscopy. The secondary antibodies used for detection of target-bound primary antibody are radiolabeled (e.g., with iodine-125) or are biotinylated and hydrogen-3 labeled biotin-streptavidin complex is used for detection. The binding is detected by autoradiography. Autoradiography is a technique for visualization of radioisotope distribution in a sample using X-ray film, the radioactive emissions (beta and beta/gamma) react with silver halide in the film resulting in formation of elemental silver that can be detected with photographic development of the film. For weak emitters, like hydrogen-3, indirect autoradiography is used. Here, a scintillator is used. The weak emissions excite the scintillator and emitted ultraviolet light produces photographic image on the film. For extended reading see [99]. In situ hybridization uses radiolabeled nucleic acid probes. ISH works on same principle as FISH (see Sect. 5.1.1) and provides information on spatial localization of a gene on a chromosome, of transcript localization within the cell and on intracellular location of viral nucleic acids. Phosphorus-32, phosphorus-33, sulfur-35, hydrogen-3, and

iodine-125 are preferably used in ISH. The tissue/cell autoradiographs are visualized using light microscope. And the number of copies of the target can be counted by (silver) grains over cells.

5.5 Electrophoretic Techniques

Electrophoresis refers to the separation of molecules carrying charge in an applied electric field. Mobility of molecules is dependent on several factors, most important of which are charge/mass ratio, net charge, molecular shape and the temperature, viscosity and porosity of the matrix through which the molecule moves. Complex mixtures can be separated at very high resolution by this process [100]. If a mixture of electrically charged biomolecules is kept in an electric field of field strength **E**, they will freely migrate towards the electrode carrying opposite charge. However, different types of molecules will migrate at quite varying and individual rates reliant on the physical characteristics of the molecule and on experimental system used. The velocity of migration, ν, of a charged molecule in an electric field depends on variables described by $\nu = E \cdot q/f$ Eq. (5.1) where f depicts the frictional coefficient and q is the molecule's net charge [101]. The frictional coefficient defines frictional resistance to movement and depends on various factors like mass and compactness of the molecule, porosity of the matrix, and the buffer viscosity in which the experiment is performed. The net charge is calculated by the number of negative and positive charges in the molecule. Charges are conferred on proteins in accordance with the amino acid side chains as well as by groups rising from post-translational modifications such as acylation, deamidation or phosphorylation. DNA has a uniform charge distribution since a phosphate group provides a single negative charge per nucleotide. Equation (5.1) elucidates that generally molecules will move at a faster pace as their net charge increases, the electric field strengthens and as f decreases (which is a function of molecular shape/mass). Molecules of the same net charges separate due to differences in frictional coefficient while molecules of same shape and mass may differ from each other in net charge. Consequently, it is often possible to achieve very high resolution separation by electrophoresis.

Gel electrophoresis Hydrated gel networks/matrices have several necessary properties for electrophoresis. They permit a high variety of stable experimental formats like vertical/horizontal electrophoresis in slab gels and capillary electrophoresis. The mechanical stability also enables post electrophoretic alterations making further experimentation possible such as electro-elution blotting, or mass spectrometric identification /finger printing of intact proteins or of proteins digested in gel slices. Supporting matrix used depends on the type of molecule to be separated and on the desirability for separation like molecular weight, charge or both [102]. The commonly used media for protein and nucleic acid are acrylamide and agarose, respectively.

Agarose gel electrophoresis of DNA Movement of DNA fragments in agarose is variable, linear DNA fragments migrate with a mobility inversely proportional to

the log^{10} of their molecular mass through the agarose gel while circular DNAs show markedly different movements where intact plasmids appear to migrate at a faster pace than the same plasmid when in linear form. Various factors affect movement of DNA fragments, like (1) agarose concentration, higher concentrations enable separation of small DNAs, while low concentrations facilitate resolution of larger DNAs, (2) voltage, and (3) electrophoresis buffer. DNA in gels can be visualized using fluorescent dyes like ethidium bromide (toxic) and other preferred non-toxic dyes like gel green [103]. Agarose gel electrophoresis technique is extensively used for investigating the DNA cleavage efficiency of small molecules and binding modes of small molecules to supercoiled DNA [104, 105] in cloning for the formation of recombinant proteins. Human kinases, for example, are used for studies related to cancer and other disorders [106], the procedure requires intensive use of agarose gel electrophoresis to investigate band size in cloning [107]. Agarose gel electrophoresis is an established technique frequently used in clinical laboratories for identification of protein abnormalities like in hemoglobin, lipoproteins, etc. in varied biological fluids (serum, CSF, urine). Electrophoretograms are assessed visually for the presence of qualitatively or quantitatively abnormal protein bands [108].

Polyacrylamide gel electrophoresis (PAGE) delivers a gentle, versatile, and a high resolution method for physical–chemical characterization of molecules and fractionation on the basis of size, net charge, and conformation. This technique allows high resolution electrophoretic separation of both proteins and nucleic acids. The addition of a small amount of acrylamide cross linked by a methylene bridge (*N*, *N′*-methylenebisacrylamide) permits formation of a cross linked gel with a highly precise porosity which is also mechanically strong and chemically inert. For separation of DNA, the ratio of acrylamide:*N*, *N′*-methylenebisacrylamide it is generally 19:1, while it is 40:1 for proteins. The polymerization reaction can be controlled thoroughly to give uniform gels of measurable and reproducible pore size over a wide range.

Discontinuous PAGE is used to increase the resolution capacity of PAGE. It uses gels of two different pore sizes: larger pore size stacking gel that provides a lower level of resistance to the movement of large molecules resulting in the accumulation of the proteins at the boundary between the two gels and the underlying lower pore size resolving gel. Different buffer ions are present in the gel compared to those in the electrode reservoir, hence the name discontinuous PAGE [109].

Native PAGE (N-PAGE) is performed in the absence of denaturants like SDS, to preserve the native or native-like structure of the protein. Under these conditions direction and rate of migration of a protein (or protein–drug complex) depends on the intrinsic charge and shape, and size of the protein/complex [110]. N-PAGE is an extensively used technique because it retains the native structure of the protein. It is used to study changes in protein conformation induced by heat, pH, binding of small molecules like caprylic acid, etc. [111]. N-PAGE can also be used to isolate a protein complex in-gel and to measure enzyme activity by histochemical staining [112]. Blue N-PAGE is a technique for isolating intact protein complexes. It has been used to investigate assembly of mitochondrial protein complexes and respiratory enzymes.

In capillary zone electrophoresis (CZE) electrokinetic separation is conducted in a cylindrical channel with a very small diameter. CZE offers a unique plug-flow (constant velocity) character of the sample material inside the capillary channel, under the influence of the electric field. This form of liquid flow produces bands of varied molecules that are free from the band broadening effects. CZE separations thus enable achieving very impressive precise and sharp bands [113].

Native isoelectric focusing (nIEF) is another variation of electrophoresis. This powerful resolving technique separates peptides and proteins based on their isoelectric points across a pH gradient. The analyte ceases to move when its isoelectric point matches a pH value in the gel [114]. nIEF has been used for separation of genetic variants, identify immunoglobulin heterogeneity and post-translational modifications in proteins.

The Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS PAGE) is the most prevalent method due to its simplicity, availability, reproducibility, and ease to use. It is extensively used to investigate the proteins in complex extracts. Modern day discontinuous SDS PAGE system is based on Laemmli's method [115]. The samples are treated with SDS, reducing agent like beta mercaptoethanol and heat to denature the higher structures of the proteins. Alterations in the compositions and pH of the stacking gel, resolving gel, and electrophoresis buffer produce a system that is capable of finely resolving proteins according to their MWs [116]. SDS PAGE has an extensive use in the determination of molecular weight of proteins. In an experiment to determine the role of mitochondrial enzyme pyruvate dehydrogenase kinase in cancer, the human gene was cloned and expressed in *E. coli*, further the protein was expressed in the bacterial system and it was concentrated in the inclusion bodies that were homogenized and analyzed by SDS-PAGE. The technique helps determine the purity/quality of the protein [117, 118]. SDS PAGE can be used to quantitate the concentration of a single protein from a heterogeneous mixture of proteins, which is commonly followed by ELISA or Western blotting [119].

5.5.1 Applications of Electrophoresis in Separation of Clinical Samples

Agarose gel electrophoresis is used routinely in clinical laboratories for screening protein abnormalities in many biological fluids. Semi-automated agarose gel electrophoresis systems deliver high resolution gel electrophoresis of urine for characterization, detection, and quantification of monoclonal proteins [120]. CZE is an essential aid to diagnosis and therapeutic follow-up of patients with plasma cell disorders [121]. CE was first employed to separate serum proteins into the classic six bands, e.g., albumin, α-1 globulin, α-2 globulin, β-1 and β-2 globulins, and γ globulin [122, 123]. Although differences in these protein fractions can be associated with a patient's health it is generally the deviations that occur in β and γ

fractions that are of the most clinical interest. The fractions have monoclonal proteins whose detection is a vital part of laboratory evaluation of lympho-proliferative diseases in patients. Large monoclonal bands are frequently present in patients with multiple myeloma, however, lower concentrations may be observed in a variety of other diseases such as leukemia, light chain disease, amyloidosis, lymphoma or monoclonal gammopathy of undetermined significance [124].

Excessive excretion of protein in urine (proteinuria) is a common abnormality instigated by a number of pathologic conditions affecting the kidney and urinary tract [125]. Electrophoretic characterization of these proteins can be helpful in determining the increased urinary protein usually achieved by AGE or CE [126]. The investigation of cerebrospinal fluid (CSF) proteins can be beneficial in the diagnosis and management of various neurological diseases including environments that cause immune responses, damaging brain diseases, or a collapse in the blood–brain barrier. Polyclonal or more importantly oligoclonal bands in the gamma regions may be produced in response to immune processes [127]. Presently the method routinely used in the clinical laboratory for the detection of protein profiles in CSF is AGE, which is laborious and requires concentration of the CSF to visualize the protein bands. Unconcentrated CSF from random patients when analyzed separated into 20 and 25 peaks [128]. Now, CE is also being used to determine proteins present in CSF and evaluating blood brain barrier in patients with neurological disorders [129, 130].

5.6 Hybridization

The term hybridization refers to the association of two mono-stranded complementary nucleic acid strands usually from different biological sources that produce hybrid double-stranded base pairing with high specificity. Hybridization-based protocols represent the most frequently used techniques in molecular biology to locate or identify particular DNA or RNA sequences in the genome [131]. It could generate a DNA–DNA, RNA–RNA, or DNA–RNA hybrid double helix according to the contingent sequence homology of the two sources. The process of hybridization includes target DNA processing, target DNA denaturation, target DNA transfer to solid carrier, probe synthesis, and probe marking (radioactive or non-radioactive). The binding of probes and complementary target sequence typically occurs on a microporous membrane via hydrogen bonds with a stable attachment of double-stranded nucleic acid [132]. The separated target DNA fragments are transferred and immobilized to nitrocellulose or nylon membranes which work well with protein and nucleic acids. Complementary single-stranded probes enable the hybridization with the specific single DNA fragments immediately after the membrane is exposed. When two complementary DNA molecules come together, rapid initiation of hybridization occurs via hydrogen bonding and proceeds in a zipper-like fashion throughout the length of the molecule. The speed of the hybridization depends on the complexity of DNA sequence where more repetitive sequence elements result in

faster hybridization. The hybridized target DNA with a specifically bound probe can be detected by autoradiography or chromogenic reaction. The design of probe is crucial as it must form a stable duplex only with those DNA molecules on the membrane that are exactly complementary to it.

Hybridization-based approaches rely on the stability of hybrid nucleic acid molecules and depend upon several factors. The buffer conditions such as thermal stability, salt concentration, pH, and concentration of denaturants are potential aspects that could influence the hybridization [133]. In addition, the molecular properties including the length of the hybrid, GC-content as well as the number of mismatches also affect the hybridization process. The optimization of above-mentioned influencing factors is essential to determine the stringency of hybridization.

5.6.1 Northern Blotting, Southern Blotting, Western Blotting, and Use of Probes in Blotting

The types of nucleic acid hybridization techniques generally used are Southern blotting and Northern blotting. *Southern blotting* is based on DNA–DNA hybridization and is used to identify specific sequences on target DNA. It is also used for the analysis of restriction fragment length polymorphisms (RFLP) and the identification of deleted, duplicated, or rearranged genomic regions (see Box 5.4). *Northern blotting* relies on hybridization of DNA–RNA or RNA–RNA to identify a specific RNA molecule in a mixture of different RNA. It enables the investigation of RNA transcript structure, processing, and gene expression in different tissues.

Another type of blotting technique is ***Western blotting***, which refers to the detection of specific protein fragments using antibodies. A combination of Southern blot and Western blot techniques is also used in the characterization of specific DNA-binding proteins and is called as *Southwestern blotting* since it involves hybridization of both protein and DNA. Several DNA-binding proteins can be characterized by this method. The molecular hybridization techniques coupled with electron, light or confocal laser scanning microscopy are used in situ hybridization assays. They are mostly applied to study viroid subcellular localization and trafficking.

Southern blotting The “Southern blot” refers to a typical method used for the detection and identification of DNA sequences from a variety of prokaryotic and eukaryotic organisms. It is a hybridization technique developed by Edwin Southern in 1975 and is still used for studying the structure of DNA with exceptional resolving power [134]. The procedure depends on the ability of denatured DNA single strands to bind tightly to microporous membrane under certain conditions.

Methodology The process of Southern blotting involves several steps including extraction and digestion of DNA with restriction enzymes, size-fractionated separation of DNA by gel electrophoresis, transfer of fractioned DNA onto nylon or nitrocellulose membrane, hybridization with a specific probe, and detection in a DNase free atmosphere [135]. In most applications, the extracted high-quality genomic DNA is cleaved into various sized fragments with one or more desired restriction

enzymes. The quantity of sample and choice of restriction enzyme plays a significant role in this technique. The digested DNA is size fractionated on a preparative horizontal agarose gel in a low voltage. A staining step is usually employed to confirm the migration of DNA bands. Adequately separated DNA fragments undergo the denaturation of the double-stranded nucleic acid. The protocol of denaturation depends upon the type of blotting membrane whether it is charged or uncharged. The denaturation is carried out by soaking the gel in a denaturing solution classically an alkali solution. However, an additional soaking in neutralization buffer is employed for blotting that transfer the DNA to uncharged membranes such as nitrocellulose or nylon. The process of blotting for the complete transfer of DNA fragments is achieved by floating the membrane on the surface of a dish of deionized H_2O until it wets completely, followed by dipping in the appropriate transfer buffer that carried the fragments on the surface of the membrane. A further depurination step is recommended to improve the efficiency of large-fragment transfer (>10,000 bases). The choice of transfer buffer also depends on the membranes [136]. A neutral transfer buffer is used for uncharged membranes and an alkaline transfer buffer for charged nylon membranes.

According to the original method, the blotting uses capillary transfer procedure and remains the most widely used method as of low cost and convenience, often take a long overnight duration to finish. Other high-speed transfers using specialized vacuum blotting apparatus or electroblotting devices have been reported lately [137]. The choice of the membrane depends on the required sensitivity and the detection method. The use of nitrocellulose usually results in low backgrounds on detection and is recommended when a high level of target is required. Perhaps it binds poorly with small molecules and transfer buffers must contain high salt concentrations to ensure efficient nucleic acid binding. The charged nylon membranes possess a higher binding capacity and are independent of the ionic strength of the transfer buffer. A hybridization buffer provides a medium that promotes the hybridization between the complementary sequence and the probe with simultaneous prevention of unmatched hybridization. A short prehybridization step in hybridization buffer is recommended before adding the labeled probe that limits the nonspecific background hybridization of genomic sequences on the membrane. Comprehensive hybridization takes a longer duration, however, rapid-hybridization buffers containing volume excluders are also available to speed up this step. The hybridized blot undergoes a washing step to remove un-hybridized probe with controlled stringency by stepwise reductions in the ionic strength of wash buffer and/or by temperature. The sequence of interest can then be detected by a hybridized probe which is labeled with radioactive, fluorescence, or other methods.

Southern blotting is an effective, cheap, and simple way of estimating DNA structure. It is sensitive enough to analyze a small portion of the primary structure of human genomic DNA, detect single-copy genes, clone genes, and study gene expression. The method is also useful to detect the presence of abnormal sequences or the absence of normal nucleic acid sequences [138].

Northern blotting refers the qualitative and quantitative evaluation of specific RNA levels from a complex mixture of RNA. This technique was originally

developed by James Alwine and George Stark [139]. It is similar to the Southern blotting except that it exploits distinct chemical properties of RNA. It involves several steps which include extraction of RNA, size separation of RNA by denaturing gel electrophoresis, transfer of fractionalized RNA to a membrane, hybridization with a specific probe, and detection [140]. The entire process of Northern blotting is long, and the target RNA species are presented as a single or several discrete bands, depending upon the nature of the probe, target RNA, and the stringency of hybridization.

Methodology Isolation of clean, intact full-length RNAs are considered the foremost and vital step in Northern blotting. Partially degraded and contaminated RNA preparations with DNA and protein affect the identification and quantitation. Perhaps, the RNA isolation is often problematic since they are extremely susceptible to digestion by ribonucleases. Owing to this fact, the RNA isolation process considered several precautions including homogenization of RNA sample sources in denaturing, reducing chemical environment along with the use of ribonuclease free environment to reduce the likelihood of sample degradation. The quantity of RNA recovered can be determined by measuring the absorbance of the diluted sample at 260 and 280 nm. However, a purification phase of extracted RNA increases the sensitivity of Northern blotting as it may contain polyadenylated messenger RNA.

The isolated and purified RNA species are separated based on size, usually through agarose gel electrophoresis. The denaturation of RNA fragments by the action of loading buffer containing formaldehyde and formamide and heating at 60 °C for several minutes helps to minimize the formation of secondary structures through complementary base pairing during electrophoresis. To keep the RNA denatured, formaldehyde or glyoxal is added to the gel matrix. Formaldehyde is present both in the gel and in the migration buffer, ensuring the inhibition of ribonuclease activity. The most commonly used electrophoresis for Northern blotting is horizontal agarose gel electrophoresis; however, PAGE can result in greater sensitivity and resolution.

The next step is the transfer of RNA from the electrophoresis gel to a solid support or membrane via different blotting methods, viz. ascending or descending capillary transfer, electroblotting, or vacuum blotting. Next, the negatively charged RNA is transferred onto a nylon membrane driven by capillary forces. The efficient transfer of RNA from gel to membrane is crucial and it may be accomplished overnight. Conversely, staining of electrophoresis gel as well as RNA sample with ethidium bromide prior to electrophoresis enable the direct visualization of transfer efficiency and transferred RNA on the membrane under UV light.

Nitrocellulose, nylon or chemically charged membranes are the commonly used membranes for northern blotting. However, the choice of membranes is limited according to the characteristics of target RNA. Both nitrocellulose and nylon membranes require crosslinking of the transferred RNA to the membrane [141, 142]. Although, chemically charged membranes have lower binding capacity than nitrocellulose or nylon, but they bind covalently [143].

The membrane transferred and crosslinked RNA undergoes a prehybridization step which confirms proper blocking and limited background signals. The

prehybridization is performed by incubating the blot for few minutes to several hours with frequent heating and agitation of hybridization solution without a probe. Several factors including the melting temperature of the duplex, the composition of the hybridization and wash solutions, and the temperature influence the formation rate and the stability of hybrids. Therefore, optimization is required for each hybridization reaction.

Hybridization is generally carried out in aqueous solutions which contains salt, SDS, sheared genomic DNA to prevent high background, and high molecular mass polyethylene glycol or dextran sulfate and ficoll to concentrate probe. The strength of Northern blotting method is its simplicity with relatively high specificity. Once prepared the blots can be stored for a long duration and enables multiple detections even with different probes. Northern blotting enables the visibility of mRNA transcript size, alternatively spliced transcripts, and could also interpret steady-state level of gene expression. It is also useful in the initial characterization of a gene including its developmental stages or the site of expression [144].

Western blotting, also called immunoblotting is a technique used to separate and identify proteins according to their MW. It evolves from similar gel electrophoresis-based nucleic acid separation methods, i.e. Southern and Northern blots. Even though the method had been used earlier, Burnette employed the use of SDS polyacrylamide gels and coined the term western blotting [145]. The principle of Western blotting typically utilizes the specific binding of proteins and its antibody. An antibody-based probe allows the detection of the target protein from a complex mixture. It is a very sensitive method that could enable the detection of proteins even in picogram quantities. The experimental protocol involves different steps such as molecular size-based separation of proteins by gel electrophoresis, transfer of the separated proteins to a microporous membrane followed by incubation with labeled antibodies specific to the protein of interest, and detection. Two types of antibodies are used in this method. One antibody specifically binds to the protein of interest and the other one is attached as marker stains to allow visualization of the bound antibody.

Methodology It is crucial to get quality protein molecules that assure the selectivity and sensitivity of the assay. To prevent denaturation and protease degradation, it is recommended to use a low-temperature environment for sample preparation. The lysis should be done as quickly as possible and repeated freeze/thaw cycles should be avoided which can have an adverse effect on overall sample quality. The lysis solubilizes the proteins from cells that enable efficient extraction and maintain antisera recognition of the protein. The lysis buffer is chosen based on the protein of interest. The presence of proteases and phosphatase inhibitors in lysis buffer results in maximum recovery of total proteins during lysis. A homogenizer or sonication with lysis buffer also supports to break down of tissues. The quantification of protein samples is recommended particularly for the calculation of lysate protein concentration for polyacrylamide gel electrophoresis. Western blot commonly uses polyacrylamide gels and buffers loaded with SDS for electrophoresis. The anionic detergent, SDS enables proteins to become denatured and negatively charged, allowing them to be separated on the basis of MW, resulting in the formation of

clear bands in each lane. The use of reducing agents also facilitates denaturation by removal of disulfide bonds. A bisacrylamide:acrylamide ratio should be standardized for optimal separation during electrophoresis. A two-dimensional gel is also possible which allows separation of proteins on the basis of isoelectric point and molecular weight and is generally used for the comparison of multiple components of proteins from a single sample.

Electrophoretic as well as non-electrophoretic transfer of proteins to membranes are suggested in the scientific literature [146]. Electrophoresis separated proteins are transferred onto a microporous membrane (western blot). The polyvinylidene difluoride or activated paper/nylon, nitrocellulose is the most commonly used membrane due to its high protein binding affinity. Because of the advantages of speed and completeness of transfer, electroblotting is the most popular procedure. It employs an electric current to pull the proteins across from the gel to the membrane towards the positive electrode using either wet or semi-dry transfer method. Wet transfer is achieved by immersion of a gel membrane sandwich in transfer buffer and the semi-dry transfer takes place by putting the gel-membrane sandwich between absorbent papers that has been soaked in transfer buffer. The former is more reliable as it prevents the gel from drying out but is more time-consuming. The factors that could affect the effectiveness of protein transfer include the type of gel, the molecular mass of protein, and the type of membrane [147].

The nonspecific binding of the primary antibody to the membrane is prevented by blocking with a dilute solution of protein such as bovine serum albumin, non-fat dry milk. The blocking buffer verifies the compatibility with specific antiserum and the membrane type because an incorrect combination of buffer and membrane can cause false positives [148]. Following the blocking, the blot undergoes multiple washes with gentle agitation using TBST or PBST (Tris-buffered saline or phosphate buffered saline with Tween 20) buffer. Subsequent incubation with primary antibody and secondary antibody with proper washings enables the formation of target protein–antibody complex that could be detected. An optimal concentration of antibodies is needed as too little can produce no visible signal while too much can produce swollen bands. Both monoclonal and polyclonal antibodies can be used for western analyses, with advantages and disadvantages in using either type. Different detection probes that enable calorimetric, radioactive, and fluorescent-based detection have been reported [133, 149]. The western blot is a semi-quantitative technique, known to be unreliable at times, difficult to optimize, and time-consuming. However, this does not diminish the functionality of the process. With experience, the western blot can be instrumental to almost any researcher and is a technique that is continually improving its efficiency (Fig. 5.12).

It is a robust method with substantially increased applications in medical research and diagnostics. It is used for different purposes and applications in biomedical sciences such as proteomics, molecular identification, and biomarker discovery [150, 151]. Western blot has been considered as the confirmatory diagnostic method to identify various infections including HIV (Human Immunodeficiency Virus), BSE (Bovine Spongiform Encephalopathy), FIV (Feline Immunodeficiency Virus), HBV

Box 5.5 Genome Sequencing

Genome sequencing is the technique by which the DNA sequence of an organism can be known to its entirety. It utilizes combination of several techniques discussed earlier. Two earliest known methods of genome sequencing also called as the first-generation sequencing are the Sanger, and the Maxam and Gilbert methods [156]. Briefly, in the Maxam and Gilbert or chemical degradation sequencing method, in a 5′-radioactively labeled DNA strand bases are chemically modified and then cleaved specifically upstream of the modified base. This generates a family of DNA fragments, each sharing a common labeled end but distinct lengths, which are separated by PAGE and the sequence is read from the gel. For example, dimethyl sulfate methylates guanine nucleotides and piperidine is used to cleave at sites immediately upstream of this modification. Due to use hazardous chemicals, Maxam and Gilbert method has not been the method of the choice. Sanger's or the chain termination method is more a PCR based approach. Single-stranded DNA templates are generated by PCR or cloning in plasmid or bacteriophage vectors. Primers are annealed to these strands and DNA synthesis is initiated using DNA polymerase. Generally, universal primers specific for a small portion of vector DNA to which template DNA was ligated is used. Several internal primers specific for template strand can also be used simultaneously. Elongation of strands complementary to single-stranded template is halted due to the presence of small amounts of dideoxynucleotides as they lack the 3′-hydroxyl group needed to form a connection with the next nucleotide. Four different families of terminated chains are generated in presence of all four dideoxynucleotides. These are separated by PAGE and visualized on the basis of label used for labeling the template and deoxy nucleotides (autoradiography or fluorescence detection). Sanger's method has been automated but suffers from the disadvantage that only few hundred base pairs of a sequence can be determined in a single experiment. Next advancement in the procedure came with pyrosequencing which is much rapid than automated Sanger sequencing. In this method, the sequence is read as the synthesis reaction at the growing strand proceeds. This is made possible by the conversion of released pyrophosphate into a chemiluminescent product by the enzyme sulfurylase. For complex eukaryotic systems cloning based sequencing methods are difficult and time-consuming. This difficulty was overcome with the shot gun sequencing method. Whole genome shotgun (WGS) method sequences random clones of starting DNA. The resulting sequence reads are than assembled by computer program by recognizing overlapping sequences and stitching the pieces together. This is very rapid and simple approach but fails for larger genomes with many repetitive sequences. For this purpose, hybrid WGS has been developed in which genome is broken down into overlapping clones that can be physically mapped (see Box 5.1) and then shot gun sequenced. The result

(continued)

Box 5.5 (continued)

is a large-scale map that tells the exact order for each piece of sequenced DNA. These sequencing approaches (as used for human genome project) required additional elements for assembly of the sequence reads into chromosomes. Two such elements were physical maps (see Box 5.1) and genetic maps. A genetic map is based on use of genetic techniques to construct a map showing distinctive features on a chromosome. These distinctive features are genes, restriction fragment length polymorphisms (RFLP), single nucleotide polymorphisms (SNPs), and simple sequence length polymorphisms (SSLPs). RFLP arises due to the presence of polymorphic restriction sites for a specific RE in the genome, where only one is amenable to cleavage. RFLP thus gives rise to restriction fragments of different lengths which upon separation by agarose gel electrophoresis, are recognized by probes (spanning the polymorphic sites) using Southern blotting (Fig. 5.13). SSLPs are repeat sequences that exhibit high degree of polymorphism (multi-allelic unlike RFLP) in the number of repeats at a given chromosomal site. SSLPs can be minisatellites also known as variable number of tandem repeats (VNTRs) that are 25 base pair in length and concentrated in the telomeric regions. A form of SSLPs are microsatellites or simple tandem repeats (STRs) that are di- or tetra-nucleotide repeats more evenly spread throughout the chromosome. Due to these reasons and also because of easier PCR typing, STRs are more commonly used as genetic markers compared to VNTRs. SNPs, as the name suggests, are genomic positions where individuals have different nucleotides. These are most abundant in the genome and can be recognized rapidly through oligonucleotide hybridization avoiding use of electrophoresis. Linkage and pedigree analysis are used to construct genetic maps.

(Hepatitis B Virus), and Lyme disease [152, 153]. Western blotting played a key role in the identification of proteins (tau) responsible for Alzheimer's disease [154, 155].

Second generation sequencing consists of next generation sequencing (NGS) or massively parallel DNA sequencing. In a sharp contrast to first-generation methods, NGS does not involve electrophoresis. A complex library of DNA templates generated by in vitro amplification is densely immobilized onto a two-dimensional surface, with all templates accessible to a single reagent volume [156]. A polymerase is used and a fluorophore or change in ionic concentration identifies the incorporation of a nucleotide in the elongating strand, the approach being called as "sequencing-by-synthesis" (SBS). Millions of SBS reaction centers each with its own clonal DNA are sequenced in parallel facilitating massive parallelization.

Third generation sequencing involves real-time single molecule sequencing and has advantages of no copy errors and read length limitations and higher accuracy [156, 157]. For example, in one of the approaches the polymerase based synthesis is observed optically in real time by fixing the polymerase on bottom of a well and

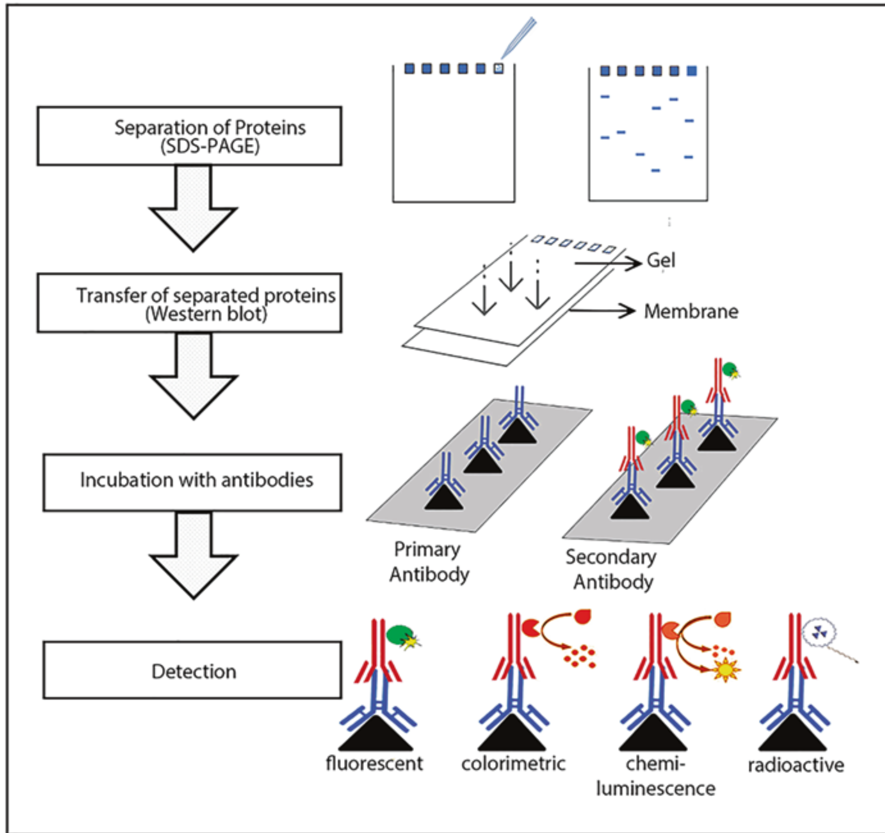


Fig. 5.12 A representative image depicting different stages of Western blot method

allowing the DNA strand to progress through a zero mode waveguide (a hole less than half the wavelength of light). This limits the fluorescent excitation to just the polymerase and its template and incorporation of fluorescently labeled nucleotide is detected with a laser and camera system.

5.7 ELISA

Enzyme linked immunosorbent assay (ELISA) is a simplified and altered version of radio-immunoassay (RIA) and is considered the gold standard for immunoassays. This sensitive technique is used for both qualitative and quantitative detection of antibodies, antigens, proteins, and hormones [158]. The antigen–antibody interaction is measured by an enzyme catalyzed color reaction detectable by simple

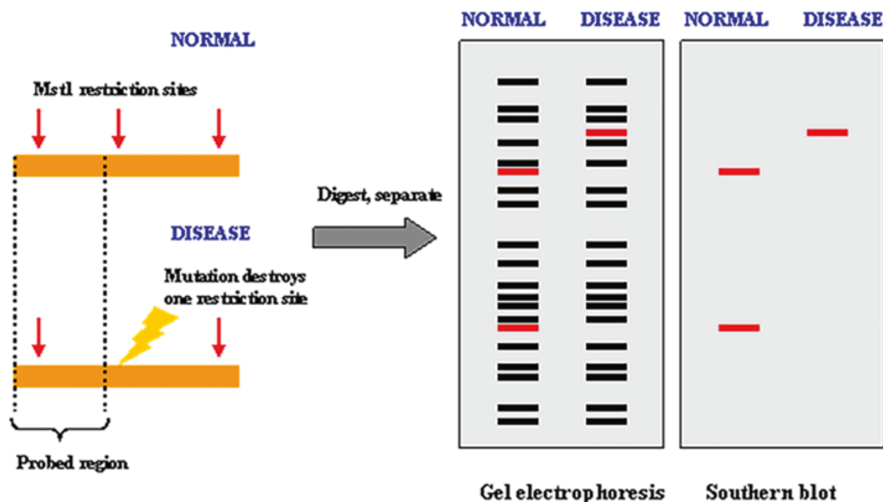


Fig. 5.13 Restriction fragment length polymorphism

colorimeter or spectrophotometer. ELISA testing is used in the diagnosis of fertility tests, HIV infection, blood typing, and many others [159].

Specimen requirements and procedure ELISAs are carried out in 96-well plates coated to bind protein very strongly. Depending upon the type of ELISA, testing requires a primary/secondary antibody, coating antibody/antigen, and substrate/chromogen. The primary detection antibody is a specific antibody that specifically binds to the protein of interest, while a secondary detection antibody is a second enzyme linked antibody that binds the primary antibody [159]. There are four steps in the procedure: coating (with antibody or antigen), blocking (with addition of bovine serum albumin), detection, and final read. Detection is usually carried out by the addition of substrate that produces color. Horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are the most commonly used enzymatic probes on secondary antibody. Hydrogen peroxide is the substrate for HRP and results in blue color. ALP measures the yellow color of nitrophenol as its substrate.

Washing of plate using buffer such as phosphate buffered saline (PBS) is done between each of the above steps. Depending on the protocol used wells are washed accordingly.

ELISA protocols usually follow serial dilution of concentration in the wells of plate. After the plate is recorded, a standard curve from serial dilution is plotted using log of concentration on *X*-axis and absorbance on *Y*-axis using a linear scale.

Direct ELISA In this type the antigen is first coated to ELISA plate. The plate is incubated for 1 h at 37 °C or can be incubated at 4 °C overnight. The next step involves the addition of an antibody linked to enzyme conjugate. This primary antibody binds directly to the protein of interest. Next the plate is rewashed to remove

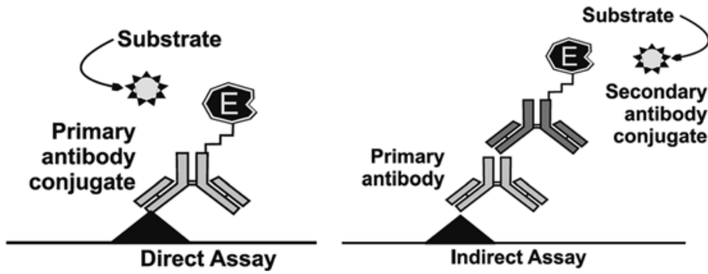


Fig. 5.14 Schematic representing direct and indirect ELISA

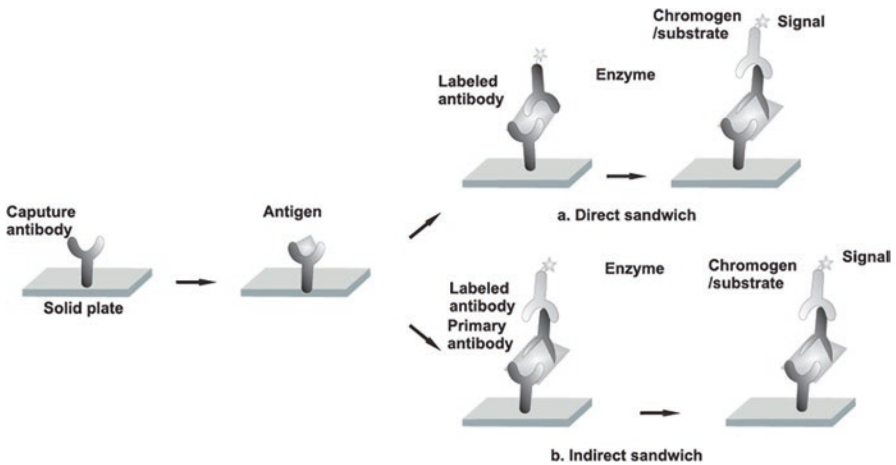


Fig. 5.15 Schematic representing sandwich ELISA

any unbound antibody, and this is followed by the addition of a substrate which produces color detected using ELISA detector. This type of ELISA is rapid but has low sensitivity as compared to indirect ELISA (Fig. 5.14) [160, 161].

Indirect ELISA has identical procedure as direct ELISA. However, it involves two antibodies, a primary detection antibody that binds to the antigen and a secondary enzyme linked antibody, that is complementary to the primary antibody (Fig. 5.14). In this form of ELISA, the antigen coated on to the microtiter well is reacted with a sample containing antibody (AB1), e.g., serum sample containing antibodies. This is followed by a wash step to remove excess AB1. Then enzyme conjugated secondary antibody (AB2) is added and incubated. This AB2 is specific for AB1 and is tagged with an enzyme. As in direct ELISA the bound antibody is detected by adding the substrate which on reacting with the enzyme results in the colored product. The color change is measured in ELISA reader as the absorbance [162].

Sandwich ELISA This type of ELISA is most convenient immunoassay arrangement and is designed for the detection of soluble antigens (Fig. 5.15). There are two main types of sandwich ELISA depending upon the number of antibodies used.

However, the principle is the same for both the types, the antigen is sandwiched between two antibodies (capture and detection antibodies). The microtiter wells are coated with the capture antibody; the plates are then covered and incubated overnight at 4 °C. For direct sandwich ELISA once, the coating is done plates are then washed once again before the addition of the antigen. After washing away excess unbound antibody, antigen is added and is specifically captured. The antigen is then detected by a second enzyme labeled antibody directly against the antigen. This type of test is useful where a single species antiserum is available.

For indirect sandwich ELISA, the antigen is detected with a second unlabeled antibody. This antibody is in turn detected using anti-species enzyme linked conjugate. It is important that the anti-species conjugate does not bind to the capture antibody. For this purpose, the species in which capture antibody is produced must be different. After the addition of the secondary enzyme conjugated antibody the plate is rewashed, and the substrate is added to produce a color change. This is the measured in the same manner by ELISA reader. Among all ELISA's sandwich ELISA has the highest sensitivity but its time-consuming and is not cost-effective [163].

5.8 Summary

For understanding the fine balance between states of health and disease, a deeper understanding of cellular and molecular mechanisms of homeostasis is required. Various analytical techniques described above have allowed discovery of these homeostatic mechanisms. We have discussed light microscopy, including light sources and theory behind bright field, phase contrast, epifluorescence microscopy, and fluorescence in situ hybridization (FISH), scanning and transmission electron microscopy as well as confocal microscopy with their usefulness as invaluable tools for contemporary studies of biological systems. Spectroscopy has traditionally been widely used as an important tool for the characterization, identification, and analysis of biomolecules. Chromatographic and electrophoretic techniques are principally used for proteins and nuclei acid characterization. Along with spectroscopic techniques like CD, their use is now fundamental in development of drugs and biosimilars. Radioisotopes and autoradiography have played an important role in discovery of biochemical pathways, as tools investigating subcellular location of proteins and nucleic acids in histology (hybridization techniques) and in determining the levels of these biomolecules in biological fluids in pathology. One such application using radioisotopes as a highly sensitive quantification technique is radio-immunosorbent assay that contrasts ELISA. As each technique has pros and cons, combinatorial use of complementary techniques aids comprehension of underlying biological processes. Undoubtedly, novel analytical methods hold promise for improving human health and environment.

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Chapter 6
Immunology in Medical Biotechnology

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Abstract Mammalian immune system is represented by a veneered defense system comprising of both innate and adaptive immune responses. There is a continuous exposure of numerous pathogens through touch, ingestion, and inhalation. These pathogens are eradicated by the immune system. Vertebrates have developed both innate and adaptive immunity which enables them to subsist constant threat posed by the environmental pathogens. This response is based on immune cells of the host or their secretions. Immune system is a network of cells, tissues, and organs working in conjunction to defend the body against the foreign invaders primarily microbes, bacteria, viruses, parasites, fungi. etc. However, when the immune system is crippled, it can unleash a torrent of diseases including AIDS, arthritis, allergy, etc. There are two major immunologic cells called as B cells and T cells. B cells produce antibodies and elicit a specific immune response. Whereas the T lymphocytes are a type of leukocytes which originate in bone marrow and mature in thymus and have specific T cell surface receptors. Immunology has revolutionized the world by facilitating advanced healthcare system, unraveled many mysterious diseases.

Keywords Immune system · B cells · T cells · AIDS · Arthritis · Allergy · Microbe

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6.1 An Overview of Immune System, Types of immunity, Cells and Organs of Immune System

6.1.1 An Overview of Immune System

Immunology, defined as the study of the immune system, is an important area of research in various fields, including biological sciences, biotechnology, biochemistry, and medicine. The immune system acts as a first line of defense in the biological systems. Inadequate and paltry functioning of the immune system leads to many serious complications like cancer, allergy, etc. Several disorders associated with cardiovascular system, neurodegeneration, and metabolism were initially viewed as non-immunological, but with the advances in science and technology, the role of immune system in these disorders became evident. Immunology is the set of all physical, chemical, and biological reactions of the organisms against the foreign substances called as antigens [1]. The word immunity has been derived from a Latin word "immunitas" which means protection from the legal prosecution. Protection from any disease or any pathogen is referred as immunity, whereas cells (WBCs, platelets), molecules, and organs (lymphoid organs, payers patches) participating in eradicating those pathogens or diseases are the essential constituents of the immune system. Recognition of the pathogen and eliciting an effector response is called as the immune response [2]. The immune system must follow three main tasks for the effective protection of an individual against any pathogen or diseases and these tasks are as follows: Detection of the presence of the potent pathogen or disease of the immune system known as immunological recognition; the second task is immune response and regulation to fight the infection without harming self. Whereas the third task is the immunological memory in which the immune system protects the body from the recurring infection by the same pathogen by making an immediate immune response against any subsequent exposure to that pathogen [3].

6.1.2 Types of Immunity

Immunity is broadly classified into two major types: (a) Innate immunity and (b) Adaptive immunity. Innate immunity is antigen independent, antigen non-specific, considered as long lasting and prevail lifelong in normal circumstances. The adaptive immunity is developed over life time. It can come when an organism is exposed to an antigen or when the host is given antibodies/vaccines for the immediate protection, e.g., placenta is a source of acquired immunity for newborn babies [4].

6.1.2.1 Innate Immunity

Innate immunity the first line of defense, consisting of anatomical and physiological barriers, chemicals in the blood and certain body cells immune system cells that attack foreign cells in the body [5]. Only vertebrates have both innate as well as adaptive immunity, whereas invertebrates are dependent on innate immunity only and there are no any adaptive immune responses found in them [6]. They key components of innate immunity are discussed as follows:

Anatomical and Physiological Barriers

These barriers provide a crucial first line of defense against pathogens which include skin, bacteriolytic lysozyme, low stomach PH, mucociliary clearance mechanisms, and other secretions. Some of these barriers are as follows:

(a) Skin

Skin is the longest organ, measuring about ~2 m^2 providing an integrity and identity to an organism. Skin is mainly formed of three different layers, epidermis, dermis, and endodermis, which are outer, middle, and innermost layers, respectively. An effective immune shield between the external and internal environment is made available by skin and mucosa. The epidermis consists mainly of keratinocytes connected by desmosomes embedded in a layer of proteins of the extracellular matrix. Keratinocytes are multifunctional as they act a physical barrier, express pattern recognition receptors, and antimicrobial peptides eventually inducing an inflammatory response and microbial destruction [7, 8]. Carefully orchestrated, researchers have divided skin into four functional barriers: (1) Microbiome barrier, (2) Physical barrier, (3) Chemical barrier, and (4) Immune barrier. Misfunctioning of any of these barriers will lead to entry of pathogens in the body and may lead to pathogenic conditions like sterile skin inflammation, skin infection, skin tumor development, etc. [9]. There should be integrity in these barriers to protect the body from entering of pathogens and eventually dismantling them.

- Outermost layer comprising of diverse microbial communities, including bacteria, fungi, and viruses and is fairly stable. These barriers, covers all surface areas of skin. Abundant gram-positive bacteria dominate the skin micro biota. The stability of these microbial communities is kept in check through a series of communication pathways [10]. Many studies have shown how these microbial communities control pathogenic bacteria [11–14].
- The chemical barrier includes acidic PH elements and compounds that together make up the natural moisturizing factor (NMF). "Sauremantel" of the skin is a term coined by Schade and Marchionini to explain the safety belt of acidity that covers it [15]. NMF are the hygroscopic compounds representing 20–30% corneocytes (dry weight) [16]. Other studies further demonstrated that the proteolysis of epidermal filaggrin forms amino acids and their

derivatives such as urocanic acid and pyrrolidone carboxylic acid as the essential constituents of NMF [17, 18].

- Stratum corneum and tight junctions are the essential portions of the physical barrier. Maturation of keratinocytes makes them move up to the epidermal layers and by terminal differentiation these keratinocytes become corneocytes leading to the formation of the stratum corneum [19]. Below the stratum corneum there is another layer called as stratum granulosum comprises of granules containing proteins such as, keratin filaments and loricrin which fill the intracellular space of stratum corneum [20]. It also contains laminar bodies with kalikreins, lipids, and corneodesmosins [21].
- The last portion of the cutaneous barrier is the immune barrier comprising of diverse cells of epidermis and dermis. The immune barrier contains many cells, including innate sentinels such as antigen presenting cells, keratinocytes, and lymphoid cells working together and ultimately maintaining the integrity [22]. This barrier senses the entry of any pathogen via pathogen associated molecular patterns and damage associated molecular proteins (PAMPs and DAMPs)and sufficient immune response is initiated by PAMPs and DAMPs subsequently, employment of circulating peers provides further interference to clear annexation.

(b) Lysozyme

Lysozyme is a protein encoded in genomes of animal kingdom and is present ubiquitously all over the animal kingdom and is an antimicrobial protein essential for host defense. All lysozymes have the same structure and have the capacity to hydrolyze the peptidoglycan of bacterial cell wall [23]. Based on biochemical structure and amino acid sequence lysozyme are categorized into three distinct types: Invertebrate type (I type), goose type (G type), and Conventional or chicken type (C type). Lysozyme is found in adequate amounts in mammals including in tears, blood, secretions urine, and milk. It is also found in mucosal surfaces where it can reach its higher concentration as high as 1 mg/ml. The bacteriolytic efficacy of lysozyme is well established [24].

(c) Stomach pH

Due to the pervasive and omnipresent nature of gastric acid among fishes, amphibians, birds, reptiles, and mammals suggests that it is evolutionary advantageous. There are many functions ascribed to gastric acid, but three main functions are critical: I) activation of pepsinogen and denaturation of proteins, ii) absorption of dietary iron and calcium are augmented by gastric acid, iii) inhibiting infectious envoys from reaching small intestines [25]. Gastric acid is secreted by a specific group of cells called as parietal cells in the stomach. HCl and pepsin are the important constituents of gastric juice and can kill bacteria within 15 min when pH is less than 3 and bacterial growth may occur when the pH is raised above four [26].

(d) Mucociliary clearance

To protect the airways, human body has developed a variety of defensive mechanisms against bacterial and viral pathogen particulates, thereby

maintaining the lungs in asceptic condition [26–29]. Anatomical barriers, cough, and aerodynamic changes are involved in lung protection; however, mucociliary clearance is the chief defense mechanism for the efficient clearance of the pathogens. Ciliated epithelial cell lining in healthy airway surfaces are enclosed with an airway surface layer, which has two constituents: mucus layer and periciliary layer. Mucus layer entraps the inhaled particles; whereas periciliary layer is a low viscous layer lubricating airway surfaces and clears the way for efficient mucus clearance by ciliary beating. The interaction between these layers, ciliary beating and airway surfaces is coordinated and results in mucociliary clearance [30].

6.1.2.2 Adaptive Immunity

Innate immunity has evolved to sense and eliminate broad variety of pathogens, but the range is limited in recognizing the common pathogenic molecular patterns. This limitation of innate immunity is fulfilled by adaptive immunity. High diversity in lymphocytes and variability in receptors for foreign substances are the important constituents of adaptive immunity [31]. The primary function of adaptive immunity is the recognition of non-self-antigens and distinguishing them from self-antigens. Manifestation of exquisite specificity towards a specific pathogen is an important characteristic of adaptive immune response. Adaptive immune response is based primarily on antigen-specific receptors expressed on the surfaces of B and T lymphocytes [32]. There are two major types of adaptive immunity:

(a) Humoral immunity:

Humoral immunity is also known as antibody mediated immunity. This type of adaptive immunity protects the extracellular spaces by producing antibodies specific for the pathogen. Pathogens move in and around cells through extracellular spaces, multiply there, and eventually get destroyed by the antibodies secreted by plasma B cells [33]. The number of circulating B cells remains constant throughout the life span; however, there are precise changes in the site of production. In the stromal cells of the bone marrow, known as niches, the first stages of B cell development take place from which the stimuli and factors needed to activate a sequence of cell signals come [34]. The classification and structure of antibodies/immunoglobulins will be discussed in a separate section.

(b) Cell mediated immunity

Cell mediated immune response is generated mainly by T cells rather than antibodies which generate a humoral immune response. Complete development of T cells occurs in thymus, and they eventually enter into the blood stream [35]. Mature re-circulating T cells that have not come across any antigen are called as naive T cells and to participate in the adaptive immune response these naive T cells must first encounter antigens. Naive T cells leave the blood on reaching the peripheral lymphoid organ and migrate to lymphoid tissue [36]. Activated naive T cells produce armed effector cells as they encounter an

antigen for the very first time in the form of a peptide: Major histocompatibility complex (MHC) on the surface of an activated Antigen presenting cell [37].

6.1.3 Cells and Organs of Immune System

The pluripotent cells of bone marrow give rise to immense cells of immune system. These cells later differentiate and give rise to common lymphoid progenitor cells and common myeloid progenitor cells. The B lymphocytes, T lymphocytes, natural killer cells all arise from common lymphoid progenitor cells, whereas and the erythrocytes, platelets, basophils, mast cells, eosinophils, neutrophils, monocytes, macrophages and dendritic cells arise from myeloid progenitor cells [38, 39].

6.1.3.1 Organs of the Immune System

The organs of immune system mainly comprise of primary lymphoid organs and secondary lymphoid organs. Their main function is the maturation of immune cells, mediate interplay of different classes of lymphocytes, antibody transportation along with other soluble factors [40].

1. Primary lymphoid organs

   The primary lymphoid organs include thymus and bone marrow. After maturation in the primary lymphoid organs the lymphocytes becomes pledged to a specific antigen and eventually cell becomes immunocompetent. T cell and B cell maturation occurs in thymus and bone marrow respectively in all mammals [41].
2. Secondary lymphoid organs

   Immune cells are present in the circulatory and lymph system throughout the body. In lymphatic system the materials entering tissue spaces are recovered by it and later travel towards the blood. Secondary lymphoid organs are organs that communicate with other cells of the immune system when antigen is detected. On antigenic stimulation these organs enlarge. Lymph nodes and spleen are most arranged organs of secondary lymphoid tissue. Mucosal associated lymphoid tissue (MALT) is a less organized lymphoid tissue. All secondary lymphoid tissue like lymph nodes, spleen, and MALT function mainly for antigens in the body [36].

6.1.3.2 Cells of Immune System

1. Agranulocytes

   Non-granular leukocytes /agranulocytes are named so because they lack lysosomal granules in the cytoplasm and are mainly represented by the lymphocytes

and mononuclear phagocytes (macrophages and monocytes) [42]. These cells are responsible for immune inflammatory response. They have the property of digesting dead or defective cells. These cells are highly motile and phagocytic cells [42].

(a) B lymphocytes

These cells got their name from bursa of Fabricus. On their maturation, they form immunoglobin or antibody receptors. After the antigens encounter a B naive cell, the cell divides into memory B cells and effector B cells called plasma cells. Memory cells have more life span indicated by its name and express the same membrane bound antibody as the original parent naive B cell [43]. Large numbers of antibodies are secreted by effector cells. The plasma cells are short lived, but can secrete more than 2000 molecules of antibody per second. These antibodies contribute to the humoral immunity and these secreted antibodies may be one of the five classes of antibody [44].

(b) T lymphocytes

T lymphocytes are named because of their maturation in thymus gland. At the time of their maturation, they expresses a T cell receptor molecule on their surface. T cells are essential for production of antibodies, cellular immune reactions, and killing of modified cells [45]. T cells have the property of recognizing antigens only when bound to special class of proteins called major histocompatibility complex (MHC) [46]. The antigenic peptide are presented on the surface of antigen presenting cells(B cells, macrophages and dendritic cells) or on virus-infected cells, graft cells or cancer cells. Thus T cells help in normal functioning of cells, and mainly form two population's helper cells (T_H) cells and T cytotoxic (T_C) cells. These T helper (T_H) cells and T cytotoxic (T_C) cells express $CD4^+$ and $CD8^+$ glycoproteins on their surfaces, respectively [47].

(c) Natural killer cells

These have resemblance of lymphocytes but lacking specific antigen receptor. Due to the lack of antigen-binding receptors, they also lack immunologic memory specificity. Natural killer cells are usually large and possess granular lymphocyte and constitute 5–10% of lymphocytes in blood. These cells produce large quantities of interferon–gamma (IFN-γ) to fight against infections specifically viral. Furthermore, these cells are involved in destruction of cancerous cells [48].

2. Granulocytes

Cells containing membrane bound granules in their cytoplasm are known as granulocytes. They possess the enzymes which have the property of killing microorganisms and removing cell debris by a simple process known as phagocytosis. These can be eosinophils, basophils, and neutrophils. These cells are categorized on the basis of nuclei segments, granules, and differentiated cells [49].

(a) Neutrophils

These are 55–70% of all the circulating leukocytes, have granular cytoplasm and segmented nucleus. After their production from bone marrow,

they remain in blood circulation for 7–10 h, then migrate to tissues and reside there for few days. Their number increase by the process called leukocytosis, which depicts the site of infection [50]. The pus cells mainly comprise of neutrophils during infection. Neutrophils are active in nature, they have different lytic enzymes and bactericidal substances that are contained in primary and secondary granules unlike macrophages [51].

(b) Eosinophils

These are blood leucocytes (1–5%) mostly present in tissues. Structurally they possess bilobed nucleus granular cytoplasm that stains mostly with eosin red dye, mostly abundant in circulation, in tissues, and when in need at the time of allergic, parasitic diseases. They are motile in nature and are quick at the site of action. They do not perform phagocytosis quickly but do degranulate in the presence of chemotactic factors and when immunoglobins like IgG or IgE is cross linked by antigen [52].

(c) Basophils

These cells have least population of all the leukocytes. These contain basophilic granules which contain histamine and heparin that are needed during allergic reactions. Structurally they are S-shaped and having bilobed nucleus [53].

(d) Mast cells

Mast cells are sessile cells and abundantly present in almost all parts of the body. Their cytoplasmic granules contain histamine and other pharmacologically active substances. Their surface F_C receptors have high affinity for IgE and their surface interaction leads to degranulation of mast cells. Basophil cells and mast cells play a boost role in allergic responses [54, 55].

(e) Dendritic cells

Dendritic cells are quite large than rest of immune cells. They are motile in nature and possess antigen presenting properties. They are nearly 1% present in lymphoid system, and are found at different sites [56].

6.2 Human Immunogenetics, Basic Principles and Clinical Relevance

Immunogenetics is defined as the study of genetic basis of immune response. Over a last decade this branch of immunology has developed uninterruptedly. Development of immunogenetics from a subdivision of HLA disease association studies to a diverse field by exploiting new methodologies like genome wide approach to identify a large no. of immune response genes; this field of immunogenetics has revolutionized human infectious disease diagnosis and treatment. Immunogenetics in human infectious diseases reflects two developments: new polymorphic gene's availability in a large assemblage and identification of new susceptible and resistance genes by taking a genome wide and mapping approaches. Although the

continuing focus on MHC of many infectious diseases; mapping and identification of the susceptible loci by using genome linkage analysis are increasing at startling rates. Large number of association studies of candidate genes has increased rapidly as more polymorphisms are identified in genes considered to have important roles in pathogenesis or protection. Various methods have been developed to analyze HLA class I and HLA class II polymorphisms and some of the methods are as follows:

1. *Serology*: HLA gene polymorphisms were studied firstly by leukoagglutination and later on by complement dependent lymphotoxicity. Antisera obtained from the multiparous or pregnant women who have developed antibodies to the HLA antigens present in the paternal haplotype of their children were primarily used.
2. *HLA class I typing*: The serological definition of HLA-A, B, C antigens can be done by two similar tissue typing assays: the National institute of health (NIH) lymphocytotoxicity test or the Amos modified technique. In lymphocytotoxicity test, lymphocytes, isolated from the peripheral blood or lymphoid tissues, are tested against a large set of complement dependent allosera and monoclonal antibodies. This test is rapid for HLA typing as compared to the others.
3. *HLA class II typing*: serological definition of HLA class II antigens is limited to the products of the HLA-DR and HLA-DQ subregions. The methodologies are based on lymphocytotoxicity with enriched B lymphocytes preparation or by two color fluorochromasia to distinguish between B and T cells (T cells don't express HLA class II antigens). Monoclonal antibodies against HLA-DR and especially HLA DQ are useful reagents for B cell typing.
4. *Cellular Typing*: There are various types of cellular typing and some of them are, mixed lymphocyte typing, primed lymphocyte test, primed lymphocyte test clone, cell mediated lympholysis, etc.

6.3 Antibody, Structure and Classification

Antibodies or immunoglobulins are Y shaped glycoproteins produced by B cells (plasma cells). They are present in body secretions/fluid and on the surfaces of specialized cells. Antibodies discern and bind to unique molecular structures known as epitopes which are present on the surface of the specific antigens [57].

6.3.1 Structure of Antibody

Antibodies are globular plasma proteins as heavy as 150 kDa. The basic structure of all antibodies remains the same, consisting of four polypeptide chains in which two are identical heavy and rest of the two are identical light chains. Two heavy chains are connected to two light chains by disulfide bonds. Light chains consist of

polypeptides about 22,000 Da and heavy chains consists of polypeptides about 50,000 or more. There are five types of heavy chains (in mammals) which are as follows: α (alpha), δ (delta), ε (epsilon), γ (gamma), and μ (Mu) which represent IgA, IgD, IgE, IgG, and IgM, respectively, whereas there are two kinds of light chains, known as lambda (λ) and kappa (κ) [58]. Each antibody can have either two **κ** or two **λ** chains but not one of each. The ratio of κ and λ is 2:1, but there are no functional differences between the types. Each antibody or immunoglobulin consists of two variable regions and one constant region. An amino terminal region (V region) is present in each heavy and light chain consisting of about 100–110 amino acids [59]. Fragment antigen-binding (Fab) region contains the variable domains of the light and heavy chains. Variable domains give rise to variable regions of the antibody which give the antibody its antigen specificity these variable regions differ in every antibody; furthermore, each Fab region contains two constant domains (one from heavy chain component and one form light chain component). Remaining constant domains from two heavy chains make the FC region (fragment crystallizable), this region interacts with different immune cells and mediates different functions e.g. opsonization. The constant domains of the heavy chains determine the antibody class and are same for all antibodies of the same class [60].

6.3.2 Classification of Antibodies

Antibodies also termed as immunoglobulins are glycoproteins produced in response to an immune reaction. These immunoglobulins are classified into five major classes based on the heavy chain they contain and discussed as follows.

1. *Immunoglobulin G (IgG):* IgG is the most predominant immunoglobulin present in human serum and is a monomer. This type of immunoglobulin is produced as a secondary immune response to a specific antigen. IgG comprises of approximately 75% of total serum Ig [61]. This is the only immunoglobulin which is able to cross the human placenta and is largely responsible for the protection of newborn during the first weeks of life. In healthy individual's serum IgG presents about 15% of total proteins besides albumin, enzymes other globulins, etc. There are four subclasses of IgG described in humans, rat, and mouse based on number of disulfide bonds, length, and flexibility of the hinge region. The four subclasses of IgG are as follows: IgG1, IgG2, IgG3, and IgG4 [62].
2. *Immunoglobulin M (IgM):* IgM subsists as a pentamer (containing five monomers) in mammals and constitutes about 10% of total serum immunoglobulin content. It preponderates in primary immune response to majority of the antigens and is the most systematic complement fixing immunoglobulin. IgM is the first immunoglobulin to be synthesized by the neonates and plays an important role in cell agglutination and pathogenesis of some autoimmune diseases [63]. IgM is the first immunoglobulin synthesized during an immune response and is predominantly found in lymph fluid and blood. Increased levels of IgM can be an indicative of exposure to antigen or a recent infection [64].

3. *Immunoglobulin A (IgA):* IgA exists both in monomeric and dimeric forms, comprises about 15% of the total immunoglobulins. Secretory IgA is abundant in mucosal secretions like tears and saliva. The primary function of IgA is to restrict the transport of external pathogens into the blood and overall circulatory system [65]. There are two subtypes of immunoglobulin A, that are IgA1 and IgA2. They both differ from each other in their concentration in the serum and molecular mass of heavy chains. Most of the immunoglobulin A is in the secreted form. IgA in secretions such as saliva, tears, colostrum, mucus, gastric acid, etc. are in the dimeric form connected by a joining peptide. IgA does not incite bacterial cell lysis through the complement system because IgA is a weak complement activating antibody [66].
4. *Immunoglobulin D:* IgD levels in the serum are relatively low as compared to the other immunoglobulins and have a short half-life. The functions of immunoglobulin D are ambiguous; because of this ambiguity the role of IgD in major antibody effector mechanisms is not known [67]. Membrane bound IgD is associated Cd76a and CD79b for signaling. When IgD leave the bone marrow they are expressed on the membranes of B cells and populate secondary lymphoid organ [68].
5. *Immunoglobulin E:* IgE levels in serum are low as compared to the total immunoglobulins. The primary function of this immunoglobulin is responsiveness towards allergic reactions and primarily defends against parasitic invasion. The IgE heavy chain includes an additional domain by which it binds to the Fc epsilon receptor I (FcεRI) found predominantly on mast cells, basophils and eosinophils with high affinity [69].The cells degranulate and release factors such as histamine, heparin, proteolytic enzymes, and cytokines leukotrienes when antigens such as poison, pollen, fungus, dust mites, spores, and pet dander attach with the Fab portion of the IgE attached to the cells. As a result, vasodilation and increased permeability of the small vessel allow fluids to break free into tissues from the capillaries, leading to the hallmark signs of allergic reaction [70].

6.4 Antibody Production and Mechanisms Involved

Antibodies are host proteins that in reaction to external particles invading body are formed by the immune system. These external particles or molecules are referred to as antigens, and their immune system molecular recognition results in selective development of antibodies capable of binding the particular antigen. B lymphocytes generate antibodies and circulate in the blood and lymph, where they bind to their particular antigen, allowing it to be cleared from circulation [71]. In a range of research and diagnostic applications, this ability of animal immune systems to generate antibodies capable of binding directly to antigens can be harnessed to produce probes for the detection of molecules of interest. Procedures were developed during the 1970s and 1980s to produce, purify, and alter antibodies for use as antigen-specific samples and have remained largely unchanged since Harlow and Lane published their classic Antibodies: A Laboratory Manual in 1988.

The term “antibody production” has both specific and general meanings. It refers, in a broad sense, to the entire process of producing a specific antibody that can be used, including the steps of preparation of immunogen, immunization, development of hybridoma, selection, screening, isotyping, purification, and labeling for direct use in a particular method. The development of antibodies includes the preparation and safe injection of antigen samples into laboratory or farm animals in order to elicit high levels of expression of antigen-specific antibodies in the serum that can then be recovered from the animal [72]. The polyclonal antibodies are directly recovered from the serum (bleeds). Monoclonal antibodies are produced to create monoclonal hybridoma cell lines that express the specific antibody in cell culture supernatants by fusing antibody-secreting spleen cells from immunized mice with immortal myeloma cells [73]. With respect to several important steps and considerations, successful antibody production depends on careful planning and implementation, mentioned below:

- Synthesizing or purifying the target antigen (e.g., hapten or peptide).
- Choosing an appropriate carrier protein that are immunogenic.
- Creating the immunogen by conjugating the antigen and carrier protein.
- Immunize animals using appropriate schedule and adjuvant formula.
- Screen serum (hybridoma) for antibody titer and isotype.

6.4.1 Antibody Purification

Purification of an antibody involves isolation of antibody from serum (polyclonal antibody), ascites fluid, or hybridoma cell line culture supernatant (monoclonal antibody). The purification methods ranges from crude to highly specific [74].

1. *Crude*: Precipitation of a subset of total serum proteins that includes immunoglobulins.
2. *General:* Affinity purification of certain antibody classes (e.g., IgG) without regard to antigen specificity.
3. *Specific:* Affinity purification of only those antibodies in a sample that bind to a particular antigen molecule.

6.5 Major Histocompatibility Complex (MHC Complex), Structure and Functions

The MHC molecules are glycoproteins encoded in a large cluster of genes located on chromosome number six. Their potent effect on the immune response to transplanted tissue and hence for this reason the gene complex was named as major histocompatibility complex. In humans these genes are called as human leukocyte

antigens (HLA). In humans this complex system contains more than 200 genes [75]. The primary function of MHC is to present antigens to T cells so that it can discriminate between self and non-self. MHC molecules are both polygenic (containing different MHC class I and MHC class II genes) and polymorphic (multiple variants of each gene) which makes it difficult for the pathogens to evade immune system. Specific regions of MHC-I and MHC-II contain predominant polymorphic sites called as domains. There is no such structural difference between the two classes of MHC molecules, however, the only difference is the manner in which the peptide is bound and presented in the binding cleft [76].

The general name given to the highly polymorphic glycoproteins encoded by the MHC Class I and MHC Class II genes involved in presenting peptide antigens to T cells is MHC molecules. They are also called as histocompatibility antigens. There are two major classes of MHC molecules like MHC class I (MHCI) and MHC class II (MHCII). These two classes of proteins play an important role in the adaptive branch of immune system. Both classes of MHC molecule share the same task of presenting peptides on the cell surface for recognition by T cells [77]. The structure of both the classes discussed as follows:

6.5.1 MHC-I

MHC-I class proteins are present on every nucleated cell except some cells in the brain and retina and enucleated red blood cells. This class of MHC is encoded by HLA-A, HLA-B, and HLA-C genes encoding HLA-A, HLA-B, and HLA-C, respectively [78].

1. MHC-I: structure and function

   MHC-I consist of α-chain which is 45 kDa and β2 microglobulin molecule which is 12 kDa and is associated noncovalently with α-chain. The α-chain is composed of three domains, α1, α2, and α3, each domain approximately 90 amino acids, a transmembrane domain of about 25 amino acids which are hydrophobic in nature followed by short stretch of amino acids (charged, hydrophilic) of cytoplasmic tail of 30 amino acids [79]. A deep groove is formed by the interaction of α1 and α2 domains. α3 and β2 are organized into β pleated sheets each formed by antiparallel β strand of amino acids, this structure is called as immunoglobulin fold. However, β2 is a protein encoded by a gene located on a different chromosome. The β2 and α3 are similar in size and organization. β2 microglobulin does not contain transmembrane region and is linked with α chain non covalently. Immunogenic peptide–MHC class I (pMHCI) complexes are presented on nucleated cells and are recognized by cytotoxic CD8+ T cells [80]. The major function of MHC-I is to bind peptide antigens and present to CD8+ T cells (T helper cells) [81].

6.5.2 MHC-II

Major histocompatibility complex class II molecules present peptides to CD4$^+$ T cells. MHC-II is normally found on antigen presenting cells which eventually initiates an immune response. These classes of MHC proteins are encoded by HLA-D region of the genome in humans. These are chiefly found on macrophages, dendritic cells, B cells, and other antigen presenting cells (APCs). Their expression on epithelial and endothelial cells is induced by interferon gamma (IFN-γ). This complex is composed of β1 and β2 subunits and thus can be recognized by CD4 co-receptors [82].

1. MHC-II: structure and function

   MHC-II molecule is a dimer composed of 133 kDa α-chain and 28 kDa β-chain associated by noncovalent linkages. Both α-chain and β-chain are composed of two domains: α1 and α2, β1 and β2, respectively. These are transmembrane glycoproteins containing external domains, a cytoplasmic tail and transmembrane segment. An open groove is formed between α and β chain at the proximal end which serves as a peptide binding cleft binding antigenic peptide [83]. The functions of MHC-II include the following:
   - The TCR–peptide: MHC class II engagement is crucial to the induction and regulation of adaptive immunity by selecting the mature CD4+ T cell repertoire in the thymus and activating these lymphocytes in the periphery [84].
   - The secure attachment to the MHC molecule with the presented peptide ensure stable peptide binding which enhance T cell recognition of the antigen, T cell recruitment, and a proper immune response.
   - Since they present antigens from exogenous sources, MHC class II molecules are critical for the initiation of the antigen-specific immune response.

6.6 Hypersensitivity Reaction

Hypersensitivity reactions are the immune system's overreaction to an antigen that does not usually cause an immune response. It is a condition in which immune system of body instead of producing protection against foreign invaders or an antigen produces a response in inflated fashion leading to the damage of the whole system. These reactions are called as hypersensitivity or allergic reactions. Hypersensitivity is classified into four types (type I, type II, type III, and type IV), first three being antibody mediated and fourth one T cell mediated (Table 6.1) [85].

Paul Portier and Charles Richet were the first scientists who were looking for the case of bathers in the Mediterranean. The bathers were reacting aggressively to the Portuguese Man of War stings. Both of them concluded that the reaction was due to toxins, and they used isolates of jellyfish toxins as antidotes. First attempt led to a disaster, followed by injecting them to dogs. The dogs reacted immediately with vomiting, diarrhea, asphyxia, and finally death. This is how term anaphylaxis was coined by them to describe this and were awarded Nobel Prize in Physiology or

Table 6.1 Types and cells involved in hypersensitivity

Characteristics	Type I	Type II	Type III	Type IV
Alternative names	Ig E mediated / anaphylactic type/ allergy	Ig G mediated/ cytotoxic type	Immune complex mediated	Cell mediated/ delayed type
Cells responsible	B cells	B cells	B cells	T cells
Cells involved	Basophils, mast cells	Red blood cells, white blood cells, and platelets	Different host cells	Different host cells
Antibody	IgE	IgG, IgM	IgG	None
Mediators	Histamine, serotonin, and bradykinin	Complement proteins, antibody dependent cellular toxicity (ADCC)	Complement proteins, neutrophils, and proteases	Lymphokines
Timing of reaction	Immediate or within 30 min	Hours to days	Hours to days	Hours to days, peaks at 48–96 h
Skin and other reaction	Wheal and flare, oedema, erythema, and bronchospasm	None, cell lysis, phagocytosis, and inflammation	Arthus reaction (tissue death or injury), vasoconstriction, and necrosis	Thickening or tissue death, granuloma formation
Target tissue	Vascular endothelium, bronchial smooth muscle	Blood or tissue cell	Vascular endothelium, epithelium cells	Infected cells, foreign tissue cells
Examples	Hay fever, insect venom, foods, drugs, vaccines	Transfusion reaction, rheumatic fever	Farmer's lung, serum sickness, malaria	Tuberculin reaction, transplant rejection

Medicine in 1913. Various types of hypersensitivity and cells involved are depicted in Table 6.1 [86].

6.6.1 Allergy and Allergens

In 1906, the term "allergy" was introduced by von Pirquet [87] who documented antigens had induced changes in reactivity in both protective immunity and hypersensitivity reactions. The word has now used synonymously with IgE-mediated allergic disease. Pirquet's intent was that the term should be generalized as a biologic response, which may either lead to a beneficial effect (immunity) or a harmful effect (allergic disease). Allergy is used to explain an unsuitable destructive immune response which occurs on an encounter with a substance like pollen or food. There are a number of reasons for the cause of allergy, whereas the symptoms may differ from mild to life threatening. A person who is allergic to a specific allergen like

pollen, when comes in contact with it an antibody response is created, and mast cells act in response by secreting histamines. Release of histamine leads to the inflammation (redness and swelling) which at times is painful and annoying [88].

An antigen is able to stimulate type I hypersensitivity in atopic individuals though IgE responses. Atopic individuals have a hereditary tendency to produce IgE antibodies against common allergens. Increase in significant Immunoglobulin E responses acts as a defensive mechanism against infections. Sensitivities may differ from one person (or from one animal) to another [89]. A very wide variety of substances can be allergens to sensitive individuals. Various sources of allergens come from by any form of direct contact with the allergen-consuming food or drink, breathing in pollen, perfume or pet dander, or brushing a body part against an allergy-causing plant (direct contact). The other causes of allergy are wasp, fire ant and bee stings, penicillin, and latex. If an allergic reaction leads to a serious consequence, that form is called anaphylaxis [90].

6.6.2 Role of Immunoglobulin E and Mast Cells in Allergy

Plasma cells which produce IgE are primarily distributed in the lymphoid tissues of respiratory and gastrointestinal tracts. On exposure to allergen, antibody is synthesized and distributed right away through the body. It quickly binds to high affinity FceR receptor on the mast cell surface. The normal concentration of IgE in blood is (0.0003 g/l) or less than 0.001%, as most of it is attached to FceR. It is retained till degranulation [91]. It has been seen that genetic factors regulate IgE biosynthesis, immune recognition of specific allergens. MHC genes and a cluster of cytokine receptor genes (IL-4R cluster) control antigen presenting cells and cytokine responsiveness, respectively. The activity of Interleukin-4 plays an important role in allergy as it instigates B lymphocytes to produce IgE, whereas mast cells are known to produce IL-4 after an encounter with antigen. Two subsets of T cells occur: Th2 cells, which secrete IL-4 and up regulate activity, whereas another subset, i.e., Th1 cells, secrete IFN-gamma, which inhibits IgE in B cells [92]. In clinical laboratories, immunoassays are used to measure level of IgE, which gives us information about severity of allergy and allergen exposure. In case of patients with chronic urticaria or anaphylaxis, IgE measurement is useless. In patients with parasitic infections and immunodeficiency conditions IgE levels are raised due to abnormal T cell regulation (Wiskott-Aldrich syndrome, Hyper-IgE syndrome, Job's syndrome) [93].

A multistep process which involves various events like, (1) Sensitization phase: To any kind of antigenic stimulus, IgE antibody is produced which binds to its receptor on mast cells and basophils; (2) Activation phase: In this phase re-exposure of same antigen leads to production of granules by mast cells and basophils; (3) Effector phase: Pharmacologically active agents are produced by mast cells and basophils leading to a complex response. A series of events take place in the immune system of individuals who suffer from an allergic reaction [94].

Antigen like pollen or dust might come into the contact with body part like mucosa of nose or throat. It is taken up by antigen presenting cells with the help of T cells and are presented to B cells, which produce antibody plasma cells. Instead of producing IgM antibodies, these plasma cells produce IgE antibodies. Then these antibodies attach to mast cells and basophils via Fc receptors, which hold cytoplasmic granules containing histamines, leukotrienes, and other chemical messengers. These granules are basically mediators. Mast cells are found in connective tissues of skin, respiratory tract, and blood vessels, while basophils are present in blood stream (1% of the blood stream). There are specific receptors present on the mast cell or basophil to whom IgE antibodies attach. They have almost 5,00,000 sites for IgE attachment. The IgE molecule can survive for a number of weeks once it is attached, and antibody reaction sites wait to interact with antigen [95]. These antibodies are harmless till a person is not exposed to antigen. Once exposed, the antigen voluntarily combines with antibodies and causes mast cells to produce histamine granules, leukotrienes and other chemicals. These reactions can lead to hives, hay fever, shock, and other allergic reactions [96].

The persistence of this type of reaction has an economic importance and it has been documented that IgE antibodies are of importance in immunity against parasitic worms. When there is a worm infection, worm antigens will respond by causing mast cells to release protein granules that augment vascular permeability and attract inflammatory cells. That's how IgE antibodies have a defensive role [97].

6.7 Tumor Immunology

When healthy cells grow in an uncontrolled fashion, that state of cell division is known as cancer. A normal healthy cell is governed by proto-oncogenes and tumor suppressor genes. A number of factors can induce proto-oncogenes into oncogenes and also affect tumor suppressor genes [98]. A malignant cell surface may carry antigens, which are unique to cancer cells and are absent in other normal cells. These antigens are called as tumor specific antigens (TSAs). There are some antigens that are present on both malignant and normal cells, but they remain masked on normal cells and unmasked on tumor/malignant cells [99]. The immune system works on the principle of the theory of immunological surveillance, which says that the immune system constantly recognizes and eliminates tumor cells. In case tumor cells get away from the surveillance, they grow into big tumors and hence called as cancer. There are different types of cancer, which are basically classified on the basis of cell type that resembles the tumor [100].

- Carcinoma: Malignant tumors associated with epithelial cells, e.g., breast, prostate, lung, and colon cancer.
- Lymphoma and leukemia: Malignant tumors associated with blood and bone marrow cells.

- Mesothelioma: Malignant tumors associated with mesothelial cell lining, peritoneum and pleura.
- Glioma: Malignant tumors associated with brain cells.
- Germ Cell tumors: Malignant tumors associated with testicles and ovary.
- Choriocarcinoma: Malignant tumors associated with placenta.

6.7.1 Acquired Immunodeficiency Disorder (AIDS)

Acquired immunodeficiency syndrome/disorder (AIDS) is an immunodeficiency disease caused by human immunodeficiency virus (HIV) called as human T-lymphotropic virus type III (HTLV III). The host individual becomes opportunistic to a number of infections leading to multiple organ failure. It was in 1981, when a case of AIDS appeared and was correlated as Kaposi's sarcoma and later in 1983, the pathogen leading to the loss of immune system was identified as retrovirus. There are several schools of thought about its origin, but it is believed to be a mutation of virus endemic to Central Africa for years. Till today there is no vaccination and cure to the disease.

6.7.1.1 Infection

The virus has an envelope coated with spiked glycoprotein (gp 120-molecular weight of 120,000). The spikes attach to CD4 receptors present on the cell surface and followed by entry into the host cell. The enzyme reverse transcriptase transcribes viral RNA into DNA, which gets integrated into chromosomal DNA, thereby taking control of the whole machinery of the cell. This DNA remains dormant as provirus [101]. The Centre for Disease Control and Prevention has classified the infection into three categories

1. *Category A:* Asymptomatic infection with swollen lymph nodes, blood count of CD4 T cells is 500 per mm^3.
2. *Category B:* CD4 T cell count falls (200–499/mm^3). Infections in mouth, throat, and vagina appears. Other severe conditions such as shingles, diarrhea, fever, leukoplakia, and cancerous or precancerous conditions may occur.
3. *Category C:* CD4 T cell count falls (200 mm^3 or lower). Symptoms appear at this stage. Infections of esophagus, bronchi, lungs, tuberculosis, eye infection, and Kaposi's sarcoma will appear.

HIV transmission is possible via body fluids and the blood is the most important source. The disease can be spread through various routes as: blood contaminated needles, breast milk, intimate sexual contact, organ transplant, blood transfusion, anal-receptive intercourse, and artificial insemination. And it cannot be transmitted by food, water, insect bite or casual contacts like playing with HIV positive patients. It has been observed the disease has 100% mortality rate.

6.7.2 Immunodeficiency Disease

The acquired immune response is mediated by B and T lymphocytes, phagocytic cells and the complement system of the body's immune system. The interactions of all these is controlled in such a way that the body functions normal. When there is an imbalance in the differentiation of cells, production of chemical messengers, a number of immunological disorders from mild to fatal arises. These disorders are called as immunodeficiency disease. Immunodeficiency disease are of two types: (1) Primary Immune deficiency disorders (immune deficiency is the cause of disease) and (2) Secondary Immune deficiency disorders (immune deficiency is a result of other disease). An account of the important immunodeficiency disease/disorders are as:

1. *X-Linked Infantile Agammaglobulinemia:* It is known as Bruton's agammaglobulinemia. A rare X-linked disorder which occurs mainly in infants (males: 1/100,000). At the age of 5–6 months, infant loses most of the IgG that had passed through placenta and suffers from recurrent bacterial infections. Infants with this kind of disorder don't respond well to antibiotics. Injecting large amounts of IgG from time to time can be effective [102].
2. *Transient Hypogammaglobulinemia:* Infants who are born premature are not able to synthesize immunoglobulin's and after their mothers transferred Ig's disappear, they suffer this kind of disorder. This may be due to the deficiency in number and function of T helper cells, which plays an important role in the synthesis of IgG. Transient Hypogammaglobulinemia may persist from few months to 2 years [103].
3. *Common Variable Hypogammaglobulinemia:* This disease affects both males and females and may occur at any age with the higher frequency in the age groups of 15–35. The individuals suffer from autoimmune disease like hemolytic anemia, thrombocytopenia, and systemic lupus erythematosus. B cells in these patients fail to produce antibody-secreting cells [104].
4. *Congenital Thymic Aplasia (DiGeorge Syndrome):* T cell deficiency disease due to defect in the embryonic development of third and fourth pharyngeal pouches during 12th week of gestation. Thymus and parathyroids fail to develop. Newborns suffer from hypocalcemia and other congenital disorders of heart and kidney. It is not hereditary, and patients are prone to recurrent infections. Treatment can be fetal thymus transplantation [105].
5. *Chronic Mucocutaneous Candidiasis:* An infection of skin and mucous membranes caused by a fungus (*Candida albicans*). It is associated with selective defect in the functioning of T cells. Patients with this kind of disease have normal T cell mediated immunity to all microorganisms except *candida*. Patients may also suffer from endocrine dysfunctioning like Addison's disease [106].
6. *X-Linked Lymphoproliferative Syndrome (Duncan's Syndrome):* This is again a rare case of disease, where individuals show abnormal response to Epstein Barr Virus (EBV) infection. The infection results into B cell proliferation, T cell

depletion, and malignant lymphoma. The syndrome was found in six maternally related males od Duncan's family and hence named after them [107].

7. *Severe Combined Immunodeficiency Disease (SCID):* It is a heterogeneous/ mixed group of immune disorders, where stem cells fail to differentiate into T and B cells, due to defects in cell function. Patients suffering from SCID are prone to all kinds of microbial infections (Viral, Bacterial, Fungal and Protozoal) [108].
8. *Phagocytic Dysfunction:* Dysfunction of complement components, lymphokines and antibodies, which help in the activation of phagocytic cells. Other causes may be the defects in the metabolic pathways leading to phagocytosis and killing of pathogen. The deficiencies are due to reduced levels of glucose-6-phosphate dehydrogenase, myeloperoxidase, alkaline phosphatase, abnormal microtubules, and lysozomal enzymes [109].
9. *Macroglobulinemia:* It is known as Waldenstroms macroglobulinemia. A large amount of Ig M is synthesized, which leads to increased viscosity in the serum, slow blood flow, nervous disorders, and bleeding. A decrease in the synthesis of other immunoglobulins is observed [110].
10. *Heavy Chain Disease:* It is characterized by the presence of large amounts of monoclonal proteins (55,000 daltons) in the urine. Patients with this kind of disorder are prone to infections and enlargement of lymphoid organs and anemia [111].

6.7.3 Immune Tolerance

It is the avoidance of an immune response against a particular antigen. For example, the immune system is tolerant to self-antigens and does not attack the body's own cells, tissues, and organs. But autoimmune disease like conditions may occur, when tolerance is lost. It can be known by different names, immune tolerance, or immunological tolerance, or immunotolerance. The immune system remains insensitive to the substances or antigens that can cause immune response. When an antigen encounters lymphocytes, an immune response is leads to its elimination or activation and finally leading to tolerance [112].

Antigens which induce tolerance are called as tolerogens/ tolerogenic antigens and are different from immunogens. Depending on the conditions in which it is displayed to specific lymphocytes, an antigen may be immunogen or tolerogen. The basic feature of a normal immune system is self-tolerance and when there is a failure of self-tolerance, it results into immune reaction against self, such a condition is called as autoimmunity and disease they cause are called as autoimmune disease [113]. It was Ray D. Owen in 1945 who observed cattle (dizygotic twins) with a common placenta shared each other's red blood cells and retained them for the rest of life. The term tolerance was not used at that time, but it was observed that the body could be tolerant to the foreign antigens. Later on in 1953, Leslie Brent, Rupert

E. Billingham and Peter Medawar performed an experiment on neonatal mice by injecting foreign cells and validated it.

Tolerance can of two types: Central (thymus and bone marrow induced) and Peripheral (lymph nodes). In general individuals remain tolerant to self-antigens, as the lymphocytes which recognize self-antigens either get killed, inactivated or their specificity is changed. During the development of T and B cells, a person may demonstrate receptors recognizing molecules (self-antigens) [114]. There is a possibility of lymphocytes to react against self-antigens causing disease. The mechanism of immune tolerance is designed to prevent such reactions. The lymphocytes when come across self-antigens become active or tolerant depends upon the properties of antigen, its maturation state and stimuli received. Some antigens show the phenomenon of ignorance and in some experimental models, self-antigens have been seen failed to reduce any response [115]. Tolerance may exploit as a biological tool as a therapeutic approach for treating autoimmune disease, allergic reactions and rejection of organ transplants.

6.7.4 Autoimmunity

It is the phenomenon when the immune system of body produces an immune response which works against its own cells, tissues and self-antigens. Paul Enrich termed this condition as “horror autotoxicus” [116]. Tolerance is such a process that as long as it remains normal, it will work for the proper normal growth and development. But once there is an imbalance in the processes, the integrity of tolerance is lost and autoimmunity is developed. The results of autoimmunity may change from mild to strong depending on the level to which self-tolerance has been affected. A pathological situation is created resulting into involvement of complement system, antibodies, etc. leading to various autoimmune disease for example, rheumatoid arthritis (RA), diabetes mellitus type 1, systemic lupus erythematosus (SLE), Hashimoto’s thyroiditis, Graves’ disease, Addison’s disease, and multiple sclerosis (MS). There can be a number of factors which are responsible for autoimmune disease. Factors can be genetic as well as environmental in nature [117]. A few detailed examples of disease cause by autoimmunity are as follows:

- *Autoimmune Hemolytic Anemia:* This disorder is categorized under immunohemolytic anemia. Antibodies are produced due to unmatched induction of erythrocyte antigens during transfusion or erythroblastosis fetalis. The destruction of red cells can be due to activation of complement system and followed by lysis of red blood cells [118].
- *Myasthenia Gravis:* It is one of the neurological disorders, where acetylcholine receptors at neuromuscular junctions are involved. Here antibodies block the nerve impulse carried across the junction by acetylcholine molecules resulting into severe muscle weakness, difficulty in chewing, swallowing, breathing, and finally dying due to respiratory failure [119].

- *Graves Disease:* This disease is due to the hyperactive thyroid gland. The patients produce autoantibodies against thyroid cell receptors for thyroid stimulating hormone. The continuous stimulation of thyroid gland by these antibodies causes hyperthyroidism [120].
- *Systemic Lupus Erythematosus (SLE):* The literal meaning is red wolf and causes red rash on cheeks. The disease attacks many organs of the body, so called as systemic. It causes fever, joint pain, and damage to the nervous system, heart, and kidney leading to death.
- M*ultiple Sclerosis:* It is believed to be T cell mediated autoimmune disease involving demyelization of central nervous system leading to chronic paralytic condition [121].
- *Rheumatoid Arthritis:* It is due to chronic inflammation of synovium., and lymphocytes cause damage of cartilage and bone. The synovial membrane becomes porous, cellular and causes erosion of joints. Abnormal antibodies called as rheumatoid factors are responsible for the inflammation and other processes [122].

6.7.5 Transplantation

Transplantation is defined as the process of transferring cells, tissues or organs from one site to another. The organ to be implanted is called as graft, the person who donates is a donor and the one who accepts is a recipient or host. Transplantation is an important clinical procedure and the first successful transplant was of cornea in 1906 [123]. Currently, transplantation procedure is done on a routine basis globally with the advent of new science and technologies.

The possibility of accepting or rejecting the organ to be implanted depends on the genetic differences between donor and recipient. The patient does not educe an immune response, if both donor and recipient are of same inbred lines or homozygous twins. However, transplants between distantly related individuals are rejected. Stem cell transplants are one of the successful clinical procedures done nowadays. There are two main reasons why stem cells are preferred: (1) These are used to treat children with inherited immune deficiencies by infusing them with stem cells, which mature fully into effective immune cells and thus saving the child and (2) Another case is of the individuals with leukemia [124]. The individuals can be treated with chemotherapy and radiotherapy but can result into destruction of stem cells in bone marrow and circulation. Here stem cells can be harvested and infused back to the patient and thus patient recovers without any graft-host reaction. One of the major immunological problems with transplantation is that the organ to be implanted is treated by immune system as foreign, recognized and attacked, leading to rejection. The degree of immune response to a graft depends upon the type of graft which can be an autograft, Isografts, Allograft or a Xenograft [125].

6.7.6 Graft Rejection

Graft rejection is the result of an immune response showed by recipient against graft due to incompatibility between tissue antigens of both donor and recipient. Based on the transplantation and rejection, there are three types of graft rejection: hyperacute, acute, and chronic [126].

1. *Hyperacute Rejection:* It is called as early rejection also and occurs within the first few hours of transplantation. There is possibility that antibodies directed against antigens may have played a part in the rejection. The antibodies cause the activation of complement system resulting into triggering of inflammatory responses (antibody mediated cell toxicity). These kinds of episode are untreatable and should be avoided before cross matching.
2. *Acute Rejection:* This kind of rejection may take few days to weeks. It is mediated by T lymphocytes, B lymphocytes, CD4 and CD8 lymphocytes, NK cells, neutrophils, and eosinophils. IL-8 attract lymphocytes and granulocytes to transplanted organ site. Acute rejection can be reversed if detected early by the administering immunosuppressive drugs [127].
3. *Chronic Rejection:* In this case there is a progressive loss of function of the transplanted organ. The damage caused is due to both immune and non-immune processes. The immune processes cause damage to vascular endothelial cells by granulocytes, monocytes, and platelets. A range of interleukins and soluble factors released by activated leukocytes damaged vessel walls, including IL-1 and platelet-derived growth factor (PDGF). All these lead to fibrosis and occlusion due to inflammatory response and proliferation of lesion [128].

6.7.7 Graft Versus Host Reaction

When there is an antigenic difference between donor and recipient, it will lead to an immune response resulting into attack on donor tissue or organ. An individual with immunodeficiency after getting an organ grafted (rich in immunocompetent cells), there is a possibility of graft versus host reaction or vice-versa [129]. It may take weeks to 2 months after transplantation. These reactions are a big problem in infants and children, in whom thymus or bone marrow is transplanted to reconstitute the immune system. Heart, lung, and liver rank second in risk category (less lymphoid tissue) followed by kidney (poor lymphoid tissue) with a rare graft versus host reaction. The immune response against a transplanted organ is very much energetic and strong than that of the response against virus/bacteria, which is seen by the high frequency of T cells recognizing the graft as foreign [130]. When an immunocompetent graft is transplanted into immunocompetent host, the immune system recognizes them as non-self and lymphocytes gets activated. They proliferate (T and B lymphocytes, macrophages), differentiate into helper and effector cells thereby attack the host cells and tissues eliciting the host versus graft reaction proliferation

in lymphoid tissues (liver and spleen) leads to hepatomegaly and splenomegaly. In the later stages of peak proliferation phase skin and gastric cavity is heavily infiltrated causing rashes and diarrhea. Immunosuppressive drugs like thalidomide are being used to treat chronic host versus graft reaction [32].

6.8 Immune Cell Therapy

It is a form of treatment which utilizes the cells of our immune system to eliminate disease. Immune cell therapy is also known as cellular immunotherapy. There can be different approaches of cell therapy like either culturing stem cells, increasing their number and infusing them into the body's immune system or genetically engineering immune cells to increase their capacity to treat various disease. There are number of scenarios where cell therapies are made to treat various disease and disorders of immune system [132].

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Chapter 7
Epigenetics and Medical Biotechnology

Mumtaz Anwar, Samia Rashid, and Zeenat Farooq

Abstract The field of epigenetics deals with the study of processes that lead to changes in gene expression without changing the underlying DNA sequence. These processes include DNA methylation, post-translational modifications of histones as well as non-coding RNAs. This field has undergone enormous progress over the years, especially during the last two decades with the introduction of high-throughput sequencing and computational biology. This magnitude of progress has enabled research in the area that uncovered its role in various key cellular processes and disease conditions alike. Epigenetics is increasingly being pursued as a discipline of study to understand diseases like diabetes, cancer, neurological abnormalities and physiological process like circadian rhythms, behaviour and homeostasis. This chapter discusses the role of epigenetics in various aspects of medical biotechnology. It includes sections on different techniques used in epigenetics, their application in medical biotechnology and role of epigenetics in various diseases.

Keywords Biotechnology applications · Technology · Stem cells · CRISPR · Genetic engineering

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7.1 Introduction to Epigenetics

7.1.1 Epigenetics: Background

Transmission of characters in a stable manner from one cell generation to another is governed by the macromolecule DNA which contains all the information necessary for synthesis of cellular proteins and hence controls all the vital cellular processes. The sequence of amino acids in the proteins is dictated by the sequence of nucleotides within DNA. This DNA, if measured from end to end in a typical human cell, measures about 2 m. Therefore, cells have developed efficient mechanisms to compact the DNA inside the small nuclear space. This compaction is achieved by the association of negatively charged DNA with the positively charged proteins called as histones. These are small, basic proteins (~12–17 kDa in size) which bind to DNA to neutralise its charge and therefore ensure efficient packaging. Various classes of histone proteins have been identified so far [1, 2]. Various other proteins also play a role in DNA compaction but those remain less well-characterised.

The prokaryotic cells do not have the same level of orderly arrangement of DNA–protein complexes as eukaryotes. They, however, contain a less organised DNA–protein complex called "nucleoid".

During the earlier part of the century, a pre-dominant concept among the researchers was that the complexing of DNA with histones only occurs for accommodation into the tiny nucleus. However, it was later realised that the complex is much more structured and organised than initially anticipated. This organisation allows for what later came to be known as the "*differential patterns of gene expression*".

7.1.2 History and Definition of Epigenetics

The term Epigenetics was coined by Waddington in 1953 to describe "changes in the gene expression that occur independently of the changes in the sequence of DNA". These "*changes*" are propagated by covalent histone modifications, covalent DNA modifications and micro-RNAs.

The original concept behind this definition has its roots in the studies carried out much earlier in the nineteenth century in cell biology and embryology. These studies laid the foundation for our present day understanding of gene expression and transmission of characters [3].

There is a long history behind the discovery of Epigenetics and in all the years that followed since its discovery, the definition has evolved a lot. Our present definition of the term reflects the understanding that although the nature of DNA is the same in many different cell types in an organism, the difference in epigenetics has the potential to give each cell type its own identity, pattern of gene expression and the power for vertical transmission. This understanding has led to a very interesting

"working definition" of the term epigenetics as "*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*" [4].

7.1.3 Evolution of the Definition of Epigenetics: Insights from Research Involved

Following the discovery of chromosomes by the German Biologist Walther Flemming in 1879, strong evidence were provided by many investigators about developmental program being governed by chromosomes. The most significant demonstration was provided by Thomas Hunt Morgan in 1911 about the "genetic linkage" of several Drosophila genes to the X-chromosome [5]. Rapid progress followed in the aftermath of this discovery in creating linear chromosome maps of Drosophila in which various gens were assigned specific positions linearly on the chromosome with respect to each other [6]. The discovery that chromosomes contained both nucleic acids and proteins sparked the debate about the contribution of both these biomolecules in carrying genetic information. Furthermore, evidence from Drosophila genetics suggested the occurrence of changes in phenotype without similar changes observed in genotype which apparently supported the notion that "cytoplasm" also plays a role in dictating "developmental plans". However, this debate was silenced by the discovery of DNA as the primary carrier of genetic information. Nevertheless, with advancements in technology which permitted cutting edge research into molecular genetics and development, the role of the "non-DNA" elements in governing development became more and more apparent and obvious. Today, each of the two elements of chromatin "DNA and proteins" is known to play key respective roles in dictating "epigenetic and developmental landscapes".

Eventually, it became both convenient and useful to define epigenetics as the mechanisms which propagate heritable changes without changing the sequence of DNA itself.

Quite often, the epigenetic and non-epigenetic components are intertwined and hence a clearer description of individual epigenetic carriers is useful to understand the entire concept (Fig. 7.1).

7.1.4 Histones and Nucleosomes

Five major classes of histone proteins have been identified. These are known as H1, H2A, H2B, H3 and H4. Two copies, each of histones H2A, H2B, H3 and H4 come together and are wrapped by ~147 bp of DNA to give rise to "nucleosome". Presence of H2A, H2B, H3 and H4 inside the nucleosome core lends the name core histones to these proteins [7, 8]. Nucleosome structure is the fundamental unit of chromatin

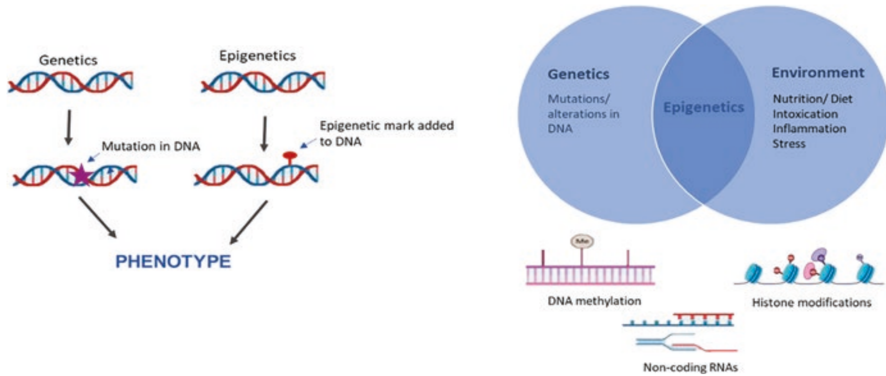


Fig. 7.1 Effect of genetic and epigenetic factors on phenotype. The figure shows the difference between the mechanism behind propagation of genetic and epigenetic factors in the effect on phenotype. (**a**) Shows that genetics affects the phenotype through mutations which alter DNA sequence, while epigenetics affects phenotype without changing DNA sequence. (**b**) Shows how genetics and environment come together to regulate epigenetics to affect phenotype. Most prominent mechanisms of epigenetic progression (DNA methylation, histone modifications and non-coding RNAs) are also depicted

organisation identified so far [9]. Histone H1 binds at the intervening DNA between adjacent nucleosomes (known as entry and exit sites) and is therefore known as the linker histone. H1 ensures further compaction of chromatin beyond nucleosomes into higher order structures like beads on a string (30 nm fibre), etc. Linker histone found in aves is known as H5 (Fig. 7.2).

7.1.5 Post-translational Modifications of Histones

In their 3-dimensional structure, histone proteins consist of a globular domain at C-terminus and an unstructured tail at their N-terminus. The globular domain is responsible for DNA–DNA and DNA–protein interactions and the tail acts as a site for different covalent modifications, collectively and famously known as histone modifications. These modification marks are mostly found in histones H3 and H4 [10]. Till date, more than 100 different types of modifications have been described, including acetylation, methylation, ubiquitination, phosphorylation, crotonylation, sumoylation [10]. In 2011, the graph of known histone modifications was raised by about 70% through identification of 67 previously uncharacterised histone modifications in a single report. Interestingly, eight of these were found to be "short-chain lysine acylations" (propionylation, butyrylation, 2-hydroxyisobutyrylation, succinylation, malonylation, glutarylation, crotonylation and β-hydroxybutyrylation) [11].

We shall describe the most prominent class of histone modifications in the next section in some more detail, which are

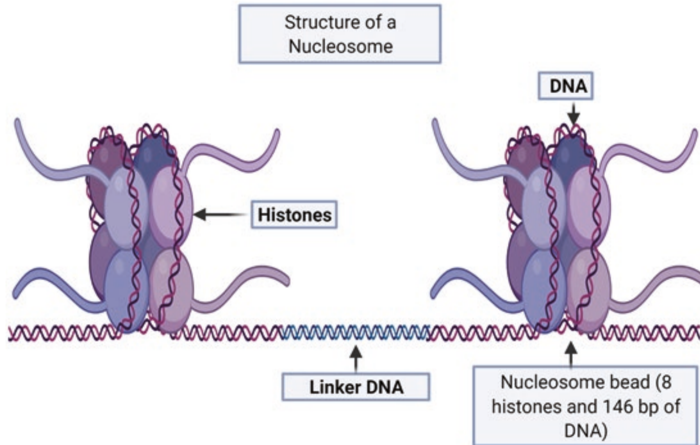


Fig. 7.2 Organisation of a nucleosome. The figure shows a nucleosome composed of two units of each core histone wrapped by 147 bp of DNA to form a nucleosome. The DNA between adjacent nucleosomes is bound by linker histone H1

- Phosphorylation of serine (S) and threonine (T) residues.
- Methylation of lysine (K) and arginine (R) residues.
- Acetylation of lysine (K) residues and.
- Ubiquitination of lysine (K) residues [11].

These modifications can act in various ways to either increase accessibility to DNA and gene expression or to decrease it.

1. Alterations in the charge of histone to change the electrostatic (negative: positive) attractions between DNA and histones, respectively. For example, acetylation neutralises the positive charge of histones, thereby decreasing DNA–histone interactions. This leads to an increase in the accessibility of DNA for processes like replication, transcription, etc.
2. Some modifications act as a signal (docking molecule) for other proteins to bind chromatin. This can influence chromatin structure and gene expression in two ways.

   (a) Modifications that act as signalling platform for proteins which promote chromatin condensation. For example, methylation on histone H3 on lysine 9 residue (H3K9) acts as a docking site for the binding of heterochromatin protein (HP1) which increases heterochromatinisation.
   (b) Modifications that act as signalling platform for proteins which promote chromatin de-condensation. For example, acetylation of H4K16 acts as a binding platform for BRD2. This protein is a transcriptional co-activator and hence enhances transcription at the de-condensed chromatin regions created by H4K16 acetylation.

We will talk about each of these modifications and their function in slightly more detail in the next section.

7.1.5.1 Histone Methylation

In this process, a methyl group is added to histones by the enzymes known as histone methyltransferases, using *S*-adenosyl methionine as the methyl group donor. Histone methylation is a neutral modification. Being non-polar in nature, methylation enhances the hydrophobic interactions inside chromatin. Methylation is the most complex histone modification because lysine can undergo three degrees of methylation (mono, di or trimethylation) and arginine can undergo two degrees (mono and dimethylation). Also, it is the most stable modification with the slowest turnover. Methylation acts as a binding site for various proteins which promote different functions, depending upon the residue being methylated and the enzyme involved. Histone methylation takes place on lysine and arginine residues on the histones H3 and H4. Methylation on H3K4, H3K36 and H3K79 plays role in gene activation and on H3K9, H3K27 and H4K20 play role in gene silencing [7, 12, 13].

7.1.5.2 Histone Acetylation

It is the most well-studied histone modification which involves addition of an acetyl group from acetyl-CoA to histones with the help of histone acetyltransferase enzymes. The negative charge on the acetyl group neutralises the positive charge on histone and hence decreases histone–DNA interactions [14]. Therefore, acetylation is involved in chromatin de-condensation and gene expression. For this reason, various transcriptional activator complexes contain histone acetylation enzymes as members of the complex like the CBP 300 and TAF II 250 [15].

7.1.5.3 Histone Phosphorylation

Phosphorylation of histones involves addition of a phosphate group on histone with the aid of enzymes known as kinases (phosphotransferases) which transfer a phosphate group from ATP to the hydroxyl side chain of the amino acid residues serine, threonine, tyrosine and histidine. Phosphorylation adds sufficient negative charge on chromatin and disrupts histone:DNA interactions to promote gene expression. In fact, various transcriptional up regulators contain phosphor-binding domains [16, 17]. However, histone phosphorylation is a versatile modification. For example, the same modification might be involved in transcriptional upregulation in one context and with condensation in another. This is best exemplified by H3Ser10 phosphorylation which performs a dual role of activation on the one hand and of chromosome condensation and segregation on another [18]. Phosphorylation status changes dynamically with the gene expression profile [19]. Phosphorylation is reversed by

another class of enzymes known as phosphatases. Phosphorylation is a very prominent post-translational protein modification mark, and it plays a role in various cellular signalling pathways by employing phosphorylation–de-phosphorylation switches like mTOR pathway, p53 signalling pathway, ISR pathway, etc. In fact, phosphorylation (including that beyond histones) is the most well-studied cellular post-translational protein modification. In non-histone proteins, phosphorylation mostly takes place on serine residues.

7.1.5.4 Histone Ubiquitination

It is a process in which ubiquitin molecules are added to lysine residues of histones. Monoubiquitination is the major form of ubiquitination in histones. However, histones H2A and H2B can also be modified by polyubiquitination. The first ubiquitinated histone to be identified was H2A [20]. H2A and H2B also hold the distinction of being the most abundantly ubiquitinated proteins in the nucleus [20, 21]. In addition, H3, H4 as well as H1 have been reported to be modified by ubiquitin but the biological function of these ubiquitinations has not been well characterised [22, 23]. Histone ubiquitinations perform several important nucleosomal functions. Chromatin immunoprecipitation (ChIP) experiments have revealed enrichment of monoubiquitinated H2A (H2Aub) in the satellite regions of genome and of H2Bub in transcriptionally active genes [24, 25].

Histone ubiquitination involves addition of the 76-amino acid protein ubiquitin to the lysine residues of histones with the help of enzymes known as ubiquitin ligases. Unlike other histone modifications, ubiquitination is the only modification in which the substrate is a protein rather than an inorganic molecule. Polyubiquitination was well known before the discovery of histone ubiquitination as a mechanism behind proteasomal protein degradation. However, histones are generally modified by mono-ubiquitination. In this case, this "*mark*" acts as a signalling platform for further modifications or downstream chromatin effector functions rather than as a "*signal*" for degradation. Histone ubiquitination mostly takes place on H2A and H2B. Histones H1, H3 and H4 have also been reported to be modified by ubiquitin, with the roles of these modifications being less well characterised [20–23] (Table 7.1).

7.1.6 DNA Modifications

In addition to histones, covalent modifications also take place on DNA itself, expanding the horizon of "*epigenetic programming*" of nuclear functions. DNA can be modified by addition of hydroxy-methyl cytosine and methylcytosine. However, hydroxymethylation remains a less significant modification, with only being identified in limited circumstances [29]. 5′ cytosine methylation involves transfer of a methyl group from *S*-adenosylmethionine to the 5′ position of cytosine residues in

Table 7.1 Outline of the major histone modifications and their effects

Modification	Charge	Effect on gene expression	Major histone modified	Major functions	References
Methylation	Neutral	Increases or decreases	H3, H4	Heterochromatin organisation, gene silencing	[26, 27]
Acetylation	Negative	Increases	H3, H4	Gene activation	[15, 28]
Phosphorylation	Negative	Increases	H2A, H3	DNA repair, gene activation	[16, 17, 19]
Ubiquitination	Neutral	Variable	H2A, H2B	Satellite DNA binding, transcriptional activation	[20–24]

A more detailed review of histone modifications has been done elsewhere

DNA and is the most well-understood DNA modification and one of the most prominent epigenetic events. This modification has been shown to play important roles in various cellular processes like genome integrity, genome imprinting, X-chromosome inactivation, development [30, 31] and diseases like CVD and chronic kidney disease [29, 32–34].

5′ cytosine methylation of DNA is carried out by two groups of enzymes.

1. *De novo methyltransferases*. This group involves the enzymes DNMT 3a and 3b. These enzymes methylate cytosine on the 5′ position, without utilisation of a template to copy the methylation pattern, hence the name. Therefore, these enzymes display high level of expression during embryogenesis and gradually diminish in adult tissues [35]. Studies performed in mice shown that mice deficient in DNMT 3b are embryonic lethal and in DNMT 3a survive for only up to 4 weeks [36].
2. *Maintenance methyltransferases*. This group involves the enzyme DNMT1 which methylates hemi-methylated DNA by utilising methylation pattern information of parent DNA strand to copy the same on daughter DNA strand following DNA replication. In this manner, it helps in the "*maintenance*" of "*DNA methylation signature*" following cell division. DNMT1 is ubiquitously expressed and is critically important for mammals as mice deficient in DMNT1 display embryonic lethality [36].

7.2 Epigenome and Diseases

7.2.1 Role of DNA Methylation in Diseases

DNA methylation is a long-term, relatively stable, epigenetic trait that contributes to maintaining the cellular homeostasis and mis-regulations in which can lead to diseases. DNA methylation predominantly takes place in the context of concentrated regions of CG dinucleotides, known as CpG islands. These islands are found

in the promoters of many genes and mostly remain unmethylated to allow gene expression (e.g., promoters of housekeeping genes like glyceraldehyde 3′ phosphate dehydrogenase (GAPDH) are rich in CpG islands in their promoters) and methylation of cytosine decreases the expression of the associated downstream genes. There are two ways in which CpG methylation reduces transcription.

1. The presence of methyl group blocks the binding of some transcription factors. For example, sp1 and sp3 transcription factors and Hif1 transcription factor in hypoxia [31, 35, 36].
2. Methyl DNA binding factors promote the binding of silencing factors. For example, MeCP2 binds methyl DNA and recruit histone deacetylases (HDACs) which promote chromatin condensation and decrease gene expression [29, 37, 38].

7.2.2 DNA Methylation and Human Diseases

Alterations in DNA methylation are responsible for a large number of human diseases, including cancer (see Table 7.2). These alterations take place as a result of the mechanisms described below.

Change in DNA methylation status. Disturbances in DNA methylation can typically lead to either overexpression of some genes like oncogenes or reduced expression of some others like tumour suppressor genes (see Fig. 7.3). A large number of mis-regulations in DNA methylation have been identified in various forms of cancer.

Cross talk between genetic and epigenetic mechanism. Another way in which CpG methylation can contribute to disease is through single nucleotide polymorphism (SNP) in CpG sites. This change links a genetic mechanism with an epigenetic mechanism and in consequence can lead to a drastic alteration of the normal function of an associated gene. For example, the gene encoding for the respiratory

Table 7.2 Changes in DNA methylation of different genes in different forms of cancer

S. No.	Gene name	Cancer type	Gene expression change	DNA methylation change
1.	P16	Renal, lung, colorectal, oral, head and neck, hepatic	Decrease	Increase
2.	P cadherin	Hepatic, breast, pancreatic, salivary gland, lung	Decrease	Increase
3.	Hmlh1 and hMSH2	Colorectal, renal	Decrease	Increase
4.	Cyclin D2	Gastric		Decrease
5.	P15	Oral carcinoma	Decrease	Increase
6.	APC	Lung, colorectal	Decrease	Increase
7.	FHIT	Lung	Decrease	Increase

Adapted with permission from Zeenat et al. (DOI: 10.5772/intechopen.97379) [40–44]

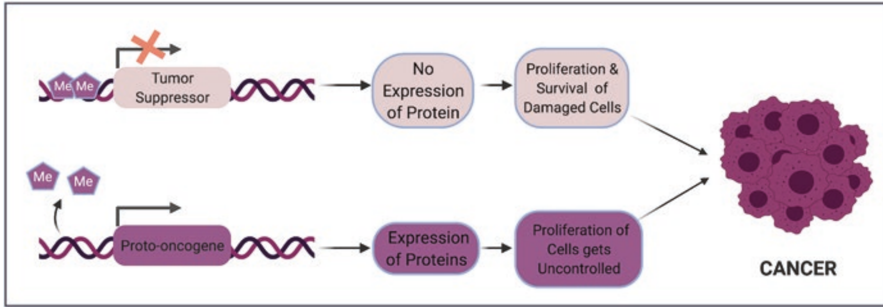


Fig. 7.3 Schematic of role of DNA methylation in cancer progression through two pathways. Hypermethylation of tumor suppressor genes allows unchecked growth and accumulation of damaged cells to generate cancer phenotype. Hypomethylation of proto-oncogenes favours uncontrolled proliferation of cells to generate cancer cell mass. Adapted and modified from Zeenat et al. (DOI: 10.5772/intechopen.97379)

chain protein NDUFB6 is a target of SNPs in a CpG site in its promoter. This gene has reduced levels of expression in type 2 diabetes. The level of expression of this gene is also found to be inversely correlated with the degree of promoter methylation in muscle biopsy samples from elderly patients [39].

7.2.3 High-Throughput Methylome Sequencing

This technique involves identification of DNA methylation pattern on a genome wide level. There are many variant techniques available to perform it but two of these techniques are the most widespread.

7.2.3.1 Bisulphite Sequencing

In essence, this technique involves treatment of DNA with a bisulphite reagent which converts non-methylated cytosine residues to uracil and leaves methylated cytosine unaltered. After performing PCR on the bisulphite converted DNA, the uracil is subsequently replaced by thymine (T) from dNTPs. Thus, by comparing the sequence of converted DNA with original DNA, the position of methylated cytosine residues on DNA can be determined. This technique is either performed on a single gene level, employing bisulphite treatment followed by PCR or at the genome wide level, employing bisulphite treatment followed by high-throughput sequencing.

Table 7.3 Shows a comparison of the variant genome sequencing techniques which employ bisulphite conversion of DNA

S. No.	Bisulphite sequencing variant	Procedural steps involved	Input DNA requirement (μg)	Coverage (%)
1.	Whole genome bilsulphite sequencing (WGBS)	Sonication of DNA, library preparation, gel-size selection, bisulphite treatment, library amplification	1–5	~95
2.	Methyl binding domain (MBD) cap sequencing	Sonication of DNA, capture 5mC by MBD, library preparation Gel-size selection, library amplification	0.2–1.0	~17.8
3.	Reduced representation bisulphite sequencing (RRBS)	Digestion with MspI, library preparation, gel-size selection Bisulphite treatment, library generation	0.01–0.03	~3.7

An outline comparison of steps involved, DNA requirement and sequence coverage has been shown

This is the only technique with a single base resolution and therefore represents the *gold standard* of studying cytosine methylation. However, the bisulphite treatment is a harsh chemical procedure which heavily degrades DNA and makes recovery difficult. A number of variants of the technique have been developed like HELP, COBRA, bisulphite pyrosequencing, etc. A comparison of some of the most commonly used variants of bisulphite sequencing is presented in the Table 7.3 [45, 46].

Methylated DNA immunoprecipitation sequencing (MeDIP seq). This technique involves immunocapture of methylated DNA fragments, utilising an antibody against 5′ methyl cytosine. In this way, DNA fragments containing methylated cytosines become enriched and can be analysed either by quantitative PCR for a single gene or by high-throughput sequencing for the entire genome. This technique is analogous to chromatin immunoprecipitation (ChIP). Although the resolution of this technique is low compared to bisulphite sequencing, it is more versatile and reproducible (Fig. 7.4).

7.2.4 Role of Methylome Sequencing in Diseases Prognosis and/or Diagnosis

Many diseases have been documented in which cytosine methylation remains a major reason for the problem. In most of these conditions, single gene methylation changes have been reported behind the problem. However, methylome sequencing could serve as an even more useful tool that yields high quality genome wide results

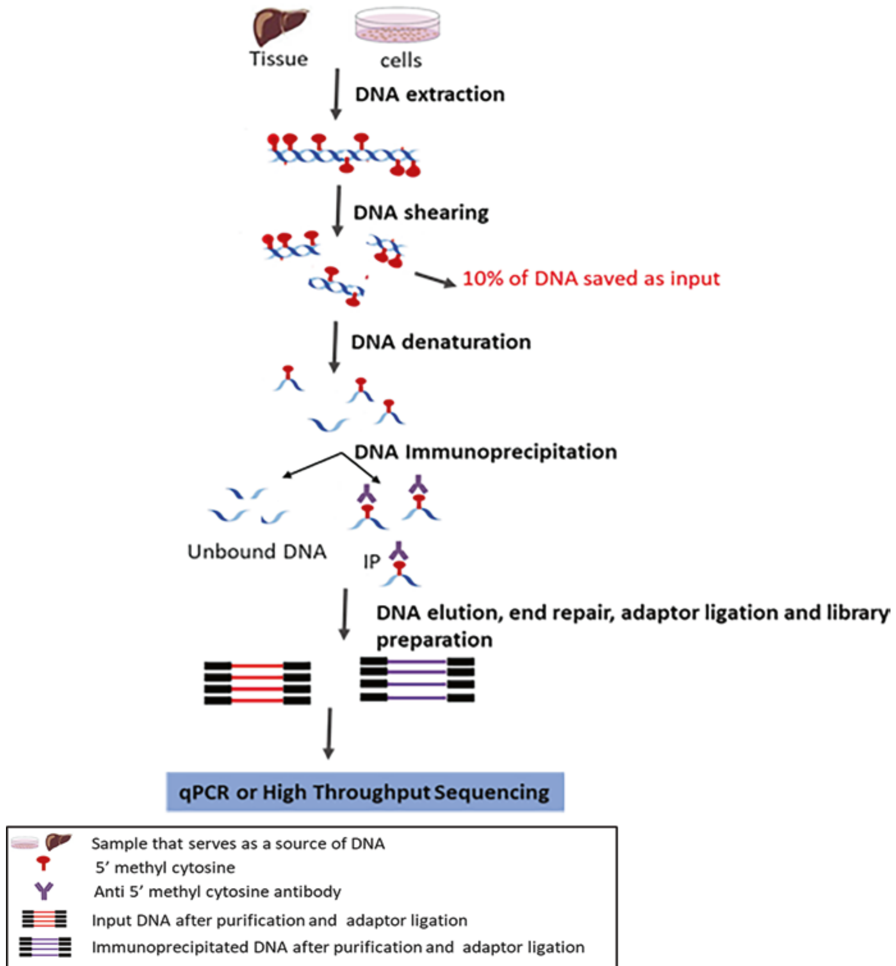


Fig. 7.4 Shows the major steps of methylated DNA immunoprecipitation. DNA is extracted from a sample followed by shearing and denaturation to generate small, single stranded DNA fragments (200–600 bp). This is followed by pull down of methylated DNA fraction with the help of antibody. Both the input DNA (a fraction of total genomic DNA used for normalisation) and immunoprecipitated (IP) DNA are purified, end-repaired (and ligated with adaptors for sequencing) and analysed after performing qPCR or sequencing

when comparing two different conditions side by side. For example, cytosine methylation of a normal versus tumour sample. This can give us an idea of the role of DNA methylation in imparting the particular disease and hence could lead to better ways of understanding the diseases and development of therapeutics [45, 47–49].

7.2.5 Chromatin Immunoprecipitation

In principle, chromatin immunoprecipitation is a technique in which chromatin is cross-linked with the help of formaldehyde followed by its isolation from the cells. This cross-linking covalently links chromatin associated proteins (like histones) to DNA. This is followed by immunoprecipitation of the sheared chromatin fragments using the antibody of interest to enrich these fragments. For example, to study methylation status of lysine residue 9 on histone H3 (known as H3K9me), crosslinked and sheared chromatin is immunoprecipitated with anti-H3K9me antibody to enrich the fragments containing this modification versus the fragments which do not. Subsequently, the level of H3K9me of an individual gene can be observed by performing quantitative PCR on that gene. This can be better understood by taking the example of the tumour suppressor gene X. In this situation, we want to study H3K9 methylation on two different tissue samples, normal and tumour for gene X. We begin by isolating chromatin from each sample and performing H3K9me ChIP. Following immunoprecipitation, we perform qPCR in both tissues for the gene X.

To study the effect of any chromatin modification on a genome wide level, Chromatin immunoprecipitation can also be followed by high-throughput sequencing (**ChIP Seq**) (Fig. 7.5).

7.2.6 Use of ChIP in Diagnostic and Prognostic Applications

In recent years, chromatin immunoprecipitation has been used in varied clinical applications for diagnostic, prognostic and research purposes. Some of the most exciting clinical applications of the technique are listed below.

1. Chromatin immunoprecipitation in cell free ChIP (cf ChIP).

   This technique relies on immunocapturing and sequencing of cell free chromatin fragments from different cells. In this technique, chromatin fragments released into the bloodstream from different cells are immunocaptured in the plasma. After washing and purification of DNA fragments isolated after digestion of chromatin, libraries are created and sequenced. Cell free (cf) chromatin maps generated for various chromatin modifications are rich in important information and such sequences are available, with plasma obtained from various volunteer donors. These maps can vary between healthy and diseased conditions and hence can serve as good molecular indicators for disease conditions. These maps can not only be used to identify differences in chromatin associated modifications (e.g., histone post-translational modifications) in different genes, but can also be used to compare similar modifications in other genomic

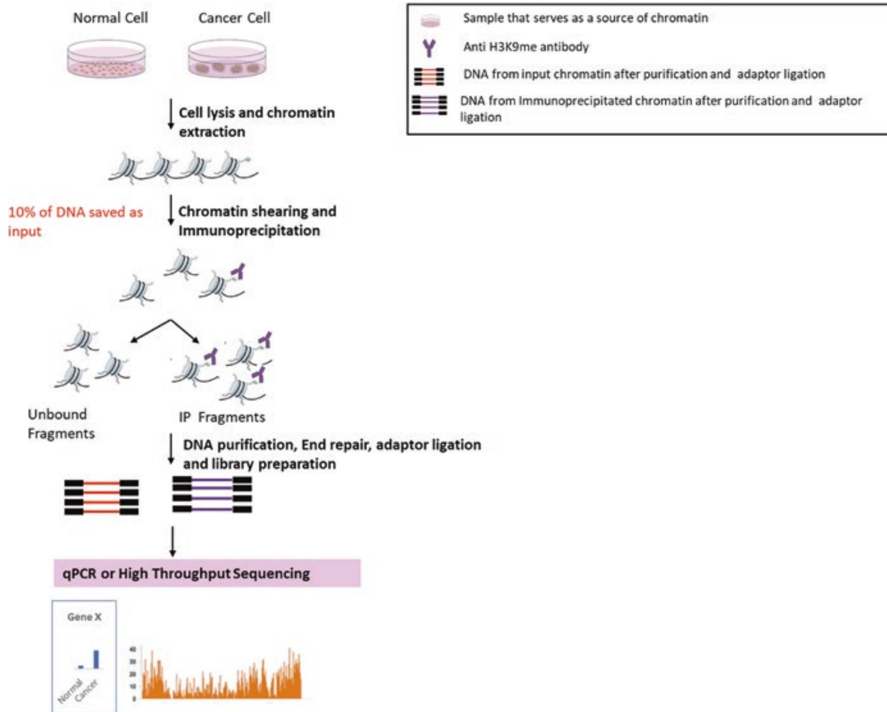
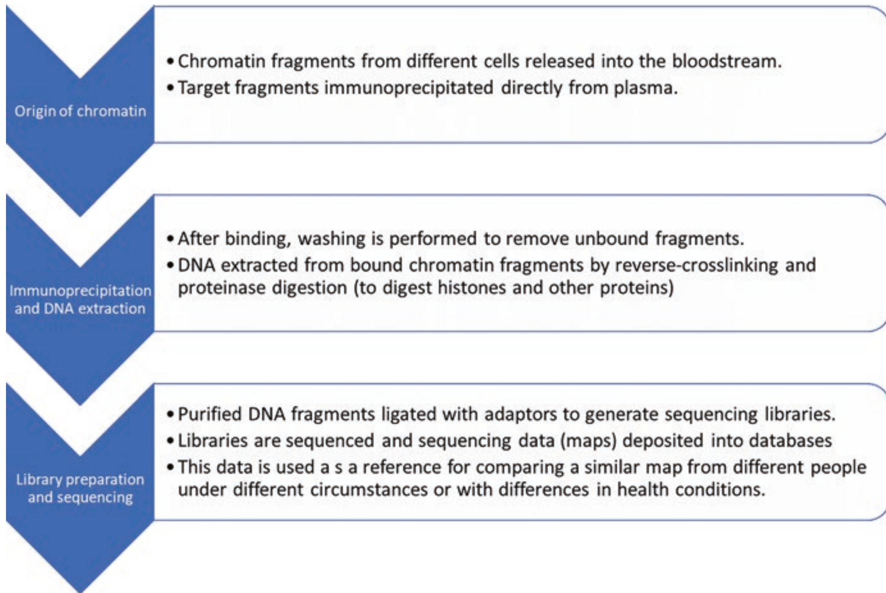


Fig. 7.5 Shows the major steps of chromatin immunoprecipitation. Chromatin is extracted from a sample followed by shearing and denaturation to generate small fragments (200–600 bp). This is followed by pull down of chromatin fragments containing the modification against which antibody is used. Both the input DNA (a fraction of total genomic DNA used for normalisation) and immunoprecipitated (IP) DNA are purified, end-repaired (and ligated with adaptors for sequencing) and analysed after performing qPCR or sequencing

regions like promoters and enhancers. Use of cell free (cf) chromatin offers a non-invasive method of patient analysis and molecular diagnosis. During the years to come, more and more **cf *ChIP seq*** libraries, corresponding to different chromatin marks would become available. This would mark a huge leap into the future of molecular diagnosis, especially epigenetic diagnosis and "*epi-therapy*" [50].



2. Study of cell type specific chromatin modifications.

   It is very useful in obtaining important information about the epigenetic status of each different cell type in tissue under different conditions (like normal versus disease). For example, studies of chromatin modifications of β-cells of pancreas in normal and diabetic individuals using ChIP can yield important information about the precise role of the said modification in β-cells in imparting and propagating type 2 diabetes [51].

7.3 Chromatin Structure and Genome Organisation

As mentioned previously, genome inside eukaryotic cells does not exist as a linear molecule but instead exists as a hierarchically packed compact structure with multiple levels of organisation. Therefore, Genome Organisation refers to the arrangement of genomic elements (DNA and proteins) in the 3-dimensional (3D) space within the nucleus. The order in which genomic elements are distributed into different chromosomes also contributes to genome organisation. Other structural genomic elements which are quintessential to this organisation are chromosome territories, compartments, topologically associating domains. The macro-scale elements termed as topologically associated domains (TADs) are demarcated by architectural proteins like cohesion and CTCF (CCCTC-binding factor) and interact through chromatin loops. The 3D genome organisation is of particular significance in

processes like replication, transcription, recombination, gametogenesis, mitosis, meiosis, development, stem cell differentiation and pluripotency maintenance [52]. In this section, we shall briefly discuss about the role of genome organisation in maintaining chromatin architecture and alterations in 3D genome in the aetiology of diseases.

7.3.1 Regulation of 3D Genome Organisation

An increase in the complexity and size of genome is observed across species, from lower to higher eukaryotes. For example, the size difference between yeast and mammalian genomes is approximately 300-fold. The most significant genomic elements which undergo a proportional increase with genome complexity are the repetitive DNA elements. In mammals, repetitive DNA and non-coding elements account for about 96% of the genomic DNA sequences (44% repetitive and 52% non-coding sequences) [53]. This leads to a proportional increase in the heterochromatic regions since repetitive DNA elements undergo hyper-recombination at higher frequencies and are therefore tightly wrapped in highly condensed, heterochromatic regions. Organisation and maintenance of such complex genomes require more extensive epigenetic programming and silencing mechanisms. Alternatively, study of these mechanisms not only provides opportunities for better understanding of genomes and their regulation but also opportunities for targeting epigenetic modifications for therapeutic purposes. This concept has immense implications in "*personalised epigenetic therapy*" [54] (Fig. 7.6).

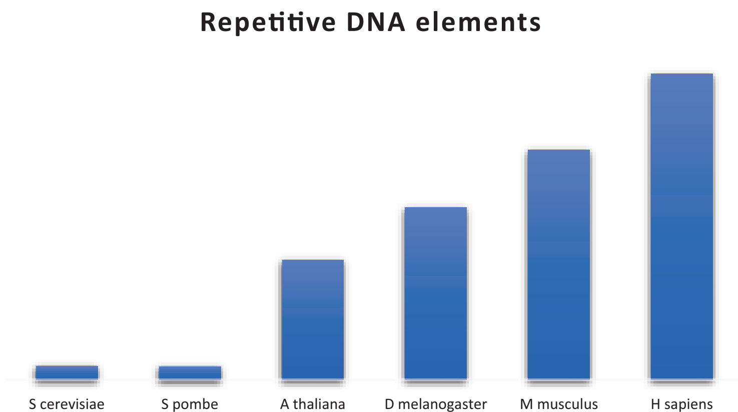


Fig. 7.6 The graph represents increase in the percentage of repetitive DNA elements across eukaryotic species, from Saccharomyces cerevisiae to humans. This increase is proportional to an increase in size and complexity in the 3D genome

7.3.2 Non-coding RNAs (ncRNA)

Non-coding RNAs are ribonucleic acid molecules that are not translated into proteins. Genomic DNA sequences from which these molecules arise are typically known as "*RNA genes*". Some of the non-coding RNAs are known for a very long time like the transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), both of which play very important roles in the process of protein translation. However, with advancements in the tools and techniques of molecular biology like "omics" and "*computational biology*" over the past two decades, various classes of non-coding RNAs have been identified with diverse range of size and functional roles. In addition to tRNAs and rRNAs, other major types of ncRNAs include small and long non-coding RNAs.

Small non-coding RNAs include a size range of 18–200 nucleotides. Major types are micro-RNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), snRNAs.

miRNAs play role in downregulation of gene expression through binding to 3′ untranslated regions (3′ UTRs) of mRNAs. A single miRNA can bind and affect the expression of several mRNAs through partial sequence complementarity. miRNAs have been identified to play various roles in disease like miR 125b in type 2 diabetes, miR 206 in oestrogen receptor alpha [55], miR 135a and 135b in colorectal cancer [56].

siRNAs are formed by the RNA interference pathway and play role in heterochromatin organisation, maintenance and transcriptional gene silencing. These have been identified and well characterised in the yeast *Schizosaccharomyces pombe* [8].

piRNAs form RNA–protein complexes with Piwi proteins and play role in transcriptional gene silencing in somatic cells. In germline cells of testes, the Piwi protein–RNA complexes are linked to transcriptional gene silencing of retrotransposons. Unlike siRNAs and miRNAs, Piwi RNAs are formed in a dicer-independent manner.

snoRNAs are small RNA molecules found in the nucleolus that mainly participate in guiding chemical modifications of rRNAs and tRNAs, thereby assisting in the process of translation.

Long non-coding RNAs (lncRNAs) have a size range of approximately 1,000 to 10,000 residues. These play important roles in gene imprinting and X-chromosome inactivation. The most well-studied examples include X inactive (Xist) and homeobox (HOX) transcript anti-sense RNA (HOTAIR).

Xist In case of human and other placental mammal females containing two copies of X-chromosome, one of the copies remains inactive and heterochromatinised (called as inactive X or Xi or Barr-body) through various mechanisms. Xist RNAs are transcribed from the X-chromosome and play a prominent role in gene silencing and heterochromatinisation of Xi.

HOTAIR is a 2.2 kb long non-coding RNA, encoded by the HOTAIR gene, located within the HOXC cluster, between HOXC11 and HOXC12 on chromosome 12. It plays a role in transcriptional downregulation of HOXD gene cluster on chromosome 2. However, the sequence and function of HOTAIR are different in the two most well-studied organisms, mice and humans [57].

7.3.3 Effect of Non-coding RNAs on Gene Silencing and Genome Organisation

Recruitment of chromatin remodelling complexes and deposition of inhibitory histone methylation mark H3K27me3 contributes to the gene silencing effect of lncRNAs like HOTAIR [58–60]. Recruitment of RNA binding proteins by lncRNAs that interfere with binding of transcription factors to gene promoters also contributes to their function. Non-coding RNAs can also contribute to or propagate DNA methylation. In fact, recent findings have shown that siRNA and miRNAs affect transcriptional silencing through DNA methylation, histone methylation (H3K9 and H3K27), histone deacetylation and recruitment of remodelling complexes [29].

7.3.4 Chromosome Territories, Compartments and Nuclear Lamins in Genome Organisation

Microscopy based studies, chromosome painting, chromosome conformation capture (3C) and its variants (4C, 5C, HiC) have shown that interphase chromosomes preferentially reside in separate chromosome territories. Within each chromosome territory, position of individual genomic elements coincides with transcriptional activity, with gene-rich, transcriptionally active regions occupying the borders of chromosome territories. Also, at a mega-base scale, similar genomic regions interact with one another. Transcriptionally active regions interact with other active regions which possess activating chromatin modifications, higher gene density and chromatin accessibility. Similarly, inactive regions interact with other inactive regions. The boundaries of topologically associated domains (TADs) can also display interactions. This can be of consequence when we study genes participating in same or related functions but belonging to different chromosomes. Overall, nuclear compartments containing TAD boundaries (active regions) are frequently found in the interior nuclear space and those containing heterochromatinised, gene poor TADs occupy nuclear periphery and preferentially associate with nuclear lamina (innermost layer of nuclear membrane) through the proteins termed as nuclear lamins [61]. Nuclear lamin A, B and C are mainly responsible for this peripheral localisation of heterochromatin TADs and absence of these proteins can result in re-localisation of heterochromatin to nuclear interior [12, 52]. Heterochromatin

protein (HP1) is responsible for maintenance of peripheral, constitutive heterochromatin. More details about the role of 3D genome organisation have been reviewed elsewhere [52].

7.3.5 Higher Order Genome Organisation and Diseases

Mis-regulations in individual components responsible for maintaining genome organisation can result in a large number of human diseases [62] (Table 7.4).

1. CTCF has been implicated in diseases like Huntington's disease, fragile X mental retardation, Silver-Russell (SRS), Beckwith-Wiedemann syndrome (BWS), cancer and myotonic dystrophy [63, 64].
2. Mutations in cohesin are implicated in Cornelia de Lange Syndrome (CLS) and Roberts Syndrome [RS] [65, 66].
3. HP1 has been shown in various studies to be involved in different forms of cancer [67, 68].
4. Absence of architectural chromatin proteins HP1, KAP-1 or HDAC1/2 hastens ATM-mediated repair [69].
5. Alterations in relative chromosome positioning in the 3D genome results can result in problems with adipocyte differentiation and cancer [70].
6. Mis-regulations in lamin A are associated with ageing [71].
7. Changes in overall nuclear architecture can affect genome stability [72].

Table 7.4 A list of papers related to some interesting aspects of epigenetics and diseases

S. No	Title of the paper	PMID
1.	The developmental origins of Well-being	15347527
2.	Epigenetic differences Arise during the lifetime of monozygotic twins	16009939
3.	Epigenetics: Connecting environment and genotype to phenotype and disease	19493882
4.	R loops: From physiological to pathological roles	31607512
5.	Impact of genetic and epigenetic factors from early life to later disease	18803962
6.	The impact of nutrition and environmental epigenetics on human health and disease	30388784
7.	Recent developments on the role of epigenetics in obesity and metabolic disease	27408648
8.	Impact of oxidative stress during pregnancy on Foetal epigenetic patterns and early origin of vascular diseases	26024054
9.	Impact of epigenetic dietary compounds on transgenerational prevention of human diseases	24114450
10.	DNA methylation: The pivotal interaction between early-life nutrition and glucose metabolism in later life	25327140

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Chapter 8
Stem Cell Technology in Medical Biotechnology

Shahnawaz Wani, Tahir Dar, Swanand Koli, Willayat Yousuf Wani, Mumtaz Anwar, and Zeenat Farooq

Abstract Stem cells are small, unspecialized, and undifferentiated cells with a chromatin conformation that is not characteristic of any particular cell type and can be programmed, upon appropriate stimulation, into different cell types. These cells provide base material for formation of many different body cells for therapeutic and research applications. There has been a revolution in the therapeutic applications of stem cell technology during the past decade and the revolutionary introduction of CRISPR-Cas9 has further increased the possibilities of their use. This chapter describes stem cell technology, its types, applications in various established pathological conditions, and ethical concerns revolving their use. It also provides insightful details about the culture conditions required for propagating and differentiating stem cells, tissue engineering, establishment of organ cultures, and limitations in establishing stem cell cultures.

Keywords Stem cell technology and Applications · Stem cell therapy · Organ culture and tissue engineering

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8.1 Introduction, Classification, and Significance of Stem Cells, Isolation and Identification of Stem Cells, and Differentiation of Stem Cells

8.1.1 Introduction

Stem cells are small, specialized, undifferentiated cells in the body with a huge potential for cell division and growth. The term is basically derived from the "*stem cells*" of plants, cells which can divide and re-divide and contribute to unabated growth. In addition to their potential for division and self-renewal, these cells have the potential for differentiating into all the cell types of the organism from which they are derived. For example, a stem cell from a mouse embryo can be cultured and differentiated into an endothelial cell, hematopoietic cell, or muscle cell under correct culture conditions with the help of agents that assist in the differentiation process toward a particular lineage [1].

Stem cells can be of different types (Fig. 8.1):

1. **Totipotent stem cells.** Cells which possess the capability or potential of forming an entire organism through cell division are known as totipotent stem cells (*totipotent* = *total potential*). These cells can differentiate into all the cell types contained within the organism and lead to formation of an entire organism. Cells of plants remain totipotent throughout their development. It is possible to use plant tissue for re-growing an entire plant of its kind. In case of animals, only the zygote is considered totipotent because it divides, differentiates, and leads to

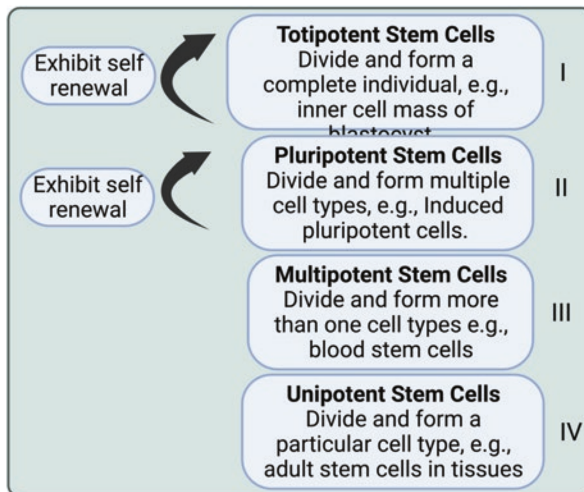

Fig. 8.1 Schematic of the hierarchical representation of different types of stem cells and their characteristic features

formation of an entire organism. Stem cells post-zygotic stage (embryoid stem cells) and tissue-specific stem cells contain varying levels of "*stemness*" and can be classified into different sub-groups.

2. **Pluripotent stem cells.** Cells which have the capability of differentiating into multiple cell types. For example, cells isolated from an embryo (embryoid cells) are pluripotent. Most of the stem cells derived from embryos of mammals are pluripotent. In fact, the term originates from the word *plural* = *pluri*, implying *different choices* for differentiation.
3. **Multipotent stem cells.** Cells which have the capability of differentiating into a set of closely related cells in a particular microenvironment. For example, a hematopoietic stem cell can form a red blood cell, white blood cell, macrophage, or any other cell type of the hematopoietic lineage.
4. **Unipotent stem cells.** Inside the body of humans and other animals, reserves of stem cells are found in different tissues which replenish the cells that are lost after completing their life span. These cells are specific to the parent tissues and differentiate only into the tissue cells in which they reside. These cells are called as unipotent stem cells (*uni* = *one*).

Based on the source of stem cells, these can be classified into adult stem cells and embryonic stem cells. As the name suggests, adult stem cells are derived from adult tissue, whereas embryonic stem cells are derived from inner cell mass of a developing embryo. There are a lot of ethical concerns surrounding the use of embryonic stem cells. While research on human embryos per se is completely banned, studies involving animal and also human embryonic cells are being carried out and are strictly regulated by institutional ethical committees and animal care committees. Oversight from federal and international agencies is also ensured for such research.

8.1.2 Isolation of Stem Cells

Cells that maintain the stemness for a specific cell type while undergoing division and replication in a controlled cultured environment are referred to as stem cell lines. To culture and propagate these stem cell lines, it is important to identify the source and extract the desired specimen from adult tissue or from an embryo. The cells isolated and enriched from the tissue source are placed in a controlled culture medium which allows the cells to undergo division and propagation but preventing them from further lineage-specific specialization. Scientists preserve the stem cell lines for long-term storage for a variety of uses and also often share them with other researchers in the field. The stem cells can be stimulated using different growth factors and modulators to induce specialization in the desired cell lineage. This process is referred to as direct differentiation. It is much easier to grow and propagate embryonic stem cells as compared to adult stem cells but with the recent advancements made in the field, scientists have made significant progress toward the establishment of both stem cell types.

8.1.3 Isolation of Embryonic Stem Cell (ESC) Lines

Embryonic stem cells (ESCs) are pluripotent stem cells that are derived from early-stage embryonic tissue. ESCs from the mouse are well characterized and most studied, although the basic protocol for the ESCs isolation and culture remains more or less similar within all species. Generally, the ESCs are harvested at the blastocyst stage (Day 5) post-fertilization from the inner cell mass referred to as embryoblast. The cells from this embryoblast are further isolated for culture and expanded to obtain viable cells in culture conditions (Fig. 8.2). Generally, the process of ESCs procurement and maintenance in vitro is highly inefficient due to the failure of primary cells to adapt and proliferate in culture conditions. The survival rate for the freshly isolated primary ESCs is variable and depends on the medium and culture environment.

8.1.4 Somatic Stem Cells

Somatic stem cells sometimes also referred to as adult stem cells are primarily located in major body organs and tissues. The protocol used for the isolation and culture of adult stem cells depends upon the tissue source and lineage of the cells from where the stem cells are procured. Currently, most of the protocols used for the isolation of somatic stem cells involve the use of fluorescent associated cell sorting (FACS) or magnetic associated cells sorting (MACS) systems. Depending on the

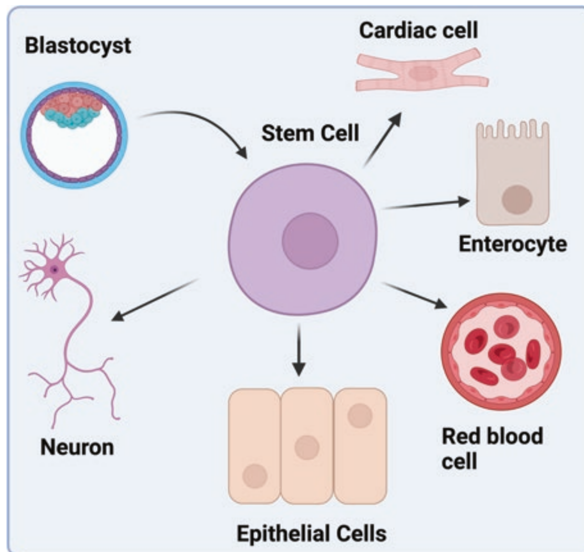

Fig. 8.2 Schematic representation of a totipotent stem cell, derived from the inner cell mass of blastocyst. The figure depicts how the same stem cell can lead to formation of all the different cell types

cell surface marker chosen, these stem cells can be further processed for the enrichment of specific cell types by either positive or negative sorting mechanisms.

The cell count and population of specific somatic stem cells enriched after isolation highly depend on the tissue or organ of origin. For example, spermatogonial stem cells are very rare and comprise about 0.01–1% of the total testis cell population depending on the species, whereas hematopoietic stem cells (HSCs) are considerably available in sizable populations hence they are easily procured, isolated, and enriched for routine bone marrow transplantation. The bone marrow transplant started in the late 1950s. After depleting the recipient's bone marrow stem cell pool, the patient receives new stem cell infusion either from his own (autologous) or retrieved from other donor patients (heterologous) whose human leukocyte antigen (HLA) type matches the recipient HLA type. HSCs used for bone marrow transplantation are either enriched directly from bone marrow or enriched via apheresis (a process of removal of white blood cells from peripheral blood supply). To mobilize the stem cells from the donor's bone marrow, granulocyte-colony stimulating factor is used.

Mesenchymal stem cells (MSCs) are adult stem cells that were isolated originally from bone marrow but later discovered to be present in various other tissue types such as adipose tissue, periodontal jaw ligaments, skin, and cord blood. The mesenchymal stem cells of bone marrow do not contribute to the formation of blood cells and do not express CD34 hematopoietic stem cell marker and are hence referred to as bone marrow stromal stem cells. The major and important source for MSCs is adipose tissue because of its accessibility and the relatively large amount present in the body. Roughly, a yield of 5000 MSCs per gram of adipose tissue has been reported in the literature. The most primitive MSCs can be obtained from umbilical cord blood or tissue (Wharton's jelly). The concentration of MSCs is higher in Wharton's jelly as compared to cord blood.

8.1.5 Culture of Stem Cells

There are specific types of media and culture protocols for stem cell culture that depend on the stem cell belonging to that specific cell lineage. Depending on the protocol used, the stem cell line can be maintained in an undifferentiated form or induced into specific cell linage and cell type using growth factors, inhibitors, and other metabolites. The stem cells are usually cultured using a feeder layer or feeder-free layer.

8.1.6 Feeder Cell Layers

Feeder layer cells are generally adherent monolayered growth-arrested, but viable and supporting cells. These cells are mostly used as a basement substratum to provide support and condition the medium used to grow the target stem cells (usually

plated at low density). Feeder layer cells are irradiated or chemically treated to limit their cell division and growth. Of the many new methods that have been reported in past years to arrest the growth of feeder cells, γ-irradiation (GI) and mitomycin C (MMC) treatment remain the preferred choice to prevent feeder cells expansion. Essentially most of the ESCs cultures are maintained on feeder cell layers. For ESCs expansion and growth, inactivated mouse fibroblasts (MEFs) were used which provide suitable substratum and necessary factors. MEFs can be freshly made in laboratories as well as are commercially available from the vendors. Briefly, embryonic Day 15 (E15) mice are retrieved from the embryo sac and the fibroblast cells are isolated which are further expanded in suitable culture for 3–5 days. These MEF cultures are subsequently mitotically inactivated using GI or MMC treatment. These MEFs can be cryopreserved either before or after treatment. Even though both treatments seem to be equally effective, some studies suggest that GI is more suitable and efficient than MMC treatment. The study by Roy et al. showed that MMC-treated feeder cells were metabolically altered, thus subsequently less efficient at maintaining target cell expansion as compared to the GI feeder layer. Alternatively, chemically fixed feeder cells are shown to support the growth and maintenance of hematopoietic stem cells, ESC, and MSCs. Mild treatment with glutaraldehyde (GA) or formaldehyde (FA) causes significant growth arrest and further immobilization of cell surface proteins of the feeder cells. The main advantage of using chemically fixed cells is that after detaching target stem cells, fixed feeder cells remain immobilized on the plate surface, hence these chemically fixed feeder cells can be reused multiple times without altering or modifying their functions. The other benefit of chemically fixed feeder cells is they barely detach from plates and do not contaminate stem cell cultures after detachment.

8.1.7 Feeder-Free Culture

There has been booming clinical interest in the use of human embryonic stem cells (hESCs) after the establishment of ex vivo culture conditions for hESCs and embryonic germ cells. However, the limitation that hESCs require to be co-cultured with the mouse or human-derived feeder cells has hindered the clinical applications for the use of hESCs. This is because there is a possibility that feeder cells might deliver or transfer animal or human viruses to hESCs. Therefore, there is a need to identify and develop a feeder-free culture system that essentially provides the same critical factors secreted by the feeder and some other cellular factors or activators supporting the signaling pathways. There are basically two types of feeder cell-free media: defined media and conditioned media. The growth factors of the media and supplements vary depending on the type of stem cell and species. A defined media is essentially a serum-free media that has been supplemented with recombinant growth factors such as leukemia inhibitory factor (LIF), bone morphogenetic protein (BMP), bovine pituitary extract (BPE), and other molecules necessary for the growth and pluripotency of stem cells. Rho-associated protein kinase (ROCK)

inhibitors such as Y-27632 and thiazovivin have been shown to increase the viability of stem cells. There are reports which suggest that long-term culture in serum-free media causes epigenetic changes in target cells to adapt to the culture environment. Therefore, the use of condition media to support the target stem cells is highly advantageous. Basically, cells in culture secrete several factors into the media that support cell growth. After the cells have grown and divided for a long time, the spent media are removed. This spent media is termed as conditioned media which can then be used as a supplement to fresh media. Even though there is a possibility and concern about the inclusion of viruses while using the conditioned media, it is far less as compared to using cross-species feeder cells. One advantage of using conditioned media is that it contains more factors than defined media.

8.2 Introduction to Stem Cell Technology: Transdifferentiation Potential of Stem Cells, Induced Pluripotent Stem Cells, Factors Involved in Pluripotency

8.2.1 Transdifferentiation

The process of direct reprogramming of one somatic cell type into another cell type, bypassing the transitional stage of induced pluripotency is referred to as transdifferentiation. Transdifferentiation is an alternative method used to generate tissue-specific terminal differentiated cells. Utilizing this process without going into the pluripotent stage, adult differentiated cells are directly programmed to induce and commit into another specific terminal cells [2]. In contrast to the ESCs and iPSCs reprogramming methodology where cells' epigenetic signatures are erased to achieve what is known as the pluripotent ground state, transdifferentiating is primarily focused on rewriting the epigenetic codes selected for the desired terminal adult cell type, thereby achieving the direct conversion between two unrelated cell phenotypes. Therefore, direct cell reprogramming is gaining popularity toward developing newer tissue engineering methods required for the treatment of tissue injuries and diseases where a limited number of cells hinder the tissue repair or tissue healing process. In some tissue damages, the proliferation rate of terminally differentiated adult cells decline which further deteriorates the injury due to the inability of tissue to heal itself. The notable examples include neurodegenerative diseases and myocardial infarctions. The transdifferentiation process takes the advantage of direct reprogramming of body cells which are in abundance and easily available, into the desired cell phenotypes which have the potential to heal the damaged body part and restore the tissue function. Hence transdifferentiation has tremendous potential and holds a promising future direction in the field of regenerative medicine.

8.2.1.1 Transdifferentiation Techniques and Mechanisms

The cellular reprogramming process can be carried out through protocols readily available in the works of literature having their pros and cons. The basis of reprogramming generally follows upregulating or transducing reprogramming factors which initiate and support the terminal cell identity, function, and phenotype. Generally, somatic cells are used for direct reprogramming without the introduction of a pluripotency state which considerably cut down the likelihood of tumorigenesis in this conversion process. The process of direct differentiation can be achieved in three different ways. (a) Few select key transcription factors can be exogenously transduced as transgenes which can overexpress and initiate the transdifferentiation process [3–6]. (b) Using targeted manipulation techniques that can directly manipulate DNA or the epigenetic signatures such as CRISPR/Cas9 can be utilized to either silence or upregulate the endogenous genes vital for transdifferentiation [7–10] (c). Several transcriptional pathways can be directly targeted using pharmacological agents activating cellular immunological response which in turn leads to a cascade of epigenetic signature remodeling or epigenetic cellular environment [11]. Currently, use of plasmids and viral vectors are popular methods used for introducing transgenes into cells although its efficiency reported is often too less. Conversely, upregulation of endogenous genes involved in transdifferentiation results in much higher direct conversion efficiencies, hence they have more potential for upscaling the transdifferentiation to a large-scale environment [7].

Transdifferentiation Through Exogenous Transgene Overexpression

Viral mediated delivery of foreign genetic material is a commonly used method to deliver transgene into target cells and initiate the process of transdifferentiation [12]. Lentiviruses, Adenoviruses, and other retroviruses are often used to induce cell transdifferentiation. The advantage of using lentiviruses and retroviruses is that they can effectively deliver the exogenous DNA into the target host genome [13]. Other viruses such as non-integrating viruses are less frequently used to initiate the transdifferentiation process due to lower efficiencies as compared to lentiviruses and it may take a longer time to achieve the same yield and number of reprogrammed viable cells. Using adenovirus, the transgene is expressed transiently, one report shows that 2.7% efficiency was observed in transdifferentiation of fibroblast to neurons [5].

The major hurdle in inducing transdifferentiation is selecting precise transcription factors (TFs). TFs modulate gene expression by regulating the gene transcription rate by upregulating or downregulating it. The TFs expression results in a change in cellular fate such as division, growth, differentiation, activation, and migration. Thus by modulating the expression levels of TFs it is possible to change the cell identity. The TFs can be used individually or in conjugation because studies have shown that TFs can work in an orchestrated manner to transdifferentiate target cells quickly and efficiently. Margariti et al. in 2012 first used OSKM (Oct4, Sox2,

KLF4, and c-Myc) to prime and initiate reprogramming of target cells for transdifferentiation process that are commonly referred to as a partial-iPSC (PiPSC) state [14]. Using this approach, the efficiency of transdifferentiation was improved by roughly 34% as compared to other studies that did not make PiPSCs but used viral-directed transdifferentiation [3, 15].

Transdifferentiation Through Endogenous Gene Regulation

Endogenous Gene Silencing with CRISPR/Cas9

Transdifferentiation can be achieved by endogenously silencing certain genes in the target cell with the help of CRISPR/Cas9 system. Target gene-specific gRNA when delivered to the cell along with CRISPR/Cas9 enzyme complex can induce DNA double-strand break and introduces mutation either by insertion or deletion caused due to error in proofreading activity, thus disrupting the gene function. Using CRISPR/Cas9 [16] disrupted Myod1 gene which drives the transdifferentiation of mouse myoblast into adipose cells [16]. CRISPR/Cas9 can also be used to enhance the normal transdifferentiation process which was shown by Rubio et al. [9] by utilizing CRISPR/Cas9 to transdifferentiate fibroblast into neuronal cells [9]. This was achieved by silencing TSC2 gene in fibroblast, loss of function of TSC2 is involved in the onset of tuberous sclerosis. The fibroblast cells were further transduced with Ascl1, Lmx1a, and Nurr1 for lentiviral mediated overexpression of these genes that convert fibroblasts to neuronal cells [9]. Thus CRISPR/Cas9 system can be used for transdifferentiation either by targeting specific gene silencing or through conjugation with other techniques to create specialized lineage-specific cells.

Endogenous Genes Upregulation by dCas9

With CRISPR/Cas9 system target gene can be disrupted, alternatively, a mutant version of Cas9 also known as dCas9 which is a nuclease-deactivated version of CRISPR/Cas9 which does not cause a double-strand break in DNA can be used to perform a different function. The dCas9 can be fused with master transactivator proteins which in turn can recruit other transcriptional machinery complexes and cause changes in chromatin structure, thereby upregulating normally silenced genes. This strategy was performed by Chakraborthy et al. [7], where they used dCas9 fused with VP64 a transactivator protein to induce transdifferentiation of fibroblast to skeletal myocytes by upregulation of Myod1 gene [7]. There are many ongoing studies toward the utilization of dCas9 in the transdifferentiation process in different cell types. Overall, several transactivators and/or repressor proteins domains can be fused with dCas9 and used to enhance or repress the target gene function. The most common fusion proteins studied so far include Vp64, VP64-p65-Rta9 (VPR), histone acetyltransferases (HATs), synergistic activation mediators (SAMs), and SunTag [7, 10, 17–19].

Transdifferentiation Through Pharmacological Agents

Viruses like lentiviruses can activate innate immune signaling pathways through Toll-like receptor 3 (TLR3) which in turn cause changes in epigenetic signatures, thus affecting the gene expression [14]. Some of these genes are an important part of the maintenance of the pluripotency network in the cell. Margariti A et al. [14] showed that treating fibroblasts cells with polyinosinic: polycytidylic acid (Poly I:C) stimulates TLR3 in human foreskin fibroblasts which transdifferentiate into endothelial-like cells expressing CD31, a key endothelial protein marker required for adhesion and monolayer formation [14]. Although the transdifferentiation efficiency was low, the cells were mimicking endothelial cell functions such as nitric oxide production and forming a “cobblestone” morphology that is a characteristic of endothelial cells. Using 5-azacytidine, a DNA methyltransferase inhibitor, [20] reprogrammed fibroblast cells into skeletal myocytes [20].

Cells metabolize 5-azacytidine which is a chemical analog of cytidine that leads to a cascade of reactions, finally incorporating it into DNA by binding it to guanine molecule. Due to differences in molecular structure azacytidine is not methylated, thus inhibiting DNA methylation which further leads to a change in the epigenetic environment, modulating target gene expression [21]. 5-azacytidine treatment to cardiac cells causes upregulation of Myod1, a skeletal myocyte-specific marker, and multinucleated myotubes typical skeletal myocyte properties [20]. Dexamethasone, a glucocorticoid capable of activating certain transcription factors, is another pharmacological agent used for promoting direct differentiation of several cell types. The mode of action of dexamethasone is through binding to glucocorticoid receptors which lead to modulation of gene expression [22].

8.2.2 Pluripotency Factors Involved in Stem Cells

In embryonic stem cells (ECSs) their pluripotency identity is attributed to the expression of trio core transcription factors Oct4, Sox2, and Nanog [23]. The pluripotent stem cells undergoing specification during the mouse embryonic development requires the genome to express Oct4 and Nanog but not necessarily Sox2, because of maternal Sox2 protein which can live long in the embryo [24, 25]. These pluripotency transcription factors regulate stem cell pluripotency and specification through their expression, colocalization, orchestrated regulation through polycomb repressive complexes (PRC), and microRNAs in the transcriptional and epigenetic modulation of genes involved in stem cells [26].

One of the Pit-Oct-Unc (POU) family of homeodomain proteins, Pou5f1 gene encodes Oct4 protein. Oct4 nuclear localization can be observed in primordial germ cells (PGCs), totipotent blastomere cells, and also in the pluripotent epiblast cells [27, 28]. In Oct4 knockout mice, the embryos fail to form pluripotent inner cell mass (ICM) but rather show differentiated trophectodermal tissue; therefore, Oct4 expression is critical for the establishment and preservation of pluripotency [29].

Precise control over the Oct4 expression is required for the maintenance of ESCs in undifferentiated form. Silencing of Oct4 by 50% can cause stem cells to enter trophectodermal differentiation [30]. Whereas overexpression of Oct4 by more than 50% could induce stem cells to mesodermal and endodermal differentiation [30].

SRY-box 2 also known as SOX2 protein is highly expressed within the inner cell mass, extraembryonic ectoderm of blastocyst before implantation [25]. Similar to Oct4 null mice, blastocyst in Sox2-knockout mice fails to develop pluripotent inner cell mass and mouse ESCs lacking Sox2 differentiate into trophectoderm [25]. This phenotypic pattern of similarity observed due to loss of either Oct4 or Sox2 can be attributed to the cooperative/synergistic mechanisms of Oct4/Sox2 required in modulating pluripotent gene regulation in several ESCs [31–35]. The differentiating phenotype observed in Sox2 knockout mESCs can be reversed through ectopic Oct4 overexpression [36].

Homeobox protein NANOG is a transcriptional factor required by ESCs to maintain a pluripotency state by suppressing cell differentiation factors. Nanog is the third transcription factor of the core ESCs pluripotent transcription factors which was identified by screening pluripotency factors that can maintain the self-renewal of mouse ESCs in the absence of leukemia inhibitor factor (LIF) [37, 38]. Similar to Oct4 and Sox2 knockout mice, Nanog null mice embryos lack pluripotent inner cell mass [37, 39]. However, Nanog knockout mouse ESCs can be sustained and established in vitro even with the loss of both Nanog alleles [37, 38]. These Nanog deficient mESCs that can be maintained in the pluripotent state in culture conditions suggest that although Nanog is required to reach a pluripotent state, it becomes dispensable once that pluripotency is achieved [38].

The fourth factor known as c-Myc is indirectly involved in the maintenance of pluripotency of ESCs. Myc is a family of regulator genes and proto-oncogenes encode for several transcription factors. The Myc module which consists of c-Myc, n-Myc, Rex1, Zfx, and E2f1 is known to be involved in self-renewal and cell metabolism [40–42]. The c-Myc gene acts as a “master regulator” of cellular metabolism and proliferation. About one-third of ESCs genes participating in the maintenance of pluripotency are bound by both the trio core transcription factors (Oct4, SOX2, and Nanog) and also with c-Myc [43]. But the mode of action for maintaining ESC pluripotency identity differs for both trio core factors and c-Myc. The trio core factors Oct4, Sox2, Nanog acting synergistically along with mediator complex can recruit RNA polymerase II (RNA Pol II) to initiate the gene transcription [44]. While c-Myc with the help of p-TEFb cyclin-dependent kinase regulates the transcriptional pause release of RNA Poly II [42]. Thus, it is considered that the core trio factors can regulate the ESCs pluripotent active genes expression via recruitment of RNA Pol II, whereas c-Myc participate in the pluripotent gene expression by aiding the release of transcriptional pause [23].

Since the trio core ESC transcription factors play an important role in establishing and maintenance of pluripotent identity of the stem cells, several molecular techniques such as chromatin immunoprecipitation have been utilized to map the genome-wide binding sites of these trio core ESC factors in both mouse and human ESCs. Many studies have shown that these core factors act synergistically and

therefore show co-binding or binding at near vicinity relative to each other at several active genomic sites [45, 46]. Overall, it is believed that these trio core transcription factors help to maintain the pluripotency identity of a stem cell (a) through activation and expression of a multitude of other pluripotency factors or genes and subsequently downregulating genes that are involved in stem cell differentiation and specification [23]. (b) Also, a feedback mechanism that regulates the expression of self-genes (Oct4, Sox2, and Nanog) and also each other, which is how the pluripotent stem cells can undergo self-renewal process but simultaneously holds differentiation potential when the need arises.

8.3 Stem Cell Technology and Therapy

Stem Cell Technology is a rapidly growing field at the intersection of biology, chemistry, and biomedical engineering. The field involves use of stem cells for correcting various health problems of individuals due to poor function or loss of function of tissues/organs. For example, patients suffering from type I diabetes mellitus experience destruction of pancreatic β-cells due to generation of auto-antibodies against them which results in loss of insulin synthesis, rise in blood sugar levels and a plethora of other problems. With the help of stem cell technology, stem cell of the individual can be isolated, cultured under in vitro conditions, and appropriately stimulated (with the help of growth and differentiation factors) to form pancreatic β-cells for re-introduction into the individual's body to correct the dysfunction. This specific discipline of stem cell technology is known as "transplantation technology or regenerative medicine." Although transplantation technology can rely on use of "donor or non-self" tissues or organs for treatment, regenerative medicine generally relies on the "regeneration potential of self-stem cells to form a particular tissue/organoid for therapeutic applications." Use of stem cell technology for therapeutic applications is also known as stem cell therapy. The field of stem cell technology is rapidly growing and has already been applied in clinical practice under various situations. The next section describes some of the most exciting applications of stem cell therapy [1, 47].

8.3.1 Applications of Stem Cell Therapy

Amyotrophic lateral sclerosis (ALS), also known as Lou-Gehrig's disease is a neurodegenerative disorder characterized by progressive degeneration of motor neurons. The most common symptoms of this disease include muscle atrophy, weakness, spasticity [48]. ALS represents the most common motor neuron disease throughout the world, with an incidence of 2–3 per 100,000 individuals worldwide. The average time from diagnosis to death for ALS is typically 3–5 years and, in most cases, death ensues because of progressive loss of motor neurons and weak-

ness of skeletal muscles, especially those responsible for breathing [49]. ALS can be familial or sporadic, with a multifactorial representation in patients [50, 51]. Adding to this, ALS can be extremely heterogenic in its presentation that makes it extremely difficult to underpin the exact cause and makes treatment difficult [52, 53]. Stem cell therapy can offer an excellent potential treatment option for ALS since research into stem cells' plasticity and differentiation into various neural cell types has been well established [54, 55]. Several types of stem cells have been studied to test their utility in treating ALS, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), neural stem cells (NSCs), and mesenchymal stem cells (MSCs). Current research on the applicability of these stem cell types in ALS and the different ways of differentiating and introducing them has been described elsewhere in detail [56].

Orthopedics and Bone Regeneration Applications of stem cell technology in orthopedics involve bone regeneration, usually required after severe accidents that lead to bone damage. The technique also has immense clinical potential in autoimmune and genetic or hereditary disorders which result in compromised formation, function, or progressive degeneration of bone or cartilage tissue. Bone regeneration technology has seen immense growth, particularly due to the boost in clinical research and practice in adult mesenchymal stem cells (MSCs) and bone marrow stromal cells (BMSCs). The adult MSCs can be typically defined as the cells having potential of self-renewal and multilineage differentiation into osteoblasts, chondrocytes, and adipocytes. Also, according to the criteria of "Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy," these cells must also possess the capacity of plastic adherence when cultured in vitro, express CD73, CD90, and CD105 biomarkers but should not express CD14, CD11b, CD34, CD45, CD19, and CD79 biomarkers [57]. BMSCs are one of the kinds of stromal cells isolated from bone marrow which, after purification, fulfill the criteria of being classified as mesenchymal cells, that is, they are able to differentiate into chondrocytes, osteocytes, and adipocytes [58] and also express the osteoblast biomarker *Runx* expression [59]. Various animal studies have shown that BMSCs are capable of healing appendicular defects [60], maxillofacial regeneration and spinal fusion [61], long one defect repair [62]. BMSCs have also been successfully demonstrated to induce posterolateral spinal fusion in humans [63]. However, despite all the successfully demonstrated applications, the field of Bone Regeneration is still in its infancy owing to drawbacks like low yield at procurement, requirement of growth factors for in vitro expansion, and increased donor site morbidity due to requirement of higher initial amounts for successful culture and regeneration [64]. Current progress and applications in the field of bone regeneration technology have been reviewed elsewhere [65].

Blood Malignancies Stem cell transplantation in case of blood cell malignancies like leukemia is given to patients for replenishing their stem cells for healthy blood formation [66]. Diagnosis of blood related malignancies in a patient is usually followed by chemotherapy to kill the "malignant precursor blood cells or stem cells"

formed inside the bone marrow. In some cases (if a patient is young, relatively healthy, and able to tolerate the radiation), chemotherapy can be combined with radiotherapy. Also, a "full body irradiation" is also carried out in some patients to destroy as many cancer cells as possible for treatment purposes. These interventions damage the bone marrow cells of the patient and hence make it necessary to replace the damaged cells with healthy ones. Stem cell transplantation is carried out after chemo- and radiotherapy. It can either be autogenic or allogenic. In an autogenic transplant, blood borne stem cells of the patient are extracted before beginning chemotherapy, are purified, expanded in vitro and later re-injected into the patient to form new bone marrow cells [67]. This method can be potentially risky because there is a probability of the "self-cells" used for re-injection to carry some mutations to become malignant in the future. However, it is more common in clinical practice and is used due to non-availability of tissue matched donor. Allogenic transplant involves re-injection of donor stem cells into a patient. This offers the advantage of having healthy, non-cancerous cells in the patient's body to form blood and other cells of the hematopoietic lineage. The clinicians usually administer immunosuppressants to the patient upon receiving donor cells in order to minimize the risk of graft versus host disease (GVHD), a form of immune condition. This application of stem technology in blood malignancies is more common in clinical practice compared to others because blood borne stem cells have a huge potential of blood cell formation upon entering the host system and populating the bone marrow. Also, administration of stem cells is carried out intravenously and the injected cells reach bone marrow through bloodstream. Upon entering bone marrow, these cells grow and divide to replace malignant marrow cells with a very high success rate. These advantages make this application highly useful and popular among clinicians and patients alike. Despite these advantages, new malignancies have been found in a large number of patients after previously suffering from blood malignancy and receiving stem cell transplantation [68]. More research into the field is required to develop better insights into the potential of this application with no to minimal adverse effects [69].

Cardiovascular Disease Cardiovascular disease remains one of the leading causes of mortality due to non-infectious causes, accounting to 30% cases. Myocardial infarction combined with low regenerative potential of cardiomyocytes is responsible for a huge number of these cases. This condition therefore calls for novel therapeutic approaches of treatment like regenerative medicine [70]. Skeletal myoblasts (SM) have been used in stem cell therapy for cardiovascular diseases. SM cells are derived from satellite cell derivatives residing in skeletal muscle fibers and therefore hold the flexibility of sharing embryonic and morphological features with cardiac muscle cells [71]. This makes availability of SM cells from autologous muscle biopsies effortlessly easy. Easy availability combined with the potential of rapid in vitro expansion, ischemic tolerability, and low risk of tumorigenesis has fueled a huge wealth of pre-clinical research into the utilization of SM in cardiac regeneration in many animal studies [71, 72] and clinical trials [73, 74] with demonstrated improved outcomes like reduction in myocardial fibrosis and infarct size. However,

this therapeutic model also suffered from limitations due to lack of electrochemical coupling between transplanted SM cells and resident cardiomyocytes because of failure to form gap junctions [75, 76]. Bone marrow (BM) derived macrophages have also been utilized for stem cell replacement therapy for cardiomyopathies [77]. The effectiveness of this model has also been demonstrated in several clinical studies [78]. Recently, research has been carried out into the utilization of cardiac progenitor cells for cardiac stem cell therapy. These are cells residing in cardiac microenvironment with the potential of regeneration upon in vitro stimulation. Research on these cells is exciting since it offers the advantage of using cardiac cells for regenerative therapy and thereby might overcome the limitation of failure of gap junction formation. It also challenges the long-standing notion that cardiac cells are post-mitotic and have almost no regenerative potential. In fact, advanced research into understanding and harnessing the research potential of cardiac progenitor cells could lead to similar research into other tissue progenitor cells [79, 80]. More current research and applications of stem cell technology in cardiovascular problems have been reviewed elsewhere [81].

8.3.2 Research in the Stem Cell Field

In addition to the various clinical applications, research in stem cell field has seen robust growth over the last decade, especially with advances in the field of genome-wide sequencing technologies. Research in stem cell field can help recapitulate the events that occur during in vitro differentiation and can hence lead to a better understanding of various physiological processes. It can also help us understand various pathological conditions in detail, by comparing normal versus abnormal development. This can be immensely helpful in developing deeper understanding of the biological processes and their mis-regulations at a molecular level which can serve as a key factor for development of effective therapeutic strategies.

Another exciting aspect of application of stem cell technology is drug testing. A vast number of drug testing modules have been developed over the years wherein stem cells are directed to develop into particular tissues to test the drug before testing it in human individuals. This form of efficacy and safety testing benefits from use of human tissues which can mimic the pathophysiological conditions in a better way than animal models. Remarkably though, it offers the advantage of avoiding any side-effects (short and long term) by excluding use of human volunteers. Many researchers across the globe agree that culture-based models should be used for testing a greater number of drugs before the drugs can be tested in human volunteers. It provides an additional blanket of safety for humans participating in the trial study. This approach is especially beneficial for testing chemotherapeutic agents.

8.4 Stem Cell Technology and Infertility: In Vitro Fertilization and Embryo Transfer

Basic and classical definition for stem cells is that these are progenitor cells that are capable of self-renewal and differentiation into many different lineages of cells. Since their discovery as pluripotent stem cells (PSCs) from mouse bone marrow cells, many other types of stem cells have been discovered and generated from other tissues and organisms. Liu et al. mention five types of stem cells in the last few decades. These include embryonic stem cells (ESCs), very small embryonic-like stem cells (VSELs), nuclear transfer stem cells (NTSCs), reprogrammed stem cells (RSCs), and adult stem cells (ASCs) [82]. Last decade has witnessed bloom of basic, translational, and clinical advances in the field of stem cell technology. ESCs and iPSCs have shown great application potential in regenerative and transplant medicine [83, 84], disease modeling, drug discovery screening, and human developmental biology [85, 86]. ASCs are seeking their future in treatment of infertility. Stem cell technology and infertility treatment have a two-way connection. On the one hand, stem cells are sought for treatment of infertility and on the other hand infertility treatment procedures turn out to be source of stem cells. Application of stem cells for infertility treatment is a diverse area wherein different types of stem cells are being harnessed to treat different causes and aspects of infertility. Infertility is defined as a condition when a couple of reproductive age cannot achieve pregnancy after having regular unprotected sex for a period of 1 year or more. It is a complex pathophysiological medical condition with either male inability (20–30%) or female inability (20–35%) affecting millions of people of reproductive age worldwide and impacting their families. Estimates suggest that between 48 million couples and 186 million individuals live with infertility globally [87]. Male infertility issues arise due to problems in the ejection of semen due to obstruction of the reproductive tract, absence or low levels of sperm due to hormonal disorders, or abnormal shape (morphology) and movement (motility) of the sperms. In females infertility may be attributed to abnormalities of the ovaries such as polycystic ovarian syndrome and other follicular disorders; uterus dysfunctionalities such as endometriosis, fibroids, septate uterus; fallopian tubes such as blocked fallopian and the endocrine system disorders causing imbalances of reproductive hormones. A breakthrough in major treatment for infertility came with birth of Louise Brown in 1978 when assisted reproductive technologies (ART) came into being and first in vitro fertilization (IVF) got successful [88]. This was the process of bringing healthy sperm and egg together in dish in a laboratory and developing embryos which would be transferred to a healthy uterus for further development into fetus. Basic technique of IVF was successful partially as infertile couples with problem in production of healthy gametes and women with unhealthy uterus had no option of having their biological babies. Stem cell technology is developing to give solutions to such IVF issues. Main steps at which stem cells resolve IVF issues are:

- Potential of generating eggs from stem cells: Several laboratory studies and clinical trials are investigating stem cells as a strategy for generating healthy gametes. Different types of stem cells differentiate into embryonic germ cells and

precursor cells, respectively [89]. Oocyte-like cells have been successfully derived from embryonic stem cells. Murine female embryonic stem cells have been shown to differentiate into oocyte-like cells that give rise to functional ovaries. Mesenchymal stem cells (MSCs) have been used as an experimental approach to restore egg production by ovaries and improve ovarian physiology in terms of follicular density in experimental models. There have also been some mechanism-based reports related to use of MSC in infertility treatment. MSCs derived from human Wharton's jelly derived express oocyte developmental genes when co-cultured with placental cells; adipose-derived mesenchymal stem cells have the ability to differentiate into granulosa, Takehara et al. reported differentiation of MSC into primordial follicles density [90]. Stem cells derived from human umbilical cord of first trimester have the potential to develop into oocyte-like structures with zona pellucida like layer. Stem cell technology has boosted infertility treatment by a technique autologous germline mitochondrial energy transfer (AUGMENT) wherein mitochondria from Ovarian germline stems cells (OGSCs) are injected into oocytes of women with poor ovarian function. This has fetched almost 18% success rate in birth of healthy babies from infertile women [91].

- Treatment of female reproductive system diseases using stem cell technology is also being studied: Gynecological disorders are associated with abnormalities in one or more of the reproductive organs: ovaries, uterus, fallopian tubes, and cervix causing premature ovarian failure (POF), polycystic ovary syndrome (PCOS), endometriosis, Asherman syndrome, and preeclampsia. Stem cell technology paves new ways for treatment of these disorders. In addition to self-renewal and differentiation, stem cells have many other beneficial characteristics which enhance their potency for infertility treatments. Bone marrow mesenchymal stem cells (BMSCs) have bioactive factor rich secretome consisting of insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and other growth factors to induce cell growth, differentiation, and immunoregulation to restore and support ovarian function. MSCs are also known for their paracrine activity and immunomodulatory effects to prevent ovarian dysfunction [92]. Placenta mesenchymal stem cells (PMSCs) have very high differentiation and proliferation potential, making them an attraction for transplantation and regenerative medicine. PMSCs reduce the levels of estradiol, FSH, and luteinizing hormone (LH) and induce the expression of FSH receptor (FSHR) and anti-Müllerian hormone (AMH) in POF mice, hence contributes to restoration of ovarian function [93, 94]. Upon transplantation, PMSCs improve ovarian function in ovariectomized rats by inducing the production of estrogen and the expression of folliculogenesis-related genes [95]. Many studies have examined the functional nature and the differentiation capacity of BMSCs. In addition to differentiating into chondroblasts, osteoblasts, and adipocytes, BMSCs also have the ability to differentiate into endometrial endothelial and granulosa cells [96]. A distressing consequence of cancer chemotherapy is ovarian dysfunction and infertility. BMCs have shown the protective effects on ovarian function and reduced ovarian failure in mice after chemotherapy. A study has reported multi-facts and mecha-

nistic revelations about BMSCs potency to improve ovarian function in cyclophosphamide-induced POF; by inducing VEGF expression, increasing estradiol levels, restoring ovarian structure, and decreasing expression of the apoptotic factor Caspase-3 [97]. Like PMSCs, remarkable effects of BMSCs might be attributed to their angiogenic and growth factor rich secretome. Their secretome is also reported to contain exosomes, a subset of membrane-bound extracellular vesicles, which enclose various proteins, lipids, and non-coding RNAs such as miRNAs. The small non-coding RNAs may play role in regulating the physiological and pathological mechanisms of stem cells and outcomes related to the ovarian treatment. Two independent studies have demonstrated a role of two different miRNAs miR-644-5p and miR-144-5p carried by BMSCs-derived exosomes in treatment of POF animal models [98, 99]. Various experts speculate that miRNA-regulated gene expression underlies BMSC-based therapy outcomes. Transplanting characteristic is another attractive feature that makes BMSCs more efficient to treat gynecological dysfunctions. BMCs migrate to the uterus and induce endometrial repair in various experimental models including even humans. Asherman syndrome treatment with BMCs has also been demonstrated in a menstruation model as well as in patients of Asherman syndrome [98]. BMSCs have also been reported to play a crucial role in improving endometrial functions by improving endometrial stromal and epithelial compartment. Zhao et al. and others have conducted studies showing increase in endometrium thickness upon infusion of BMSCs into uterus in a rat model [99, 100].

- Male reproductive dysfunction in majority of the cases is attributed to impaired spermatogenesis. Spermatogenesis is a process of sperm formation from male germline stem cells, called spermatogonial stem cells (SSCs). Reproducing these events in vitro has not been successful yet and there is no cellular replacement therapy available for men who suffer from azoospermia. Transplantation of mouse SSCs from an infertile donor to a favorable testicular environment could restore fertility of the infertile male donor [87]. This represented case of azoospermia due to missing stem cell factor on Sertoli cells. Similar treatment of defective testicular environment in men has not yet been studied. The main issue for Azoospermic males is defect in germ cells. Intensive efforts are being made to develop male gametes from SSCs. Optimal isolation and purification of SSCs is an important first step for the downstream applications. SSCs are isolated using two step enzymatic digestion of testis tissue from non-human primates and humans [101]. Enrichment of germ cells is attained with different methods like use of antibodies followed by FACS or MACS in human and donkey testis. MACS has also been successfully employed for the enrichment of human spermatogonia using antibodies against GPR125 and SSEA4. Other studies report obtaining 87% purity of human SSCs using OCT4-antibody. Property to adhere to the culture plate or to extracellular matrices (ECM) is alternative method to enrich SSCs; differences in velocity sedimentation or density gradient centrifugation can be used to separate somatic and germ cells. Percoll density gradient for SSCs enrichment in human testis cells led to ~87% pure population of SSCs [102]. Establishment of an efficient in vitro culture system to maintain both the

self-renewal and proliferation capacity of human SSCs is crucial for their potential clinical applications and has been achieved for 2 months in case of human SSCs. Efficient in vitro culture systems replicating the process of male germ cell development and spermatogenesis have several important applications and is being termed as in vitro spermatogenesis (IVS). IVS would also allow experimentations as genome editing of germ cells or correction of genetic causes of infertility to serve benefits to research and ethical issues.

- Generation of stem cells from healthy embryos during IVF and healthy implantation via stem cell technology: The ability to successfully derive human embryonic stem cells (hESC) lines from human embryos following in vitro fertilization (IVF) opened up a plethora of potential applications of this technique. The main source for human embryos has been "discarded" or "spare" fresh or frozen human embryos following IVF. It is a common practice to stimulate the ovaries of women undergoing any of the assisted reproductive technologies (ART) and retrieve multiple oocytes which subsequently lead to multiple embryos. Of these, only two or maximum of three embryos are transferred, while the rest are cryopreserved as per the decision of the couple. In case a couple does not desire to "cryopreserve" their embryos, then all the embryos remaining following embryo transfer can be considered "spare" or if a couple is no longer in need of the "cryopreserved" embryos, then these also can be considered as "spare." Improving implantation rates in IVF has been the center of focus as failure rates are high due to implantation failures. Since stem cell technology has been used in many pathologies as myocardial infarction and spinal cord injuries, endometrial receptivity, using stem cells can be enhanced. Rate of implantation failures are so high that "recurrent implantation failure (RIF)" term was coined for couples who failed to achieve pregnancy in three consecutive IVFs from good embryos. Almost 10% couples in Europe and the USA are affected by RIF, and it is estimated that RIF has a prevalence of 15–20% in IVF [103]. Very crucial aspect of implantation failure is appropriate endometrial thickness (Eth) and a thin endometrium (<7 mm) is associated with low pregnancy rates [104]. Unfortunately, 0.6–0.8% of patients do not reach minimum thickness for embryo transfer [105] due to various issues like inflammatory causes (acute or chronic endometritis/CE); iatrogenic (repeated curettage, polypectomy); hysteroscopic (myomectomy or laparoscopic) where the cavity is opened and the irrational use of clomiphene citrate or individual uterine structural pattern. Endometrial stem cells can provide therapeutic resources in endometrial atrophy, thinned endometrium, and Asherman syndrome. Their properties to maintain normal chromosomal number after several passages, the ability to differentiate into multiple cell lines under standard culture and immunosuppressive properties (inhibits LT, LB and NK make endometrial mesenchymal cells (enMSCs) a source of excellence in certain regenerative therapies). These immunomodulatory properties are explained by the release of inflammatory cytokines in the tissue [106]. Transplantation of EnMSCs to uterus has been studied. EnMSCs, for their properties of high clonality, multipotentiality, regenerative capacity, immunomodulatory, angiogenic and low immunogenicity are an alternative in severe endometrial

lesions. There was a highly significant increase in endometrial thickness after the inoculation of enMSCs, expressing the high regenerative capacity of the intervention. Finally, the endometrium thickness and the standardization of histopathology and immunohistochemistry in post-treatment with enMSCs resulted in higher clinical pregnancy rates in a population with repeated implantation failures, representing a reliable strategy in assisted reproduction.

Stem cells have been used in animal experiments to repair and improve injured endometrium. Though understanding of adipose-derived stem cells (ADSCs) in endometrial injury repair and their further therapeutic mechanisms is incomplete. Benefits of ADSCs in restoration of injured endometrium were demonstrated by utilising a rat endometrial injury model. It was shown that 30 days after ADSCs transplantation, injured endometrium was significantly improved, with increased microvessel density, endometrial thickness and glands when compared with the model group. Furthermore, the fertility of rats with injured endometrium in ADSCs group was also improved and had a higher conception rate [107].

Stem cells are initially undifferentiated cells that display a wide range of differentiation potential with no distinct morphological features. Stem cell therapy method recently has become a novel procedure for treatment of tissue injury and fibrosis in response to damage. Currently, there is massive interest in stem cells as a novel treatment method for regenerative medicine and more specifically for the regeneration of human endometrium disorder like Asherman syndrome (AS) and thin endometrium. AS also known as intrauterine adhesion (IUA) is a uterine disorder with the aberrant creation of adhesions within the uterus and/or cervix. Patients with IUA are significantly associated with menstrual abnormalities and suffer from pelvic pain. In addition, IUA might prevent implantation of the blastocyst, impair the blood supply to the uterus and early fetus, and finally result in the recurrent miscarriage or infertility in the AS patients. It has been evidenced that the transplantation of different stem cells with a diverse source in the endometrial zone had effects on endometrium such as decline in the fibrotic area, elevated number of glands, stimulated angiogenesis, enhanced thickness of the endometrium, better formed tissue construction, protected gestation, and improved pregnancy rate. This study presents a summary of the investigations that indicate the key role of stem cell therapy in regeneration and renovation of defective parts [108]. However, there are still issues as regards the efficacy and safety of SC related infertility treatment as no clinical proofs are available in humans.

8.5 Limitations and Ethical Considerations of Stem Cell Technology

Multiple abilities of self-renewal and differentiating caliber into any gametic or somatic cell without losing standards of normal cell characteristics, stem cells have come to become single point of focus and hope for medical professionals, scientists,

and patients. Stem cell therapies are being developed for genetic disorders, and biomaterials including human tissues are being developed for efficient treatment of common and rare diseases [109, 110]. However, the endless endeavors of stem cell researchers have landed this technology in certain ethical concerns and safety issues. Ethical issues are born due to the need for balance between concepts of saving life or respecting life. Human embryonic stem cells (hESC) represent the worst form of imbalance between the two concepts and hence are center of ethical controversies in stem cell technology. hESCs are derived from the spare pre-implantation embryos which either could be cryopreserved to develop into fetus or implanted to become humans [111, 112]. But to yield stem cells from pluripotent inner cell mass of the would-be humans (embryos), these need to be dismantled. This is where ethical concern regarding hESCs rises and hESC technology is not allowed to grow at its pace. This ethical dilemma has seeked legal intervention in different legislations throughout the world. Some countries including UK allow use of hESCs for research but not for therapeutic applications. While other countries like Italy exhibit more stringent stances, as it prohibits all hESC-based research [113]. The USA banned production of any hESCs line that requires the destruction of an embryo and research using hESCs lines is limited on usage of lines created prior to August 9, 2001. Such legalities and their diverse executions have hampered progress of hESCs technology internationally and development of cell-based clinical therapies globally. Giving a pause to enthusiastic caliber of hESCs, the realistic approach emphasizes to safety issues regarding hESC-based therapy for their clinical use. The pluripotency of hESCs turns out to be a double-edged sword as tumors can generate from these cells upon implantation in vivo [114–116]. Besides scientific ethics and safety concerns, hESCs have few non-medical impacts related to social and psychological aspects of life. Allowing destruction of embryos might lead to de-sensitization of human values. If fear of taking other life disappears, we might end up in increased crime against our life and security of other lives. Embryos might be used to grow tissues only and misuse of organs for commercial benefits will create lacuna of morality in our society. Further, use of hESCs for only research purpose instead of reproductive purpose faces challenges. However, Dworkin's views seem to be a balanced approach toward hESCs technology which states that "Embryos shall not be considered as humans but are valuable enough to begin or extend a human life", condemning creation of embryos for research purposes. As the philosopher John A. Robertson says, "In taking such a stance, persons define or constitute themselves as highly protective of human life" [117]. Robertson notes, however, that this same symbolic respect for life can be expressed through allowing embryos to be created so that others' lives can be prolonged, or deaths averted. The discovery of iPSCs overcame this concern. Safe autologous generation of iPSCs and storage in tissue repository and stem cell banks gave an edge to safer generation of stem cells. But other ethical controversy of human cloning came into being for iPSCs technology. Therapeutic use of iPSCs has certain safety issues as well.

Stem cell characteristic features, such as longer life span, apoptosis resistance and growth regulators and control mechanisms resemble cancer cells. The potency for malignant transformation is a key obstacle to the safety of stem cell based

therapeutics. The risk of tumor formation is further enhanced by other intrinsic and extrinsic risk factors. The site of administration (i.e., the local environment of the stem cell in the recipient) and the need for in vitro culturing contribute to the tumorigenic potential.

8.5.1 Genetic Modification

Genetic modification/reprogramming is required for manufacturing certain types of some stem cells (e.g., iPSC) prior to their clinical application. Genetically modified retroviruses and lentiviruses have been used for such modifications to generate mouse or human iPSCs. The use of viruses raises safety issues of cancer occurrence due to integration of therapeutic vectors activating oncogenes [118, 119].

8.5.2 Bystander Tumor Formation

Stem cells might act as activators of the existing tumor cells.

8.5.3 Immune Responses

Administration of stem cells may affect the host immune system. The administered cells may directly induce an immune response or may have a modulating effect on the immune system. Both ESC-derived cells and especially MSCs have been reported to be immune-privileged and have a low immunogenic potential. An immune suppressive effect of MSC has also been observed in an animal model of rheumatoid arthritis In addition, MSCs have been shown to suppress lymphocyte proliferation to allogenic or xenogenic antigens leading to acceptation of allo/xenotransplants in animal models. In clinical studies MSCs have been used to facilitate the engraftment of HSC and decrease GVHD [120–122].

8.5.4 Biodistribution

Biodistribution of the administered stem cells is a matter of concern. Preferred distribution of MSCs is known for specific tissues, e.g., the bone marrow, muscle, or spleen and tissues facing pathophysiological stress like ischemia or cancer. The mechanism underlying the migration of MSC remains to be clarified [120].

8.5.5 Unwanted (De)differentiation

For clinical use, iPSCs or ESCs must undergo in vitro differentiation prior to administration. However, what if dedifferentiation of stem cells occurs post administration? Dedifferentiation or redifferentiation into another cell type has been already described [123] but the clinical consequences remain unclear. MSC differentiation into unwanted mesenchymal cell types such as osteocytes and adipocytes has been described [124]. Encapsulated structures containing calcification and/or ossifications in the heart have been seen in animals treated with BM-derived MSC for (induced) myocardial infarction [124]. Thus, unwanted differentiation is a theoretical risk; however, the factors contributing to this risk are unknown.

8.5.6 Purity and Identity

Another critical issue is purity of the desired stem cell population. Contamination with other types of cells or undifferentiated cells could cause undesirable effects. Cross contamination of HT1080 human fibrosarcoma cells in MSC led to non-reproducible results on spontaneous transformation events of MSC and publications were retracted since the reported observations could not be reproduced [125–127]. These examples illustrate that even relatively simple risks should be considered. The primary concern being unwanted differentiation upon transplantation in vivo. Stem cells might differentiate into undesired tissues after being implanted for a specifically desired tissue. Safety concerning tumor tissue generation is another unwanted threat of stem cell technology. Earlier, reports show that adipose tissue stem cell-based therapy developed loss of vision in patients treated for macula degeneration promoted metastasis upon implantation [128, 129]. However certain regulatory guidelines laid by the Food and Drug Administration (FDA) define safe and effective protocols of stem cell-based therapies. These guidelines state that minimal laboratory manipulation shall be done to stem cells desired for treatment purposes and shall be intended for homogeneous use without requiring premarket approval to come into action and shall only be subjected to regulatory guidelines against disease transmission. In 2014, a radical regulatory reform in Japan passed two new laws that permitted conditional approval of cell-based treatments following early phase clinical trials preconditional to submission of safety data from at least ten patients. These laws deny earlier "fast track approvals" where treatments were classified according to risk [130]. To date, the treatments that acquired conditional approval include those targeting spinal cord injury, cardiac disease, and limb ischemia [131]. Now, the regulatory authorities demand "Good Manufacturing Practice," use of Xeno-free culture media, recombinant growth factors for safety protocols for cellular products. The balanced approach has led to many clinical trials to study application of hESCs. Exploitation of hESC-based therapy for the treatment of diabetes mellitus has begun in 2014 [117, 132], subretinal transplantation of

hESC-derived retinal pigment epithelial cells (hESC-RPE) for treatment of macular degeneration, dental pulp regeneration, periodontal tissue regeneration, Parkinson's disease [133].

8.6 Organ Culture: Agar Gel, Grid Method, Plasma Clot: Tissue Engineering

Organ culture refers to the explantation of organs or part of organs in vitro, so as to grow a new organ same as the parent organ. In newly developed organ, the parent in vivo characteristics of various tissue components with their anatomical relationship and function are preserved within the culture, in vitro [134]. In tissues lined with squamous epithelium, such as skin or esophagus, or in bladder lined with transitional epithelium, the epithelium follows a similar pattern of differentiation as in the organs in vivo. Hormone-dependent tissues remain hormone sensitive and responsive, and endocrine organs continue to secrete specific hormones. Finally, in fetal tissues, morphogenesis in vitro closely resembles that seen in vivo. Applications of organ culture are diverse in research and medicine. Animal studies cannot mimic state of human physiology to the extent that valid conclusions can be drawn for different experiments. Moreover, animal ethics restrict use of certain drugs or procedures and also limit number of animals for studies. Organ culture thus seems to be a better approach [135]. Modern culture approaches, such as three-dimensional (3D) cultures or organoids or organs-on-a-chip have been designed to better replicate the tissue microenvironment resembling natural tissue histology, physiology and responses to different stimuli. These are grown in a defined three-dimensional (3D) environment in vitro as mini-clusters of cells that self-organize and differentiate into functional cell types, mimicking the structure and functionality of an organ in vivo (hence, also called "mini-organs"). Organoids can be derived from either embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), neonatal/adult stem cells (ASCs), or explants from human tissues obtained upon biopsy or surgery. Self-organization within the organoid occurs through spatially restricted lineage commitment and cell sorting, which requires activation of various signaling pathways mediated by intrinsic cellular components or extrinsic environments such as extracellular matrix (ECM) and media [136].

Organ culture techniques are described below:

1. **Clotted Plasma Substrate.** It is a watch glass technique introduced by Fell and Robison to grow organ rudiments or whole organs. Organs are grown on surface of a clot consisting of chick plasma and chick embryo extract, kept in a watch glass. This was the classical standard technique for morphogenetic studies of embryonic organ rudiments. The method has been used to study the action of hormones, vitamins, and carcinogens in adult mammalian tissues. A modification of this method was introduced by Rudnick and later adopted by Gaillard. It consisted of culture vessel containing an embryological watch glass, a plasma

clot and closed with a glass lid sealed on with paraffin wax. Clot consists of two parts of human plasma, one part of human placental serum, and one part of human baby brain extract mixed with six parts of a saline solution. The plasma clot method had several disadvantages. Liquefaction of media led explants to lie in a pool of medium. Due to the complexity of the medium used, biochemical investigation could not be made possible.

2. **Agar Substrate.** To address the problems of plasma clot technique, agar gels were introduced by Spratt. The agar method has been successfully used for developmental and morphogenetic studies like the watch glass technique. The medium used for this method is composed of a salt solution, serum as well as the embryo extract or a mixture of various amino acids and vitamin with 1% agar. The explant has to be subcultured every 5–7 days. The method is largely used for the study of developmental aspects of normal organs and tumors. Although the agar does not liquefy, it requires transplanting the cultures for any study purposing. This disadvantage was overcome by the use of fluid media combined with a support which prevented the cultures being immersed.
3. **Grid Method.** The difficulty of immersing the cultures was overcome by Trowell's grid technique using metal grids, made of tantalum wire gauze. This has been replaced by more rigid continuous sheet of stainless steel or titanium. The dimensions of grids are 25 × 25 mm, with the edges bent over to form four legs, and height about 4 mm. The grids are ideal to grow harder tissues like skeletal tissues. For softer tissues, such as glands or skin, explants need to be placed on strips of lens paper and then deposited on the grids. Finally, the grids with their explants are placed in the culture chamber filled with medium up to the level of the grid. Grid technique was originally developed to maintain adult mammalian tissues having higher requirement for oxygen than fetal organs. Therefore, culture chambers are enclosed in containers which are perfused with a mixture of carbon dioxide and oxygen. This method has proved efficient for preserving the viability and histological structure of the adult tissues, such as prostate glands, kidney, thyroid, and pituitary.
4. **Tissue engineering.** Tissue engineering is an interdisciplinary field that merges engineering and life sciences for the development of organ or tissue substitutes to either restore or replace the lost function. It involves implantation of suitable cells isolated from donor tissue and biocompatible scaffold materials to construct bioartificial tissues in vitro. Combinations of cells and biomaterials must have the ability to reorganize themselves based on the nature of biomaterial and implanted cells. Optimum strength of adhesion between cells and substrate, controlled surface chemistry, porosity, and biodegradability of scaffolding biomaterial are required to aid migration and deposition of extracellular matrix materials by the implanted cells. There are two main methods to produce engineered tissue: First, cells are seeded on scaffolding matrix in vitro and thereafter cells are allowed to lay down on matrix to produce the foundations of a tissue for transplantation. The second approach involves using scaffold only to deliver growth factors or drugs, which upon implantation help cells from the patient body to get recruited to the scaffold site and form tissue upon and throughout the matrices.

These two approaches can be combined as well. To switch cells between growth and differentiation, strategies are required to allow interaction and integration with tissue and cells through incorporation of appropriate physical and cellular signals. For this, biologically active proteins and DNA are involved. After successful generation of the constructs, they must be intimately integrated into the host's vascular system for efficient nutrient supply and waste removal. This need is fulfilled by scaffold matrices to fill the tissue void, provide structural support, and deliver growth factors and/or cells that have the ability to form tissues within the body upon transplantation. The source of cells is also an important choice for success of tissues implanted [137]. The production of an engineered tissue in vitro requires the use of cells to populate matrices and produce matrix resembling that of the native tissue. The most favorable choice for such purpose comes from the use of cells taken from the patient. However, the patient cells are likely to be in a diseased state. Therefore, the use of stem cells, including embryonic stem (ES) cells, bone marrow mesenchymal stem cells (BM-MSCs), and umbilical cord-derived mesenchymal stem cells (UC-MSCs) has been focused upon. Previously, certain procedures have been successful using primary chondrocytes for the replacement of damaged cartilage [138, 139] skin cell sheets for damaged skin [140]. Certain larger and more complex tissue reconstructions, notably the bladder, have also been successfully performed [141], offering hope for more complex tissue engineered procedures in the future.

8.7 Applications of Organ Culture and Tissue Engineering in Medical Biotechnology

Tissue engineering has emerged as a chimera of apparently unrelated disciplines, i.e., biotechnology, engineering, and bedside medicine, with a common goal to solve pathological issues through artificially facilitated tissue regenerative processes. Tissue replacement, generation of prosthesis for lost extremities, biomechanical targeted muscle prosthesis, iron lung and heart pumps are few examples of artificial organs developed by tissue engineers to serve humanity. The discovery of human stem cells (SCs) has been proven the basic foundation for onset of tissue engineering era for the creation of biological substitutes in order to restore, maintain, or improve tissue and organ functioning. Currently, the trachea and the main bronchus replacement are promising in clinical phase trials. Biotechnologically developed artificial esophagus, intrathoracic organ, is in wet-lab phase [142]. Although self-regenerative capabilities of various organs have been exploited in medicine for decades, medical sciences have been working hard to accelerate the search for novel ways to direct tissue regeneration. Progress is often slow, as regenerative potentials, structural and functional requirements vary from organ to organ and range from the highly regenerative liver to the ominously resistant central nervous system [143]. Clinical research in tissue engineering is steadily advancing toward applications in operating theaters. Development of tissue engineered heart valves, reconstruction of functional intact distal airways, [144]. mesh chest-wall

prosthesis, interposition of artificially made vascular grafts, synthesis and functional implantation of tubular structures, like trachea and blood vessels, are the widening scope of tissue engineering in clinical applications. Besides, SCs find greater applications of tissue engineering in medical sciences. Injection of myocardial SCs, for myocardial infarction therapy, bone marrow or lymphatic SC replacement in hemopoietic malignancies offer good examples [145]. Three-dimensional (3D) and four-dimensional (4D) printing is a recent shot of tissue engineering which enables the use of intelligent materials to construct patient specific scaffolds and improve the extent and rate of targeted tissue regeneration. Bone tissue engineering is an important application of tissue engineering with printed polymers which increase strength to heal the bone tissue [146, 147] and then printed [148]. Poly(ε-caprolactone) (PCL) is the most commonly used polymer for 3D printing of scaffold for bone tissue [149] to improve the properties of the printed constructs. Besides, various types of SCs are reported for bone tissue engineering applications. Based on their potential to differentiate into bone cells, mesenchymal stem cells (MSCs) isolated from bone marrow or adipose tissue are the most frequently used for bone engineering [150, 151]. Combined with human umbilical vein endothelial cells (HUVECs), 3D printed scaffolds improve vascularization at the injury site and generate a tissue engineered bone tissue. This technology was updated to printing the cells and the scaffolds together [152]. These printed tissue engineered products have been successfully implanted in rabbit femurs at the defect site in order to study their effect on bone regeneration [153]. MSCs are incorporated with the PCL based scaffolds and are reported to improve the bone regeneration when applied to rabbit femurs [148].

Microtechnology is another shoot of tissue engineering with promising applications for liver system development. It is developed to mimic the complex in vivo microenvironment and microlevel ultrastructure of the organ using two-dimensional (2D) and three-dimensional (3D) culture conditions. Microtechnology based liver tissue engineering uses 3D culture methods, to maintain liver functions and recapitulate native liver [154]. Three-dimensional cell culture models when combined with bioengineered constructs lead to generation of tissue architecture which isolated 3D cell cultures are unable to generate. Lone organoids with self-renewal capacity can develop into early structures and mimic early development, but full-fledged tissues are yielded with implantation of bioengineered constructs. Human forebrain tissue with self-organizing capacity has been generated using floating microfilaments comprising of poly(lactide-co-glycolide) copolymer (PLGA) to generate elongated embryoid bodies. Likewise, microfluidic chip technology combined with natural alginate hydrogels has been developed to construct 3D liver tissues mimicking hepatic plates. These 3D cultures are capable enough to change in the bile secretion pathway via effector mechanisms associated with various receptors and efflux transporters [155]. Thus, tissue engineering gives a firm hope of developing physiologically relevant and active bionic organs and such systems of developing organs and tissues may have further applications, including drug development and disease exploration.

8.8 Summary

Stem cell technology (SCT) is a multifaceted technology and rapidly evolving to offer a utility in various scientific fields especially biomedicine. It involves combined efforts from cell biologists, geneticists, and clinicians and presents immense potential to help us treat various conditions including but not limited to malignant and non-malignant diseases. Since stems cells are multipotent with excellent ability for self-renewal and differentiation into multiple lineages, they become ideal targets for manipulation in vitro. Stems cells can be cultured in a controlled manner to offer utility in different fields. Stem cell technology has a diverse range of applications, which makes this cutting-edge technology most valuable in advancing healthcare and medicine, offering tremendous promise to treat, besides others, difficult diseases like Parkinson's disease, diabetes, and spinal cord injury. In the absence of substantiated research, however, the potential for harm to patients—as well as to the field of stem cell research in general—may outweigh the potential benefits.

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Chapter 9
Pharmaceutical Biotechnology: The Role of Biotechnology in the Drug Discovery and Development

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Abstract Biotechnology has made a great impact on the drug discovery and development process and improved human health and well-being in an unprecedented manner. It happened due to better understanding of the pathological signaling pathways, which allowed identification of the potential drug targets. Besides, advancements in cell and molecular biology techniques made the researchers able to screen the drugs in a timely manner and to gather mechanism of action and the toxicity of the drugs more efficiently. This decreased the failure rate of the drugs and improved therapeutic outcomes. This chapter provides a brief overview of the overall processes involved in drug discovery and development. Thus, our aim here is to provide readers a perspective on how biotechnology is increasingly becoming a reliable tool in the drug industry with a significant role in rational drug design.

Keywords Drug development · Biotechnology · Clinical research · Target validation · Preclinical research

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9.1 Drugs: Definition, Types, Properties, and Classification

In recent years, pharmaceutical biotechnology has emerged as a rapidly growing field due to its vast applications in the discovery and development of drugs [1–4]. The current trends in the market suggest that biotechnology-based therapeutics are surging up with the discovery of novel nucleic acid products and vaccines to target the disease progression and immune system of the patient [5]. Clinical drug discovery requires a robust understanding of the principles underlying health and diseases. It also requires sound understanding of the role of the molecular signaling mechanisms governing the functions of related biomolecules in pathology [2, 3]. Biotechnology also plays an important role in enhancing biomolecule synthesis and purification, determining the product shelf life, stability, toxicity, and immunogenicity studies of the drug molecule [6]. It is actively participating in developing novel drug delivery systems including liposome and nano-technology based therapeutics in the patients [3, 6, 7].

9.1.1 The Critical Steps Involved in Drug Discovery

There are multiple yet sequential steps involved in the drug discovery process [5, 8]. The process begins with the identification of the drug targets and often concludes at the build of the sound preclinical data with the opportunity to file an Investigational New Drug (IND) application to the US Food and Drug Administration (FDA). The processes involved between the target identification and preclinical discovery such as assay and reagent development, hit-to-lead generation, lead optimization, extensive lead characterization, and clinical candidate selection require robust collaborative efforts and availability of enough resources to carry forward the project [4]. As the process moves from one step to another, failure rates are also decreased gradually [5]. The pharmaceutical and biotechnology companies are always trying to bring up the discovery portfolio with large number of early projects to build pipelines. This enables the companies to end up with mature projects, which will help them to thrive [5, 8]. Even after spending huge amount of money and spending time and efforts on multi-layered projects, still the process of discovery is always remaining at high risk of failure. More importantly, if the steps at the beginning of the process are not planned properly and if there would be any caveat remained in applying any criteria, the entire process will show up in highly expensive preclinical and clinical phases [9]. This will add up the cost of the final product tremendously. Here, the valid questions to ask are how to make the entire process less risky to increase the success rate? If we will be able to accurately find the answer of these questions, we hope that we will be able to shed down huge cost associated with the entire drug development process [10]. Ultimately, this will not only bring medicines fast in the market but will also put less economic burden on the patients. What will be the starting point in the pharmaceutical company to initiate the effective drug

discovery process? There may be multiple starting points such as by bringing clarity in the entire research and development culture of the company in terms of well-defined specific goal and enhanced collaborations, utilizing specific and up to date technologies, changing corporate culture and make use of the availability of alternative drug discovery and developments tools [5]. More importantly, recent advances in the drug discovery show the utilization of advanced drug discovery technologies often easier to implement and better as a starting point. Indeed, latest technological advancements have proven the above points by accelerating the entire drug discovery process [9].

Biotechnology has emerged as a major platform and a niche in the discovery and development of drugs. For example, it did not only provide ability to decipher the complexities of pathologies but simultaneously bring novel treatments and vaccines to the clinic [11]. On the other side, gene therapy is ready to treat the previously called untreatable genetic diseases. Thus, at this point of time with the advancement and expansion of biotechnology in drug discovery, we are more hopeful than before. How biotechnology led to the paradigm shift in drug discovery and development? This is being owed due to the simultaneous advancement of molecular and cell biology sciences [11]. This enables us rapid and reliable testing of new molecules using in vitro models compared to time consuming work at organ or whole animal level. Thus, an early part of the drug discovery process heavily relies on discovering and developing of the molecular drug targets using purified recombinant proteins as well as genetically modified cell lines. And this brings the era of high throughput drug screening where large number of molecules are screened in short period of time with even limited budget [11]. This entire drug development process would even be impossible to imagine if there was no improvement of the understanding of the molecular basis of the disease process through the developments of newer biotechnology tools [12].

9.2 Drug Metabolism, Efficacy, and Drug Interactions

Here, we briefly summarized the sequential steps involved in the entire drug discovery process.

The first step in the path of drug discovery is target selection. Although it appears as step one, it is a multi-layered process, which requires a multidisciplinary work and collaborations. Here, the focus is to identify targets against which to develop the small molecules for the interventions [13]. Therefore, the overall aim in this step is to find out the potential modulator which affects the underlying cellular and biochemical disease pathways operating the disease processes. This is followed by the second step, the lead discovery. During the lead discovery process the entire focus is geared towards identification of a collection of small organic molecules which inhibit the selected drugs targets. These small molecule inhibitors are called “hits.” Later, these “hits” are further modified chemically to enhance and improve their potency and selectivity towards the identified molecular target [13]. The premise of

this final modification is to further narrow down selectivity of the compounds to a few where the structure–activity relationships may be promiscuous and defined. This will further enhance a degree of in vivo activity of the lead compounds. Following the chemical modifications, these lead molecules enter the next phase called lead optimization [9]. This is the longest and most resource intensive phase. Here the sequential steps of chemical synthesis of analogues will be done, which will be followed by their biological testing to further optimize the lead molecule in terms of their selectivity, potency metabolic and pharmacokinetic behavior, bioavailability, and finally activity in relevant in vivo animal models of disease [9, 13]. At this point there is a great opportunity to investigate and to minimize any anticipated toxicology. After this intensive phase, the molecule is ready for preclinical pharmacology and followed by clinical testing. Due to multifaceted and prolonged steps involved, the drug development process takes several years to complete. It demands a serious investigation to establish the safety profile of the compound, along with the optimization of the drug dose to be used and finally its efficacy in treating the disease [12].

9.3 Advantages of Sustained Release Technology in Drugs

Despite the plethora of scientific and technological progress, the entire process of drug discovery and drug development still demonstrates a high degree of uncertainty and the process is typically based on serendipity [4]. This leads to most of the time high rates of failure at clinical level. Therefore, there is an urgent requirement to make an entire process foreseeable. In this regard, the role of biotechnology in the drug industry is booming [2]. For instance, the Human Genome Project had a breakthrough role in changing the fate of entire drug discovery process and the ways new medicines are discovered and thus set up the foundation of the biotechnology era in bringing the new medicine at fast pace. The significant contribution of this project is perceived in a way that it will transform pharmaceutical research and drug discovery processes and improve the lives of the patients [2, 5].

9.4 Molecular Complexes and Their Stability

9.4.1 Traditional Small Molecule Drug Development

Here, biotechnology as a tool allows the researchers to focus the drug discovery process particularly on a single molecular therapeutic target. For example, the tools and techniques allowed in this regard are molecular biology, biophysical and biochemical methods. These methods are utilized to identify specific drug targets along with target validation, target protein expression, and generation of specific drug

screening assays. Although recombinant technology has its own niche in the drug discovery process, it is important to mention here that it is not involved in the synthesis of the leads and development of the candidate compound [2, 14].

9.4.2 Protein Therapeutics Development

In this process, which may include not only the traditional small molecule, but also the important role of recombinant technology in the expression or synthesis of protein medicine. In this regard the notable example of protein therapeutics is antibodies, which will be discussed further at length in this chapter [2, 14].

9.5 Evaluation of Toxicity of a Drug, Approval of New Drugs, Clinical Trials, and Post marketing Surveillance (PMS)

Before the development of the concept of drug target, most of the pharmaceutical research used to be specifically focused on the identification of pharmacological effects of the potential therapeutics [5]. Moreover, the activity of a given therapeutics was described as targeting a disease. Later, to search the specific therapeutics to treat the disease and simultaneously increasing the success at drug development level, the term “target” was coined. So, what is a target? The term, target, is a recently evolved concept, which describes a specific protein or a molecular entity [2, 5]. More precisely, it is a molecular or a cellular or even a biochemical unit which can be affected or regulated by a specific action of the therapeutics. As per the present rough estimate, the number of targets affected by the drugs in the clinic is between 120 and 500 [5]. Interestingly, if we talk about the most successful drugs in the market, the estimated number of targets is 43.4 [5, 14, 15]. On the other side, there are about 7% drugs in the clinical practice with no known targets [5, 14]. As we explained above, targets are molecular entities which make them specific candidate to be regulated by the medications during diseases. Biotechnology has played a significant role to understand the precise role of these targets both in homeostasis and in pathology. Intriguingly, studies show that during inflammatory conditions, where multiple pathologies share the common underlying disease mechanism, targeting that common molecular entity could result in beneficial outcomes [16]. Thus, once we know the specific target in disease condition, we may predict both efficacy and specificity of the potential therapeutics. We may even predict the adverse effects and the toxicity profile of the medications. In this way disease could be managed without serious adverse outcomes [15, 16].

9.5.1 The Role of Genomics and Proteomics in Identification of Targets

Mapping of the human genome followed by the development of proteomics flooded the information of the role of genes and proteins in health and diseases. Both genomic and proteomic studies also provided the precise targets of the drugs, which were hidden previously. Besides, development of latest scientific tools and techniques due to the advancement of biotechnology also enhanced the discovery of target genes [15]. However, this also poses a change to researchers to validate the so many drug targets, which are available to consider. Advancement of the research provides us a good estimate of the “druggable genome” and suggests the number of the genes as drug targets or “druggable genes” are somewhere around 5000 [17, 18]. Besides, the number of diseases modifying genes are estimated about 3000 [17, 18], and estimate of overlapping between above two groups is somewhere between 600 and 1500 [17]. Please note that the above estimate is excluding the recombinant protein drugs and the soluble, extracellular targets for antibodies, used as the therapeutics [5].

9.5.2 Proteins as Potential Drug Targets

In human proteome we may find a family of proteins such as protease, G-protein coupled receptors, and family of kinases [19]. Medication are available clinically to target currently 130 families of proteins, in which about half of the drugs target six gene families [18]. These six gene families are G-protein coupled receptors, zinc metallo-peptidases, nuclear hormone receptors, serine/threonine and tyrosine protein, serine proteases, and phosphodiesterases [18, 19]. On the one hand it may look tempting that evaluation helped us in that aspect, however, realistically it brings complexities due to the reason that homology would remain very high particularly at the conserved domains of the active sites [5]. Besides, there is a tendency that a drug molecule, which is a member of one target family may be able to bind and inhibit the related molecules. Thus, it is understandable how much time and effort are required to develop very specific therapeutics. Besides constraints in targeting the family of proteins, it brings its own advantages on the other side. For example, chemical libraries can be developed with leads variants for protein families [20]. This will increase a significant propensity of a “hit” with another member of the family during high throughput screening [21]. Further, inadvertent or chance cross-target most of the times may turn out to be advantageous [20]. This can be conceptualized with the help of example such as imatinib mesylate (Gleevec), which is a tyrosine kinase inhibitor [5, 22]. It is initially developed as a drug to target bcr-abl. However, it was revealed that it targets and inhibits four distinct families of kinase such as bcr-abl, c-kit, the two PDGF receptors, and ARG kinase [5, 23]. The drug’s action on these other kinases may appear as off-target action, however, the

prevailing understanding of the mechanism of action of the therapeutics suggests that all the afore mentioned targets are required for the inclusive efficacy of the medication [24]. In another example, an antibody abciximab (ReoPro), which is in clinic, targets a glycoprotein IIb/IIIa (GP IIb/IIIa) [25]. Later studies show that it may also bind to the vitronectin receptors avb3 and the Mac-1 receptors [5]. Studies suggest that those off-targets are clinically important and beneficial [26]. These observations demand a very careful pharmacological analysis of the medications to delineate their mechanism of actions [15]. It is worth to mention here, these further clinical assessments should not remain to be restricted at only developmental stage of the drug, rather it would be useful if it may be applied to the approved medications as well [15].

9.6 Drug Delivery Systems: Introduction and Types, Targeted Drug Delivery, Vehicles for Targeted Drug Delivery

Both the academic researchers and physician scientist are heavily involved at early stage of the drug development as well as on the clinical trials [27]. Besides, they are involved in the optimization of the treatment schemes following drugs approval [27, 28]. These clinical studies revealed how the drugs work in human body and provide relevant information about the possible targeting of the pathology particularly in disease sub-set responder and non-responders. Therefore, these data set not only provide a novel mechanism of action of the drug in question, but also provides feedback on medical need in the patients who failed to show response to the drugs [29]. Therefore, not only non-responder class but even partial responders may emerge as novel subpopulation which requires a new drug with a different mechanism of action to treat. It is important to mention here that to diagnose a disease using a conventional single approach may not be enough in the above set of population [28]. This requires novel ways to get deeper knowledge of pathology due to the involvement of multiple molecules. In this regard gene array and other advanced biotechnology tools are warranted to accurately characterize the sub-set of patients and later to identify the novel targets to treat the disease. For instance, the use of novel anti-TNF class of drug therapies is currently under active investigation to treat rheumatoid arthritis in patients [28, 29].

9.6.1 The Role of a Target Product Profile in Drug Discovery Process

The target product profile is the exact expectations of the drug candidate in the process of the development [30]. If we talk about the small molecules, this profile includes the anticipated clinical indications, the proposed mechanism of action of the drug, the drug target, drug specificity, affinity, in vitro and in vivo potency, pharmacokinetic profile, biopharmaceutics characteristics such as drug absorption, distribution, metabolism and elimination [5]. Besides, chemical accessibility, safety profile of the drug, and biomarker requirements are also being considered for the target product profiling [30]. For the therapeutic antibodies, the criteria include suggested clinical indications, the target of the antibody, the mechanism of the action of the antibody, epitope affinity, specificity, constant region isotype, expression rate in mammalian cells, effector functions, specificity in formulations, unwanted effects, pharmacokinetic and biomarker requirements [31].

It is necessary to mention here that biomarkers are important to decrease the risk in drug discovery projects [9]. It is worth remembering that when two discovery projects are competing for scarce resources, the one with predefined knowledge of biomarkers should be preferred [5] because it has a better chance of being successful [9]. The next logical question arises who provide the input of biomarkers? The answers include pharmacologists, molecular and cell biologists, regulatory scientists, and clinicians. Next to the biomarkers, is the implementation of plan of how and when each of the profile parameters is assessed. It is imperative to understand here that the problems in pharmacokinetic parameters of the drug such as absorption, distribution, metabolism, and elimination (ADME) are used to contribute about 40% of the clinical drug failure. However, early implementation of in vitro and in vivo tests reduced this failure rate to about only 10% [31]. Besides, the biochemical and cellular assays, reagents, surrogate antibodies, reference compounds, and animal models are cornerstone requirements of the successful target product profile. Therefore, target product profiles develop and thus evolve throughout the life of a discovery project [31]. However, the following three points are significant [5, 32]:

1. If require any changes to the target profile, it only occurs according to the thorough review of the up to date scientific data.
2. When progress from one project transitions to another, the target product profile is the only reliable standard to follow for approval. Since organizations vary within their own criteria of defining target profile, thus, transition criteria will be solely based on the specific target product profile.
3. Each specific component of the discovery project such as chemistry or antibody technology, proof-of-concept research, ADME assays, etc. should be at the appropriate pace relative to each other. For example, if one activity of the project is going on at a faster rate than others, required tests may not be feasible, and thus proof-of-concept test may be conducted too late. This will be very difficult

to continue the project due to the lack of scientific foundations. Therefore, it is required an appropriate and balanced resourcing to manage all arms of the projects including a sound planning and collaboration. Thus, it is difficult to conceive an idea of successful drug discovery project without a target product profile, with the notion that all the concerned departments and relevant people are determined to work as unit [5, 32].

9.7 Advantages of Targeted Drug Delivery System

Initial discovery of the potential drug targets differs substantially from one another in terms of validation [13, 20]. The academic pharmaceutical or biotechnology researchers contribute immensely in the investigation of the biology of a target [20]. Following the target identification including from the genome database require complete validation to its role in normal physiology, and the intended target disease, before moving forward towards expensive drug discovery cycle [33]. The pharmaceutical and biotechnology fields have generated novel platforms and technologies that provide additional key information helpful to take decision regarding the forward move of the target. It is clearly defined that thorough target validation is of utmost necessity to prevent high rate of failure of the discovery [33]. However, already validated and researched drug targets most of the time may need very minimal process of validations and thus save time as well as money [13]. However, as general rule in the process in industry researchers are always inclined to confirm published reports on drug target validation because of the developments and validation of reagents and assays in the laboratory [33]. Once the targets become validated, it would signal to move on to the in vivo pharmacology and biomarker development phase of discovery cycles [34]. One of the important reasons of these studies is the fulfillment of the regulatory requirements, which ask specifically about the thorough understanding of the mechanisms of drug actions before moving forward to clinical studies [35]. This phase of the discovery also elaborates the unwanted effects of the drugs including the toxicological effects [33].

As suggested by Jurgen Drews [36], former president, Global Research of the Roche Group, the target validation is not a onetime process, rather it is continuous process throughout life cycle of the project [5]. He laid out the following four-point criteria of the target validation [5, 33, 35, 36].

1. The manipulation of a potential target by genetic or pharmacological means should consistently lead to phenotypic changes that are consistent with the desired therapeutic effect.
2. The observed effect should be dose-dependent (this is approachable once early drug leads with enough specificity are available). Alternatively, conditional knockout (see below) could be used.

3. The desired phenotypic changes must be inducible in at least one relevant animal model. If possible, several animal models should be used, all of which reflect at least some important aspects of the human pathogenesis of the respective disease.
4. The specific mechanism of action of the new therapeutic agent and any possible off-target activities should be known. This would allow for appropriate benefit and risk assessment.

9.8 Genomics in Drug Discovery

One of the most promising tools emerged in recent years is the use of the gene-deleted or gene knockout mice model in target validations [14, 37]. Upon deletion of the target gene in the mice and if the mice will be viable, this technology will completely block the biological effects of the target in mice [34]. Gene knockout technology will also provide any causative role of the target on the development of disease, if any, in the mouse model [38]. These mice will provide the overall role of the target on the physiological outcomes [37]. This will ultimately provide the relevant data on the biological functions of the target [14, 34].

Studies also show the role of the knockout mice model in the investigation and identification of "druggable" genome [38]. Although this is still in infancy but has tremendous potential to provide information on novel druggable targets. Besides, these knockout models serve as a valuable tool to identify prophylactic intervention, where the gene is already deleted before the induction of the disease [14, 38]. Next to the whole-body knockout, research has shifted heavily in conditional knockouts where they are deleting the gene only in specific tissue or organ in the mouse body [39]. For example, the endothelial specific knockout mouse models are catalyst in the identification of drug targets to prevent and treat vascular inflammatory diseases such as sepsis, acute respiratory distress syndrome, and atherosclerosis [38, 40]. Thus, these conditional knockout mouse models are more relevant for therapeutic interventions [41]. The disease can be induced in the adult mouse with the gene in the "on" state, and the gene is later turned "off" by a generic agent that triggers a genetic switch. These models are providing clinically relevant data with high precision and reproducibility. They also helped to lower the cost of the drug research by decreasing overall time of the target validation [41].

In vivo research will further complement in vitro cell culture research using primary or modified cell lines [42]. So, while the researcher will be waiting to get the genetic knockout mice, the basic premise of the research would have been done on cell lines. This will further validate later the mouse models [43]. This will provide a robust data sets and complete function of the drugs targets under in vitro and in vivo environment. In vitro target identification technology will also enhance the development of novel biochemical assays to screen the novel drug molecules in short span of time. This will provide quick guide on the biology of the targets including their mechanism and side effect profile [42].

In recent years the small-interfering ribonucleic acid (siRNA) technology has emerged as a powerful tool for target identifications and validation both in vivo and in vitro. siRNA technology is employed to deplete the genes in animal models and cell lines [44]. Unlike gene deletion technology, which is irreversible, the siRNA technology deletes gene in a reversible manner [40]. This technology is deeply immersed in the laboratories both in academic and industry research focusing on the drug discovery [40]. High number of peer reviewed published articles are coming up regularly and further validating the potential role of this technology in biotechnology and drug research [44].

9.8.1 The Role of Academic Research in the Drug Target Identification and Validation

Academic researchers are actively involved in drug discovery research and utilizing heavily both in vivo and in vitro models [40, 45]. Their research provides insights on the role of the targets in health and diseases [46]. Academic scientists are meticulously involved to understand the signaling pathways that regulate the targets in the molecular and cellular environments [45]. Researchers investigate both upstream and downstream signaling, which will be very helpful in understanding the molecular circuitry of the drug targets [46]. This will be further helpful in the prediction of adverse profile of the target. Thus, a close networking between academic and industry scientists is required to fully yield the potential of their work and decreasing the time and cost of the drug discovery [45, 46].

9.8.2 Potential Role of Technologies in the Process of Target Identification in Drug Discovery Process

Statistics show that drug discovery failure rate is high and only less than 1% of projects yield the compounds for further clinical development [47]. Data show that only 10% of those 1% compounds will end up to be approved by the FDA to treat diseases in the clinical setting [48]. This gives a picture that there is very high risk of failure compared to success in drug discovery process [47]. Recent industry trends further show that the success rate is higher in protein-based therapeutics including antibodies-based discoveries as compared to small molecule inhibitors [47]. This data guides the companies to invest in balance way to enhance their success in the process. It also brings into attention that companies should also invest to build up their technology and expertise to be involved in wide range of the projects [47, 49].

9.8.3 Antibodies vs. Small Molecule Inhibitors as Therapeutic Agents

The therapeutic antibodies are emerging class of drugs and hold a great promise in the management of variety of diseases [50]. As of December 2019, 79 therapeutic monoclonal antibodies have been approved by the US FDA, but there is still significant growth potential [51]. Antibody-based therapeutics provide predictable responses, faster delivery due to the intravenous route of injection, and require short period of time to validate the target. On the other side, small molecules possess their own line of advantages including oral delivery due to better bioavailability; however, less efficacious compared to therapeutic antibodies [51]. Regarding the discovery risk, antibodies developments have a lesser risk of failure compared to small molecule drugs due to differences in their specificity and the structure and binding to their unique epitopes. For example, antibodies interact with their larger region of the target molecule surface, thus providing a higher rate of selectivity and affinity. Small molecule drugs often displayed more conserved binding to their target sites and thus less advantageous compared to antibody therapeutics. Due to the profound specificity and inability to cross plasma membranes, antibodies have minimum "off-target" effects and thus less toxicity. This brings another advantage of them over the small molecule drugs [51].

9.8.4 Example of Antibody Therapeutics

Although antibodies as therapeutics gained clinical popularity and approval in recent years, the foundation was laid down more than a century ago when Behring and Kitasato received the Nobel Prize for passive immunotherapy [52, 53]. Later, in their ground-breaking work, Milstein and colleagues after isolating monoclonal antibodies provided the cue that a paradigm shift in the medicine would soon be going to be witnessed [54, 55]. Since then with advancement of the technology allowed the development of initially murine and chimeric, and followed by humanized antibodies, and now we are having fully human antibodies are actively involved in diagnosis and treatment of the previously thought treatable diseases. Today, more than 80 antibodies are approved by the FDA as potential therapeutics for the treatment and the management of cancers, rheumatoid arthritis as well as for the prevention of transplant rejection [50, 54].

As the understanding of the antibody-based therapy grew, it was recognized that mouse and mouse-sequence-containing antibodies are not efficacious due to identification of them by the human immune system as foreign. Later, recombinant human antibodies are generated and gained popularity clinically and were also better accepted by the human system. These antibodies were developed by employing advanced molecular and cell biology techniques such as phage display [55] and transgenic mice with a human immune repertoire [54, 56].

One of the major breakthroughs happened when FDA approved Adalimumab (Humira), a fully human monoclonal antibody in 2002 and by the European Medicines Evaluation Agency (EMEA) in 2003 for management of rheumatoid arthritis in patients [57, 58]. Adalimumab was discovered using phage display technology [57, 59]. This antibody is an immunoglobulin G1 (IgG1), which contains highly selected heavy and light variable domains of human origin [59, 60]. Adalimumab has a high specificity and an affinity for TNF but not with other inflammatory cytokines, such as lymphotoxin. It exerts its pharmacological effects by blocking the interaction of TNF with p55 and p75 TNF receptors [57, 58].

9.8.5 Biomarkers

Good biomarkers play cardinal role in a drug discovery project in the company. These biomarkers are acting as connection between animal models and patients and are significant in assessing the impact of the drug molecule on the target [61, 62]. Recent literature shows that changes in a biomarker need to be measurable objectively such as by using gene array technique, enzyme-linked immunosorbent assay (ELISA), etc., in response to the drug intervention [5, 61].

By determining the effect on potentially known downstream biomarkers, we can determine if the drug is affecting the intended target or not [1]. Besides, biomarkers for other pathways help to assess the specificity of the effect [62]. It has been shown that when the therapeutic molecule has the required effect on the biomarkers, however the clinical assessment measures failed to show any effects, this observed discontinuity is either may be due to the dose of the drug or it could be due to inappropriate duration of the treatment [1, 5]. When the biomarkers indicate complete target inhibition, but the disease still does not respond, it is likely that the target is inappropriate for the disease [1, 5].

9.9 Summary

Drug discovery is always a risky process for companies. However, using proper approaches and strategies including latest available technologies, a company may energize its discovery research to maximize the success rate. To check on the failures, companies also require focusing on their available resources including regular training of the researchers on latest techniques, internal decision-making capabilities, choosing the appropriate projects and establishing the goals and improving project management and collaborative environments. Besides, industry-wide data collection on the overall success and failure, will help to set the priorities, and streamline the projects. This will provide the team with the direction where project will be moving and researchers will learn from successes and from the failures in a timely manner and will be able to take an informed decision.

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Chapter 10
Diagnostic and Therapeutic Biotechnology

Ashvinder Raina, Vignesh Villingiri, Shafqat Jehan, and Syed Abdul Qadir

Abstract The term vaccination (Vacca meaning cow) came into existence with the discovery of smallpox vaccine by Edward Jenner and involves utilization of material like live avirulent microbes, attenuated microbes, peptides, and recently also nucleic acids. The history of vaccination goes back to the eighteenth century, but it was not until the twentieth century that it became possible to make immunological marker-based vaccines. The twenty-first century came with its own challenges like vaccine development for individuals with pre-existing medical conditions and compromised immune function and opportunities in vaccine development like edible vaccines, nano-particle vaccines, DNA/RNA vaccines, CRISPR based vaccines. Most recently in this field, an RNA-based vaccine for SARS CoV-2 has revolutionized and re-oriented the area of vaccination technology with hopes for vaccine development for previously uncurable conditions like many forms of cancer looming over the horizon. This chapter describes in detail different types of vaccines, technology behind their synthesis, use of plants, animals, and microbes in vaccination technology, and utilization of vaccination technology in immunization against various diseases with examples. The chapter also focuses on the molecular basis of diagnostic biotechnology, with focus on use of antibodies in molecular diagnosis and therapy.

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10.1 Vaccination and Immunization

Immunization refers to developing immunity artificially by inducing some effector functions of the immune system. From a clinical point of view the immunization may be active or passive depending upon the biological material used to induce the immune system. Active immunization occurs when an inactivated, avirulent, or modified pathogen is administered into the body with the intention of developing immunity in the form of B and T cell memory against the active virulent pathogen. These memory cells readily attack the pathogen whenever the infection occurs. Active immunization is also called as vaccination. In most of the cases of vaccination there is an intended attempt to expose the body to the pathogen which results in the primary response, hence upon the subsequent encounter with the pathogen under consideration would cause the secondary immune response mediated by antibodies and effector cells and individual is protected.

Passive immunization involves the administration of antibodies against the pathogen which lead to the immune defense against the infection without any lag phase but no memory cells are developed in this process. Yet again humans have understood the importance of vaccines in healthcare at this time of SARS-CoV-2 pandemic. It was not until the discovery of the first vaccine, for smallpox discovered by British physician Edward Jenner in 1796, prior to which smallpox caused death of millions of people. This vaccine was resultant of the experiment of Edward Jenner in which he took out the fluid from cowpox blister and scratched it into the skin of 7-year-old boy suffering from smallpox. The boy recovered from smallpox after appearance of small blister at the site of scratching. Jenner again inoculated the boy's smallpox and observed that the boy did not develop the disease indicating that fluid from the benign cowpox virus resulted in the immunity against smallpox. This breakthrough discovery by Edward Jenner led to appearance of new terms "vaccine" and "vaccinology." Jenner described thus developed vaccine against smallpox as "variolae vaccinae." For this remarkable contribution, he has been also called the "Father of Vaccinology." The term "vaccine" came from the Latin word *vacca*, which means cow [1].

Eradication of smallpox through vaccination is one of the best success stories. Discovery of smallpox vaccine by Edward Jenner in 1798 led to speedy immunization and control in developed countries; however, the developing countries are still facing a lag in this scenario.

In 1876, Robert Koch worked with *Bacillus anthracis* and inoculated the same in animal which caused the disease, this led to the foundation for the popular germ theory of the disease. Afterwards Robert Koch extended the same protocol to other pathogens expecting the vaccination protocol would be for them also, but many pathogens do not exhibit the cross-reactive counterparts similar to cowpox and

smallpox. Serendipitously Robert Koch encounters the way to attenuate the pathogens such that it remains immunogenic but loses its disease-causing ability. Many pathogens experience attenuations when these are inoculated in a different host. The pathogen becomes less virulent in such host or in suboptimal condition of culturing in laboratory. The concept of attenuation developed over the centuries.

The method of Variolation was just like one use poison to develop immunity against its toxic effects. Jenner's idea of using an animal pox virus to protect from human virus smallpox was based on the concept that an animal virulent virus becomes attenuated in human [2].

As these technologies evolved with time, in 1881 it became reality to make anthrax vaccine for animals and rabies vaccine for humans in 1885. Rabies virus is highly virulent in humans but when rabies virus is grown in rabbit, a less virulent strain of the live rabies virus is developed that could be employed as vaccine. Around the same timeline it was known that Cholera bacteria when exposed to very high temperatures still retains the immunogenicity I avian hosts and led to the immune response upon exposure to live bacteria. This kind of reports led to the development of live attenuated vaccines.

In 1980 Smallpox was announced as eliminated in 1980, whereas poliomyelitis is on the verge of worldwide eradication and measles has been controlled in almost all over the world. It is now well-known fact that vaccines work very well for acute infections, whereas chronic infections like HIV pose major challenges concerning efficacy and safety [3].

However nowadays various categories of vaccines have emerged such as: live attenuated, vector vaccines, adjuvants, recombinant and DNA vaccines, peptide vaccines, and subunit vaccines.

10.1.1 Live Attenuated Vaccines

Live attenuated vaccine consists of live, whole bacterial cells, virus particles, or pathogens treated in such a way that results in less virulent form so that they lack the ability to cause the disease but exhibit immunogenicity. Mutation causing attenuation is induced when the pathogen is cultured in suboptimal conditions for prolonged period of time. Suboptimal conditions are different from the physiological conditions in a potential host; hence, the culturing conditions would cause the selection of only those pathogens which have modulated themselves with the suboptimal conditions, making them less virulent yet immunogenic.

Attenuating a virus by introducing it into a less potential host in which it is unable to replicate well, for example, infecting an animal with human virus or multiple replications cycles in tissue culture. This process is known as passaging. Influenza vaccine was developed by passaging the influenza virus in embryonated chick eggs for long period of time. Similarly, the polio vaccine was developed by passaging the polio virus in Monkey kidney epithelial cells. Likewise, the attenuated strains of the viruses causing measles, rubella, mumps, and yellow fever have also been created by passage through non-human hosts or cells.

Live attenuated vaccines have proven to be quite success in humans with some potential issues regarding safety and efficacy. For example: mumps vaccines had a doubtful safety and efficacy; the first-ever licensed rotavirus vaccine had to be withdrawn as it caused intussusception. Furthermore, some live attenuated vaccines are to be used in specific conditions only, e.g., polio vaccine is to be given throughout the year in mass campaigns in tropical region where the exposure to the polio virus occurs throughout the year so that the potential susceptible population can be reduced and also breaking the transmission circle of the virus [3].

Research finding in cold-adapted attenuated vaccine against Influenza virus revealed that this type of vaccines is more effective than inactivated influenza vaccines. Hence the Cold-adapted attenuated influenza vaccines are a promising defense against influenza [4].

Examples of live attenuated vaccines are developed for: Smallpox virus, polio virus, yellow fever, measles, mumps, etc.

Advantages

- As whole organism is administered into the body of an individual, epitopes for both B and T cells are presented causing more potent immune response with no chance of reversion. Epitopes are recognized by pathogen recognition receptors on the surface of antigen presenting cells resulting in generation of co-stimulatory signal pathways. Both cell mediated and humoral response are induced. Innate immune response is initiated with the secretion of cytokines inducing inflammatory response.
- In the process of attenuating the virulent pathogen, the machinery of replication is still preserved, hence large number of pathogens accumulate in the body, reducing the dosage and increasing the cost-effectiveness.
- Lesser chance of animal virus contamination.
- As whole pathogen is introduced into the individual's body, there is ample stimulation to induce immunity life-long in one or two doses, which also make them cost-effective as less doses/boosters are required.
- Live attenuated vaccines are very stable, hence no need of cold chain.
- May be effective against intracellular pathogens.

Disadvantages

- Reversal of attenuating mutations in the viruses may cause the diseases.
- Vaccine might get contaminated with animal viruses.
- Cold chain required for transport.

10.1.2 Adjuvants

Recombinant biotechnology and genetic engineering have aided in replacing the vaccines based on live attenuated pathogens and facilitated the advancement toward the vaccines derived from highly purified antigens which have decreased the potential side effects as compared to live attenuated vaccines. Though such purified

antigens can be less immunogenic or non-immunogenic at all especially among the population with differing immunity in children, adults, elderly individuals, and individuals with chronic diseases. In such cases there is a crucial need of amplifying the immune responses.

Adjuvants are the components that when used with the vaccines can enhance the immunogenicity of the antigen given as vaccine. The word "adjuvant" came from Latin word "adjuvare" which means "to aid or help." Adjuvants are used with vaccine only when needed due to various regulations put to control their use. It is crucial to determine the mode of action of an adjuvant before using it in order to fulfill the regulations as well as licensing. Alums or aluminum salts are widely used to alleviate humoral immune response as adjuvants but its mode of action is still under investigation [5–7]. A few examples of FDA approved adjuvants are: Oil in water emulsion composed squalene (MF59) with trivalent inactivated influenza (TIV) vaccine; MPL and QS-21, a natural compound extracted from the Chilean soapbark tree (AS01B) used with recombinant zoster vaccine RZV (Shingrix); and CpG, a synthetic form of DNA that mimics bacterial oligodeoxynucleotide and viral genetic material (CpG-1018) used with Hep B (Heplisav-B).

Simultaneously, an all-inclusive understanding and evaluation of unwanted reactogenicity linked with the adjuvant supported vaccines is mandatory to develop appropriate prophylactic and therapeutic vaccines [8].

10.2 Recombinant DNA Vector Vaccine

In the 1990s, the time when it was reported that a plasmid containing gene insert from Influenza virus especially a nucleoprotein could elicit the protection against the virus by inducing cytotoxic response mediated by cytotoxic T lymphocytes, the beginning era of DNA-mediated immunization was marked. Yankauckas and team demonstrated that plasmid DNA with influenza virus nucleoprotein is administered to mice through intramuscular injection results in activation of nucleoprotein specific cytotoxic T cells and production and specific antibodies. This immune response lasted for over 1 year and protected mice from subsequent infection with live influenza virus [9].

Recombinant DNA vector vaccine involves the use of DNA encoding the immunogenic protein, that is used as vaccine. Interestingly the individual who receives such vaccine produces the immunogen after vaccination and that immunogen is presented via endogenous pathway. Cytotoxic T cells are activated in large numbers that can combat with the intracellular antigens. DNA vaccine are developed for pathogens which are not compliant or are not successful with other types of vaccines, e.g., HIV, HepC, Mycobacterium leprae, and P. falciparum.

Vaccinia virus is a commonly used DNA vector in which the genes which are non-essential for its entry into the host cells can be replaced with the gene inserts encoding the antigenic proteins from the pathogen of interest. These gene insert might include the DNA sequence encoding signal sequence that directs the

particular protein to the desired destination such as membrane, e.g., the virus envelope protein. Vaccine development for HIV using its envelope glycoprotein and Plasmodium falciparum using proteins from its sporozoite stage is under investigation.

Human adenoviruses are also exploited at recombinant DNA vectors for vaccine development due to some characteristic features: it can replicate in host cells even in the presence of large copy number of viral DNA, large gene insert can be incorporated without hindering the replication process, naturally virus is nonpathogenic to human, adenovirus is easy to culture in vitro. Adenoviridae family of viruses is characterized as being non-enveloped with icosahedral shaped capsid enclosing double stranded linear DNA genome of 30–40 kb size. There are 57 human adenoviruses classified into seven classes. The receptors for adenoviruses are expressed on almost all human cells which results in its uniform distribution in whole body [10]. Adenovirus based vaccine can be made competent replication or replication defective by swapping E1A and E1B genomic region with antigen expression cassette from pathogen rendering it to unable to replicate [11].

Adenovirus with gene inserts of antigens from herpesvirus, measles virus, HepC virus, and rabies virus is capable of inducing cell mediated, humoral as well as mucosal immunity.

One discouraging fact about adenovirus is that it is tumorigenic in nature although the tumor associated sequences can be deleted from the genome.

Furthermore, the insect borne alphaviruses Semliki Forest virus (SFV) and Baculovirus have also been investigated as recombinant DNA vectors. Alphaviruses are capable of producing large amount of mRNA as indicated by animal studies that recipient of such vaccine showed high level of viral protein from the pathogenic virus whose gene of interest was inserted in the recombinant DNA vector derived from SFV alphavirus. Similarly, the animal studies with influenza virus in which the mice administered the influenza nuclear protein inserted into SFV recombinant vector developed prolonged cell mediated and humoral immune response. Avipoxvirus are also among recombinant vectors used to insert the glycoproteins from various human viruses such as HIV.

All the above-mentioned recombinant DNA vector vaccines develop potential cell mediated and humoral responses in animal studies. Canarypox virus derived recombinant DNA vaccine has showed promising results and has entered human clinical trials in which these vaccines have shown strong stimulation of immune response.

Baculovirus has been used to produce Hep C virion like particles that can be employed as vaccine. Envelope protein from Hep C virus is very difficult to express in animal cells, whereas insect viruses express these proteins quite successfully. Animal studies with HepC virion like particles have shown to invoke strong humoral and cell mediated responses.

DNA vaccines have potential applications in therapeutics of infectious diseases, autoimmune diseases, cancer, and hypersensitivity disorders [12].

Advantages
- As whole organism pathogen is not used as vaccine, there is very less chance of potential side effects or reversion.
- Large quantities of immunogen produced within host cells evoke strong immune response.
- Depending upon the nature of vector used site specific expression of immunogen can be driven.
- Immunogen produced with post-translational modification and conformation can elicit both helper and cytotoxic T cells responses by endogenous pathway as well as cross-presentation of antigen.

Disadvantages
- Some vector may cause tumorigenicity.
- Accidental replication of vector may produce major side effects.
- In response to first dose the vector protein can cause generation of anti-vector antibodies which can further lead to unresponsiveness to booster doses.

10.2.1 Peptide Vaccine

Some peptides are capable of inducing both B and T cell responses as they contain epitopes that are recognized by both cell types. In case of peptide vaccines, the exact molecular information about the peptide is known, hence the chances of pathogenic reversion are negligible. Furthermore, as the size of the vaccine peptide is small, there are very thin chances of pathogenic components getting purified along with the vaccine. Peptide vaccines can be derived both naturally, synthetically and via modification using recombination DNA technology in those cases where natural peptide is non-immunogenic. A peptide is identified, purified, and then mixed with the adjuvant which alleviates its uptake via antigen presenting cells which present the antigenic peptide via MHC-1 and MHC-II to helper T cells and cytotoxic T cells, respectively [13].

There is an interesting rationale behind recognizing a peptide as vaccine. B cell epitopes are hydrophilic sequence which points toward their existence on the external surface of protein. Conversely, T cells recognize MHC bound to the peptide as one entity, hence the vaccine peptide must contain recognition sequence for both T cell receptor and MHC. Involvement of MHC further complicates the recognition process as each MHC can bind differently to the same peptide at different sites eliciting different T cell responses. As MHC halotype is very unique for an individual, hence one peptide may be recognized very well in one individual but poorly in individual expressing some other MHC haplotype [14].

Natural peptides from influenza virus, pertussis, and cholera have been used as vaccine with uncertain results. Modified hemagglutinin (HA) glycoprotein of influenza virus has been employed in modified form as peptide vaccine. The natural peptide is not immunogenic and is made immunogenic by introducing a supermotif

into the sequence that allows the binding with TAP antigen transporter. Addition of supermotif increases the presentation of antigenic peptide and enhances T cell responses.

One major problem with pathogenic peptides is that these peptides vary among different pathogenic strains making them non-immunogenic. Hence it becomes crucial to identify the peptide sequence which is invariable among pathogenic strains so that those peptides can be further modified and employed as peptide vaccines. This approach of vaccine development is under investigation in highly variable pathogens such as influenza, HIV, HepC, and P. falciparum.

Rather peptide vaccines have been developed in three therapeutic areas: infectious disease, Alzheimer's disease, and cancer [15].

Advantages
- Molecular details of the peptide vaccine are known, hence there are very thin chances of pathogenic reversion.
- As peptides are small in size, hence there is possibility of contamination by pathogenic fragments and side effects.
- As peptides are small molecules, these can be introduced into various delivery vehicles such as nanoparticles.
- Peptide vaccines are very stable.

Disadvantages
- As peptides are small and perceived as hapten, they represent small non-conformation antigenic determinants, hence may not be recognized by peptide specific B cells.
- Small peptides are non-immunogenic, hence require conjugation with carrier proteins.
- Due to the small size the peptide vaccines are more likely to get wasted in tissues more readily than the bigger sized proteins. This loss of vaccine peptide may be so fast that immune response is not generated. In such cases the peptide vaccine is given with adjuvant to increase the protection against degradation.

10.2.2 Subunit Vaccines

To avoid the complications of reversions and potential side effects that result when whole organism is used in vaccine, designing vaccines using particular part of the pathogen can be advantageous. The idea is to use the immunogenic part of pathogen. This kind of vaccine is known as subunit vaccine. The component of pathogen that acts as vaccine must possess the epitopes so that its immunogenicity remains intact.

10.2.2.1 Viral Subunit Vaccine

One of the primitive efforts to develop subunit vaccine involved the purification of surface protein of Hep B virus known as HBsAg with the rationale being that this purified protein induced production of complimentary antibodies upon immunization of the host. However, the process of purification of the immunogenic subunit is quite laborious if sufficient amount is to be purified. In this situation recombinant DNA technology proved to be a bonus in case Hep B and Hep C viruses which are difficult to be grown in vitro and to purify sufficient amount of protein is a challenge. This technology also allowed the researchers to modify the desired protein with specific intentions. Modification involved the fusion of the viral protein with gene inserts that allow the better and probe based purification of the expressed proteins. The gene of interest in natural or modified form is cloned into a suitable expression host in large quantities. In recombinant DNA technology, to generate a subunit vaccine, the DNA fragment that encodes the viral subunit is into the genome of a suitable host such as Saccharomyces cerevisiae, *Bacillus subtilis*, or *Escherichia coli*. These host microorganisms can be induced to overexpress the gene insert corresponding to the viral polypeptide specifically and the overexpressed protein in bulk can be isolated in sufficient quantities without any complication of co-expressed proteins as only the desired gene is induced for overexpression. The recombinant HBsAg vaccine against HepB virus was developed using this method. The HBsAg gene was cloned into the genome of Yeast host. The HBsAg gene is expressed and released into the cytosol of the yeast cells which makes it easier to purify. The influenza vaccine was also developed using same strategy. Hemagglutinin protein of Influenza virus is used as vaccine against influenza. Similarly, various vaccines for veterinary use were developed for use in protecting livestock against diseases like foot and mouth disease.

However, in bacteria the immunogenic molecules are polysaccharides such as lipopolysaccharides rather than proteins. Such macromolecules are not immunogenic in isolated form, hence are needed to be conjugated with carrier proteins. As an example, Haemophilus Influenzae Type B (HiB) and pneumococcus bacteria are enclosed by polysaccharide capsule and these polysaccharides are responsible for pathogenicity of these bacteria. The polysaccharide available on the surface of the bacteria is easily accessible for isolation and purification. The polysaccharide antigens act as Thymus independent antigens (Ti antigen) which induce the IgM production from B cells in the T cells independent manner [16] and hence incapable of generating T cell response and memory cells. Furthermore, many polysaccharides do not produce any response at all in children below 2 years of age. This issue can be tackled by conjugating the polysaccharide with protein to alleviate this non-immunogenic nature of polysaccharides. Conjugated carrier protein provides the T cell epitopes to generate the T cell response. The vaccine such produced has better efficiency and is named as conjugate vaccine. Examples of such carrier proteins are: diphtheria, tetanus toxoids, their modified forms, outer membrane proteins (OMPs) from different bacteria and bovine serum albumin (BSA), core antigen of HepB virus (HBcAg), and the nucleoprotein of influenza virus (NP).

One of the best examples of the conjugate protein is Haemophilus Influenzae vaccine Hib for infants. This vaccine consists of Hib polysaccharide conjugated with Diphtheria toxoid.

With the advancing research development of novel multifunctional carriers and hence the possibilities for the development of new novel vaccines have increased to a great extent. These novel carries could be nano-carriers including liposomes, polymeric nanoparticles, and inorganic nanoparticles [17].

Advantages

- One of the major advantages of recombinant subunit vaccines is that the production process is less laborious and cost-effective.
- Subunit vaccines pose less side-effect as purified immunogenic component is employed.
- Can be used for patients with immunocompromised conditions such as cancer, autoimmune diseases.
- Does not induce the development of diseased state [17].

Disadvantages

- Native conformation of epitopes may get altered in the process of cloning and overexpression, hence some of the antibodies formed might not induce the immune response.
- The antigenic polysaccharides with glycosylated residues cannot be processed in this process as glycosylation does not occur in bacterial hosts. For the proteins that require post-translational modifications such as glycosylation the hosts like yeast, plant, and animal cells are used [17].

10.3 Applications of Recombinant Antibodies, Enzymes, and Hormones in Therapy and Treatment

10.3.1 Recombinant Antibodies

Antibodies constitute the most rapidly growing class of human therapeutics and the second largest class of drugs after vaccines [18]. The term monoclonal antibody refers to a single specificity antibody derived from a single B cell clone and initially these were created by fusing B cells (from immunized mice) with lymphoma cells. In clinical practice, however, the administration of murine antibodies induces human anti-mouse antibodies that may lead to allergic reactions and reduced efficacy. Repeated administration of murine antibodies can cause human anti-mouse antibody reaction (HAMA), reducing antibody half-life and has severe side effects including anaphylactic shock [19]. These difficulties have been partially overcome by recombinant technology to develop less immunogenic monoclonal antibodies [20]. The use of a number of molecular biology techniques, mostly recombinant DNA technology, and the increased understanding of the antibody structure and

function led to the development of chimeric and humanized mAbs [21]. Techniques like phage display techniques, generation of transgenic animals, allowed the development of fully human antibodies. Antibody phage display is an in vitro technology to generate recombinant antibodies. In particular for pathogens like viruses or toxins, antibody phage display is an alternative to hybridoma technology, since it circumvents the limitations of the immune system [22]. Nowadays, more IgG based biotherapeutic agents have achieved an obvious efficacy in the treatment of various diseases including cancer, infections, immune disorders.

10.3.1.1 Cancer Treatments

Antibody-based therapeutics against cancer are highly successful and currently enjoy unprecedented recognition of their potential [23]. Bevacizumab (Avastin), an anti-vascular endothelial growth factor antibody, and cetuximab (Erbitux), an anti-epidermal growth factor antibody and anti-HER2/neu antibody trastuzumab (Herceptin) are the approved monoclonal antibodies for treating tumor malignancies in combination with standard chemotherapy regimens. Bevacizumab is significantly known to prolong the survival of patients with metastatic cancers of the colorectum, breast, and lung [24].

10.3.1.2 Inflammation and Airway Diseases

Monoclonal antibodies are considered as a treatment option for respiratory diseases like asthma. Omalizumab, a humanized IgG1/k monoclonal antibody, targets the Fc region of IgE, and by binding to free IgE in blood and body fluids, it neutralizes the ability of IgE to bind to its receptor (FcεRI, high-affinity receptor and FcεRII, low-affinity receptor). On top of inhibiting the cross-linking on mast cells, this induces the downregulation of IgE receptor expression on other immune cells such as basophils and dendritic cells [25]. Omalizumab was the first biological therapy developed for asthma. Mepolizumab is a humanized IgG1/k monoclonal antibody toward IL-5, binding to it with high affinity and preventing its linkage to IL-5Rα. Reslizumab is a humanized IgG4/κ monoclonal antibody specifically interacting with the epitope IL-5 uses to bind its receptor IL-5Ra, thereby blocking its bioactivity [25].

IL-4 and IL-13 can both induce Th2 cells and epithelial cells to produce eosinophil-promoting factors (i.e., IL-5 and eotaxins) and stimulate eosinophils to migrate to sites of inflammation from blood. Dupilumab is a fully human monoclonal antibody to the interleukin-4 receptor α subunit, IL-4R alpha, which is utilized by two cytokines IL-4 and IL-13 [26].

10.3.1.3 Pathogenic Infections and Toxins

The Centers for Disease Control and Prevention (CDC) classifies several bacterial toxins or the pathogens producing these toxins as category A or B agent as they are a high risk to public health. Neutralizing antibodies block the interaction of the toxin to its cellular target, by binding to the cell binding domain of the toxin. Neutralization of the toxicity is also possible by antibodies directed against other domains like the translocation domain or the enzymatic domain.

The pathogenesis of anthrax is closely associated with the bacterium's lethal (LT) and edema (ET) toxins. Raxibacumab was the first anthrax anti-toxin agent approved by the FDA, followed by Anthrax Immune Globulin Intravenous (AIGIV) and then ETI-204. Each agent is directed at the component of LT and ET, and all three agents were reported to be effective when compared to placebo controls in antibiotic-treated animal models of live *B. anthracis* infection. Raxibacumab is a recombinant, fully human, IgG1λ monoclonal antibody against B. anthracis PA [27, 28]. This is derived from a phage display library from Cambridge Antibody Technology (now Medimmune) [29].

Palivizumab are the recombinant antibodies that are approved for the treatment of viral infections and toxins. Palivizumab for the treatment of respiratory syncytial virus (RSV) bronchiolitis is a classical humanized antibody [30].

10.3.2 Recombinant Enzymes

Enzymes are used as anti-inflammatory agents, digestive aids, mucolytics, thrombolytics, anticoagulants, oncolytics, and antimicrobials for the clinical management of several disorders [31].

Pancreatic exocrine insufficiency (PEI) is one of the well-known causes of malabsorption syndrome. An insufficient secretion of pancreatic enzymes and bicarbonate secondary to different pancreatic diseases and upper gastrointestinal and pancreatic surgery leads to maldigestion and malabsorption of nutrients. Together with symptoms of maldigestion, PEI is associated with nutritional deficiencies leading to osteoporosis, low-trauma fractures, sarcopenia, and increased mortality [32]. Oral pancreatic enzyme replacement therapy (PERT) is the mainstay of treatment for EPI. Pancreatic enzyme replacement therapy (PERT) improved pancreatic exocrine insufficiency (PEI). PEI-related malabsorption and weight maintenance in patients with cystic fibrosis, chronic pancreatitis, pancreatic cancer, and post-surgical states. In patients with chronic pancreatitis, PERT improved PEI-related symptoms and quality of life measures.

Severe cardiovascular disease conditions lead to acute myocardial infarction and stroke. One of the reasons for this is formation of blood clots inside the vessel. Thrombolytic therapy plays a pivotal role in cardiovascular disease management. An effective therapy should be based on rapid clot dissolution so as to minimize tissue damage and boost survival rates [33]. Thrombolytic enzymes have become

more substantial for treating cardiovascular diseases since they could lyse the fibrin clot within the blood vessel [34]. Treatment of acute myocardial infarction by intra-coronary perfusion of thrombolytic enzymes can improve both cardiac function and long-term survival [35].

Gaucher disease (GD) is an autosomal recessive lysosomal storage disease, caused by deficiency of the enzyme glucocerebrosidase, required for the degradation of glycosphingolipids [36]. Enzyme replacement therapy with recombinant GBA is the mainstay of treatment for GD, which became the first successfully managed lipid storage disease [37]. In cancer chemotherapy, L-asparaginase has long been a useful adjunct in the treatment of acute lymphoblastic leukemia [35].

10.3.3 Recombinant Hormones

Hormones are biological substances which are secreted by cells in very small quantities in response to particular stimuli and they exert their function by "*messaging target cells*" to carry out or stop a particular function. For example, the hormone insulin is secreted by β-cells of the pancreatic islets as a result of increase in glucose levels of the blood. This insulin travels through the blood to its target cells (liver cells) to covert the excessive glucose into glycogen for use as a fuel for later use and regulate the levels of free glucose in the blood. By their chemical nature, hormones can be peptides (made of a few amino acids) like oxytocin (birth hormone), proteins like insulin, or lipids like cortisol and testosterone. Most of the hormones utilized for therapeutic purposes were derived from animals in earlier days. Recombinant hormones are synthesized by cloning the sequences coding for the hormone (in case of peptide or protein hormones) and expressing it in a heterologous system like bacteria. This is done in order to produce the hormone at a massive scale without the need for sacrificing the animals. This technology also offers the advantage of using the hormone with the exact same sequence as that produced in the recipient organism. This prevents the necessity of utilizing closely related animals for obtaining the hormone which generally have slight differences in the primary amino acid sequence. It also eliminates the chances of adverse immune reactions. More details on this recombinant human insulin can be found in Chap. 1, Sect. 1.1 of this book.

10.3.4 Summary

Various recombinant therapeutic proteins have been approved for the treatment of a variety of diseases. As an adjunct to standard treatments, they have proved to be useful in patients with more severe disease. Although the clinical benefit of the antibody has been demonstrated in numerous studies, other factors like secondary complications, cost-effectiveness have to be considered. Currently, therapeutic proteins are being gradually improved for efficacy, safety, quality, and cost and new

targets are being explored, but there are no new concepts similar to those leading to the development of recombinant proteins and identification of unique high-affinity binders out of billions of different molecules [21].

Immunogenicity of mAbs has implications for their pharmacokinetics and safety. Early studies of mAbs in humans require careful consideration of the most suitable study population, route/s of administration, starting dose, study design, and the potential difference in pharmacokinetics in healthy subjects compared to patients expressing the target antigen [38]. Hundreds of mAbs, including bispecific mAbs and multispecific fusion proteins, mAbs conjugated with small-molecule drugs, and mAbs with optimized pharmacokinetics, are in clinical trials [23].

Although these exciting data from clinical trials provide optimism for the development of various therapeutic recombinant proteins, a deeper understanding of mechanisms is necessary to overcome major problems including resistance to therapy, access to targets, and individual variations.

10.4 Heterologous Protein Expression in Diagnostics and Therapeutics

Heterologous expression is the ligation of either complementary DNA (cDNA) or RNA (cRNA) which codes for a particular protein from one species into the cell of another species so that the hosts' cellular machinery is used as a factory to expresses the added foreign protein. Immortalized cells that are cultured in the laboratory can be transfected with cDNA stably or transiently, depending on how the foreign DNA is integrated into the host genome. Stable DNA expression allows permanent overexpression of the foreign protein, whereas transient DNA expression lasts only for about 24–72 h.

Recent developments in the field of genetic engineering have made possible the production of therapeutics and vaccines for humans and animals in the form of recombinant proteins [39, 40]. With the dawn of our ability to clone and express foreign genes in the heterologous host came a remarkable capability to make almost any protein in abundant quantity to be used as therapeutic or diagnostic agents. It led to the finding that proteins expressed in different hosts are different in many ways, especially in their post-translation modifications. Various types of cells derived from various species have been used to express these foreign proteins. Some of the most common examples are frogs (Xenopus laevis), insects (Spodoptera frugiperda), bacteria (Escherichia coli), and yeast (Saccharomyces cerevisiae). But for many proteins which require proper post-translational modifications like membrane proteins, e.g., transporters and channels, mammalian host cells are appropriate. Post-translational modifications which can fully occur in mammalian cells include glycosylation, deamidation, isoprenylation. A number of mammalian cell lines have been used for heterologous expression of membrane proteins: the HEK293 cell line generated by transformation of normal human embryonic kidney cells with adenoviral DNA [41], the CHO cell line from the ovaries of the Chinese hamster

(Cricetulus griseus) [42], and the COS-7 cell line which is derived from the kidney of the African green monkey (Cercopithecus aethiops) [43]. One of the major obstacles in this field is the production of a large number of recombinant proteins in both the medical field and research. Therefore, researchers apply both prokaryotic and eukaryotic cell systems to overcome the problems associated with the production of these recombinant proteins [44].

These genetically engineered heterologous recombinant proteins form a new class of drugs that can be used for many diseases like cancer, hypertension, genetic disorders, and AIDS for which we have no possible treatment or cure. These heterologous proteins have the advantage that they are our own molecules, unlike chemical drugs, and hence more suitable with our biological systems. Expression systems for the production of heterologous proteins are discussed in next section [40].

10.4.1 Expression Systems

Currently, expression systems that are used for the production of heterologous proteins are bacteria, yeasts, molds, mammals, plants, and insects. Prokaryotic cells such as bacteria were first to be exploited for the purpose of genetic engineering technology. The most common member used for cloning recombinant DNA and subsequently, for the production of heterologous proteins was Escherichia coli [45]. Bacterial expression system has several advantages which include rapid multiplication, high-level expression, simple and inexpensive nutritional requirements, and a fast and easy transformation process. However, it has some limitations as well such as misfolding of heterologous proteins and intracellular aggregation, lack of post-translational modification, and protein degradation due to proteases [46].

Another part of expression systems is the eukaryotic cells which include yeast and mammalian cells. The most common mammalian cell lines are Chinese hamster ovary (CHO) cells, which are currently used to produce biopharmaceutical compounds, monoclonal antibodies, and Fc-fusion proteins. In addition to this, baby hamster kidney, human embryonic kidney 293 and NS0, SP2/0 (mouse-derived myeloma) cell lines are also in practice [47]. The major advantage of the mammalian system is that this system includes proper protein folding, post-translational modifications, and glycosylation of recombinant proteins in the correct sites which is important for protein stability [48]. Whereas the major drawback of the mammalian expression systems is that it grows slowly and the relevant nutrient requirement is costly.

Other eukaryotic cells that are widely used for the expression of various proteins in vaccine and pharmaceutical products are the yeast cells. The mechanism of protein expression in these microorganisms is very closely associated with those in mammalian cells. As compared to bacteria, yeast cells offer some major advantages that include post-translational modification, growth speed, secretory expression, and straightforward genetic manipulation. In addition to this linearized foreign DNA can be inserted into the host chromosome with high efficiency via cross

Table 10.1 Basic characteristics of different systems for the expression of heterologous proteins

		Heterologous expression system		
S. No.	Specific feature	*Pichia pastoris*	*Escherichia coli*	CHO cells
1.	Doubling time	60–120 min	30 min	24 h
2.	Cost of growth medium	Low	Low	High
3.	Complexity of growth medium	Minimum	Minimum	Complex
4.	Expression level	Low to high	High	Low to moderate
5.	Extracellular expression (secretion of expressed, heterologous protein)	Secretion occurs into medium	Secretion occurs into periplasm	Secretion occurs into medium
6.	Protein folding	Refolding may be required	Refolding usually required	Proper folding
7.	Post-translational modifications	Yes	No	Yes

recombination phenomena to get stable cell lines for extended expression of proteins [49]. The most common yeast which has been used for the production of hepatitis B and human papillomavirus vaccines, both of which produce a protective immune response against wild-type viruses is Saccharomyces cerevisiae [50]. The expression proteins produced in S. cerevisiae are often N and O-hyperglycosylated, which can affect protein immunogenicity [51]. Recently, other methylotrophic yeasts like Hansenula polymorpha and Pichia pastoris (P. pastoris; syn. Komagataella phaffii) are developed for various protein expressions. Among these, P. pastoris has become the foremost popular for its cost and expression host system. This yeast can produce high yields of heterologous proteins with the high similarity of glycosylation thereto of occurring in mammalian cell [52].

The basic characteristics of various host systems for the expression of heterologous proteins are summarized in Table 10.1.

10.4.2 Heterologous Protein Production in the Yeast Pichia Pastoris (Tables 10.2, 10.3, 10.4, and 10.5)

10.4.3 Heterologous Protein Production in the Yeast Kluyveromyces lactis

Kluyveromyces lactis is both scientifically and biotechnologically one of the most important non-Saccharomyces yeasts. Its biotechnological significance builds on its history of safe use in the food industry and its well-known ability to produce enzymes like lactase and bovine chymosin on an industrial scale [53].

Table 10.2 Common *Pichia pastoris* expression vectors for the production of secretory proteins

S. No.	Vector	Strain	Marker gene	Recombinant protein
1.	**pJL-SX**	MS105	*FLD1*, *Amp*	Formaldehyde dehydrogenase
2.	**pBLHIS-SX**	JC100	*His4*, *Amp*	Leukocyte protease inhibitor
3.	**pPIC9K**	GS115	*His4*, *Kan*, *Amp*	Xylanase
				Staphylokinase
				Porcine circovirus type 2
				Endo-1,3(4)-b-D-glucanase
4.	**pPICZα**	GS115	*Shble*	Human interferon gamma
		SMD1168		Human chitinase
		GS115		Human topoisomerase I
		X-33		C-reactive protein
		SuperMan5		Insulin
5.	**pHIL-S1**	KM71	*His4*, *Amp*	Camel lactoferricin
		GS115		Rabies virus glycoprotein
		GS115		*Rhizopus oryzae* lipase
6.	**pGAPZα**	X-33	*Shble*	Human gastric lipase
		GS115		Acyl homoserine lactonase
		SMD1168		Variable lymphocyte receptor B

Table 10.3 Common *Pichia pastoris* expression vectors for the production of intracellular proteins

S. No.	Vector	Marker gene	Strain	Recombinant protein
1.	**pJL-IX**	*FLD1*, *Amp*	MS105	Formaldehyde dehydrogenase
2.	**pBLHIS-IX**	*His4*, *Amp*	KM71	L1–L2 proteins of HPV virus type 16
3.	**pPICZ**	*Shble*	X-33	Aquaporin
			KM71	Membrane protein
			KM71	Dengue virus envelope glycoprotein
4.	**pGAPZ**	*Shble*	GS115	GTPase RabA4c
			GS115	Xylose isomerase
			GS115	β-Galactosidase
5.	**pHIL-D2**	*His4*, *Amp*	GS115	Prostaglandin H synthase-2
			GS115	CatA1 and SODC
			KM71	*Rhodococcus* nitrile hydratase
			GS115	Feline serum albumin

Kluyveromyces lactis is both scientifically and biotechnologically one of the most important non-Saccharomyces yeasts. Its biotechnological significance builds on its history of safe use in the food industry and its well-known ability to produce enzymes like lactase and bovine chymosin on an industrial scale.

Table 10.4 Common *Pichia pastoris* expression vectors for the production of recombinant subunit vaccines

S. No.	Vector	Construct	Strain	Targeted disease
1.	**pPICZαA**	Gp350	GS115	EBV infection
		PIMP-V1 and PIMP-V2	KM71	Malaria
		RBD219-N1	X-33	SARS
		F protein	GS115	Newcastle
		Tc52	GS115	Chagas
		CFP10-Fcγ2a	GS115	Tuberculosis
		ESAT6-CFP10-Fcγ2a	GS115	Tuberculosis
		ESAT6-Fcγ2a	GS115	Tuberculosis
		CFP10-HspX-Fcγ2a	GS115	Tuberculosis
		ESAT6-HspX-Fcγ2a	GS115	Tuberculosis
2.	**pPIC9K**	VP2–VP5–Fc	GS115	Infectious bursal
		CHIKV-C-E3-E2-6K-E1	GS115	Chikungunya
		OmpA	GS115	*P. mirabilis* infection
		Apa	GS115	Tuberculosis
		HBHA	GS115	Tuberculosis
		Glycoprotein D	GS115	HSV-2 infection
		OmpA-Fc	GS115	Bordetellosis
3.	**pPICZ-A**	DENV-3 E	KM71	Dengue
		BoNT Hc	X-33	Botulism
4.	**pPink-HC**	P1-3CD	PichiaPink	Hand–foot–mouth disease

Table 10.5 Common *Pichia pastoris* expression vectors for the production of recombinant biological molecules

S. No.	Vector	Strain	Product	Usage
1.	**pPICZαA**	X-33	Plectasin	Antibacterial peptide
		KM71H	Class I chitinase	Antifungal peptide
		GS115	Hispidalin	Antimicrobial peptide
		X-33	CecropinA-thanatin	Antimicrobial peptide
		X-33	Goat chymosin	Hydrolysis of κ-casein
		SuperMan5	Proinsulin	Treatment of diabetes mellitus
		X-33	IL-3	Multipotent hematopoietic cytokine
		X-33	IL-15	Differentiation and proliferation of T, B, and NK cells
		GS115	Cyanate hydratase	Detoxification of cyanate and cyanide
		X-33	Human antiplatelet scFv antibody	Treatment of atherosclerosis
		X-33	α-Amylase	Starch saccharification
		KM71H	Bromelain	Edematous swellings
		X-33	Keratinocyte growth factor	Epithelialization phase of wound healing
		X-33	Streptokinase	Thrombolytic medication
		X-33	TFPR1	Adjuvant

Table 10.5 (continued)

S. No.	Vector	Strain	Product	Usage
2.	**pPIC9K**	GS115	Bovine IFN-α	Prevention and therapy of viral diseases
		SMD1168	Apidaecin	Antibacterial peptide
		GS115	hPAB-β	Antibacterial peptide
		GS115	*Pisum sativum* defensin 1	Antifungal peptide
		KM71H	Ch-penaeidin	Antimicrobial peptide
		GS115	Human serum albumin	Maintaining osmolarity and carrier in blood
3.	**pGAPZA**	X-33	Lycopene and β-carotene	Feed supplements
		X-33	PAF102	Antifungal peptide
4.	**pJ902**	KM71H	Bovine lactoferrin	Transferrin and antibacterial protein
5.	**pGAPZαB**	GS115	Tachyplesin I	Antibacterial peptide

The yeast Kluyveromyces lactis has been studied for decades and has a well-established track record of safe use in various food industry applications [53]. Since the 1950s, K. lactis has been used as a source of lactase (β-galactosidase), an enzyme that degrades milk sugar (lactose) and is necessary for production of lactose-free dairy products. Dried and inactivated K. lactis was also used as a protein supplement in food in the 1960s. In the 1980s, K. lactis was one of the first yeasts for which a transformation system was established [54], leading to its development as an efficient host for heterologous protein expression. As an expression host, K. lactis is best known for its use in commercial production of the milk clotting enzyme bovine chymosin [55]. This protein was the first heterologous enzyme originating from a higher eukaryote that was produced at low cost in a microorganism, and the process developed for its industrial-scale production was widely recognized as a major biotechnological achievement. In 1993, Swinkels et al. [56] first reviewed the use of K. lactis as a host for heterologous protein expression. At that time, eight proteins had been successfully secreted from K. lactis. Today, over 40 proteins have been produced with K. lactis, illustrating its utility as an alternative yeast expression system (Table 10.1). These proteins originate from bacteria, fungi, viruses, plants, and mammals, emphasizing the ability of K. lactis to efficiently produce a diverse range of heterologous proteins.

10.5 Utilization of Single Cell Organisms, Plants, and Animals for Production of Therapeutics and Nutraceuticals

Nutraceutical is a term derived from nutrition and pharmaceutical, and are sometimes termed “functional foods” [57]. Nutraceuticals have received high interests due to their potential nutritional and safety profile, other than therapeutic capability

[58]. Nutraceuticals have the ability to control the DNA damaging factors in cancer cells and regulate DNA transcription in tumors. Nutraceuticals help in combating some of the major health problems such as obesity, cardiovascular diseases, cancer, osteoporosis, arthritis, diabetes, cholesterol, etc. [58–60, 62].

The food products used as nutraceuticals can be categorized as dietary fiber, prebiotics, probiotics, polyunsaturated fatty acids, antioxidants, and other different types of herbal/natural foods [62]. Nutraceuticals are found in a mosaic of products emerging from (a) the food industry, (b) the herbal and dietary supplement market, (c) pharmaceutical industry, and (d) the newly merged pharmaceutical/agribusiness/nutrition conglomerates. It may range from isolated nutrients, herbal products, dietary supplements, and diets to genetically engineered "designer" foods and processed products such as cereals, soups, and beverages [61, 63].

Nutraceuticals are derived from various natural sources such as medicinal plants, marine organisms, vegetables, and fruits [59].

10.5.1 Nutraceutical from Microorganisms

Direct extraction strategies are limited by the availability and cost of raw materials, the quality control of supplies, and the low content and purity of nutraceuticals. To address these issues, microbial-based metabolic engineering is an appealing approach and has achieved great progress on production of value-added nutraceuticals in very recent years.

The rapid elucidation of biosynthetic pathways for natural products and the genetic amenability of microorganisms have enabled the development of microbial hosts for production of various nutraceuticals. Microbial platforms like the most widely used Escherichia coli and Saccharomyces cerevisiae have been engineered as versatile cell factories for production of diverse and complex value-added chemicals such as phytochemicals, prebiotics, polysaccharides, and poly amino acids [64]. Some of the biggest advantages of E. coli include its fast growth and ease of genetic manipulation; for S. cerevisiae its Generally Regarded As Safe (GRAS) status and its ability to functionally express plant metabolic enzymes [64]. Due to this, these organisms have generally been developed as platform organisms for de novo or semi-de novo production of almost all kinds of nutraceutical compounds (Table 10.6).

10.5.2 Nutraceutical from Plants

Plants are a rich source of bioactive compounds to maintain or improve human health nutraceuticals, phytonutrients/secondary metabolites [65]. However, the presence of intrinsically low levels of the beneficial phytonutrients in the available genotypes of crop plants is not always at par with the recommended daily allowance

Table 10.6 List of nutraceuticals/compounds produced by engineered heterologous hosts

S. No	Compounds	Hosts
1.	**Terpenoids:** Lycopene, b-Carotene, Zeaxanthin, Astaxanthin	*E. coli*
2.	**Prebiotics:** Galactooligosaccharides, 2-Fucosyllactose	*Lactococcus lactis*, *E. coli*
3.	**Flavonoid derivatives:** Sakuranetin, Kaempferol 3-O-glucoside, Anthocyanin, Daidzin, 3-Hydroxydaidzein	*E. coli*, *S. cerevisiae*
4.	**Polyphenolic compounds:** Apigenin (Flavone), Chrysin (Flavone), Pinocembrin (Flavanone), Naringenin (Flavanone), Curcumin (Curcuminoid), Caffeic acid	*E. coli*, *S. cerevisiae*
5.	**Poly amino acids:** Poly-e-L-lysine, Cyanophycin, Poly-g-glutamic acid	*Streptomyces* sp. M-Z18, *Bacillus subtilis* BL53, *E. coli*
6	**Polysaccharides:** Heparosan, Hyaluronan, Chondroitin	*Streptomyces albulus*, *Sclerotium rolfsii*, *E. coli*
7	**Alkaloids:** Indolylglucosinolate (Glucosinolate), (S)-reticuline (Benzylisoquinoline alkaloid)	*E. coli*, *S. cerevisiae*

(RDA) for different phytonutrients (nutraceuticals). Molecular engineering of crop plants has offered a number of tools to markedly enhance intracellular concentrations of some of the beneficial nutrients, levels that, in some cases, are closer to the RDA threshold [66]. Some of these include increases in: vitamin C in corn, protein level in potato, lysine in corn and rice, folates in rice, corn, tomato, etc.

The boom in plant biotechnology at the end of the last century has created the possibility to engineer plants to manufacture new compounds, including small molecules and biologics that originate from non-plant sources [67, 68].

10.5.3 Nutraceuticals from Animals

10.5.3.1 Live Biotherapeutic Products (LBP)

Live biotherapeutic products (LBPs) are defined as live organisms designed and developed to treat, cure, or prevent a disease or condition in humans. LBPs can include genetically modified organisms (recombinant LBPs) if they have been engineered by adding, deleting, or altering genetic material within the organism [69, 70].

LBPs exclude vaccines, filterable viruses, oncolytic viruses, and organisms used as vectors for transferring genes into the host. Most probiotics are regulated as dietary supplements and cannot make claims to treat or prevent disease [71, 72]. While there are many probiotics approved as nutritional supplements and some engineered bacterial strains have been studied in the clinic but the FDA has not approved a live biotherapeutic product for medicinal use to date [70].

Table 10.7 List of some of the chassis organisms that are engineered and used in clinical development for live bacterial therapeutics (LBT)

S. No.	Organism	Therapeutic indication
1.	*Listeria monocytogenes*	Non-small cell lung cancer
2.	*Lactococcus lactis*	Gastrointestinal inflammation in primary immunodeficiency
3.	*Escherichia coli*	Phenylketonuria (PKU)
4.	*Escherichia coli*	Solid tumors

Escherichia coli Nissle 1917 (EcN) has been used as a probiotic since its isolation over 100 years ago. In its unengineered form, EcN has been used to treat various gastrointestinal conditions, including inflammatory bowel disease and irritable bowel syndrome. EcN is believed to impede the growth of opportunistic pathogens, including Salmonella spp. and other coliform enteropathogens, through the production of microcin proteins or production of iron-scavenging siderophores [70, 73, 74]. Recently many studies have reported engineering EcN to respond to the environment within the human gastrointestinal tract. Various genetically modified EcN strains have been developed as intestinal-acting antimicrobial agents and evaluated in pre-clinical models [75] (Table 10.7).

10.6 Molecular Markers and Their Role in Disease Diagnosis

10.6.1 Introduction

Any biological substance such as a nucleotide sequence or protein which is specifically associated with and exclusively expressed in a diseased state (pathophysiology) is referred to as a molecular marker. These molecular markers help in early identification of a disease, assessing disease severity, monitoring disease progression, or may even act as a prognosis marker for the patients undergoing treatment therapies. Most of the molecular markers can easily be isolated from blood, hence they act as a better alternative to the invasive methods widely being used for disease identification.

Broadly, molecular markers can be classified as either biochemical or molecular markers. Biochemical markers are generally protein markers which are characterized by the change in amino acid sequence, one such most important protein being alloenzyme, identified through techniques such as isozymes or protein banding pattern. Conversely, variations or polymorphisms in the DNA sequence are identified through RFLP, SNP, AFLP and such markers are known as molecular markers.

Adequately validated biomarkers can contribute to improve patient care by [76] facilitating early detection of subclinical disease, [77] improving the diagnosis of acute or chronic syndromes, [78] stratifying patients' risk, [79] selecting the most appropriate therapy for a given patient, and/or [80] monitoring disease progression and response to therapy.

10.6.2 Molecular Markers in Cancer

10.6.2.1 Current Cancer Markers

Cancer, i.e., uncontrolled proliferation of cells, is one of the biggest cause of mortalities observed worldwide. The major shortcomings of such cancers are their poor prognosis. With the advancements of molecular research in cancer, new molecular markers associated with cancers are emerging, which besides being acting as a marker for presence or absence of cancer can also be used as a prognosis marker. Though molecular markers are associated with a number of cancers now, but only few such markers act as biological markers. However, extensive research in this field is still going on [76]. Few examples where molecular markers are currently being used in cancer are discussed below.

Nucleic-acid-based markers Since DNA can be easily isolated from various body fluids, it has been used to analyze tumor markers such as oncogene mutations, microsatellite instability, hypermethylation of promoter regions, and viral DNA in cancer patients. Polymerase chain reaction (PCR) is one of the most sensitive techniques used for the identification of DNA mutations associated with cancer [77, 78]. Tp53 mutations were identified in the urine of bladder cancer patients.

DNA methylation In this approach, hypermethylated promoter regions of cancer-associated genes are identified. Since hypermethylation can completely block transcription [79], hence most of the cancers use this mechanism to inhibit translation of tumor suppresser genes. CpG islands are methylated in promoter regions, which can be rapidly and accurately detected using PCR assays [79]. In this procedure, DNA is treated with bisulfite, which converts unmethylated cytosines to uracil, whereas methylated cytosines are protected and not converted. This approach has been used to detect cancer cells in the saliva of patients with oral cancer [80], in the sputum and Bronchoalveolar Lavage Fluid of patients with lung cancer [81–83] and in the serum of patients with lung [84], head and neck, and colorectal cancers [85]. For example, methylation of genes that encode INK4A (also known as p16; a cyclin-dependent kinase inhibitor that is encoded by CDKN2A), DAPK (death-associated protein kinase, which is involved in resistance to apoptosis), and MGMT (methyl *O*-guanine methyltransferase, which is involved in DNA repair) has been associated with lung, and head and neck cancer. These genes were found to be methylated in serum samples of over 50% of lung, and head and neck cancer patient [84, 86]. Similarly, methylation of APC, which is involved in WNT signaling, was observed in the serum and plasma DNA of early-stage lung cancer and esophageal cancer patients [87]. The list of cancer-associated methylated genes is constantly expanding [83].

Other example includes methylation of glutathione-*S*-transferase placental enzyme 1 (GSTP1), which was found to be elevated in prostate cancer cells compared with normal tissue.

Viral DNA Few viral DNAs are also now used as molecular markers for virus-associated cancers. For example, the human papillomavirus (HPV) has been associated with cervical carcinoma, so testing for the presence of HPV DNA is a useful approach for identifying women who are at risk of developing this cancer [88, 89].

RNA-based approaches Several mRNA-based approaches have also been developed to detect cancer cells in clinical samples. Reverse transcriptase-PCR (RT-PCR) is among the most common approaches that is used to identify and quantify mRNA levels in tissue samples [90, 91]. For instance, RT-PCR is clinically used to quantify mRNA levels of tyrosinase, a marker expressed only in benign moles and melanoma cells and its presence in serum can be indicative of cancer [92, 93]. Similarly, cytokeratin mRNA is a common marker of epithelial cells, so its detection can also indicate the presence of cancer cells. Elevated CEA levels, indicative of colorectal cancer, high PSA, indicative of prostate cancer are also detected using RT-PCR assays.

Protein markers Several protein-based assays have also been developed to detect cancer cells. This approach is important, because post-translational protein modifications, some of which are cancer-cell specific, cannot be determined from genomic information. Protein-based assays typically detect proteins that are overexpressed or structurally altered in cancer cells, compared with normal cells. However, these approaches are generally not suitable for larger clinical studies yet. One of the most common protein-based screens for cancer is to measure serum levels of PSA, as high serum levels of this protein are associated with prostate cancer. PSA levels, however, cannot be used as a sole indicator of cancer, and results must be correlated with other clinical factors as well. Certain protein markers can be used to assess therapeutic response and to track reoccurrence of the disease. Examples include increased expression of oncofetal proteins such as the human glycoprotein hormone (β-HCG) and α-fetoprotein (AFP) for testicular cancer and hepatocellular cancer, CEA for colon cancer progression. In fact CEA, a protein usually restricted to fetal development, was one of the first serum based marker used for colorectal cancer progression [94]. Other biomarkers, such as PSA for prostate cancer and cancer antigen (CA)125 for ovarian cancer, are also extensively used [95–98]. Telomerase—a ribonucleoprotein that is involved in telomere maintenance—represents a promising molecular marker for cancer [99–101].

Some of the sophisticated techniques for protein marker identification like serial analysis of gene expression (SAGE) for pancreatic cancer marker—mesothelin, matrix-assisted laser-desorption-ionization time-off light (MALDI-TOF) mass spectroscopy or liquid chromatography–ion-spray tandem mass spectroscopy (LC-MS/MS) have revolutionized protein analysis. Bioinformatic technologies are also used along with to identify cancer-specific proteomic patterns and to detect cancer in other samples. So, new high-throughput techniques are necessary not only to identify new markers, but also to analyze known markers in a cost-effective fashion [102, 103].

Molecular Markers in Upper Tract Urothelial Carcinoma

Upper tract urothelial carcinoma (UTUC), a rare and aggressive form of carcinoma, is usually associated with high rates of recurrence and death. Lack of significant clinical and pathological prognostic methods in patients makes it very difficult to start with the invasive treatment options. Hence, identification of tissue-based molecular markers as prognostic tools could be of great value for better treating the disease. However, despite rapid increase in studies on tissue-based markers in this disease in the recent years, still extensive research is further required to identify a promising biomarker. Table 10.8 summarizes the molecular markers currently being studied for UTUC along with their function and prognostic values [104].

Molecular Markers in COPD

Recently, many analytical biomarkers have been explored in COPD [105], such as plasma fibrinogen [106], CRP [107], interleukins IL-6 [108] and IL-8 [109], total bilirubin (important due to its antioxidant capacity), serum amyloid protein (SAA), surfactant protein D (SP-D), club cell secretory protein 16 (CCSP-16), and matrix metalloproteinases MMP-8 and MMP [110]. Among these, fibrinogen, CRP, and total bilirubin could be assumed as the most promising systemic/inflammatory biomarkers for predicting mortality in COPD patients. Moreover, plasma fibrinogen was the first biomarker drug development tool qualified for use in COPD under the FDA's drug development tool qualification program. High sensitive CRP was the first biomarker to be investigated in COPD [110].

Table 10.8 Tissue-based molecular markers used in various conditions

Tissue-based markers	Function
Cell cycle regulation	
P53	Tumor suppressor gene function
cyclins	Regulates cell cycle progression (G1/S transition)
p21 and p27	Loss of function is associated with tumor development
Cell growth and proliferation	
Ki67	Associated with cell proliferation
HER-2	Role in tumor cell growth and development
NF-kB	Associated with inflammation, angiogenesis, and cancer
Cell adhesion/EMT	
E-cadherin	Calcium dependent glycoprotein essential for epithelial tissue integrity
N-cadherin	Expressed by mesenchymal cells
Catenins	Role in stable cellular adhesion
Apoptosis	
Bcl-2	Anti-apoptotic protein
Caspase	Proteins that induce cell death by apoptosis

Molecular Markers of Glioma

Gliomas are the most malignant and aggressive form of brain tumors, leading to majority of brain cancer related deaths. The current mode of treatment is radiation therapy along with temozolomide with less survival time. Recent advances are being made to understand the biology of gliomas so that better diagnostic and treatment options could be made available. One such advancement is toward the identification of molecular markers in glioma. These molecular markers are of great importance clinically, since it can help in early and precise detection of gliomas, hence can help in better and early treatment of such malignant cancer [111]. Following are the few molecular markers currently being used for gliomas.

CD133 CD133 (or AC133) a membrane bound glycoprotein encoded by PROM1 gene, is involved in cell differentiation, epithelial to mesenchymal transition, and also acts as a marker for human neural stem cells [112, 113]. CD133+ cells have high telomerase activity, a possible sign of stem cell activity. CD133+ cells along with nestin and amount of PROM1 genes have been observed as a marker for GBM in few patients.

Nestin Expression studies have found that nestin expression is correlated with higher grade gliomas and lower patient survival rates at either protein or mRNA expression levels, while other studies have shown that nestin expression has no effect on prognosis of the patient [114, 115].

ALDH1 activity Aldehyde dehydrogenase 1 (ALDH1) has been shown to play important role in stem cell maintenance, hence it could serve as functional marker of cancer stem cells including gliomas [116]. ALDH1 can be detected by Aldefluor through FACS [117]. However, the validity of using ALDH1 activity as a marker for GSC has not been well validated across a spectrum of tumors. Furthermore, ALDH1 is a relatively nonspecific marker and is expressed by normal astrocytes as well [118].

Low proteasome activity Low proteasome activity correlated with poorer survival in GSC patients, hence in future, it might serve as a promising functional marker in these patients [119].

Besides these, CD15, ABC transporter proteins, label retention also have the potential for molecular markers in gliomas. However, extensive search is still required.

Conclusion The emerging role of molecular markers in the prognosis/diagnosis of diseases is revolutionizing in better understanding the pathogenesis of a disease. Besides early detection, these markers can also provide accurate diagnosis. Also, these markers can be used to track the therapy of diseases. A wide array of diseases are currently being diagnosed and a lot many more diseases need to be explored.

10.7 Applications for Probes in Disease Diagnosis of Humans

Globally, genetic defects are drastically adding up to the burden of human diseases. More and more people are getting affected with genetic diseases contributing to increased childhood deaths. Screening and treatment of such defects at a younger age can help provide better treatment to such diseases. Hence, new methodologies are coming up to help detect such defects at early stages. Over the years, extensive research has been done in the molecular analysis of human genetic disorders. One such technique which helps in early and accurate measurement of genetic diseases is DNA probing.

DNA molecule consists of two complimentary strands held together with hydrogen bonds between the nitrogenous basis. Such bonds when heated, become denatured, i.e. the two strands separate and when allowed to cool, it forms double helical structure again. This property of DNA is being explored in DNA probing. A single strand of DNA is labeled, and when it is added to a complex mixture of nucleic acids, it binds at the complementary sites of another DNA/RNA molecule and the gene/region of interest can be identified as described in Fig. 10.1.

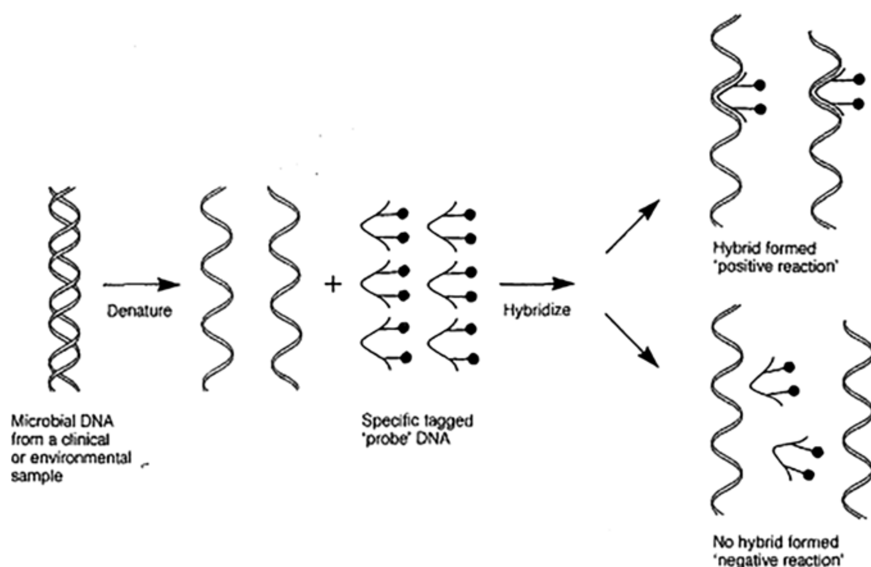

Fig. 10.1 Steps of DNA probe hybridization: This figure shows the identification of specific nucleotide sequences. —● this shows a tag for the probe detection. ^{3}H, ^{25}I, ^{32}P radio-labeled metal atoms can be used as probes

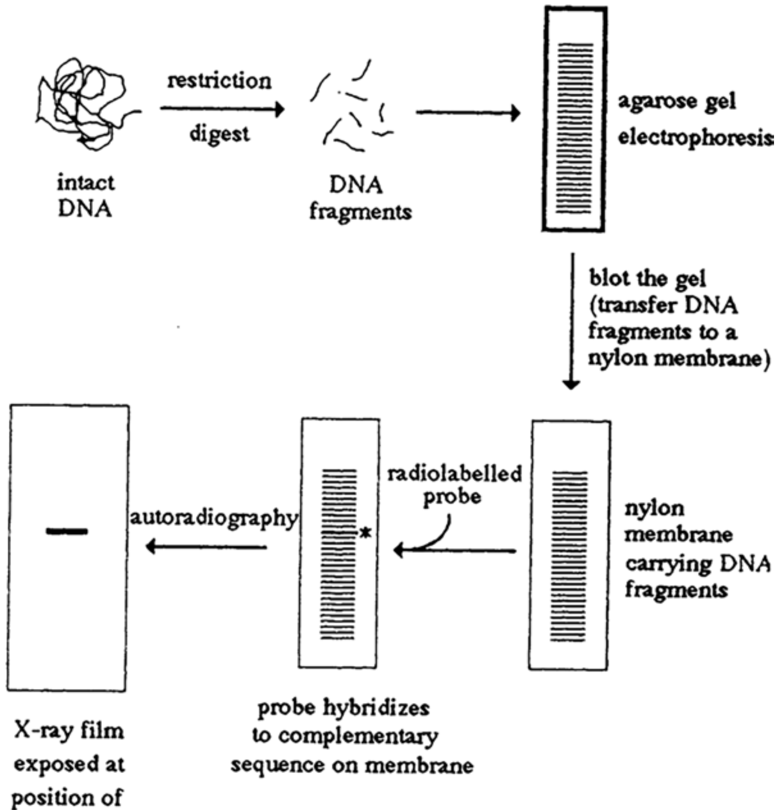

Fig. 10.2 Steps in autoradiography: This figure shows the layout that radioisotopes can be used to label DNA probes that can be easily detected by autoradiography

10.7.1 Synthesis of DNA Probes

10.7.1.1 Isolating DNA Probes

DNA probes are broadly classified as cloned DNA fragments (isolated from genomic library or a cDNA library) and synthetic oligonucleotides (short, 15–50 nucleotides long chemically synthesized desired DNA sequences).

10.7.1.2 Labeling DNA Probes

For the detection of region of interest (hybridized complementary nucleic acid sequences), DNA probes must be labeled. Following are the widely used methods for DNA probe labeling:

1. Radioisotopes. Traditionally, radioisotopes are used to label DNA probes that can be easily detected by autoradiography, i.e., overlaying the filter containing

the samples with X-ray film or by scintillation counting. It is the most sensitive method of DNA labeling which can detect up to 0.1 pg of DNA [120, 121]. ^{32}P is the most commonly used radioisotope. Recently, nonradioactive methods of labeling and detection systems are also being developed, which are much safer but are less sensitive than radioisotopes. DNA probes (cloned DNA fragments and PCR fragments) are usually labeled by random priming method, where one of the deoxyribonucleoside 5′-triphosphates (dNTPs) in the reaction mixture is labeled which becomes incorporated in the entire sequence of newly synthesized strand. On the contrary, oligonucleotides based DNA probes are usually labeled by attaching a ^{32}P-labeled group to their 5′ or 3′ end. Most frequently, the enzyme T4 polynucleotide kinase is used to transfer the ^{32}P-labeled γ-phosphate group from [γ-^{32}P] ATP to the 5′ end of the oligonucleotide. Unfortunately, this method has certain limitations, i.e., short life of radioisotopes, especially ^{32}P and safety concerns associated with radioisotopes (Fig. 10.2).

2. Biotin–Avidin Systems. It was the first alternative system developed to eliminate the use of radioactivity [122]. Here biotinylated deoxyuridine 5′-triphosphate or deoxythymidine 5′-triphosphate is incorporated into the DNA probe as primary label. Following hybridization, usually horseradish peroxidase attached to avidin or enzyme labeled antibodies are added to the solution as a second detector group. A colorimetric product is produced when a substrate binds to the avidin-biotin moiety of hybridized DNA, and the intensity of color is measured spectrophotometrically. Several variations of this technique have also been developed such as fluorometer (photobiotin) [123].
3. Enzyme-Conjugated probes. In this method enzyme, such as alkaline phosphatase, is attached to the DNA [124]. This technique is preferred mainly for the detection of HSV and enterotoxigenic E. coli (ETEC) [125]. However, compared to radioisotopes, this technique is less sensitive.
4. Antibodies. Mainly, two types of antibodies are used for DNA probe reactions. The first set of antibodies recognize antigenic moieties chemically coupled to nucleotides contained within the probe. For example, *N*-acetoxy-*N*-2-acetylaminofluorene can be attached to guanine residues in the probe DNA, as a target for polyclonal anti-*N*-acetoxy-*N*-2-acetylaminofluorene antibodies coupled with enzymes such as horseradish peroxidase [126]. Use of monoclonal antibodies is the second approach [127].

10.7.2 How Are DNA Probes Detected?

DNA probes are usually detected by two methods: Southern blotting and restriction fragment length polymorphisms (RFLPs).

Southern blotting It is the most commonly used technique to detect region of interest using DNA probes. Briefly, following steps are involved in Southern blotting. The genomic DNA is cut into random fragments with restriction endonuclease. These fragments are run on agarose gel to separate different lengths of fragments.

This is followed by denaturing the gel by soaking it in alkaline solution, transferred on to nylon membrane binding covalently. It is necessary to transfer the DNA from the gel to a solid support because attempts to hybridize a probe to DNA in the fragile gel would cause it to disintegrate.

Then the membrane is incubated with radiolabeled probe (denatured if double stranded), excess probe is washed off and bound probe is detected by autoradiography. Complimentary fragments of DNA to which probe is bound appear black on the film. Precautions should be taken to avoid mismatched hybridization (Fig. 10.3).

Restriction fragment length polymorphism (RFLP) Usually, gene probes hybridize to fragments of equal length in all subjects. However, occasionally, it may bind to fragments of different lengths. Such a difference is due to change in genetic sequence which is known as polymorphism which is detected using restriction fragment length polymorphism technique (RFLP). RFLPs could arise due to two reasons, first there is a change in single base pair, thus affecting the site of restriction enzyme which is consequently present in some individuals and not others. Second reason could be due to insertion or deletion of a sequence at the restriction enzyme site as a result it is no longer able to cleave and different length fragments are obtained. Since polymorphism changes the genetic sequence at a particular site, the restriction endonuclease is no longer able to cleave at the different site and as a result, fragments of different lengths are obtained and such a technique is known as restriction fragment length polymorphism. RFLPs provide a useful technique for mapping of DN sequences, identification of mutant genes running through the families and prenatal detection of inherited diseases.

10.7.2.1 Applications of DNA Probes

Clinically, DNA probes are extensively being used to identify and detect known genetic defects and also defects whose genetic sequence is not known. These methods are discussed below:

1. **Inherited diseases including a defect in single known gene**. If a probe is available for a known mutated gene in a disease, then that probe can be used to determine if that particular mutation is present in an individual or not. This technique can be used for carrier detection, prenatal (fetus) [128], or postnatal diagnosis. Techniques for identifying such mutations are described below:

   (a) **Detecting gross gene deletions.** Large deletions of the genetic lesions can be easily detected using DNA probes. Defect clinically diagnosed using this technique is deficiency of human pituitary growth hormone (hGH) where GHI gene region, encoding for hGH is deleted. Hence this can be easily identified using hGH cDNA probe or GHI gene fragment probe [129].

   (b) **Detecting Point Mutations**

   RFLPs. Point mutations are much more difficult to detect. This is because most of the times such mutations do not change the sequence of restriction

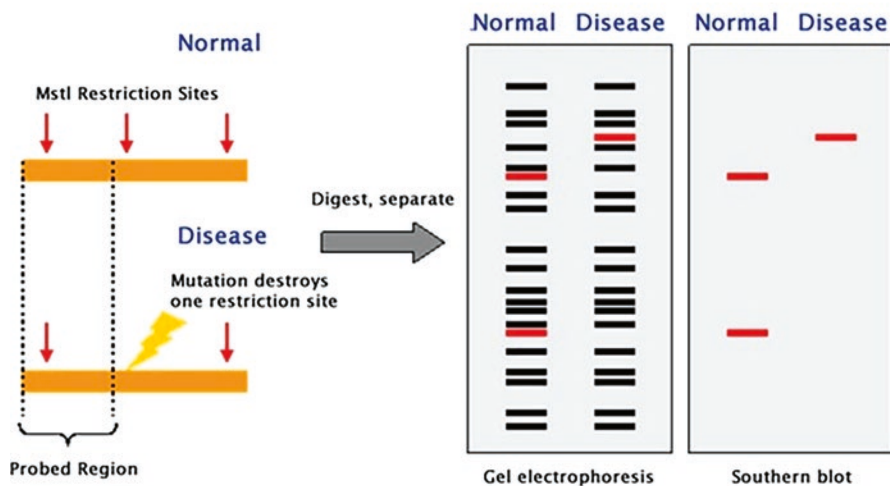

Fig. 10.3 Steps in Southern Blotting. This figure shows the layout of steps used in southern blotting

site, hence RFLP is not generated. Very rare it is that a point mutation also creates an RFLP. The classical example for this is sickle cell anemia where a point mutation in P-globin gene changes the sixth codon sequence resulting in valine coding instead of glutamic acid. The normal coding region of P-gene contains restriction site for MstXS (CCTGAGG) which in sickle cell anemic patients is disrupted due to which this enzyme is not able to cleave. This can easily be detected using southern blotting technique and patients can be identified [Fig. 10.3] [130].

However, as discussed earlier, few point mutations do not create different RFLPs, such RFLPs are detected directly using allele-specific oligonucleotides (ASOs) and ribonuclease A (RNase A) protection.

Allele-specific oligonucleotides (ASOs). Short nucleotides sequences (138–140 nucleotides long) of normal and mutant sequences are synthesized, labeled and used to probe blots of genomic DNA of individuals to be tested. Experimental conditions are so adjusted that perfectly matched hybrids are stable and hybrids with mismatch are unstable. Hence, normal nucleotide probes will give signal with heterozygotes and homozygotes for normal sequence. Whereas mutant oligonucleotides will hybridize with heterozygous and individuals with homozygous mutated sequence. Nucleotide probes should be short so that single base pair mismatch has significant effect on the stability of the hybrids. However, nucleotides shorter than (138–140) nucleotides may have a complimentary sequence elsewhere in the genome, resulting in nonspecific fragments. Best results are obtained if genomic DNA of the patient is first amplified using PCR, transferred to the nylon membrane and then detected using "normal" and "mutant" probes. This also avoids the problem of nonspecific binding [Fig. 10.3].

This technique is widely used for prenatal diagnosis of a number of inherited diseases such as phenylketonuria (phenylalanine hydroxylase deficiency), hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency), etc. [130].

Ribonuclease A (RNase A) protection. Here, instead of DNA, single stranded RNA probes are synthesized (by transcribing the DNA sequence containing point mutation) which is then allowed to hybridize to the solution of genomic DNA of the individuals to be screened (Fig. 10.2). The mismatched RNA–DNA hybrids will be degraded by RNase A digestion but perfectly matched DNA–RNA hybrid will be protected from the action of RNase A. These protected products are then electrophoresed on denaturing polyacrylamide gels and autoradiography is detected. The sensitivity and specificity of this technique are also increased by first amplifying the region of genomic DNA containing mutations. Various mutations in huge number of diseases have been identified using this technique including the gene involved in hypoxanthine phosphoribosyltransferase (HPRT), which is defective in people with Lesch-Nyhan syndrome [131].

Linked RFLPs. RFLP technique can also be used to indirectly identify point mutations resulting in a disease. For instance, normally, P-globin gene is found on a HpaI fragment of either 13 or 7.6 kb, depending on whether a polymorphic HpaI site is present (Fig. 10.3). The polymorphism is said to be linked to the P-globin gene, because it lies on the same chromosome. Hence, it is possible to track the P-globin gene by probing Southern blots of HpaI-digested genomic DNA with a P-globin gene probe [132]. This technique can be used for carrier detection and prenatal diagnosis,. Such technique is very useful for identifying inherited diseases involving known genes such as P-thalassemia.

DNA probes can also be used to identify inherited diseases involving a single unknown gene [133].

DNA Probes and Cancer

Using DNA probing techniques, a large number of proto-oncogenes have been identified in tumor biology, which otherwise has the potential to transform into malignant tumors. For example, many mutations in Ki-ras proto-oncogenes have been identified in colon carcinomas which can be detected by Southern hybridization, using ASOs for the normal and mutated Ki-ras genes. Similarly, few colon cancers are characterized by the deletion of tumor suppressor genes, hence such patients have distinct RFLPs and can be identified. It is hoped that such DNA-based tests might be valuable in some situations for diagnosis, for the assessment of prognosis and for the detection of residual cancer cells after treatment.

DNA Probes and α-Antitrypsin Deficiency and Pulmonary Emphysema

One of the major causes of fatal liver cirrhosis is the genetic disorder in which plasma protease inhibitor a-1-antitrypsin is defective [134, 135]. Condition worsens when this severe deficiency syndrome develops chronic

obstructive pulmonary emphysema. Around 30 different phenotypes of this protein have been identified [136]. Before the advent of DNA probes, Pi-typing of blood sample obtained from fetoscopy was the only technique for prenatal diagnosis of a-1-antitrypsin deficiency, a procedure that carries a fetal mortality of approximately 6% [138]. At the protein level, the deficiency is caused by substitution of a glutamic acid for lysine at residue 342 in human a-1-antitrypsin [139], i.e., the amino acid substitution of G to A. DNA probes specific for this region are now used to diagnose this disease, hence reducing the chances of fetal mortality.

Using DNA probes for diagnosis of prenatal a-1-antitrypsin deficiency has various advantages: less invasive and relatively safe than fetoscopy, less amniotic fluid or cultured amniotic cells requirement, and directly identifies the mutation site in the gene.

DNA Probes and Infectious Diseases

Besides detection of specific human genes, probes are also being used to detect the nucleic acid genomes of microorganisms in tissue or biological fluids. For example, in a technique known as in situ hybridization, viral DNA can be detected in formalin-fixed DNA sections or paraffin embedded biopsies. This technique is useful where serological tests for virus are not available, or where virus cannot be isolated from clinical samples by tissue culture methods, or where the viral infection load is less or where virus establishes latent infection. Detection of human papillomavirus, cytomegalovirus, and HIV [140] are the examples of in situ hybridization in clinical use. Such probes are also used to distinguish pathogenic and non-pathogenic strains of bacteria. For example, enterotoxigenic Escherichia coli (ETEC) and normal commensal E. coli can be distinguished by growing them on agar layered on nylon membranes, breaking the bacteria and denaturing their DNA with sodium hydroxide solution, fixing the DNA to the membrane by UV irradiation, and then hybridizing the immobilized DNA with a DNA probe for sequences that are found only in ETEC [141]. This procedure is better than the conventional techniques involving immunological or cytotoxic properties of the virus. Similarly, probes are being extensively used for protozoa and helminths [142], Leishmania tropicalis [143], P. falciparum [144] and fungi (Aspergillus nidulans), a filamentous fungus, could be detected by DNA hybridization [145].

To conclude, DNA probes have revolutionized the detection of genetic disorders which otherwise was responsible for huge mortality rates. These probes have led to early detection of genetic diseases and hence in their better treatment. Besides genetic diseases which are currently being diagnosed using this technique, DNA probes are also been extensively used to identify few cancers, parasitic and viral infections. Hence, it could be assumed that DNA probing technique holds a great potential in the clinical field.

Note: For Tables 10.1 to 10.5, please refer to Karbalaei et al [146].

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Chapter 11
Nanotechnology and Nanomedicine

Tahir Bashir Dar, Adil Rafiq Bhat, Fleury Augustin Nsole Biteghe, Ab Raouf Bhat, and Zaria Malindi

Abstract Nanotechnology (often referred to as nanoscience) is an amalgam of various scientific fields, that includes but is not limited to chemistry, biology, engineering, material science, and advanced technology, at nanoscale. This concept of science got recognition after a famous talk by physicist Richard Feynman at an American Physical Society meeting at the California Institute of Technology (CalTech) on December 29, 1959. His talk entitled "There's Plenty of Room at the Bottom," in fact, proved to be the brainchild of what we know as nanotechnology today. Nanotechnology, nowadays, is fast advancing to make an impact in various fields from healthcare to electronics and might be extremely beneficial particularly in revolutionizing drug delivery

In loving memory of
Prof. G. Mohiuddin Bhat.
Tahir Bashir Dar is Lead Contact.

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systems and diagnostics in healthcare. In fact, the intrinsic limitations of traditional cancer therapies, have paved the way for the development of a novel treatment approach herein referred to as nanotechnology, using nanoparticles. Because of their versatile nature and nanoscale size (1–100 nm), nanoparticles have primarily been able to ameliorate the balance between the toxicity and therapeutic efficacy of conjugated or encapsulated anticancer agents (e.g., chemotherapeutic drug), by tilting it toward more beneficial treatment efficacy. In addition, nanotechnology has also been recently utilized and has already emerged with profound application in a range of sectors from food packaging to food processing, cosmetics to textiles, and warfare to environment. The basics of nanotechnology and its diverse applications in different fields are summarized in the following six sections.

Keywords Nanotechnology and applications · Nanomaterials · Nanorobots · Nanomedicine

11.1 Origin of the Field, Everyday Applications of Nanotechnology: Nanoparticles in Food Packaging, Textiles, Cosmetics, and Disinfectants

The imagination, creativity, and interest of humans toward growth has given rise to new science and technology. The twenty-first-century frontier, nanotechnology is the technology in nanoscience which utilizes the matter of dimensions 1–100 nm that enable novel size-dependent applications with understanding and control of nanomatter [1]. The applications cover a wide range of fields from physics, chemistry, and biology to electronics, engineering, and medicine. Thus, two preconditions for nanotechnology are (1) scale issue for controlling the size and shape at nanoscale and (2) doing with novelty and advantages from nanoscaled matter. Therefore, nanoscience is the convergence of materials science, physics, and biology, that deals with the manipulation of matter at atomic to molecular scales, whereas nanotechnology is the ability to observe, measure, control, manipulate, assemble, or manufacture materials of nanoscale [2].

The 1925 Nobel Prize laureate Richard Zsigmondy in chemistry proposed the concept of nano meter for the characterization of particle size and first studied the gold colloids under microscope. But it was Richard Feynman, the American Nobel Prize laureate, who introduced the concept of nanotechnology in 1959. While presenting the annual lecture at the California Institute of Technology, Caltech entitled "There's Plenty of Room at the Bottom" Feynman hypothesized the concept of manipulation of matter at molecular or atomic level and further to construct machines of molecular level [3]. Since then, his hypothesis has led to novel ways of thinking; for these reasons Feynman has been considered the father of modern nanotechnology. Soon after about a decade of Feynman's lecture, Norio Taniguchi, a Japanese scientist, in 1974 used and defined the term "nanotechnology" as a

technology that consists of processing, separation, consolidation, and deformation of material by one atom or molecule. He used nanotechnology to pronounce the semiconductor processes which occur at nanometer order [4].

Many scientists are showing interest in the field and mainly two approaches have been developed to describe the possibility for nanostructure synthesis, that is, top-down and bottom-up. The two developed approaches differ from each other in quality, speed, and cost as well. Top-down refers to breaking down the bulk material in order to get nano-sized particles and it can be attained by utilization of modern techniques like lithography or precision engineering that have been developed with optimization in industries from last decade. Bottom-up, on the other hand, is to build up the nanostructures (1–100 nm) from the bottom, atom by atom by physical or chemical methods using the controlled use of self-assembly of atoms or molecules. Eric Drexler of Massachusetts Institute of Technology (MIT) used concepts of Feynman and Taniguchi, and developed the idea of nanoscale as "molecular nanotechnology" and proposed it in his books on nanotechnology *Engines of Creation: The Coming Era of Nanotechnology* and *Unbounding the Future: The Nanotechnology Revolution* [5, 6]. The former led to the theory of "molecular engineering" and in the latter he proposed the term "assembler" or "nanobots" that has ability to build copy of itself or other items with arbitrary complexity for applications in medicine.

In the 1980s, however, the golden era of nanotechnology started when the scanning tunneling microscope (STM) was invented by Gerd Binnig and Heinrich Rohrer at IBM Zurich Research Laboratory which won them the Nobel Prize in Physics in 1986, that uses the electron wave functions of the atoms in the sharp tip of STM and the conductive surface atom on applied voltage [7, 8]. STM was utilized to image surfaces at atomic scale and to create structures by manipulating atoms and molecules with the use of tunneling current for selective bond making or breaking. The invention of STM has led to the development of scanning probe microscopes (SPM) and atomic force microscope (AFM), which have been largely used in nanotechnology nowadays [9, 10]. In 1985 Kroto, Smalley, and Curl claimed the discovery of fullerenes (stable sphere form of carbon) [11]. The nanotechnological science further advanced when another Japanese scientist Iijima et al. developed "carbon-nanotubes" or hollow graphitic nanotubes by using transmission electron microcopy (TEM) having potential applications as energy storage material, catalysis, field emitters and molecular electronic components due to the strength and flexibility of these nanotubes [12]. The accidental C-dots (carbon dots sized ≤10 nm) reported by Xu et al. (2004) and due to their abundance and inexpensive nature have shown interesting properties in bioimaging, biosensors, catalysis, energy conversion, photovoltaic devices, nanoprobes, and drug delivery [13, 14].

The potential applications of nanotechnology in many bio-related fields like biomedicine for diagnosis, molecular imaging, and drug delivery have shown astounding results. A number of nanomaterials containing medical products are already available in the U.S. market, such as in nanopharmaceuticals, regenerative medicine and nanomaterial for drug delivery; other functional nanostructures have been used in biomarker detection as nanobiosensors or nanobiochips [15, 16]. Other potential benefits can be antibacterial coatings of medical devices, better surgical tissue healing, reduced inflammation, and recognition of circulating cancer cells. The nano-oncology as an attractive application of nanotechnology has seen considerable

progress in improving the efficacy of chemotherapeutic drugs, as functional molecules, cytotoxic agents, or antibodies can be directly targeted to tumor sites with improved response rates [17]. Nanotechnology has been utilized to produce efficient and cost-effective energy, like producing less pollution during material manufacturing, developing solar cells with efficient electricity production at affordable costs, cleaning groundwater from organic chemicals or air from volatile organic compounds (VOCs), that results in the improvement of the environment. Human life has been well influenced by nanotechnology with diverse potential benefits; in the food and cosmetics industries, nanomaterials are used for their improvements in production, packaging, bioavailability, shelf life and used as food sensors to detect food quality and safety as well. Some metal-oxide nanoparticles show effective antimicrobial activity against food-borne bacteria [18, 19]. However, the safety of nanomaterials due to their complex nature makes the assessments challenging which may be due to lack of reliable toxicity data; thus, the potential to affect human health remains a concern. New emerging nanoinformatics can provide supplementary platform for analytical design of nanoparticles to overcome the in vitro barriers and help to brighten the future of nanotechnology. Nanotechnology can be used in different fields with potential applications from medicine to food processing and cosmetics, and they are summarized in the following sections.

11.1.1 Nanoparticles in Food Packaging

The global food industry needs to package and transport the commodities hygienically to preserve and protect that from alteration and meet the demands (safety, healthy, and freshness) of the end customer with food safety regulations. The packing of food has evolved with the innovative approach of material science and technology. In twenty-first century, the food packing has been controlled by nanotechnology, nanomaterial sciences as it has significantly addressed the concerns of safety, real-time quality, and stability of food products. Further, it has been employed to enhance the shelf life within packaging by considering the release profile of preservatives and antimicrobials. Packaging based on nanoparticles has attracted industries as well as consumers. These nanomaterial-based innovations in the food packaging mostly explore the possibilities of incorporation into food products, incorporation in packaging materials, and the application in food processing [20]. To meet the standards of effective food packaging, advanced nanoaugmented polymers will help to enhance the benefits of already available polymeric materials. Improved packaging materials will diminish the negative interactions between the food and packaging, which will ultimately positively impact human health, improve biodegradability, and reduce toxic gas emissions. Nanomaterials can be broadly devised into intelligent, active, and improved packaging. Smart packaging offers excellent functionality by means of communication and the marketing by providing dynamic feedbacks of packaged food. Different communication methods like

nanosensors, freshness, and temperature indicators and oxygen sensors have already been started to be used [21].

The nanosensors which are usually applied on the labels or coatings of packing help to detect spoilage, pathogens, and chemical components in the packaged food, thus providing the idea of food condition in time [22]. Gas sensors provide the information of gas analyte in the packaging. The limited oxygen availability packages are chosen over the free excess packages, the concentration is detected by O_2 irreversible sensors (provided in the packaging), which work on the principle of absorbance changes produced in the analyte. CO_2 sensitivity and cross-sensitivity to O_2 optical gas sensors, mainly dry optical sensors that have pH-sensitive dye indicators, can be investigated in food packaging applications [23]. The freshness indicators introduced into the packaging provide the food quality information while storage and transit. The indicators are sensitive to the spoilage compounds of the metabolites (volatile sulfides and amines) which are generated during the spoilage of any food product [24]. The information is usually visual. Esser et al. developed a sensor by using carbon nanotubes that detects CO_2, volatile compounds, and ethylene that is emitted during fruit ripening [25]. Environmental factors like temperature and humidity are mainly responsible for the diminished shelf life of the food products during. Time-temperature indicators and humidity indicators have been developed and used to monitor the thermal history and actual status of the quality and safety of food products. Use of color or iridescent technology for humidity and temperature have been developed to check the food quality [26, 27].

The active packaging has been designed with the incorporation of components with antimicrobial and antioxidant releasing ability. The incorporation of agents like antibacterial agents, vapor absorbers, preservatives has effectively enhanced the shelf life with quality regulation of the food items [28]. Different nanoparticles like silver (Ag), copper (Cu), gold (Au), zinc (Zn, ZnO), TiO_2, MgO, and SiO_2 [29, 30] have been explored in the packaging materials due to their antibacterial properties, improvement of mechanical and physical properties, surface reactivity (i.e., strength and elongation), photocatalytic disinfecting ability or photoactivated biocidal activity. Nanoparticle-based biodegradable films, membranes, and coatings have resulted in enhanced shelf life, control of undesirable compounds, and improvement in mechanical and physical properties of packaging material. Nanoparticles have been applied in improved packaging or polymer matrix of packaging to increase the elasticity, stability of moisture and temperature parameters, gas barrier traits, which has improved the mechanical and barrier characteristics of polymers used for packaging [31, 32].

The nanotechnical food packaging has offered several advantages than the conventional one by presenting improvements in various properties like enhanced durability, flame resistance, temperature resistance, better recycling property, low cost, and reduced waste. These developments validate the candidature of nanomaterials in food packaging applications for cheese, cereals, meat, meat products, and confectionery. Further, it can be applied to coatings in fruit juices and milk products or as coextrusion processes in manufacturing carbonated bottles. The application of

nanotechnology in the food industry is globally growing and predictions are that more nanofood products will be in the market in the next few years.

11.1.2 Nanoparticles in Textiles

The textile industry is experiencing a transformation through modernization in the wearable technology as designers are now creating functional materials with integration of modern communication devices, and introduction of nanomaterials into garments and designer clothes has made it even better. The demand of durable and functional clothing from customers has created opportunities for designers to integrate nanomaterials into the textiles or textile substrates. These nanomaterials, without altering the comfort and durability of the substrate, have shown advantages like wrinkle-freeness, electrical conductivity, induced stain repellence, water repellence, sensing, odor control, color, and increased strength [33–36].

Cotton fiber, being the widely used in textiles, provides excellent absorbency with softness. However, due to the relatively low strength and durability, easy creasing, and flammability; its use in nonclassical applications is limited [33]. On the other side, synthetic fibers can be resistant to crease-stains and provide good antimicrobial applicability but simultaneously lack comfort when compared to cotton. Since the 1940s, the developmental process started for combined fiber production from both natural and synthetic fibers that offer advantages with novel functions [34]. A new area "nanoengineered functional textiles" in the clothing technology has been working for the demands of customers with improved color, texture, and functionality [35, 37–39]. The advantage of the use of nanomaterials is creating of function or introducing flexible electronics and optical devices into these textiles without changing the comfort properties of the substrate [40]. The integration of such materials and technologies provide platform with a response in chemical with mechanical, electrical with thermal, optical as well as magnetic stimuli. The engineered materials may include sensors, data transmission, or processing units with no allergic reaction to the body and provide other applications which include medical monitoring of body function and metabolism [36, 41]. Further, the development of nanoengineered textiles or introduction of nanoparticles has shown a large expansion in the properties of textiles like, by modifying the surface to make it wrinkle resistant, water and oil repellant, and with improved appearance provides potential UV blocking and enhanced strength. The electrically conductive textiles being flexible, and lightweight have been designed for energy storage [42], with color-tunable optical fibers provided with digital components like OLEDs for electromagnetic shielding [43]. Other inclusions to the textiles include antibacterial properties, odor control, sensing devices for moisture, temperature, pressure with much advanced application toward the controlled drug release. Nanotechnology can thus lead to evolution of textiles which are customer friendly as well as climate neutral with reduced greenhouse emissions and fair recyclability. However, nanoengineered

textiles with nanoparticles remain questionable due to its impact on environment and any pollution it may cause.

11.1.3 Nanoparticles in Cosmetics

The application of nanotechnology has also stretched to the field of cosmetics, referred to as "nanocosmetics" and is considered as an emerging technology. The pronounced effect of nanotechnology can be seen by the alteration in the properties of cosmetics like, transparency, color, solubility, and reactivity which has made these materials attractive to the industries [44]. The use of these nanomaterial altered cosmetics provides better skin penetration with UV protection and enhanced color quality. The different types of nanomaterials which have been employed in cosmetics are, liposomes, fullerenes, nanosomes, solid lipid nanoparticles, nanocrystals, dendrimers, nanocapsules, nanoemulsions, hydrogels, cubosomes, nanosilver, and nanogold [45–47]. The applications of these nanocosmetics have provided various products with effective results; UV filters and sunscreens are provided with titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles to prevent skin from UV damage. Skin cleansers, moisturizing creams, and disinfectants contain nanosilver like in soaps, toothpastes, and wet wipes to clean the skin and remove unpleasant smell of body and are also regarded as effective disinfectants for skin protection. Moisturizers are being provided with nano structures like liposomes to carry vitamin E which helps to overcome skin dehydration, drying, scaling, and breaking away of the skin surface. Antiaging products have been regarded as the main application of nanocosmetics. The aging of skin can be characterized by thinning, loss of skin elasticity, dehydration, wrinkling with appearance of spots and loss of gloom of the skin. Skin creams use stem cell derived proteins, carried effectively by liposomes which also carry cosmetic components, that help to prevent skin aging [48]. The creams are incorporated with nanoparticles to restore the natural suppleness of skin; nanosized UV filters and antioxidants have been used to produce antiwrinkle creams [46]. The unique properties of nanoparticles in cosmetics have two distinct sides, one capturing hearts and appealing benefits due to enhanced properties and other as a concern regarding their safety for environment and with a potential untoward effect on the human system.

11.2 Nanomaterials as Drug Delivery Systems

Over the last two decades the use of nanotechnology for drug delivery has witnessed an explosive growth in research, broadly labeled as nanomedicine. Problems such as nonspecific delivery and inadequate distribution and accumulation of therapeutics remain major challenges to drug developers. To overcome the limitations of conventional delivery nanotechnology could help with large-scale issues such as

biodistribution to smaller-scale barriers such as intracellular trafficking through targeting cell-specific antigen, transport of molecules to specific organelles, and other different approaches. Bioengineered nanomaterials hold substantial promise to improve disease diagnosis and treatment specificity. To facilitate the promising nano-enabled technologies its realization and clinical manifestation, the US National Science and Technology Council (NSTC) in year 2000 launched the National Nanotechnology Initiative (NNI) and delineated well-defined initiatives and grand challenges for the field. These projects have supported significantly in the field of nanomedicine, further to investigate and improve nanotechnology of which nanoparticles (NPs) promotes a paradigmatic shift in nanoparticle-based drug delivery, now constitute a significant portion of reported research and advancement. NP are generally low-cost technologies having high potential which can be used for noninvasive administrations, due to its highly tunable formulations drug loading, better targeting and release efficacy than conventional drugs.

NPs have an immense potential to improve the stability and solubility of encapsulated cargos, promote transport across cellular membranes, and prolong half-life in circulation to increase safety and efficacy [49] For these characteristics, NP research biology has been extensive, generating promising results both in vitro and in preclinical animal models. However, because of the translational gap between animal and human studies, the number of nanomedicines available to the patients is significantly below projections for the nanotechnology field [50]. This gap is due to lack of understanding of the differences in physiology and pathology between animal model and humans; more specifically how these differences influence the behavior and functionality of nanomedicines in the body need to be further explored [51]. Moreover, it is not only the differences across species that limits clinical translation, but other factors may also play a role. Also, heterogeneity among patients can also limit the success of nanomedicines, and there is currently only limited research on the interactions between nanomedicines in different patient populations studied so far. That is the reason, most of the nanomedicines that are approved, some are recommended as first-line treatment options, and many show improvements in only a small subset of patients [52]. This potentiates the need to understand the underexplored heterogeneity important for this field of science for both disease biology and among patients, which alters NP efficacy because the growth, structure, and physiology of diseased tissue alter NP distribution and functionality. In earlier times NP iterations were unable to overcome these biological barriers to delivery. Nowadays, NP designs have applied advancements in controlled synthesis strategies to incorporate complex architectures, bio-responsive moieties, and various targeting agents to enhance delivery [53–55]. Therefore, NPs can be utilized as more complex systems by including in nanocarrier-mediated combination therapies to overcome drug resistance mechanisms, maximize the therapeutic efficacy against specific macromolecules to alter multiple pathways, or target particular phases of the cell cycle.

In this part of chapter, we will focus on the delivery of precision medicine therapeutics, because in the future these medicines will greatly influence precision NPs. As the impact of NP characteristics on therapeutic responses are explored the biological barriers that have limited the widespread success of NP applications and the

distribution and delivery trends are the areas need to understand in detail. These advances in engineering of NPs for specific applications are of particular importance as new opportunities arise for the clinical translation of NP-based precision therapies in cancer medicine, immunotherapy, and in vivo gene editing.

11.2.1 Different Types of NP Classes

There are three broad categories in NPs depending upon the composition that is lipid-based NPs, polymeric NPs, and inorganic NPs.

(a) Lipid-based NPs include various subset structures but are most typically spherical platforms comprising at least one internal aqueous compartment with one lipid bilayer surrounding outside. Lipid-based NPs are the most common class of FDA-approved nanomedicines due to their formulation simplicity, self-assembly, high bioavailability and biocompatibility, and ability to carry large payloads that can be controlled to modulate their biological characteristics [56, 57]. Another prominent subset of lipid-based NPs is referred to as lipid nanoparticles (LNPs); which are liposome-like structures widely used for the delivery of nucleic acids. They differ from traditional liposomes primarily because they form micellar structures within the particle core, a morphology that can be altered based on formulation and synthesis parameters [58].
(b) Polymeric NPs are another class which can be produced from natural or synthetic constituents, as well as monomers or preformed polymers—allowing for a wide variety of possible structures and characteristics. Some polymers which are often copolymerized for these applications include poly(ethylene glycol) (PEG) and poly(dimethyl siloxane) (PDMS). Dendrimers are hyperbranched polymers that are best examples having complex three-dimensional architectures for which the mass, size, shape, and surface chemistry can be highly controlled. Overall, in terms of functionality, polymeric NPs are ideal candidates for drug delivery because they are biodegradable, water soluble, biocompatible, biomimetic, and stable during storage. Their surfaces can be easily modified for additional targeting [59].
(c) Inorganic NPs are the ones which are made from inorganic materials such as gold, iron and silica. They are used to synthesize nanostructured materials for various drug delivery and imaging applications. Due to their magnetic, radioactive or plasmonic properties, inorganic NPs are uniquely qualified for applications such as diagnostics, imaging, and photothermal therapies. These NPs have good biocompatibility and stability, and fill niche applications that require properties unachievable by organic materials. However, limitations in their clinical application are low solubility and toxicity concerns, especially in NP formulations using heavy metals [60, 61].

11.2.2 Translocation Across Biological Barriers

Various factors such as size, shape, charge, and surface coating determine what happens to nanoparticles (NPs) in the circulation, including clearance, and how the NPs interact with local barriers such as the tumor microenvironment or mucus layers. A few general trends have been noted that include (1) spherical and larger NPs marginate more easily during circulation, whereas (2) rod-shaped NPs will extravasate more readily; and (3) uncoated or positively charged NPs are cleared more quickly by immune cells such as macrophages. In terms of local distribution, in general, rod-shaped, neutral, and targeted NPs tend to penetrate tumors more readily whereas positively charged, smaller and coated NPs can more easily traverse mucosal barriers. In this part, we discuss strategies used by NPs to overcome biological barriers on the systemic, local, and cellular scale to perform their functions.

11.2.3 Cellular Heterogeneity

One of the big challenges to NP delivery is that drug-resistant cells contribute to cellular heterogeneity. Likewise, resistance against to platinum(II)-based drugs, for example oxaliplatin and cisplatin, which induce apoptosis by distorting DNA structure, can arise if cancer cells increase their rate of DNA repair or overexpress membranal efflux pumps [62]. Therefore, the best way to overcome this is to engineer NPs capable to cross these barriers. For example, micelles deliver NPs more effectively to the nucleus, and thus the cell has less chances to attain drug resistance [63].

11.2.4 NPs in Precision Medicine

To overcome the many limitations of traditional approaches one-size-fits-all improved therapeutic outcomes need to be developed for patient-specific treatments in a clinical setting, the field referred to as precision medicine. As NPs overcome many of the current limitations to delivery thereby improving the potency and therapeutic efficacy of precision medicines, they may allow more patients to qualify for clinical trials and benefit from individualized therapies. For example, NPs can be used to modulate immune activation or sensitize cancer cells to therapeutics or tumor suppression, helping to homogenize these currently heterogeneous environments to increase the number of patients who respond to or qualify for precision treatments [64, 65].

11.2.5 NPs Are Adaptive to Tumor Microenvironment and Therapy for Cancer

NP systems have a great potential to improve therapeutic efficacy; their design is versatile and can be tailored specifically to the tumor microenvironment. Since cancer remains the second leading cause of death worldwide [66], NPs are actively being researched to targeting cancer cells. Preexisting chemotherapeutics have various mechanisms and sites of action. For example, some disrupt DNA within the nucleus (such as doxorubicin and platinum drugs), and others work within the cytosol or affect organelles such as the mitochondria [67]. Each drug must be delivered to its site of action at therapeutic levels in order to work properly, indicating a need for NP trafficking to these sites. Moreover, the development of effective cancer therapies is very challenging partially because of heterogeneous and complexity among the patients. In order to best treat individual cancer patients, both therapeutics and their delivery systems can be personalized for a given patient. Thus, targeted chemotherapeutics have been developed that can treat patients who express specific biomarkers with the help of precision medicine approach.

11.2.6 NPs for Immunotherapy

Although immune checkpoint inhibitors have shown significant promise for cancer treatment, there are still challenges with efficacy, patient variability, and off-target effects when immunomodulators are used. Some immunotherapeutics, such as proteins, have limited delivery potential when administered freely, and thus NPs have the potential to significantly improve delivery by protecting immunotherapeutics and enhancing their interaction with immune cells [67].

11.2.6.1 Immune Activation

Recently, NPs have been extensively explored in vaccines against SARS-CoV-2 (which causes COVID-19), with multiple successful late-stage clinical trials. Well-known companies such as Moderna and BioNTech use LNPs to encapsulate mRNA that encodes for a COVID-19 antigen [68].

11.2.6.2 Immune Suppression

NPs are modulated to be used as immune suppressant in various diseases such as rheumatoid arthritis and systemic lupus erythematosus which result from incorrect immune regulation, hyperactivation. In these autoimmune diseases, T cells and B cells are sensitized to self-antigens [69]. Autoimmune diseases are typically treated

with general immunosuppressants, which can cause serious side effects. Conditions caused by immune overactivation could benefit from more targeted immunotherapies. For more long-term effects, genetic engineering reprogramming immune cells at the genomic level could be effective [70].

11.2.6.3 NPs for Genome Editing

Recent advances in CRISPR, transcription activator-like effector nuclease (TALEN), and zinc-finger nuclease (ZFN) technologies are making it increasingly easy to engineer the genome for widespread use in biomedical research, drug development and discovery, and gene therapy [71]. Alternative methods of CAR T production could reduce the complexity of antigen delivery to T cells using NPs, including the delivery of CAR-encoding DNA in vivo and the delivery of CAR-encoding mRNA to produce transiently modified T cells [72]. Intracellular targeting of most NP-based systems for genome editing are formulated by electrostatic complexation of nucleic acids with cationic materials, which are delivered intracellularly through mechanisms including receptor-mediated endocytosis and phagocytosis. Companies are currently developing LNPs for in vivo delivery to treat several liver diseases, including amyloidosis, α1-antitrypsin deficiency, and hepatitis B virus infection. With precision NP design, gene editing holds promise to cure diseases and significantly improve patient lives.

11.3 Nanomedicine in Cancer Therapy and Surgery

Traditional cancer therapies including surgery, radiotherapy, and chemotherapy have been unsuccessful in controlling metastatic tumor lesions, likely due to tumor recurrence, side effects, and negligible therapeutic efficacies [73, 74]. With this in mind, immunotherapy (which mobilizes host immunity to selectively destroy tumors) was developed with the intent to (1) improve therapeutic efficacy, (2) establish durable antitumor immune responses, and (3) minimize side effects [75, 76]. The clinical benefits of immunotherapy has widely been demonstrated, and led to the US Food and Drug Administration (FDA) approval of many therapeutic agents including ipilimumab (anti-cytotoxic T-lymphocyte antigen-4) and pembrolizumab (anti-programmed cell death-1) [75–79]. Unfortunately, though the addition of immunotherapy increases response rates, it does not benefit the majority of patients (less than 30%), partly due to several factors related to low intrinsic tumor immunogenicity and development of immune suppressive microenvironment [80–83]. Therefore, new therapeutic strategies are urgently need. Nanotechnology, using multifunctional nano-sized particles (size between 1 and 100 nm), has the potential to be such a therapy [84–86].

Nanoparticles can be categorized as organic (e.g., polymers, dendrimers, liposomes) or inorganic (e.g., silica, quantum dots, gold, silica) [52, 87]. These nanoscale agents are highly versatile in virtue of their small size, shape, and surface charge and can be manipulated to (1) increase tumor-specific delivery of cytotoxic payloads, due to leaky tumor vasculature [86], (2) prolong the serum half-life of polyethylene glycol (PEG)-modified nanoparticles compared to the free drug [88], (3) improve tumor targeting by functionalizing nanoparticles with ligands, peptides, or antibodies [87], (4) indirectly control the subcellular localization and kinetics of payload release (e.g., pH, light, temperature), and (5) utilize their theranostic properties to perform dual diagnosis and therapeutic treatment [52, 87, 89, 90]. These diverse properties make nanoparticles clinically relevant, as they allow noninvasive assessment of therapeutic response, providing valuable information to refine treatment dosage and scheduling. For instance, Doxil, a clinically approved liposomal modified version of doxorubicin, was reported to considerably improve objective response rates (ORR) and reduce cardiotoxicity compared to the free drug, as a result of increased serum half-life and specific tumor accumulation [91]. Of late, the NCT01696084 clinical trial revealed for the first time the synergistic potential of the FDA approved (2017), VYXEO (bilamellar liposomes entrapping two different drugs: cytarabine and daunorubicin) in treating acute myeloid leukemia (AML) [15]. Moreover, Aghighi et al. [92] revealed the noninvasive diagnostic ability of ferumoxytol in quantifying tumor associated macrophages (TAM) in patients bearing lymphoma and bone sarcoma tumors using magnetic resonance imaging (MRI). These results clearly highlight the nanoparticles potential in improving conventional cancer therapies and targeted immunotherapies. It is for these reasons that this chapter will firstly aim to highlight the therapeutic benefits of nanotechnology with respect to cancer imaging and theranostic treatment. Thereafter, we will discuss the contribution of nanotechnology in enhancing antitumor immunity, when combined with targeted immunotherapies.

11.3.1 Cancer Nanomedicine and Image-Guided Surgery

Surgical resection of nonmetastatic palpable tumor remains one of the most common therapeutic options to treat most cancers (63–98%), including lung, bladder, breast, and colorectal cancer [93, 94]. However, these surgical interventions are mostly performed using the naked eye, and residual positive tumor margins equipped with metastatic potential are often left unresected; this can cause clinically observed relapses, thus compromising long-term patient survival [93, 95–97]. Therefore, it is of paramount importance to completely remove the tumor tissue, as recurrence in about 30% of breast cancer patients has been associated with increased incidence of positive tumor margins following lumpectomy (breast-conserving surgery). To mitigate these undesired effects as well as the diagnostic limitations of conventional

imaging procedures (e.g., RT, MRI, PET, ultrasound) in providing high-resolution tumor contrast images, image-guided surgery (IGS) was developed [98–100]. IGS refers to diagnostic modality relying on tumor-specific accumulation of light-sensitive fluorophores to specifically distinguish neoplastic tissues from their healthy counterparts [101]. The clinical utility of IGS using the prodrug 5-aminolevulinic acid (5-ALA) was reported by Stummer et al. [102] following the tumor-specific accumulation of its fluorescent protoporphyrin IX (PpIX) byproduct, which contributed to an increased detection and total gross glioblastoma resection rate (from 36 to 65%) compared to the control [102]. This latter result was corroborated by Valdes et al. [103] who achieved improved resection of meningioma tumor tissues using 5-ALA, which gained FDA approval in 2017 for neurosurgical interventions of brain tumors [102, 103]. Nevertheless, the diagnostic potential of 5-ALA can be compromised by other endogenous fluorophores (e.g., heme, flavin, lipofuscin), capable of competing with 5-ALA for visible light absorption, thereby reducing light-tissue penetration while increasing background fluorescence [101]. To bypass these limitations, IGS using near infrared (NIR) dyes was developed [93]. The advantage of NIR dyes are that (1) they are activated using longer wavelength (700–800 nm) that can penetrate deeper into the tissue (several millimeters), (2) they generate real time higher resolution images, and (3) they produce minimal autofluorescence, as most endogenous fluorophores cannot absorb at these wavelengths [93, 98, 104]. IGS using NIR dyes has been widely applied clinically using the FDA approved indocyanine green (ICG) for the detection of solid tumors or peripheral lymph nodes assisting tumor dissemination to distant organs [93]. For example, Lieto et al. [100] reported that ICG-based IGS was able to improve the detection and removal of primary liver tumors and metastatic lesions, that could not be detected by naked eye surgery. Similarly, Cho et al. [101] showed ICG's ability to specifically detect intracranial neoplasms including gliomas, meningiomas, chordomas, and craniopharyngiomas. However, ICG-guided intraoperative interventions can be limited, as a result of reduced serum half-life or tissue inflammation that can cause unspecific accumulation in nonmalignant tissues [93, 105]. Palliative approaches to undermine these effects were developed by Souchek et al. [98] entrapping both ICG and Cy7.5 dyes within chemically modified hyaluronic acid nanoparticles. During this study, compared to free ICG and Cy7.5, ICG and Cy7.5 encapsulated in nanoparticles were shown to exhibit superior tumor-specific detection signals in poorly vascularized xenograft model of prostate tumors [98]. Likewise, Tanner et al. [93] reported superior tumor-specific accumulation and contrast enhancement of ICG-based nanoparticles compared to their drug-free counterparts using a xenograft model of breast cancer. Moreover, Philp et al. [104] have highlighted the promising imaging properties of the self-assembling porphyrin-like lipid nanoparticles known as porphysomes (100–150 nm diameter) in easing the detection and removal of endometrial primary tumors, as well as abdominal and metastatic lymph nodes. While these nanoparticles are clearly able to improve tumor detection, their therapeutic benefits still have to be assessed by evaluating their capacity to reduce tumor relapses and prolong patient survival

post-IGS. Alternatively, these NIR dyes can be used for theranostic treatment, representing a light-dependent strategy that can be used for fluorescent diagnosis and therapeutic purposes depending on the type of wavelength used and the malignancy [74, 76].

11.3.2 Contribution of Nanotechnology in Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) for cancer is performed by irradiating a photosensitizer (PS) in the presence of molecular oxygen to process to singlet oxygen ($^1O_2$) and levels of ROS high enough to cripple the cancer's resistance mechanisms and induce cell death through necrosis, apoptosis, autophagy, vascular damage, and/or local and systemic inflammation [73, 106, 107]. PSs are ideal therapeutic compounds because they are harmless at ground state, and only upon light activation do they get activated to exhibit their cytotoxic properties. When exposed to its peak absorption wavelength, the PS enters an excited triplet state through "intersystem crossing." The interaction of the now excited PS with nearby oxygen molecules induces ROS production [73, 75]. Some PSs currently under investigation for their theranostic promise include hypericin, 5-ALA, chlorin e6 (Ce6), and NIR compounds, such as IR700. Hypericin is derived from Saint John's wort (*Hypericum perforatum L.*) and absorbs UVA (315–400 nm, with peak absorbance at 351 nm) and visible light (548–593 nm, with peak absorption at 563 nm) and emits at 594–642 nm, with peak emission at 600 nm; it has shown to be effective in eliminating cancerous skin and has recently been used in phase-III clinical trial to treat cutaneous T-cell lymphoma (CTCL) [75, 108–110]. Ce6 is a second-generation PS that displays peak absorption at 399 nm and peak emission at 668 nm [111, 112]. 5-ALA has an excitation wavelength of 410 nm and emits at 628 nm and has shown to be effective in treating several dermatological diseases [113]. Additionally, 5-ALA is desirable as it allows for repeated doses with no damage at the application site, and it has shown favorable cosmetic results in addition to functional recovery of the skin at the application site [114]. However, of late, NIR compounds are gaining popularity for several reasons. For example, they cannot be interfered with by normal daytime visible and UV light; they are less susceptible to light absorption and scattering; and they provide better penetrability into the deeper layers of the skin, resulting in superior tumor-to-background ratios and thus higher therapeutic potency with fewer off-target effects [115, 116]. In particular, IR700 has recently been widely investigated. It absorbs light at 658–758 nm, with peak absorption at 689 nm [117, 118]. PDT has thus far been approved for clinical trial, with promising results for the treatment of several malignant and nonmalignant skin diseases, including melanoma, squamous cell carcinoma, thyroid, breast cancer, esophageal cancer, actinic keratosis, and condyloma acuminatum, among others [73, 120–126]. Moreover, this approach is considered to be minimally invasive [127].

However, despite its benefits, PDT depends on the passive accumulation of the PS compound in tumor tissue; the technique thus lacks specificity and healthy tissue surrounding the tumor can be damaged. This “scattergun effect” can lead to side effects, such as off-target necrosis and severe photosensitivity [75, 76]. In addition, poor biodistribution of the PS agent and incomplete cellular uptake lead to suboptimal treatment. There are several strategies that can be employed to overcome this limitation and ensure the active targeting of relevant biomarkers. One is the use of antibody technology, which enables us to greatly reduce systemic and dose-limiting toxicity through the use of photoimmunotherapy (PIT). This will be discussed in the following section. The other is the use of nanoparticles as a drug delivery system, which will be also be discussed in detailed in the sections to follow.

Nanomedicines have been studied for their ability to refine site-specific drug delivery and their potential to enhance the biodistribution, cellular uptake, and immunotherapeutic effectiveness of various agents, including PSs [84]. This technique offers several valuable advantages in PDT, in which PSs can be either encapsulated within or conjugated to nanocarriers [52, 128–130]. A key advantage of encapsulating cytotoxic agents is that they are protected from degradation as it circulates in the blood [131–133]. Furthermore, nanoparticles provide the possibility to control drug release—ensuring that off-target effects are minimized—and they allow for hydrophobic compounds to be circulated in the blood [74, 134]. In one study, nano-PDT using pyrolipid-loaded inorganic nanoparticles showed improved immunotherapy outcomes by elevating proinflammatory cytokines in the serum and increasing the infiltration of CD4+ and CD8+ T cells into the tumor [135]. It has also been shown that PDT nanotherapy, using Ce6 and other compounds, has the ability to amplify immunogenic cell death (ICD) [136–138]. ICD occurs when destruction of a cancer cell releases damage-associated molecular patterns (DAMPs), such as calreticulin (CRT), adenosine triphosphate (ATP), group box-1 protein (HMGB-1), and heat shock protein 70/90 (HSP70/90), which are normally expressed intracellularly but when exposed extracellularly are able to initiate an immune response against the tumor [39, 75, 110, 139, 140].

Although rare, nano-PDT has, furthermore, been associated with the abscopal effect, whereby local tumor treatment at the primary site is able to elicit an immune response resulting in the regression of distant growths and a reduction in the number of untreated metastases [141]. When used in combination with immunotherapies such as anti-CTLA-4 and anti-PD-L1, nano-PDT has been shown to augment ICD and the abscopal effect, thus mediating reduced tumor growth, reduced tumor burden, and increased survival [74]. In one mouse study, ICG and the toll like receptor-7 (TLR7) agonist, imiquimod, were coencapsulated in polymeric nanoparticles to form a dual nanotherapy. The authors observed increased dendritic cell maturation and elevated IL-12p70, IL-6, and TNFα secretion, leading to reduced metastasis [142]. Another study used the pyrolipid photosensitizer in combination with the chemotherapeutic drug, oxaliplatin, coencapsulated in a core–shell nanoscale coordination polymer. The results indicated that ICD and a resultant antitumor immune response were triggered [143, 144]. These findings are in accordance with those

reported by Muhanna et al. [90] and Muhanna et al. [120] demonstrating the theranostic potential of porphysomes in a clinically relevant animal model of thyroid cancer and head and neck cancer. In addition to these uses, pilot clinical trials examining the theranostic potential of gold (Au) nanoparticle-Ce6 PDT are currently underway [145]. In summary, while PDT alone has already shown some promise as a minimally invasive therapeutic strategy to combat cancer, by incorporating nanoparticles, we might be able to refine the drug delivery process, minimize adverse effects, and maximize clinical outcomes.

11.3.3 Photoimmunotherapy

Monoclonal antibodies (mAbs)—such as trastuzumab, developed for the treatment of HER2-positive breast cancers—are able to selectively identify biomarkers such as tumor-associated antigens, making them promising tools for targeted cancer immunotherapy, whereby antibodies are used to direct modulatory effects to a specific cellular target [146, 147]. Cell death is induced upon antibody-antigen coupling and may occur through (1) antibody-dependent cellular cytotoxicity (ADCC), in which immune response are activated via the fragment crystallizable (Fc) region, (2) complement-dependent cytotoxicity (CDC), in which cell lysis is induced via the Fc region, or (3) inhibition of an aberrant signaling pathways, resulting in, for example, suppressed proliferation and survival pathways. Alternatively, drugs may be delivered in the form of an antibody–drug conjugate (ADC) [148–150]. This ability of mAbs to selectively target cell surface markers unique to a specific disease modality enables us to overcome the problem of passive and off-target drug accumulation, for example, that associated with PDT. Instead, targeted drug delivery to cells of our choosing is possible, thus eliminating systemic side effects and damage to neighboring tissue. Several ADCs have been developed and approved for cancer immunotherapy [76, 151–153].

ADCs are preferable over naked mAbs because the latter are limited in their efficacy in so far as the inhibition of a particular receptor is able to induce cell death or elicit an immune response. Conjugation of a drug to a ligand also increases the construct's serum half-life, which allows for the unbound conjugate to be redistributed and specifically internalize in target cells [119]. Furthermore, because cancers can become resistant to directed mAbs, attaching a drug molecule greatly increases the likelihood of successful treatment, as ADCs allow for directed and controlled drug release and, subsequently, relatively rapid cell death; the combination of selectivity and controllable drug release means that lower dosages of ADCs compared to mAbs can elicit comparable responses. Resistance to a particular ADC will therefore develop significantly later than that to the constitutive naked mAb, thus widening the therapeutic window and allowing for repeated drug application [154].

Photoimmunotherapy (PIT) utilizes the cytotoxic effectiveness of PDT and enhances it using the targeting precision of immunotherapy to form an

antibody-photoconjugate (APC). Recent work in this field has demonstrated that it is a promising cancer therapy. Thus, while PDT depends on the passive accumulation of drugs in the tumor tissue, PIT is an active targeting strategy [155, 156]. This greatly reduces the risk of off-target accumulation and prevents the damaging scattergun effect of PDT, thus reducing systemic toxicity. In 2011, Hussain et al. provided proof of concept for SNAP-tag-based PIT using the anti–epidermal growth factor receptor (EGFR) antibody fragment, scFv-425. They generated fusion proteins conjugated to the Ce6 PS to target and kill EGFR-positive epithelial cancer cells and were able to demonstrate selective binding of their construct to EGFR-positive cells as well as the unique destruction thereof [157]. Research in this area has lately been increasing significantly, and several groups have shown the effective use of PIT—and in particular, NIR-PIT—in various types of cancer, including breast cancer, glioblastoma, and melanoma, among others [156, 158–161]. In 2015, Kishimoto et al. used panitumumab-IR700 APCs to assess their effectiveness in prolonging survival in mice [162]. In vivo, they observed a reduction in tumor size, which occurred selectively in EGFR-positive tumors. In 2016, Amoury et al. developed a multiplex panel of photoimmunotheranostic agents against triple-negative breast cancer biomarkers, that is, chondroitin sulfate proteoglycan 4 (CSPG4), EGFR, and epithelial cell adhesion molecule (EpCAM), which were coupled to the PS IR700 [156]. They were able to demonstrate effective tumor imaging in selected cell lines and in ex vivo biopsies, as well as potent phototoxicity in targeted cells. Moreover, they found that when used in combination, all three constructs amplified the efficacy of the therapeutic response. In 2016, Ito et al. investigated the effectiveness of human epidermal growth factor receptor 2 (HER2)-specific photoimmunotherapy with IR700 [155]. They generated trastuzumab-IR700 and pertuzumab-IR700 theranostic constructs and used cell culture models and xenograft mouse models to demonstrate the effectiveness of the constructs in initiating tumor cell death, particularly when applied in unison. In 2017, Zhen et al. demonstrated the antitumor efficacy of NIR-PIT through indirect cancer-associated fibroblast (CAF) destruction using ferritin nanoparticles, which served as a photosensitizer carrier conjugated to target and kill CAFs overexpressing antagonist receptor ligand (fibroblast activation protein alpha) [163]. This nano-PIT was able to amplify antitumor immunity through enhanced T-cell infiltration following the destruction of the extracellular matrix, which acts as a physical barrier, as well as the depletion of C–X–C motif chemokine ligand 12 (CXCL12), which can activate immune suppressive mechanisms [163]. With these accumulative findings, a phase II clinical trial was successfully initiated and revealed the safety and efficacy of cetuximab-IR700 based NIR-PIT in treating both localized and metastatic lesions of squamous cell head and neck cancer patients [117]. Based on these promising results, a phase III trial (NCT03769506) has begun to compare the efficacy of cetuximab-IR700 against routinely used methotrexate and docetaxel [117].

11.3.3.1 Conclusion and Future Directions

Research in nanomedicine continues to increase, with nano-PIT offering solutions to many of the obstacles facing conventional diagnostic and therapeutic methods. The standard methods for tumor diagnosis and evaluation are biopsy and morphological examination. Unfortunately, these methods are invasive and lead to late diagnosis as they rely on pathological changes that typically indicate late stage tumor progression, such as tumor size and gross morphology [155, 162]. However, in PIT, due to the innate fluorescent properties of photosensitizers, the same agent can be used to detect and image a tumor, monitor accumulation of the PS, treat cancer, and track treatment-induced changes (such as size, location, and receptor expression). Tumors can be both assessed through real-time monitoring and treated noninvasively simply by changing the wavelength of the light to which the agent is exposed [107, 157, 164]. Ultimately, PIT is a theranostics approach, allowing for both diagnosis and treatment at an early stage.

In developing future therapeutic strategies, researchers should aim to clinically assess how they might improve the efficacy of PDT, as Chen et al. did in their 2015 report in which they developed a nanoparticle system composed of nanoscintillator and mesoporous silica containing photosensitizers, which could be indirectly activated using X-ray photons to efficiently damage tumor cells to a greater extent than PDT [165]. This nanosystem was very advantageous in that it enhanced tissue penetration (in virtue of X-ray properties), permitting treatment of disseminated tumors [165]. Similarly Zhang et al. [166] reported the radio-enhancing potential of hafnium oxide (NBTX3) nanoparticles in shrinking colorectal tumors more effectively than RT alone. This NBTX3-mediated killing efficacy was achieved through RT-induced genotoxic stress and immune mediated tumor destruction of both irradiated and nonirradiated tumors (abscopal effect) [166]. This promising results led to the clinical usage (2019) of NBTXR3, which once administrated intratumorally in soft tissue sarcoma, produces superior therapeutic efficacy following RT activation compared to RT alone [52, 167, 168]. Notably, Hu et al. [169] showed that NBTX3 was able to synergize with immune cell blockage agents such as anti-PDL-1 and anti-CTLA-4 to significantly reinvigorate previously exhausted cytotoxic T-lymphocytes to amplify antitumor immunity against lung and colorectal xenograft model of human cancers.

These aforementioned studies give us some insight into the direction and future possibilities of nanoparticle-mediated PDT, immunotherapy, and PIT. Nanomedicine might enable us to enhance current strategies by improving drug biodistribution and uptake efficiency, increasing the selectivity of drug uptake, offering highly controlled drug release, and allowing for dual therapies through coencapsulation. Moreover, when used in unison with PDT and immunotherapy, nanoparticle therapy shows potential in its ability to intensify to effects of ICD and the abscopal effect. With the favorable findings thus far in this field, the incorporation of nanomedicine into PDT, immunotherapy, and PIT hold promises as an efficacious anticancer treatment.

11.4 Nanorobots

Nanorobots or nanobots are tiny nanoscale devices (robots) typically of the size ranging from 0.1 to 10 μm and designed specially to carry small molecular components. This tiny size is comparable to the size of a virus (0.1 μm) or a human cell (10 μm). They have a unique ability to be programmed to carry out specific functions. One of the most common use of nanorobots can be found in assembling nucleic acids in these tiny robots, a technique called DNA origami.

Nanorobotics includes technologies that can manipulate biological cells or components and/or include assembling tiny nano-sized materials. These nanorobots are essentially nanoelectromechanical devices that can be controlled, sensed, programmed, and directed to perform a desired function. Since there are different electric and mechanical aspects to this unique technology, designing efficient nanorobots is not easy and is still in its infancy; however, the fact that its size is comparable to animal cells, its success can have significant impact especially in the field of medicine. A typical example of its importance in medicine can be deduced from its ability to be programmed to carry a specific drug to its target site and help carry out a specialized function at a precise location. For instance, a nanorobot can be loaded with an anticancer drug and delivered to a tumor site and thereby help reduce the burden of tumor growth directly by causing cell death, without any potential off-site targets. However, despite the tremendous potential of these nanorobots to have a significant impact in translational medicine, its size is still a vital factor to further its utility in medicine. Since bacteria range in size about 1 μm, which is too large to enter cells, the size of a nanorobot would have to be much smaller. Till date, a smallest nanorobot, about size 0.12 μm with an ability to cross cell membranes, was created by Professor Ruyan Guo and Amar Bhalla at the University of Texas at San Antonio. This holds the *Guinness Book of World Records* title for this smallest nanorobot.

11.5 Bioactive Nanomaterial in Bone Grafting and Tissue Engineering

Bone fracture, osteoarthritis, osteoporosis, rheumatoid arthritis, and other musculoskeletal disorders are the most prominent clinical problems related to bone and skeletal defects [170]. Presently, musculoskeletal related diseases resulting in inflammation (e.g., rheumatoid arthritis) or tissue degeneration (e.g., osteoarthritis) mostly account for the observed disability associated diseases worldwide [170, 171]. Interestingly, a 2019 Global Burden of Diseases (GBD2019) study performed by the Institute for Health Metrics and Evaluation (IHME), revealed that musculoskeletal disorders induced a worldwide burden of about 150075329.9 disability-adjusted life-years (DALYs: representing a measure of healthy life deprivation due to premature death or disability related diseases), mostly comprising rheumatoid

arthritis, osteoarthritis, low back/neck pain, gout, as well as other musculoskeletal disorders [171]. Therefore, understanding the bone anatomy becomes important.

The bone can be defined as a very dynamic anatomical scaffold of the human body supporting much of the body weight such as the connective tissue [170, 172]. Additionally, it is (bone) endowed with many regulatory functions involving mineral homeostasis and hematopoiesis. The bone is majorly composed of minerals (70%) and minimally of collagen (30%) and bone derived cells including osteoblasts, osteoclasts, lining cells, progenitor cells, and adipocytes accounting for 2% [173]. Moreover, the bone is able to operate self-repair of small defects throughout lifetime through a coordinated physicochemical and mechanical actions of mesenchymal stem cells (MSCs), osteogenic and immune cells [174, 175]. In contrast, larger bone defects which may be caused by trauma, infection, surgical excision, congenital malformations or tumors are unable to achieve similar self-repairs [175, 176]. Thus, finding novel therapeutical approaches becomes pertinent to overcome this clinical hurdle.

Bone grafting represents such a therapeutic option, clinically used as a gold standard treatment for bone repair [174, 175, 177]. Among bone grafting, two methods defined as allografting (derived from genetically unrelated subject) or autologous grafting (obtained on the same individual at a different site) can be distinguished based on the graft donor's origin [174, 175, 177]. The principle of these bone grafting methods is to use living tissues (e.g., MSCs) together with a bone scaffold (natural or synthetic possessing a mineralized matrix) to enable bone healing, once transplanted within the damaged bone site. Despite the therapeutic efficacies of the latter, they may suffer some limitations, related to the development of immunogenicity (possible immune rejection in host of allogenic tissue), quality of donor's bone or shape (which should match with defect site), insufficient access of graft tissue, morbidity/pain at the donor site (in autologous grafting), and high transplant failure rates (more than 50%) [174, 175, 177]. Considering what precedes, it becomes evident that alternative therapeutic approaches using synthetic materials are needed to address these unmet medical needs. Of late, nanotechnology using naturally or synthetically derived nanomaterials having a nano size inferior to 100 nm has shown some promising results in bone tissue regeneration (BTE) as well as in other biomedical applications, where they are used either as a drug delivery carriers or as a diagnostic and therapeutic agents like in cancer particularly [74, 89, 173, 174]. The therapeutic properties of nanomaterials were exemplified by several reports, demonstrating the abilities of gold nanoparticles (GNPs) to reach BTE clinical fruition, based on their capacity to promote mineralization; osteoconductive and osteoinductive differentiation of osteoblastic precursor cell lines through successive ERK/MAPK pathway activation and increased osteogenic gene expressions [174, 178]. However, it is worth mentioning that the type of scaffold used can profoundly influence the success of bone regeneration [179, 180]. Ideally, the best scaffolds should aim (1) at providing structural support by acting as an extracellular matrix (ECM) on which osteogenic progenitor cells can easily adhere, proliferate, and differentiate, (2) have minimal cytotoxicity and immunogenicity, (3) be porous and possesses biodegradable properties allowing for diffusion of nutrients, growth

factors, and metabolite necessary for new tissue formation, and finally (4) exhibit stable mechanical properties sustaining healing and the recapitulation of bone defects functional activities [179, 180]. To this end, many bioactive and biodegradable scaffolds such as hydrogels have been developed and used in combination with nanomaterials to achieve successful BTE. This section will discuss on the recent advances on the therapeutical usage of nanoparticles and bioactive nanomaterials in BTE.

11.5.1 Biomaterials Used in Bone Repair

Biomaterials in bone tissue engineering (BTE) consist of diverse natural and artificial materials used to regenerate bone tissues [173]. Depending on their origin, they can be categorized either as (a) natural (e.g., autologous bone graft, naturally obtained hydroxyapatite or algae derived calcium phosphate), (b) synthetic (e.g., ceramic derived material like tricalcium phosphate and hyaluronic acid), or (c) a combination of materials such as ceramic with polymers [181, 182]. While natural and synthetic biomaterials may possess good osteoconductive properties, their bone regenerative capacities might be limited as a results of poor osteoinductive capacity [181]. Efforts to overcome this limitation have led to their combination (biomaterials) with specific biological molecules including growth factors, hormones, drugs, or the development of better 3D scaffolds known as hydrogels [179, 183, 184].

11.5.2 Hydrogels

Hydrogels are defined as three-dimensional scaffolds which can be synthesized physically or chemically through cross-linking of hydrophilic polymers [179, 181, 184]. Similar to soft tissues, they have a high water retention potential and possess a porous versatile framework which best mimic the extracellular matrix (ECM) microenvironment that is very conducive for chondrocytes and osteoprogenitor cell adhesion, and growth and differentiation to osteoclasts [179, 181, 184]. Moreover, hydrogels have received widespread interest, because of elasticity easing their adaptation or adjustment to surrounding tissues, thus consequently minimizing an immunogenic response, as they do not require complex surgical interventions that other therapies need [179, 181, 184]. Besides the latter, they are equipped with the ability to entrap various molecules, drugs, or proteins and can timely control their release as required, while increasing their circulation half-life. Based on these observations, our next step is to introduce different subclasses of hydrogels.

11.5.3 Naturally Derived Hydrogels

Hydrogels can be synthesized using naturally or derived proteins such as fibrin, collagen, and gelatin or using carbohydrate molecules known as chitosan, hyaluronan, and alginate [185]. Their therapeutic usage mainly relies on their ability to facilitate cellular interactions and their biodegradable properties. For example, chitosan, a natural deacetylated polysaccharide from chitin found within the exoskeleton of crustacean shells (e.g., crabs), yeasts, and fungi, has been used in bone regeneration [186]. However, its therapeutic usage in bone regeneration might be compromised by its reduced mechanical and bioactivity [179, 186]. Efforts to bypass this hurdle, led to its combination with other biopolymers (e.g., collagen, alginate, and silk fibroin) or other molecules such as vascular endothelial growth factor (VEGF), which improved its bioactivities manifested through increased biomineralization and protein absorption [186]. For example, Poth et al. [187] demonstrated that chitosan–tripolyphosphate (CS–TPP) loaded nanoparticles coated with bone morphogenic protein-2 (BMP-2) were able present BMP-2 to titanium-coated bone implants and promote ectopic bone growth. In spite of their improved bioactivities, chitosan composites are not able to improve its biodegradation rates or thermal stability [181, 186]. Furthermore, hydroxyapatite (HA), calcium phosphate, and collagen accounts for most of the bone tissue [173]. Recently, Zhang et al. [188] demonstrated that aspirin-loaded chitosan nanoparticles with an asymmetric collagen chitosan membrane improved osteogenic potential through continued aspirin release at the defect site. Likewise, multiple studies reported the beneficial effects of collagen loaded nanoparticles as a scaffold enhancing osteogenic potential [173, 181]. Conversely, collagen may be limited by its poor mechanical strength which can compromise bone regeneration [181]. In comparison to collagen, polyethylene glycol (PEG) gelatins are less immunogenic and possess superior mechanical strength, with adequate degradation rates and cellular integration favoring osteogenic cell proliferation and differentiation [181].

11.6 Emerging Applications: Nanotechnology and Electronics, Nanotechnology and Industry, Nanotechnology and Environment, Nanotechnology and Warfare

11.6.1 Nanotechnology and Electronics

Nanoelectronics devices have critical dimensions ranging from 1 to 100 nm [189]. The application of nanotechnology in electronic components is known as nanoelectronics. The phrase refers to a wide range of technologies and materials that have the common feature of being so tiny that inter-atomic interactions and quantum

mechanical characteristics must be thoroughly investigated. Hybrid molecular/semiconductor electronics, one-dimensional nanotubes/nanowires (e.g., silicon nanowires or carbon nanotubes), and advanced molecular electronics are some of the examples in this category. Recent silicon MOSFET (metal–oxide–semiconductor field-effect transistor, or MOS transistor) technology generations, such as 22 nm CMOS (complementary MOS) nodes and subsequent 14 nm, 10 nm, and 7 nm FinFET (fin field-effect transistor) generations, are already within this management. Because current candidates differ considerably from classic transistors, nanoelectronics is sometimes seen as a disruptive technology.

11.6.1.1 Devices Made of Nanoelectronics

Current high-tech manufacturing methods are based on classic top-down approaches, with nanotechnology having previously been quietly implemented. When it comes to the gate length of transistors in CPUs or DRAM devices, the crucial length scale of integrated circuits is already at the nanoscale (50 nm and lower).

Computers and displays: Nanoelectronics has the potential to make computer processors more powerful than those made with traditional semiconductor production processes. New kinds of nanolithography, as well as the usage of nanomaterials such as nanowires or tiny molecules in place of standard CMOS components, are currently being investigated. Carbon nanotubes (CNT) and/or silicon nanowires might be used to create low-energy displays [189]. These nanostructures are electrically conductive, and their tiny dimension of a few nanometers allows them to be utilized as field emitters for field emission displays with exceptionally high efficiency (FED) [190].

Memory storage: In the past, electronic memory systems depended heavily on the creation of transistors. However, research into crossbar switch-based circuits has provided an alternative, allowing for extremely high-density memory to be created utilizing programmable interconnections between vertical and horizontal wire arrays. Nanoelectronic memory became commercially available in the 2010s. SK Hynix started mass-producing 16 nm NAND flash memory in 2013, while Samsung Electronics started mass-producing 10 nm multilevel cell (MLC) NAND flash memory in 2014. TSMC began producing SRAM memory using a 7 nm technology in 2017.

Production of energy: Nanowires and other nanostructured materials are being studied in the hopes of producing cheaper and more efficient solar cells than traditional planar silicon solar cells. The development of more efficient solar energy is thought to have a significant impact on meeting global energy demands.

Diagnostics in medicine: The construction of nanoelectronic devices that might detect concentrations of biomolecules in real time for use as medical diagnostics, and therefore the area of falling under nanomedicine, has sparked a lot of attention [191, 192]. A distinct line of study called nanosensors aims to develop nanoelectronic devices that can interact with single cells in order to aid fundamental biological studies [193].

11.6.2 Nanotechnology and Warfare

Nanotechnology in warfare is a subclass of nanoscience in which molecular systems are planned, manufactured, and constructed on a nanoscale (1–100 nm). The broad application of such technologies, particularly in the areas of combat and defense are huge, which has opened the path for future weaponization research. Materials science, chemistry, physics, biology, and engineering are few of the scientific areas that nanotechnology brings together [194]. Advances in this field have resulted in the categorization of such nanoweapons, which include small robotic devices, hyperreactive explosives, and electromagnetic supermaterials [195, 196], With this technical advancement led to the implications of related dangers and ramifications, as well as regulatory measures to counteract these consequences. These consequences raise concerns about global security, societal safety, and the environment. Because of the potential advantages or risks of its usage, legislation may need to be continually reviewed to keep up with the dynamic growth and development of nanoscience. Regulating or anticipating such consequences via legislation would exclude or "avoid irreparable harm" from deploying defense-related nanotechnology [197].

Nanotechnology has been used extensively in combat and defense in the past. Several countries, including China, the UK, Russia, and, most importantly, the USA, have supported military uses of this technology during the last two decades. The U.S. government has long been seen as a national leader in nanotechnology research and development, but it is now being challenged by foreign competition as recognition of the technology's importance grows. As a result of the growth of this domain, military interests in the use, or misuse, of its power have a prominent platform. Recent military nanotechnological weapons research has focused on the development of defensive military equipment with the goal of improving existing designs of lightweight, flexible, and durable materials. These cutting-edge designs include elements that improve offensive tactics by using sensing devices and manipulating electromechanical features. Various examples related to nanotechnology and warfare are given below.

Battle suit for soldiers: The Institute for Soldier Nanotechnologies (ISN), form a collaboration between the US Army and MIT, let the US Army and MIT to focus financing and research development only on creating armor to improve troop survival.

Expanded materials: Sol–gel ceramic coatings have helped "materials that cannot tolerate high temperature" by protecting metals from wear, fractures, and moisture, as well as permitting adaptability to a variety of forms and sizes. Integrating fibre-based nanomaterials into structural elements like missile casings can reduce overheating and improve the materials' dependability, strength, and ductility.

Devices for communication: Soldiers and vehicles with tiny antenna beams, tags for remote identification, acoustic arrays, micro GPS receivers, and wireless communication are anticipated to be equipped with nanotechnology intended for improved communication. Nanotechnology makes it simpler to communicate about

defense issues since it uses less energy, is lighter, has higher power efficiency, and is smaller and cheaper to produce. Aerospace applications include solid oxide fuel cells that can supply three times the energy, surveillance cameras on microchips, performance monitors, and cameras that weigh as little as 18 g.

Mini-nukes: Small nanotechnologies are being used by the USA, as well as other nations like Russia and Germany, to create nuclear "mini-nuke" explosive devices. This weapon would weigh 5 pounds and have the explosive power of 100 tons of TNT, allowing it to obliterate and threaten mankind.

11.6.3 Nanotechnology and Industry, Nanotechnology and Environment

Nanotechnology is expected to be a major engine of technology and industry in the twenty-first century, promising higher-performing materials, intelligent systems, and new manufacturing processes with far-reaching implications for society. Modern fabrics will become "smart" in the mid-term, thanks to embedded "wearable electronics," and such innovative goods have a great potential, particularly in the sphere of cosmetics, as well as various possible uses in heavy industry. Nanotechnology is having an influence on consumer goods; nanomaterials are currently present in a number of products, many of which people are unaware include nanoparticles, and products with unique functionalities ranging from easy-to-clean to scratch-resistant. To name a few examples, lighter car bumpers, is more resistant clothing, radiation resistant sunscreen, stronger synthetic bones, lighter mobile phone display, longer shelf life of glass packaging for drinks, and various more durable sports balls [196].

Global climate change and pollution, such as air, soil, and water, are issues that need to be addressed in order for the environment to be sustainable. Even in large industrialized countries, climate change is still a contentious issue. Beyond technology's scope, direct proof from study, public awareness, and government should all work together to combat climate change. One of the most pressing issues confronting humanity is environmental conservation. We have inadvertently harmed our environment by producing and discarding plastics, contributed to climate change by extracting and burning fossil fuels, and contaminated our air and streams with human-made products over the years. Meanwhile, current technology can provide direct management of pollution from several sources, including heavy metals, organic chemicals, biological or radiation risks, oil spills, and microplastics, among others. The use of nanomaterials has recently substantially enhanced environmental technology, such as detection and cleanup [198]. This has shown how environmental nanotechnology is developing and adapting.

Bulk environmental materials, such as metals, metal oxides/sulfides, polymers, and carbons, are expected to have fascinating physiochemical characteristics down

to the nanoscale due to their large surface area-to-volume ratio. As a result, as compared to their bulk counterparts, varied functions and greater reactivity/selectivity might be predicted. Manipulation of nanomaterials in terms of morphology (particle size and shape), microstructure (pore and surface), and composition can expand the capabilities of environmental nanotechnology (heterojunction and fixing). The new conspicuous characteristics, such as electrical, thermal, optical, magnetic, and electrochemical qualities toward adsorptive and/or catalytic performances, therefore present new opportunities, and difficulties [199]. Environmental nanotechnology has had a significant influence on all aspects of the area, from detection through cleanup. Despite significant amount of research on nanoparticles have been carried out and published, the mature production of desired nanomaterials and their safe uses are not always possible. There are also problems for environmental nanotechnology, in addition to the generic challenges of nanotechnology.

Nanomaterials and its associated nanotechnology have opened huge potential for traditional environmental technologies. As a result, environmental nanotechnology is on the increase. Several effective applications have been proven, including new sensing and monitoring, selective adsorption on nanosorbents, nanomembrane separation, and environmental nanocatalysis. These serious concerns, however, continue to pose significant obstacles. It is necessary to do mechanistic research on both nanotechnology and environmental processes. The environmental risks of nanomaterials should also be fully studied by delving into the processes at the nano–bio interfaces. Environmental nanotechnology participation and initiatives would contribute to long-term sustainability.

11.7 Summary

Nanotechnology encompasses research and development of matter at atomic scale, that is aimed at improving our knowledge and application in biomedicine, energy storage, electronics, environment, and many others. It has soon been recognized as technology that is emerging to make a significant impact in multiple fields. One of the major utilities of nanotechnology has been its application in imaging, diagnostics, and drug delivery systems in health care. Although, for example, nanotechnology has considerably improved the clinical efficacy of surgical interventions and targeted immunotherapy, where it has achieved complete cures, while prolonging survival of metastatic patients, the clinical utility of nanotechnology may suffer various hurdles relating to the lack of specific tool and technologies enabling appropriate patient stratification. However, despite being in its infancy, nanotechnology is hailed as being a compelling technology that is multifaceted and has potential to present tremendous useful applications in energy conservation, cleaning the environment, electronics, textiles, cosmetics, and solving major health issues.

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Chapter 12
Laboratory Protocols in Medical Biotechnology I

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Abstract Medical biotechnology is a science that studies and includes research and development of technology used in the medical and pharmaceutical companies. Medical biotechnology utilizes living cells to do research and manufacture pharmaceutical products which help to prevent and treat diseases in humans. The medical biotechnology field has helped to develop drugs or vaccines to treat diseases. This chapter provides a brief account of medical biotechnology and describes the safety measures used in the medical biotechnology laboratory, preparation of various solutions required to carry out experiments involved in medical biotechnology, and some of the commonly used techniques involved in molecular biology. This chapter also discusses about the sterile methods that are very important for efficiency of results. Good lab practices are an integral part of conducting any research safely and so are in the medical biotechnology laboratory. Engineering controls help to limit exposure to hazards, and personal protective equipment, commonly referred to as "PPE," minimize exposure to hazards that cause serious workplace injuries, illnesses and can protect a researcher's body and cover many of the common routes of exposure and contaminations. In a medical biotechnology laboratory, working with biological material can sometimes pose a danger to laboratory staff and the environment. For this reason, the staff must correctly handle material according established standards. These standards comprise four biosafety levels (BSLs) that

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typically apply to biological materials. Medical biotechnology uses substances and produces by-products and mixtures of substances which can be hazardous to employees, contractors, and other people. Control of Substances Hazardous to Health (COSHH) is the law that requires employers to control substances that are hazardous to health. One can prevent or reduce workers' exposure to hazardous substances by finding out what the health hazards are; deciding how to prevent harm to health; providing control measures to reduce harm to health; making sure they are implemented; keeping all control measures in good working order; providing information, instruction, and training for employees and others; providing monitoring and health surveillance in appropriate cases; and planning for emergencies. While working in a laboratory, different concentrated stock and diluted solutions are used. Stock solutions are more stable than working solutions. Stock solutions are used to save preparation time, conserve materials, reduce storage space, and improve the accuracy with which working lower concentration solutions are prepared. Nucleic acids like deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) central to biotechnology and medicine because it provides the basic blueprint for all life; nucleic acid is a fundamental determinant of how the body functions and the disease process. DNA and RNA are also used to help to know different diseases and correcting genetic defects, treating diseases, preventing spread of diseases, and more. For most molecular diagnostic methods, nucleic acids must be isolated in pure form for downstream testing. Isolation of nucleic acids serves several purposes. Assessment of the amount and quality of isolated nucleic acids should be performed prior to testing. Several methods are available; UV spectrophotometry is an excellent method to simultaneously assess the amount and purity of nucleic acids. SDS-PAGE and western blot methods helps to separate and analyze the proteins. Histochemistry is an important technique that is used for the visualization and distribution of various chemical components of tissues through the use of stains, indicators as well as microscopy. Immunohistochemistry is (biology–chemistry) the analytical process of finding proteins in cells of a tissue microtome section exploiting the principle of antibodies binding specifically to antigens in biological tissues. In summary, proper instructions and safety are to be followed in the medical biotechnology laboratory. Appropriate and set protocols provide individual sets of instructions that allow scientists to recreate experiments in the laboratory. They provide instructions for the design, applying different methods and implementation of experiments that include the safety bias, reporting and troubleshooting standards for experiments.

Keywords Lab practices · Biosafety and laboratory hazards · DNA · RNA · Electrophoresis · Western blotting · Histochemistry and immunohistochemistry

12.1 Importance of Observing Healthy Lab Practices. Role of Gloves, Eyewear, and Lab Coats, 70% Ethanol

Using correct lab safety products are always suggested while handling science lab equipment. Gloves are highly recommended to guard your hands.

There are a variety of reasons to wear your laboratory gloves.

12.1.1 Defend Your Hand Skin

Working with several chemicals and blood samples of disease patients in a science laboratory can infect your hands. So always wear gloves whenever you handle infectious materials, chemicals, radioactive substances, and so on.

12.1.2 Avoid Stains

Gloves can prevent staining incidents. When you are managing lab equipment with bare hands, there is a risk that your skin may harm due to spillage of substances and fluids, which won't hurt the skin immediately but also can leave stains that may further cause burn or rashes.

12.1.3 Suspension of the Detrimental Effect

Whenever there is a spillage of blood samples or any other infectious liquids on your hands, you can immediately take off your gloves and wash your hands. There is always a chance that those fluids can seep through the gloves to your skin. Yet, if you are not wearing gloves, you will be exposed to harm immediately.

12.1.4 Use Appropriate Gloves According to Specific Applications

Different types of gloves are compatible for various applications.

For example,

1. Nitrile gloves for chemicals and infectious materials.
2. Neoprene gloves for oils and solvents.
3. Rubber gloves for mild corrosive materials.
4. Coated fabric gloves for moderate concentrate chemicals.

12.1.5 Eye Wear

Safety eyeglasses are crucial for lessening eye hazards. Defensive eyewear is mandatory in all science laboratories.

12.1.6 Lab Coats

Lab coats are also a mandatory laboratory practice. Lab coat should not be worn outside of the lab. Lab coats must be 100% cotton in science laboratory. Lab coats made of synthetic fibers are not allowed. Researchers who work with liquid pyrophorics, open flame, or high volumes of flammable liquids must wear fire-resistant lab coats.

12.1.7 70% Ethanol Solution

Using 70% ethanol in the science laboratory is recommended for disinfecting surfaces, equipment, and so on. 70% Ethanol alcohol solution kills microorganisms by dissolving the cell wall. The cell wall of Gram-negative bacteria consists of a thin layer of peptidoglycan that is easily destroyed by the alcohol.

12.2 Role of Biosafety Cabinets. Meaning of Biosafety Level (BSL), Difference Between BSL2 and BSL3 Laboratories

According to U965, biosafety is defined as “the discipline which addresses the safe handling and containment/control of infectious microorganisms and hazardous biological materials.” Based on the hazardous nature or risk group, different infectious microorganisms require specific laboratory practices, safety equipment and laboratory facilities. Biological safety cabinets (BSCs) are specially designed biocontainment equipment that are used to safely work with contaminated materials, for example, pathogens. Such equipment is also termed as biosafety cabinet or sometimes microbiological safety cabinet. This serves the purpose of providing safety to personnel handling, environment, and the product. The principle is that workspace is enclosed however properly ventilated such that its air circulation is not only well regulated but sufficiently filtered to carry out the practices of microbiology. BSCs use HEPA (high-efficiency particulate air) filters that are basically 0.22 μM porous membranes, enough (99.97% efficient) to block the entry of most of the microorganisms including aerosols. Based on what kind bio-contaminant or pathogen or

biosafety level you are dealing with, US Centers for Disease Control (CDC) the BSCs are classified into three types.

Biosafety Cabinet I: Provides protection to personnel and environment, however, doesn't protect the products being handled. Air regulation is such that the inward flow of air can contribute to contamination of samples. It is equipped with an exhaust blower to pull the air through-usually to the outdoors and finally BSCs of this class are either ducted or unducted.

Biosafety Cabinet II: It provides personnel, product, and environmental protection. BSII are the most widely used BSCs in clinical, hospital, life science, research, and pharmaceutical laboratories. Based on the air intake velocity; the amount of air recirculated over the work surface and the exhaust system, it is of four types:

1. Class II Type A1: Face velocity of air is 0.38 m/s, Airflow: 70% recirculates, 30% exhausted, Positive pressure in Plenum.
2. Class II Type A2: Face velocity of air is 0.51 m/s, Airflow: 70% recirculates, 30% exhausted, Negative pressure in Plenum.
3. Class II Type B1: Face velocity of air is 0.51 m/s, Airflow: 30% recirculates, 70% exhausted, Negative pressure in Plenum.
4. Class II Type B2: Face velocity of air is 0.51 m/s, Airflow: 0% recirculates, 100% exhausted, Negative pressure in Plenum.

Biosafety Cabinet III: This type is used to work with microorganisms assigned to BSL-4. It ensures the maximum protection to personnel and environment. The enclosure is gastight, and all materials enter and leave through a pass-through box. It includes Heavy duty rubber gloves attached and may be connected to a double-door autoclave. HEPA filtered air is supplied, and exhaust air is double HEPA filtered. Interior pressure is managed (124.5 Pa).

Based on the hazardous nature or risk group, different infectious microorganisms require specific laboratory practices, safety equipment and laboratory facilities. On that basis, laboratory facilities are designated as follows:

- Biosafety Level 1—Basic.
- Biosafety Level 2—Basic.
- Biosafety Level 3—Containment.
- Biosafety Level 4—Maximum containment.

Risk assessment by professionals is required to assign the biosafety level:

1. *Biosafety Level-1*: Suitable to work with well-characterized microorganisms that do not cause disease unhealthy adults and present minimal potential hazard to lab and environment. Ideal for Risk Group-1 agents that includes examples like *Lactobacillus*, *Bacillus subtilis*, and *E. coli* K-12 that pose no or low individual and community risk. It includes following of good microbiological practice and procedure. Minimal safety requirements are needed. Such labs exist in basic teaching and research.

2. *Biosafety Level-2*: Suitable for work involving agents that pose moderate hazards to personnel and the environment. Ideal for Risk Group-2 Agents like *Hepatitis B virus*, *Salmonella*, *Toxoplasma* that pose moderate individual risk, low community risk. Practices must be BSL-1 plus limited access. Under personal safety, biosafety cabinets (BSCs) or other approved containment devices are a must. PPEs like lab coats, gloves, face protection are needed. Protective clothing must be removed when personnel leave laboratory area. Facility provided must be BSL-1 plus the availability of a mechanism for decontamination (autoclave). Such types of labs are associated with primary health services, diagnostic services, research.
3. *Biosafety Level-3*: Suitable to work with agents that cause serious or potentially lethal disease in humans. Ideal for Risk Group-3 agents like *M. tuberculosis, SARS-CoV, Yersinia pestis* that pose high individual risk, low community risk. Practices must be performed in BSL-2 plus limited access. Under safety, biological safety cabinet and PPE are required similar to BSL-2. Respiratory equipment must be worn if risk of infection through inhalation is possible. The facility provided must have BSL-2 barriers with access through self-closing double doors, with corridors separated from direct access to lab. Single-pass negative directional airflow, that is, air handling systems to ensure negative airflow (air flows into the lab). Air pumped into lab is not recirculated in the building.
4. *Biosafety Level-4*: Suitable to work on agents that cause life-threatening disease in humans or animals. Suitable for Risk Group-4 that includes *Ebola virus*, *Marburg virus*, *Lassa virus*, and so on they are readily transmittable and cause serious human or animal disease. Practices require BSL-3 plus controlled access with safety equipment like full-body air-supplied, positive pressure personnel suit in addition to BSC. Facility must have dedicated air and exhaust system, decontamination procedures for exit, separate building, a recommended absence of windows (or sealed and resistant to breakage). Such labs are used to handle and study dangerous pathogens.

12.3 Laboratory Hazards: Health and Safety Regulations (COSHH-Control of Substances Hazardous to Health)

Awareness about the laboratory hazards and risks are among the essential prerequisites for a lab worker, be it the janitor, manager, or scientist. Depending on the type of work being carried out in the medical lab, it could be accordingly hazardous and risk-filled. Understanding those hazards and ways of their prevention is essential for a researcher. Modern-day laboratory hazards and risks can be classified into three categories based on their nature:

1. Chemical hazards; Medical laboratories are associated with handling different sorts of hazardous organic and inorganic chemicals that are toxic, readily reactive, corrosive, and flammable. Therefore, it is always recommended not to eat or drink in lab and don personal protective equipment (PPE) that includes

full-sleeve lab coats, gloves, goggles, masks, and so on. Moreover, a well-ventilated lab is mandatory to prevent lab mishaps like explosions from happening.

2. Biological hazards; Such hazards represent the most terrifying hazards that not only put lab workers at risk but under unfortunate leakage could subject environment to unforeseen dangers example the leak of deadly Anthrax (1979) in Russia. Biological hazards include infectious microbes, diseased animals, invasive plants, or GMOs that have the ability to pose any sort of risks to health and environment. However, with proper lab cleaning, sufficient storage and well-established protection/containment facilities, we can prevent biohazardous mishaps from happening.
3. Physical hazards; It includes the hazards posed facility or establishment, for example noise that can be disturbing enough to cause accidents, improper lab infrastructure like frictionless workplaces that readily slip the persons or equipment. Heating devices could also pose threats or improperly working machines that make noise or discharge currents. Sharp edges of glasses, machines, doors, or equipment also need to be kept in check from hurting the working personnel. Proper housekeeping and maintenance teams must work to make workplace safer and more efficient.

Keeping in view the necessity to control the exposure of hazardous substances to health, Nick Brown introduced The Control of Substances Hazardous to Health Regulations (COSHH, 2002) in UK under European Communities Act 1972, Health and Safety at Work etc. Act 1974. COSHH states the requirements or rules that are imposed on the employers to safeguard their employees from getting exposed to potential health risk substances. By saying substances, it includes any form of health hazardous materials like liquids, solids, fumes, fibers, dust, gases, pathogenic biological agents that could compromise the health safety of the workers. Under COSHH, employers are supposed to prevent exposures (provision of PPEs), ensure controlled measures (workplace cleaning and containment), risk management training to workers, handling the emergencies, ensure hazard surveillance in the facility, constant health-concerned supervisions. COSHH also expects the employees to cooperate with the employers in creating and maintaining a healthy and secure workplace. COSHH assessment includes the identification of the potential health hazardous substance at workplace, then understand its severity and ways of preventing its exposure.

12.4 Importance of Preparing Stock and Working Solutions, Concentration Terms and Calculations. Preparation of Stock Solutions of Reagents and Buffers

12.4.1 Preparation of working solutions from stock solutions

In simple words, stock solutions are concentrated solutions with accurately known concentrations made for the purpose to be used in future by diluting to any desired work-specific concentration. Importance of making stock solution includes saving

space, time and resources with evidently efficient outcomes. One of the highlighted benefits is the stability of concentrated solutions since they don't allow the bio-contaminations to grow. Stock solutions are commonly made in terms of percentage solutions (%), molar solutions (*M*) and most commonly *X* (times) solutions. Important tips while preparing stock solutions include following a standardized recipe, this way its less tedious and save time. Another one is recommended use of online tools or calculators to determine what goes how much to get desired concentrated solution. Finally, labelling is a must step, since you do not want to misplace or misput wrong ingredients in your working solution. Once the stock solution is ready, it's pretty easy to get a desired working solution from it as all it takes is just a formula known as dilution formula that is based on law of conservation of mass and goes like this:

$$M_{(\text{dilution})} \times V_{(\text{dilution})} = M_{(\text{stock})} \times V_{(\text{stock})}.$$

Let's say we need to prepare a 50 mL of 1 M solution from a 2 M stock solution.

Since we need to prepare 50 mL dilution of 1 M from 2 M stock, we want to figure out how much volume of stock needs to be diluted with solvent (water) to yield a 50 mL 1 M solution.

$M_{(\text{dilution})} = 1$ M
$V_{(\text{dilution})} = 50$ mL
$M_{(\text{stock})} = 2$ M
$V_{(\text{stock})} = ?$ mL

Therefore, we get; ? mL = $M_{(\text{dilution})} \times V_{(\text{dilution})}/M_{(\text{stock}),}$ that is, 25 mL.

That means we have to add 25 mL of stock and dilute it with 25 mL of water, to make 50 mL 1 M solution.

12.4.2 Importance of preparing stock and working solutions

Stock solution is a concentrate, that is, a solution to be diluted to some lower concentration for actual use. A stock solution is prepared by weighing out an appropriate portion of a pure solid or by measuring out an appropriate volume of a pure liquid, placing it in a suitable flask, and diluting to a known volume. The stock solution is used as such or used it as a component in a more complex solution and the solution that we make from the stock solution is called a working solution.

12.5 Advantages of preparing stock solutions

A stock solution is a known concentrated solution that is often times diluted to some lower concentration for laboratory use. Using a stock solution has several advantages, these include the following:

Reduced storage space: A single stock solution can be prepared and stored rather than storing several vials of working solutions.
Reduced preparation time: Working solutions can be prepared by diluting a stock solution.
Greater stability: Because stock solutions do not support bacterial growth they are more stable than working solutions.
Conserves precious materials.
Improves the accuracy of experiments.

12.6 Extraction of DNA from Bacterial Cell, Blood, and Liver Tissue (Fig. 12.1)

12.6.1 Cell Lysis and Protein Digestion

- Pellet cells and remove supernatant. Resuspend cell pellet in 390 μL of the cell lysis buffer. Animal tissue should be homogenized in appropriate amount of cell-lysis buffer to achieve uniform cell suspension.
- Add 10 μL of proteinase K (10 mg/mL). Mix by inversion and incubate overnight in 37 °C water-bath.

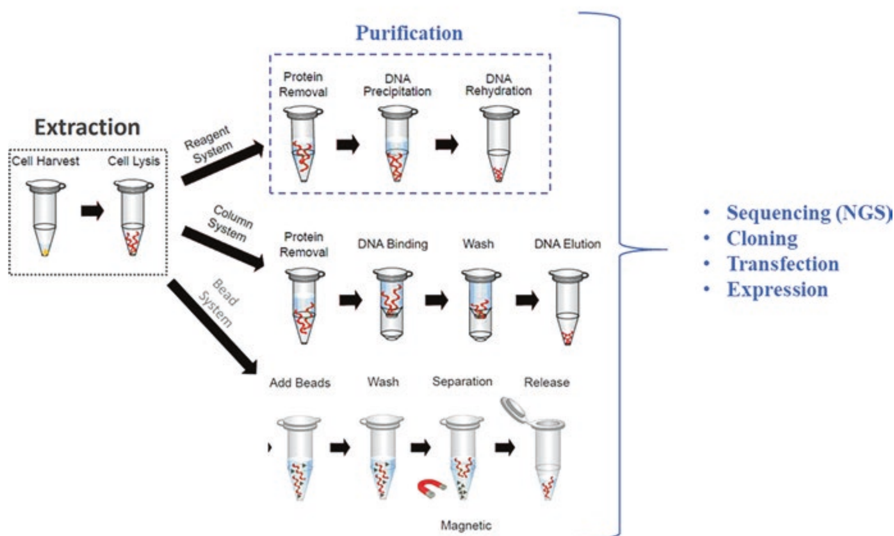


Fig. 12.1 Outline representation of different methods employed for extraction of nucleic acid from a sample containing cultured cells. The method is also applicable to other sample types like blood and tissue material, with additional processing steps at the beginning (also see Fig. 12.3)

12.6.2 Purification of DNA Via Phenol–Chloroform Extraction

- Add one volume of phenol–chloroform–IAA to lysed cell extract.
- Mix solution gently and spin down for 15 min, 16,000 *g* at room temperature in a tabletop centrifuge. After centrifugation, precipitated proteins are visible as a cloudy layer between the aqueous and organic layers.
- Transfer the 300 μL of top (aqueous layer) to a new tube containing chloroform–IAA mix. Discard the tube with the bottom layer and the cloudy layer into a liquid waste container.
- Mix the tube containing DNA–chloroform–IAA solution gently and spin down for 2 min at 16,000 *g* at room temperature in a tabletop centrifuge.
- Remove the top (aqueous layer) to a new tube and discard the tube with bottom layer into liquid waste container.

12.6.3 Ethanol Precipitation of DNA

- Add sodium acetate to DNA solution at a final concentration of 0.3 M.
- Add three volumes of chilled 100% ethanol.
- Mix gently by inverting the tube 5–6 times and spin down for 25 min at 16,000 *g* at 4 °C.
- A pellet (opaque to translucent) should be visible at this point (glycogen can be added to aid pellet visualization). Remove supernatant carefully without disturbing the pellet.
- Add 300 μL of 70% ethanol and spin down for 15 min at 16,000 *g* at 4 °C. Remove 70% ethanol carefully without disturbing the pellet.
- Repeat wash.
- Dry the pellet at room temperature, until all residual ethanol has evaporated.
- Dissolve the pellet in appropriate amount of TE or desired buffer and incubate overnight at 4 °C.

Solution I: Cell lysis buffer

- 100 mM NaCl

10 mM Tris-HCl pH 7.5
25 mM EDTA pH 8.0
0.5% SDS

12.7 Extraction of Plasmid from Bacteria (Fig. 12.2)

- Inoculate a single bacterial colony into 4 mL of sterile LB medium containing antibiotic in round bottom tubes. Vortex to completely resuspend the colony in medium. Incubate on a shaker overnight at 37 °C at 250 rpm.

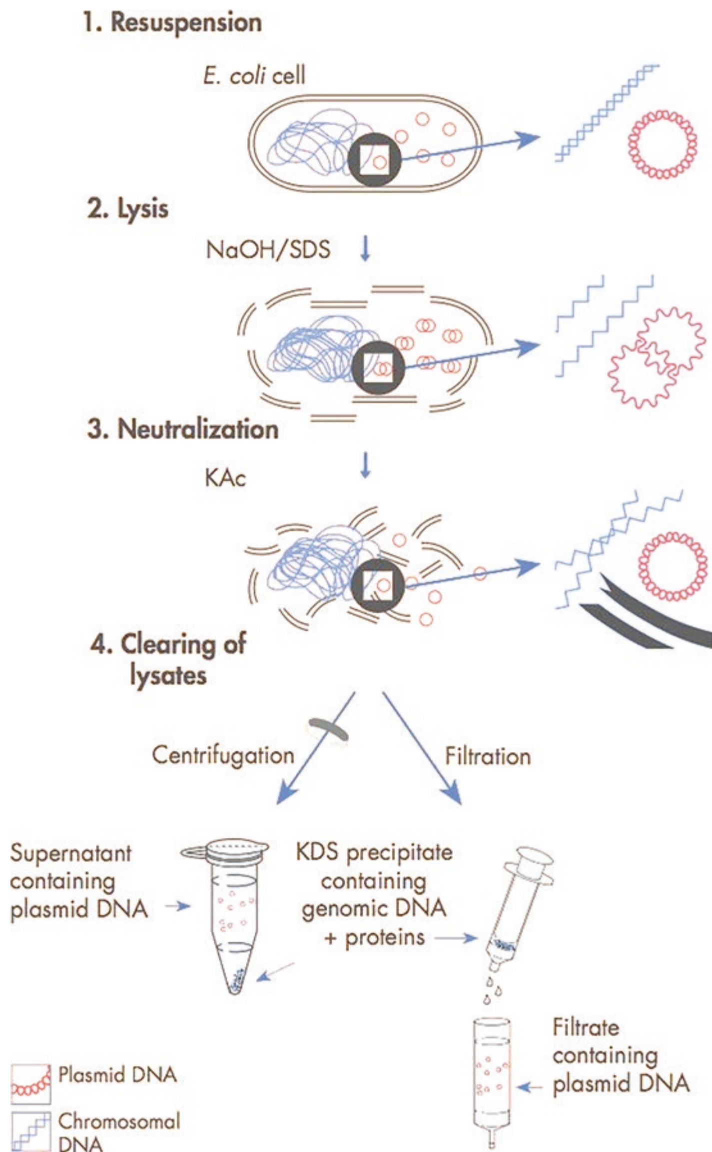


Fig. 12.2 Principle of alkaline lysis, another most commonly used method of lysing bacterial cells prior to plasmid purification

- Transfer 1.5 mL culture to microfuge tube and spin down for a minute in tabletop centrifuge at 16,000 *g* to pellet the cells. Remove supernatant and save the pellet.
- Add 1.5 mL of culture to the cell pellet and spin down for a minute, remove supernatant, and save the pellet.
- Completely resuspend the cell pellet in 200 μL solution I and let sit 5 min at room temperature.

- Add 200 μL solution II, mix by flicking the tube and place on ice for 5 min. Do not exceed 5 min.
- Add 200 μL solution III, invert 5–6 times to mix completely. Place on ice for 5 min.
- Spin down for 20 min at 4 °C to pellet cell debris and chromosomal DNA.
- Transfer 500 μL of supernatant to a fresh tube containing 500 μL isopropanol, mix and let sit for 2 min at room temperature to precipitate the plasmid DNA.
- Spin down at room temperature for 5 min to pellet plasmid DNA.
- Resuspend gently in 200 μL 0.3 M solution III (dilute solution III to 0.3 M).
- Add 3 volumes of ethanol to precipitate. Spin down for 5 min at 16,000 *g* at room temperature and carefully remove the ethanol.
- Add 3 volumes 70% ethanol to wash the precipitated plasmid DNA pellet. Spin down for 5 min at 16,000 *g* at room temperature and carefully remove the ethanol.
- Let the ethanol evaporate at RT or 37 °C for 20 min.
- Add 40 μL TE to plasmid DNA pellet and incubate at RT for 20 min or 4 °C.

  Solution I: 50 mM Tris, 10 mM EDTA pH 8, 100 μg/mL RNase.
  Solution II: 0.2 M NaOH, 1% SDS.
  Solution III: 5 M potassium acetate solution, pH 4.8.

29.5 mL glacial acetic acid.
Add potassium hydroxide pellets to pH 4.8.
Make volume to 100 mL with autoclaved water.
Store at room temperature (do not autoclave).

12.8 Extraction of RNA from Blood and Liver Tissue (Fig. 12.3)

12.8.1 Solutions Required for Extraction of RNA

1. Denaturing solution: 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% (w/v) *N*-lauroylsarcosine, 100 mM β-mercaptoethanol.
2. Diethyl pyrocarbonate (DEPC)-treated water: 0.2% DEPC in double distilled water. Leave overnight and autoclave.
3. Column wash buffer: 100 mM NaOH, 5 mM EDTA solution.
4. Equilibration buffer: 500 mM LiCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) SDS.
5. Wash buffer: 150 mM LiCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) SDS.
6. Elution buffer: 2 mM EDTA, 0.1% (w/v) SDS.

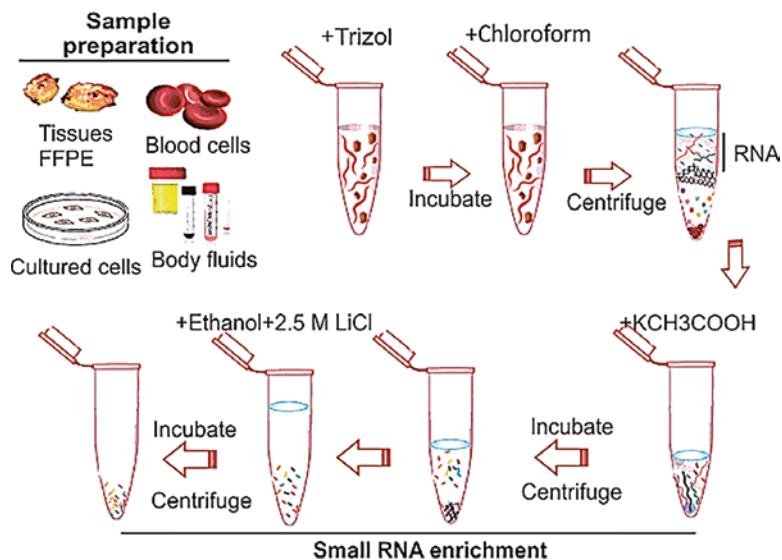


Fig. 12.3 TRIzol RNA extraction method from different biological samples

12.8.2 Solutions Needed for Analysis of RNA

1. 10× MOPS buffer: 200 mM MOPS (pH 7.0), 50 mM sodium acetate, 10 mM EDTA (pH 8.0).
2. RNA loading buffer: 1 mM EDTA (pH 8.0), 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue, 50% (v/v) glycerol.
3. 10× SSC: 1.5 M NaCl, 150 mM trisodium citrate.

Any work involving the use of RNA must be carried out using RNase-free reagents, solutions, and laboratory-ware. Most of the protocols are available for RNA extraction and a single-step isolation method for total RNA is outlined below. The total RNA isolated is comprised mainly of transfer RNA (tRNA), ribosomal RNA (rRNA), and it can be used for gene-expression studies, reverse a small amount of mRNA transcription–polymerase chain reaction (RT-PCR) work, and S1 nuclease or ribonuclease protection assay. The isolation of RNA with high quality is a crucial step required to perform various molecular biology experiments. TRIzol Reagent is a ready-to-use reagent used for RNA isolation from cells and tissues. TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. Addition of chloroform, after the centrifugation, separates the solution into aqueous and organic phases. RNA remains only in the aqueous phase. After transferring the aqueous phase, RNA can be recovered by precipitation with isopropyl alcohol. But the DNA and proteins can be recovered by sequential separation after the removal of aqueous phase. Precipitation with ethanol removes DNA from the interphase, and an additional precipitation with isopropyl alcohol removes proteins from the organic phase.

Total RNA extracted by TRIzol Reagent is free from the contamination of protein and DNA. This RNA can be used in Northern blot analysis, in vitro translation, poly(A) selection, RNase protection assay, and molecular cloning.

12.8.3 RNA Isolation from Blood

Buffy coat-TRIzol method

- Centrifuge the tube at 1600 *g* for 10 min at 4 °C.
- Remove the supernatant (plasma) by pipetting, add 1 mL RBC lysis buffer into the tube. Mix gently and incubate at room temperature for 10 min.
- Centrifuge the tube for 5 min at 10,000 *g*, 4 °C.
- Remove the supernatant, add 1 mL RBC lysis buffer to the tube, and mix it gently using a clean pipette tip.
- Centrifuge the tube at 10,000 *g* for 5 min at 4 °C.
- Remove the supernatant. Wash the pellet once with 1 mL PBS buffer, mix the sample gently, and centrifuge at 10,000 *g* for 5 min at 4 °C. Discard the supernatant.
- Add 1 mL TRIzol reagent, use a pipette to mix uniformly, and incubate for 15 min at room temperature (RT). Transfer the mixture of blood white cells and TRIzol reagent to a −80 °C freezer.

The methods applied to RNA extraction: the TRIzol reagent single-step method [1], TRIzol reagent are composed of monophasic solutions of phenol and guanidine isothiocyanate and can maintain the integrity of the RNA while disrupting cells and dissolving cell components. Finally, measure the RNA concentration with Nanodrop ND-1000 spectrophotometer (Fig. 12.4) and quality may be checked by bioanalyzer (Agilent Technologies, CA, USA).

12.8.4 RNA Isolation from Liver Tissue

- Liver tissues must be stored in RNA later for RNA isolation at later stages.
- Homogenize 100 mg of tissues crushed in a mortar and pestle with liquid nitrogen. Powder formed after crushing collect the tissues in 1.5 mL sterile microcentrifuge tubes.
- Next, add 800 μL of TRIzol for homogenizing the sample by gentle pipetting and vortexing thoroughly. Incubate the homogenized sample for 5 min at room temperature to allow complete dissociation of nucleoprotein complex.
- Centrifuge at 13.3 rpm for 5 min at 4 °C to remove cell debris.
- Transferred the supernatant in another tube, added 0.2 mL of chloroform per mL of TRIzol. Vortex for 15 s, incubate at room temperature for 2–3 min.

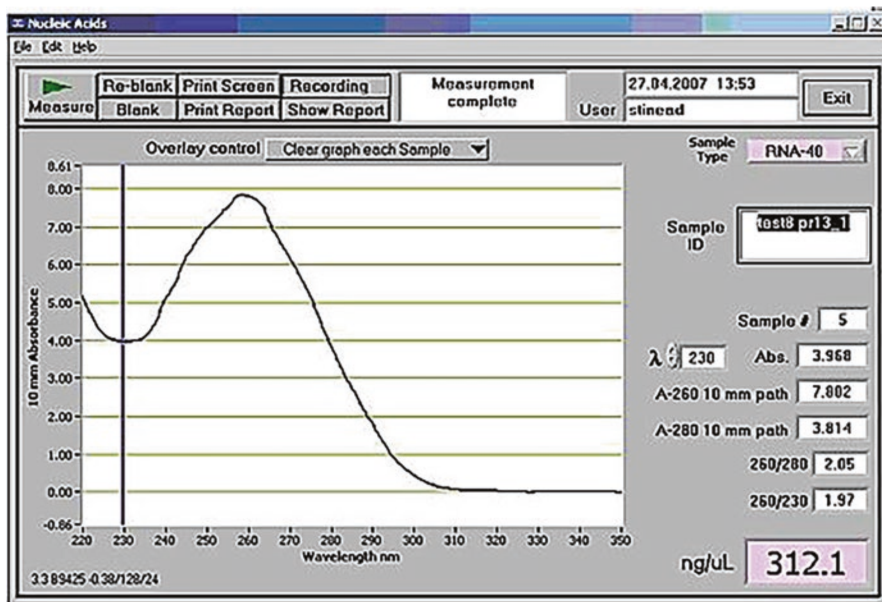


Fig. 12.4 Measurement of RNA concentration by Nanodrop spectrophotometer

- Centrifuge at 12,000 *g* (750 rpm) for 15 min at 2–8 °C. Out of the three phases, that is, lower phase, interface, and upper colorless phase that contains RNA, collect the upper phase a in new tube.
- Precipitate with chilled isopropyl alcohol (0.5 mL/mL of TRIzol). Incubate it for 10 min at 10–30 °C.
- After centrifugation at 12,000 *g* for 10 min at 2–4 °C, wash pellet with 75% ethyl alcohol, 1 mL/mL of TRIzol. Vortex and centrifuge at 7500 *g*/5 min at 2–8 °C and repeat the washing step twice.
- Air-dry the tubes, once dried dissolved in 20–50 μL RNase-free water or DEPC-treated water and incubate the tubes on a heating block for 5–10 min at 56 °C.
- Using 10 μL pipette, measure OD at 260 nm and 280 nm to determine sample concentration and purity; thus, the quantity and purity of total RNA must be determined with a Nanodrop for downstream analysis (Fig.12.4).

12.9 Estimation of DNA Using Diphenyl Amine (DPA) Method (Fig. 12.5)

Aim: The aim is to estimate the concentration of DNA by diphenylamine reaction (Fig. 12.5).

Diphenylamine test for DNA

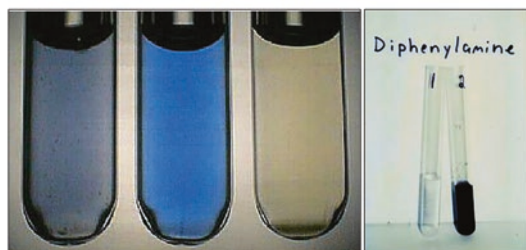
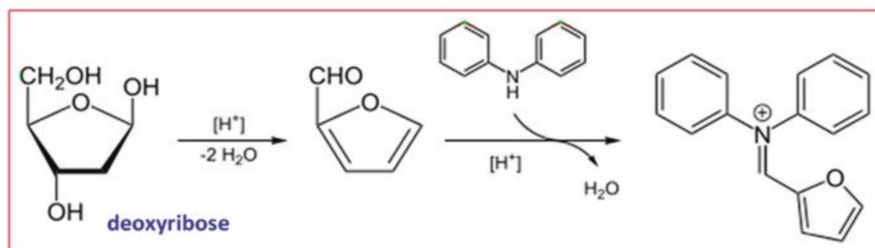


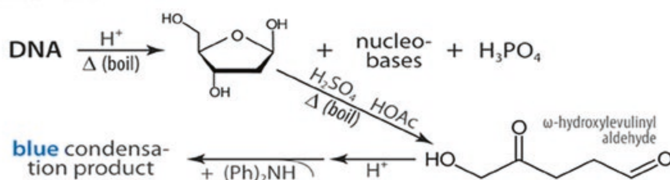
Fig. 12.5 Representation of the DNA estimation by Diphenyl amine (DPA) method

The principle of this method is that it is a general reaction given by deoxypentoses. The 2-deoxyribose of DNA, in the presence of acid, is converted to ω-hydroxylevulinic aldehyde, which reacts with diphenylamine to form a blue-colored complex, which can be read at 595 nm.



Acidic conditions convert deoxyribose to a molecule that binds with diphenylamine to form a blue complex.

With DNA:



Requirements:

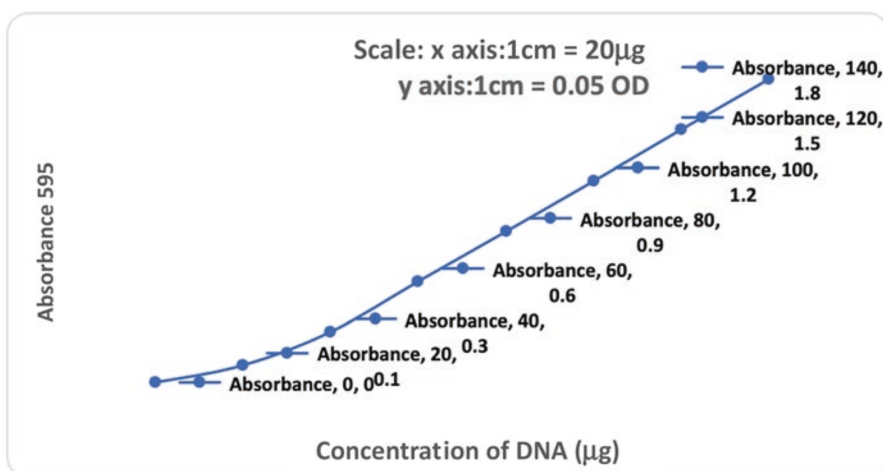
1. Standard DNA solution: Dissolve DNA (200 μg/mL) in 1 N perchloric acid/buffered saline.
2. Diphenylamine solution: Dissolve 1 g of diphenylamine in 100 mL; of glacial acetic acid and 2.5 mL of concentrated H_2SO_4. This solution must be prepared fresh.
3. Buffered saline: 0.5 mol/L NaCl; 0.015 mol/L sodium citrate, pH 7.

Procedure

1. First pipet out 0.0, 0.2, 0.4, 0.6, 0.8, and 1 mL of working standard in to the series of labeled test tubes.
2. Pipet out 1 mL of the given sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 2 mL of DPA reagent to all the test tubes including the test tubes labeled 'blank' and "unknown."
5. Mix the contents of the tubes by vertexing/shaking the tubes and incubate on a boiling water bath for 10 min.
6. Then cool the contents and record the absorbance at 595 nm against blank.
7. Then plot the standard curve by taking concentration of DNA along *X*-axis and absorbance at 595 nm along the *Y*-axis.
8. Then from this standard curve calculate the concentration of DNA in the given sample.

Result: The given unknown sample contains ——— µg DNA/mL.
Example: Observations and calculations

Volume of standard (200 µg/mL) DNA (mL)	Volume of distilled water (mL)	Concentration of DNA (µg)	Volume of DPA reagent (mL)		A595
0	1	0	2	Incubate in boiling water bath for 10 min and cool	0
0.2	0.8	20	2		
0.4	0.6	40	2		
0.6	0.4	60	2		
0.8	0.2	80	2		
1	0	100	2		
1.0 Unknown	0	To be estimate	2		



12.10 Agarose Gel Electrophoresis of Extracted DNA and RNA (Fig. 12.6)

Agarose gel electrophoresis is used to resolve DNA or RNA fragments on the basis of their molecular weight. Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes ranging from 100 bp to 25 kb. Migration of smaller fragments are faster than larger ones; the migrated distance on the gel varies inversely with the logarithm of the molecular weight. The size of fragments can therefore be determined by calibrating the gel, using known size standards, and comparing the distance the unknown fragment has migrated (Fig. 12.6).

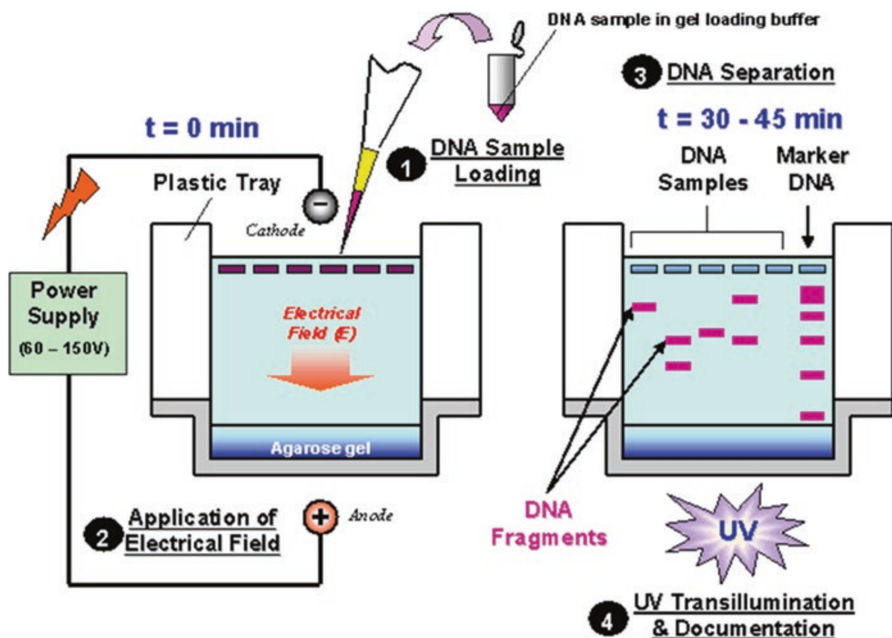
Gel Preparation

- In a flask weigh out the appropriate mass of agarose using a w/v percentage solution. Depend upon the sizes of the DNA fragments in a gel to be separated the concentration of agarose need to be adjusted, with most gels ranging between 0.5 and 2%. Add buffer volume but should not be greater than 1/3 of the capacity of the flask.
- In agarose-containing flask add running buffer, swirl to mix. The most common gel running buffers are TAE (40 mM Tris–acetate, 1 mM EDTA) and TBE (45 mM Tris–borate, 1 mM EDTA).
- Melt the agarose–buffer mixture. This is most commonly done by heating in a microwave, At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.
- Add ethidium bromide (EtBr) to a concentration of 0.5 μg/mL. Alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5 μg/mL EtBr for 15–30 min, followed by destaining in running buffer for an equal length of time.
- Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath.
- Place the gel tray into the casting apparatus. Place an appropriate comb into the gel mold to create the wells.
- Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box. Alternatively, the gel can also be wrapped in plastic wrap and stored at 4 °C until use.

Note: EtBr is a suspected carcinogen, care must be taken upon handling and must be properly disposed of as per institution regulations.

Separation of DNA/RNA Fragments

- Add loading dye to the DNA/RNA samples to be separated.
- Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel. Gel loading dye is typically made at 6× concentration (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol).
- Program the power supply to desired voltage (1–5 V/cm between electrodes).



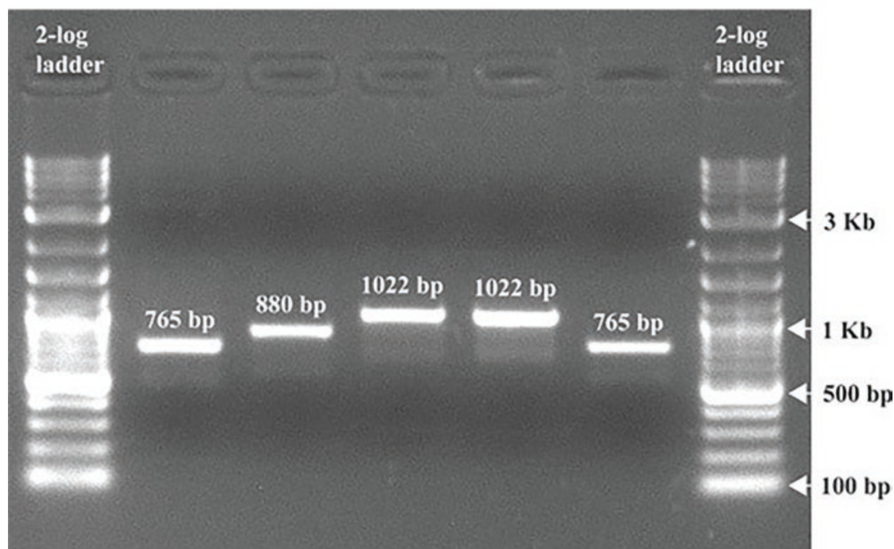


Fig. 12.6 An example image of a gel postelectrophoresis. EtBr was added to the gel before electrophoresis, followed by separation at 100 V for 1 h. The gel was exposed to UV light and the picture taken with a gel documentation system. (Adapted from Lee et al. [2])

- Cover the surface of the gel and fill with enough running buffer. It is important to use the same running buffer as the one used to prepare the gel.
- Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working.
- Remove the lid. Slowly and carefully load the DNA sample(s) into the gel. An appropriate DNA size marker should always be loaded along with experimental samples.
- Replace the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Double-check that the electrodes are plugged into the correct slots in the power supply.
- Turn on the power. Run the gel until the dye has migrated to an appropriate distance.
- When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
- Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
- Remove the gel from the gel tray and expose the gel to UV light. This is most commonly done using a gel documentation system. DNA bands should show up as orange fluorescent bands. Take a picture of the gel.
- Properly dispose of the gel and running buffer per institution regulations.

12.11 Extraction of Proteins from Bacterial Cell and Animal Tissue

12.11.1 TCA (Trichloroacetic Acid) Protein Extraction Method

- Pellet 1 million cells and remove supernatant. Resuspend the cell pellet in 1.5 mL of chilled 10% TCA. Incubate on ice for 30 min.
- Centrifuge at 9000 *g* for 10 min at 4 °C.
- Remove supernatant without disturbing the cell pellet.
- Gently resuspend the cell pellet in TBS solution.
- Centrifuge at 9000 *g* for 10 min at 4 °C.
- Remove supernatant without disturbing the cell pellet.
- Gently resuspend cell pellet in 30 μL of lysis buffer.
- Sonicate for 10 cycles at 4 °C (30 s on and 30 s off).
- Boil samples at 70 °C for 10 min.
- Centrifuge for 5 min at 16,000 *g* at room temperature and remove the supernatant to new tube.
- Use 1:15 diluted supernatant for protein estimation by Qubit method.

Lysis buffer solution (100 μL)

- 10 μL of 1 M DTT.

25 μL of 4× sample buffer (Invitrogen).
65 μL of water.

12.11.2 RIPA Protein Extraction Method

- Pellet one million cells and discard supernatant. Wash the cell-pellet once with 1× PBS and discard the 1× PBS.
- Gently resuspend the cell pellet in 30 μL RIPA buffer containing added protein and phosphatase inhibitors. Incubate on ice for 20 min after resuspension.
- Sonicate for 10 cycles at 4 °C (30 s on and 30 s off).
- Centrifuge for 10 min at 16,000 *g* at 4 °C and carefully remove the supernatant to new tube.
- Estimate protein by method of choice.

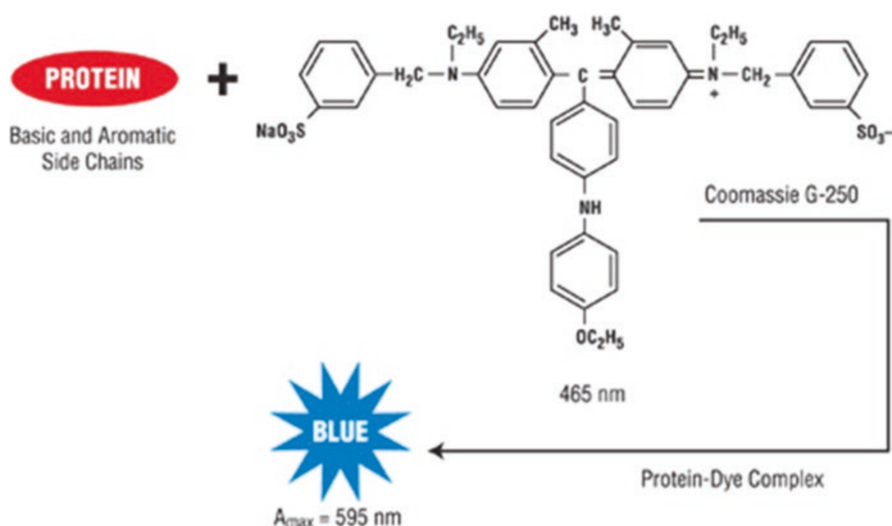
RIPA buffer

- 50 mM Tris–HCl pH 8.0.

150 mM NaCl.
1% NP 40.
0.5% Sodium deoxycholate
0.1% Sodium dodecyl sulphate (SDS)
1 mM Sodium orthovanadate.
1 mM NaF.
Protease inhibitor tablet (Roche).

12.12 Estimation of Protein Using Bradford Method and Use of BSA as a Standard

The concentration of proteins in any biological sample can be determined by spectrophotometry by using Bradford assay which is very easy to perform. The traditional method for calculating protein concentration of an unknown sample is to use a standard curve that is generated from known protein standards. Protein assays often use standards to determine the quantity of other proteins, by comparing an unknown quantity of protein to known amounts of the protein standard being used. As a result, it has become acceptable to use readily available proteins such as bovine serum albumin (BSA) and gamma globulin as standards. Using either BSA or the bovine γ-globulin (IgG) as reference proteins, Bradford protein assays do show significant protein-to-protein variation; hence, the calculated result is an estimation of protein concentration [3]. The protein standard is measured, and its absorbance and concentration are plotted on a graph to create the standard curve. This curve is then used to determine the concentration of the unknown protein.



12.12.1 Principle

The Bradford assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye–albumin complex solution is constant over a ten-fold concentration range.

12.12.2 Procedure

Reagents

- Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol, add 100 mL 85% (w/v) phosphoric acid.
- Dilute to 1 L when the dye has completely dissolved, and filter through Whatman #1 paper just before use.
- (Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent).

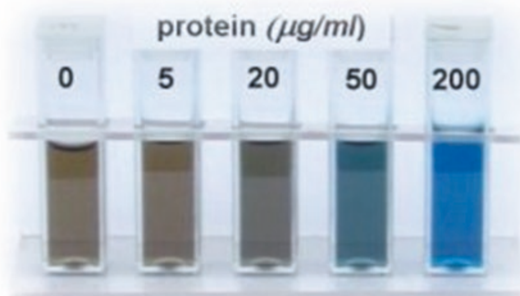
- The Bradford reagent should be a light brown in color. Filtration may have to be repeated to get rid the reagent of blue components.

Assay

- Turn on and warm up the spectrophotometer before use.
- If necessary, dilute unknowns to obtain between 5 and 100 μg protein in at least one assay tube containing 100 μL sample or one well in 96-well plate.
- Prepare standards containing a range of 5–100 μg protein (albumin or gamma globulin are recommended) in 100 μL volume.
- Add 5 mL dye reagent and incubate for 5 min.
- Measure the absorbance at 595 nm.

Analysis

- Prepare a standard curve of absorbance versus micrograms protein and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any in the experiment.



The Bradford method is able to detect microgram amounts of protein. By this test an accurate measurement of protein in different samples such as foods, plant and animal tissues can be done.

12.13 SDS-PAGE and Western Blotting of Extracted Proteins (Fig. 12.7)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis involves separation of proteins on the basis of their size using polyacrylamide gel systems. This is achieved by using the anionic detergent SDS to coat the proteins and impart the same negative charge per unit area on all proteins. In western blotting proteins are transferred to a membrane followed by detection using antibodies of interest [4].

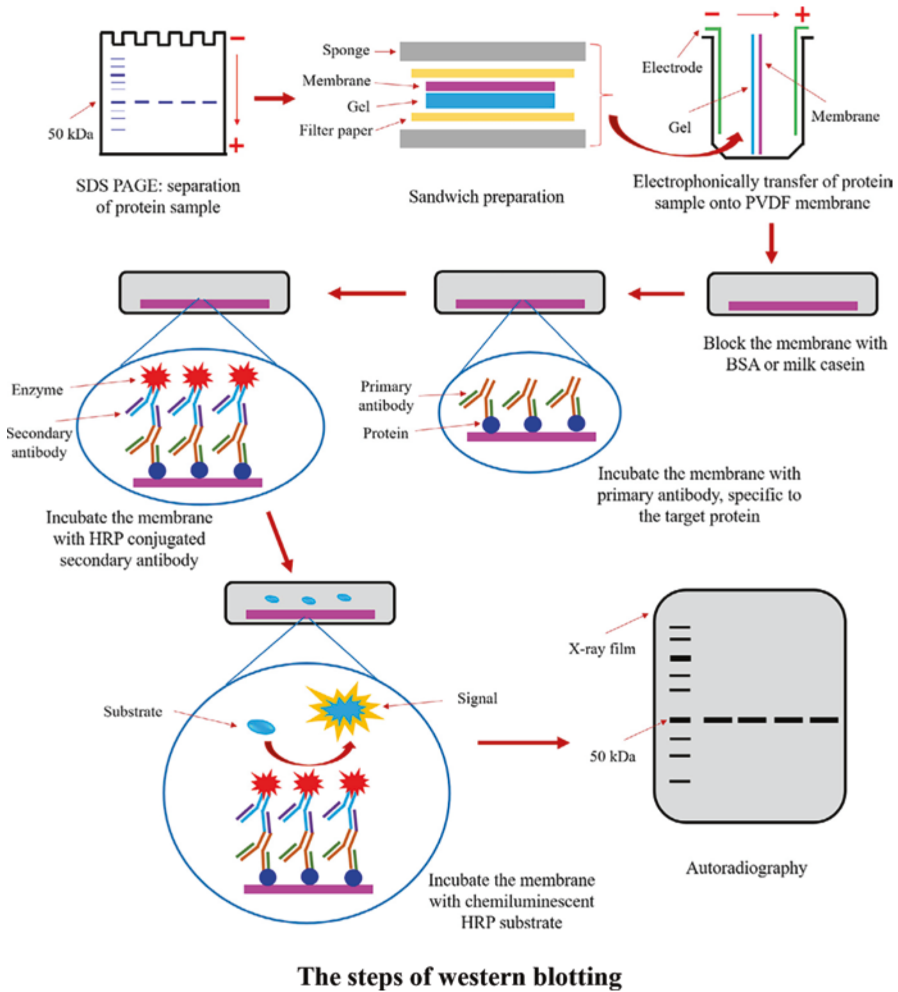


Fig. 12.7 Schematic representation of SDS-PAGE and Western blotting

12.13.1 Day 1

- Add one part of the loading buffer to three parts of the protein sample and boil sample for 5 min. Centrifuge at 16,000 *g* for 5 min at room temperature in a tabletop centrifuge.
- Load equal amount (30–50 µg) of samples on precasted SDS-PAGE gel in running buffer along with molecular weight marker. SDS-PAGE gel percentage depends on the size of protein of interest.

- Separate proteins at 80 V for 20 min and increase the voltage to 120 V until the exit of dye front from the gel.
- Place the gel in 1 × transfer buffer for 15 min and presoak the PVDF membrane in methanol for 5 min.
- Create a sandwich of gel and PVDF membrane between wet Whatman papers and sponges in the transfer cassette. Avoid air-bubbles between the gel and PVDF membrane.
- Place the assembled cassette with gel on the anode and blot on cathode side in the transfer tank filled with prechilled transfer buffer. Transfer at 30 V in cold room for 1 h or overnight at 10 mA.
- Remove the membrane and label the membrane with separated proteins face-up with pencil.
- Incubate membrane in blocking buffer for 1 h at room temperature with gentle shaking.
- Incubate membrane in primary antibody for overnight at 4 °C with gentle shaking.

12.13.2 Day 2

- Remove the primary antibody and wash the membrane with wash solution for 10 min with shaking.
- Repeat wash three times.
- Incubate membrane in secondary antibody for 1 h at room temperature with gentle shaking.
- Remove the secondary antibody and wash the membrane with wash solution for 10 min with shaking.
- Repeat wash three times.
- Develop the membrane depending upon the conjugate attached to the secondary antibody.

Transfer buffer
- 25 mM Tris.

190 mM glycine.
0.05% SDS.
20% v/v methanol.

Running buffer
- 25 mM Tris.

190 mM glycine.

TBS-T (Tris-buffered saline with Tween 20) buffer
- 25 mM Tris.

150 mM NaCl.
0.1% Tween 20.

Blocking solution

- 5% nonfat milk or bovine serum albumin in TBS-T buffer.

Histochemistry

Perform tissues sectioning and fixation in phosphate-buffered 10% formaldehyde for the histological analysis. Each formaldehyde-fixed sample from tissue sections must be embedded in paraffin, cut into 5 μm-thick sections and stained with hematoxylin–eosin (H–E) or any other stain. The extra remaining portions of the tissue need to be collected in RNase-free tubes and snap-frozen in liquid nitrogen for other expression studies [5, 6].

12.13.3 Histopathological Studies: Haematoxylin and Eosin (H&E) Staining

For histopathology, liver tissue must be collected in formalin. Perform sectioning on formalin-fixed, paraffin-embedded liver (2.5 μm thick sections). The slides staining for H&E, dewaxed sections in xylene and rehydrated through graded alcohol (100%, 90%, 80%, and 70%) to water followed by staining with hematoxylin for 3–5 mins and then were wash in running water. Sections were then given 1 dip in 0.3% hydrochloric acid and again wash in water then 3 dips in ammonia. Next wash the sections with alcohol and then stained in eosin. Again, dehydrate with graded alcohol (70%, 80%, 90%, and 100%). Finally, dip in xylene and mount in DPX mounting medium. Analyse slides under the microscope at different magnification (20× and 40×).

12.13.4 Histopathological Studies: Masson Trichrome (MT) Staining

For histopathology, collect liver tissue in formalin. Perform sectioning on formalin-fixed, paraffin-embedded liver tissue sections (2.5 μm thick). For MT stain dewax the slides sections in xylene and rehydrate through graded alcohol (100%, 90%, 80%, and 70%) to water. Keep slides in Boyin reagent in a hot oven for an hour, then wash in water and further stain in Beibrich scarlet for 2–5 mins and again wash by water. Then use 5% phosphomolybdic for 1 min and wipe off, then 2.5% aniline blue for 2 min and then wash away with distilled water. Further put 1% glacial acetic acid on slides for 1 min and then wash with alcohol. Blot the slides on filter paper and mount in DPX mounting medium. Analyze slides under the microscope at different magnifications (20 × and 40 ×).

12.13.5 Immunohistochemistry

For immunohistochemistry (IHC), collect liver tissue in formalin. Perform the IHC on formalin fixed, paraffin-embedded liver tissue sections (2.5 µm thick). Next, dewax sections in xylene and rehydrate through graded alcohol (100%, 90%, 80%, and 70%) to water. Block the endogenous peroxidase by incubating the tissue sections in 15% hydrogen peroxide (H_2O_2) for 10 min and then kept either in boiling sodium citrate buffer (pH 6.0) or tris EDTA buffer (pH 9.0) for 10 min for antigen retrieval, cool in running water and three washes in Tris buffer saline (TBS, pH 7.6). Next, wipe the excess buffer all around the tissue. Add PolyExcel HRP conjugated, from any commercial kit available. Use one drop of protein blocker and place on the tissue section for 10 min and then drain. Incubate sections overnight with primary antibodies for normally 1:100 dilution at 4 °C, wash thrice with TBS for 5 min each then a drop of target binder, place (secondary antibody) on tissue for 10 min at room temperature in a humid chamber. After washing thrice with TBS, apply a drop of horseradish peroxidase (tertiary antibody) for 10 min and wash again (Poly Exel HRP conjugated, from any commercial kit). Then, add a cocktail of diaminobenzidine (DAB) buffer and chromogen (by mixing in 1 mL of buffer a drop of chromogen) and apply as a chromogen for 5–10 min and then wash with running water. Counterstain sections with Mayer's hematoxylin for 1 min, wash and mount in DPX mounting medium. Analyze the slides under the microscope at different magnifications (20× and 40×) [6–8].

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Chapter 13
Laboratory Protocols in Medical Biotechnology II (Contemporary Principles and Practices of Bacterial and Human Cell Culture)

Shabeer Ahmad Rather, Swati Sharma, Naveed Pervaiz Bhat, and Riyaz Ahmad Rather

Abstract Cell culture is a technique in which eukaryotic cells are grown in the laboratory under controlled physiological conditions with complex growth requirements, whereas microbial culture is the method of growing microorganisms under optimal conditions with simple growth requirements. Both culture techniques address the basic scientific and translational research queries posed by certain scientific beliefs. Owing to the importance of cell and microbial culture in research and development, it is imperative to carry out these techniques with the highest competence and purity so that the reproducibility and homogeneity of the results are maintained. This chapter elucidates the basic principles and techniques of microbial and cell culture. Purification techniques, media formulation, growth requirement of cells, and decontamination measures are thoroughly discussed. Since the outcome of the research needs to be analyzed and interpreted to reach a convincing conclusion, the basic statistical overview and the methods involved in the analysis of research data are also described herein.

Keywords Microbial culture · Yeast culture · Human cell culture · Contamination · Shelf-life · Statistical analysis

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13.1 Introduction

Cell culture is a technique wherein cells are grown under controlled physiological conditions like pH, nutrients, temperature and moisture [1]. This technique is useful in studying eukaryotic and prokaryotic cell physiology and biology. Culture technique has facilitated a greater knowledge among researchers about, microbial classification, drug interaction, vaccine production, monoclonal antibody generation, and biotransformation. To study the behavior of organisms under conditions with no systemic changes, it was used as a method of choice in the early twentieth century and was based on the need of two branches of medical sciences, virology and oncology and with the advancement of medical sciences led its interest in various other applications [2]. Cell culture(s) have been an important tool used in the separation of intracellular cell components of bacteria, viruses, and protozoa to understand the underlying mechanism of microbial infection. Further, infectious agents requiring a living host can only be replicated in cell cultures, hence, making us understand the disease-causing mechanism. Culture methods help to generate data with a high level of reliability and continuity that cannot be justified while studying in an organ system [3]. In this chapter, we will discuss in detail various types of microbial and mammalian cell culture, their types, media formulation, reagents used, and storage conditions.

13.2 Basic Principles of Microbial Cell Culture

The biosphere of the Earth is dominated by microbial populations. This illustrates that microorganisms are vital for plants and animals to sustain. Cell culture in the form of microbes or mammals is the primary tool used in biological studies. Consistency and reproducibility of the cell cultures make them an optimum choice for conducting any sort of experiment like metabolic, molecular, or drug assays. Cell culture has proved to be an imperious model system in every biological research.

13.2.1 Importance of Cell Cultures and Requirements

Microbial cells require optimum environmental conditions for their growth. Parameters inclusive of physical and chemical conditions are imperative for continuous and adequate growth of the microbial cells. The physical and chemical parameters include nutrients in precise amounts, optimum temperature, and pH along with other basic conditions like sterilized growth environment. Like other higher organisms, microorganisms also need all the required sources of nutrients for carrying physiological and metabolic activities. Healthy metabolic activities are

important to maintain growth and energy levels. Any change in the media formulation can have a deleterious effect on the growth of the microorganism. Hence, choosing the right media always benefits the growth and multiplication of the microbe. Based upon the nutritional requirement, the bacterial growth media are classified into various categories like synthetic, semisynthetic, or completely natural. Synthetic media is widely used in the industrial setup at a pilot scale. Commercially available semisynthetic nutrient media like trypticase soy broth (TSB), nutrient broth (NB), yeast extract (YE), brain–heart infusion broth (BHI), potato dextrose agar (PDA), and casein digest are used in the laboratory setup for basic biological research. For large scale industrial production a chemically undefined complex medium is preferred for microbial growth because of its simple availability and cost-effectiveness [4]. Here are some basic constituents of the bacterial growth media which are used to cultivate microbial cells in the laboratory.

Carbon source: Carbon is the main energy source of all cells, hence it is vital for the growth of any organism. Simple, noncomplex, and easily available sugars such as glucose, lactose or sucrose are good sources of carbon. Some complex polysaccharides such as starch, glycogen, cellulose, and molasses can also be used. A carbon source provides energy and helps in building the carbon reservoir of the cell. Accumulated carbon is helpful for the synthesis of compounds required to carry out physiological processes.

Nitrogen source: Nitrogen is the second most nutrient constituent present at higher concentrations in the bacterial growth media. Nitrogen accumulates in cells in the form of amino acids, nucleotides or nucleic acids. Cells need nitrogen for the formation of proteins, nucleic acids, and other cellular components. Ammonium salts, urea, amino acid mixtures, and plant-tissue extract are good sources of nitrogen.

Micro or macro elements: These elements are required in small or trace amounts in a nutrient medium. Elements such as magnesium (Mg), copper (Cu), zinc (Zn), manganese (Mn), and cobalt (Co) are required in small quantities. These elements either act as cofactors of enzymes or are required for cell component synthesis. For example, phosphorus is needed for the synthesis of lipids and adenosine triphosphate (ATP); sulfur is needed for amino acid synthesis and vitamins; Mn, Co, and Zn act as cofactors for different enzymes. Collectively micro- or macroelements have a pivotal role in media formulation.

Water: It is the base requirement for making any liquid culture media. It acts as a foundation for mixing all media components uniformly. Water also helps to contain the pH of the media and maintains a consistent environment required for growth. Furthermore, agar can be added to the liquid media as a solidifying agent whose quantities can be varied according to the requirement of the microorganisms cultured.

13.2.2 Growth Curves

Microorganisms primarily reproduce by binary fission or budding. The division and growth of cells result in an increase in cell mass and nucleic acid content. This growth consequence in a surge in cellular elements and ultimately rise in cell number is witnessed. However, in coenocytic microorganisms nuclear divisions are not facilitated by cell divisions therefore, the cell size augments without any increase in cell number [5].

The initiation of cell growth is instigated by the DNA molecule which is attached to the membrane at a point where the replication initiates. The two DNA molecules are attached to these points adjacent to each other on the membrane surface. The synthesis of a new membrane takes place between these two points resulting in the movement of the DNA molecule in opposite directions. The new cell wall and the membrane make a septum between the two chromosomal compartments. On being completely formed, the septum breaks into two. These events that happened in a particular period is called generation time. Hence, the generation time is the time required by a bacterial cell to divide or double its population. Generation time varies across microbes, it could be as short as 15 min or as long as several days. Throughout the growth cycle, the changes in growth can be measured by observing a change in cell number or cell mass [6].

Microbial growth parameters or kinetics are studied in a batch culture which is a closed system. In batch culture, a single batch of media is used to feed the microorganisms. During incubation fresh medium is not supplied or added therefore, the nutrient concentration happens to decline, while the waste product concentration swell. Batch culture exhibits an impounding mechanism illustrating different growth phases and principles. For example, microbial cells exhibit growth dynamics which consists of an initial lag phase, exponential phase, stationary phase, and followed by the death phase (Fig. 13.1). In all these phases growth is measured in terms of change in the number of living cells versus time or change in the cell biomass versus time. The change in mass or cell number versus time generates a growth curve. The growth curve is obtained as a logarithm of the number of living cells on one axis against the incubation time on another axis. In this way, the growth curve exhibits four different phases of microbial growth, namely, lag phase, exponential phase, stationary phase, and death phase [5].

Lag phase: Microorganisms when inoculated into the fresh medium adapt to new environmental conditions; hence, no immediate growth or division is observed. This initial phase of adaptation is called as lag phase. There is no division of cells or increase in cell mass in this phase, rather the cells synthesize new components required for the cell division. The lag phase is essential for the energy-deficient cells as it replenishes nutritional contents like ATP and essential cofactors or synthesizes ribosomes required for protein synthesis. The length of the lag phase can vary according to the type of microorganism and the nature of the culture medium used. The inoculum used is a part of an old preserved culture or frozen at a very low temperature, may take some time to adjust to the new environment, and may show some

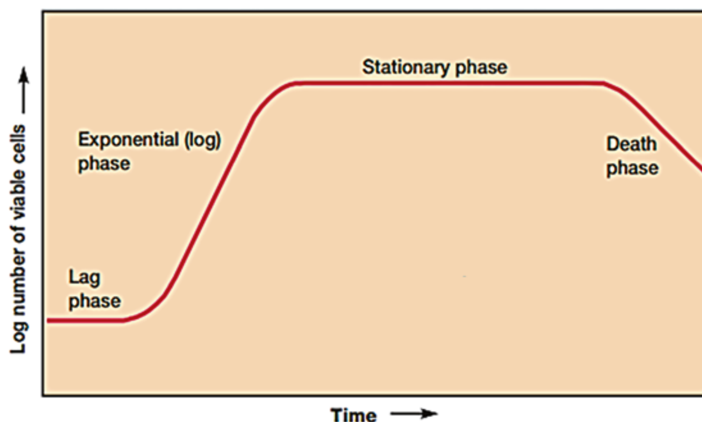

Fig. 13.1 Microbial growth curve in a closed system. (Adapted from Pelczar. Microbiology Concepts and Applications. New York, NY: McGraw-Hill)

delays in growth and therefore an extended lag phase can be observed in such cases. However, the freshly prepared culture used as an inoculum would exhibit a rapid exponential phase when transferred to a medium having the same composition. Thus, a shorter lag phase or null lag phase is observed [5].

Exponential phase: The exponential phase is also termed the log phase. The lag phase proceeds with the exponential phase. The exponential phase is marked by the doubling of the population where every cell population is doubled after every division. One can easily determine the final number of cells after a specified period or certain cell divisions if the initial number of cells at the start of the exponential phase is known. Mathematically it can be expressed by

$$N = N_0 2^n,$$

where N_0 is the initial cell concentration of the inoculum; n is the number of generations that occurred within a specified time and N is the final cell concentration of the cells after n divisions.

In this phase, microorganisms divide and grow at the maximum rate; however, factors like genetic makeup, the nature of the medium, and the physical conditions provided for growth can impact the growth rate of bacteria. A constant growth rate is achieved during the exponential phase and the microorganisms divide and double at regular intervals. Since the cells divide at slightly different intervals of time than the previous one, the growth curve rises smoothly rather than steeply. During the exponential phase, the physiological and chemical properties change uniformly; therefore, exponential phase cultures are preferred during physiological and biological studies. Hence, the cellular constituents are synthesized at constant relative rates. A change in nutrient constituents or environmental conditions leads to disruption in uniform growth.

Death phase: Continuous cell division and utilization of media components lead to nutrient deprivation and toxic waste buildup. In such a scenario decrease in the number of living cells is observed. Predictable cell viability decreases uniformly during this phase. It is been advocated that no 100% cell death is observed; however, most of the cells go in a dormant phase which could be revived upon providing suitable conditions. Such type of count is called viable, but noncultivable and such cultures can resume growth later when favorable conditions are provided to them.

13.3 How to Culture Bacterial Cells

Sterilization: The culture media and the vessels used in its preparation should be thoroughly sterilized, to restrain the growth of unwanted microorganisms and other contaminants. Normally sterilization is carried in small or big autoclaves depending upon the volume of media to be sterilized. The whole process of sterilization is carried out at 120 °C for 15–20 min under 15 psi pressure.

Environment: Factors like pH, ionic concentration, temperature, light or dark exposure, and aeration are the basic environmental factors needed for the growth of the bacteria. All these factors vary in different microorganisms and each microbe uses optimal growth conditions for multiplication. To maintain these conditions, automated incubators are used. These incubators are automatic and can accommodate a large volume of cultures.

Aeration and mixing: Aeration and proper mixing of media components are very important for the growth of microbes. Barring anaerobes, microorganisms need a constant supply of oxygen to sustain their growth. Further, aeration is also important as it helps in gaseous venting. Mixing is essential for the even distribution of nutrients across the microbial mass. In small cultures, aeration and mixing are done manually or in a shake flask incubator. However, in big cultures process fermenters are used to ensure proper mixing and aeration. Continuous or periodic stirring of the media in a fermenter is achieved by mixing cells and media formulation. Inlet and outlet of fermenter make gaseous flow constant while baffle system attached to the fermenter makes an axial mixing steady.

13.3.1 Isolation of Pure Cultures

Microorganisms grow in natural habitats as a composite population. The separation of these microbes always results in the isolation of a mixed set of microorganisms. Owing to this reason, it is very complex to isolate and study a specific type of microorganism. Hence it is imperative to isolate the microorganisms in pure cultures for further study. Therefore, a pure culture of a single microbial type is needed. Multiple pure culture techniques have been developed which have still immense importance

in microbiology. Techniques like streak plate, pour plate and spread plate are currently widely used for the isolation of pure cultures of the microorganisms.

Streak plate: The microbial cells or inoculum containing a mixture of cells is placed at the edge of an agar plate. The inoculum is streaked all over the surface of the plate. The inoculum is streaked on the surface of the solidified media over successive quadrants of the media. The streaking is done in such a way so that bacterial cells are separated from each other and grow as a single colony. Usually, the fourth quadrant yields the discrete growth of individual colony-forming units. This way the streak plate procedure helps to isolate pure colonies from a mixed population. The isolation rate of the single-cell colony depends on the spatial separation of the cells.

Pour plate: In the pour plate technique, the inoculum is diluted several times before inoculation. This way microbial density is reduced and desired individual colonies are obtained. The mixed microorganism sample is added to a molten agar medium whose temperature is set around 40–45 °C. The sample dilution(s) are mixed with the molten agar and mixed thoroughly. The mixture is poured onto agar plates and incubated under the desired conditions. Once the agar hardens and each cell is fixed in place and forms an individual colony. This technique helps find viable plate count plus new colonies growing on the surface, can be used as an inoculum for growing pure cultures. This technique is widely adopted to count the number of viable colonies.

Spread plate: Spread plates helps to isolate microorganisms from a small sample culture volume. The inoculum is diluted with the distilled water and spread over the surface of an agar plate resulting in a growth of discrete colonies. These colonies grow evenly on the surface of agar the plate, providing an optimum concentration of cells is plated. This technique can be employed for obtaining viable plate counts where several colony forming units can be calculated to get the cell count present in the initial sample used [8].

13.3.2 Determination of Growth Curve of E. coli *by Spectrophotometer*

Bacteria population is diverse and ubiquitous, hence their contribution is every aspect of life is no hidden fact. Because of their rich diversity, ease to handle and fast growth, their role in research as model organisms is acknowledged. Investigators intend to study growth rates of microorganisms for metabolic, physiological or drug effect studies. This is achieved by tracking all four growth parameters or phases of microorganisms under consideration. The period between these phases can be used to plot the growth curve of bacteria that can help to estimate certain growth kinetics like generation time or initial and final cell numbers.

A spectrophotometric or turbidometric method is the simplest method of determining the growth curve of bacteria in liquid or broth cultures. In this method, the

growth of the bacteria is determined by measuring the optical density of the bacterial culture using a spectrophotometer. With an increase in the microbial cell growth, turbidity of the culture increases and the transmittance of light through the culture decreases, increasing the optical density (OD) of the culture. It is one of the easiest methods used to analyze growth trends in bacteria because it uses a spectrophotometer to trace changes in OD over time.

Sterile broth (uninoculated) is prepared and the OD is set zero at 600 nm to standardize the OD_{600} of media without cells. This step is needed to standardize the turbidity of uninoculated media so that calculations quantify the growth changes, nullify the absorbance (if observed any) of media. Primary media inoculated with the sample organism is taken immediately and OD_{600} is estimated and recorded as time "zero." Thereafter readings of the inoculated culture are taken every hour till the reading become constant. The readings are plotted on the graph with OD_{600} as Y-axis and time on X-axis. Spectrophotometric determination of growth curves helps in tracing the growth rates without counting the colonies on the media plates and is an efficient method to study the standard growth curves.

13.3.3 Antibiotic Sensitivity Test of Microbes by Antibiotic Doses

Antibiotic sensitivity test (AST), also known as susceptibility test is a method that determines the effectiveness of an antibiotic on infecting microorganisms. Based on the effectiveness of the antibiotic, the microorganisms are classified as either susceptible, resistant or intermediate [9]. An antibiogram created by AST evaluation helps in predicting suitable empiric treatments against pathogens. The examination helps in dose formulation and therapy for specific infections caused by the pathogens. The evaluation of antibiotic sensitivity reduces the complications caused by blind trials hence, facilitate an effectual use of antibiotics inpatient treatment protocol. The classical methods used to carry out AST are diffusion and dilution methods; however, newer technologies like PCR or microarray embedded with classical ones have improved the efficacy of this test.

A diffusion method introduced by Bauer and Kirby is the gold standard for carrying AST on bacterial pathogens. In this method, the target bacteria are inoculated onto the agar plate and the antibiotic-treated sheet or disc is placed on the inoculated agar plate. The plates are incubated overnight at 35 °C. The antibiotic gets diffused into the agar plate, forming an inhibition zone and the size of the inhibition zone reflects the concentration of the antibiotic required to kill the bacteria. The zone of inhibition depicts the minimum antibiotic concentration to inhibit bacterial growth. This method is simple, cost-effective and can be used for routine testing in clinical laboratories. However, it has the limitations of having poor effectiveness in slow-growing and fastidious bacteria and is influenced by temperature, pH, solubility, and so on [10].

The dilution method started in the 1870s is one of the primary methods used to test the microbiological susceptibility of clinical isolates. The two basic dilutions are carried by either broth or agar. Broth medium involves the dilution of antibiotics in twofold in different centrifuge tubes and the test pathogen is allowed to grow at 35 °C overnight. The growth of the culture is determined by measuring the OD or turbidity of the culture over time. Whereas in agar dilution method antibiotic is mixed with the agar media and the growth of the bacteria is allowed on these antibiotic containing agar plates [11]. To enhance the efficiency of these methods, multiple improvisations have been carried out. For example, microdilution one such modification involves the culturing of bacteria in 96-well microtiter plates containing different concentrations of antibiotics. The growth of the culture and minimum inhibitory concentration (MIC) is determined by various optical instruments [12]. Dilution methods too have certain limitations like chances of false-positive results are common, cross-contamination, inefficient procedure to distinguish viable and nonviable bacterial count are associated challenges with these methods.

Etest or Epsilometer testing developed by Bolmstrom and Eriksson helps perform multiple antibiotic tests on a single platform. In this method plastic strip containing a predefined antibiotic concentration is kept on agar plates streaked with the bacteria. The culture plates embedded with an antibiotic strip are incubated under optimal growth conditions and inhibition zone formation is observed. This is the FDA-approved method that is more accurate, reliable, and convenient. However, the test efficacy is affected by pH sensitivity, storage of strips, and inaccurate response toward some bacterial agents like ciprofloxacin, rifampicin, penicillin, and ofloxacin [13].

Several commercial systems have developed convenient automated preparation systems, microdilution and reading panels, thus reducing technical errors and tedious preparations. Different automated systems have their defined panel capacity, detection time and are integrated with advanced software for speedy performance and online processing [14]. Some molecular approaches have been employed to check the resistance genes among pathogens. For instance, genotypic methods like PCR, DNA microarray, and loop-mediated isothermal amplification (LAMP) are currently used as rapid tools for identifying resistance genes in pathogens [10].

13.4 How to Culture Yeast Cells

Yeasts are considered model systems for eukaryotic studies as they exhibit fast growth and have dispersed cells. They are one of the oldest organisms having a broad spectrum of applications [15]. Investigations carried on the yeast *Saccharomyces cerevisiae* has significantly enhanced our understanding of major cellular processes like biosynthetic pathways, regulation of the cell cycle, aging, and cell death. *Saccharomyces cerevisiae* is inexpensive to grow and does not require special growth conditions and many ready-to-use strains are now commercially available. These features make yeast a good model organism for research and

development. Generally, yeast is grown in yeast growth media known as YPD. The "Y" stands for yeast extract, which contains essential water-soluble compounds derived from a previous population of yeast cells, "P" refers to peptone, which acts as the source of carbon and nitrogen. Peptone is a mixture of peptides and amino acids prepared by proteolytic digestion of the proteins. "D" is dextrose or glucose, which is a good carbon source. YDP is a rich medium that supplies all the essential nutrients required for the growth of yeast cells. However, multiple modifications have been carried out in YPD to attain an effective growth of the yeast cells. These changes are done based on the type of yeast to be cultured. Reports suggest that yeast can grow in broth cultures or on solid media.

13.4.1 Yeast Growth Phase

Yeast follows the growth kinetic pattern as most of the microbial cultures would follow under optimum environmental and physical conditions. A small inoculum of yeast is required to initiate the growth of a yeast cell. On inoculation, the cells become acclimated to the new environment and prepare for further growth by synthesizing new metabolites. The cells enter into the log phase or exponential phase, during which the number of cells increases exponentially. A steady state of growth is observed where the growth and division rate slows down, commonly known as the stationary phase, and gradually the cultures enter the death phase [16].

13.5 Human Cell Culture

Cell culture is a process in which cells are grown outside their natural environment under the controlled conditions. It encompasses the processes of cell isolation from the animal tissue and subsequent growth in a controlled artificial condition that is suitable for its growth [17]. The primary cells of humans are directly isolated from tissues. They retain their characteristic morphology as well as the function of the tissue from which they are derived [18]. The immediate process of cell culture begins with a primary culture technique. Cell monolayers are grown in culture media flasks, supplemented with specific nutrients and growth factors. As the cells grow to sufficient confluency, they are passaged to secondary, tertiary, or until a continuous cell line is established. This process is called subculturing. After the first subculture, the primary cells start to become a cell line or subclone.

To isolate cells from a tissue containing many cell types, the first step is to digest the extracellular matrix that keeps the cells organized. This is done by treating the tissue sample with some proteolytic enzymes such as trypsin or collagenase. Proteolytic enzymes disrupt the rigid membrane present around the cells woven around the extracellular matrix and release the cells. Other specific agents like

ethylenediaminetetraacetic acid are also added that chelates Ca^{+}. The latter ion is a very important adhesion molecule in cell–cell adhesion. The tissue is teased and thoroughly pipetted into suspension cells. Multiple methods based on cell properties are available that are involved in the separation of different cell types. For instance, cells of different sizes can be separated by centrifugation, adherent cells can be separated from non-adherent ones based on their tendency to adhere to plastic or glass surface. The most common method to grow cells differently is by utilizing specific or differential culture media. This is because different cell types have different growth requirements for growing and survival. After the particular culture reaches a suitable stage, the cells are identified morphologically and then characterized using specific markers [19]. Since primary cells are directly derived from the tissues, hence they show a functional resemblance with the original tissue. Because of this reason primary culture is prioritized when investigating some specific cellular features. However, their limited self-renewal and differentiation process is the major challenge faced by the investigators while using primary cell cultures. Therefore, immortal cell lines have become an amazing asset for innumerable applications such as testing drug metabolism, cytotoxicity, gene function studies, developing vaccines, antibodies and biological compounds. They are financially savvy, simple to handle, and can be kept in culture for a longer time frame.

Even though it is easy to propagate the cell lines, but they may undergo alteration in the morphology and functions on successive divisions. This may lead to genetic manipulation and the emergence of an entirely new cell strain completely different from the original cell type. Further, serial passaging also leads to genotypic as well as phenotypic variation with time. Even genetic drift may also cause heterogeneity abruptly in cell lines. Thus, cell lines are not always true representatives of primary cells and may therefore convey altered results [20].

13.5.1 Adherent and Nonadherent Cells

Cells are cultured in media either in the presence or absence of solid support. The presence or absence of solid support decides the cell adhesion with the support. Based on cell adherence, cell culture is classified into the adherent culture or suspension culture. In Adherent culture, the cells grow as monolayers adhered to an artificial substrate [21] whereas, in suspension culture, cells do not adhere to the substrate and grow as free-floating in the culture medium. Most of the cell lines derived from vertebrates are anchorage-dependent, but some cell lines like hematopoietic cell lines, are cultured in suspension. Adherent cell culture is visualized easily under the microscope but requires interval passaging. On the other hand, passaging of suspension culture is quite easy, but cell counting and determination of cell viability is the time needed to follow growth patterns.

13.5.2 Culture Media

Mammalian cells are mostly cultured in incubators under controlled conditions. The temperature is typically set at 37 °C, CO_2 concentration at 3–5% and humidity at 95%. However, the composition of the cell culture media is the most essential requirement to achieve good experimental reproducibility. The media is selected based on cell type, experimental aim and accessibility of resources in the research facility. Various cell types have exceptionally explicit development necessities; consequently, the most reasonable media for every type of cell culture should be experimentally standardized. But generally, for adherent cells, it is good to start with MEM while RPMI-1640 for suspension [22].

13.5.3 Composition and Importance for Cell Growth

Cell culture media comprises all those components that are required for the survival and growth of the cells. Media components are the sole sources of nutrients for the growth of cells. They contain essential nutrient components such as glucose, fructose, amino acids, ions, and hormones with or without serum. The media also consists of those additional components that are required for inducing particular desired cellular behavior like proliferation and attachment. Primarily, the basic composition of media includes sources of carbon and nitrogen, for example, glucose, glutamine, and other amino acids, buffers, signaling molecules like growth factors, vitamins, and inorganic salts [22]. Each component of culture media performs a specific function. For instance, glucose and galactose serve as the fundamental sugar substrate [23, 24]. However, alternate sugar substrates like fructose, maltose, sucrose, and pyruvate are also used in some animal cell culture media, even though they are metabolized less efficiently while limiting the ideal growth [23, 25]. Glutamine and some other amino acids are added to the culture media as nitrogen sources. All 13 essential amino acids are compulsory for culturing mammalian cells. Nitrogen is an important component of nucleotides and proteins required for the functional and structural properties of the cell [26]. Buffer solutions and balanced salt make up the basic requirement for specific media formulations. They play a vital role in regulating the osmolarity and pH of the media [22]. In all serum-free media formulations, insulin has been demonstrated to be compulsory. Glucocorticoids, triiodothyronine (T3), and other hormones are also extensively used in serum-free media for increasing intracellular cAMP levels in specific cells. Cells are unable to synthesize adequate amounts of vitamins and subsequently, significant supplements are needed in tissue culture. The vitamins are necessary for the growth and propagation of cells. For decent growth and proper propagation, seven vitamins are required which include choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine [26–28]. B vitamins are essential for cell biochemistry. Tricarboxylic acid intermediates along with trace elements such as copper, zinc, and selenium, and are

needed for the proper growth of cells [30]. In addition to all this, additional components are further required in certain cell lines to complete their growth media. Such components and supplements are mandatory in sustaining, proliferating, and maintaining normal metabolism [31, 32]. Conventionally, supplements and growth factors are provided as bulk as fetal bovine serum. Proteins act as transporters for various low molecular weight constituents and also encourage cell adhesion [29]. For instance, bovine serum albumin is frequently utilized as a carrier of lipids. Currently, for animal component-free cultures, multiple recombinant proteins such as albumins are available [33].

13.5.4 Feeder Layer

Feeder layer cells generally include the growth-arrested adherent cells, which are biologically active and viable. This technique was pioneered by Puck and Marcus in 1955 [34]. The feeder layer acts as a foundation medium on which different cells are grown at a low clonal thickness. Technically the proliferation of feeder layer cells is done by either irradiating them with the particular wavelength of the light or by treating them with certain chemicals. The feeder cells are capable of enhancing the survival and development of fastidious cells that require the assistance of some growth factors and receptors. Some cell types are dependent on a feeder layer for survival and multiplication, however, few types of cells have been developed feeder-free. The latter is achieved by covering the culture dishes with extracellular matrix proteins like fibronectin, collagen, laminin, or a combination of the extracellular network segments (Matrigel), enhanced with a medium molded by feeder cells.

13.6 Biological Contamination

According to Lincoln and Gabridge, cell culture contamination continues to be a major problem at all levels of cell culture research, ranging from simple to highly sophisticated laboratories [35]. Contamination may be because of chemicals such as media impurities or by biological agents like viruses, bacteria, mycoplasma or fungus. Nowadays, testing approaches have been improved and protective tools are easily available that minimize contamination to a greater extent. While it is difficult to eliminate the media contamination, but a thorough understanding of the contamination source or agent may minimize contamination significantly. There is a consciousness among the researchers toward the risk and effects of contamination in cell culture settings, but, still, it remains the worst nightmare of every scientist working on cell lines.

It is reported that in the mid-1990s around 11–15% of laboratories in the USA working on cell lines, were infected with mycoplasma species [36]. In another study, the mycoplasma contamination in research laboratories was reported to be

23% [37]. In 2010, cultures that were commercially tested from biopharmaceutical sources showed fungal and bacterial contamination in approximately 8.45% of cases [38]. Hence, continuous efforts like the introduction of complete aseptic techniques are put in place to minimize the serious contamination issues. Contamination is not just a simple problem, it leads to loss of valuable resources, time, manpower and money. In addition, it affects the reliability of a researcher and scientists. In biopharmaceutical work, contamination can even lead to the discarding of the entire production setup. Therefore, it is imperative to understand the major contaminants and their sources so that their prevention is formulated.

13.6.1 Major Contaminants in Cell Culture

The major contaminants in the cell cultures include bacteria, fungus, mycoplasma, viruses and cross-contamination by other cell types. Based on ease of detection, biological contaminants can be divided into two types; bacteria and fungi which are easy to detect, whereas viruses, mycoplasmas, and cross-contamination which are difficult to detect.

Bacteria and Fungi

Bacteria and fungi are present everywhere in the environment. They are fast-growing and can inhabit and flourish in the culture media very quickly. Because they are smaller in size and their growth rates are faster, these contaminants become the biggest problem for scientists who deal with cultures. Bacteria can be detected in a culture within a few days of contamination. Contamination caused by the bacteria can be visualized under a microscope or can be perceived by changes in culture characteristics such as turbidity or cell detachment. Yeast contamination is generally observed by looking at the medium as it changes the growth medium to cloudy or turbid. Similarly, molds appear as a furry mass due to branched mycelium and can be seen floating in the medium.

Mycoplasma

Mycoplasma is the most widespread and severe of all the biological contaminants found in cell cultures because of the smaller size and least detection rates. Technically mycoplasma is bacteria, however, they possess certain unique features which distinguishes them from eubacteria. Their size range from 0.15 to 0.3 μm and are much smaller than most bacteria, giving them the advantage of sliding through the filtration membranes used for sterilizing the media. They grow larger and remain unnoticeable even at higher densities. Mycoplasma does not have a cell wall and is therefore resistant to most antibiotics. They are enormously harmful to cell cultures. They can cause damage to the shape and metabolic activities in host cells, cause chromosomal aberrations and can incite cytopathic reactions. Approximately 25–40% of mycoplasma contamination has been found in laboratories operating in Europe. While Japan has witnessed as high as 80% contamination cases [36]. The

presence of mycoplasma species can be detected by using advanced methods of testing like diamidino phenylindole (DAPI) staining or PCR simultaneously [37].

Viruses

Viruses also do not show any visible signs of presence in culture media. Their contamination does not lead to any turbidity or pH change in the culture medium. Viruses have very strict growth conditions for their hosts; hence, they do not exert any serious effect on cell culture. They can cause harm to the host cell if derived from the same cell species. However, the most important worry for their contamination is the risk of infection to laboratory people.

Other Mammalian Cell Types

Cross-contamination is an undesirable and unwanted transfer of cells from one culture to another. This leads to the wrong conclusions of the experiment. This critical problem has been taken into consideration only recently [39, 40]. It is one of the main reasons that almost 15–20% of cell lines that are currently in use are misidentified [41, 42]. Such cross contaminations are harder to detect with conventional methods. However, using differential media, applying Giesma-banded karyotyping and isoenzyme analysis are few authentic methods to spot cross-contamination. Recently methods like DNA fingerprinting, cytogenic examination or bcr–abl genotyping are few advanced methods used to check cross-contamination. While dealing with multiple cell lines at the same time utmost care is warranted.

13.6.2 Sources of Biological Contaminants in the Lab

Contaminants enter a culture system as a physical, chemical, or biological element of the environment. Usually, laboratory personnel are the main source of contamination in most laboratories. Coughing, sneezing, and chatting are the potential ways that generate contaminants in the laboratory. Even microbes accompanying the laboratory personnel can reach the culture. Even the air currents created by the movement of personals working in the laboratory could be the reason for contamination. The equipment like pipettes, vortex mixers, or centrifuges used in a laboratory can produce huge quantities of microbial-laden particulates and aerosols. Similarly, microbes and fungi are observed frequently when using instruments like water baths, refrigerators, and microscopes. Ill-maintained incubators or media components can result in serious contamination issues. Uncertified culture media, reagents, and plastic ware may also pose a significant risk of contamination. Simultaneously, working with many types of cell lines may lead to cross-contamination. To use cell culture successfully, all apparent cell culture apparatus must be stabilized and sterilized.

13.6.3 Measures to Contain Contamination

Numerous measures are undertaken to prevent physical, chemical, and biological contamination in the laboratory. It is important to keep the laboratory surrounding clean and personals working in such laboratories must maintain hygiene around the workplace. It is a must to wear sterile lab wares while working in the culture laboratory. Since commercial testing techniques are significantly better than those of previous times, it is fundamental to utilize only those materials that are guaranteed for the use of cell cult some under given points should be taken into consideration to minimize the chances of contamination.

Disinfection
Disinfection is an important tool used to minimize the chances of contamination in the laboratory. Solvents having an antimicrobial effect, are used as disinfectants. It is recommended to wear suitable personal protective equipment (PPE) while using disinfectants. Major disinfectants used in the laboratory include sodium hypochlorite, ethanol and isopropanol. Sodium hypochlorite or bleach is a good general-purpose disinfectant and beneficial against viruses. However, it is corrosive and therefore should not be used on metal surfaces. Likewise, ethanol or isopropanol used at 60–70% (v/v) concentration is effective against bacteria and some viruses.

Working Within the Biological Safety Cabinet
The biological safety cabinet should be cleaned with 70% sterile alcohol before making it on. The cabinets are provided with ultraviolet lamps, therefore should be treated with UV radiation for approximately 15–30 min. Before placing the items in the chamber, the airflow should be initiated and continued for at least 20 min. Every required item that is kept on the cabinet shelf must be wiped with 70% alcohol. Any vessel, tube, flask or glassware must be brushed with airflow and mild heat, before using in the culture process. It helps in the elimination of particulates brought together with the items out of the work area.

Pipetting and Prevention of Aerosols
Serological pipettes are essential tools and therefore must be enveloped separately to maintain sterility. To avoid contamination, the pipette aid parts should be disassembled and disinfected regularly. In addition, plugged pipettes should be used whenever needed. During a media transfer, drawing of liquids in pipette plug should be avoided. Physical touch on the serological pipette tips or the point of entry must be avoided. Bubbles should not be created in the medium or pipette to avoid generating contaminating aerosols.
The best way to minimize the contamination is to take all precautions while working in the cell culture laboratory. Adhering to best laboratory practices and working with cell culture grade chemicals and specialized instruments are important for successful cell culture experiments. Use of lab coats, gloves, and hoods should be encouraged. The laboratory disinfection process should be carried out regularly to avoid microbial growth.

13.7 Significance of Making Aliquots of Reagents

An aliquot is a homogeneous subsample that is taken from a whole or original sample. In other words, an aliquot is the fractional part of an entire sample volume or partial piece of the whole sample. For example, if we have a stock solution of 30 mL, and in our experiment, we need 3 mL of this solution. The minimal required volume (3 mL) will be our aliquot from 30 mL of stock solution. Aliquots can be prepared fresh or can be stored over months at appropriate storage conditions. Aliquoting helps to maintain the integrity of the specimen and reduces the risk of contamination while sharing reagents within or outside the laboratory. Prealiquot sampling is a great way of minimizing source contaminations. Secondly, aliquoting keeps samples stable for a longer time as individual tubes undergo a minimum number of freeze and thaw cycles. There is always a risk of contamination when a sample is open and closed multiple times.

13.7.1 Shelf Life of Reagents

The shelf life of a reagent is the length of the time that a reagent can be stored without losing its function or activity. Certain environmental factors like light, temperatures, humidity, pH, and bacterial contamination can cause reagents and chemicals to degrade. To maximize the shelf life of chemical or reagents, they must be stored as per the recommendation of the manufacture. The in-house reagents must be stored in such a way so that the chances of degradation are minimal. Chemical reagents could be stored at room temperature or lower temperatures in the fridge. The opened containers must be handled and stored properly because atmospheric contaminants may enter the container and react with the reagent component making its use unhealthy. Older reagents and chemicals that are not working properly should be disposed of as per the material safety data sheets and standard operating procedures of the laboratory.

13.7.2 Effect of Freeze–Thaw on Shelf Life

The process of thawing and freezing are intricate procedures that result in a sequence of changes in the physical and chemical properties of the reagent [43]. The quality of a reagent is widely characterized by its colour, texture or appearance. These parameters may be affected if multiple freeze–thaw cycles are undertaken. Freezing inhibits enzyme activity and helps in reducing the deteriorating chemical reactions thus increasing the shelf-life of the product [44]. However, fast freezing leads to protein damage and there is a minimal recovery rate of activity after the freezing–thawing process [45]. Inappropriate freezing, storage and thawing lead to chemical,

physical, and microbiological deterioration of the products [46]. The process of defrosting affects the shelf-life of the products. Defrosting if carried out at an ambient temperature maintains the shelf-life of the product in an ambient way. It is observed that there is a 25% reduction in the shelf-life of the products if defrosting is carried out above 5 °C as thawing process [47].

13.8 Statistics in Data Handling and Interpretation

Statistics is a science of decision making under unpredictability, supposed from numerical and measurable scales. It is the study of assembling, structuring, summarizing, and analyzing data and making inferences after observing a given part of the data. The decision making process must be based on data, neither on one's opinion or belief. Statistics are presented as the "classified facts representing the condition of a data set that can be stated in numbers or any other tabular or classified arrangement" [48, 49]. The application of statistics is used in various disciplines in problem-solving and data interpretation. Computational methods embedded with classical statistical tools help in elucidating numerical data. It helps to determine the population parameters like mean, standard deviation, hypothesis testing, inferential statistics, p-value, and standard error of the mean. Successful data interpretation and analysis is very important in research. Unvalidated data with no statistical analysis touch makes data unreliable and doubtful [50, 51].

Data interpretation must be done with absolute honesty as it plays an essential role in the usefulness of research findings, otherwise the research output might become futile. Interpretation has two major dimensions viz. continuity in research and establishing underlying conclusions. Interpretation helps in comparing the earlier and new findings, serves as a guide and helps the researchers in the continuity of research and establishing concrete facts. Data may be interpreted by scientists in different ways based on their knowledge and experience. Interpretation becomes an interactive process by collecting and analyzing data concomitantly. These interpretations could be presented in the form of tables, graphs, plots, or diagrams.

The basics of statistics include the measurement of central tendency and the measure of dispersion. The central tendencies are mean, median, and mode, and dispersions comprise variance and standard deviation. Mean, median and mode in statistics give the central tendency of the observations and these are used to summarize the position of the observations in a given data set.

13.8.1 Mean

The mean is an average value from the set of observations. It includes every subject from the study and is affected by inconsistent low and high scores that sometimes alter the expected information of the analyzed data. It is obtained by counting all the scores and dividing them by the number of scores. Mean is generally denoted by X:

$$\overline{X} = x_1 + x_2 + x_3 + x_4 + x_5 + x_6 \ldots \frac{X_n}{N},$$

where $\overline{X}$ is the mean of the sample, x is an individual number of the sample, and N is the total number of samples. x_n is the total sum of all the samples.

Mean is the most important tool used in data interpretation as it forms the basic foundation in understanding and carrying out all the complex statistics. It is the “central gravity” of the data and takes information from all the subjects of the study and provides the generally desirable results which is a better representation of data. From the measures of central tendency, mean resists any variation between different observations [52].

13.8.2 Standard Deviation

Standard deviation is the statistical measure that is mostly used to determine the variability of the data [53]. It is the estimate of the spread of data from the mean and is represented mathematically as the square root of the variance [54]. The standard deviation will be high if the data points are away from the mean that is more spread out data and vice versa. Standard deviation is high for volatile stocks and low for stable stocks. The normal curve and the mathematical relationship of the data are determined by statisticians using standard deviation. The effect of outliers is the biggest drawback of standard deviation. Standard deviation is calculated by the following equation:

$$\sqrt{\frac{\sum_{i=1}^{n}\left(x_i - \overline{x}\right)^2}{n-1}},$$

where x_i is the value of the ith point in the data set; x bar is the mean value of the data set and n is the number of data points in the data set.

13.8.3 Standard Error

The standard error is the measure of accuracy and efficiency of the samples in an observation that gives the precise distribution of the samples in a data set [55]. It is very useful in testing hypotheses and regression analysis. It forms the basis in inferential statistics in the establishment of confidence intervals. The standard error helps in identifying the extent of variation and determines the measure of the mean population [56]. The standard error is expressed using the following equation:

$$\sigma\bar{x} = \frac{\sigma}{\sqrt{n}},$$

where σ is the population standard deviation; n is the size of sample, that is, the number of observations in the sample; and $\bar{x}$ is the mean of the sample.

13.9 Use of Excel Spreadsheet in Data Handling

Spreadsheets are commonly used software tools that are used for data entry, storage, analysis, and visualization [57, 58]. They are the easiest tools to store and preserve data in a useful way. They present a convenient graphical interface for plotting data tables and basic data quality control assignments. They play an essential role in the Operational Research/Management Science (MS/OR) practitioner's toolkit. For sophisticated end-user modelers, it provides access to the power of MS/OR. The spreadsheet is an expeditious fourth-generation programming language that could be an appropriate option for projects needed for fast disposal of higher production [59]. Spreadsheet programs facilitate the entry and storage of data information in large worksheet formats which resembles the SPSS data editor in appearance and function and have the potential of entering equations in the worksheet which are useful in doing calculations within cells. Various programs like Microsoft Excel, Lotus 1-2-3, and Quattro are applicable in arranging a large database in spreadsheets. Preliminary models of spreadsheet software were devised particularly for accounting exercises which were simple in their functions.

Since spreadsheets are the most common form in which data are presented to a statistician. Microsoft Excel continues to be the most widespread business analytical standard due to its universal and continual accessibility, user friendly and ease to use [58]. Moreover Excel appears to be the most used program by biologists and biomedical researchers [59].

Microsoft Excel has a capacity of holding more than one million rows (1,048,576) in addition to 16,000 columns (16,384) of a database in a worksheet. An analyst can slice and dice spreadsheet data organized into rows and columns through pivoting, filtering, sorting. It also gives access to create formulas and do statistics for carrying data. The spreadsheet is comprised of columns and rows in which each row stands for a new set of data and different types of data, like text, number, and date are represented in columns that are labelled accordingly. The individual elements are known as cells which can be given an equation and determine its value equal to some function of the other cells. It is the choice of the user to select different graph types which include a bar, line, pie, column, area, surface, and bubble, and may also merge some of these graph types. The particular graph type can be selected which efficiently give the intrinsic nature of the data.

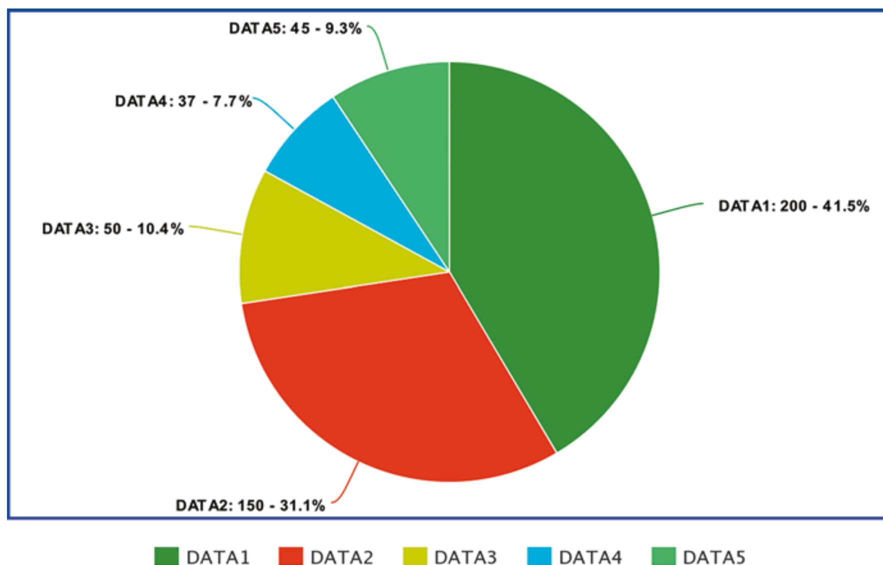

Fig. 13.2 Representational diagram of a pie chart. Each color-coded slice represents a set of the proportion of data

The graphs can be constructed while plotting the dependent and independent variables on the *X*- and *Y*-axis designated with numerical magnitude. Moreover, the order of the data, the positioning of the title, axis, chart, subtitles, legends, scales, major ticks, and minor ticks can be entered or modified with the least difficulty. Excel enables to insert of trend lines and carry out linear regression of the data by just clicking on the chart data. Post-graph preparation changes in any data in the worksheet result in an instant recalculation of all formulas and the graph gets updated to reflect the new values. Most of the spreadsheet programs display results graphically, giving a quick insight into the data [58].

13.9.1 Pie Chart

A pie chart or circle chat is a type of statistical graphic which is divided into slices, where each slice illustrate a numerical proportion (Fig. 13.2). The area of every slice presents the magnitude of each data set it represents. Each slice is colour coded to give a differential look to the data present within the slices. Three-dimensional pie chart, exploded pie chart, waffle chart, and sunburst pie chart are some subtypes of the pie chart. Despite having some limitations, pie charts are widely used for presenting or comparing different data sets under one statistical frame.

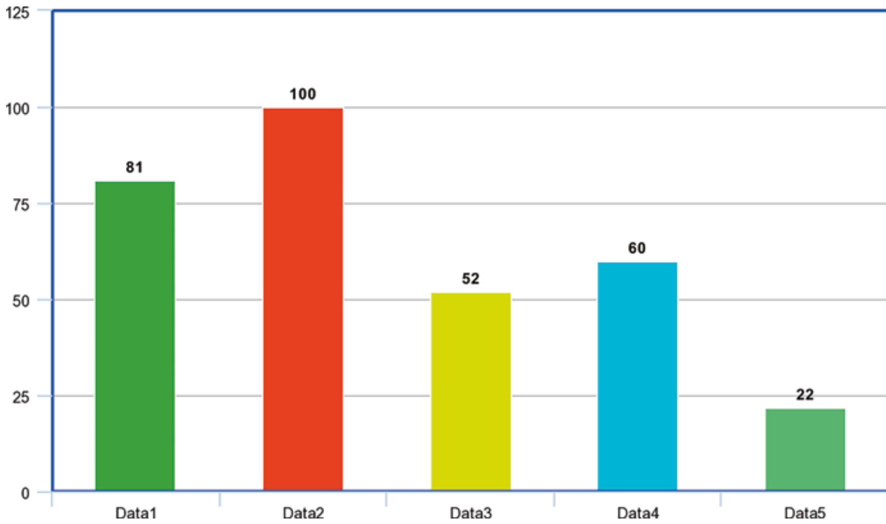

Fig. 13.3 Representational diagram of a bar chart. Data is presented in vertical bars with values plotted on the *Y*-axis and the name of the variables on the *X*-axis

13.9.2 The Bar Chart

A bar chart is another type of data presentation method used by professionals. The bar chart presents data in vertical bars with values plotted on the *Y*-axis and variables on the *X*-axis (Fig. 13.3). The bars are constructed in rectangular shape and the height of each bar corresponds to the value plotted. There are multiple variants of column charts like clustered, stacked and multiple column charts. Columns are constructed on all three dimensions using *X*, *Y*, or *Z* axes.

13.9.3 Line Chart

The line chart is a type of data representation in which data points are connected by a line. The line chart is mostly used to display trends concerning time (Fig. 13.4). It is also helpful in displaying trends for multiple series. Inline charts the *X*-axis displays time and *Y*-axis shows numeric values. Stacked line charts and 3D line charts can also be displayed; however, 3D line charts do not present data well in three dimensions.

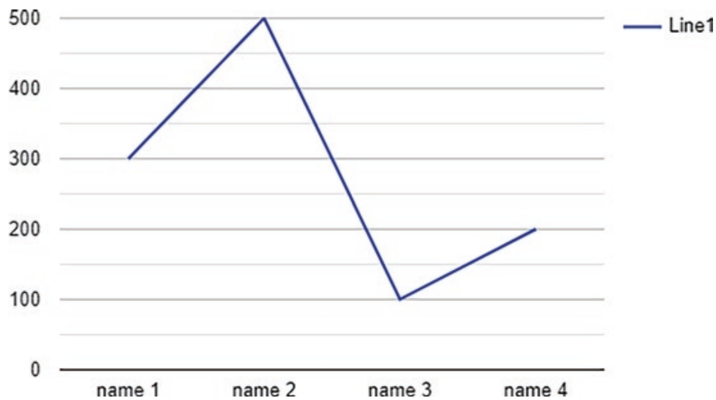

Fig. 13.4 Representational diagram of a line chart

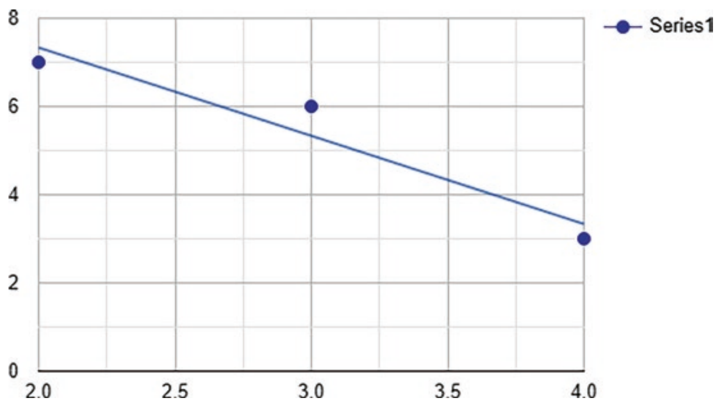

Fig. 13.5 Representational diagram of a scatter chart

13.9.4 Scatter Chart

Scatter charts are used to display a comparison of two series of data over time and category. Like line graphs scatter plots use horizontal and vertical axes to plot data but have a different basis (Fig. 13.5). Scatter plots show the relationship between two variables, that is, how a variable changes concerning another. It displays a positive correlation if there is a similar increase and a negative correlation if the decreasing trend is observed. Scatter charts are displayed in different formats. Data points are shown as markers that are connected or unconnected by smooth or straight lines.

13.10 Skills of Data Collection, Recording, and Report Writing

Data collection consists of gathering information and measuring it on different parameters. Data collection is established in a systematic fashion that allows the researcher to question and evaluate the outcomes. While collecting and analyzing data, it is ensured that the accuracy and validation of the data are preserved [60]. Various physical devices and tools are used for data recording. Some of them are as simple as paper, pencil or pen, cameras, video cameras and voice recorders or any other tool that is needed as per the nature of the study. Using organizational tools, data are recorded, organized and saved efficiently. The use of technology with manual efforts makes data compilation and analysis trouble-free. Data should be handled and organized as soon as it is collected or recorded. A model for recording and analyzing data should be prepared beforehand. Different analyzing approaches for recording and analyzing qualitative and quantitative data should opt. For large data sets with multiple coworkers working on a single project, it becomes difficult to record data and compile it. For this reason, data management applications like laboratory information management systems (LIMS) or electronic laboratory notebooks (ELNs) are widely used to compile and organize big data.

There are multiple ways to present unorganized or analyzed data. One of the presentation forms is a “report.” A report is a detailed write-up of the work concerning all aspects of the project. It is written for an obvious purpose and a particular audience. The reports present precise information about the workflow, data sets and analysis. Reports can be written in structured or unstructured formats, though no universal template exists. However, a mostly structured one is preferred as it mentions sections and subsections so that the information is easy to locate and follow.

13.11 Summary

Microbial cell culture is used in research for various applications. Essential experiments like cloning and recombinant protein expression are widely studied using microbial cultures. They are also used in various clinical applications like isolation and detection of pathogens, which help in disease diagnosis and clinical management. Bacterial growth can be carried in broth or solid nutrient agar using pure culture techniques to avoid contamination. Before growing any microbe, it is important to characterize and evaluate the media components as per the growth requirements of the microbes. Different microbes have different growth requirements ranging from simple to complex, exhibiting similar growth kinetic parameters consisting of lag, log, exponential and death phases. The growth kinetics monitoring is vital for maintaining a resourceful microbial growth. The simplest method to measure or monitor microbial growth is by measuring the OD at regular intervals of incubation using a spectrophotometer.

Like microbial culture, eukaryotic cell culture has also proved a significant means for biological research. Cells require to grow with a complex nutritional requirement under a controlled physiochemical environment, with the highest care to avoid contamination from physical or biological agents. However, decontamination procedures like the use of disinfectants and aseptic techniques exist which can limit or stop any kind of contamination threat. Currently, the application of cell culture is utilized in the field of biological and medical sciences in exploring the mechanisms involved in cell-to-cell communication, protein interactions, hormone regulation, as well as other cellular and biochemical processes.

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Chapter 14
Ethics and Medical Biotechnology

Aqeel Ahmad, Prabjyot Jhatu, Ahmed Abu Fayyad, and Mohammad Tauseef

Abstract Science is a human activity that encourages human beings to explore nature and to seek deeper and comprehensive knowledge for the betterment of the living beings on the planet earth. Sometimes scientific endeavors or their outcomes raise some ethical questions in the society. Scientific community has always tried to comply with the ethical recommendations prompted by authorities or community organizations. However, there will be severe consequences if the researchers deviate from the set forth ethics in their scientific quarries. Biotechnology improved the life of human beings tremendously by providing diseases resistant crops to effective medicines. However, just like its high potential in human lives, if not properly regulated ethically, its harms are also significant. Today, biotechnology has come on the center stage of ethical debates due to its high probability of misuse. This chapter provides an overall view on the ethical aspects of biotechnology in scientific research and will specifically focus on the biosecurity issues.

Keywords Biotechnology · Biosecurity · Dual use research · Bioethics · Genetic engineering

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14.1 Biotechnology and Society: Introduction to Model Systems, Use of Animal Models for Human Diseases. Recombinant DNA Technology

Many fields in scientific research had initially been taboo. For example, the dissection of human cadavers to learn the anatomy and to train the medical doctors disturbed church and the public as well, initially [1]. But later it became an inherent part of medical education [1]. This remains the case when a new scientific endeavor is seen in a moral perspective. But sometimes this act of science is not that much innocent [2]. When someone touches the human embryo, cut a gene and insert a new one into human, or sell designer babies, then eye brows raising is natural and these scientific practices raises the serious ethical issues in the human society. The field of biotechnology, which allows researchers to manipulate the genes for variety of justifiable reasons but if goes in wrong hands can brings harms and destruction as well [3]. In this industry every advancement and research are conspicuous to press, governments and public for scrutiny [2]. Though biotechnology has given us tremendous potential for developing new drugs, deciding extremely personalized treatment regimen and creating cure for incurable diseases, it also triggered ethical debates as it handles and manipulates life's core material—DNA [3, 4].

Apart of morality, unintended consequences of biotechnology engineering products or techniques are also a concern for ethics defining organizations [5]. Introducing new species into ecosystem can fairly be dangerous not only to man but the kingdoms of flora and fauna [2]. Corporates having mega financial interests may ignore a limiting ethical alert to achieve the business goal, that may prove potentially harmful in future [2]. On the other hand, politically or otherwise motivated organizations can be in search of some breakthrough that could help in making or deploying biological and toxin weapons [6]. Civil society is also raising voice about biotechnology-induced inequality in the society. For example, $475,000 cancer therapy or $20,000 yearly expense for a biologic is obviously a challenge for sociologists, economists, and health insurers [7].

In this context researchers, reviewers, funders, and policy makers all have burden of keeping the freedom of scientific research intact while finding ethically acceptable ways to do this [2].

14.2 Biotechnology and Human Welfare: Introduction of Transgenics, Creation of Genetically Engineered Organisms, Biosafety, Biowar, Biopiracy, and Human Cloning

For the purpose of simplicity, problems of ethics in biotechnology can be divided into two subgroups [2, 8].

1. Misuse and dual-use problem.
2. Unintended consequences dilemmas.

14.2.1 Misuse and Dual-Use Problem

The rule of the modern science is to share every new knowledge that emerges in any research [8]. Fears could not overrule this. But in this global village we are constantly watching some eyes tickling on opportunities to misuse this human asset [2, 9, 10]. A very popular case is of Ron Fouchier in Netherland in 2011 [9, 11–13]. He showed that after some genetic modifications (mutations), lethal Avian Flu virus (H5N1), which is not air transmissible, can become so. This research was prevented to be published, although it was published later [9–13]. The Dutch government saw a risk in letting this published in an open platform, and labeled sending this paper to *Science* as an "export" [8, 14]. Government also wanted future H5N1 transmissibility studies to be approved as well [10, 14]. By doing so the Dutch government interpreted European Union regulations aimed at preventing the proliferation of weapon of mass destruction [10, 13]. Its document has a list of dual-use items [10]. It not only monitors export, trade, and transfer of virus materials but interestingly also apply to knowledge that helps in making any bioweapon [8, 10, 15]. Foucher's lawyers argued that his research work is excluded as an exception in Annex as information present in paper was already in public domain [8]. Finally, Fouchier got an export license for his work [8]. Court asserted the delay in dissemination of scientific knowledge by these proceedings but marked this as outweighed by the need of preventing bioweapon proliferations [8, 16].

Eminent bioethicist Arthur Caplan has hard stand on publishing sensitive biologics strategies to open platforms [2, 17]. He says, "We have to get away from the ethos that knowledge is good, knowledge should be publicly available, that information will liberate us. Information will kill us in the techno-terrorist age". The openness of science publishing is crucial for dissemination of science research and advancement, but on every case sensitivity and reach should be decided by the fraternity [2, 17].

Another most talked about case is Anthrax attack in the USA just after 7 days of 9/11 event in 2001. Some letters reached media companies and congressional offices loaded with anthrax spores [18, 19]. Five people died, and 17 others fell ill. FBI suspected and raided a scientist who later sued government and settled with $5.8 million [19, 20]. Later, another scientist, Dr. Bruce Edwards Ivins, associated with US Army Medical Research Institute of Infectious Diseases, Maryland, was suspected who allegedly committed suicide in 2008. Interestingly, Dr. Ivins was the man who had helped develop an anthrax vaccine to protect American troops [19, 20].

If we see earlier in 2002, newspaper across the world highlighted the creation of a virus in a test tube in Stony Brook University, NY, USA [21]. Scientific community was amazed, and public was shocked as to why a virus being created when everyone is wanting to get rid of this globally. Behind this was a German-origin organic chemist, Eckard Wimmer, who chose to study poliovirus in 1968 [21]. In the 1960s, molecular biology of poliovirus proliferation and mechanism of its pathogenesis was not understood [21]. His work in 2002 reduced poliovirus to a chemical entity [21, 22]. He wrote in his own paper, "We were probably among the

first to realize the potential dangers associated with this technology—the possible misuse of viral synthesis in bioterrorism. The US Défense Research Project Agency (Arlington, VA, USA) took the same stand and provided funding for our project, an endeavor we considered as a wake-up call" [21].

This breakthrough was rightly a concern from biosecurity pint of view. Although other more virulent viruses are complex in nature and difficult to be created like this, but technique used by Wimmer could be misused by determined exploiters [22]. Further, advanced aerosolization techniques will make this agent more virulent to target population [22].

14.3 Bioethics, Ethics of Genetically Engineered Crops and Animals. Public Concerns and Controversies Associated with Biotechnology

Now consider a novel technique that is going to remove animal models from experiments and hence are going to reduce drug or vaccine development time drastically [23]. Recent advances in tissue engineering and microfabrication technologies have enabled us to develop organ-on-a-chip (OOC) or microphysiological systems (MPS) [23, 24]. An organ-on-a-chip is a multi-channel 3D microfluidic cell culture chip that simulates the activities, mechanics and physiological response of entire organs and organ systems, a type of artificial organ [24]. These 3D model chips can mimic animal or human physiology [25]. Its applications will be in drug development, drug and toxicology screening and disease modelling etc. In the last 4 years, the rate of development of these systems have increased exponentially. For example, lung-on-a-chip microdevice places two layers of living tissues—the lining of the lung's air sacs and surrounding blood vessels—across a porous flexible boundary [23]. Now this alveolar lining can be challenged experimentally just like the real lungs of an animal and record the responses. Apart of other models like heart-on-a-chip and nephron-on-a-chip, human-on-a-chips are also available [24]. This is multiorgan microphysiological system that can integrate and reproduce complex interactions between different organs or tissues in the body. Here the concern is till now, researchers of wrong and terrorizing intentions needed a whole of logistics and a lot of time to develop a potent toxin [23, 25]. Can this technology be misused by them to extremely fasten their process with less infrastructure [23]? If yes, then how can we prevent them by doing so?

Lastly, a report by British Medical Association—"Biotechnology [26], Weapons and Humanity II," says, the combination of human genome studies, the development of vectors capable of introducing harmful material to cells and new ways to disrupt genes should raise concerns about potential misuse [26, 27]. It also warns that a bioweapon can be a genetically targeted one. It means that a disease agent can be tailored to be activated only when a person of an ethnic group, having targeted SNP, is infected [26, 27].

14.4 Ethical Considerations of Genetic Testing in Humans

In 2003 the National Academies published "Biotechnology Research in an Age of Terrorism." This is also called "Fink Report" [28]. The committee was chaired by Gerald Fink. The committee's first recommendation is as follows [29]:

> *We recommend that national and international professional societies and related organisations and institutions create programs to educate scientists about the nature of the dual-use dilemma in biotechnology and their responsibilities to mitigate its risks* [28, 29].

The Fink committee suggested that following classes of research must raise concern in the scientific community [28]:

- Research that demonstrates how to render a vaccine ineffective.
- Research that confers resistance to therapeutically useful antibiotics or antiviral agents.
- Research that enhances the virulence of a pathogen or render a nonpathogen virulent.
- Research that increases the transmissibility of a pathogen.
- Research that alters the host range of a pathogen.
- Research that enables the evasion of diagnostic/detection modalities.
- Research that enables the weaponization of a biological agent or toxin.

Fink committee recommended to resolve a national committee in the USA, to "provide advice, guidance, and leadership for the system of review and oversight we are proposing" [28, 30]. This led to chartering of National Science Advisory Board for Biosecurity (NSABB), by Executive Office of the President "to provide advice, guidance and leadership regarding biosecurity oversight of dual-use research, defined as biological research with legitimate scientific purpose that may be misused to pose a biologic threat to public health and/or national security" [29]. The board has advisory capacity to [28, 29] the following:

- Secretary of HHS.
- The director of NIH.
- The heads of all federal agencies and departments that conduct or support life sciences research.

Currently in the USA, any academic institution involved in recombinant DNA research and funded by National Institute of Health, must prove to be complied with federal oversight guidelines. Recombinant DNA advisory committee is also sitting to oversee this [31].

14.5 Intellectual Proprietary Rights (IPR) and Patenting. Statutory and Legal Obligations of Biomedical R&D Organizations

14.5.1 Regulations in India

In India multiple authorities and organizations form a complex network to regulate issues regarding genetically engineered products [2]. Genetic Engineering Approval Committee (GEAC), Institutional Biosafety Committees (IBSC), Recombinant DNA Advisory Committee (RDAC) and Review Committee on Genetic Manipulation (RCGM), operate collaboratively as regulatory regime. The RDAC and RCGM report to the Department of Biotechnology; and the GEAC reports to the Ministry of Environment and Forests [2].

RDAC monitors the developments in biotechnology at national and international levels and submits recommendations for the safety regulations. IBSC is constituted by every organization involved in research in genetically modified organisms. It needs approval from department of Biotechnology. Every R&D project must have an investigator who informs about status and results of experiments to IBSC [2]. RCGM under the Department of Biotechnology, Ministry of Science and Technology monitor the safety of ongoing research projects. It provides biosafety clearance on the recommendation of the IBSCs [2]. GEAC functions as a body in the Ministry of Environment and Forests. It is responsible for approval of activities but with environmental angle only. GEAC will be replaced by National Biotechnology Regulatory Authority (NBRA) if it is cleared in the parliament [2].

Being many organizations in place, India needs to do far more to increase the level of awareness about Dual Use Research Concern (DURC), at the funding and publication stages [32].

14.6 Examples of Revolutionary Breakthroughs in Biotechnology: Insight into the Technologies Involved

Biotechnology is about manipulation of genetic material. A material which nature gave us through lacs of years. Its integrity with nature is tested and optimized with time [33]. Now if change or delete a part of DNA in a species. We must be sure that it will not cause any disruption in the balance in the living world. Sometimes our manipulation, when incorporated in the ecosystem, may lead to unintended consequences. Majority of fears may prove to be exaggerated whereas some are with substance [33].

A naturally occurring phenomenon is there, in which gametogenesis and reproduction is manipulated by some genetic elements to increase their transmission to next generations. This phenomenon is called “gene drive” [33]. This gene drive can also be synthetic. That means, scientists can exploit this drive to spread a trait,

rapidly in a species to modify or eliminate that target species. This is being considered as an efficient mechanism to control insect-borne diseases and pests in crops. Effect on non-target species, evolving an ability to come out of that drive and disbalance of ecosystem are some of risks associated with it [33]. One of the examples works is: an intracellular parasite, *Wolbachia*, was inserted into *Aedes aegypti*. This mosquito is a carrier for dengue, Zika, and chikungunya viruses [33]. Through cytoplasmic incompatibility, the strain of *Wolbachia* spreads throughout mosquito populations. Since the release of this strain of mosquito in Cairns in Australia, the city has become dengue free [34]. At another front, development of gene drive systems intended to reduce reproduction and potentially eliminate invasive rodents from some islands is also in progress [33, 35].

As mentioned above, one of the risks associated with synthetic gene drives is spilling over of gene drives into nontarget populations and species. The US National Academy of Sciences recommends that, before any release of gene drive into wild, risk of horizontal gene transfer should be evaluated [27]. However, the Royal Society in 2018 has recommended to the UN Convention on Biological Diversity (CBD) to avoid moratorium on gene drive research internationally [33]. Later, the moratorium was reworded to include consultation with potentially affected local communities and indigenous groups before any planned release of gene drive [33]. Note that even armed with strategies to reverse a gene drive, we may find gene drives difficult to control once they spread outside the lab [36].

Another field where prominent scientists and journals are calling for a moratorium is, using gene editing (CRISPR/Cas9) tool in viable embryos. CRISPR-Cas9 is a genetic technique that enables us to edit DNA with unprecedented precision and ease [37]. Through this we can change human germ line to get rid of genetic diseases. Notably these changes will be permanent and inheritable throughout the progeny tree so that it causes a permanent change in human gene pool [37].

In 2015, a meeting was convened, by four prominent academies of global repute, in Washington. There, a group of scientists met to discuss the moratorium on making inheritable changes to human genome. The organizers were National Academy of Sciences of the United States, the Institute of Medicine, the Chinese Academy of Sciences, and the Royal Society of London. These academies have no regulatory powers, but their recommendations have weight in the scientific community [38]. Several speakers put plans for correcting DNA sequence in egg and sperm for various hereditary diseases. But many pointed out that there was no current demand for making any heritable changes to the human genome [38]: firstly because diseases caused by erroneous genes are rare, secondly, if single gene defect is known, IVF (in vitro fertilization) can give a healthy child through genetic screening and healthy embryo implantation. It was concluded that this issue must be revisited on regular basis with better risk assessment and improvement of technologies [38]. On the other hand, Dr. George Church mentioned correctly in December 2015 issue of *Nature* that “Banning human-germline editing could put a damper on the best medical research and instead drive the practice underground to black markets and uncontrolled medical tourism, which are fraught with much greater risk and misapplication” [39].

Now head to another meeting in Harvard in 2016, where 150 scientists, lawyers, and entrepreneurs met to discuss whether we should construct human genome from scratch. This skill also includes ability to sequence and synthesize the genome of a genius say Einstein. The agenda also included, Could we synthesize a modified human genome which is resistant to all-natural viruses? What if the same technique makes others capable to create modified viruses who can shatter this resistance. Will it not be a start of genome-engineering arms race [40]?

The Convention on Biological Diversity (CBD) is a multilateral treaty and an international legal instrument having three main goals [41].

- Conservation of biological diversity.
- Sustainable use of its components.
- Fair and equitable sharing of benefits arising from genetic resources.

Article 8 of CBD deals with invasive alien species (IAS). Stating that member states must prevent the introduction of, control or eradicate any IAS which threaten ecosystems, habitats, or species [41].

14.7 Summary

Scientific advancement is a precious asset of mankind. This journey of knowledge and problem-solving technologies must continue at their pace. The need is to make research and development fraternity aware for the accountability and ethics. Authorities may require policies to be implemented internationally, that can manage risks in genetic manipulations and anticipate potential ill points in any project. Further biodiversity and eco-balance must be valued in prior while deciding about any project that can have long term effects.

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Chapter 15
Careers and Opportunities in Medical Biotechnology

Ashish Sawhney, Mohd Imran, Saba Zulfiquar, and Mohammad Tauseef

Abstract This chapter presents a general overview of biotechnology as a science and as an industry of immense economic value. The many industrial applications of biotechnology have been enumerated and then we have gone to discuss the dynamic sector of medical and pharmaceutical biotechnology in some detail. We have discussed the economics of pharmaceutical biotechnology particularly focusing on the reasons why the products and services it creates have a price tag so high that they become almost inaccessible for so many who need those. Additionally, an attempt has been made to introduce the relatively much newer concepts of bio-industry and bio-economy. We have further briefly presented information about the top 5 global biotechnology companies, incidentally all happen to belong to the medical and pharmaceutical biotechnology sector, and have finally discussed about being an entrepreneur in the world of biotechnology and what that entails.

Keywords Medical biotechnology · Pharmaceutical biotechnology · Bio-industry · Bio-economy · Biotechnology companies · Entrepreneurship in biotechnology · Biotechnology start-ups

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15.1 Introduction to Bio-Industry and Bio-Economy, Economics of Pharmaceutical Biotechnology and Bioindustry

The definitions of the terms “bio-technology,” “bio-industry,” and “bio-economy” are not strictly etymological definitions the way we know and use these terms and so, their definitions are idiomatic [1–3]. Moreover, for “bio-industry” and “bio-economy,” there is no consensus on a single definition [4]. Depending on what source or sources one looks up, the scope of their definitions differs and that can be a cause of confusion during the initial attempts to try to understand those [3]. More on the definitions of these two terms will come a little bit later in this section [2, 4].

At the center of anything bio-technological is a living system, which can be a single- or a multicelled organism, or something that is derived or extracted or isolated from a living system, but if stored and used appropriately, can carry out the same function outside as it does natively [5]. One can use these living systems or derivatives from them the way they occur in nature, raw and unaltered, or these can be modified using genetic engineering for efficiency, productivity, and quality [5, 6]. With the emergence of synthetic biology, we can even engineer living systems and derivatives of living systems with desirable attributes and build the same de novo in a laboratory [6, 7]. The “technology” part refers to techniques and processes, that employ these living systems or derivatives from them (devised based on principles from cell biology, cytology, genetics, embryology, biochemistry, immunology, and microbiology) to make products or create services of added value for end uses [6–9].

In addition to the living systems, the techniques and processes utilizing those may also be modified with the same goal as that for modifying the living systems themselves. Broadly, end uses of products made and services created are either their application as tools for exploratory research or for consumption. Techniques and processes, once perfected and optimized, can also be productized. For instance, RNA and DNA preparations are now used routinely in biomedical and other life science research laboratories [10]. Such products are heavily used in exploratory research and because of their demand, there is a whole dedicated sector of biotechnology-based industry that develops and manufactures these products (to support exploratory research) [10, 11]. A major advantage of this is intra- and inter-laboratory comparability of experimental results because techniques and processes employed are product-based, not homemade [12].

15.2 Recent Achievements and Advances in Medical and Pharmaceutical Biotechnology

As for products and services for consumption, depending on what it is that we make, it has led to the creation of several biotechnology-based niches [10]. There is medical and pharmaceutical biotechnology and biotechnology applied to production of foods and beverages, to agriculture for plant crops and livestock used as food, animal feed or fodder, and for several nonfood uses [13]. Biotechnology is also applied to cultivation of plants in extreme environments which are unconducive and unamenable to typical ways of plant cultivation, for example, to grow plants in deserts [13], to control pests, to production of several nonfood items (e.g., biodegradable plastic and biopesticides), to production of living systems (e.g., microorganisms) or products of living systems (e.g., enzymes) which are used as biocatalysts in large-scale production of chemicals, pharmaceuticals, foods, beverages, animal feed, paper, textiles, and biofuels [1, 14]. Bioremediation and maintenance of biodiversity are other areas where biotechnology is applied. Lastly, biotechnology unfortunately can be and is applied for the sinister purpose of developing and producing biological weapons [1, 10, 13].

Through the human genome project, we learnt that biotechnology can be employed to sequence the full complement of genetic material/genes of anything living [15, 16]. This capability, while mostly used for scientific curiosity to sequence genomes of more and more organisms and to study inter-individual variations, has found good use in human medicine [15]. Businesses providing personal genomics services is a relatively new, but growing biotechnology-based sector of the larger industry [16, 17]. Personal genomics-based businesses can sequence, analyze, and interpret the genome of an individual [16]. This information about an individual can be used to figure ancestry and parentage, but more importantly can be used in genetic screening/testing to determine any susceptibility to genetic disorders or the likelihood of passing on one, in pharmacogenomics to develop personalized treatments, and in diagnostics to identify disease-causing pathogens [15].

15.3 Promising Areas of Research in Medical Biotechnology

All the biotechnology-based applications, employing either living systems or derivatives from them and mentioned earlier, are its industrial applications and the resulting products or services are of commercial value [10, 18]. These applications make individual sectors of the larger bio-industry. In other words, bio-industry is the full complement of biotechnology-based industrial sectors [10, 19]. Another term which sounds more like an expanded form of the term bio-industry is "bio-based industry." The two, however, have some differences [2]. Bio-industries use living systems or their derivatives in processes to make value-added products or services. Bio-based industries, on the other hand, are more expansive in terms of what they employ as

their feedstock [10]. They use living systems and derivatives from them, but they also use by-products, residues, and organic waste from plants and animals [10]. All these materials are collectively called biomass [20]. Also noteworthy are the products and services from bio-industries and bio-based industries. Bio-industries, being based on biotechnology, give us the wide range of products and services already mentioned [1, 3]. Bio-based industries, on the other hand, give us food and nonfood ingredients (not foods and beverages), biofuels, biomaterials, and bio-based chemicals which are then used to make various (bio-based) products for consumption [4, 21]. So, while based on input raw materials used, it can be said that bio-industries are a subset of bio-based industries, but based on their product portfolio, they appear discrete [3]. Further, the methods of biotechnology can be used to enhance living systems and so it can be implied that by-products, residues, and organic waste from such enhanced systems may already be value-added and when those are used as feedstock in biorefineries, we are able to make more of something and/or of a better quality of it (e.g., a modified strain of an alga that makes a better biofuel, which upon combustion releases more energy per unit mass or volume than the original strain) [22].

Just as with any other industry, bio-based industry (including bio-industry) too engages in buying (goods and services that it requires as inputs) from and selling to individuals, businesses, organizations, and governments [22]. Economic activity of the bio-based industry is known as bio-economy [21, 22]. To rephrase, bio-economy is an economy or economic model in which all activities and interests revolve around living systems [1, 22], derivatives from them, and their by-products, residues, and organic waste. Tenets of a bio-economy are utilization of renewable resources (thereby addressing resource depletion of fossil fuels), do so sustainably, and with the most minimal impact on our environment [21, 23]. Having said that, this is just one way of defining "bio-economy [3, 23, 24]." Bio-economy strategy documents of different countries reveal different definitions of the same term which reflects their existing needs as a country and that in turn shapes their vision of what and how their local bio-economy is going to be like and that is the reason one notices so much malleability in its definition in the literature [3, 22–24]. There is growing interest to transition from our present model of a fossil economy to that of a bio-economy considering the damage the former has done to the quality of our air, water, and soil [23, 24]. We have reached at a stage that the detrimental effects of the rampant and indiscriminate use of fossil fuels is now hitting us in a very real way and almost daily and almost everywhere on the planet [23]. Certain parts of the world are affected more than others, but no one is untouched. All of us are at different points of a "damage done" continuum, if you will, and so are affected differently at any moment [24].

With an ever-increasing population, the majority of which suffers from ill-health, the demand for food and energy has been increasing beyond the capacity of our traditional ways of producing those [25, 26]. Demand for safer (that does not elicit an immune response to the drug itself) and more effective drugs has also risen concomitantly [25, 26]. Biotechnology, especially since the arrival of genetic engineering in the early 1970s, and bio-industry have been helping mankind by shouldering

this burden of increasing demand for food, energy, and medicines [26, 27]. They have been giving us products and services, some of which have been revolutionary (e.g., insulin, personal genomics), to make the quality of our lives better and its expectancy longer [26, 27]. It is no surprise that biotechnology and bio-industry were and continue to be viewed as an important source of economic activity and growth [25, 26]. This potential of growth and their importance for man has attracted substantial investments in research and development and prototyping, in productizing promising technologies, and in creating businesses that will manufacture those products for or provide those services to consumers [27]. As is mostly the case, technologically advanced countries are at the frontline in making such investments in biotechnology and bio-industry too and so it is only fair that they are the principal beneficiaries of their investments [1, 27]. Over time, these first businesses enter other countries for business expansion [27]. Often, countries help other countries to start research and development activity locally and set up their own bio-industries and create their local bio-economies [2, 28]. Whether it be for business expansion or for local capacity building, such crossing of borders helps to demonopolize control over technology and democratize biotechnologies and bioindustries [2, 28].

Products of pharmaceutical biotechnology, classified as biologics or biopharmaceuticals, are not just interesting from a scientific aspect, but also from an economic point of view [28, 29]. Even though they haven't been around as long as their synthetic chemistry-based counterparts, the small molecule drugs, they have already earned a bad reputation of being costly and hence making treatments expensive [29]. An example is an analysis by the Boston Consulting Group in which they looked at ten pharmaceutical companies and found out that the average cost of producing a single pack of the formulation of small molecule drugs was around $5 compared to around $60 for biologics [30]. This has ramifications for us as a society because if treatment costs are high (and if they keep getting worse), we risk marginalizing populations who can't afford to pay those and that means a really large number of people worldwide [30]. So, effectively, medicines and treatments become inaccessible to people for whom they were developed in the first place, which is paradoxical [30, 31]. It is not hard to imagine that something, a product or a service or an application, is costly to the consumer because it is costly to make or create it in the first place. But what is unique about the development and manufacturing of pharmaceutical biotechnology products that makes them so much more expensive than small molecule drugs [31, 32]?

First, while the overall process of development of small-molecule drugs and biologics is the same, it is still more expensive to move biologics from bench to market [33]. The manufacturing process of biologics is generally more complex than that for small molecules, for example, to make aspirin only five ingredients are required, but to make insulin the list of its raw materials is much longer. Then, some of the steps are just more expensive to do in case of biologics vs. small molecules, for example, large-scale cell culturing, harvesting, and purification to make a biologic is costlier than chemical synthesis and purification of a small molecule [34]. Adding further to the cost are certain steps that cannot be bypassed in case of biologics and which simply are not part of the workflow of producing small molecules,

for example, testing for viral contamination of cell cultures. Further, biologics making is not as tolerant (to variations in raw materials and process) and consistent as synthetic chemistry schemes. This can cause structural variability in an already structurally complex biologic which in turn can lead to unpredictable clinical effects and treatment outcomes (e.g., an unanticipated immune response) [30, 34] in patients.

Second, the world of biologics development and manufacturing does not have a lot of players trying to capture a market share for a particular biologic [30]. Third, making biosimilars of known biologics (that can bring down costs), is almost as hard as making the respective reference molecules [33, 35]. Further, manufacturers of biologics are not mandated to disclose their manufacturing process in their patent filings and as a result it becomes very difficult for prospective manufacturers of biosimilars to unambiguously characterize the original molecule they are trying to replicate [30, 33, 35]. Moreover, biosimilars of biologics must be clinically tested as rigorously as their respective reference molecules (not the case for generic small molecule drugs) [30].

Fourth, there are costs beyond development and manufacturing [33, 35]. Treatments with biologics are usually performed in hospitals [36]. They are administered directly to patients by competent medical personnel and then the patients are monitored [37]. Either patients have to show up as out-patients at a hospital or have to be admitted to a hospital to get biologics administered, they cannot be given for self-administration [36, 37]. Also, because they cannot be self-administered they are not sold by retail pharmacies and so are not available to the patient for buying [36, 37]. What complicates it more is that hospitals completely control the cost of administering biologics and if there is no external oversight and regulation, how and what hospitals charge for their service is a black box [37]. All this adds to the cost [30, 35].

15.4 Major Global Biotechnology Companies, Self-Employment Avenues in Biotechnology

We enlisted several applications of biotechnology earlier in this chapter. Because of the immense value of their products and/or services to mankind, these applications have grown into industrial sectors [38, 39]. These sectors are of a very high economic value, by themselves and collectively [39, 40]. The major biotechnology-based industrial sectors include medical and pharmaceutical biotechnology, industrial biotechnology, agricultural biotechnology, food biotechnology, and environmental biotechnology [38, 40]. The interesting thing, however, is that when we want to learn about the “major global biotechnology companies,” they all fall under just one sector of the biotechnology pie and that is medical and pharmaceutical biotechnology [38, 40].

15.5 Medical and Pharmaceutical Biotechnology in the USA: Industrial and Research Avenues

Most companies in the medical and pharmaceutical biotechnology sector are of a hybrid nature in terms of their product portfolio [40, 41]. That just means they have both kinds of products, synthetic chemistry-based drugs ("pure" pharmaceuticals) as well as biotechnology-based drugs (classified as biologics or biopharmaceuticals), that they develop, manufacture, and sell [40]. So, their "major-ness" on the world stage is based on the overall size of the company.

Major multinational biotechnology behemoths are Johnson & Johnson, Roche, Novartis, Pfizer, Merck & Co., Abbott Laboratories, Abbvie, Amgen, GlaxoSmithKline, Bayer, AstraZeneca, Novo Nordisk, Bristol-Myers Squibb, Eli Lilly & Co., and Sanofi [41, 42].

Johnson & Johnson, with a market cap of over $444 billion [43] (during the week of 6/28/2021) and with $82.584 billion in revenue [43] (at the end of 2020), is the top biotechnology company in the world [43, 44]. It was ranked 36 in 2021 (based on financials ending 12/31/2020) on the Fortune 500 list of the largest US corporations by revenue [43–45]. The company has 250 subsidiaries that collectively have operations in more than 60 countries [45]. Next is an almost 125 year old company, F. Hoffman-La Roche AG, or simply Roche. It is a Swiss company headquartered in Basel. Its revenue at the end of 2020 was CHF 58.323 billion and had a market cap of CHF 264 billion at December 31, 2020 [46]. After Roche is another Swiss player, a relatively much younger player though, Novartis. It came into existence in 1996. Its revenue in 2020 was $48.659 billion [47] and a market cap upward of $206 billion [48] (during the week of 6/28/2021). Next is Pfizer, with a market cap slightly over $220 billion [49] (during the week of 7/4/2021) and an annual revenue of $41.908 billion in 2020 [50]. Not only is Pfizer one of the biggest biotechnology companies in the world, it is one of the biggest companies in the world [51, 52]. In 2021, it ranked 77 on the Fortune 500 list of corporations in the USA by revenue [45]. When COVID-19 struck the world in 2020, and pharmaceutical companies had to take a litmus test [52, 53], Pfizer took the test and proved to the world that it is worthy of the accolades of being one of the biggest companies in the world and a world major in medical and pharmaceutical biotechnology [54]. Pfizer, in partnership with the German biotechnology company BioNTech, successfully developed an mRNA-based vaccine for COVID-19 at an unprecedented speed [52, 54]. By early December of 2020, the duo was the first in the world to have secured authorizations for widespread use of their vaccine during emergency from the MHRA in the UK and the FDA in the USA and a recommendation from the CDC in the US for its use in anyone 16 years of age and older [52]. The duo's relatively prompt response during the COVID-19 pandemic to develop a vaccine for it is noteworthy and also cemented their respective positions as serious and major players in medical and pharmaceutical biotechnology on the world stage [55, 56]. Merck & Co. is next. It had a market cap of around $197 billion [55] (during the week of 7/4/2021) and had booked total sales of $47.994 billion in 2020 [17]. Merck & Co. ranked 65 on

the Fortune 500 list of corporations in the USA by revenue in 2021 [45]. Its holding company is the German Merck which has the distinction of the world's oldest operating pharmaceutical and chemical company.

Having looked at names in "big biopharma," let us switch gears and dwell on entrepreneurship and self-employment opportunities in the larger biotechnology industry. Here, we must realize that no big company started as a big company [57]. They too started small, some a few decades ago and others over a century ago and were started by people from amongst us. So, at the time of their inception, they too were start-ups that employed their respective founders. Over time, some grew to the size they are today while some others closed doors [57]. Since COVID-19 gripped the world, two little-known biotechnology companies in the medical and pharmaceutical space gained a lot of popularity worldwide, not just in technical and scientific circles, but in the speech of the next person on the street [58]. They are BioNTech and Moderna, headquartered out of Germany and United States, respectively [59]. Both started in the last 15 years, BioNTech in 2008 and Moderna in 2010 [60]. While BioNTech already had a commercial presence before they came out with their vaccine for COVID-19 (with Pfizer), for Moderna, their COVID-19 vaccine is their only product in the market to date [60].

The good thing about starting your own company is you choose its scientific and research focus and direction and then push the boundaries of science in that area [61]. In addition, you get to set the tone and culture of your venture that is motivating for everyone in the team and where everyone has a sense of belongingness and shared responsibility toward the overarching goal of the company. But, this is just one side of the coin that is entrepreneurship [61].

15.6 Private–Public Initiatives in Biomedical Sciences, Biotechnology Parks

The biotechnology industry, now, is more conducive for entrepreneurship and self-employment. In recent years, large pharmaceutical companies have been downsizing their R&D workforce and have shifted from running in-house R&D to scouting for and investing in and/or acquiring potential smaller or start-up biotechnology companies with a strong R&D of their own. This leaves them to channel their efforts in the marketing, sale, and distribution of products. This is a growing trend. Such acquisitions, take-overs, partnerships, tie-ups, collaborations, or codevelopment are a win–win for both parties [62]. For the large company, this expands their product portfolio and for the smaller company, this means more money for their scientific pursuits, access to more resources, and expansion of their reach because of the larger company's platforms and positioning. With such support, start-ups are willing to invest manpower and resources into more risky areas that have a serious commercial potential that they would not have touched otherwise [62]. A pertinent example of this is the codevelopment of the mRNA-based vaccine for COVID-19 by BioNTech and Pfizer [63].

Principal investigators in academia, who, over the course of their training and professional career, developed one or more innovative and novel products, technologies, or services in their laboratories and also have an entrepreneurial bent, have gone on to found businesses with their students, postdoctoral fellows, or collaborators. An advantage of doing this is that people involved in such start-ups can tap into additional funding streams of the government (e.g., grant mechanisms for small businesses) through their company [62]. In recent times, several research-intensive universities have set up comprehensive business incubation facilities to encourage and facilitate translation of basic science research to productization and commercialization. Entrepreneurs can also emerge out of big biopharma [9]. Scientists running research programs in such companies can capitalize on their own research findings and turn their ideas into start-ups developing products or services based off of those [62]. Regardless of where an entrepreneur comes from, academia or a large company [9], the theme is that such people have been fully invested in scientific research in a certain area for years before they were ready to take the plunge to be an entrepreneur themselves, understand the landscape very well, and have a good reason to believe that the idea or ideas they are spinning out have a very high chance of being successful at meeting a previously unmet need [7, 9, 56].

Another avenue for entrepreneurs is that once you can successfully create and run your first company you are more appealing to the corporate world. Large companies and other start-ups, and even universities and state and federal governments may like to have you as a consultant or an expert advisor or even as a board member [62, 64]. Because of your scientific and technical expertise and your understanding of the industry, venture capital and investment banking firms may like to on-board you as an analyst for them [62]. With the right skills and network, you can even be a serial entrepreneur [61, 62].

The other side of the entrepreneurship coin is the hard part of being an entrepreneur. It is cliché to say that starting a business and being an entrepreneur is not for the faint of heart, but it does not stop being true [62]. People in academia, or those working in the life science industry, or anyone with an idea about a product, service, or application (that seems commercializable) and with keenness and aptitude for being an entrepreneur, sooner or later, may take a leap of faith and start their own venture [62, 65]. Mostly, the task of founding one's own company starts much earlier than its official founding and it is not uncommon for thoughts and ideas behind it to go through one or more periods of dormancy during this "prefounding" stage [62, 65]. But once you get serious and are ready to focus and dedicate your time and efforts to starting your own business, the first thing to do is to assess your idea for its viability and competitiveness in the commercial space and its fundability from the initial stages until, at the very least, it becomes self-sustaining [62]. Following this, you must formulate your idea and the assessment you did into a palatable and convincing business plan to present to potential early team members, investors, and to apply to funding mechanisms of the government [61, 62]. You then move to test your plan by undertaking proof-of-concept studies. The original plan will now go through several iterations as it is tested multiple times and in multiple ways and the result of the process is that one develops a better understanding of things in the

context of being able to translate the idea from bench to market [23, 61]. With high hopes in your original business plan, your early team members and investors are eagerly waiting for the outcome of these proof-of-concept studies to make their own assessment of how much further they want to go with you on this plan [23, 61, 65]. At this stage, if the business plan, now more embellished than before, becomes more enticing, you are able to attract a substantial capital investment making it possible for you to attract more talent and set up facilities with necessary infrastructure to move things further in the direction of materializing what was just an intangible idea when you had first started [29, 61, 65]. Because of the almost uncountable number of variables linked to starting one's own business and being self-employed, it makes a lot of sense to work in an established big biopharmaceutical company or in a start-up early in your career [39, 61]. Not only can one gain first-hand experience of conducting research in a company setting, but also of building, running, and managing a business [39, 61]. This kind of path prepares you better to found and run your own business later in your career [39, 61].

Considering the numerous directions one gets pushed or pulled into on an almost daily basis and being face to face with insecurities, uncertainties, and risks in a very visceral way, it is not hard to believe that not everyone is wired to be a founder and start a business [66, 67]. To be a founder, a strong scientific background is a non-negotiable requirement and for most people, they build their scientific background and a research focus during their years in graduate and/or professional school supplemented by postdoctoral training or residency [66, 67]. These years are the bedrock of their scientific and technological pursuits as an entrepreneur. As far as attitude goes, a founder must mindfully be a bulldog and have a tunnel vision about the goal of the start-up venture which is to develop a product, a service, or an application that, first and foremost, adds value to its users and so, is of commercial value as well [67]. Anything else, whatever it may be, is simply a distraction from this goal [62]. Additionally, because the start-up is the founder's brainchild and his employer, one must be ambitious, confident (being able to handle and manage self-doubt and doubts of those you are enlisting), and passionate about his goal and his vision. Moreover, professional and intellectual honesty, integrity, and observing and following ethical standards and practices in all your dealings are a must to be able to sustain the momentum of running what you started for long [62]. Also, because a founder must convince a lot of people about himself, his company, his goal, and his vision, he must be strong-willed and resilient. Then there is a gamut of soft or transferable skills one needs as an entrepreneur: organizational skills, analytical and problem-solving skills, being able to function as part of a team of people with diverse educational and cultural backgrounds, written and oral communication skills, interpersonal skills, being composed and perseverant even in failure, leadership skills, strategic planning skills including risk assessment and management, financial planning skills, fundraising skills, networking skills, negotiating skills, and project and personnel management skills [66, 68]. The good news is that during graduate and postdoctoral training, there are numerous opportunities to pick up and develop one or several of these soft skills and so this is something that can be done in parallel to one's training to be a scientist [62]. Moreover, while it seems one

needs to know so many things to be an entrepreneur, that is not actually the case, and one must start to build a team from a very early stage that collectively has all the expertise and skills that the business requires and will require as things evolve [61, 62, 68]. Having said that, being a generalist will be more helpful in this situation, rather I should say that the ability to recognize when to be a generalist and when to be a specialist is a good skill to have as a founder [39, 61]. Nevertheless, founders must be willing to wear any hat as the need arises, at times, more than one at the same time. But as they build their team, they will have the right people to handle supporting matters while the founder focuses on the core areas [39, 42, 61, 62].

15.7 Summary

Biotechnology is all about techniques and processes involving living systems or derivatives from them either in their naturally occurring or altered or a purely synthetic form to create products and services for exploratory research or for consumption [69]. The industrial application of biotechnology to make products and services of commercial value has resulted in the formation of several biotechnology-based sectors, all of which are collectively referred to as bio-industry [70]. The prominent biotechnology-based industrial sectors are medical and pharmaceutical biotechnology, industrial biotechnology, agricultural biotechnology, food biotechnology, and environmental biotechnology [69]. Bio-based industry is a related term but is more expansive than bio-industry in what it employs as feedstock to make products and services [22]. All economic activities and interests centered around living systems have created a new bio-economy model, different from our existing fossil economy model and there is a growing interest worldwide to transition to this new model because of the glaring damage caused by the latter to our atmosphere, hydrosphere, and lithosphere [71, 72].

It is an understatement that biotechnologies and bio-industries have helped mankind. In the absence of biotechnology, it would have been impossible to meet the demands of our planet's growing population for food, energy, and medicines. The demand has led to expansion of bio-industries and an increase in their economic activity and there is still more potential to address many unmet needs [13]. Due to this potential of growth in bio-industries, they have already attracted massive investments from technologically advanced nations [13]. Unfortunately, the developing world is still behind in recognizing this potential [13].

Even though the application of biotechnology has led to the creation of several industrial sectors, it is noteworthy, however, that all the major global biotechnology businesses only belong to the medical and pharmaceutical biotechnology sector [13]. Products of pharmaceutical biotechnology, classified as biologics or biopharmaceuticals, are of high medical and economic value [57]. Even though they are not as old as their small molecule counterparts, they have earned themselves a bad reputation because of the high price tag attached to treatments employing them, making such treatments unaffordable and beyond the reach of most of the world population [57].

The economic potential in the pharmaceutical biotechnology sector of the bioindustry is directly linked to our world's growing population and the concomitant rise in the number of people suffering from ill-health [22, 28]. This has led to the rise of many multinational companies in this sector like Johnson & Johnson, Roche, Novartis, Pfizer, Merck, and others. In addition to big biopharma, this sector also has a good segment of start-up businesses [43]. These start-ups, a lot of times, are the hotbeds of some breakthrough developments. BioNTech from Germany and Moderna from the United States have recently exemplified this point with the development of the first usable mRNA-based vaccines [59]. Even the big biopharma, of late, has been more interested in investing in such promising start-ups as against setting up their own R&D in-house [10]. The recent collaboration between Pfizer and BioNTech really illustrates the interest of Pfizer and Pfizer-like behemoths in high potential start-ups. Having said that, starting a business and being an entrepreneur is not easy [59]. It is doable, but it is hard work. It is very demanding in terms of the technical or scientific background and soft skills that the founders must have or build a team to support their efforts as early as possible in their journey [24]. Even though anyone with a good idea and an entrepreneurial bent can start a business, people who have been involved in first hand scientific research, whether in industry or academia, and know well about areas with unmet needs, have a better chance in being successful as entrepreneurs [9, 24].

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