RECOMBINANT DNA TECHNOLOGY IN GENOMIC RESEARCH

Abstract

Recombinant DNA technology joins genetic materials from different organisms through genetic engineering, enabling scientists to modify DNA, create transgenic organisms, and produce therapeutic proteins. It begins with the background, acknowledging the foundational discoveries of ligases, vectors, restriction enzymes, plasmids, and host organisms with techniques such as TALENs, mega-nucleases, zinc finger nucleases, and CRISPR-Cas9, which are acknowledged as influential tools in genetic engineering. Genetically modified organisms (GMOs) are utilised to improve crop traits such as increased yields, resistance to pests and herbicides, and tolerance to environmental stresses. The Flavr-Savr tomato is highlighted as one of the pioneering genetically engineered crops that demonstrated extended shelf life and resistance to spoilage through gene silencing. beyond agriculture and into biomedical applications, notably in the production of therapeutic proteins. Different hosts, including bacteria, yeast, insect cells, and plant cells, are employed to produce commercial products with improved qualities. The focus is on mammalian cell lines in the biopharmaceutical industry for creating high-quality proteins, such as monoclonal antibodies, used in therapies for various diseases and vaccine production. The insertion of specific antigens of pathogens into harmless vectors stimulates an immune response, leading to the production of neutralising antibodies against targeted pathogens, which offers a safe and effective means of disease prevention and control, particularly in veterinary medicine for diseases like bovine viral diarrhoea and respiratory syncytial virus. Two types of gene therapies are widely used in gene delivery: somatic and germline gene therapy. Engineered bacteria, fungi, and plants are employed to degrade pollutants in the environment, making waste treatment more effective and sustainable. The future prospects of recombinant DNA technology are promising, and the technology is involved in ongoing advancements in health, agriculture, and environmental management.

Keywords: Genetic Engineering, Recombinant DNA, Genetically Modified Organisms, Gene, Vectors, DNA Cloning, CRISPR-Ca9 Technology.

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I. INTRODUCTION

The word recombinant is the adjective of "recombination" which means "to join together" while DNA is the shortened form for deoxyribonucleic acid which is a molecule containing all genetic codes that make an organism, Recombinant DNA is the joining of two or more genetic materials, from the same organism or different organisms (**Figure 1**). Let's for a moment think of the body as a big machine with different parts coordinating different functions to allow the machine to function properly. If a certain part gets extracted, if for instance, the blade of an excavator- the machine may move but will no longer have the ability to clean the road, by this, one can understand the special function of the blade on the machine. Sometimes, if the blade gets damaged, it is replaced by a blade from another machine of the same kind or a different one. This is the same way recombinant DNA technology works. With the use of recombinant DNA technology, scientists are able to modify and mix DNA molecules from various sources. It is sometimes referred to as genetic engineering or gene splicing. In order to develop new genetic combinations, DNA fragments are cut and rejoined. By changing an organism's gene, scientists can improve crop harvests, create useful medications, and investigate the genetic causes of illnesses. When an organism's gene is altered or manipulated, it is termed a "transgenic organism". The development of genetically modified organisms (GMOs), the manufacturing of therapeutic proteins, and the investigation of gene control and function are all made possible by this technology. The power to alter DNA holds great potential for solving major world problems including food safety, healthcare, and sustainability in the environment.

In admiration of the great potential of the technology, scientists are also concerned about its potential threats to humans and the environment from three main angles: the worry of off-target effects, the selectivity of edits (edits may take up some cells), and the editing of germline which could have an impact on future generations. This insecurity became more tense when the latest and most promising technique, CRISPR-cas9 technology, developed in 2012 was utilized by Dr. He Jiankui of China and his team to modify the germline of two infants in an effort to confer resistance to HIV infection. As a result, the biosecurity law on genome editing was proposed. The goal of this chapter is to provide readers with a thorough grasp of recombinant DNA technology, including its historical development, methodologies, numerous uses, and related ethical and safety issues

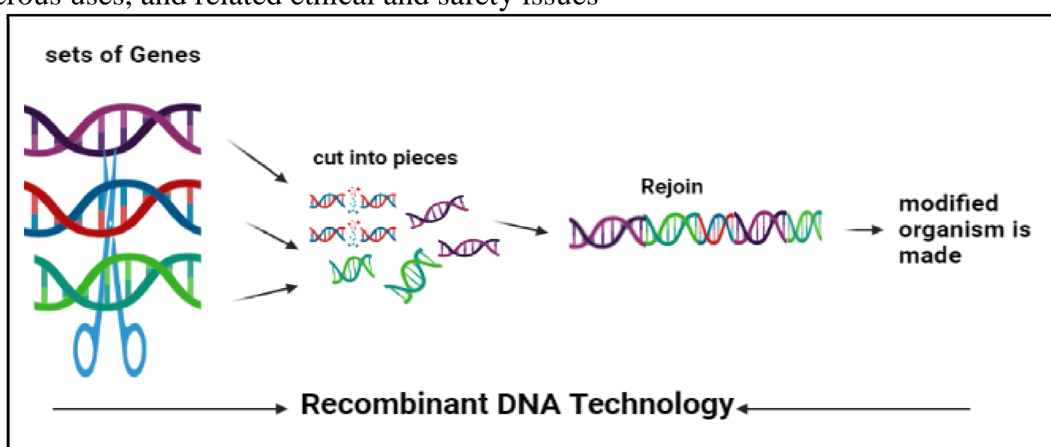

Figure 1: A Diagrammatic Illustration of Recombinant DNA

II. BACKGROUND OF RECOMBINANT DNA

Because DNA forms the basis of contemporary genetics, many scholars have cited James Watson and Francis Crick's discovery of the DNA molecule in the early 1950s as the inception of recombinant DNA technology. However, given that we are discussing Recombinant DNA, invents involving the development/discovery of ligases (molecular glues), vectors (molecular vehicles) restriction enzymes, plasmids, and host organisms are key in discussing the rise of Recombinant DNA [1].

The first Recombinant DNA was created in the late 1960s and early 1970s through the collaborative efforts of Gelbert, Lehman, Richardson, and Hurwitz laboratories. Subsequently, Werner Arber, in 1968 hypothesized the presence of restriction enzyme. This hypothesis was validated in an experiment involving the isolation of two restriction enzymes from E. coli. The first site-specific type-II Restriction enzyme, known as Haemophilus influenzae (Hind II), was also successfully purified by Hamilton Smith and his colleagues in 1972. During this same era, Stanley Cohen and Herbert N. Boyer jointly developed the first recombinant plasmid known as pSC101 in 1973. In a joint meeting between the United States and Japan on plasmids, both men presented their research. They discussed the characteristics of DNA ends produced by EcoRI cleavage and the steps involved in plasmid uptake by bacteria. This milestone in molecular biology was followed by the development of a technique called Southern Blotting named after Sir Edwin M. Southern who invented it. It allows for the detection and analysis of specific sequences in a DNA sample. The English scientist Frederick Sanger and his colleagues created the Sanger's sequencing method, often known as the "chain termination method," in 1977. This technique is used to identify the arrangement of nucleotide bases in a fragment of DNA that is typically smaller than 1,000 base pairs in length. Sanger sequencing is regarded as the "gold standard" for verifying DNA sequences since it has a 99.99% base accuracy rate [2].

Prior to the invention of a method that can create multiple copies of DNA molecules, in 1971 Kjell Kleppe, a researcher in Khorana’s laboratory, described the replication of a segment of DNA by a two-primer system The method for DNA amplification known as Polymerase chain reaction “PCR” invented by Kary Mullis is the easiest, most efficient, and most promising technique used in laboratories for DNA fragments of varying lengths. It uses a thermal cycler to manipulate DNA molecule and is based on the principle that DNA denatures at a certain temperature and anneal at a given temperature. Recombinant DNA has many applications including the creation of Genetically modified organisms (GMOs) and many techniques are discovered from this evolution. These techniques include: Transcription Activator-Like nucleases (TALEN), mega-nucleases, zinc fingers nucleases (ZFN) and Cluster regularly interspaced palindromic repeats (CRISPR) and its associated proteins, cas9 [3].

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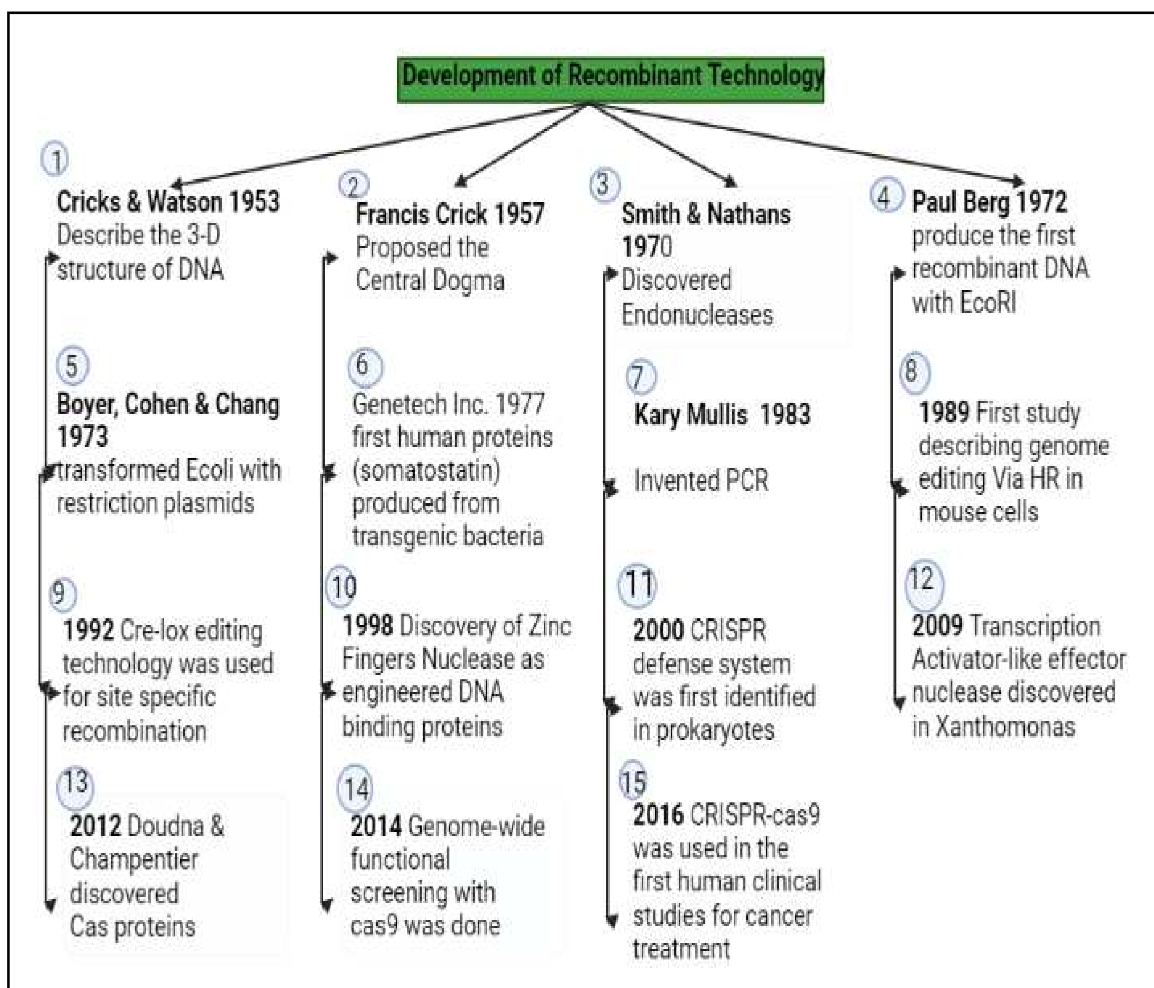

Figure 2: An Illustration of Brief History of DNA Technology

III. TECHNICAL RESOURCES USED IN RECOMBINATION OF DNA

Several techniques are used to enhance recombinant DNA technology. Restriction enzymes and ligases are common tools and techniques in these processes.

1. **Restriction Enzymes:** In order to manipulate a DNA molecule, we need to cut, remove, and insert. Cutting of the molecule is the function of enzymes known as Restriction enzymes. As the name suggests, a restriction enzyme is restricted to cut at a designated location on the molecule hence, the location is termed a “Restriction site” Since the discovery and isolation of the first restriction enzyme in 1972 many different restriction enzymes have been discovered with each having its own restriction site.

   All restriction enzymes carry unique nucleotide sequences which are the primary composition of DNA molecule subunits i.e. Adenine(A), Guanine(G), Cytosine(C) and Thymine(T) and each is made of four or six base pairs that are arranged in such a way that when read back and forth it produces the same sequences. This type is arrangement is referred to as palindromic [4, 5, 6]. That restriction enzymes are isolated from bacteria and it is evidenced that they exist in the bacteria genome as adaptive immunity. Restriction

enzymes are extracted from bacteria genomes and use in laboratories to manipulate a DNA fragment.

The names of restriction enzymes are written based on their background. For instance; *Eco*RI is the name of a restriction enzyme derived from Escherichia coli bacteria, where **"E"** indicates the genus name, **"co"** indicates the species name, **"R"** represents the specific strain of the bacteria, and **"I"** denotes the order of discovery. Both genus and species names are indicated in Italics [7, 8].

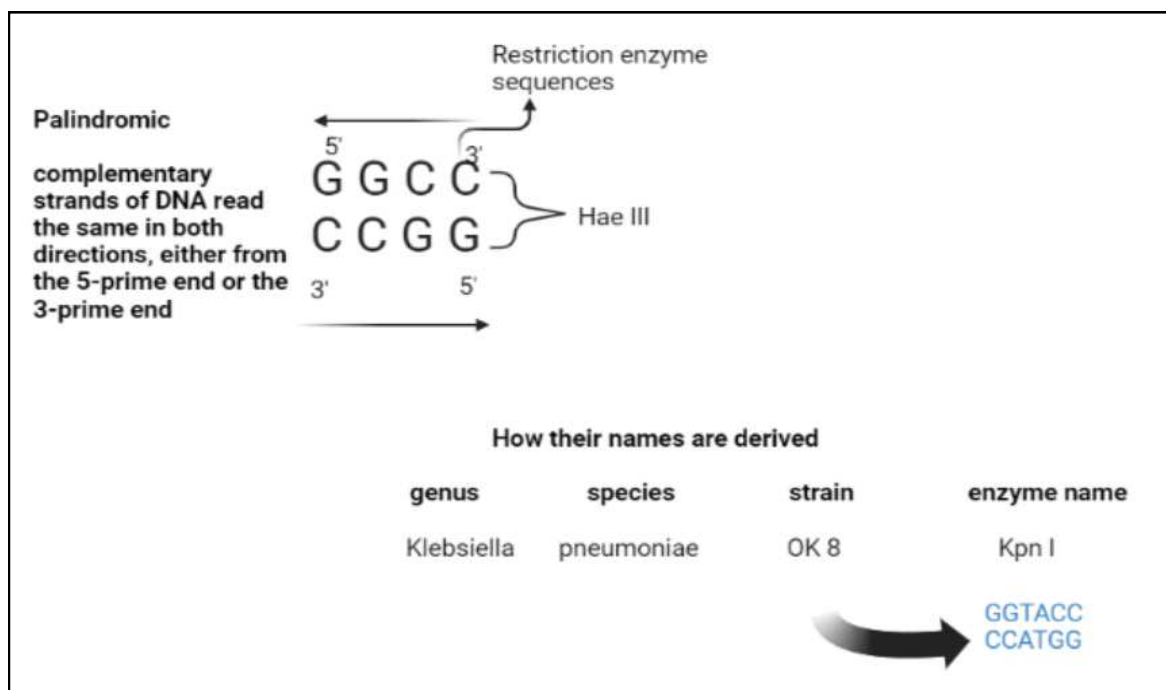

Figure 3: Palindromic Sequences and Nomenclature of Restriction and Enzymes

2. **Understanding Restriction Enzymes' Actions:** Only one or a few restriction sites are recognized by each restriction enzyme. A restriction enzyme will create a double-stranded cut in the DNA molecule once it locates its target sequence. The cut often occurs in a neat, regular pattern at or near the restriction point [9]. In a process known as hydrolysis, a restriction endonuclease that recognizes a sequence cut through the DNA molecule by breaking a chemical connection by adding a water molecule to the bond between adjacent nucleotides. Bacteria disguise their recognition sequences to stop this kind of DNA degradation from happening to their own DNA. In the recognition sequence, methylases add methyl groups (—CH3) to adenine or cytosine bases as a way of shielding the restricted region from endonucleases. The restriction-modification system of a bacterial species is made up of the restriction enzyme and its related methylase [9].

3. **Pattern of Cleavage:** Endonucleases or restriction enzymes cut DNA in a staggered manner to form "sticky ends" or cohesive ends. They can also produce "Flush ends," also known as blunt ends or non-coherent ends. Sticky ends are produced when a restriction enzyme splits the two DNA strands at various points, often two or four nucleotides apart, leaving short single-stranded overhangs at each end of the DNA fragments. These are known as sticky or cohesive ends [10], because when base pairing between them is

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revealed, it can reassemble the DNA molecule. Some sticky-end cutters (such as Bam HI) provide 5′ overhangs, whereas others only leave 3′ overhangs (such as PstI). A flush or blunt end is an end of a double-stranded DNA molecule where both strands terminate at the same nucleotide position with no single-stranded extension (e.g. HpaI) [11].

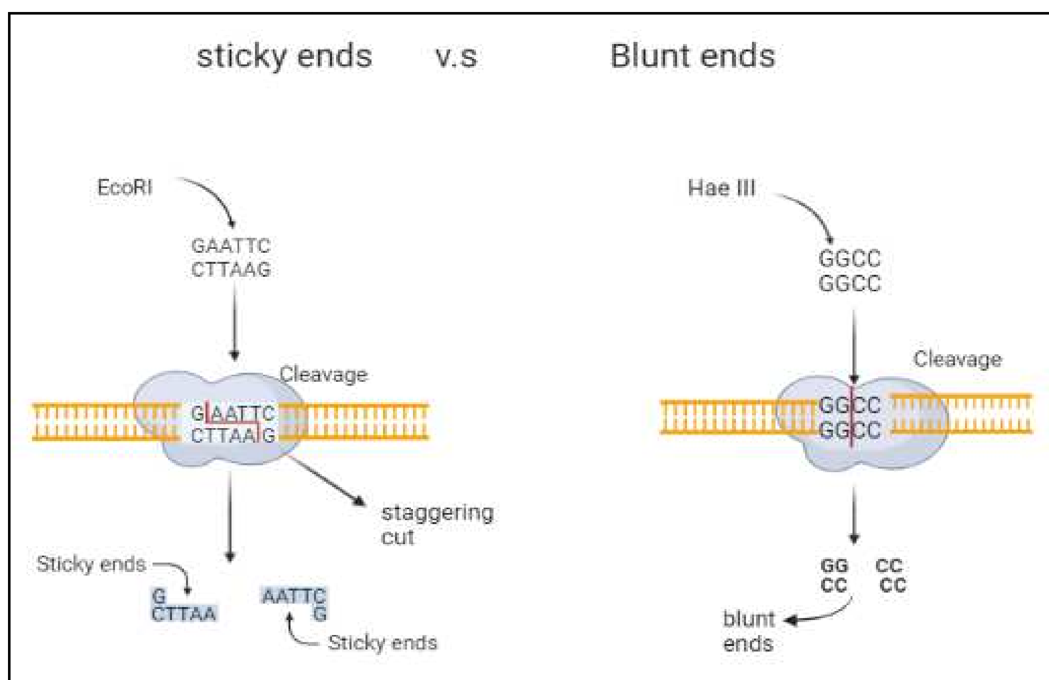

Figure 4: Sticky and Blunt Ends Cleavage

4. **Types of Restriction Enzymes:** On the basis of subunit composition, cleavage location, sequence specificity, and cofactor requirements, restriction enzymes are often divided into four kinds. Though there may be more from the perspective of molecular biologists. They are grouped into types I, II, III, and IV traditionally.

Type I enzymes are complex, multi-subunit, combination restriction-and-modification enzymes that cut DNA at random about 1000bp away from the recognition site and possess methylase activities as well. These enzymes have recognition sequences of about 15 bp in length. They require Mg++ ions and ATP for their functioning. Such types of restriction endonucleases cleave the DNA about 1000 bp away from the 5′-end of the sequence TCA.

Type II enzymes cut DNA at specific spots near or inside their recognition sequences (**Table 1**). They are the most frequent category used in the lab for regular DNA examination and gene copying because they produce separate restriction pieces and recognizable gel striping designs. Type II enzymes are a collection of unrelated proteins of multiple different types, not a single group of connected proteins. (New England Biolab inc, 2023). Type III endonucleases are not employ by genome cloning does not. They are the intermediary restriction endonucleases between Type-I and Type-II enzymes. They cleave the DNA at certain locations near recognition sequences, and need Mg++ ions and ATP to do it [12].

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Table 1: Mostly used Type II Restriction Enzymes

Type	Description	Co Factor(S)
Type I Restriction enzymes	• First to be identified • Holds little relevance since they do not generate efficient fragmentation of DNA • They are multifunctional and have 3 subunits (DNA recognition, DNA cleavage and an ATP-dependent translocation) • Cleave up to 1000 bp away from recognition sequence	AdoMet, ATP & Mg^{2+} ions
Type II Restriction enzymes	• Cleavage occurs at both digestion & restriction sites • Utilize Mg2+ as co factor • Make blunt & sticky ends • one subunit	Utilize Mg^{2+} as co factor
Type III Restriction enzymes	• Cleaves 25 bp away from recognition site • They are multifunctional and have 2 subunits for modification, restriction digestion and methylation	Use AdoMet and ATP for restriction

No	Microorganism	Enzyme	Recognition Sequences	Types of Cuts
1.	Escherichia coli RY13	EcoRI	GAATTC	Sticky ends
2.	Haemophilus influenzae Rd	Hin dIII	AAGCTT	Sticky ends
3.	Klepsiella pneumonae OK 8	KpnI	GGTACC	Sticky ends
4.	Bacillus amyloliqueaciens	BamHI	GGATCC	Sticky ends
5.	Escherichia coli Restriction enzyme V	EcoRV	GATATC	Blunt ends
6.	Serratia marcescens I	Smal	CCCGGG	Blunt ends
7.	Providencia stuartii	PstI	CTGCAG	Sticky ends
8.	Nocardia otitidis	NotI	GCGGCCGC	Sticky ends
9.	Streptomyces achromogenes	SacI	GAGCT	Sticky ends

IV. CHEMICAL GLUE

Ligase joins broken pieces of DNA covalently through the catalysis of phosphodiester bond formation between nucleotides that make up the DNA molecule in an energy-driven manner. It is otherwise referred to as molecular glue due to its similarity in function as a “glue”. To repair a DNA molecule, a bond is formed by ligase between the sugar-phosphate backbones. The functions of ligase include repair, replication, and joining of DNA [13].

Isolation of ligase is done in vitro to create recombinant DNA (Sharma, 2022). Since the discovery of ligase from Ecoli in 1967 independently by the Gellert, Lehman, Richardson, and Hurwitz Institute, DNA ligase has become an important tool in molecular biology and hence, several others were discovered the years after from different organisms. With DNA ligase, only identical DNA segments with complementary ends

may be linked. Okazaki fragments are linked together naturally throughout the replication process by a process called ligation.

1. **The Building Block and Working Process of DNA Ligase:** Having the knowledge of the basic mechanisms of ligase gives insights into its role in recombinant DNA technology and its application in genetic engineering. Ligase enzyme seals the nicks on duplex DNA through the catalysis of phosphodiester bonds between 5' to 3' hydroxyl terminal. It is a cofactor in DNA repair pathways and functions in base excision repair, nucleotide excision repair, double-stranded break, and joining of Okazaki's fragments found on the lagging strand. Ligase interacts with nucleotide cofactor to form a covalent enzyme nucleoside monophosphate through ATP or NAD^+. The full mechanisms can be found in a review by (Allan *et al*. 2006) where they explained four major phases and are summarized as thus; 1. Adenylation of DNA ligase 2. DNA binding and acetylation transfer 3. Phosphodiester bond formation and 4. AMP release and DNA ligation [14].

   Firstly, during the initiation, a phosphoamide bond (P-N) is formed between an active site lysine and 5' phosphate of AMP of DNA and RNA ligases, or GMP of mRNA capping enzymes. In phase 1, nicotinamide mononucleotide (NMN) is produced by NAD+ while inorganic pyrophosphate is generated by the catalysis of nucleotide-triphosphate. A pyrophosphate linkage is created at one end of the process when the 5' phosphate group of nicotinamide mononucleotide phosphate is transferred from an active site lysine to a phosphorylated DNA 5' end. Phase 2 of the process is completed when the 5'AMP activates the DNA substrate's 5' phosphate, resulting in the creation of phosphodiester bonds. Phase 3 involves the breaking of the 5' phosphorylated DNA end by the hydroxyl group near the DNA strand, which displaces AMP and covalently joins the strands [15].

2. **Ligases Classification:** Based on the need of ATP or NAD^+ as cofactors, ligases are divided in two groups. Those working with the use of ATP are referred to as ATP-dependent while those using NAD^+ are NAD^+ dependent [16]. As the name implies ATP-dependent DNA ligases use ATP as energy source, they variation among them in substrate specificity due to diversity in cellular functions. Beside ATP, they may require additional coenzymes such as Mg^{2+} or Zn^{2+} **(Table 1)** The transfer of AMP moiety from ATP to one of the substrates leads to pyrophosphate leads to pyrophosphate (ppi) and covalent bond formation resulting in phosphodiester bond. Members of this category includes: DNA ligase I, II, III, IV, LagA, LagB, T4 Ligase, and T7 Ligase in mice with Breast Cancer Susceptibility Gene 1(BRCA1-deficient mammary tumors), DNA Ligase 3 (LIG3) deletion also increases Poly(ADP-ribose) Polymerase Inhibitor (PARPi's) effectiveness, indicating LIG3 as a possible therapeutic target. Due to its catalytic role in mitochondria [17].

   DNA ligase III (Lig3) has been shown to be essential for cell survival. DNA ligase IV (Lig4) catalyzes the final step of DNA end ligation in non-homologous ends joining the DNA repair pathway and further disclosed that Drosophila melanogaster DNAlig4 mutant strains exhibited shorter lifespans and decreased resilience to food restriction. The multiple presence of complexes containing DNA ligases involved in A-EJ by using the CRISPR-cas9 system to construct a mouse CH12F3 cell lines in which the absence of Lig4 was compensated by the removal of either Lig1 or nuclear Lig3. These cell lines contained only one type of DNA ligase (Lig3 or Lig1) within their nuclei but surprisingly

discovered that both Lig1 and Lig3 complexes were proficient in facilitating A-EJ for class switching recombination (CSR) at the IgH locus, as well as chromosomal deletions between double-strand breaks (DSBs) induced by CRISPR/Cas9 within the same chromosomes [18].

3. **T4 and T7 Ligase (ATP-Dependent):** T7 is the most extensively used DNA ligase is one that was developed from the T7 bacteriophage. It weighs 41KDa and possesses two domains namely: six alpha helices surrounding three antiparallel beta sheets known as ATP binding sites and another containing antiparallel beta sheets and an alpha helix [19]. The enzyme does not perform blunt end ligations thus, the enzyme discriminates between cohesive (active) and blunt (not active) fragment DNA ligations, as it only performs sticky end ligations. Because of their short genome, easily comprehended functional genomics, speedy life cycle, and high potential for bio-application, modified T7 phages can be created using techniques which includes explicit genetic modification in vitro, CRISPR, and recombineering [19].

   T7 DNA Ligase requires ATP for activity. T4 bacteriophage is the source of T4 ligase. Although ligation in RNA molecules is low, it has great fidelity, can effectively combine blunt and cohesive ends, and fixes single-stranded nicks in duplex DNA or DNA/RNA hybrids. T4 can be utilised in TA cloning, adapter ligation cloning, and restriction cloning. three basic steps are involved with T4 ligase: [20].

   Firstly, the addition of adenosine monophosphate (AMP) molecule to the ligase from a high-energy co-factor like ATP or NAD^+ in a process called Enzymatic adenylation. secondly, the AMP molecule is then moved to the 5' phosphate at the location of a nick and finally, 3' OH engages in a nucleophilic attack on the 5' phosphate in order to create a phosphodiester bond since AMP is present on it, this is termed Nucleophilic assault on the 5' terminus [21].

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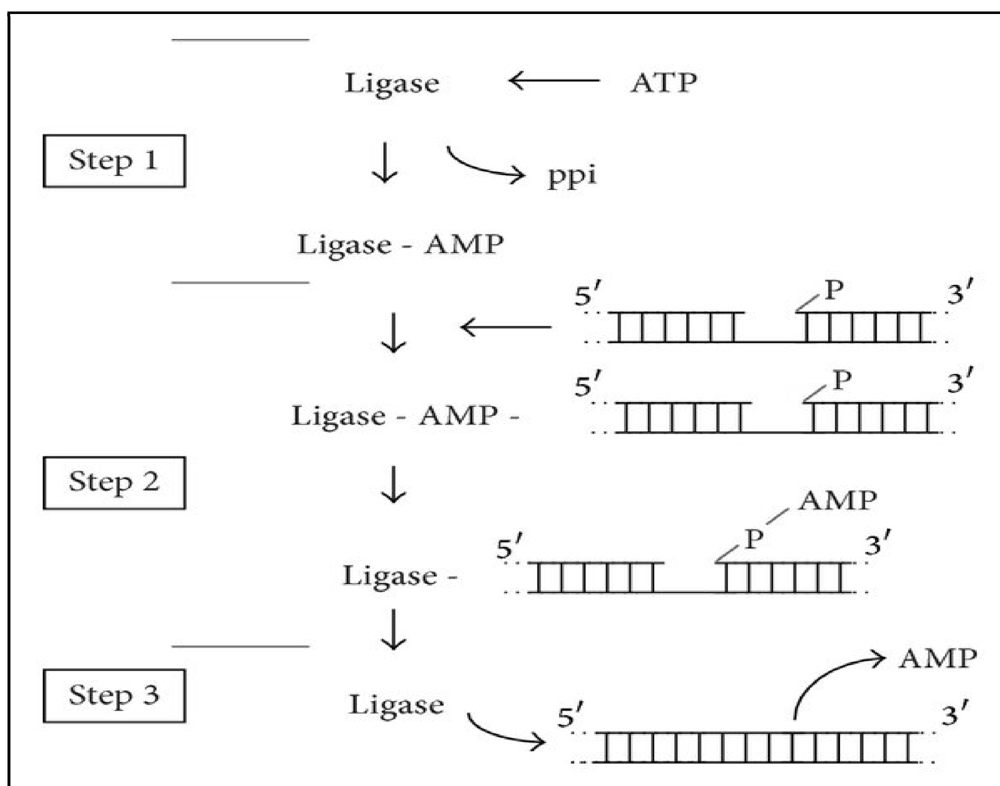

Figure 5: The Figure Illustrates A Three-Step Procedure using ATP-Dependent DNA Ligases. AMP is Transferred to A Lysine Residue in Step 1, Followed by The 5′-PO4 End in Step 2. in Step 3, A Second DNA Strand's 3′-OH End Attacks the 5′-PO4, Releasing AMP

4. **NAD^+ Dependent DNA Ligases:** While ATP-dependent ligases are considered ubiquitin, NAD^+ dependent ligases are found in bacteria, some entomopox viruses and mimi viruses. NAD+-dependent DNA ligases have two functional domains: an N-terminal cofactor binding domain and a C-terminal DNA-binding domain. Ecoli DNA ligases, which employ NAD+, is frequently used tools in recombinant DNA technology alongside T4 ligases (ATP dependent) [22].

5. **DNA Ligase in Repair and Replication:** Unwanted events can occasionally cause abrupt changes in DNA molecule, causing mismatches, base removals, and base insertions which subsequently leads to mutation. Thankfully, the cell uses ligase enzymes to repair itself in both single-stranded break (SSB) and double-stranded break (DSB) situations, depending on the extent of the damage. Most species employ ATP-dependent ligases in these activities, but Eubacteria requires NAD for repair [23].

   In eukaryotes, single-stranded breaks may be repaired via both homologous recombination (HR) and non-homologous ends-joining (NHEJ) mechanisms, however in bacterial cells, only homologous recombination is used. Cells use several mechanisms including, mismatch repair (MMR), DNA damage repair pathway (Nucleotide excision repair (NER) or Base excision repair (BER)) (Khan Academy). With the exception of directly undoing DNA damage, two DNA repair pathways - base excision repair (BER) and nucleotide excision repair (NER) - involve successive activity of several repair proteins. In DNA recombinant technology, these functions of the cell are manipulated to

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introduce a foreign gene in the organisms' genome. For instance, in homologous direct repair (HDR), scientists introduce two mechanisms, after CRISPR-cas9 cleavage: (1) synthesis dependent strand annealing (SDSA) and double strand break repair (DSBR) [24].

V. HOW LIGASES ARE APPLIED IN RECOMBINANT TECHNOLOGY

Ligation is a fundamental step in DNA cloning and Recombinant technology. A ligated DNA fragment may have cohesive or blunt ends relative vector to which it is applied. A vector, often described as "molecular vehicle," is used to transport segment(s) of DNA into a host cell. A vector can exist as a plasmid, Bacteriophage, Cosmids, Phagemids, Bacteria Artificial chromosome (BAC), Yeast Artificial chromosome (YAC) and depending on the process, it can be classified as cloning vector, expression vector or integration vector [25].

A fragment is ligated as foreign DNA which is then transformed into a host such as bacteria. The transformed bacteria are cultured and used to construct a gene library as in Genomic or cDNA libraries. Both types of gene libraries preserve genes for long-term applications such as gene delivery, and genetic engineering purposes. In order to study protein interaction and develop therapeutic agents for Biotechnological and Biomedical research purposes, a combination of several DNA fragments encoding different protein domains or entire proteins are enhanced. In a technique known as Site-Directed mutagenesis a modification by insertion of a foreign piece of DNA is made in the genome of an organism in the overlapping region with the aid of polymerase chain reaction (PCR). Ligation of DNA fragments is used in sequencing methods and are also utilized as adapters for Next Generation sequencing (NGS) library collections [26].

VI. FACTORS INFLUENCING THE ACTIVITIES OF DNA LIGASES

Five main factors accountable for ligation optimization to improve efficiency:

1. **The First is Temperature:** the DNA ligase enzyme can function best by lowering the temperature, and while the optimal temperature for the DNA ligase enzyme is 25°C, reducing it to 4°C can further improve efficiency despite the longer time required [27].

2. **The Second Factor is the Buffer Solution:** to prevent degradation of ATP in the ligase buffer, which is crucial for DNA ligation, it is recommended to aliquot the buffer from each new DNA ligase stock, as repeated freeze-thaw cycles can break down ATP [28].

3. **The Third Step is Heating at a Proper Temperature:** when performing cohesive-ended ligation, combine the vector and insert fragments first, and subject the mixture to a 5-minute heat treatment at 65°C. This step disrupts any potential interference from cohesive-end interactions within the vector or insert, thus improving the efficiency of the desired vector/insert interaction.

4. **The Fourth Step Involves pH:** the best pH range for DNA ligation is between 7.6 and 8.0. The mixture may deviate from this range based on the DNA fragments' preparation method. To verify the pH, pipette around 0.2 µL of the mixture onto narrow-range pH

paper. The pH can be changed by adding 0.2 µL droplets of either 1 M HCl or 2 M Tris base if necessary.

5. **The Fifth Step Requires that the Restriction Enzyme Should be Added Before Ligation:** in cases where the vector fragment obtained during the preparative digest, adding the corresponding restriction enzyme to the ligation reaction will selectively digest any remaining intact vector, effectively preventing its transformation and enabling the intended insert to be successfully integrated. To avoid this, the addition of 1 µL of the enzyme, made 5-10 minutes before the transformation, is adequate [29].

VII. RECENT ADVANCEMENTS IN LIGASE RESEARCH

1. **Enhanced DNA Ligases:** DNA ligase holds a strong place in molecular techniques. Recently, researchers have modified ligases to improve their activity and efficiency in joining DNA fragments. Engineering ligase for improvement is necessary, some have huge limitations in joining double-stranded breaks for instance T4 ligases.

   Modified T4 ligase enzyme fusing T4 DNA ligase with 7 different DNA-binding proteins, the researchers were able to significantly enhance its performance in joining DNA fragments with cohesive or blunt ends. Using agarose gel-based screens evaluated the activity of the engineered ligases. They observed that engineered Ligase-cTF out-performed the normal T4 DNA ligase by ∼160% in blunt end ‘vector + insert’ cloning assays, and p50-ligase showed an improvement of a similar magnitude when it was used to construct a library for Illumina sequencing.

   Additionally, it was reported that [30], that the ability of coronafacic acid ligases (CfaLs) to catalyze the coupling of coronafacic acid to amino acids. CfaL enzymes can produce jasmonyl-L-isoleucine (JA-Ile) (a mimic of the plant hormone), despite having low similarity to the plant enzyme Jar1, which is responsible for the ligation of JA and L-Ile in plants. suggests that Jar1 and CfaL evolved independently to catalyze similar reactions: Jar1 producing a compound essential for plant development, and the bacterial ligases (CfaL) producing analogues that are toxic to plants. They proved that CfaL enzymes can be utilized to synthesize a diverse range of amides without the need for protecting groups and employed structure-guided mutagenesis to engineer improved variants of CfaL [31].

   DNA ligases I, III, and IV, have distinct functions within the nucleus and mitochondria that help to seal nick in the backbone to ensure genome stability. Mutations in the LIG1 and LIG4 genes have been linked to rare DNA ligase deficiency syndromes, with DNA ligase I associated with increased proliferation, DNA ligase III indicating reliance on an alternative DNA repair pathway, and DNA ligase IV conferring radio resistance through enhanced repair of DNA double-strand breaks. An enhanced DNA inhibitors in future researches could help minimize the inefficiency. The enhanced ligases give scientists more dependable and effective tools for working with DNA fragments, decreasing the likelihood of experiment failure and enabling more exact and precise genetic engineering [32].

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2. **Other Techniques and Tools:** Making many, exact copies of a specific segment of DNA is known as DNA cloning. The gene or other DNA fragment of interest is first extracted using a conventional DNA cloning method and then put into a circular piece of DNA known as a plasmid. Most often, a particular restriction enzyme is used to create sticky ends on the extracted DNA fragment and plasmid to ensure adequate compatibility [33].

The beginning of gene cloning can date as far back as in 1953 when Francis Crick and James Watson describe the structure of DNA as being double helical and its function in genetics. This was a breakthrough in molecular biology that give light about the possibility manipulation of DNA. John Gurdon showcased the feasibility of cloning an organism by successfully cloning South African frogs. He achieved this by utilizing the nucleus of fully developed adult intestinal cells. This groundbreaking experiment provided evidence that cells maintain their genetic potential even after they specialize, aligning with Spemann's hypothesis in 1962.

In the early 60s, Werner Arber discovered restriction enzyme and made a hypothesis of how they bind to DNA, and this postulation was validated by Hamilton Smith who purified a restriction enzyme from a bacterial and showed how they cut DNA based on sequence matching. This idea was first applied to genetics and show the importance of restriction enzymes in genetic maps construction. For this work, all three won the shared the Nobel Prize in physiology in 1978. This event was followed by the discovery of first DNA ligase (T4 Ligase) by Gellert, Lehman, Richardson, and Hurwitz laboratories in 1967. A Stanford university Biochemist discovered the first recombinant DNA in 1972. This was followed by the development of plasmid by Herbert Boyer and Stanley Cohen in mid 1970s. From this experiment, plasmids became the most widely used tool for gene cloning and not so long after this, Karry Banks Mullis, an American Biochemist discovered a technique called Polymerase Chain Reaction (PCR) – a method used to rapidly amplify DNA molecules. It was and still is one of the biggest achievements in molecular biology. PCR is the most widely used technique today [34].

In 1996, the first mammal, Dolly (a sheep) was cloned A cell from a six-year-old Finn Dorset sheep's mammary gland and an egg cell from a Scottish Blackface sheep. Today, there are several methods developed from early discoveries are available for DNA cloning. It is conceivable to isolate a single area of a genome, make almost endless copies of it, and overnight figure out the nucleotide sequence.

When the Human Genome Project was at its peak, enormous facilities with automated machinery were continuously producing DNA sequences at a rate of 1000 nucleotides per second. By using comparable approaches, an isolated gene may be freely modified (engineered) and returned to the animal or plant's germ line to become a useful and heritable component of the genome [35].

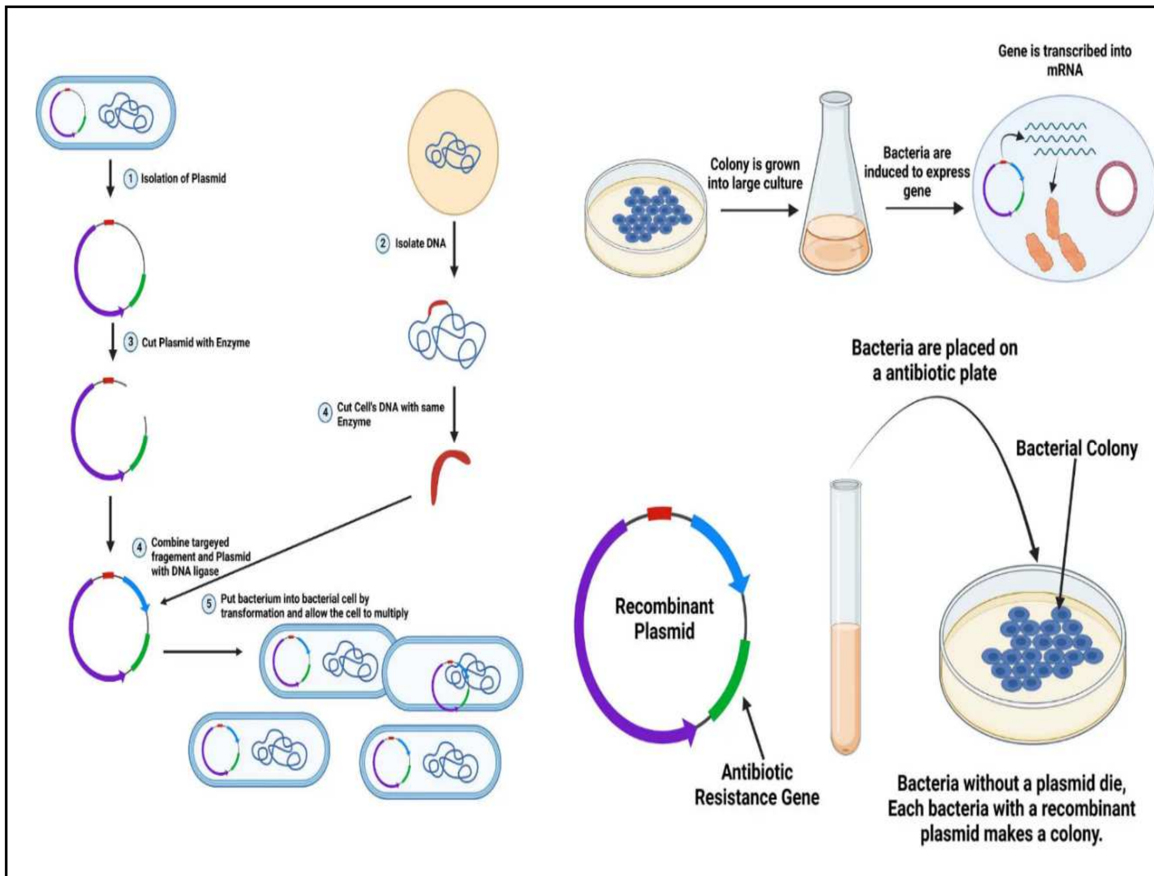

Figure 6: Diagram of Gene Cloning

3. **Vectors and Types:** A vector is a DNA molecule that contains the desired gene and transports it to the host cell. It is also known as a molecular vehicle. There are several molecular vehicle types used in cloning, and the choice of a particular type depends on the number of copies of the gene, the cloning technique, or the size of the insert. For instance, depending on the technique used, a bigger gene of interest with a high copy number will require a vector with a high carrying capacity, such as a BAC. To make many copies, the recombinant DNA is often introduced artificially into the host organism [36]. Certain criteria are considered when selecting a vector, which are listed below:

   - Multiple cloning sites unique for Restriction enzymes
   - Possess selectable markers genes
   - Should easily be separated from host organism when needed
   - Self-replicative
   - Origin of Replication

   A detailed description of these features is seen in (Figure 7). Six types of vectors are described below:

4. **Plasmids:** Plasmids are genetic materials found in bacteria. They are circular, extra-chromosomal DNA present in each cell in approximately 1-20 copies. Plasmids can replicate independently without using the host cell's machinery and its molecular weight

ranges from 106 to 108, and 40 to 50 genes may be encoded by it. Plasmids come in several varieties. They may be divided into two categories based on their capacity to interchange genetic material, namely: those that can transmit genetic material across bacterial cells are known as conjugative, and those that cannot are referred to as non-conjugative. Non-conjugative plasmids are transferred into bacterial cells with the aid of conjugative plasmids. Regardless of their ability to conjugate, they are also self-replicative. The table below describes the types of each plasmid type under discussion [37].

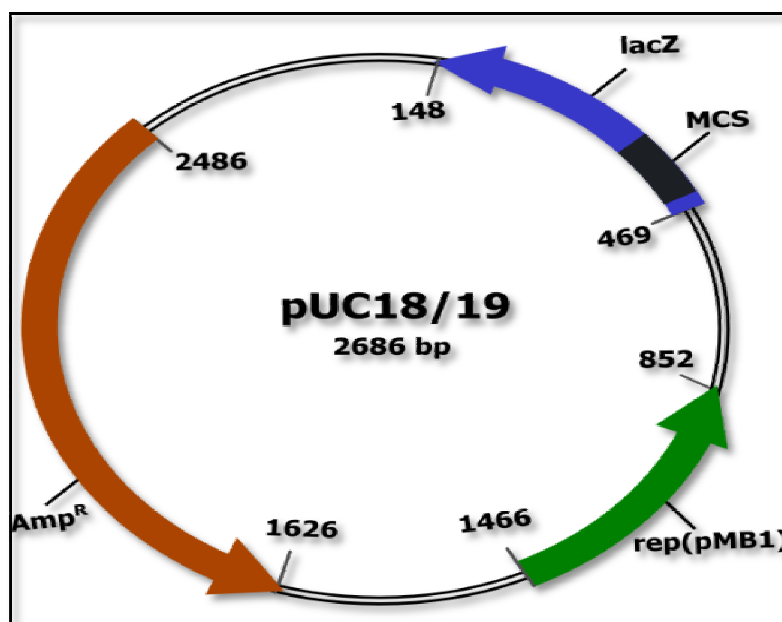

Figure 7: Vector Structure

5. **Bacteriophage:** Bacteriophages are group of viruses that infect bacteria and Archaea. Also known as Phages which when translated from Greek means ‘to eat’ i.e., bacteriophages are bacteria eaters. Upon infection, they insert their genomes within the bacteria cell which becomes a part of the bacteria genomes and function as a surveillance system. Phages are small, straightforward creatures made up mostly of genetic material (nucleic acid) that is encased in a protein cap. They are grouped into several viral families such as Siphoviridae, Myoviridae, Ackermannviridae, and Podoviridae. The basic structure of a phage entails a filamentous form, icosahedral head with tail, and icosahedral head without tail [38].

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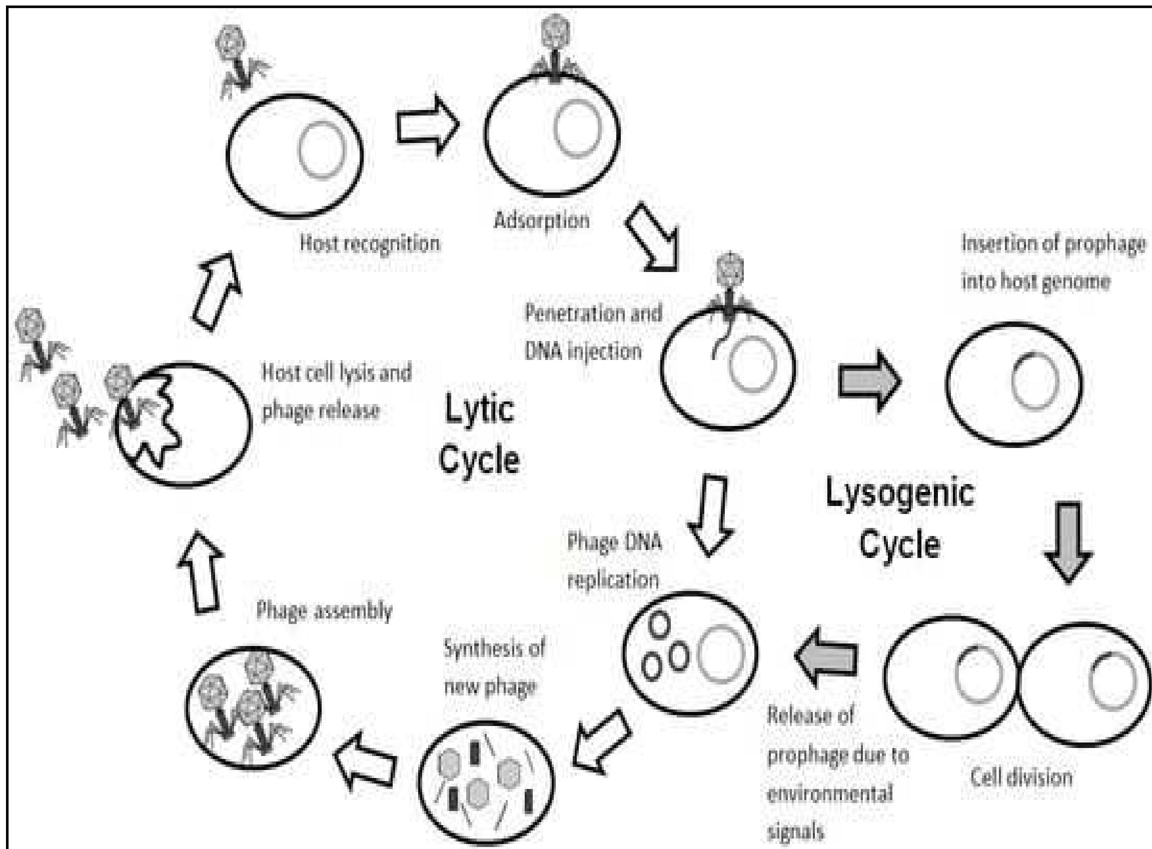

Figure 8: LYTIC and Lysogenc Cycles of Bacteriophage

6. **Cosmids:** A cosmid is a hybrid plasmid with a bacterial "ori" and lambda phage-derived "cos" sequence. It is a vector that combines linearized DNA plasmid ends with cos-sites of λ DNA, forming a circular structure. Cosmids lack lambda DNA structural and regulatory genes, preventing DNA breakdown or incorporation into host cells. Cosmid DNA consists of two single-stranded sections, creating a duplex. It does not encode phage proteins or host cell lysis and relies on plasmid DNA for independent replication. It has cloning sites and selectable marker genes. Cosmid DNA is tightly packed inside the bacteriophage's protein coat to create dormant phage particles [39].

7. **Bacteria Artificial Chromosome (BAC):** BAC vectors are plasmids with E. coli F factor replication origin, storing up to 300 kb of DNA fragments per cell. The BAC system is a large insert cloning technology used to create DNA libraries for genetic data processing. It transforms and clones bacteria, typically E. coli, using a fertility plasmid (F plasmid). BAC inserts generally range from 150 to 350kbp, although current sequencing techniques like Shotgun and Next Generation sequencing have mostly supplanted them [40]. BAC possess the following key characteristics:

   - Bacterial artificial chromosomes (BACs) are ideal for cloning and altering large gene sequences.
   - stable maintenance: enabling the retention of cloned genes during propagation
   - low copy numbers: BACs often live-in bacterial cells in low copy numbers, reducing the danger of gene overexpression or toxicity during gene cloning research.

- F-factor origin of replication: enabling autonomous cloning and efficient insertion and modification of target genes.

8. **Yeast Artificial Chromosomes (YAC):** Are enhanced cloning vectors derived from human DNA and are used to clone sequences in yeast cells. enable comprehensive examination of DNA spans from 50 to 2000 kb, enabling restriction mapping, physical mapping, and gene or coding sequence analysis. Upon insertion into the yeast cell, multiple copies of the DNA are produced as the yeast cell grows and divides. YACs are essential for cloning entire sequences of large genes or complexes larger than traditional bacterial cloning vectors like plasmids, bacteriophages, and cosmids [41]. The components of YAC vectors are:

   - **Centromere Region:** A region in yeast that enables proper vector segregation during cell division as along with selected marker genes.

   - **Autonomous Replication Sequences (ARS):** These are the sequences that allow the YAC to make multiple copies of itself within the yeast cell.

   - **Telomere Sequences:** prevent YAC DNA degradation and fusion.

   - **Selectable Markers:** Genes that provide antibiotic resistance or enable yeast cells to grow rapidly under certain conditions.

   - **Multiple Cloning Site:** a region having restriction enzyme recognition sites to facilitate the incorporation of DNA fragments**.**

   - Other major types of cloning vectors are described in (***Table 2***)

Table 2: Different Types of Vectors and Their Features

Vector	**Insert Size**	**# of Clones Required in the Library**	**Special Characters**
Plasmid	Up to 15kb	2.13 x 10^5	Versatile and easy to manipulate
Lambda λ	20kb	5×10^5	Stable inserts and easy to construct library
Cosmid	45kb	2×10^5	Easy to construct libraries (combination of plasmids and phage)
Yeast Artificial Chromosome (YAC)	1Mb	10^4	Maintains large DNA inserts in yeast cells
P1-derived Artificial Chromosome (PAC)	120kb	10^5	Large DNA insert capacity.
Bacteria Artificial Chromosome	>500	2×10^4	Accommodates large genomic DNA

(BAC)			fragments
OPO® TA Vector	3kb	10^6	Topoisomerase I-based cloning method

VIII. DNA ISOLATION AND PURIFICATION TECHNIQUES

The first step involved in accessing a specific DNA of interest is extraction ("to draw out" all DNA components) followed by isolation ("to set apart" the specific DNA of interest). There are many Five general steps are followed in DNA isolation and purification:

1. **Lysing of Cell Structure:** this is done to expose the cellular components and can be done using physical methods (e.g., free-thaw cycles, homogenization, sonication, vertexing), chemical methods using detergents (e.g., SDS or CTAB) and enzymatic methods such as lysozyme for bacterial cell, cellulase for plants or chitinase for fungus cell depending on the starting materials, there are more enzymes used [42].

2. **Separation of Soluble DNA from Cell Debris:** Cellular lysates requires debris removal before nucleic acid purification to minimize unwanted materials like proteins, lipids, and saccharides. Clearing is typically achieved through centrifugation, filtration, or bead-based methods.

3. **Precipitation:** The supernatant includes dissolved DNA that must be concentrated before it can be purified. Usually, the DNA made to precipitate in ethanol or isopropanol, generating a visible pellet out of the solution.

4. **Washing:** To eliminate any leftover contaminants and residual salts, the DNA pellet is washed with ethanol or isopropanol. Washing ensures that the DNA sample is clean.

5. **Resuspension:** involves resuspending purified DNA in a suitable buffer for downstream applications like PCR, sequencing, and cloning, ensuring it is ready for use in various genetic analyses such as PCR, sequencing, cloning etc.,

   A diagram of this protocol is seen in (

   Figure 9:) and other methods are in (***Table 3***)

   NOTE: These steps are general and may vary from depending on the isolation protocol and source of DNA.

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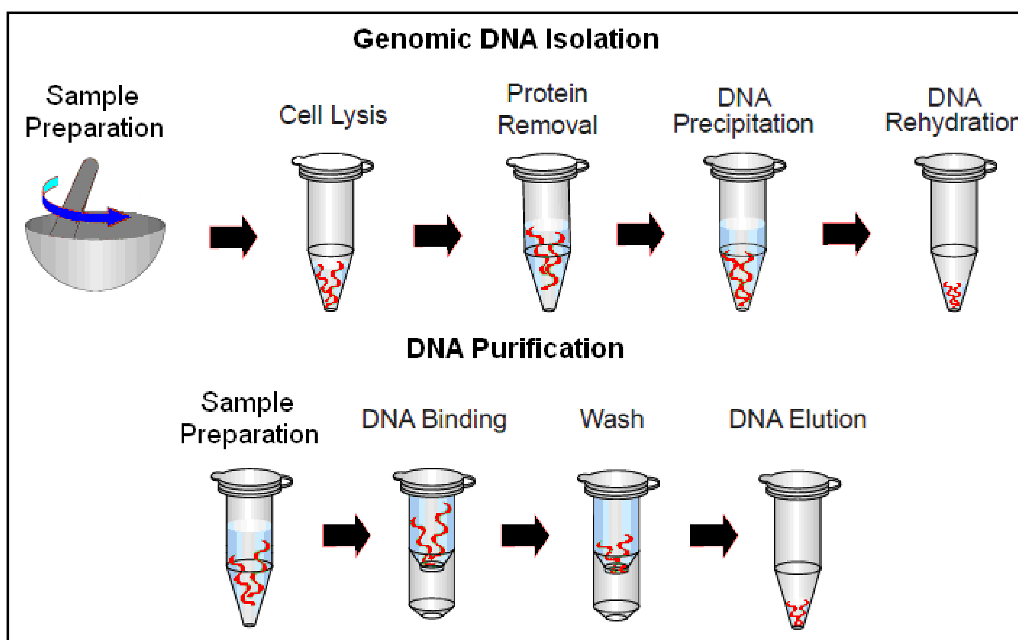

Figure 9: Gene Isolation and Purification Process

Table 3. Methods of Gene Isolation

No	Method	Description
1.	CTAB DNA extraction	Cetyl Trimethyl Ammonium Bromide is a chemical for plant extraction. It is Liquid-liquid based
2.	SDS DNA extraction	sodium dodecyl sulfate is an anionic detergent that digests nuclear and cell membrane proteins
3.	Silica-gel-based techniques	silica gel as a solid phase is immobilized in a tub used to digest samples and help separate it in solid phase
4.	Paper DNA extraction	Utilizes organic substances such as phenol and chloroform.
5.	Magnetic bead DNA extraction	Selective DNA purification method (molecules that have an affinity for DNA (such as silica or paramagnetic particles), are added to the lysed sample)

IX. DNA SEQUENCING AND AMPLIFICATION METHODS

The process of determining order arrangement of sequences in a genome is called sequencing while amplification is to make multiple exact copies of a DNA molecule. The most widely used amplification method is known as polymerase chain Reaction (PCR). PCR involves a series of repeated temperature dependent cycle that allows exponential amplification of targeted DNA segment. PCR is a crucial step because it increases the copies of the desired gene to make the process more feasible. It consists of 20-30 cycles per round of PCR [43]. The first step is to design two primers flanked by sequences of gene of interest and follow the following steps:

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1. **Denaturation:** high temperature is used to separate the double stranded DNA molecule into single stands. A temperature between 94-98° C is enough to break the H-bonds between complimentary base pairs.

2. **Annealing:** lowering the temperature between 50-65° C allows the primers to bind to the complimentary sequences on each single stand creating an origin of replication for DNA polymerase (e.g., Tag polymerase) to synthesized new strands.

3. **Elongation:** When the temperature is increased to 72° C, DNA polymerase begins to lay down new nucleotides using the primers as template in 5' to 3' direction resulting in two double stranded molecules per cycle.

4. **Termination:** lowering the temperature to 4°C∞ help to hold the DNA and maintain stable temperature.

Variations occur in the annealing temperature is due to the following reasons factors**:**

- Length and GC content of primers
- Presence of secondary structures such as hairpin loops, self-complementarity

PCR is carried in a a machine called thermal cycler. In normal PCR, DNA Tag polymerase is often used due to its ability to withstand Temperature as high as 95° C and function at a low temperature compare to other enzymes. See the list of PCR cocktail mixture in **(Table 4).** Other types of PCR include reversed transcription PCR (RT-PCR), and Real-time PCR (qPCR).

Table 4: Materials Require for PCR Process

Primers (forward 5' and reversed 3')	Building of new nucleotides and are 20 – 25 nucleotides in long
Deoxynucleotidyl phosphate (dNTPs)	Aid in strand extension (usually beyond 20-25 nt)
$MgCl_2$	Promote correct positioning nucleotides by catalyzing the formation of phosphodiester bond b/w nt during synthesis
DNA polymerase 80^0 C (e.g., Tag polymerase)	Build new nucleotides to grow new strands
Cocktail mixture volume	25mL
Double distilled water (H_2O)	Use for storage of mixture to avoid impurities
Template DNA	Original DNA from which new ones are synthesized

- **RT-PCR:** Reversed transcription PCR is a two steps PCR process that utilize an enzyme called reversed transcriptase which converts mRNA template into ds cDNA followed by amplification. This reaction occurs at 37° C for an hour (Shomu's Biology, 2013). RT-PCR is use in cancer research, forensic science, Gene expression analysis, DNA fingerprinting, detection of diseases etc.

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- **Quantifying DNA with the use of Real TIME PCR:** Real-Team PCR, referred to as quantitative PCR (qPCR), like other techniques is use in the detection and quantification of DNA. Here the techniques allow the measurement of starting amount of DNA. As the process progresses, short labeled segments of DNA known as fluorescence probes are added to the growing strands, so that when it binds to complimentary sequences on the template strand during elongation, it is cleaved by the DNA polymerase, resulting in the separation of the fluorescent dye from the quencher while the florescence release its intensity to be measured after each cycle. During Real-Time PCR, the amplification process is monitored throughout [44].

  Cycle threshold (ct) is the cycle at which the significant amount of DNA has be amplified. It is a value used to quantify the initial amount of target DNA in the sample. Two reactions are observed during detection in qPCR:

  - **SYBR Green Dye:** This dye does not emit fluorescence when in a free state. It produces fluorescence when bind to double stranded DNA in a sample solution. The intensity of fluorescence is proportional to the amount of ds DNA present.

  - **TagMan:** TagMan is also a probe-based method named after Tag polymerase are single stranded DNA hydrolysis probes containing 5’ reporter dye and 3’ quencher designed to be used in conjunction with PCR primers. They are labeled with reporter fluorophore such as FAM, HEX or VIC and are about 18-22bp. RT/qPCR is faster, accurate, and uses DNA probes with fluorescent, chemiluminescent, colorimetric, or radioactive markers to identify amplicons.

- **Sequencing:** Genome sequencing is carried out to know the level of communication among chemical bases (A, T, G, C) that makeup the gene. For instance, which stretch of nucleotides produce genes responsible for regulation. Without sequencing, researchers wouldn’t have been able to tell that the Human genome is composed of 3 billion base pairs and how they connect to create human being as whole. Sequencing was a challenge during the peroid known as the first-generation era as traditional methods could not cater for large DNA and accurate results, and as well as cost-effectiveness.

  Technologies improvement had it that the Human genome which cost over 3 billion and 13 years to finish, can now be done for about $1,000 dollars within a day. DNA sequencing is the highest resolution method for determining complex gene families, such as the HLA (Human Leukocyte Antigen system) genes in tissue transplant patients and donors. Databases with donor sequences can be used to construct virtual crossmatches with unrelated donors. A single primer creates copies of one strand of DNA, but full-length copies cannot determine the sequence. To determine the sequence, specific nucleotides, termed dideoxynucleosides, are used in the mix. UK Prime Minister David Cameron's 100,000 Genomes initiative in 2012 quickly achieved its goal, creating Genomics England, surpassing the initial initiative of 2003 [45].

- **Sanger Sequencing:** Sanger sequencing, developed by Nobel Laureate Frederick Sanger and his friends in 1977, is a method for determining DNA nucleotide order. It

is also referred to as chain termination method, carried out by means of automatic machine or manually and the protocol requires three basic steps, namely; chain termination, gel electrophoresis and analysis.

- **Chain Termination using PCR:** It is based on the use of the modification of nucleotides with dideoxynucleoside phosphate (ddNTPs). ddNTPs lack the 3'-OH that plays a key role in extension during replication. Because of this characteristic, during the normal PCR process, when ddNTP is added to the growing strand, it inhibits the extension process, leaving the chain at a specific location, hence the term "chain termination". A low amount of ddNTP is enough to stop the stretch and is capable of producing millions to billions of base pairs.

- **Gel Electrophoresis:** After chain is termination, the DNA is separated based on sizes using a technique called gel electrophoresis. Electrophoresis use electric current to run the DNA in a gel. It has a positive (+) end and a negative (-) end and wells to pour the mixture. Since DNA is a negatively charged molecule, the gel migrates from the negative end to positive end and the rate of migration is based on size. The smaller fragments migrate faster due to less friction compare to larger fragments. The resulting DNA fragments are arranged and observed from smallest to largest.

- **Analysis and Sequence Detection:** The last step comprises examining the gel to identify the sequence of the input DNA. As DNA polymerase only rely on synthesizing DNA in the 5' to 3' orientation, originating at a specific primer, each terminal ddNTP will correspond to a particular nucleotide in the initial sequence. For instance, the shortest fragment will finish at the first nucleotide from the 5' end, the second-shortest fragment will terminate at the second nucleotide from the 5' end, and so on. And so, reading the band from small fragments to largest helps to derive the original strand sequences in the 5' to 3' direction. Manually, Manual Sanger sequencing involves reading all four gel lanes simultaneously to determine terminal ddNTP identity while each band is read in automated to determine each ddNTP identity [46].

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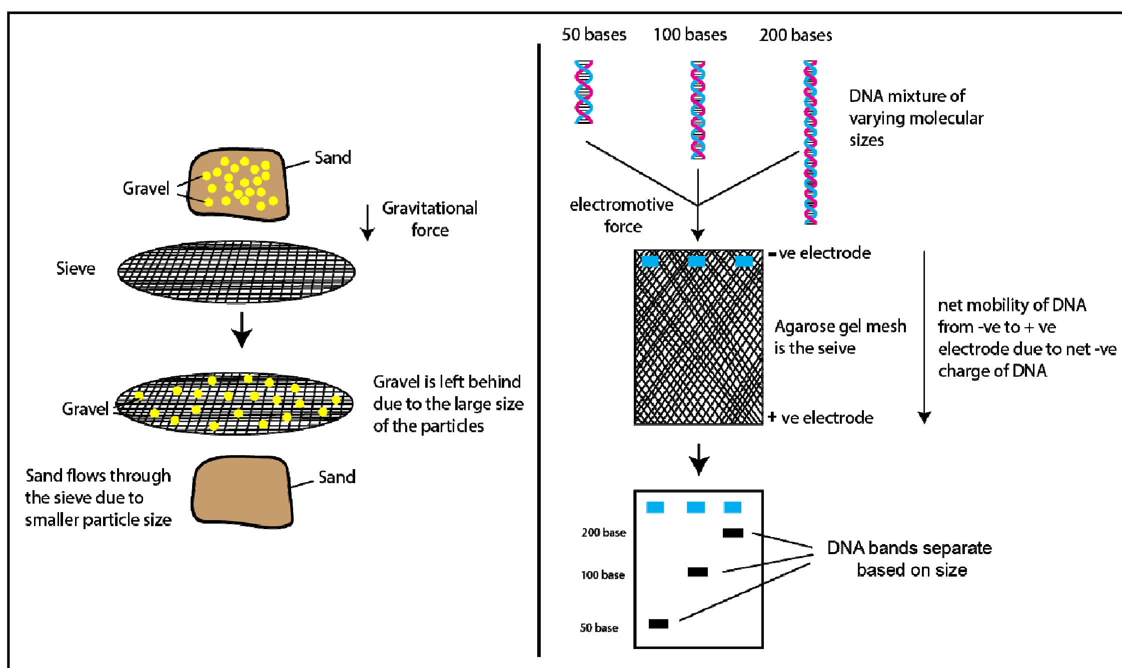

Figure 10: Diagram of Gel Electrophoresis

X. APPLICATIONS OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology has improved lots of things for our comfort. The usefulness of genes can be expressed in almost every aspect of human wellbeing, including the improvement of biopharmaceutical industries, vaccines, genetically modified organisms (GMOs) and crops with enhanced traits for better yield and so on. It offers sustainable solutions for problems of environmental pollution and climate change. Recent advancements of Recombinant DNA technology in Agriculture, medicines and environmental science are discussed below.

1. **Genetically Modified Crops:** Genetically modified organisms such as Agrobacterium tumefacien, Baculoviruses, etc., are used to produce genetically modified crops. Crops are modified to give high yields, resistance to pests and herbicides, and also phytophthora-resistance. Flavr Savr tomato pioneered this milestone by being the first crop to be engineered in 1994. To achieve this, researchers silenced the gene responsible to produce polygalacturonase, an enzyme naturally found in tomato that breaks down a substance known as pectin that holds plant cell together which when becomes overproduced in the tomato leads to spoilage.

   The antisense RNA was used to control the expression of polygalacturonase in ripening tomatoes conferring prolonged flavor, delayed ripening, and resistance to herbicides. GM crops have advanced in many areas abiotic stress tolerance etc., (**Table 5).** According to a research, 88% of corn and 93% of soybeans are genetically modified in the United States. Additionally, Bt genes obtained from the bacterium thuringiensis were used to modify GM food crop known as Bt-bringal, making it a pest resistant crop [46].

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Table 5: Some Commonly used Engineered Crops

No.	Modified crops	Traits	Use
1	Apple	Delayed browning	Food
2	Potato	Reduced acrylamide Blackspot bruise tolerance Late blight resistance	Food
3	Sweet pepper	Virus resistance	Food
4	Rice	Insect resistance	Food
5	Sugar Cane	Drought tolerance	Food
6	Soybean	increased oleic acid production Stearidonic acid production	Animal feed Soybean oil
7.	Maize	Animal feed high-fructose corn syrup corn starch	Food

2. **Production of Therapeutic Proteins:** Recombinant DNA (rDNA) technology greatly influences biomedical applications, notably in the production of mutant proteins derived from native ones. This is performed by altering the appropriate gene to boost protein synthesis, allowing the creation of commercial products with better qualities.

   Therapeutic applications usually entail exploiting human genes produced in diverse hosts like bacteria (e.g., Escherichia coli), yeast (e.g., Saccharomyces cerevisiae, Pichia pastoris), Insect cells (e.g., Sf9 cells) or Plant cells (e.g., Nicotiana benthamiana) within a cultured environment. To acquire a pure gene, mRNA is turned into cDNA. Bacteria are generally selected by researchers owing to their Familiar Genes, Fast Growth and High Yield, affordability and ease of handling. However, proteins that require post-transcriptional changes, such as the addition of phosphate (phosphorylation) or sugar molecules to proteins or lipids (glycosylation), demand the use of eukaryotic vectors.

   Mammalian cells, such as HEK293 (Human Embryonic Kidney 293), CHO (Chinese Hamster Ovary) and PER.C6 (Human Retinal Pigment Epithelium) cell lines are widely used in the biopharmaceutical industry for the production of therapeutic proteins, including monoclonal antibodies and other complex biologics since they have the aptitude to create high-quality proteins mimicking natural ones. Recombinant proteins providing a better understanding of critical biological processes and play a significant role in laboratory procedures like Enzyme-Linked Immunosorbent Assay (ELISA), western blot, and immunohistochemistry, enabling the design of enzymatic assays and studying cellular reactivity to stress and illness. They are used as therapies for various diseases like diabetes, cancer, and infectious diseases, and creating vaccines like the FDA-approved Hepatitis B vaccine.

3. **Vaccines Production:** Recombinant vaccines play a crucial role in preventing and controlling various diseases in both animals, such as cattle, and humans, offering valuable tools for disease management and eradication efforts. vaccines, including those for bovine viral diarrhea (BVDV) and bovine respiratory syncytial virus (BRSV)**,** utilize genetic engineering to insert specific antigens of the pathogens into harmless vectors [47]. These

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vectors, such as DNA-based or RNA-based constructs, efficiently deliver the genetic materials encoding the antigens to the host cells, stimulating an immune response and inducing the production of neutralizing antibodies against the targeted pathogens. This approach offers a safe and effective means of disease prevention and control in cattle and other animals, demonstrating the potential of recombinant vaccines in veterinary medicine.

Scientists choose the target antigens for vaccines based on the pathogens responsible for causing clinically and economically significant diseases in the target species, such as cattle. In the case of cattle, pathogens like bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), Leptospira sp., E. coli, Clostridia sp., and others are common targets for vaccination. The selection is based on their crucial role in disease development and vaccinating against them can offer protection and prevent infection**.** Genetic engineering has also been exploited manufacture in large quantities pharmaceuticals such as insulin, human growth hormones, follistim (used to treat infertility), human albumin, monoclonal antibodies, antihemophilic factors, vaccinations, and many more. Mouse cells, hybridomas cells fused together to produce monoclonal antibodies have been transformed to produce human monoclonal antibodies.

4. **Gene Therapy:** Gene therapy refers to the method wherein a genetic defect is treated by inserting a functional gene or section of DNA in cells. The functional gene inserted into an individual or an embryo compensates for the non-functional gene present. The newly inserted DNA corrects the effect of the mutated gene. The two types of gene therapy are somatic and germline therapy:

5. **Somatic Gene Therapy:** Here the desired gene is transferred to a somatic cell. The somatic gene therapy targets body tissues. Therapeutic DNA is transferred to a somatic cell, which is any cell other than gametic cells, Example of cells include muscle cells, liver brain etc**.** In somatic gene therapy, gene delivery is carried out in two ways:

   - **Ex-Vivo Therapy** refers to treatments or interventions that are conducted outside the living organism, typically in a laboratory setting. In this approach, cells, tissues, or organs are removed from the patient's body, treated or modified in some way, and then reintroduced back into the patient's body.

   - **In-Vivo Therapy** refers to treatments or interventions that are performed inside a living organism, typically within the body of the patient. This means that the therapy is applied directly to the patient, and the effects occur within the patient's body. It involves administering drugs, procedures, or therapies that target and affect the patient's organs, tissues, or cells while they are still in their natural physiological environment.

6. **Germline Gene Therapy:** Targets germinal or reproductive cells. These cells produce male and female gametes therefore the inserted gene passes to the future generations. The transfer can also be done during early embryonic development. **E.g**., during in-vitro fertilization, then the desired gene can be inserted in all the cells of a developing embryo [48].

See table 8

- **Gene Augmentation Therapy:** Here a functional gene is introduced, which produces sufficient levels of proteins to compensate for non-functional genes.

- **Gene Inhibition Therapy:** This is used when a gene activity is altered and needs to be suppressed.

Table 6: Common Methods of Gene Delivery

Methods of Gene Delivery	
Viral vectors systems	Direct methods
Retroviral • 7-10kb in size	Micro-injection
Adeno virus • About 36kb • Linear genome & double stranded	Gene gun
SV40 vector • Approximately 5kb	Electroporation

7. **Bioremediation and Waste Management:** The use of genetically modified organisms (GMOs) to degrade or remove pollutants from the environment, making them more environmentally friendly and sustainable. Recombinant DNA technology allows scientists to engineer these microorganisms to enhance their ability to degrade specific pollutants.

- **Oil-Eating Bacteria:** Certain bacteria can naturally break down hydrocarbons found in oil spills. researchers can enhance the metabolic pathways in these bacteria, making them more efficient in breaking down and digesting oil. Some examples are *Pseudomonas putida, Acinetobacter baylyi, Alcanivorax borkumensis*

- **Heavy Metal-Absorbing Plants:** Introducing genes responsible for metal-binding proteins into plants, scientists can create GMOs with improved abilities to remove heavy metals from contaminated soil. This group includes, Arabidopsis thaliana, Brassica juncea (Indian mustard) - engineered Brassica juncea to express metal-binding proteins, such as metallothioneins, and Populus spp – can degrade organic pollutants, such as trichloroethylene (TCE), a common groundwater contaminant [47, 48].

Engineered microorganisms can also be employed in waste treatment processes to break down organic waste more effectively or produce valuable byproducts. Some examples include:

- **Sewage Treatment:** Certain bacteria and fungi can degrade organic matter present in sewage. Some Examples are:

  - **Phage-Assisted Continuous Bioprocessing:** Scientist are studying the use of bacteriophages to target and control the growth of specific bacteria in sewage treatment systems.

  - **Modified Algae for Nutrient Removal:** Algae play a vital role in nutrient removal from wastewater, especially for nitrogen and phosphorus

- **Biodegradable Waste:** Engineered bacteria are used to produce biodegradable plastics, such as polyhydroxyalkanoates (PHA). These GMOs can be used to treat organic waste, and once they have accumulated sufficient PHA, they can be harvested, providing a sustainable source of bioplastics.

XI. FUTURE PROSPECTS OF RECOMBINANT DNA

DNA technology is likely to have profound effects on society. Its applications have shown continuous improvement in health, agriculture, and environmental management since its birth in the 1950s. In agriculture, the use of genetically modified bacteria, plants, or fungi for phytoremediation or bioremediation has been instrumental in preventing crop loss due to drought, pests, and diseases

Similarly, recombinant DNA techniques have broadened our understanding of disease mechanisms, leading to adapted solutions for various illnesses, including diabetes, cancer, and pandemics. A notable current progress is the harnessing of phytochemicals as radioprotectors to be used alongside radiation therapy. This advancement may increase the chances of patient survival for cancer types that have proven to be radio-resistant while minimising side effects. The completion of the Human Genome Project (HGP) stands as one of the most significant advancements in Recombinant DNA technology, providing much more detailed insight into gene variation. The technology keeps evolving, enabling scientists to continuously explore new ideas through the manipulation of three billion base pairs [45, 46, 47, 48].

1. **Pricised Gene Editing:** Gene editing techniques like CRISPR-Cas9, holds great promise in treating genetic diseases caused by specific faulty genes. The ability to precisely edit genes within an individual's genome can potentially correct genetic mutations responsible for inherited disorders, offering a potential cure or long-term therapeutic relief. Some off-target activity can lead to mutation of this techniques can be develop highly specific and efficient gene-editing tools that can accurately target and correct disease-causing mutations without causing unintended side effects.

2. **Synthetic Biology and Bioengineering:** Recombinant DNA technology holds promising potentials for advancements in synthetic biology and bioengineering. systems and organisms with tailored functions to address various challenges, including biodegradation of pollutants, production of biofuels, and synthesis of complex molecules.

XII. CONCLUSION

Recombinant DNA technology has emerged as a powerful tool with diverse applications in various fields, revolutionizing biopharmaceuticals, agriculture, vaccines, gene therapy, and environmental management. In the biopharmaceutical industry, mammalian cells like HEK293, CHO, and PER.C6 have paved the way for the production of high-quality therapeutic proteins, enabling better disease management and improved laboratory

procedures. Genetically modified crops have demonstrated increased yields, pest resistance, and enhanced traits, contributing to food security and sustainability.

Vaccines developed through genetic engineering offer effective prevention and control of diseases in both animals and humans. Recombinant vaccines utilize harmless vectors to deliver specific antigens, stimulating immune responses and the production of neutralizing antibodies against targeted pathogens. This approach shows promise in veterinary medicine, particularly in controlling diseases in cattle. Gene therapy holds the potential to treat genetic disorders by inserting functional genes into somatic cells, compensating for non-functional ones. It offers hope for targeted and long-term therapeutic relief for patients with inherited disorders. Additionally, the future prospects of precise gene editing techniques like CRISPR-Cas9 offer the possibility of accurately targeting disease-causing mutations without unintended side effects, further enhancing the potential of gene therapy.

Moreover, recombinant DNA technology contributes to environmental management by utilizing genetically modified microorganisms for bioremediation and waste treatment. These organisms efficiently degrade pollutants, making them more environmentally friendly and sustainable.

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