

1. What is the significance of this research topic?

Recombinant DNA (rDNA) is considered one of the most important technologies of the 20th century and started the biotechnology revolution. rDNA technologies allow the production of new proteins, and biochemicals by inserting DNA fragments using appropriate vectors into other organisms to develop for example human insulin or engineered vaccines like hepatitis B vaccine. Once introduced into the cell, multiple clones of the incorporated DNA fragments are produced and harvested. The introduced genes can be turned on to create the desired protein. rDNA has been applied since the late 70s in research, agriculture, engineering, medicine, food and many other areas. By 2001 over 80 rDNA-based products were approved for treating disease and for vaccination and a further 350 recombinant DNA-based drugs were tested. In 2021, all COVID-19 vaccines with FDA approval, were produced using recombinant DNA technology containing either mRNA derived from recombinant plasmids or a recombinant adenovirus. Since 2016, the number of investigational new drugs (INDs) applications to the FDA, which include genetically modified therapies have increased exponentially [1]. Today according to D. Eisenman and al., current major developments in recombinant (or synthetic) nucleic acid molecules can be divided in 3 revolutions:

- **Clinical applications of mRNA-based technology:** two days after SARS-CoV-2 sequence was posted on line, Moderna in collaboration with NIAID¹, developed a vaccine in 2 days.
- **CAR T Cell therapy:** patient cells are collected and reengineered to produce chimeric antigen receptor proteins or CARs. CAR T cells multiply in the patient's body after being reinjected and bind to cancer cells to kill them[2].
- **Drugs for rare diseases:** although more than 30 million people in the United States are affected by 7,000 rare diseases, by comparison for a specific rare disease, only few patients are identified, and to cure such diseases is not a strong financial incentive for pharmaceutical companies. Recent advancements in recombinant DNA technology have enable the creation of drug discovery platforms in which a single product can be adapted for many uses. Recently FDAA approved two novel drugs: Luxturna to treat patients with a rare form of inherited vision loss and Zolgensma to treat children less than 2 years old with spinal muscular atrophy (SMA).

Polymerase chain reaction (PCR) has been a major breakthrough in rDNA technology, and has become the ubiquitous molecular biology workhorse. Every few minutes, at each PCR cycle, the number of double-stranded copies of the original DNA is double, i.e., 2^n at cycle n . PCR since its inception in 1987 by Mullis and al., has been successful due to its overall simplicity, low cost and reduced error rate. Over the years, its operational procedures have been highly optimized (real-time PCR) and many modifications have been made to the original PCR to extend its capabilities, major PCR variations, are:

- **Amplified fragment length polymorphism (AFLP):** uses selective amplification on a subset of DNA fragments to compare fingerprints of genome of interest (criminal and paternity tests).
- **Colony PCR:** is an effective high-throughput method to determine presence of genetic constructs in colonies of yeast or bacteria.

¹ National Institute of Allergy and Infectious Diseases

- **Asymmetric, inverse, long range PCR and others:** variations of PCR methods for amplification of specific DNA fragments.

One of the most significant applications of PCR technique is the generation of sequencing templates that allows to study DNA molecule in details for research [3][4][5].

2. Who is working in this area?

There are many centers of excellence in genetic and genomic research across the country both on the East and West coasts [5]:

- Johns Hopkins has been a leader in these fields starting in the 1950s with Victor McKusick, who is considered the “father of medical genetics”. The department of genetic medicine has numerous affiliations with different facilities, labs and other centers, and its major research areas are identifying genes that influence complex traits, studying the genes and proteins that regulate other genes, and finding molecular bases of single-gene disorders.
- Broad Institute is a genomic research center open for collaboration between MIT, the Whitehead institute, Harvard and Harvard affiliated hospitals.
- Cold Spring Harbor Laboratory has several cell biology and genomics research programs in: RNA interference (RNAi), small-RNA technology; DNA replication, RNA splicing, deep sequencing, and single-cell sequencing.
- National Human Genome Research Institute (NHGRI): recently Dr. A. Phillippy from NHGRI, completed the full decoding of the human genome sequence, filling the remaining gaps in the sequence.
- There are many other universities involved in relevant research areas. Historically in the industry, companies like GlaxoSmithKline (GSK), Genentech, Roche, have been on the forefront of rDNA applications for vaccines, drugs and therapies. Illumina is a leader for gene sequencing, and CRISPR Therapeutics for gene editing.

3. What methods are used to study the concepts described in the paper?

There are two major processes to replicate a specific DNA sequence:

- In rDNA, the segment of DNA to insert into the receiving DNA molecule is cut with an endonuclease, restriction enzymes, cleave DNA at a specific nucleotide sequence (cutting the phosphodiester bonds), and the DNA strand can be joined to another complementary DNA strand by the “sticky ends” of each DNA. These strands are joined together using a DNA ligase which catalyzes the formation of phosphodiester bond within a polypeptide. In vivo cloning using *E. coli* as host, *EcoRI*, a restriction endonuclease, cleaves the sequence GATTC between G and A on both DNA strands. The same restriction enzyme is used in the donor DNA with the gene producing the protein. Cut DNA plasmids and donor DNA are mixed together and the complementary strands of both type of DNA unite randomly. Several pairing combinations are possible and in particular plasmid to donor fragment. After transformation, bacteria cells are then checked and only the ones with the right insert in the right orientation are selected. Once inside the bacteria cells, the recombinant plasmid is replicated by mitosis. The introduced gene can then produce the target protein by transcription or translation [6].

- While rDNA involves the replication of DNA in-vivo, Polymerase Chain Reaction (PCR) replicates DNA in vitro by copying an existing sequence. The tubes containing the mixture reaction are subjected to repetitive temperature cycles, 30 or 40 cycles on an automated cycler which can heat and cool the tubes quickly. The process of PCR is divided in 3 major steps:
 - **Denaturation at 94°C:** the DNA to be copied is heated, hydrogen bonds cannot be maintained at temperature greater than 80°C, and the DNA paired strands separate.
 - **Annealing at 50°C:** short single-stranded sequences complementary, primers, that flank the DNA to be amplified, bind to matching subsequences along the DNA template strand. The shorter the primers, the easier they bind and the higher the annealing temperature, the more specific it is. The temperature is then decreased allowing the hydrogen bonds between nucleotides to reform.
 - **Extension at 60°C:** *Taq polymerase* binds to primed single-stranded DNAs and catalyzes reaction using deoxynucleotide triphosphate or dNTP. Using dNTP during extension phase, provides nucleotides to the template DNA side and extend it. In chain-termination PCR, chain-terminating dideoxynucleotides (ddNTPs) are added to the reaction mixture. ddNTPs lack the 3'-OH group required for next phosphodiester bond formation and extension stops.

This 3-steps cycle is repeated and at each cycle, the number of copies is doubled. After the sequencing reactions, the mixture of strands of different length are separated via gel electrophoresis. The strands are loaded onto a gel matrix, and an electric current is applied, DNA molecules being negatively charged, are pulled toward the positive electrode. Smaller DNA segments move faster, as a result DNA segments are separated by size. The last step consists in determining the order of the nucleotides in the sequenced gene. Each of the four ddNTPs is tagged with a different fluorescent color, by reading the gel bands from smallest to largest, the 5' to 3' sequence of the original DNA is reconstituted [7][4].

4. Does the review article lead to new questions or hypotheses in this technical area (by the authors, by other researchers)?

Despite the promising and spectacular applications of rDNA, PCR or molecular cloning technologies, there are challenges to translate gene modification into product of quality and in case of therapies products accepted by the human body. Researchers have pointed to risks and potential threats that will need to be addressed in the future (some of them already solved):

- PCR can only amplify reliably relatively short sequences.
- If two genes have the same end sequence, the wrong gene could be amplified.
- The PCR is in-vitro, the cloned DNA has yet to be reliably introduced in a living organism.
- Post-translational modifications.
- Unfavorable cell stress response activation.
- Instability in proteolysis.
- Low solubility.
- Resistance in expressing new genes.
- Technology running amok like destruction of an ecosystem, cross contamination, deadly viruses.

- Preservation of biodiversity.
- Need to define ethical guidelines and legal frameworks.
- Need to produce the required molecules in affordable manner.
- Better integration of cellular factories of microorganisms into pharmaceutical process.

5. What are some practical applications of the research discussed in the article?

As mentioned earlier, there are various molecular techniques based on PCR technology: AFLP, or identification of microsatellites, short tandem repeats (STR), single nucleotide polymorphisms (SNPs), restriction fragment length polymorphisms (RFLPs), mitochondrial DNA polymorphisms (mtDNA). Customized cloning methods have been developed to address specific problems like increasing assembly length, multidomain fusion proteins, or construction of functional genes expressing fluorescent labels.

6. How does this topic relate to other areas of cell biology, bioengineering, or medicine?

Application of rDNA, PCR or CRISPR are numerous and cross-domains [8][9][10][11][12]: protein production, gene therapy, vaccines, growth hormones, antibodies, anticancer drugs, also:

- **In agriculture and food:** production of important enzymes, production of products with less toxicity, increased yield, and nutritional values, production of products with specific savor, with increased resistance to weather vagaries, and resistance to parasite.
- **In gene therapy:** for treatment of genetic human, or cardiovascular diseases.
- **In environment:** genetic engineering has been used in various challenging environmental situations like in bioremediation.
- **Sequencing methods overall:** have been critical in understanding the mechanisms of cellular physiology, in analysis of gene expression, detection of infectious diseases by amplifying limited availability of biological samples in which the presence of the pathogen is not always detectable with other techniques, in genetic diseases and diagnostic. Sanger sequencing, another cloning technique, was a critical tool for the human genome project.

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