RECOMBINANT DNA TECHNOLOGY AND GENETIC ENGINEERING: A SAFE AND EFFECTIVE MEANING FOR PRODUCTION VALUABLE BIOLOGICALS

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ABSTRACT:

Recombinant DNA is artificially created from two or more DNA incorporated into a single molecule. Genetic engineering, recombinant DNA technology, genetic modification/manipulation and gene splicing are terms that are applied to the direct manipulation of an organism's gene. The development of these new technologies have resulted into production of large amount of biochemically defined proteins of medical significance and created an enormous potential for pharmaceutical industries. The biochemically derived therapeutics is large extracellular proteins for use in either chronic replacement therapies or for the treatment of life threatening indications.

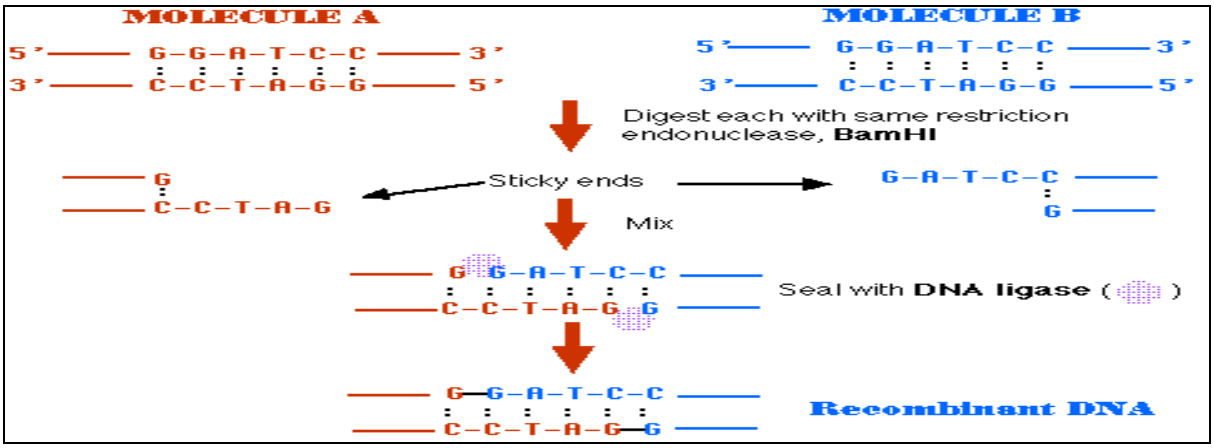
Keywords: Recombinant DNA, genetic Engineering, ligase, therapeutics

INTRODUCTION:

Genetics is the science of genes, heredity, and the variation of organisms. In modern research, genetics provides important tools in the investigation of the function of a particular gene, e.g. analysis of genetic interactions. Within organisms, genetic information generally is carried in chromosomes, where it is represented in the chemical structure of particular DNA molecules. Genes encode the information necessary for synthesizing proteins, which, in turn play a large role in influencing, although, in many instances, do not completely determine, the final phenotype of the organism. Developmental biology studies the process by which organisms grow and develop. Originating in embryology, today developmental biology studies the genetic control of cell growth, differentiation and "morphogenesis," which is the process that gives rise to tissues, organs and anatomy. Model organisms for developmental biology include the round worm Caenorhabditis elegans, the fruit fly Drosophila melanogaster, the zebrafish Brachydanio rerio, the mouse Mus musculus, and the weed Arabidopsis thaliana.

Recombinant DNA is DNA that has been created artificially. DNA from two or more sources is incorporated into a single recombinant molecule. Treat DNA from both sources with the same restriction endonuclease (BamHI in this case). BamHI cuts the same site on both molecules 5' GGATCC 3' 3' CCTAGG 5'. The ends of the cut have an overhanging piece of single-stranded DNA. These are called "sticky ends" because they are able to base pair with any DNA molecule containing the complementary sticky end. In this case, both DNA preparations have complementary sticky ends and thus can pair with each other when mixed.

Making Recombinant DNA (rDNA): An Overview



DNA ligase covalently links the two into a molecule of recombinant DNA. To be useful, the recombinant molecule must be replicated many times to provide material for analysis, sequencing, etc. Producing many identical copies of the same recombinant molecule is called cloning. Cloning can be done in vitro, by a process called the polymerase chain reaction (PCR). Here, however, we shall examine how cloning is done in vivo[1, 2].

Cloning in vivo can be done in: Unicellular microbes like E. coli, Unicellular eukaryotes like yeast and, In mammalian cells grown in tissue culture. In every case, the recombinant DNA must be taken up by the cell in a form in which it can be replicated and expressed. This is achieved by incorporating the DNA in a vector. A number of viruses (both bacterial and of mammalian cells) can serve as vectors. But here let us examine an example of cloning using E. coli as the host and a plasmid as the

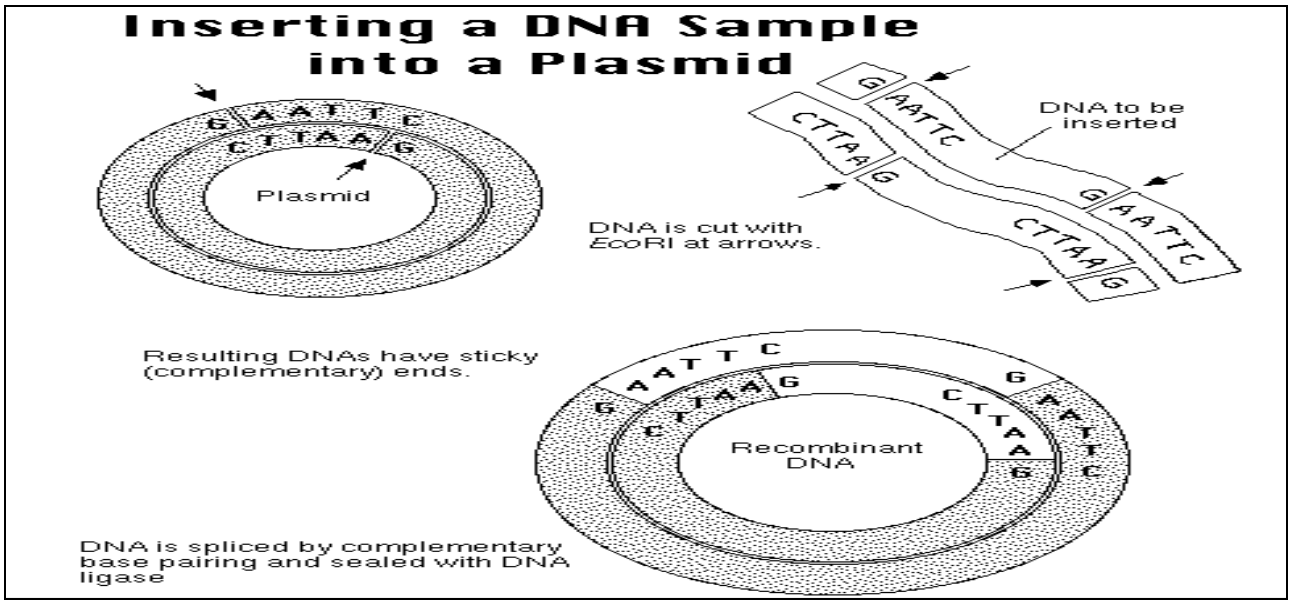
vector. Basic genetic engineering (GE) takes donor DNA from one organism or type of cell and places it into the DNA of another organism or type of cell. It includes following steps:

1. Isolation of gene
2. Preparation of target DNA
3. Insertion of DNA into plasmid
4. Insertion of plasmid back into cell
5. Plasmid multiplication
6. Target cells reproduction
7. Cells produce proteins

1. Isolation of Gene: The gene for producing a protein is isolated from a cell. The gene is on the DNA in a chromosome. Special DNA cutting proteins are used to cut out certain sections of DNA. The gene can be isolated and then copied so that many genes are available to work with.

2. Preparation of Target DNA: In 1973, two scientists named Boyer and Cohen developed a way to put DNA from one organism into the DNA of bacteria. This process is called recombinant DNA technology. First, a circular piece of DNA called a plasmid is removed from a bacterial cell. Special proteins are used to cut the plasmid ring to open it up[3].

3. Insertion of DNA into Plasmid: The host DNA that produces the wanted protein is inserted into the opened plasmid DNA ring. Then special cell proteins help close the plasmid ring.



4. Insertion of Plasmid back into cell: The circular plasmid DNA that now contains the host gene is inserted back into a bacteria cell. The plasmid is a natural part of the bacteria cell. The bacteria cell now has a gene in it that is from a different organism, even from a human. This is what is called recombinant DNA technology.

5. Plasmid multiplication: The plasmid that was inserted into the bacteria cell can multiply to make several copies of the wanted gene. Now the gene can be turned on in the cell to make proteins.

6. Target Cells Reproduction: Many recombined plasmids are inserted into many bacteria cells. While they live, the bacteria's cell processes turn on the inserted gene and the protein is produced in the cell. When the bacterial cells reproduce by dividing, the inserted gene is also reproduced in the newly created cells.

7. Cells Produced Proteins: The protein that is produced can be purified and used for a medicine, industrial, agricultural, or other uses.

Gene Cloning: ***Gene cloning is a process by which large quantities of a specific, desired gene or section of DNA may be cloned or copied once the desired DNA has been isolated[4].***

Method of Gene Cloning:

1. The gene or DNA that is desired is isolated using restriction enzymes.

2. Both the desired gene and a plasmid are treated with the same restriction enzyme to produce identical sticky ends.

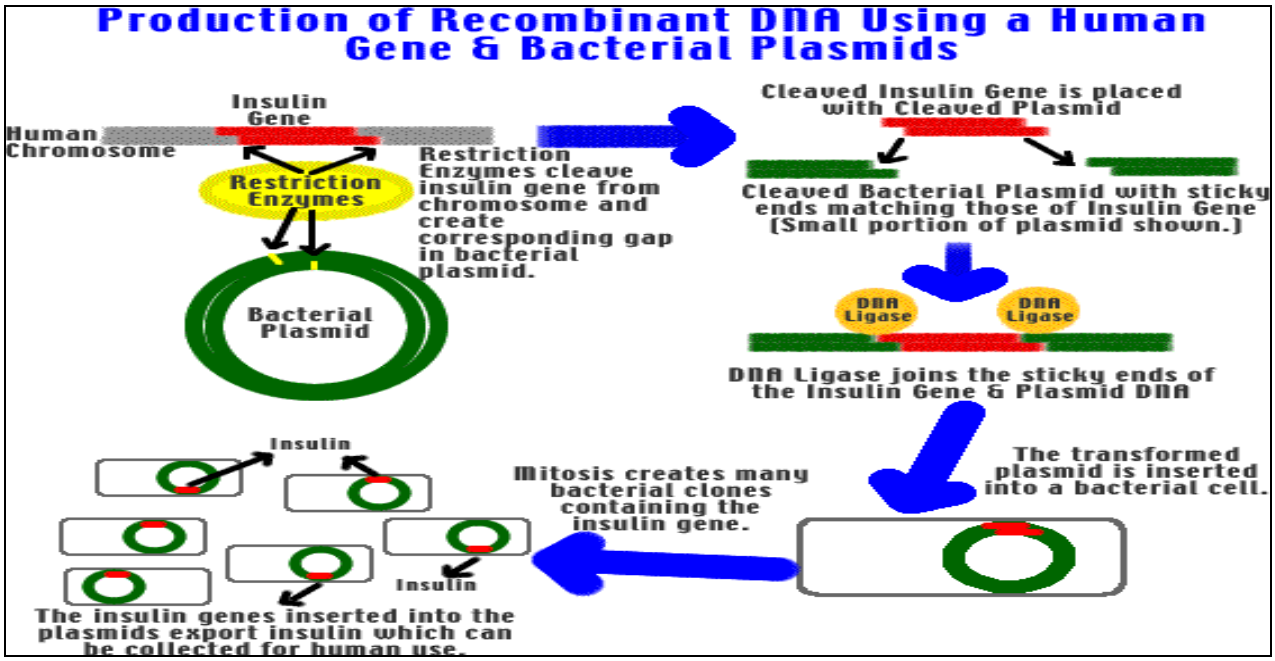
3. The DNAs from both sources are mixed together and treated with the enzyme DNA *ligase* to splice them together.

4. Recombinant DNA, with the plasmid containing the added DNA or gene has been formed.

5. The recombinant plasmids are added to a culture of bacterial cells. Under the right conditions, some of the bacteria will take in the plasmid from the solution during a process known as transformation.

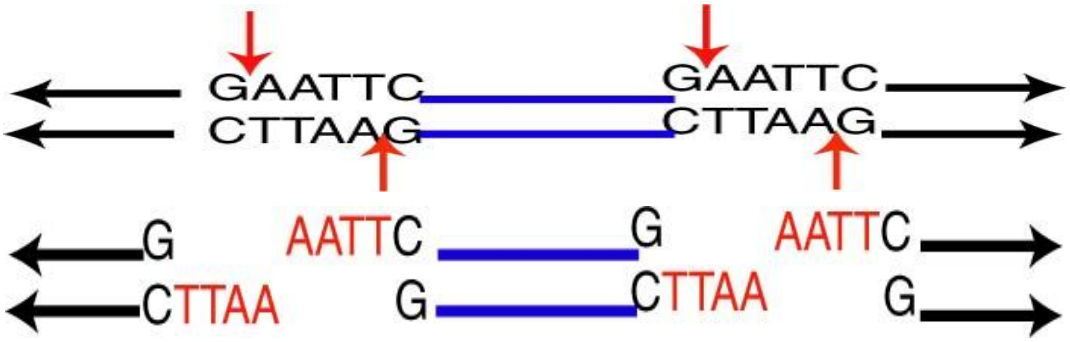
6. As the bacterial cells reproduces (by mitosis), the recombinant plasmid is copied. Soon, there will be millions of bacteria containing the recombinant plasmid with its introduced gene.

7. The introduced gene can begin producing its protein via transcription and translation.



Gene Cloning Tutorial:

Step 1: In order to clone a gene the first step is to isolate it using restriction enzymes. These enzymes recognize specific regions on the DNA molecule. The region of DNA shown below is from *Rhodobacter sphaeroides*. The gene of interest lies in the region of the chromosome indicated in blue. The base sequences are the ones that the restriction enzyme EcoRI recognizes. Note that reading from left to right in the top strand is the same as reading from right to left in the bottom strand. Use EcoRI to cut the sugar-phosphate backbone at the points indicated by the red arrows.

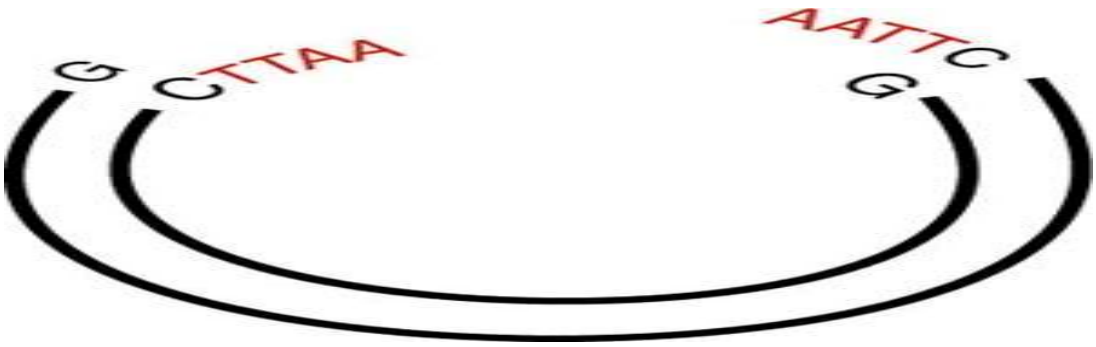


Unpaired bases result when EcoRI cuts a DNA molecule. Note that the gene of interest is bounded by fragments of DNA containing unpaired bases or "sticky ends". If the temperature is lowered and DNA ligase is added these unpaired bases can reanneal following the rules of base pairing.

Cut Molecule

Reannealed Molecule

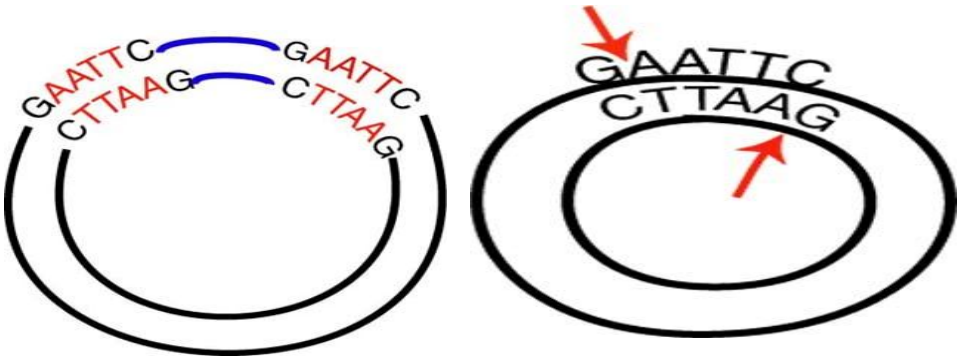
Compare the two molecules. Note the base pairing



When pK19 is cut by EcoRI it has "sticky ends" that are complementary to those made by cutting *R. sphaeroides*. Like *R. sphaeroides* the "sticky ends" can reanneal if DNA ligase is added. This would return the plasmid to it's original ring structure

Step2: Cooled, added DNA ligase and the molecules can reanneal. Resulting in a variety of recombinant forms.

One of interest is the plasmid containing the *R. sphaeroides* DNA.



pKC105 pK19

The host plasmid pK19 only has a single EcoR1 site. Inserting the *R. sphaeroides* DNA disrupts the base pair sequence in the region of the plasmid chromosome that codes for the alpha peptide[5, 6].

Cloning a Gene (Polymerase Chain Reaction): Clone: Making exact genetic copies of whole organisms, cells or pieces of DNA are called clones. A clone is a copy of a plant, animal or micro-organism derived from a single common ancestor cell or organism. Clones are genetically identical. A gene is said to be cloned when its sequence is multiplied many times in a common laboratory procedure called polymerase chain reaction (PCR). PCR copies the cell's natural ability to replicate its DNA and can generate billions of copies within a couple of hours.

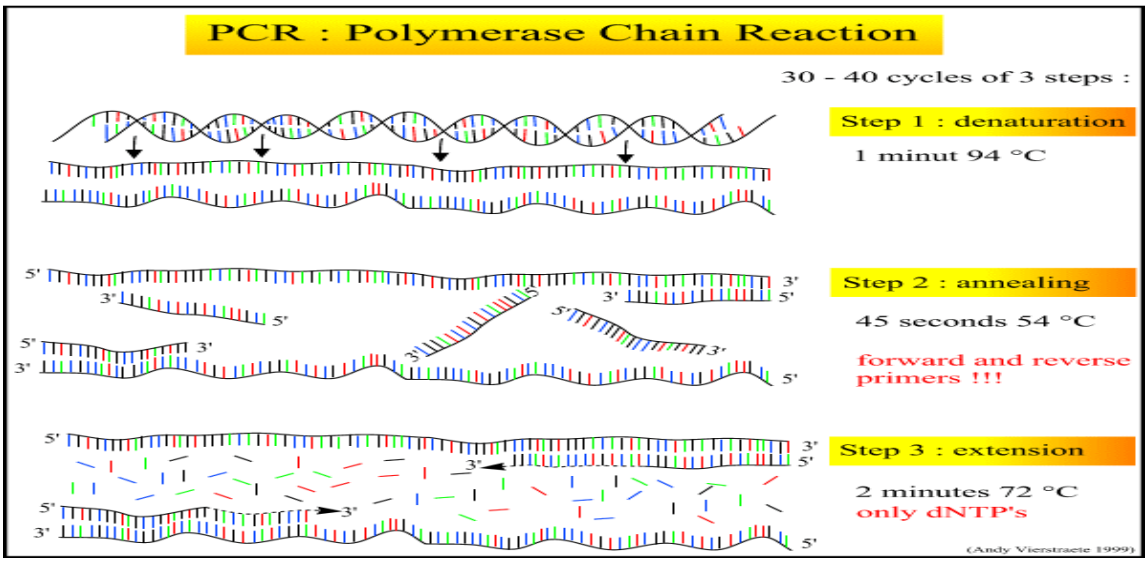
There are four main stages:

1. The DNA to be copied is heated, which causes the paired strands to separate. The resulting single strands are now accessible to primers (short lengths of DNA).
2. Large amounts of primers were added to the single strands of DNA. The primers bind to matching sequences along the DNA sequence, in front of the gene that is to be copied. The reaction mixture is then cooled which allows double-stranded DNA to form again. Because of the large amounts of primers, the two strands will always bind to primers, instead of to each other.
3. DNA polymerase was added to the mixture. This is an enzyme that makes DNA strands. It can synthesise strands from all the DNA primer combinations and dramatically increases the amount of DNA present. One enzyme used in PCR is called *Taq* polymerase which originally came from a bacterium that lives in hot springs. It can withstand the high temperature necessary for DNA strand separation and therefore, can be left in the reaction and still functions.
4. The above steps were repeated until enough DNA is obtained.

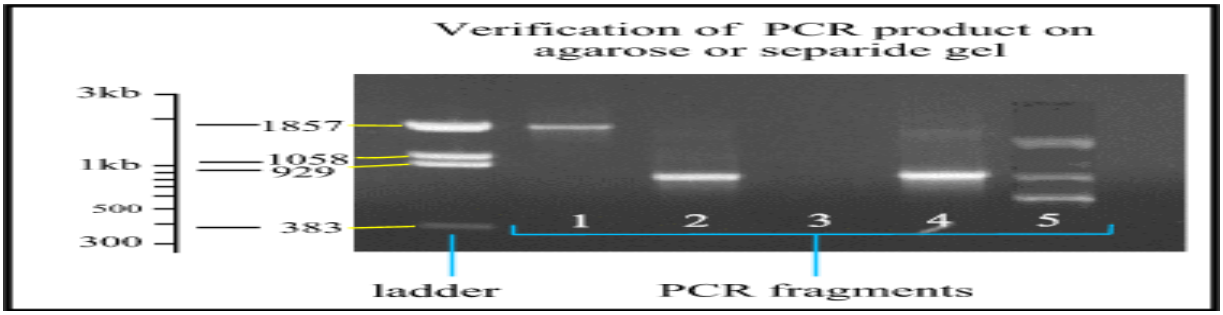
This whole process is automated and happens very quickly. The reaction occurs in a small tube which is placed inside a specialised machine which can make the big temperature adjustments quickly.

Principle of the PCR: The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing.

The cycling reactions: There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler[7, 8], which can heat and cool the tubes with the reaction mixture in a very short time. Denaturation at 94°C, Annealing at 54°C, Extension at 72°C.



The different steps in PCR



Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. Let us suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, and three cycles will result in 8 copies and so on[3].

To check whether gene is copied during PCR and to check its right size: Before the PCR product is used in further applications, it has to be checked if: There is a product formed, the product is of right size, only one band is formed.

Principle of sequencing: The purpose of sequencing is to determine the order of the nucleotides of a gene. For sequencing, we don't start from gDNA (like in PCR) but mostly from PCR fragments or cloned genes[9].

The sequencing reaction: There are three major steps in a sequencing reaction (like in PCR), which are repeated for 30 or 40 cycles.

Denaturation at 94°C: During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).

Annealing at 50°C: In sequencing reactions, only one primer is used, so there is only one strand copied (in PCR: two primers are used, so two strands are copied). The primer is jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension at 60°C : This is the ideal working temperature for the polymerase (normally it is 72 °C, but because it has to incorporate ddNTP's which are chemically modified with a fluorescent label, the temperature is lowered so it has time to incorporate the 'strange' molecules[10, 11].

Mechanism of extension: The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, come loose again and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3'side (adding dNTP's or ddNTP's from 5' to 3', reading from the template from 3' to 5' side, bases are added complementary to the template). When a ddNTP is incorporated, the extension reaction stops because a ddNTP contains an H-atom on the 3rd carbon atom (dNTP's contain a OH-atom on that position). Since the ddNTP's are fluorescently labeled, it is possible to detect the color of the last base of this fragment on an automated sequencer.

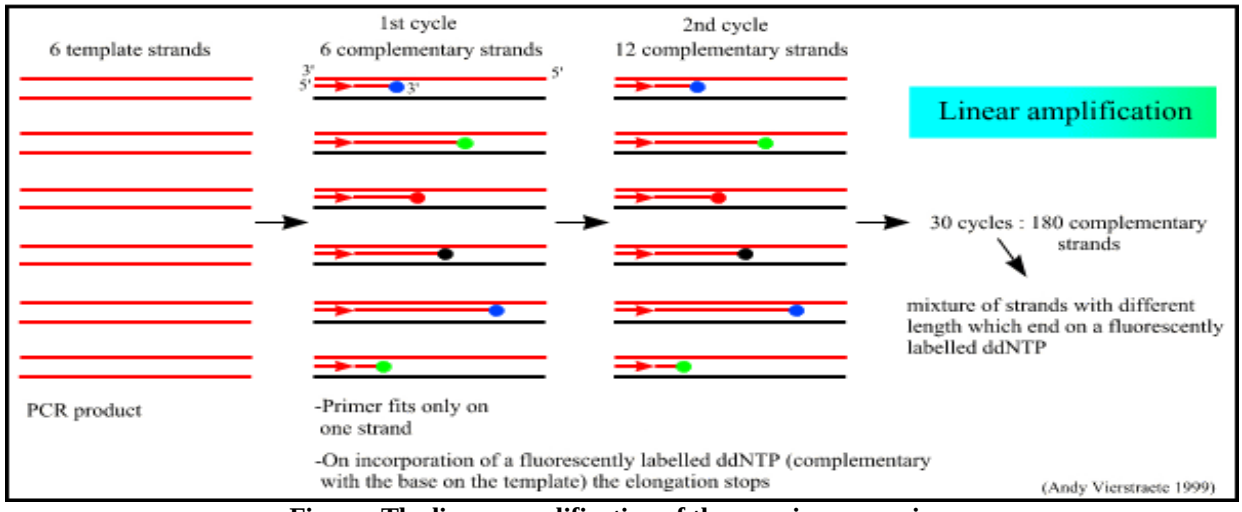
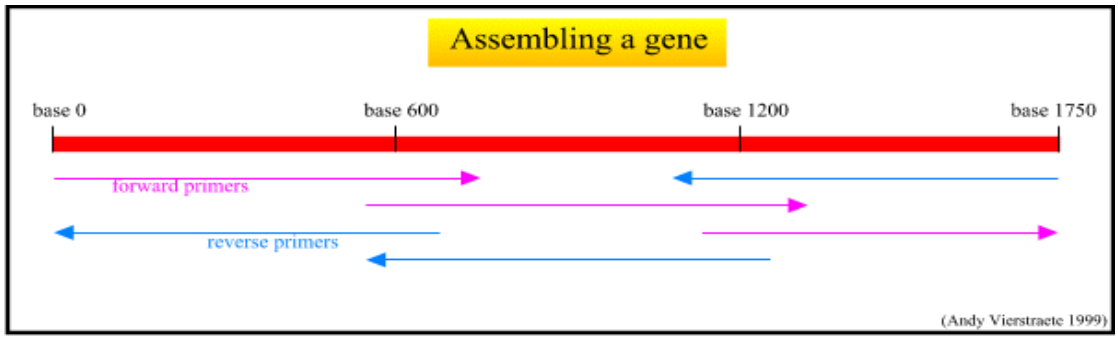


Figure: The linear amplification of the gene in sequencing.

Separation of the molecules: After the sequencing reactions, the mixture of strands, all of different length and all ending on a fluorescently labelled ddNTP have to be separated; This is done on an acrylamide gel, which is capable of separating a molecule of 30 bases from one of 31 bases, but also a molecule of 750 bases from one of 751 bases. All this is done with gel electrophoresis[12, 13]. DNA has a negative charge and migrates to the positive side. Smaller fragments migrate faster, so the DNA molecules are separated on their size.

Detection of an automated sequencer: The fluorescently labelled fragments that migrate trough the gel, are passing a laser beam at the bottom of the gel. The laser exits the fluorescent molecule, which sends out light of a distinct color. That light is collected and focused by lenses into a spectrograph. Based on the wavelength, the spectrograph separates the light across a CCD camera (charge coupled device). Each base has its own color, so the sequencer can detect the order of the bases in the sequenced gene.

Assembling of sequenced parts of a gene: For publication purposes, each sequence of a gene has to be confirmed in both directions. To accomplish this, the gene has to be sequenced with forward and reverse primers. Since it is only possible to sequence a part of 750 till 800 bases in one run, a gene of, for example 1800 bases, has to be sequenced with internal primers. When all these fragments are sequenced, a computer program tries to fit the different parts together and assembles the total gene sequence.



Applications of PCR: PCR has replaced cloning for many purposes: Particularly the sequencing of DNA. It is faster and requires no vectors, which can mutate as they reproduce. It can be used forensically, to amplify tiny amounts of DNA from criminal evidence; or clinically, to detect DNA sequences linked to inherited disorders[14, 15].

Limitations of PCR: Only relatively short sequences can be amplified reliably. Anything more than 10,000 base pairs are unlikely to be amplified. You need to know the right primer sequences to use, at both ends of the sequence you want to amplify. If two related genes have the same end sequences[16], you might amplify the wrong gene. You only obtain a DNA fragment. To see this DNA at work inside a living organism, some type of cloning has to be done.

CONCLUSION

The advances in recombinant DNA technology had occurred in parallel with the development of genetic processes and biological variations. Recombinant DNA is artificially created from two or more DNA incorporated into a single molecule. Genetic engineering, recombinant DNA technology, genetic modification/manipulation and gene splicing are terms that are applied to the direct manipulation of an organism's gene. The development of these new technologies have resulted into production of large amount of biochemically defined proteins of medical

significance and created an enormous potential for pharmaceutical industries. The biochemically derived therapeutics is large extracellular proteins for use in either chronic replacement therapies or for the treatment of life threatening indications. Recombinant DNA technology has also an important role in forensic science in identification of criminals, DNA profiling to study kinship analysis and in paternity testing.

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