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An Overview Of Polymerase Chain Reaction

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Abstract: The majority of disease diagnosis nowadays is laboratory-based. Molecular diagnosis is now the primary diagnostic modality for the majority of rare, complex, and unique disease presentations due to recent advancements in medical science and molecular biology. The most generally recognised and often used diagnostic modality with very high specificity and sensitivity for accurate diagnosis is PCR, one of many molecular methods that are currently being utilized extensively throughout the world. A single or a few copies of a DNA fragment can be amplified across several orders of magnitude using the PCR technique in molecular biology, producing thousands to millions of copies of a specific DNA sequence. Today, PCR is a widely used and frequently essential technology in biological and medical research labs for a wide range of uses. The PCR technique consists of three main steps: denaturation, annealing, and extension. A rising range of disorders can be investigated and diagnosed with the use of PCR. PCR finds applications in bioscience, diagnostics, forensic labs, and numerous other domains. In this paper we discussed about the basics of PCR in relation to its working principle, types, methods, application and advantages in diagnosis.

Key Points: pcr, dna sequence, pcr technology, current scenarios.

1. INTRODUCTION:

A single or small number of copies of a DNA fragment can be selectively amplified using the polymerase chain reaction (PCR) technology to produce millions or more copies of a specific DNA sequence. Therefore, PCR is generally referred to as a "molecular photocopier" or, to put it more succinctly, "DNA replication in a test tube." Kary Mullis of the United States1 created the polymerase chain reaction in 1983, and in 1993, he was granted the Nobel Prize in Chemistry for this invention. However, Gobind Khurana had already detailed the fundamentals of replicating a DNA fragment using two primers in 19712.When it comes to sensitivity, specificity, and speed, the PCR outperforms the other diagnostic tests in the toolbox. It is possible to identify the pathogen's DNA or RNA without having to wait for the results of the in vitro culture. However, the ophthalmologist's meticulous assessment, which aligns with the clinical diagnosis, determines the test's value. Although polymerase chain reaction was first used in research, it has now become a potent diagnostic tool, particularly for identifying the presence of infectious pathogens that can be challenging to grow on culture media. If slow-growing known sources of ocular infections are present, they might be highly helpful in detecting their presence.[1]

1.2 HISTORY:

Many have characterised the history of the Polymerase Chain Reaction (PCR) as a prime example of collaborative effort between diverse experts. A summary of some of the things that happened prior to, during, and following its development. By publishing “a radically different structure” for DNA on April 25, 1953, James D. Watson and Francis Crick established the study of molecular genetics. Two strands of complementary base-paired DNA that run in opposing directions form a double helix that makes up their structure. As they wrap up their study, they note, “We have noticed that the particular pairing we have

postulated suggests an immediate possibility for a copying mechanism for the genetic material." The Nobel Prize is given to them in 1962. The investigation of DNA replication mechanism by Arthur Kornberg starts in the mid-1950s. He discovered the initial DNA polymerase in 1957. A primer must already be present for the enzyme to start duplicating the template strand, which makes it rather limited in its ability to create DNA. Remarkably intricate, though, is the entire process of DNA replication, including different proteins for each of the following functions: opening and maintaining the DNA helix; generating primers; synthesising new DNA; removing primers; and joining the pieces. The 1959 Nobel Prize is given to him. Participating in the discovery of the Genetic Code in the early 1960s is H. Gobind Khorana. Following that, he starts a significant attempt to fully synthesise a human gene. In order to do this, he develops a number of novel methods for creating and utilising synthetic DNA oligonucleotides. Sequence-specific oligos are employed as DNA polymerase primers and templates in addition to serving as the gene's building blocks. Khorana receives the Nobel Prize in 1968 for his contributions to the genetic code. A novel type of bacteria was isolated from a hot spring in Yellowstone National Park in 1969, according to Thomas Brock. After being given the moniker Thermostatically (Taq), it develops into a common source of enzymes that can tolerate temperatures greater than those produced by E. Coli. It is reported in 1970 that E. coli's DNA Polymerase I has been altered. The 'forward' nuclease activity of this enzyme is eliminated by protease treatment. This means that rather than degrading DNA, the overall activity of the resultant Klenow fragment is biassed towards its synthesis. Concerned about the yields of DNA they were producing, Khorana's project researchers started investigating "repair synthesis" in 1971.[6]

In a publication on the previous method, he concludes by describing how replication of a particular DNA segment could be achieved with a two-primer system: "one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed with the primer." Replication of the repair process will be finished by adding DNA polymerase. The initial duplex should produce two molecules as a consequence. Every time a new dose of the enzyme was administered, the entire cycle could be repeated." The two-primer replication scheme may or may not be mentioned in relation to the unpublished studies mentioned in another work, however no findings are displayed there. A battle over patents involved a close examination of these early PCR forerunners. In 1971, Donald Glaser, Peter Farley, and Ronald Cape found Cetus Corporation in Berkeley, California. Initially, the business looks for microbes that can make substances that are utilised to make chemicals, food, medicines, or vaccines. They start working on biotechnology-related projects after relocating to Emeryville, which is close by. These projects mostly include the cloning and expression of human genes, but they also involve the creation of diagnostic tests for genetic mutations. T. aquatics yields a DNA polymerase that is isolated in 1976. It is discovered that at temperatures higher than 75°C, it still functions. Frederick Sanger describes a technique for ascertaining the DNA sequence in 1977. The method uses DNA polymerase, an oligonucleotide primer, and modified nucleotide precursors that, in a sequence-dependent manner, prevent the primer from extending farther. In 1980, he receives the Nobel Prize. Thus, the scientific community knew by 1980 all of the components required to achieve PCR amplification. When sequencing DNA and producing cDNA for cloning and expression, DNA polymerase was frequently used to lengthen oligonucleotide primers. The most popular technique for labelling DNA probes for Southern blotting was to employ DNA polymerase for nick translation. Kary Mullis is hired by Cetus Corporation in 1979 to synthesise oligonucleotides for a variety of internal research and development initiatives.

These oligos serve as primers for cDNA synthesis and DNA sequencing, screening probes for cloned genes, and building blocks for the synthesis of new genes. Mullis synthesises these oligos by hand at first, then assesses early iterations intended for automated synthesisers. Mullis has created oligo probes by May 1983 for a Cetus study that aims to examine a mutation that could cause a genetic disorder in humans. When Mullis learns of issues with their work, he imagines a different approach based on Sanger's DNA sequencing methodology. As Mullis realises how hard it is to target a single region of the genome using that approach, he thinks about putting another primer on the other strand. Mullis starts experimenting with his concept later in 1983. He hopes that the polymerase can carry out continuous replication on its own, thus he does not need heat cycling in his initial experiment. Subsequent tests conducted in that year focused on short portions of a cloned gene and involved repeated heat cycling. Mullis finds these experiments successful, but he can't persuade other scientists of this. Cetus has its annual meeting in Monterey, California, in June 1984. Presenting their findings and discussing upcoming projects are its scientists and consultants. Mullis displays a poster that details the oligonucleotide synthesis process used in his lab and includes some of the findings from his PCR tests. The only person who expresses interest is Joshua Lederberg, a consultant for Cetus. A disagreement unrelated to PCR causes Mullis and another Cetus researcher to get into a violent brawl later on in the conference. Soon after, the other scientist quits, and Mullis is no longer in charge of the oligo synthesis lab. There might not be much longer for him to work at Cetus. Mullis is under pressure to present

his proposal to the team working on the genetic mutation test in September 1984 by Tom White, the vice president of research at Cetus and a personal acquaintance. Together, they spend the ensuing months devising tests that would provide solid evidence of PCR's effectiveness on genomic DNA. Unfortunately, agarose gel electrophoresis does not show the expected amplification result, which raises questions about the reaction's specificity to the targeted region. When the amplification products are subjected to Southern blotting analysis in November 1984, it is evident that the anticipated 110 bp DNA product is present in increasing amounts. The first discernible signal allows the researchers to start figuring out the reaction's ideal circumstances. The amplified products are subsequently cloned and analysed, demonstrating that only a small portion of the amplified DNA represents the intended target and that erroneous nucleotides are infrequently incorporated during replication by the polymerase that is being utilised.[2]

The results are initially utilised to file patent applications, as is customary in the industry. Mullis drafts an application for the fundamental concept of PCR and numerous other possible uses, and the PTO requests that Mullis include further results. The whole development team, including Mullis, files an application on March 28, 1985, with a greater emphasis on using PCR and OR to analyse the SCA mutation. On July 28, 1987, both patents are approved following revision. The development team starts using PCR on additional targets in the spring of 1985. A variable region of the HLA DQα gene is the target of primers and probes. The predicted PCR result is directly visible on agarose gel electrophoresis, indicating that this reaction is far more specific than the one for the β-hemoglobin target. In order to determine new alleles by PCR, the amplification products from different sources are additionally cloned and sequenced. Simultaneously, the first PCR determination of novel alleles and the original OR assay sequencing occurred. Concurrently, the original OR assay method is substituted with the broader ASO method. The team focuses on using a thermos table DNA polymerase early in 1985 as well (each heating step destroys the enzyme employed in the initial reaction). Out of all the published works, just two—from Taq and Bst—have been described. Because of its greater depth, the Taq polymerase report is selected for testing. The Bst polymerase turns out to be inappropriate for PCR, which was a fortunate choice. Throughout that summer, Mullis makes two unsuccessful attempts to isolate the enzyme and hires a group from outside Cetus to produce it. Randy Saiki discovers that the polymerase, which Susanne Stoffel and David Gelfand at Cetus created in the autumn of 1985, supports the PCR process right away.

The process of reporting PCR to the broader scientific community continues after patents are filed. In April 1985, an abstract is presented for a meeting in Salt Lake City, where Saiki makes the initial announcement of PCR in October. A ‘concept’ article from Mullis and a ‘application’ paper from the full development team are scheduled for publication. Mullis sends his work to the journal Nature, but it is rejected since it does not contain any results. The other manuscript is submitted to Science on September 20, 1985, and is accepted in November. It primarily describes the OR analysis assay. The second publication, which emerges on December 20, 1985, rapidly adds details on the PCR procedure following Mullis’ report’s rejection in December. Mullis presents PCR at the Cold Spring Harbour Symposium in May 1986. A modified version of his initial "idea" manuscript is published by Mullis considerably later. The fact that the first non-Cetus report utilising PCR is turned in on September 5, 1986, shows how swiftly other labs are adopting the method. On September 8, 1986, and November 13, 1986, respectively, the Cetus development group publishes their comprehensive sequence analysis of PCR products and their usage of ASO probes.

At a conference in Berlin on September 20, 1986, Henry Erlich announces the use of Taq polymerase in PCR. The article is submitted for publication in October 1987 and published early the following year. PCR using Taq polymerase is covered by a patent that was submitted on June 17, 1987, and issued on October 23, 1990. In order to develop tools and reagents for PCR, Cetus and Perkin-Elmer create a joint venture in December 1985. Though they are built, Complex Thermal Cyclers are never sold because they cannot conduct the Klenow-based amplifications. A press release announcing the commercial availability of the “PCR-1000 Thermal Cycler” and “AmpliTaq DNA Polymerase” is released on November 19, 1987. Simpler Taq-based PCR equipment are produced. John Sninsky at Cetus starts using PCR in the spring of 1985 to tackle the challenging task of measuring the quantity of HIV circulating in blood. April 11, 1986, saw the announcement of a feasible test, which is published in May 1987. It is now possible to check donated blood for the virus and track the effectiveness of antiviral medications. Another member of the development team, Norm Arnheim, ends his vacation at Cetus in 1985 and starts a full-time job at USC. He starts looking into the possibility of amplifying materials with a single copy of the target sequence using PCR. In 1989, his laboratory began analysing meiotic recombination products directly on single sperm using mutiplex-PCR. Both the genetic typing of preimplanted embryos and the research of ancient DNA depend on these single-copy amplifications, which were initially performed during the characterisation of Taq polymerase. 1986 saw the application of PCR to the investigation of criminal evidence by Edward Blake, a forensics expert employed by Cetus, Bruce Budowle of the FBI, and Cetus researchers. Using the

HLA DQα assay, Saiki performs a blind analysis on a panel of DNA samples coded from historical instances. The proof and those responsible match when the code is breached. Blake used the method practically right away in "Pennsylvania v. Pestinikas," the first criminal case to use PCR. As part of Cetus's "Ampli-Type" kit, this DQα test is created and later used in early forensic evidence testing techniques. The earliest DNA fingerprinting assays were created and used by Alec Jeffreys in 1989. To boost sensitivity, PCR is used. Through additional modification, the widely accepted practice for national DNA databases like CODIS will be the amplification of highly polymorphic VNTR sites. Since PCR may retest previously collected evidence, the guilty go to prison while the innocent are gradually freed. When it comes to amplifying DNA from human hair, Russ Higuchi succeeds in 1987. This research aims to create techniques for amplifying DNA from severely deteriorated samples, including Ancient DNA and forensic evidence. An episode of Star Trek: The Next Generation debuts on January 30, 1989. A virus attacking the ship's doctor's DNA is causing her to age quickly; however, the virus can be stopped when the doctor's DNA is recovered from a hair found in her stateroom. The mainstream media now covers PCR. Taq Polymerase (and PCR) receive their first "Molecule of the Year" award from the journal Science on December 22, 1989. Later on, the "Taq PCR" study emerges as the most cited biological publication for a number of years.

The United States Government chastises Randy Saiki in a scathing letter for publishing a report on "chain reactions" without the necessary previous inspection and approval by the U.S. Department of Energy, following the release of the first PCR article. In response, Cetus explains how PCR and the atomic bomb are not the same. The sale of Cetus to Chiron, a nearby biotechnology company, is announced on July 23, 1991. As part of the sale, Hoffman-La Roche receives USD $300 million for the PCR patents. Many Cetus PCR researchers have moved to Roche Molecular Systems, the company's new subsidiary. On October 13, 1993, Kary Mullis, who had left Cetus in 1986, received the Nobel Prize in Chemistry. On the morning of his victory speech, he is almost imprisoned by Swedish authorities for "inappropriate use of a laser pointer."

1.3 PCR TECHNOLOGY:

PCR Working:

The PCR process consists of three essential steps: denaturation, annealing, and extension. In the first stage, high temperatures are used to denature the DNA. Primers anneal to the DNA template strands in step two, allowing for prime extension. In step three, extension occurs at the end of the annealed primers, resulting in a complementary DNA strand. The third step in the PCR cycle practically doubles the amount of DNA. A DNA segment can be amplified using PCR by heating the material until the DNA denatures and splits into two single-stranded DNA molecules. The enzyme "Taq polymerase" then synthesises - or makes - two new strands of DNA using the original strands as templates. This process culminates in the replication of the original DNA, with each new molecule comprising one old and one new strand of DNA. Then, each of these strands can be The fundamental PCR concept is straightforward. It is a chain reaction, as the name implies: one DNA molecule yields two copies, then four, then eight, and so on. Polymerases, enzymes capable of stringing together individual DNA building pieces to form long molecular strands, carry out this constant doubling process. Polymerases require DNA building blocks, which are nucleotides made up of the four bases adenine (A), thymine (T), cytosine (C), and guanine (G), to function. They also require a tiny segment of DNA known as the primer, to which they connect the building blocks, as well as a longer DNA molecule to serve as a template for making the new strand. If these three elements are provided, the enzymes will create perfect replicas of the templates. PCR is a technique for producing many copies of a certain nucleic acid strand. It is a method of selectively amplifying a specific section of DNA. The segment may be a small fraction of a big and complex mixture of DNAs, such as a specific exon of a human gene. It can function as a molecular photocopier. In around 2 hours, PCR can amplify enough DNA for gel electrophoresis. The template DNA—a cooked bacterial colony—does not need to be well purified. The PCR result can be digested by restriction enzymes, sequenced, or cloned. A single DNA molecule, such as one from a sperm, can be amplified using PCR. The polymerase chain reaction is dependent on the ability of DNA copying enzymes to withstand high temperatures. Because of its simplicity and usefulness, PCR has changed the way practically all research requiring the manipulation of DNA fragments may be conducted.5 In Mullis' initial PCR method, the enzyme was utilised in vitro. Heating the double-stranded DNA to 96°C resulted in two single strands. However, at this temperature, E.Coli DNA polymerase was damaged, necessitating the replacement of the enzyme after each cycle's heating stage. Mullis' original PCR method was inefficient since it required a significant amount of time, a large volume of DNA polymerase, and constant monitoring throughout the PCR process. 6 Make two fresh copies, and so on 7. The annealing phase takes place at a lower temperature, 50-60°C.[16]

This makes it possible for the primers to hybridise to the corresponding complementary template strands, which is an extremely helpful tool in forensic chemistry. The desired number of identical copies of the

original template strands is then produced using the freshly created primer DNA strand that was coupled to the template. The Taq polymerase ends the annealed primers with nucleotides that are accessible. Taq polymerase extends the primers at approximately 72°C for two to five minutes. As one might anticipate, DNA polymerase I is unstable at the high temperatures needed for PCR, therefore it cannot be utilised to extend the primers. When compared to other methods, the PCR cycle and process are incredibly quick, and each cycle doubles the amount of copies of the target DNA strand. Thirty cycles would only take six hours to finish, assuming the maximum amount of time for each stage. Primers repeatedly bind to complementary sites in the freshly synthesised strands as well as the original DNA template during the denaturation, annealing, and polymerase extension processes. This process results in the extension of the primers, which creates new copies of DNA. The sequences between the PCR primers are eventually represented at a theoretical abundance of 2n, where n is the number of cycles, and the total amount of DNA fragments that contain these sequences increases exponentially as a consequence.8, 5, 8 Because the Taq DNA polymerase, a thermostable DNA polymerase, was added once at the start of the PCR reaction. The productivity, specificity, automation, and usefulness of the polymerase chain reaction have all been significantly enhanced by the thermostable characteristics of the DNA polymerase activity, which were isolated from Thermusaquaticus (Taq), a species that grows in geysers that reach temperatures above 110°C. Every time the mixture is cooled to allow the oligonucleotide primers to bind, the catalyst for the extension is already present because the Taq enzyme can tolerate repeated heating to 94°C.10. Samples are typically incubated at 72°C for 5 minutes following the final cycle in order to fill in the protruding ends of freshly synthesised PCR products. Both the reaction mixture preparation and the cycle condition setup need to be done carefully to guarantee success. Since the plateau happens when the reagents are exhausted and accumulate, raising the cycle number above around 35 has minimal beneficial impact. How well the primers are able to identify and bind to sequences other than the intended target DNA sequences determines how specific the amplification will be.

1.4 ESSENTIAL COMPONENT OF PCR:

- Primer
- Enzyme
- Buffers

The essential components of PCR are given below:

• Thermal cyclers (thermocyclers)
• Target DNA (DNA template)
• Two primers(forward and reverse primers)
• Taqpolymerase (themusaquaticus)
• Buffers
• Deoxy nucleotide triphosphates (d NTP's)
• Monovalent \bivalent cation
• Nucleotides (A\T\G\C)
• Water

Buffer: It is composed of magnesium chloride, which provides the necessary cofactor magnesium divalent cations. Magnesium binds to nucleotides so that the polymerase enzyme can recognise it, and it is required at concentrations of 1–5 mM. It assists the enzyme by functioning as a co-factor. At pH 8.3, 50 mMkcl, 1.5–2.5 mMMgcl, and 10 mM Tris is the most widely used buffer.Other PCR buffers that are utilised include DMSO, PEG 6000, and glycerol formamide.Initials: These are forward and reverse primers, with typical lengths ranging from 16 to 30 nucleotides. Primers cause an individual DNA sequence to be amplified and restrict the range of DNA sequences that can be replicated7. Primers are synthetic DNA strands that are no longer than 50 nucleotides and serve as markers for the start and finish of the region that needs to be amplified. The polymerase then assembles the complementary sequence from each primer. A nucleotide in the template causes the enzyme to add a T nucleotide; a G nucleotide in the template causes the enzyme to add a C nucleotide to the primer8. Two factors that are taken into account for a primer are its length and its actual sequence4. The primers are determined by the following factors: primer length; melting point; specificity; complementary primer sequences[7]

1.5 PROCESSES INVOLVED IN PCR:

There are 3 main processes viz.

1. Denaturing \ melting
2. Annealing
3. Elongation

Denaturing \ melting: The process begins with heating the sample to 94–96 °C for 10–20 minutes, causing two complementary strands to separate. This process takes approximately 5 minutes, during which the thermally stable polymerase enzyme is used. The product of all three steps is then visualised using UV transillumination. If bands are visible, they correspond to the target sequence of the original DNA sample; if none are visible, the original DNA sample is absent.

Anneling: The ideal annealing temperature is one that is two degrees Celsius below the melting/denaturing temperature. Primers attach to single DNA strands during this one to two minute process, which is sped up by lowering the temperature. Each strand of DNA is annealed with an oligo-nucleotide primer complementary to either or both of the target sequences when the temperature is dropped to 50–65 C.

Elongation: In this process, Taq polymerase (Thermusaquaticus polymerase) stabilises the temperature at which DNA polymerase fills in the gaps in the strands. During five to fifteen minutes, the polymerase is heated to 72 C. It then begins to fill in the nucleotide gaps in each primer by moving in a 3' to 5' orientation. DNA polymerase can polymerise thousands of bases in a minute. The PCR process produces an amplified result known as an amplicon, which is amplified on a logarthimic scale. In 45 minutes, one cycle takes one to three minutes, and millions of copies are produced during the process.

1.6 THE PCR PROTOCAL:

A total of 14 PCR reactions will be carried out by you. In order to minimise the differences across the treatments (varying annealing temperatures plus controls), prepare a single reaction cocktail and aliquot it into separate PCR tubes in the manner described below:

1. Make the cocktail and swirl it around carefully.
2. To use as your control tubes, transfer 49 ml of the cocktail into two PCR tubes.
3. Fill the negative control tube with 1 ml of nucrease-free water (i.e., no template), and the positive control tube with 1 ml of E. coli DNA (i.e., known positive template). Write the quantity of E. coli DNA you added, expressed in nanograms, in your notebook.
4. After that, you'll mix one E. Coli colony with the cloned ribosomal gene into the leftover cocktail.
5. Select one single colony and incorporate it into your drink using a pipette tip. Make sure you just remove a visible portion of the colony—never any of the agar.
6. Repeatedly vortex the cocktail.
7. Divide the mixture into 12 PCR tubes, aliquoting 50 ml for each[8].
8. Until every team is prepared to put the reactions in the thermocycler, keep your reactions chilled.
9. Put the tube caps on, but don’t seal them until the machine has been used. The tubes could shatter if you force the caps on without any support.
10. Put the machine’s tube strip in. The lower annealing temperature will be on the left, as you can see.
11. Snap the hot top cover closed, being careful not to overtighten it enough to squish the tubes.
12. Select the software that you want to use? In this instance, 16SGRAD is the programme you will be running.
13. Conditions of the thermocycler for 16Saradaga: To ensure that the cells are lysed and the DNA is denatured, heat the mixture to 95 degrees Celsius for three minutes. 30 iterations of the subsequent profile:

Denaturation: 30 seconds at 95°C **Annealing:** 40 to 60°C for one minute (each tube’s precise temperature will be given in the lab). **Extension:** 68°C for three minutes, followed by 68°C for five minutes to ensure that all extensions are finished After that, the machine will maintain the tubes at 4°C till the reaction tubes are taken out.

Due to the thermocycler’s approximate 3-hour run duration, you won’t have time this week to see or prepare your PCR results for sequencing. Your reactions will be taken out of the machine and stored at –20 C by us. The week after next is your oral presentation week, during which you will run gels on your PCR results. The week after that, in Week 6, you will get your templates ready for DNA sequencing. Finalised for next week.

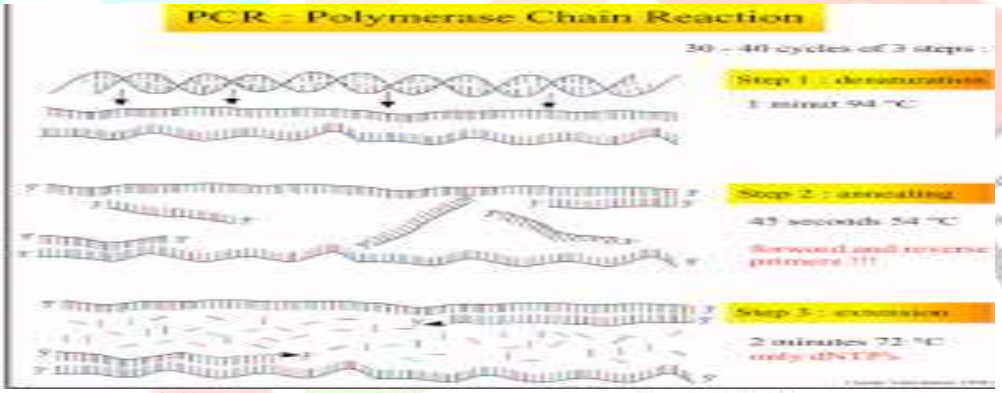
Your PCR products' electrophoresis To see your PCR results and see how the various annealing temperatures affected the PCR process, use agarose gel electrophoresis.

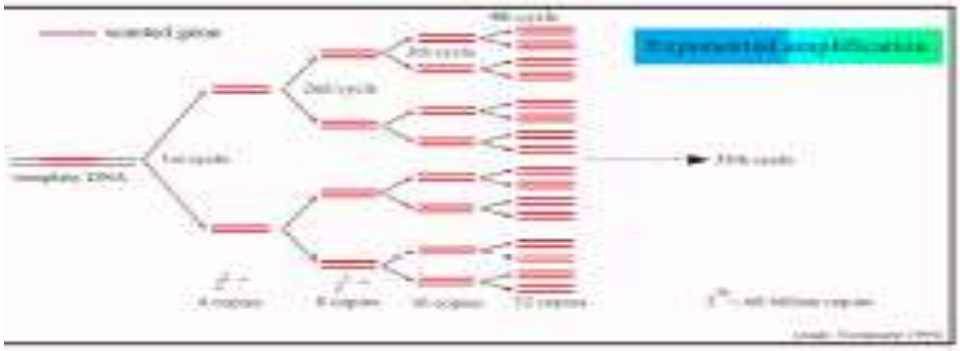
Examine Agarose gel electrophoresis in the DNA fingerprinting exercise for the first week.

1. To ensure that every sample is loaded onto a single gel, pour a gel using two combs.
2. Get your samples ready for the gel to be loaded. Your full reaction cocktail cannot be used in the gel since you will be sequencing your PCR products. Rather, you will use just 10 ul from every reaction for the gel, following the steps 3 through 5 listed below.
3. Cover the top of an Eptube rack with a piece of parfilm. Here, you'll blend your samples together.
4. Apply fourteen 2 ul areas of 10X loading dye onto the parafilm.
5. Gently pipette pump 10 ul of each PCR reaction into a different loading dye spot.
6. Fill the leftmost well in each of the two lanes of wells with five ul of the 1 kb ladder standard.
7. Insert the 14 PCR reactions. Make sure you record the sequence in your lab notebook, along with the location of the control reactions, and maintain the order from the lowest to the maximum annealing temperature.
8. Run the gel, taking care not to run the top of the gel into the lower half of the gel's wells. There will only be a 30-minute maximum run time.
9. Use the UV transilluminator and digital camera to see and take pictures of your gel.

1.7 ANALYSIS OF PCR PRODUCT :

Analysis of PCR results can be done in numerous ways. A. Using a chemical dye such as ethidium bromide to stain the amplified DNA product14. B. Applying fluorophore labels to PCR primers and nucleotides prior to PCR amplification14. In place of ethidium bromide, SYBR Green I may alternatively be utilised (1). Its benefit is that, in addition to being less expensive than a probe, it may be used with multiple pairs of distinct primers. B. Electrophoresis on Ag Agar Gel 0.9% agarose in 40mM tris-base pH-8.3, 20mM acetic acid, and 1mM extracted from 0.9% agarose gels using QIA (quick gel extraction kit) make up an agarose gel. Gel is immersed in a buffer following the gel electrophoresis procedure.[3]





1.8 LIMITATIONS Of PCR :[14,15]

1) Target DNA can be amplified in a reaction tube together with minuscule amounts of DNA from laboratory personnel' skin and even from a tiny particle in the air.

2) Under typical PCR settings, TAQ polymerase is incapable of proofreading.Around once per 20,000 base pairs, it adds an erroneous nucleotide.

3) Although researchers have successfully extended PCR amplification to larger fragments up to 50,000 bp or smaller, the typical size of fragment that can be amplified by ordinary Taq polymerase is less than 2000 bp.[9]

1.9 DIFFERENT TYPES OF PCR:

1. Multiplex PCR
2. Nested seminested PCR
3. Touchdown PCR
4. RT-PCR
5. Hot start PCR
6. Colony PCR
7. Inverse PCR
8. Allele specific PCR
9. Asymmetric PCR
10. Arbitrary PCR
11. Core sample PCR
12. Degenerate PCR
13. Assembly PCR
14. Dial-out PCR
15. Digital PCR
16. Traditional PCR
17. In-silico PCR
18. Inter sequence PCR
19. Ligation-mediated PCR
20. Methylation- specific PCR
21. Miniprimer PCR
22. Nano particle PCR
23. Overlap-extension PCR
24. Quantitative PCR
25. Solid phase PCR
26. Suicide PCR
27. Thermal asymmetric interplaced PCR
28. Semiquantative PCR
29. Conventional PCR
30. After exponentional PCR
31. Standard PCR
32. Qualitative PCR

1.9.1 Multiplex PCR:

The multiplex PCR approach is utilised to identify exonic/intronic sequence5 in particular genes by detecting many pathogens in a single sample. Because primers are designed to bind to a particular DNA sequence, their designs differ. Here, in multiplex PCR, different base pair lengths are required to form distinct bands because multiple DNA genes of varying sizes are targeted in a single reaction to minimise costs and time consumption while simultaneously recognising numerous pathogens11. Infectious agents such as bacteria and viruses can be found using this method17. Primers pairs increase the probability of primer-dimer amplification and DNA laer fragment discrimination10. Besides buffers, Taq polymerase additive is included in multiplex Pcr, which lessens amplicon competition. The perosamine synthetase gene-based Brucella diagnostics is one instance of a multiplex PCR technology used in the medical field. Brucellatargettingbcsp 31, omp 2b, omp2a, and omp 31 are the principal species of genes identified by the application of PCR technique employing two sets of primers B4/B5-JPF/JPR for the diagnosis of active human brucellosis in Egypt. One 19-primer multiplex PCR was used to identify B.neotomae, B.ceti, and B.microti. Multiplex PCRs have been established for the simultaneous detection of M. tuberculosis and Brucella species.

1.9.3 Nested-seminested PCR:

For one locus point, two sets of primers are utilised in this instance. The second set is a complementary sequence that will be shorter than the first set, which is an amplified sequence. The initial augmented product. The purpose of nested PCR is to minimise product contamination caused by primer binding sites that are not predicted to amplify. Its disadvantages include the possibility of contamination and the necessity for extreme caution when doing it. Primers made to anneal at different temperatures and the addition of ultra-pure oil of two mixtures5 can both be used to reduce these contaminations. In order to identify Brucella in human blood samples, nested/seminested PCR was employed. Although this approach is more focused, it has drawbacks such as "primer and dimerization"17 cross reaction. As of yet, no one species has had a cross-reaction with PCR products.

1.9.5 Touchdown PCR:

During the cycling process of touchdown PCR, the annealing temperature is progressively lowered. The primers' Tm is 5 to 10°C higher at the start of the cycling stage than the annealing temperature. Even if the temperature is high, only the most specific base pairings between the primer and template will be favoured, which means that only particular products will be amplified. During the amplification step, the temperature is gradually lowered in consecutive cycles until the annealing temperature is 2 to 5°C below the Tm. At the lower, more permissive annealing temperatures, the specified products—which are already amplified and present in excess—will be amplified preferentially. The increment/decrement time/temperature feature included on all Techne thermal cyclers makes programming easy even though it may initially seem complicated.

1.9.2 Real Time PCR:

This extremely reproducible, quick, sensitive, and targeted data automation method is also known as quantitative PCR (qPCR)5. This approach has been used to create Brucella species that target the 16S23S genea. Less contamination is likely to occur.

It can be detected by using two different methods:

1. SYBER Green dye(or) fluorochromes (or) inter calculating agents
2. Taq man probes (or) fluroprobes. Other forms of probes include

a. Minor groove DNA binder probes

b. Hybridisation probes

c. Sunrise probes

d. Scorpion probes

e. Molecular beacons

f. FRET (fluorescent resonance energy transfers).[10]

Comparing these fluoroprobes to intercalculating agents, they are more costly and sophisticated. Each cycle's rise in DNA corresponds both propotional to the hybridization of probes and propotional to the increase in fluorescence emitted. With the exception of molecular beacons, all probes have the same performance attributes. Real-time PCR offers a number of benefits, including:

1. Input target DNA quality control by quantitative means.
2. Quick turnaround times thanks to quick and effective real-time PCR on 96- or 384-well plates.
3. Removing the post-amplification stages.
4. High reproducibility is achieved by using standardised PCR procedures with consistent amplification specifications.
5. A high degree of reliability is contributed by the availability of reliable and quality-controlled PCR reagents.

1.9.4 Hotstart PCR:

For the polymerase chain reaction (PCR) amplification of nucleic acid templates, HotStart PCR SuperMix offers suitable chemicals. The mixture includes recombinant Taq DNA polymerase, Mg++, dNTPs, and anti-Taq DNA polymerase antibody at amounts appropriate for PCR amplification. In order to make use of about 50% of the total reaction volume for the addition of primer and template solutions, HotStar PCR SuperMix is supplied at a 2X concentration. There are enough reagents for 100 50 µl amplifcation procedures. The anti-Taq DNA polymerase antibody prevents polymerase activity, enabling ambient temperature setup and an automatic hot start. The productivity and specificity of PCR are enhanced by antibody-mediated hot starts. The HotStar PCR SuperMix is present in an inactive form due to specific antibody binding, and it is reactivated following a denaturation phase in PCR cycling at 94°C. The recommended storage temperatures for HotStar PCR SuperMix are -20°C and 4°C. Thawing the mix before constructing the PCR is not necessary when the mix is stored at 4°C. Frequent freeze-thaw cycles may lower activity or performance.[11]

1.9.6 Colony PCR:

This colony PCR is intended to assess the success of the cloning process by detecting the presence of the insert and determining its size:

1. Write the names of the eight testable colonies on the bottom of each petri dish. White people ought to colonise these places. The β-galactosidase enzyme, which converts X-gal in the media into a blue substance, is produced by blue colonies. White colonies are unable to manufacture this enzyme, but these colonies can. Because we have put DNA directly into the middle of the lacZ gene, β-galactosidase synthesis is stopped in while colonies. This process, known as "blue/white screening," dictates that blue colonies should not include an insert whereas white colonies have.
2. To transfer colonies into a fresh petri dish, mark the bottom with eight squares.
3. Fill 0.2 ml strip tubes with 15 µl of water for every colony. Put the colony number on them. For every colony, prepare 5 ml of LB broth cultures and add 10 ul of ampicillin (25 mg/ml).
4. Using a 20 µl pipet tip, gently contact a colony and dip the tip a few times into the 15 µl of water. Afterward, streak the new medium onto the corresponding square and place it in the LB broth. For every colony, repeat.
5. In the PCR machine, heat the strip tubes for five minutes at 95°C. After that, immediately place tubes on ice.
6. Reagents for PCR should be defrosted. Complete the PCR sheet by adding 1 µl of colony DNA to each reaction and 10 µl of total volume. Make use of primers M13F and M13R, whichh amplify across the insert by binding to the plasmid DNA on each side of it. This will assist in figuring out the insert's size.
7. Using all of the reagents but the DNA, prepare a master mix in accordance with the calculations above. After adding the DNA produced in step 4 (1 µl each), dispense the master mix onto strip tubes (9 µl each). Put tubes in the PCR machine after labelling them.[4]

Equipment Necessary

1. 20 & 10 µL micropipette
2. PCR Thermocycler

Materials Necessary:[13]

1. Plates from cloning
2. LB Broth
3. PCR reagents
4. 8-strip or single 0.2 ml PCR tubes
5. Sterilized DI H2O
6. 10 and 20 µL pipette tips
7. M13 F and M13 R primers

2. APPLICATIONS OF PCR:

•Classification of organisms

•Detection of pathogens

•DNA fingerprinting

•Drug discovery

•Genetic matching

•Genetic engineering

•Pre-natal diagnosis

•Genotyping

•Molecular archaeology

•Mutagenesis

•Mutation detection

•Sequencing

•Cancer research

✓ **Applied Research:**

•Pre-natal diagnosis

•DNA

•Genetic matching

•Detection of pathogens fingerprinting

•Gene therapy

✓ **Basic Research:**

•Drug discovery

•Classification of organisms

•Genotyping

•Molecular Archaeology

•Molecular Epidemiology

•Molecular Ecology

•Bioinformatics

•Genomic cloning

•Site-directed mutagenesis

•Gene expression studies

- ✓ **Sequencing:**
  - •Bioinformatics
  - •Genomic cloning
  - •Human Genome Project

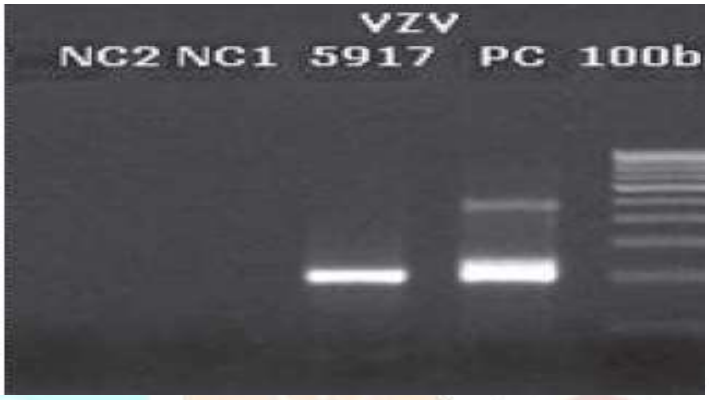
- ✓ **Genetic Engeneering:**
  - •Bioinformatics
  - •Genomic cloning
  - •Human Genome Project

- ✓ **Molecular Identification :**
  - •Molecular Archaeology
  - •Molecular Epidemiology
  - •Molecular Ecology
  - •DNA fingerprinting
  - •Classification of organisms
  - •Genotyping
  - •Pre-natal diagnosis
  - •Mutation screening
  - •Drug discovery
  - •Genetic matching
  - •Detection of pathogens

2.1 CURRENT SCENARIO OF PCR APPLICATION:

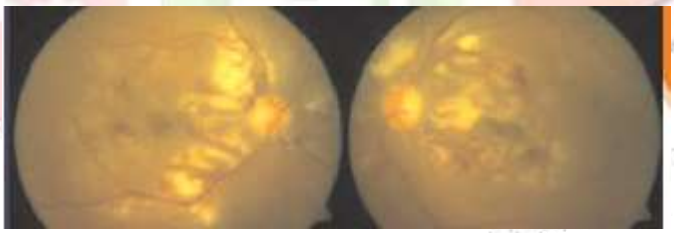
Case Scenario 1:

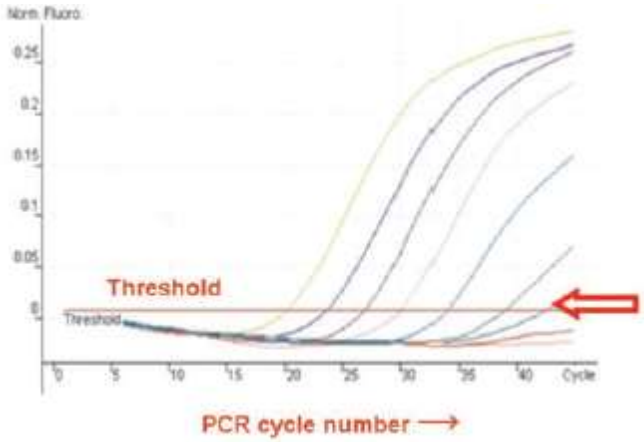
A 28-year-old male patient appeared with abrupt visual loss. Upon assessment, his greatest corrected visual acuity in the right and left eyes was 6/6, N6 and 6/36, N18, respectively. Upon slit-lamp examination, the right eye's results were within normal limits, whereas the left eye displayed cells in the anterior vitreous. On fundus inspection, the right eye's condition was within normal bounds. A hyperaemic disc, scattered necrotizing retinitis, and inferior haemorrhages were observed on a fundus examination of the left eye. A PCR study was conducted on his aqueous aspirate. gel of agarose Electrophoretogram displaying the outcomes of a nested PCR that targeted the Varicella Zoster Virus's ORF 63 gene [Lane 1: Negative control] Negative Control in Lane Two 1 Lane 3 AC tap with Lab #5917 affirmative (product amplified by 102 bp) Positive (102 bp amplified product) - Lane 4 Positive control (Oka vaccination strain DNA) 100 bp ladder molecular weight marker in Lane 5.[4]



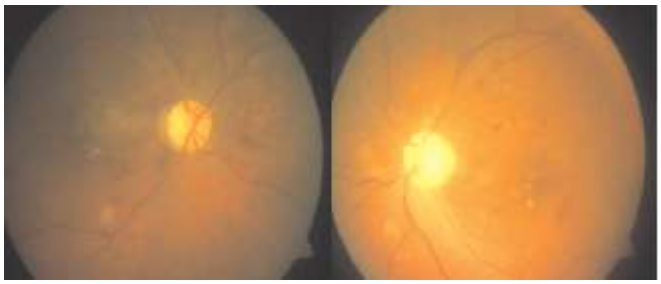
2.3 Case Scenario 2:

A 48-year-old man arrived complaining of gradually declining vision in both eyes during the previous 15 days. He informed us about the history of chikungunya fever 15 days prior to the commencement of vision issues. He had received a diagnosis of neuroretinitis from another consultation. His right eye's best corrected visual acuity was 2/60,





Nine copies of the viral RNA were found by RT-PCR for the chikungunya virus. The identified Chikungunya virus load is indicated by the red arrow, which indicates its copy number.



Fundus photo of the same patient following two months of oral steroid-based antiviral therapy. In his right eye, his greatest corrected visual acuity was 6/18,N18, and in his left eye, it was 6/24,N18.

3. ADVANTAGES OF PCR:

1. The capacity to track the real-time progress of the PCR reaction
2. The capacity to measure ampticon of each cycle processively, allowing for extremely precise quantification of sample beginning material amount.
3. An expanded dynamic range of identification.
4. Amplification and detection take place beyond PCK manipulation in a single tube.
5. No particular product could be seen in the reaction carried out without a hot start.
6. A manual hot start with unaltered DNA polymerase produced observable results.
7. A stronger product band and a decrease in nonspecific products were obtained using PCR using modified DNA polymerase.[12]

4. CONCLUSION:

Numerous significant discoveries in science have resulted from the development of PCR and real-time PCR. While both techniques are still often employed in lab settings, real-time PCR is gaining ground and is swiftly overtaking other approaches as the most economical and efficient way to analyse DNA results. Real-time PCR is becoming more and more common in several clinical laboratory disciplines, such as microbiology, virology, and genetics. The numerous applications for which this technology is employed are far more numerous than those that have been covered in this course. The development and promise of real-time PCR technology is just getting started, as more platforms and procedures are being established for it. The idea and procedure will remain the same, though, therefore it's critical that laboratory workers comprehend and become knowledgeable about this technology.

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