Assoc. Prof. Dr. Nihat TELLİ

PRINCIPLES of POLYMERASE CHAIN REACTION, TECHNOLOGY and APPLICATIONS in THE FOOD SCIENCE

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PREFACE

With the unravelling of the genetic structure of living organisms, studies carried out to determine the location of genes in this structure, to understand their functions and to determine their relationships continue at a great pace. In accordance with this purpose, it has become necessary to develop methods that will facilitate and accelerate the achievement of the determined goal. In line with the developments; biotechnological methods have been very useful and have started to fill and eliminate the gaps of the techniques used for many years.

Nowadays, it is possible to detect the DNA sections investigated by using biotechnological methods from samples and to reproduce them under *in vitro* conditions. In addition, the diagnosis, typing, species determination, determination of resistance to antimicrobial drugs and identification of the sources of microorganism-specific DNA sections by epidemiological studies have become applicable. One of the most important of these methods developed is Polymerase Chain Reaction or PCR. Today, PCR has taken its place as an important method among biotechnological methods in the field of food microbiology due to its effective sensitivity and frequently used for the reported purposes.

This book contains basic information about PCR technology and its applications in the field of food technology. I hope that the book will be useful to all readers. I would like to thank everyone who contributed to the writing and preparation of the book.

May 2024

Assoc. Prof. Dr Nihat TELLİ

1. HISTORY OF POLYMERASE CHAIN REACTION

The discovery and development of PCR has often been compared to the internet revolution in terms of its transformative impact on science and society. Although this similarity carries the risk of exaggerating its impact outside the scientific environment, the comparison is favourable in understanding the subject in several ways. Firstly, the extent of the impact of both technologies on daily life is such that it is difficult to imagine life without them. Similarly, these technologies have gone beyond their initial simple design structures and created a wide range of opportunities, the limits of which are difficult to imagine today. In addition to shaping a new scientific jargon in the fields that their technologies address, the literacy of this language has also become important over time. Considering the history of science, it is hard to believe that the PCR technique, which forms the basis of the human genome project and molecular biology laboratory protocols, was discovered relatively recently (Bartlett and Stirling 2003).

As with the discovery of DNA structure in the 1950s, the discovery of PCR is the subject of claims that have yet to be fully elucidated. PCR as we know it today has its origins in research carried out at Cetus in California in the early 1980s. Kary Mullis states that the idea for PCR began in 1983 while travelling on the San Francisco-Mendocino highway. This idea is recognised as the origin of the modern PCR technique, which is now used worldwide and forms the basis of important PCR patents. For his contributions to the development of PCR, Kary Mullis was awarded the 1993 Nobel Prize in Chemistry (Bartlett and Stirling 2003, Broll 2010, Williams 2010).

The original concept of PCR, like many groundbreaking ideas, was a fusion of various pre-existing components and techniques were already established practices within the toolkit of molecular biologists at the time. The novelty of Kary Mullis' concept, however, was to use the juxtaposition of two oligonucleotides complementary to opposite strands of DNA to specifically

amplify the region between them, and to achieve this in a single method. Thus, the product of polymerase activity in one cycle would be added to the template pool for the next cycle, and a chain reaction would ensue (Bartlett and Stirling 2003).

Although the discovery of PCR and its widespread reputation as a highly significant invention is generally attributed to Kary Mullis, the undeniable efforts of scientists in Henry Erlich's laboratory are at the heart of its now recognised successful applications. For example, the isolation of a thermostable polymerase enzyme from a thermophilic bacterium isolated from thermal sources was critical to the application of the technique. Therefore, it would be more accurate to say that PCR was fully developed by Henry A. Erlich, Kary Mullis and Randall K. Saiki working for Cetus in the United States of America (Bartlett and Stirling 2003).

Over time, some arguments have arisen suggesting that PCR was the focus of prior scientific studies and that its technique isn't entirely novel. In the objections to Hoffman La Roche's PCR patents, it was claimed that there was at least one *prior art* idea, that is, the original invention of PCR was known before Kary Mullis' work in the mid-1980s. However, it is known that in the late 1960s Khorana et al. used a method described as *repair replication*. The similarities between the repair replication method and PCR are (1) binding and extension of primers to templates, (2) separation of the newly synthesised strand from the template, and (3) re-binding of the primer and repeating the cycle. Regardless of the ongoing debates, it is clear that much of the work that made PCR such a widely used methodology originated from the laboratory work of Mullis and Erlich (Bartlett and Stirling 2003, Broll 2010).

The most important feature of PCR is that it allows working even with a small fragment of the DNA under investigation. With the PCR method, it is possible to amplify millions from a target DNA fragment. The method is based on the enzymatic synthesis of the region restricted by these two primers by

using a pair of synthetic oligonucleotide primers with an average length of 18-25 bases, which are specific to the two ends of the region to be amplified and complementary to the base sequences in this region (Bartlett and Stirling 2003).

The DNA polymerase originally used for PCR was extracted from the bacterium *Escherichia coli*. Although this enzyme was a valuable tool for a wide range of applications and initially revolutionised DNA sequencing technologies, it also had significant disadvantages. In PCR, the double-stranded DNA product must be heated to denature after each round of synthesis. This rendered the DNA polymerase from *E. coli* irreversibly ineffective. Therefore, there were technical difficulties such as manual addition of fresh enzyme aliquots at the beginning of each cycle. A stable DNA polymerase was needed for the DNA denaturation step, which was carried out at about 95 °C (Bartlett and Stirling 2003).

DNA polymerase enzyme obtained from *Thermophilus aquaticus* bacteria isolated from hot water sources was started to be applied as a solution to this problem as it does not inactivate at high temperatures. Subsequently, Gelfand and colleagues purified and then amplified this polymerase, enabling a complete PCR amplification without opening the reaction tube. Furthermore, since the enzyme was isolated from a thermophilic microorganism, it worked optimally at a temperature of about 72 °C, allowing the DNA synthesis step to be performed at higher temperatures than possible with the *E. coli* enzyme. This made it possible to replicate the DNA strand with higher accuracy and to eliminate the non-specific products that had hampered previous attempts at PCR amplification (Bartlett and Stirling 2003).

Despite the aforementioned developments, the PCR technique was still not an easy and fast method. In particular, it required manual transfer between water baths at different temperatures. The development of the first thermocycle machine (Mr Cycle), which replicates the temperature changes required for the PCR reaction without the need for manual transfer, was another important

development. Following the purification of Taq polymerase, Cetus and Perkin-Elmer introduced the closed DNA thermal cyclers, which have since become widely adopted in laboratories worldwide. With the diversification of its application areas over time, PCR became a frequently used method in scientific studies in the 20 years following its discovery, as shown in Fig. 1. Today, it remains foundational to a wide array of scientific research activities (Bartlett and Stirling 2003).

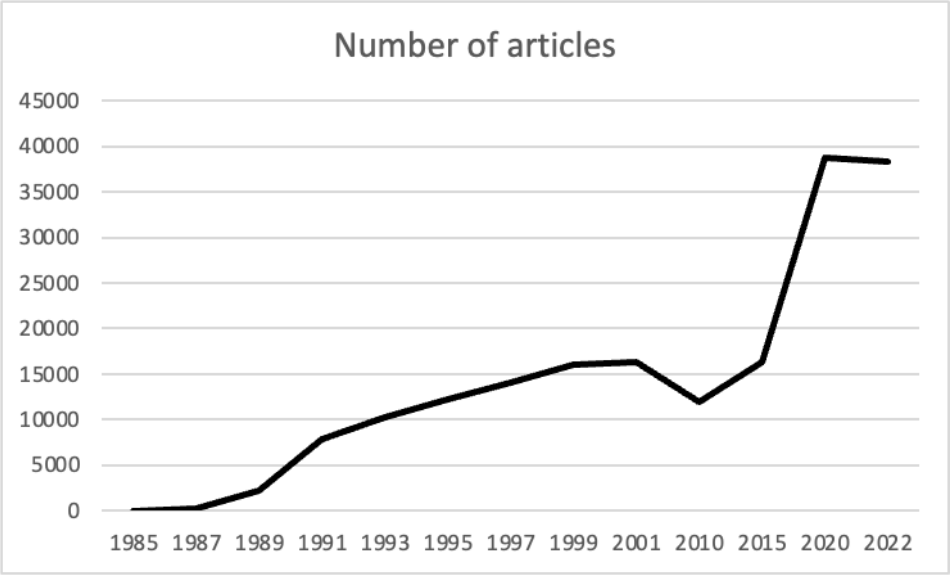


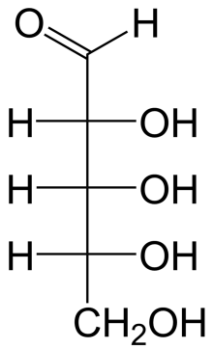
Figure 1. PubMed search results for articles with the term PCR

2. NUCLEIC ACIDS

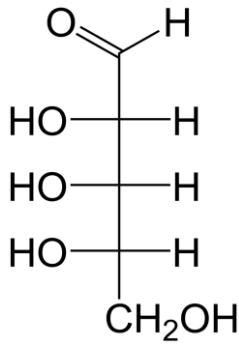
Nucleic acids are molecules responsible for the storage and expression of genetic information. There are two different nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), with different chemical structures. Nucleic acids are formed by the combination of many structural units such as sugars, purine and pyrimidine bases, nucleosides, nucleotides and polynucleotides in an orderly manner (Yeşilkaya et al. 2002, Neidle and Sanderson 2008).

2.1. Sugars

In both RNA and DNA, pentoses, in other words sugars, constitute the basic structures of nucleic acids. RNA contains ribose (Fig. 2) and DNA contains 2-deoxy ribose sugars. The atoms of sugar units in nucleic acids are labelled with an exponent such as $1'$, $2'$,...$5'$ in order not to be confused with the atoms of nitrogenous bases. The hydroxyl group attached to the carbon atom of nucleic acid sugars $3'$ reacts with the phosphate group attached to the carbon atom of the following nucleic acid sugar $5'$ to form an ester bond. Phosphodiester bonds between sugar units form the linear backbone of nucleic acids (Champe and Harvey 1998, Yeşilkaya et al. 2002, Neidle and Sanderson 2008).



D-Ribose



L-Ribose

Figure 2. Ribose molecular structure

Ribose is a simple sugar and carbohydrate with the molecular formula $C_5H_{10}O_5$ and the linear form composition H-(C=O)-$(CHOH)_4$-H. Its chemical name (3*R*,4*S*,5*R*)-5-(Hydroxymethyl) tetrahydrofuran-2,3,4-triol. It is a monosaccharide found extensively in nature. The ribose molecule is a pentose containing five carbon atoms. The naturally occurring form of D-ribose is a component of ribonucleotides from which RNA is formed, and hence this compound is essential for the encoding, decoding, regulation and expression of genes. As a result of phosphorylation, it can become the subunit of ATP, NADH and many other metabolic compounds (Neidle and Sanderson 2008). Deoxyribose (Fig. 3), also known as D-deoxyribose and 2-deoxyribose, is a member of the aldopentoses, which contain five carbon atoms and harbour an aldehyde group.

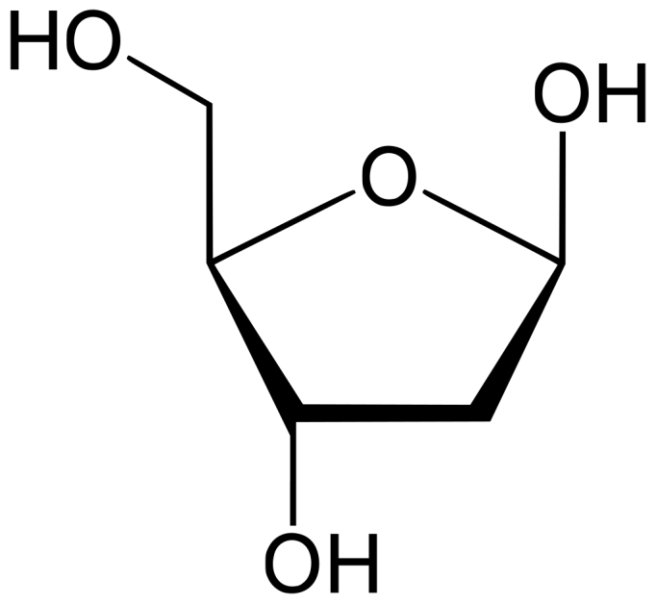


Figure 3. Deoxyribose molecular structure

Deoxyribose is formed by the replacement of the hydroxyl group at position 2 of the pentone ribose sugar with hydrogen, i.e. this results in the loss of one oxygen atom. The replacement of the hydroxyl group also changes the ring structure from C3'-endo to C2'-endo. This molecule was discovered by Phoebus Levene in 1929. It is an important building block of DNA nucleic acid. Chemical name *2R,4S,5R*)-5-(Hydroxymethyl) tetrahydrofuran-2,4-diol with the molecular formula $C_5H_{10}O_4$ (Neidle and Sanderson 2008).

2.2. Purine and Pyrimidine Bases

Chemical degradation studies performed on materials extracted from cell nuclei have revealed that high molecular weight nucleic acid is actually composed of acid units called nucleotides. Four distinct types were isolated; guanylic, adenylic, cytidylic and thymidylic acids (Neidle and Sanderson 2008). Nitrogenous bases are also called purines and pyrimidines. Purines arise from the fusion of two ring structures, one consisting of six atoms and the other of five atoms. There are four purine bases (adenine, guanine, hypoxanthine, xanthine).

-Adenine (6-amino purine)

Adenine (Fig. 4) is a molecule which is one of the two purine bases. It is found in the nucleotides of DNA and RNA nucleic acids. Adenine stabilises the structure of the nucleic acid in which it is present by hydrogen bonding to thymine in DNA and to uracil in RNA. Adenine forms adenosine, a nucleoside, by binding to ribose, and it forms deoxyadenosine by binding to deoxyribose. When three phosphate groups bind to adenosine, adenosine triphosphate (ATP) is formed. ATP is used in cell metabolism as one of the basic molecules that provide energy transport between reactions. Its chemical formula is $C_5H_5N_5$ (Neidle and Sanderson 2008).

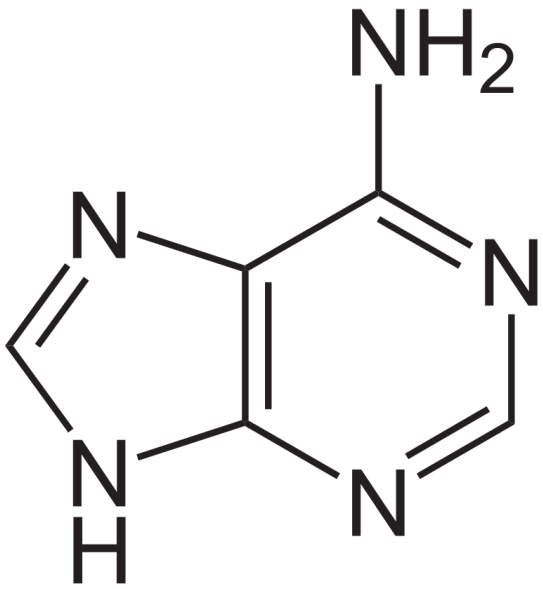


Figure 4. Adenine molecular structure

-Guanine (2-amino-6-oxy purine)

Guanine (Fig. 5), a purine derivative with the formula $C_5H_5N_5O$, forms a triple hydrogen bond with cytosine in Watson-Crick base pairing. It contains a pyrimidine-imidiazole ring system paired with double bonds and its unsaturated bi-cyclic state is planar. The guanine nucleotide is called guanosine. Although thymine is usually observed in DNA and uracil only in RNA, guanine, together with adenine and cytosine, is found in both RNA and DNA. Guanine has two forms, the essential keto form and the rare endo form (Neidle and Sanderson 2008).

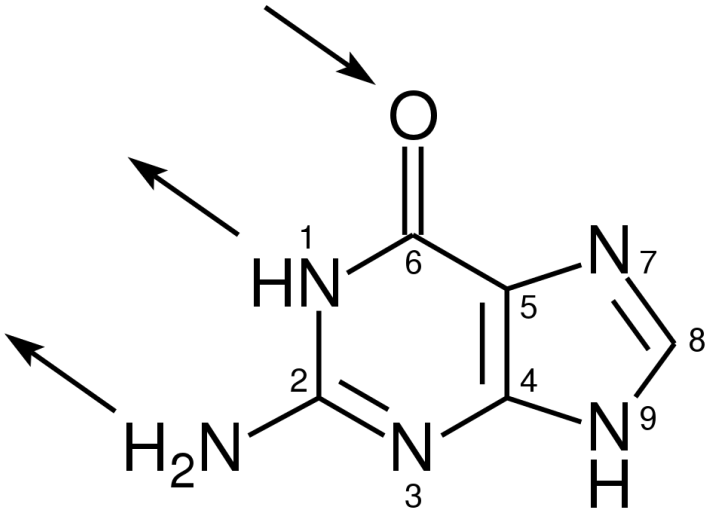


Figure 5. Guanine molecular structure

-Hypoxanthine (6-oxy purine) and xanthine (2,6-dioxy purine)

Two of the many bases that are mutagenic. Both are deamination products. Hypoxanthine is formed from adenine and xanthine from guanine. Adenine and guanine are present in the structure of both DNA and RNA. Hypoxanthine and xanthine do not participate in the structure of nucleic acids yet they serve as vital intermediaries in both the synthesis and degradation of purine nucleotides (Lehninger et al. 2000, Yesilkaya et al. 2002).

Pyrimidine bases consist of a single ring with only six members. Pyrimidine bases are uracil, thymine, cytosine and orotic acid (Lehninger et al. 2000).

-Uracil (2,4-dioxy pyrimidine)

Although uracil, a non-canonical base (Fig. 6), occurs naturally in RNA, it may also be present in small amounts in DNA. The uracil present in DNA can arise as a result of spontaneous deamination of cytosine (leading to U:G

mispairing) or from the incorporation of dUMP during replication instead of dTMP leading to U:A pairing. Uracil is one of the four bases in the structure of RNA. Its International Union of Pure and Applied Chemistry (IUPAC) nomenclature is Pyrimidine-2,4(1H,3H)-dione and its chemical formula is $C_4H_4N_2O_2$ (Olinski et al. 2010).

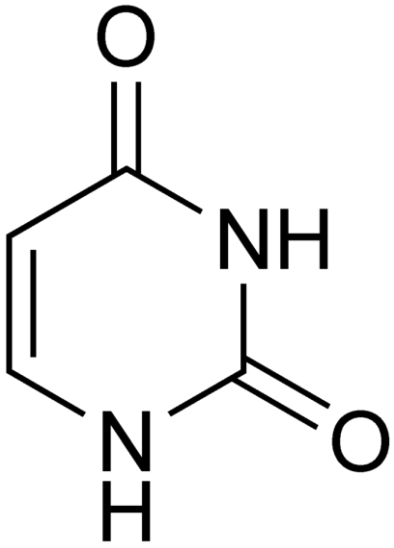


Figure 6. Uracil molecular structure

-Thymine (2,4-dioxy-5-methyl pyrimidine)

Thymine, whose chemical formula is $C_5H_6N_2O_2$ (Fig. 7), is a molecule that is one of the bases of nucleic acids in DNA. It has two weak hydrogen bonds with adenine and can form a base pair. When combined with deoxyribose, it forms thymidine. Thymidine combines with one, two or three phosphoric acid groups to form thymidine monophosphate, thymidine diphosphate and thymidine triphosphate respectively. In RNA, uracil is present instead of thymine. Its IUPAC nomenclature is 5-Methylpyrimidine-2,4(*1H*,*3H*)-dione with a molecular mass of 126.11 g mol^{-1} and a boiling point of 335 ºC (Lehninger et al. 2000).

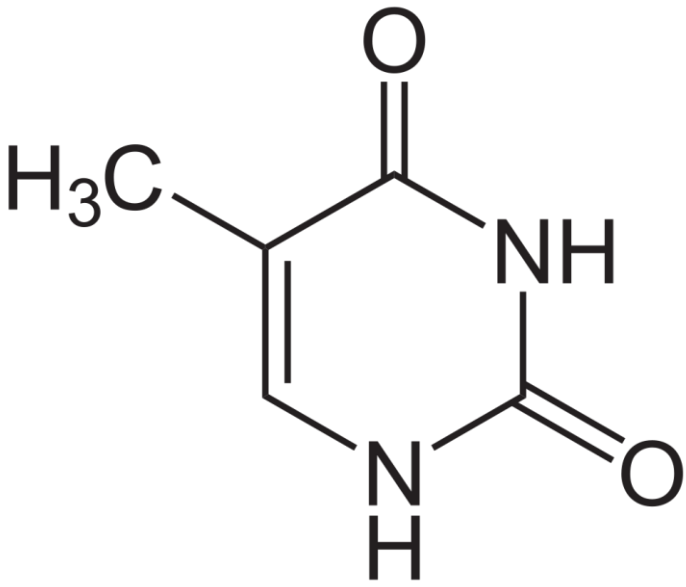


Figure 7. Thymine molecular structure

-Cytosine (2-oxy-4-amino pyrimidine)

Cytosine (Fig. 8) is a molecule that, together with guanine, adenine and thymine (uracil in RNA), is one of the main nitrogenous bases in DNA and RNA. Its chemical formula is $C_4H_5N_3O$. It is a pyrimidine derivative consisting of a heterocyclic aromatic ring and two substituents. The nucleoside of cytosine is cytidine, which forms a base pair with guanine linked by three hydrogen bonds according to Watson-Crick base pairing (Neidle and Sanderson 2008).

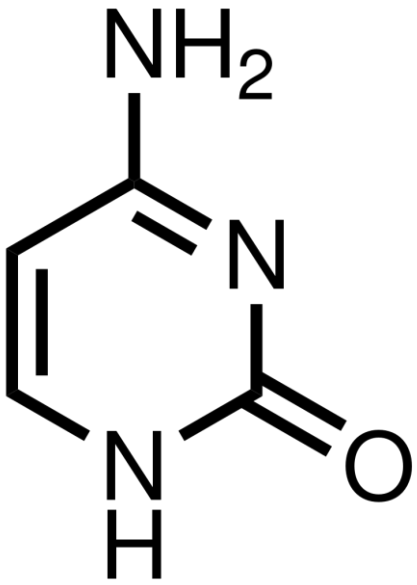


Figure 8. Cytosine molecular structure

-Orotic acid (2,4-dioxy-6-carboxy pyrimidine)

Orotic acid (Fig. 9) is a precursor of pyrimidine nucleotides involved in many biochemical reactions. It serves as a substrate for RNA polymerase and UDP sugars and carbohydrate-containing macromolecules (e.g., glycogen, glycoproteins and glycolipids) (Cihak and Reutter 2012). Orotic acid is a pyrimidinedione and a carboxylic acid. At the time of its discovery, it was thought to be a part of the vitamin B complex and was called vitamin B13. The compound is synthesised in the body via a mitochondrial enzyme, dihydroorotate dehydrogenase, or a cytoplasmic enzyme of the pyrimidine synthesis pathway. It is sometimes used in some dietary supplements to increase bioavailability. Cytosine is found in both DNA and RNA, uracil only in RNA. Thymine is normally found in DNA but is sometimes incorporated into tRNA, as is uracil. Orotic acid does not participate in the nucleotide structure but is the first co-product formed in the synthesis of pyrimidines (Lehninger et al. 2000, Yesilkaya et al. 2002).

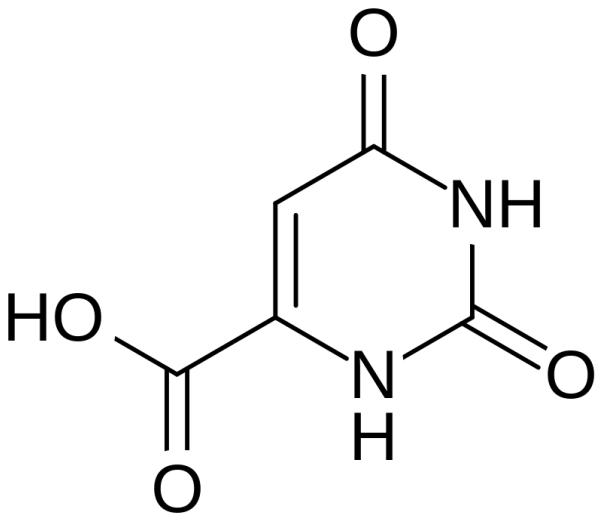


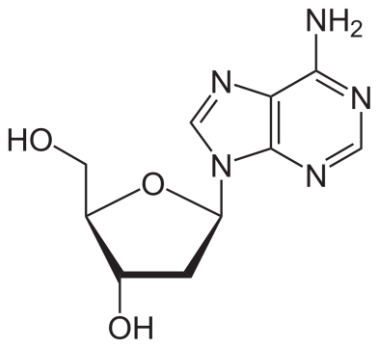
Figure 9. Orotic acid molecular structure

2.3. Nucleosides

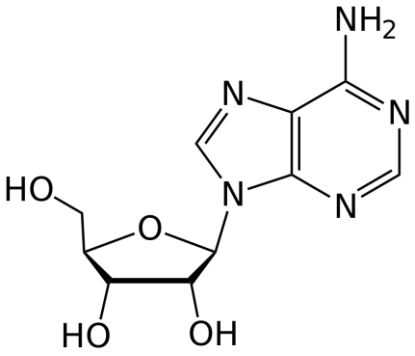
If one of the sugars ribose or 2-deoxy ribose is added to a purine or pyrimidine, compounds called nucleosides are formed. In nucleosides, the first carbon atom of the sugar is bond with the ninth nitrogen atom of purine bases and the first nitrogen atom of pyrimidine bases by N-glycosidic bond. The resulting nucleosides are named by adding the suffix -osine to the purine bases in the case of purines and -idine to the pyrimidine bases in the case of pyrimidines. Unless otherwise stated, nucleoside sugars are assumed to be ribose sugars. The fact that the sugar is a 2-deoxy ribose sugar is indicated by the d- sign before the nucleoside name (Mathews and van Holde 1996). Fig. 10 shows the chemical structure of adenosine and deoxyadenosine (Wikipedia 2024).

A nucleoside consists simply of a nucleobase (also called a nitrogenous base) and a five-carbon sugar (ribose or 2'-deoxyribose), whereas a nucleotide

consists of a nucleobase, a five-carbon sugar, and one or more phosphate groups. In a nucleoside, the anomeric carbon is attached to N9 of a purine or N1 of a pyrimidine via a glycosidic bond (Mathews and van Holde 1996).



Deoxyadenosine



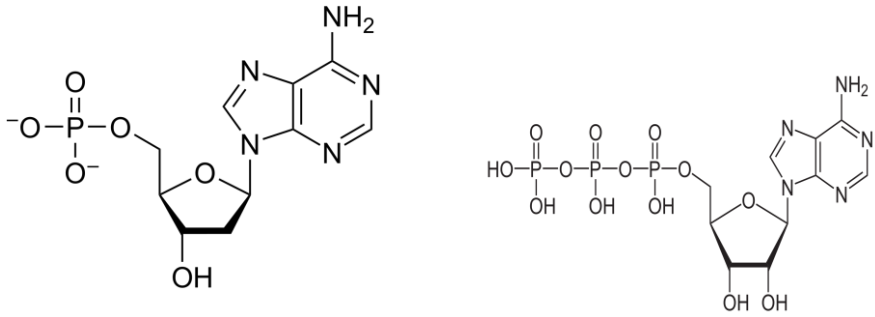
Adenosine

Figure 10. Chemical structure of deoxyadenosine and adenosine

2.4. Nucleotides

Nucleotides are formed by the addition of one or more phosphate groups to the ribose or deoxyribose sugars of nucleosides. They serve as monomeric units of nucleic acid polymers such as DNA and RNA, which are essential biomolecules in all life forms. They play a central role in metabolism at the cellular level. Nucleotides are organic molecules consisting of a nitrogenous base, a pentose sugar and a phosphate. Usually the phosphate group is attached to the 5' carbons of the sugar by an ester bond. If there is more than one phosphate group, they are connected to each other by a high-energy acid anhydride bond. In this case, there is no need to write the carbon atom number in front of the nucleotide name to express the location of the phosphate group. However, in case of bonding in a position different from 5' carbon atoms, the position of the binding point is indicated before the nucleotide name. For

example, for cyclic adenosine mono-phosphate 3' - 5', the phosphate group is attached to the nucleoside sugar at carbon atoms 3' and 5' (Minniti et al. 1998, Yesilkaya et al. 2002, Neidle and Sanderson 2008). Fig. 11 shows the basic chemical structure of nucleotides and adenosine triphosphate.



Nucleotide Adenosine triphosphate

Figure 11. Chemical structure of nucleotide and adenosine triphosphate

2.5. Polynucleotides

Nucleotides polymerize in a 3'-5' direction, forming polynucleotides through phosphodiester bonds. Polymerisation of ribonucleotides leads to RNA formation and polymerisation of deoxy ribonucleotides leads to DNA formation (Briani et al. 2007, Neidle and Sanderson 2008).

2.5.1. Ribonucleic acid (RNA)

Ribonucleic acid (RNA) is formed by the polymerisation of purine and pyrimidine ribonucleotides by 3'-5' phosphodiester bonds. Although it is similar to DNA, it has some important differences. The sugar unit to which purine and pyrimidine bases and phosphate groups are attached is ribose instead of 2-deoxyribose in DNA. While adenine, guanine and cytosine are present in ribonucleotides, thymine is absent except in some cases. Thymine is replaced by

uracil. RNA is organised as a single helix, DNA as a double helix. However, if a suitable complementary sequence is found in front of it, it can be twisted in the form of a hairpin and mimic the structure of a double helix chain (Chen et al. 1999, Neidle and Sanderson 2008).

The information in RNA depends on the sequence of purine and pyrimidine bases in a single chain. In the cells of all prokaryotic and eukaryotic organisms, RNAs are grouped into three main classes: messenger RNA (mRNA), transporter RNA (tRNA) and ribosomal RNA (rRNA). Each class is distinguished from each other by size, functional and general durability (Chen et al. 1999). Fig. 12 shows the chemical structure of RNA (Wikipedia 2024).

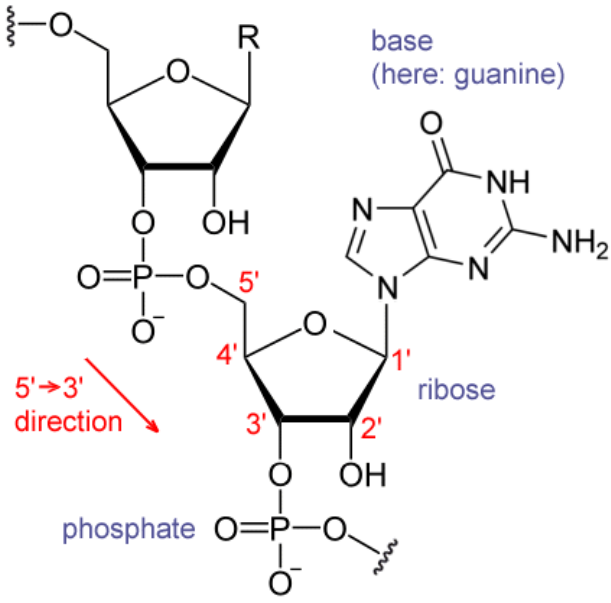


Figure 12. Chemical structure of ribonucleic acid

Each nucleotide in RNA contains a ribose sugar and the carbons are numbered 1', 2', 3', 4' and 5'. A base, usually adenine, cytosine, guanine or uracil, is attached to the 1' position. There is a phosphate group between two

riboses. The phosphate group is attached to the 3' position of one ribose and the 5' position of the other ribose (Lee and Gutell 2004, Neidle and Sanderson 2008).

RNA synthesis is a reaction catalysed by the enzyme RNA polymerase, usually using DNA as a template. The binding of the enzyme to the site on DNA to be written into RNA constitutes the initial step of synthesis. The helicase activity of RNA polymerase causes the DNA double helix to open. The enzyme proceeds in the 3'-5' direction in the DNA template. It then synthesises the RNA chain with a complementary sequence in the 5'-3' direction. The stage at which RNA synthesis is terminated is determined by a specific sequence on the DNA. Unlike DNA-dependent RNA synthesis as described, enzymes with RNA-dependent RNA polymerase activity use an RNA strand (e.g., some RNA viruses) as a template for RNA synthesis. However, it is also known that most RNA-dependent RNA polymerases are involved in the RNA interference pathway (Hansen et al. 1997, Ahlquist 2002, Nudler and Gottesman 2002, Neidle and Sanderson 2008).

2.5.2. Deoxyribonucleic acid (DNA)

The structure of the DNA molecule (Fig. 13), which constitutes the genome of living organisms, was described by James Watson and Francis Crick in 1953. DNA molecule is a polymeric nucleic acid macromolecule consisting of a five-carbon sugar, phosphate group and nitrogen-rich purine and pyrimidine bases (Varshavsky 2006, Neidle and Sanderson 2008).

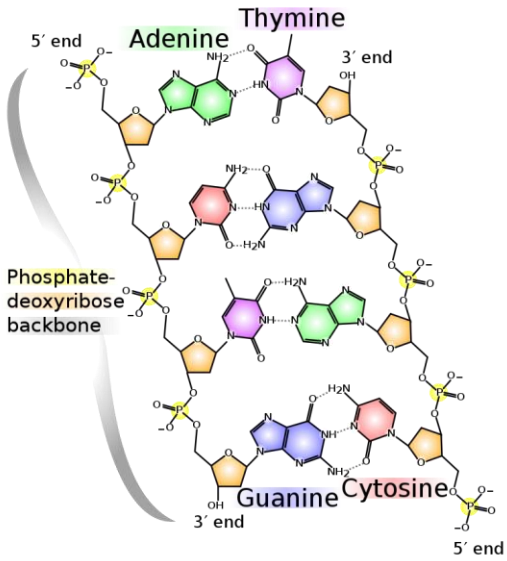
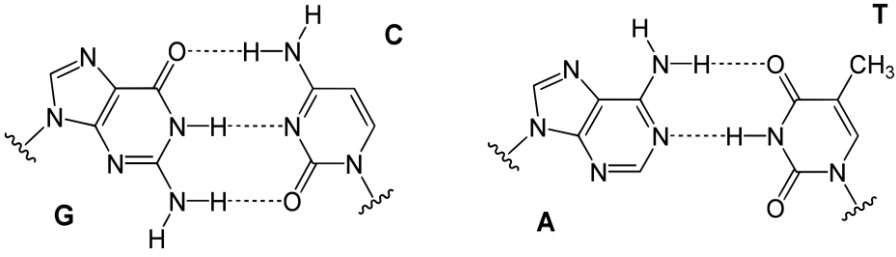


Figure 13. Chemical structure of deoxyribonucleic acid

DNA molecules have a double helix structure formed by two chains consisting of a sugar phosphate backbone from the 5' end to the 3' end, wrapped anti-parallel to each other around each other's axis. This structure is called "double helix" structure. The adenine nucleotide in one DNA chain forms a base pair with the thymine nucleotide in the other chain and the guanine nucleotide forms a base pair with the cytosine nucleotide in the other chain. Base pairs are held together by hydrogen bonds (Fig. 14) (Varshavsky 2006, Neidle and Sanderson 2008).

The polynucleotide chains that make up DNA form helices that rotate in either the right or left screw direction. The geometry of the sugar-phosphate skeleton is more favourable to the former. Normal DNA is therefore orientated in the right-helix direction. This form of DNA is called B-DNA. In B-DNA, the distance between the overlapping base pairs of the double helix is 0.34 nm. The step corresponding to a complete turn of the helix is 3.4 nm. In B-DNA, there

are 10 bases in each turn. In this form of DNA, the outer part of the helix has one large and one small indentation in a regular order. Thus, the molecules required for DNA to function can bind to these places. A-DNA, which also has a right-helix structure, is a slightly more compact molecule than B-DNA. The strand is 2.3 nm long and contains 11 bases. DNA can be found in this form in some environments. Z-DNA is a helix organised in the left screw direction. Here, the bases appear to zigzag in succession, making angles in opposite directions. It is not yet known whether this form is found in nature. In this form, the helices form one deep indentation instead of two (Wu et al. 1981, Yesilkaya et al. 2002, Neidle and Sanderson 2008).



A GC base pair with three hydrogen bonds

An AT base pair with two hydrogen bonds

Figure 14. Hydrogen bonds between base pairs

The flow of genetic information takes place as DNA → RNA → Protein. This flow is known as the basic mechanism of life (central dogma) and the same mechanism applies to all living organisms except retroviruses (Banaszynski and Wandless 2006, Neidle and Sanderson 2008).

Genetic information is hidden in the sequence of bases along the DNA chain. This is called the "genetic code". Three consecutive nucleotides in the DNA chain form a code and this code determines the amino acid sequences in the protein. Since there are four nucleotides in the structure of DNA and three of them next to each other form a code, 64 different codons form the genetic code according to the 3-of-4 combination (4^3). With only 20 amino acids contributing to protein structure and 64 possible codons, multiple codons can encode for the same amino acid, leading to many proteins being determined by more than one codon. This is called "code degeneracy". Three of these 64 codons (UGA, UAA, UAG) do not code for any amino acid. These codons are defined as stop codons. The transfer of the information in DNA to RNA is called transcription and the conversion of this information into amino acid sequence is called translation. With transcription, the information on the DNA chain is transferred to the mRNA molecule. mRNAs undergo processing in the nucleus to mature into spliced mRNA. Mature mRNAs are transferred from the nucleus to the cytoplasm and translate the information they carry into proteins in ribosomes. Ribosomes are composed of a special RNA known as rRNA and many structural proteins that can integrate with it. The molecule that establishes the molecular relationship between the base sequence encoded in mRNA and the amino acid sequence of the protein is the tRNA molecule. The anti-codon region on the tRNA forms a base pair with the codon on the mRNA, and the codon-specific amino acid brought by the tRNA is added to the carboxyl end of the polypeptide chain synthesized by peptide bond. The reading of codons on the mRNA continues until a stop codon is encountered, at which point protein synthesis is terminated. The synthesised protein undergoes post-translational modifications (e.g., addition of side groups, truncation and removal of some regions, packaging into a three-dimensional structure) and becomes a functional protein and fulfils its function in the cell (Rodnina et al. 2002, Weitzke and Ortoleva 2003, Lavrov et al. 2005, Yusupova et al. 2006).

3. HISTORICAL DEVELOPMENT OF MOLECULAR BIOTECHNOLOGY

Molecular biotechnology is a discipline in modern biology in which molecular methods are transferred to technological developments. The emergence of the science of the era was undoubtedly made possible by the relentless struggle, honourable sacrifice and groundbreaking contributions of many scientists. A brief history of these developments is given in Table 1 (Glick et al. 2022).

Table 1. Historical Development of Molecular Biotechnology

Year	Developments
1917	Karl Ereky coined the term biotechnology.
1940	A. Jost used the term genetic engineering.
1943	Penicillin was produced on an industrial scale.
1944	Avery, Macleod and McCarty showed that DNA is a genetic material.
1953	Watson and Crick determined the structure of DNA.
1961	Biotechnology and Bioengineering journal was founded.
1961-66	The entire genetic code has been decoded.
1970	The first restriction endonuclease was isolated.
1972	Khorana et al. synthesised an entire tRNA gene.
1973	Boyer and Cohen founded recombinant DNA technology.
1975	Kohler and Milstein described the production of monoclonal antibodies.

1976	The first guidelines for the conduct of recombinant DNA research were published. Techniques for DNA sequence determination were developed.
1978	Genentech has produced human insulin via *E. coli*.
1980	The US Supreme Court has ruled that genetically modified microorganisms can be patented.
1981	The first commercial and automated DNA synthesisers are sold. First monoclonal antibody-based diagnostic kit approved for use in the USA.
1982	The first animal vaccine produced with recombinant DNA methodologies has been approved for use in Europe.
1983	Genetically modified plasmids were used for the transformation of plants.
1988	PCR method was published. A patent was granted for a genetically modified mouse susceptible to cancer.
1990	The Genome Project is officially launched. Human somatic cell gene therapy was approved in the USA. Recombinant chymosin was used in cheese making.
1994	Genetic and physical maps of human chromosomes were published. The FDA announced that genetically modified tomatoes are safe.
1995	The first genome sequence of *Haemophilus influenzae* has been completed.
1996	Annual sales of the first recombinant protein erythropoietin exceed $1 billion. The complete DNA sequence of *Saccharomyces cerevisiae* is determined.
1996	Commercial cultivation of genetically modified crops has begun.
1997	Cloning of a mammal (sheep) with a differentiated cell nucleus was performed.
1998	The FDA has approved the first antisense drug.
1999	The FDA approved a recombinant fusion protein (diphtheria toxin-interleukin-2) for cutaneous T-cell lymphoma.
2000	Arabidopsis genome sequenced. Annual sales of monoclonal antibodies exceeded $2 billion. The development of Provitamin-A producing rice was announced.
2001	The human genome has been sequenced.

2002	Full human gene microarrays (gene chips) were released as commercial products.
2002	The FDA has approved the first nucleic acid test system to screen donor whole blood for HIV and HCV.
2004	The Sargasso Sea metagenome has been sequenced on a large scale.
2005	NCBI announced that 100 gigabases of nucleotides were found in the GenBank sequence database.
2006	A recombinant cancer vaccine was introduced to protect against cervical cancer.
2008	Two billion acres of genetically modified crops have been planted.
2009	The FDA has approved the first drug produced in a genetically modified animal (goat).
2009	The first clinical trial using embryonic stem cells was performed.
2010	The first synthetic cell was created.
2012	Doudna and Charpentier demonstrated targeted cleavage of DNA with the CRISPR-Cas system.
2013	The US Supreme Court has ruled that isolated genes are not eligible for patenting.
2014	Patents were granted for CRISPR-Cas systems and methods for altering the expression of gene products.
2015	The FDA approved the first transgenic animal (salmon) for human consumption.
2016	The National Bioengineered Food Disclosure Act, passed by the US Congress, mandates the labelling of genetically modified foods.
2017	The FDA has approved *in vivo* gene therapy for the treatment of an inherited form of retinal dystrophy.
2018	The first RNA intervention therapy (to treat hereditary transthyretin amyloidosis) was approved in the USA and Europe.
2018	In China, the issue of the birth of genome-edited babies has begun to be taken seriously.

2019	Genetically modified crops have started to be grown on 190 million hectares in 29 countries.
2019	The safety and efficacy of CRISPR-Cas genome editing to treat β-thalassaemia and sickle cell anaemia are beginning to be tested at the clinical level.
2019	The Philippines became the first Asian country to receive safety approval for genetically modified Golden Rice.
2020	Results from the first CRISPR clinical trial suggested that the technique was safe.
2020	First report of genome-edited mitochondria published.
2020	It has been suggested that the COVID-19 pandemic has stimulated the development of innovative molecular diagnostic tests, treatments and vaccines.
2021	A trial version of genetically modified mosquitoes was created in the USA.

Along with the above-mentioned developments, the advances observed over time in the application areas of molecular-based diagnostic methods have led to the application of these techniques in many fields.

3.1. Classification of Molecular Based Diagnostic Methods

Molecular-based diagnostic methods can typically be classified into two categories: classical and amplification-based diagnostic methods (Aldemir and Uçan 2001).

3.1.1. Classical methods

Classical methods encompass liquid phase, solid phase, and in situ hybridization techniques.

3.1.1.1. Liquid phase hybridisation

The term molecular hybridisation refers to the combination of two anti-parallel helices resulting from the weak hydrogen bonding of the four basic bases on DNA. In liquid phase hybridisation, both the target nucleic acid and

the probes are in the liquid phase and hybridisation takes place in the liquid phase. Liquid phase hybridization generates more stable hybrids and has a lower error rate compared to solid phase hybridization. This form of hybridization is employed in hybrid capture and branch DNA techniques (Sambrook et al. 1989, Stoler and Michael 1995, Liu et al. 2004).

3.1.1.2. Solid phase hybridisation

In solid phase hybridisations, the target nucleic acids are first fixed to a support layer to form a solid phase and hybridisation takes place in the solid phase (Sambrook et al. 1989, Stoler and Michael 1995, Viljoen et al. 2005).

3.1.1.3. In situ hybridisation

In situ hybridisation is a technique for the detection and localisation of specific DNA and RNA sequences in cells, preserved tissue sections or whole tissue by hybridising the complementary strand of a nucleotide probe to a specific sequence. Hybridised hybrid cells can be visualised by autoradiography for radiolabelled probes or by the development of a histochemical chromogen for non-isotopically labelled probes (Jensen 2014).

In situ hybridisation is important in determining how the distribution of specific nucleic acids is related to the protein products of the target gene. Moreover, one of its significant advantages is that it enables the determination of relationships with cellular structures through immunohistochemistry. In situ hybridisation technique data have provided important scientific contributions in many different research areas such as viral infections, gene mapping, cytogenetics, gene expression, prenatal diagnosis and development (Fig. 15) (Jensen 2014).

The basic steps of this hybridisation method for the determination of nucleic acids in tissues, organs and cells are as follows;

- Processing of tissues (fixation and cutting)

- Inhibition of non-specific binding
- Hybridisation
- Washing and imaging.

For tissue fixation, 4% paraformaldehyde in phosphate buffered saline (PBS) may be preferred. Carrier nucleic acids can be used with heated parafilm to prevent non-specific binding with probes and to provide a clean background. In hybridisation, RNA-RNA, RNA-DNA, DNA-DNA hybrids are obtained using labelled probes. In the final stage, depending on the type of labelling, enzymatic or radioactive imaging is used. While alkaline phosphatase and peroxidase enzymes are commonly utilized in enzymatic labeling, radioactive labeling with ^{32}P is also frequently employed (Sambrook et al. 1989, Stoler and Michael 1995).

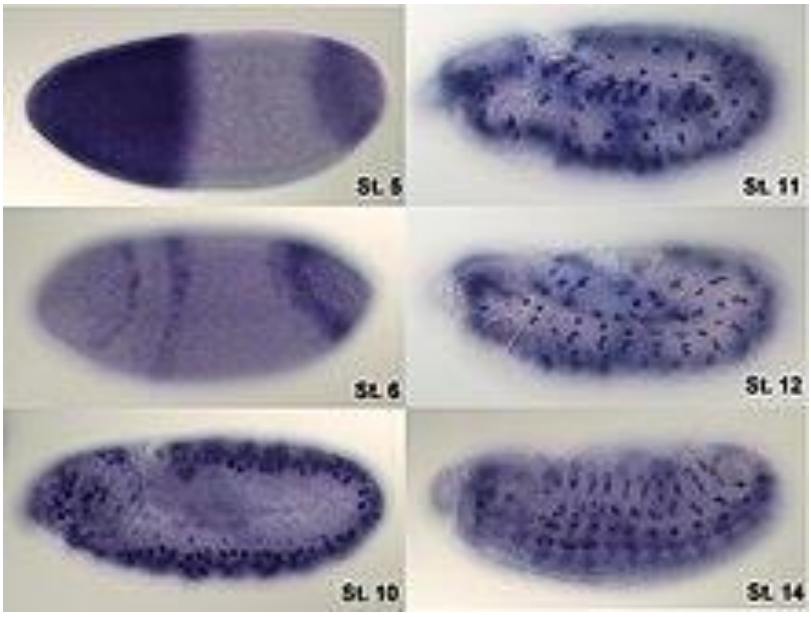


Figure 15. In situ hybridisation of *Drosophila* embryos for RNA derived from the hunchback gene

3.1.2. Amplification techniques

Amplification techniques can be broadly classified as follows.

3.1.2.1. Probe replication techniques

It is possible to classify probe replication techniques as QB Replicase method and Ligase Chain Reaction. The QB Replicase Method harnesses the remarkable speed and efficiency of the QB replicase enzyme to rapidly amplify a chain of 200 nucleotides to extremely high quantities in just 13 minutes. This innovative technique revolutionizes nucleotide amplification, offering unparalleled speed and scalability for molecular biology applications. Ligase Chain Reaction is based on the binding and replication of two probes hybridised side by side on a single strand of DNA by DNA ligase enzyme (Aldemir and Uçan 2001).

3.1.2.2. Signal amplification techniques

The QB Replicase Method capitalizes on augmenting the quantity of markers through the strategic binding of numerous probes to various regions of the nucleic acid.

3.1.2.3. Target nucleic acid amplification techniques

These techniques can be classified as Self-Sustaining Sequence Replication (3SR), Standard Displacement Amplification (SDA) and Polymerase Chain Reaction (PCR). 3SR technique is a technique developed based on the transcription-based replication system. SDA, conducted under isothermal conditions and employing specific primers, DNA polymerase, and endonuclease, achieves substantial amplification of nucleic acids within a concise two-hour timeframe (Aldemir and Uçan 2001).

4. POLYMERASE CHAIN REACTION

Developed in 1985 by Henry A. Erlich, Kary Mullis and Randall K. Saiki of Cetus in the United States of America, the method is based on the reproduction of nucleic acids under appropriate *in vitro* conditions. PCR defined as a kind of *in vitro* cloning (Taylor 1991, Bartlett and Stirling 2003, Birben 2006, Amanda Fairchild et al. 2006).

4.1. Uses of Polymerase Chain Reaction

PCR method can be used in many different fields due to its advantages such as easy applicability and fast results. These areas can be summarised as follows;

- Sequence analysis and mapping of DNA
- Assignment of unknown sequences
- Research in the human genome project
- Diagnosis of genetic diseases
- Prenatal diagnosis
- Oncological research
- DNA fingerprint analyses
- Genetic typing of forensic samples
- Determination of tissue type for tissue transplantation
- Determination of seed purity in agriculture
- Diagnosis of various species in nature in systematic and evolutionary studies
- Cloning experiments
- Mutagenesis studies
- Fossil DNA studies

- Comparison of gene expression
- Diagnosis of microorganisms whose isolation, identification and culture are very difficult or impossible
- Detection of toxins of toxin-forming agents that are difficult to detect
- Identification of bacteria resistant to antimicrobial drugs
- Determination of subtypes of microorganisms
- Diagnosis of microorganisms in foods
- In epidemiological research
- Performing genetic tests in *in vitro* fertilisation
- Investigation of DNA-protein interactions (footprinting) (Birben 2006, Valones et al. 2009).

4.2. Basic Components of Polymerase Chain Reaction

Optimization of the PCR reaction can be complex due to different parameters. These parameters are;

(1) Quality of DNA template

(2) Concentration of DNA template

(3) Primer design

(4) Primer concentration

(5) Magnesium concentration;

(6) dNTPs concentration;

(7) Quality of PCR buffer systems

(6) DNA polymerase availability and concentration;

(7) PCR thermal cycling protocol

(8) Quality and concentrations of other additives

(9) Correct selection of the technique to be applied (Grunenwald 2003).

There are five main ingredients required for a PCR cycle: target DNA, a pair of synthetic primers flanking the region to be amplified from right and left, deoxy-nucleotide-triphosphates (dNTPs), high temperature resistant DNA polymerase enzyme, buffer solution providing appropriate pH conditions (Grunenwald 2003, Ceyhan 2005, Hernandez-Rodriguez and Ramirez 2012, Hedman et al. 2013).

4.2.1. Extraction of DNA

Target DNA refers to the DNA region to be amplified or the target DNA containing this region. DNA or RNA obtained from any source (e.g. blood, serum, semen, body fluids, tissue fluids, tissue fragments, water, meat, organs) can be used for PCR (Tekelioğlu 1999). One of the most important features of PCR is that the target DNA to be amplified does not need to be of high quality both qualitatively and quantitatively. DNA separated from each other by exposing the samples to high temperature can be used directly without any purification. If RNA is to be used as the target strand, RNA is converted to complementary DNA (cDNA) using reverse transcriptase enzyme before the reaction starts. cDNA serves as a template for PCR and the reaction is continued with the addition of Taq polymerase enzyme to the medium as in DNA (Kimmel and Berger 1987, Vosberg 1989, Erlich et al. 1991, Bartlett 2003, Pearson and Stirling 2003).

DNA extraction is carried out in laboratories specially designed for extraction procedures, taking precautions against contamination. Today, phenol extraction-ethanol precipitation, Snounou, Wataya, Chelex, sodium phosphate and freeze-thaw methods are commonly used (Lawyer et al. 1989, Coote 1990). The most important point to be considered in this process is that the DNA to be

amplified should be functional. Therefore, care should be taken not to fragment the DNA during the preparation phase. In addition, it should be ensured that DNA polymerase inhibitors (e.g. EDTA) are not present in the environment during the extraction of DNA. In PCR processes, a very small amount of sample is required. For example, genomic DNA from a single semen cell may be sufficient for PCR. Generally, 0.1-1 µg of genomic DNA is used for 50 µL reaction solution (Tekelioğlu 1999, Bartlett 2003, Birben 2006).

4.2.2. Primer design

Primers are auxiliary oligonucleotides used to amplify the target DNA by PCR. Primers are small DNA sequences consisting of an average of 18-25 oligonucleotides that initiate DNA synthesis to replicate the target DNA. They can be shorter or longer under special conditions. Primers, which are single-stranded, short DNA molecules, have a complementary structure with the end of a certain part of the DNA matrix (Broll 2010).

Primers, which do not consist of repeated sequences and can be easily prepared synthetically, vary according to their intended use. Accordingly, the base sequences on the primers should be exclusive to a single region on the target DNA, avoiding any presence in other sequences of the target DNA. The presence of 50-60% G+C bases in the structure of the primers helps to establish stronger bonds with the target DNA. In addition, such associations reduce nonspecific binding that may occur during high-temperature replication. Another important consideration in primer selection is the sequence and number of primers. The 3' ends of the primers should not be complementary to prevent primer-dimer formation. In cases where primer-dimer is formed, primers detract from the reaction activity. This causes an undesirable polymerase reaction that competes with the targeted PCR product. It is necessary to avoid three G or C nucleotides near the 3' end of the primer because they may increase the synthesis of undesirable products by causing non-specific primer annealing. In order to protect the primer-dimer, the 3' end must be designed with

complementary components. (Saiki et al. 1988, Triglia et al. 1988, Saiki et al. 1989, Broll 2010). Gene region primers specific to some foodborne pathogens are given below as examples.

Table 2. Certain primers for common foodborne bacterial pathogens

Bacterial pathogen	**Primer (F/R)**	**Primer sequence (5' to 3')**
B. cereus	*cerAB*	GAGTTAGAGAACGGTATTTATGCTGC GCATCCCAAGTCGCTGTATGTCCAG
C. jejuni	16S *rRNA*	AATCTAATGGCTTAACCATTA GTAACTAGTTTAGTATTCCGG
C. perfringens	*pls*	CTCATCTCCCATAACTGCACTATAATTTCC AAGTTACCTTTGCTGCATAATCCC
Shiga toxin producing E. coli	*stx-2*	TGTTTATGGCGGTTTTATTTG ATTATTAAACTGCACTTCAG
E. coli	*uspA*	CCGATACGCTGCCAATCAGT ACGCCAGACCGTAGGCCAGAT
EHEC O157:H7	*stx 1*	CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG
L. monocytogenes	*hlyA*	CTAATCAAGACAATAAAATC GTTAGTTCTACATCACCTGA
Salmonella	*oriC*	TTATTAGGATCGCGCCAGGC AAAGAATAACCGTTGTTCAC
Shigella	*rfc*	ATCAGGTGTCGTAATTTTA GGGCTAAGTTCCCTC
S. aureus	*sec*	CTCAAGAACTAGACATAAAAGCTAGG TCAAAATCGGATTAACATTATCC
V. vulnificus	*vvhA*	GACTATCGCATCAACAACCG AGGTAGCGAGTATTACTGCC

V. cholerae	*hlyA*	TGCGTTAAACACGAAGCGAT AAGTCTTACATTGTGCTTGGGTCA
Y. enterocolitica	*yadA*	TGTTCTCATCTCCATATGCATT TTCTTTCTTTAATTGCGCGACA

Primer concentration is recommended as 0.1 to 0.5 mM. Higher concentrations lead to the formation of non-specific bands called primer-dimers. Primer dimers are small DNA products and are bands formed when primers bind to each other or when the DNA polymerase enzyme binds non-specific nucleotides to the ends of unused primers. The points to be considered when designing primers can be listed as follows;

- The optimal length of primers typically ranges from 18-22 nucleotides for prokaryotes and 24-28 nucleotides for eukaryotes. However, it's important to note that specific techniques may necessitate deviations from these guidelines. For instance, in the Random Amplified Polymorphic DNA (RAPD) technique, primer length is recommended to be 8-10 nucleotides.
- The specificity of the primers should be determined in various ways. Long primers increase specificity. Non-specific binding can be prevented by increasing the binding temperature.
- A-T and G-C ratios should be approximately 50%-50% or similar to the target DNA.
- Primers must be synthesised and written continuously from the 5' end to the 3' end.
- The concentrations of the primers in the PCR reaction should be between 0.1 and 0.5 mM (Broll 2010).

Primers are also included in some PCR kits. Apart from this, primers can be synthesised in the desired sequence by certain companies or they can be

synthesised in laboratories with a DNA synthesiser. Primers can be stored at -20 ºC under appropriate conditions until they are used. The storage period of primers is at least one year in liquid and 24 months after lyophilisation (Aldemir and Uçan 2001, Broll 2010).

4.2.3. Deoxynucleotide triphosphate (dNTPs) mixture

Deoxynucleotide triphosphates, all of which are called dNTPs, consist of deoxyadenine triphosphate (dATP), deoxythymine triphosphate (dTTP), deoxyguanine triphosphate (dGTP) and deoxycytosine triphosphate (dCTP) (Fig. 16) (van Pelt-Verkuil et al. 2008).

Figure 16. Commercial dNTPs set

Primers and dNTPs provide the raw material required in the test medium for amplification of the target DNA. The concentrations of the stock dNTP solutions must be determined spectrophotometrically and the pH values must

also be neutralised. After these procedures, the solutions are diluted to obtain working solutions. Working solutions should be stored at -20°C when not actively in use. It is recommended that the working stock solution should contain 1 mM of each dNTP. During PCR cycles, dNTP stability should be at 50% of the total dNTP after approximately 50 cycles. Each dNTP should be utilized at an equal concentration to mitigate the risk of obtaining inaccurate results (van Pelt-Verkuil et al. 2008).

4.2.4. DNA polymerase enzymes

DNA polymerase enzymes use nucleotides in the medium to synthesise a copy of the target DNA strand to be copied in the 5'→3' direction. In the first PCR applications with the Klenow method, the temperature-sensitive polymerase enzyme of *E. coli* was used. However, since the DNA polymerase enzyme obtained from *E. coli* was not resistant to temperature, it was necessary to add fresh enzyme at each step of the reaction. This negatively affected the practicality of the process. This problem was eliminated by obtaining DNA polymerase enzyme from *Thermus aquaticus*, which lives in hot water sources and is very resistant to heat. This enzyme, called Taq polymerase, maintains its structure at temperatures up to 95 ºC. Taq polymerase enzyme can remain active at the lowest and highest temperatures applied during a PCR cycle (denaturation, hybridisation, polymerisation) (Innis et al. 1988, Lawyer et al. 1989, Erlich et al. 1991, Rakshit 2010).

The optimum operating temperature of Taq polymerase is 72 ºC. It is recognized that the PCR cycle exhibits reduced activity during the denaturation stage temperature, and the extension rate, typically 30-65 base pairs per second at 70-80 ºC, markedly declines below 55 ºC and above 85 ºC. In addition, the half-life is below 5 minutes at 100 ºC and 40 minutes at 95 ºC. Taq polymerase has an exonuclease activity during polymerisation. Moreover, the selectivity and sensitivity of PCR are enhanced at the temperatures at which this enzyme operates, as the likelihood of primers binding to non-target sites is substantially

diminished. The recommended amount of DNA polymerase (Fig. 17) is 1.0-2.5 IU / 100 µl. It has been reported that if the optimum amount is exceeded, non-specific products may be formed, and if it is low, synthesis of the desired product may be insufficient (Innis et al. 1988, Lawyer et al. 1989, Erlich et al. 1991, Rakshit 2010).

Figure 17. Commercial DNA polymerase

In recent years, thermostable polymerase enzymes isolated and purified from different bacterial species have been tested. These include Vent polymerase isolated from *Thermococcus literalis*, Tth polymerase from *Thermus thermophilus*, Pfu polymerase from *Pyrococcus furinsus* and Tma polymerase from *Thermotoga maritima*. The exonuclease activity of Vent polymerase enzyme 3'-5' has the ability to increase the specificity and it shows a better activity than Taq polymerase in the presence of excess dNTPs and MgCl ions. In addition, the half-life of Vent polymerase enzyme is longer (Rakshit 2010).

Tth polymerase is also very specific and has DNA-bound DNA polymerase activity as well as RNA-bound DNA polymerase activity (reverse transcriptase). In terms of these properties, Tth polymerase is superior to Taq polymerase. It is known that the DNA synthesis capacity of Pfu polymerase enzyme is 12 times higher than Taq polymerase and the probability of error is lower. However, its activity at high temperatures is lower than Taq polymerase (Innis et al. 1988, Lawyer et al. 1989, Erlich et al. 1991, Rakshit 2010).

4.2.5. Reaction solution and magnesium concentration

The pH of the medium and the presence of divalent cations are crucial factors for polymerase enzyme activity. The concentration of $MgCl_2$ is usually between 0.5-5.0 mM and the optimum concentration can be determined experimentally. Mg^{+2} ions have functions such as forming a soluble complex with dNTPs, stimulating polymerase activity, providing binding between primer and DNA and stabilising the replication complex formed by DNA polymerase by combining with the target-primer complex. Since theoretically nucleotides bind to magnesium in a 1/1 ratio, it is recommended to use a concentration of Mg^{+2} twice the total nucleotide concentration in the test. Low concentrations of Mg^2+ result in either no or minimal product formation, whereas high concentrations lead to the production of non-specific products (Birben 2006, van Pelt-Verkuil et al. 2008).

The recommended reaction buffer solution for PCR is 10-50 mM tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.01% NP_4O and 0.01% Tween 20. KCl facilitates primer binding, but excessive amounts inhibit the Taq polymerase enzyme. Non-ionic detergents such as Tween 20, gelatin or bovine serum albumin are used to stabilise Taq polymerase (Perssing 1991). In order to prevent evaporation that may be caused by the design of old model thermocyclers, the PCR solution should be covered with mineral oil in PCR processes. The use of mineral oil also helps to prevent cross-contamination. Since oils can inhibit heat transfer during the reaction, excessive amounts

should be avoided. For this purpose, it is sufficient to add 70 µl of mineral oil to 100 µl of reaction solution and 40 µl of mineral oil to 50 µl of solution. New types of thermocyclers have largely eliminated the need for mineral oil as the reaction tubes are heated from the top (Perssing 1991, Steffan and Atlas 1991, van Pelt-Verkuil et al. 2008).

4.3. Application Stages and Principles of PCR Process

The PCR reaction involves the separation of two strands of DNA at high temperature (denaturation), followed by binding of synthetic oligonucleotides to the target DNA (hybridisation), chain elongation (polymerisation, synthesis of double-stranded DNA), and a certain number of repetitions of all these cycles (Fig. 18).

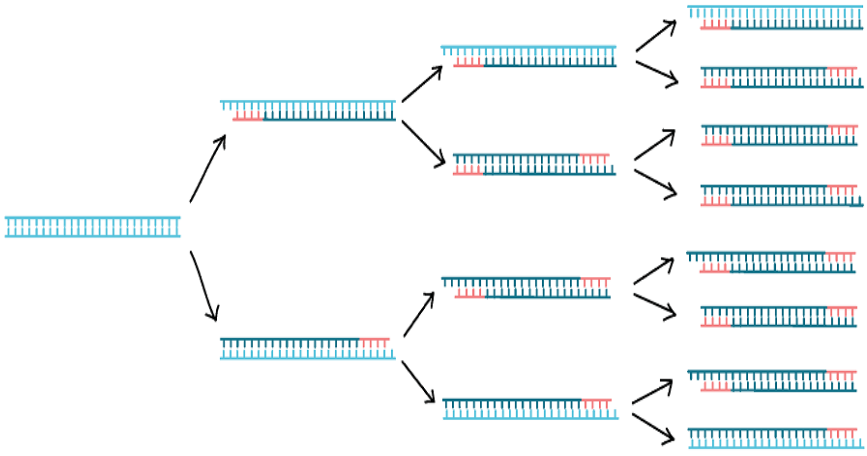


Figure 18. Steps of the PCR cycle

The automation of the PCR technique is achieved with the help of PCR devices called thermocyclers (Fig. 19), which perform the heating and cooling processes during each cycle according to software programmes. Nowadays, thermocyclers are commercially available from different companies, in which temperature, incubation time and number of cycles can be programmed. PCR

grade tubes of different volumes and numbers can be placed in thermocyclers. In these devices, the temperature can be programmed between 4 and 100 ºC and the eppendorf tubes can be kept at this temperature for a long time by setting to 4 ºC at the end of the reaction processes (Deacon and Lah 1989, Leonard et al. 2003, Viljoen et al. 2005).

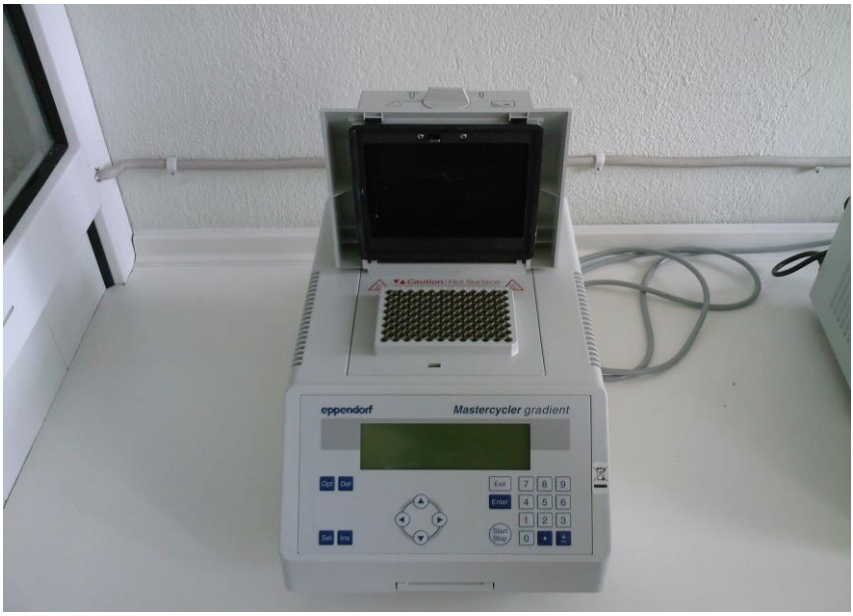


Figure 19. Thermocycler device

4.3.1. Denaturation step of DNA

At this stage, the double-stranded target DNA is separated from each other (Fig. 20). Double-stranded DNA is converted into a single helix by breaking hydrogen bonds. In the denaturation step, although high temperatures are more suitable for G+C rich target chains, commonly used denaturation temperatures are 30 seconds at 95 ºC or 15 seconds at 97 ºC. In thermocycler devices, denaturation of DNA is performed by applying these temperatures. In addition, if the denaturation temperature is too high or the duration is too long, the

enzyme activity is adversely affected (Erlich et al. 1988, Koneman et al. 1993, Viljoen et al. 2005, van Pelt-Verkuil et al. 2008).

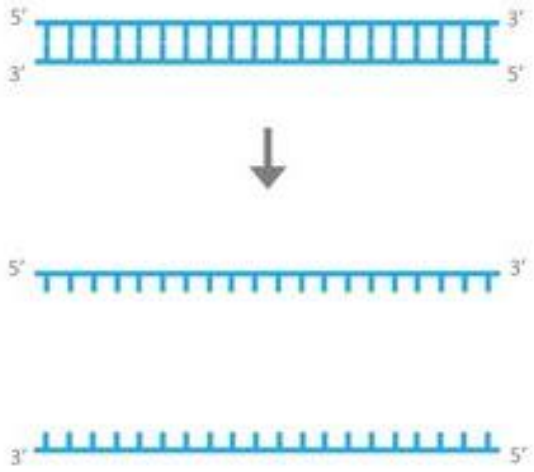


Figure 20. DNA denaturation

4.3.2. Primer binding (hybridisation, annealing) step

At this stage of PCR, oligonucleotides, called primers and specific for the DNA to be amplified, bind to the complementary sequence on the DNA single strand obtained in the denaturation step (Fig. 21).

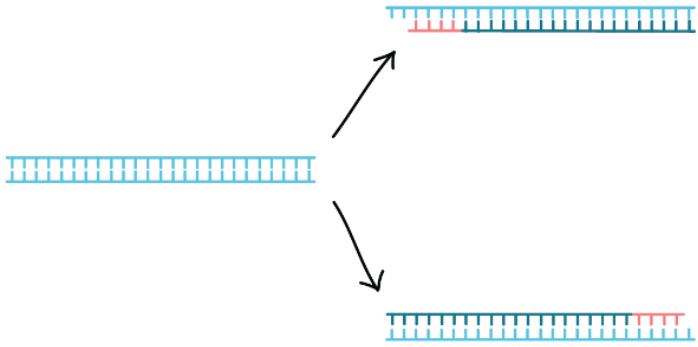


Figure 21. Primer binding

The thermocycler, programmed to bind the two types of primers to their specific complementary sites on the single-stranded target DNA, reduces the temperature to 37-65 ºC. The 5' end of one of the primers binds to the 3' end of one of the target DNAs and the other primer binds to the 3' end of the complementary end of the second single strand DNA in accordance with the direction of the DNA polymerase (5' → 3'). These processes take approximately 0.5-1 minute to complete (Aldemir and Uçan 2001, Viljoen et al. 2005, van Pelt-Verkuil et al. 2008).

The temperature and length of time required for primer binding depends on the nucleotide structure, length and concentration of the primers in the PCR solution. The binding temperature is usually 5 ºC below the melting temperature of the primers and gives the best results at 55-65 ºC. The optimal binding temperature is calculated by the amount of bases G-C, A-T and the number of bases. There are three hydrogen bonds between G and C bases, while there are two hydrogen bonds between A and T bases. The bonding temperature between each G-C base is calculated as 4 ºC and the bonding temperature between A-T bases is calculated as 2 ºC. For example; If it is accepted that there are 11 G-C

and 9 A-T in a primer structure consisting of 20 bases, the optimal binding temperature is calculated as follows.

$$11 \times 4 + 9 \times 2 = 62\ ^{o}C$$

The optimal binding temperature is obtained by subtracting 5 ºC confidence margin from the calculated 62 ºC (Aldemir and Uçan 2001, Viljoen et al. 2005, van Pelt-Verkuil et al. 2008).

4.3.3. Extension of primers (polymerisation, extention, elongation) stage

The primer extension step is usually carried out at 72 ºC. After the primer binding step is completed, the complementary strands to which the primers hybridize are synthesized by DNA polymerase (Fig. 22).

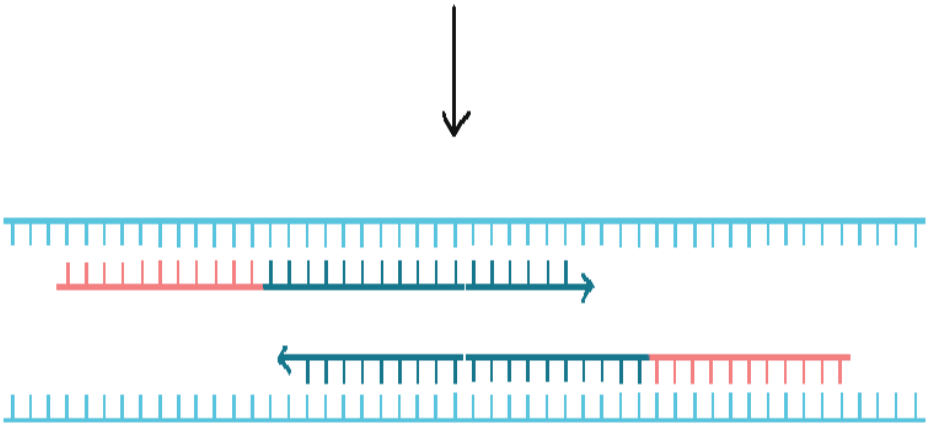


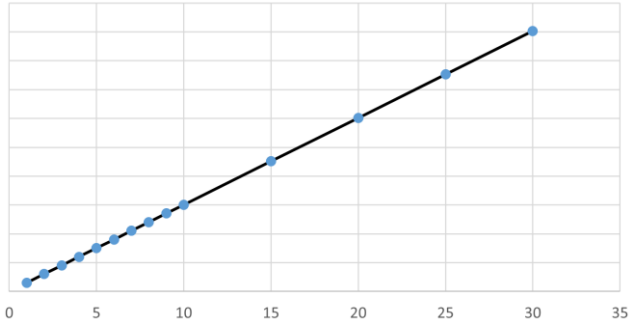
Figure 22. Extension of primers

In PCR processes, a one-minute extension time for each cycle is usually sufficient, but this time can be increased if the DNA region to be amplified is longer. For the amplification of longer DNA fragments, it is recommended to add one minute per kb on average. At this stage, Taq polymerase enzyme shows activity in the direction of $5' \rightarrow 3'$ and makes a copy of the target DNA sequence by using the nucleotides in the medium starting from the $3'$ ends of the primers (Viljoen et al. 2005, Birben 2006, van Pelt-Verkuil et al. 2008).

The reaction temperature is increased again, and the final elongation step is performed at 72 ºC. The PCR process, which consists of three main steps—denaturation, annealing, and extension—lasts approximately 10-15 minutes per cycle. These steps are repeated 25-30 times, with the denaturation step typically occurring at 95 ºC to ensure the DNA strands are fully separated before each annealing and extension cycle. Thus, a single target DNA segment is amplified approximately 33.6 million times according to the formula 2^n (Table 3) (Viljoen et al. 2005, Birben 2006, Amanda Fairchild et al. 2006, van Pelt-Verkuil et al. 2008).

Table 3. Amount of DNA Amplified During PCR Cycles

Cycle	Number	Increase graph
1	2	
2	4	
3	8	
4	16	
5	32	
6	64	
7	128	
8	256	
9	512	
10	1,024	
15	32,768	
20	1,048,576	
25	33,554,432	
30	1,073,741,842	



In a PCR process, it is expected that after 20 PCR cycles the DNA sought will have increased exponentially 2^{20} times. However, this number can only be reached if the product in each cycle is 100 %. In each cycle, different factors prevent the formation of 100 % product and this situation becomes more apparent in later PCR cycles. In this way, after 25-30 cycles, the imbalance between the increase in the amount of DNA and the decrease in the amount of enzyme limits the reaction. During this process, the activation of the enzyme also decreases (Viljoen et al. 2005, Birben 2006, Broll 2010, van Pelt-Verkuil et al. 2008).

4.4. Evaluation of PCR Results

The amplified product as a result of PCR consists of a DNA fragment of a certain length. After the end of the thermocycler stage of PCR processes, it is important to reveal the amplified reaction products.

It is possible to mention four different basic applications for the interpretation of PCR results.

4.4.1. Staining of amplified products

The amplification products in Eppendorf tubes were loaded onto a 2% agarose gel stained with ethidium bromide, SYBR Green, or GelRed (Fig. 23) using loading buffer. Once the gel had been poured into the tank and allowed to cool, it is filled with either Tris-Borate-EDTA (TBE) or Tris-Acetate-EDTA (TAE) buffer until the gel is completely covered.

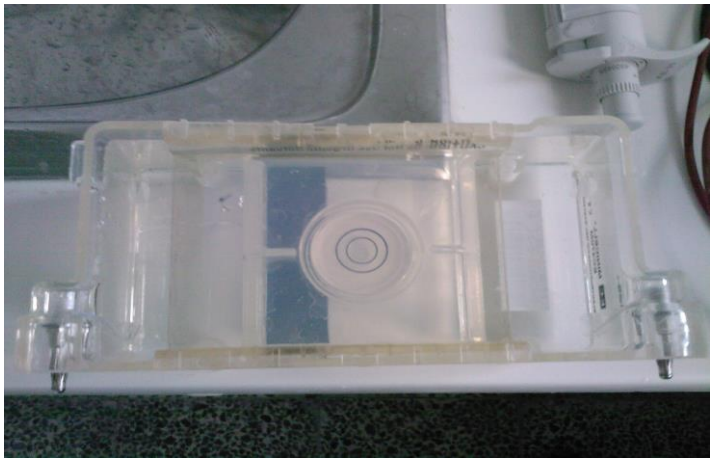


Figure 23. Agarose gel used in PCR procedures

Approximately 1 µl of loading buffer (prepared by combining 250 g bromphenol blue, 33 ml pH 7.6 Tris, 60 ml glycerol, and 7 ml distilled-sterilized water to create 100 ml of loading buffer) is added. The sample solution is mixed with the loading buffer (5 µl), and the resulting mixture is loaded onto the gel. Following the loading process, the bands are separated via electrophoresis (Fig. 24).

Figure 24. Electrophoresis step in PCR processes

The PCR results are evaluated by visualising the separated, i.e. executed, bands with a UV transilluminator (Fig. 25). In addition, polyacrylamide gel electrophoresis (PAGE) method, which is more sensitive than this technique but difficult to prepare and control, can also be used. After PAGE, the bands formed are made visible by staining with some techniques (e.g. $AgNO_3$ staining method). The resulting bands are evaluated by comparing them with the positive control DNA samples. Although very simple and easy, the sequence specificity of the PAGE method is poor. Nevertheless, PAGE method is more effective in the separation of small DNA fragments (5-500 bp) compared to agarose gel containing ethidium bromide (Arda 1997, Viljoen et al. 2005).

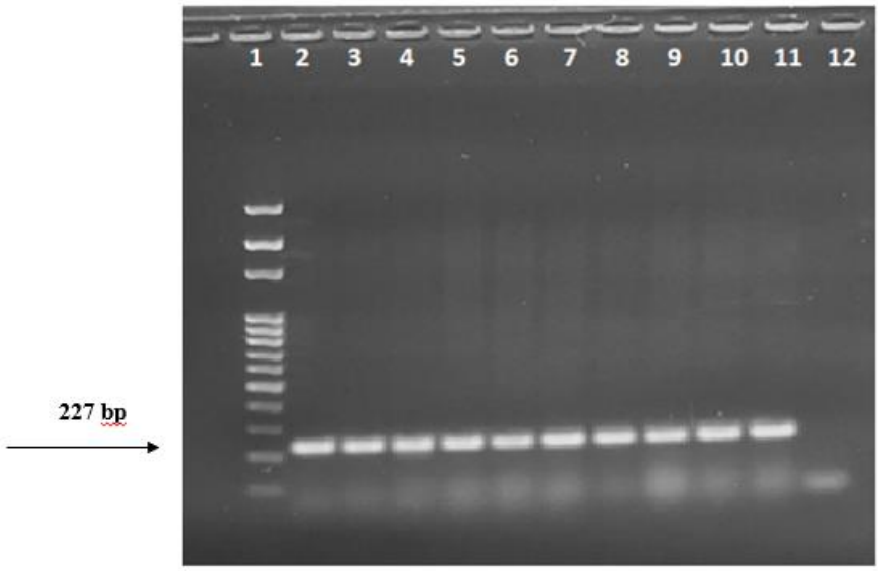


Figure 25. Visualisation of bands in UV transilluminator in PCR processes

4.4.2. Southern blot analysis

The amplified products are subjected to a separation according to their molecular weight by agarose gel electrophoresis. After this step, they are transferred from the agarose gel to solid media (e.g. nitrocellulose filter or nylon). This step is called the electrotransfer step. After denaturing and fixing on solid media, they are subjected to hybridisation with specific probes labelled with substances such as ^{32}P and biotin. They are then evaluated by autoradiography. If biotin is used, it is evaluated according to the colour index. The presence of black bands on the film indicates that the target DNA is amplified. Although the sensitivity and specificity of the Southern blot technique are high, the processing steps are time consuming (Arda 1997, Sabelli 1998).

4.4.3. Solution hybridisation technique

In the solution hybridisation technique, amplified DNA products and labelled probes are brought together in a hybridisation solution with an appropriate concentration of NaCl. The mixture is denatured at 95 ºC to separate the DNA strands from each other. The mixture is cooled down to 50-60 ºC and binding of specific probes to DNA strands is performed. This mixture is subjected to PAGE and separated. In the gel, DNA-RNA hybrid molecules are located at the beginning because they are large and slow moving, while small molecules (e.g. unbound probes, single stranded DNA) are localised at the opposite end because they are faster. The following procedures are carried out and evaluated as in southern blot analysis (Tenhunen 1990, Arda 1997).

4.4.4. Other techniques

Different techniques have been developed to detect amplified products (amplicons). In DNA molecules hybridised with specific probes, the activity of the polymerase enzyme in the direction of $5' \rightarrow 3'$ cleaves the hybridised probes, resulting in a reduction in the size of the probe DNA with isotopes at the $5'$ ends, which can be detected radiographically.

The hybridization protection assay has also been evaluated for the same purpose. In this technique, digital readout systems are also available and the time is much reduced. There has been a trend towards the use of nonisotopic probes to detect amplified products. Biotin or digoxigenin labelled probes are easier to prepare and can be used for longer periods (Arda 1997).

5. PCR FOOD ANALYSIS LABORATORY DESIGN

Although the laboratory design required for PCR analyses depends on the type of analysis performed, it is recommended that food analysis laboratories be divided into three sections (pre-PCR, thermocycler, post-PCR) (Fig. 26) (Schubbert 2010).

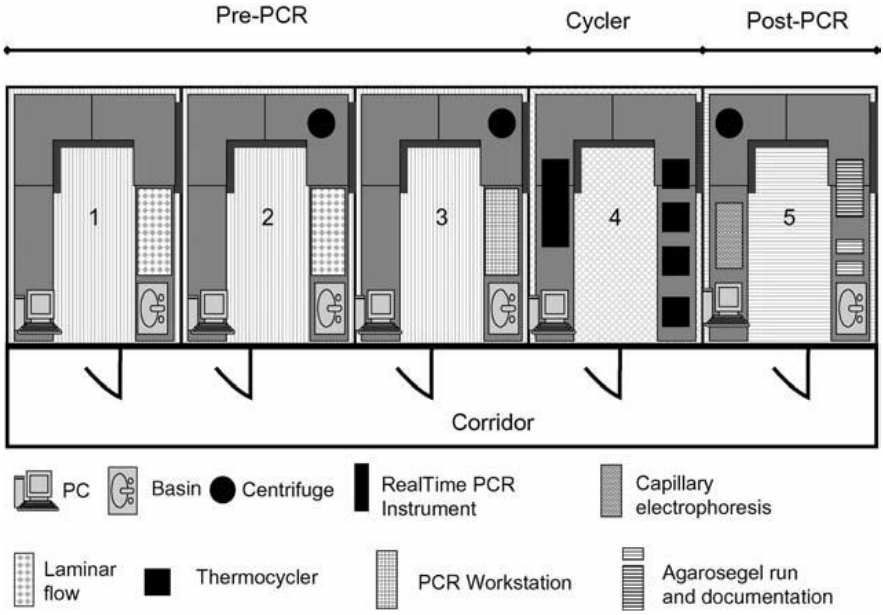


Figure 26. Sample design of food PCR laboratories

5.1. Pre PCR Section

In food PCR laboratories, at least three independent rooms are required to perform the pre-PCR steps.

- *Room 1 (Sample registration chamber)* : In this room, the registration, classification and barcoding of the samples to be analysed are carried out.
- *Room 2 (DNA extraction chamber)* : This is the room where DNA extraction for PCR analysis is performed. All working steps must be performed with nuclease free and filtered pipette tips. Gowns and gloves

should be worn to protect both laboratory personnel and samples from contamination. The room temperature should be maintained at certain conditions with air conditioning. A certain part of the room should be reserved for working with samples containing small amounts of DNA. Laminar flow should be used to ensure biosafety in pathogenic microorganism analyses**.**

- *Room 3 (PCR set-up chamber)* : This is the chamber where the PCR reaction is prepared and set-up is performed. Pipetting should be performed using sterile filter tips at all relevant stages. If possible, it is recommended that all PCR reagents be prepared on a different bench from the area where genomic DNA is being worked with in order to prevent DNA contamination. Air conditioning should be installed to maintain the room temperature under certain conditions. Cleaning-disinfection of the benches with appropriate reagents should be carried out after each working phase. In cases of suspected contamination, all surfaces, tools and equipment should be cleaned and the relevant chemicals and working solutions should be replaced (McDonagh 2003**,** Schubbert 2010).

5.2. Thermocycler Section

In the thermocycler section where the PCR cycle takes place, care should be taken to ensure that there is no contamination from the areas where pre-PCR procedures are carried out. For this purpose, no plastic material or solution should be transferred between both sections. It should be ensured that the room temperature is maintained under certain conditions with air conditioning (McDonagh 2003, Schubbert 2010).

Since the development of the PCR technique, there have been significant improvements in thermocycler equipment that enable changes in DNA strands in response to temperature fluctuations. In early applications of PCR, water

baths of the appropriate temperature were used for each step in the procedure. This was accomplished by the technician moving samples through 30 or more cycles to obtain sufficient DNA copies. Today, thanks to automation technology, PCR reactions can be run in thermocyclers to reliably yield a high number of specific DNA sequences in a very short time (Amanda Fairchild et al. 2006).

Standard thermocyclers use a large heating block in which the microcentrifuge tubes are placed. In this type of instrument, the heating and cooling functions of the blocks involved in each reaction cycle are regulated by a computer. An oil or wax coating was used to prevent the sample from escaping from the tube, especially during the denaturation phase of the reaction. These standard procedures have become unpreferable due to the time required to heat the entire block to the appropriate temperature at each stage and then cool it down. The time required for the heat block to reach each temperature uniformly is considerably longer when combined with the slow heat transfer rates to the microcentrifuge tubes. Such thermocyclers are now considered inadequate for the demand for high speed and accurate amplification of PCR products (Amanda Fairchild et al. 2006).

RapidCycler technology is an example of equipment designed to provide the rapid temperature cycling required for PCR reactions. The RapidCycler utilises heat transfer through high velocity bursts of hot air to carry out the temperature processes from the initial heating of the DNA sample to the annealing of primers and the extension of the new double helix of DNA by the polymerase. Within the cavity of the reaction vessel there is an overall temperature equilibrium and rapid heat exchange takes place within the sample. This is made possible by loading the samples individually into microcapillary tubes or thin-walled microcentrifuge tubes for the reactions to take place. This process allows for a smaller total volume in each reaction tube. In this way, it is also important to save on reaction components such as polymerase and primers.

After completion of the PCR cycles, the samples are loaded onto an agarose gel containing ethidium bromide, GelRed, or SYBR Safe, and visualized under a UV light source (Amanda Fairchild et al. 2006).

5.3. Post PCR Section

The post- PCR section is the area where PCR products are processed using agarose gel electrophoresis, capillary electrophoresis and other procedures. A separate pipette set, plastic material, gloves and apron are required for these procedures. For laboratories using genetic analysers such as laser and charge-coupled cameras as detection systems, air conditioning installation is required. It is important not to transfer all materials from the post- PCR section to the pre-PCR section. For re-amplification of PCR products, the PCR master mix should be prepared in the pre-PCR section, transferred to the post-PCR section, and then supplemented with the PCR product for subsequent amplification (McDonagh 2003, Schubbert 2010).

6. PCR: COMMON CHALLENGES AND TROUBLESHOOTING

During PCR process, some problems that cause misinterpretation and erroneous results can be encountered. The biggest problem encountered in PCR is the contamination of amplified DNA during a new reaction. Sources of contamination can be biological samples such as blood, tissue and food, sampling technique, laboratory personnel, laboratory equipment, liquid nitrogen, ice, tissue homogeniser, pipettes, pipette tips, reaction tubes, reagents, filters, centrifuge, centrifuge tubes, thermocycler, UV transilluminator and electrophoresis chamber (Viljoen et al. 2005).

Measures that can be taken to prevent contamination are reported below.

- If very small amounts of amplified DNA remain in the pipette or contaminate a stock solution, it is possible that these fragments may be treated as target DNA. To avoid this problem, amplification products should not be handled or stored near PCR solutions.
- There should be a PCR room where only PCR pipettes, pipette tips, tubes and solutions are stored and handled.
- New gloves should be used in the PCR work area.
- Preparation of the target DNA should be done in a separate location and away from the PCR working chamber if possible.
- The negative control, which does not contain target DNA, should be run together with each PCR to determine whether there is any possible contamination.
- DNA-plasmid should be prepared and pipetted in another laboratory as a positive control.

If contamination occurs despite all these precautions, the problem can be solved with a UV light source. Primers are more resistant to UV light than the target DNA. Therefore, the PCR solution can be exposed to UV light before

adding the DNA sample. The UV light should destroy any contamination or render it unamplifiable. If contamination of the primer or Taq polymerase is suspected, the remaining parts should not be used (Viljoen et al. 2005).

The desired result is not always achieved with every PCR procedure. Problems such as the formation of double or more DNA bands or the formation of little or no reaction product can often occur.

- Little or no reaction product;
    - Too low quality or too low quantity of target DNA,
    - Inappropriate denaturation, binding and elongation time or temperature,
    - It depends on the formation of secondary structures in the DNA area that will combine with the primary.

In light of these circumstances, the PCR protocol should be carefully assessed and refined as needed. In this context, it may be considered to change the time and temperature during the denaturation, binding and amplification steps or to readjust the buffer composition. In addition, with the help of a positive control, the polymerase, dNTP mixture and, if possible, primers should be reviewed for suitability.

Challenges encountered during, prior to, or following PCR reactions have contributed significantly to the advancement of novel PCR methodologies. Indeed, in order to minimise or eliminate these problems, PCR procedures have been modified at certain stages. These modifications have led to the development of many PCR methods from past to present (Viljoen et al. 2005).

7. APPLICATION of PCR-BASED METHODS

Numerous PCR methods have been devised to enhance specificity, adapt to RNA targets, or serve diverse research objectives.

7.1. Multiplex PCR

Multiplex PCR is an adaptation of PCR that allows simultaneous amplification of many sequences. This technique is used for the diagnosis of different diseases in the same sample. For example, different pathogens can be detected in a single sample with multiplex PCR (Hernández-Rodríguez and Ramirez 2012). In the multiplex PCR method, a large number of target DNA can be amplified at the same time by using primers specific for different target sites on each microorganism gene that can be found in the sample examined in the amplification tube. By observing the amplification products of different sizes specific to each primer pair on agarose gel, the presence and identification of different types of microorganisms in the examined sample can be performed simultaneously (Hendolin et al. 1997). This technique has been used in the diagnosis of infections caused by many pathogens such as *Neisseria meningitidis, N. gonorrhoeae, Streptococus pneumoniae, Haemophilus influenza, Moraxella catarrhalis, Mycoplasma pneumoniae, Coxiella burnetti, Chlamydia psittaci, Legionella pneumonia, E. coli* (Table 4).

Table 4. Infections Diagnosed with Multiplex PCR

Infection	Factors investigated
Meningitis	*N. meningitis, S. pneumoniae*
Chronic otitis media with effusion	*S. pneumoniae, H. influenzae, M. catarrhalis*
Atypical pneumonia	*M. pneumonia, C. burnetti, C. psittaci*
Respiratory tract infection	*L. pneumophila*, Influenzae
Diarrhoea in children	Rotavirus, Adenovirus, *E. coli* O157 verotoxigenic strains
Sexually transmitted infections	*N. gonorrhoeae, C. trachomatis*

7.2. Broad range PCR or consensus PCR

Broad range PCR or consensus PCR technique, which is another version of the PCR method, has been tested for the detection and identification of a significant number of bacterial, viral and parasitic pathogens that may be involved in the etiology of sepsis and meningitis in a short time. In this method, amplification is performed with a pair of primers capable of amplifying the gene region common to all bacteria or all species of a genus. Subsequently, the specific agent is diagnosed either by base sequence analysis of the amplicon or by hybridisation with a specific probe specific for a particular pathogen (Shanson 1999, Jordan et al. 2000).

As common target molecules, phylogenetically informative genetic molecules (large-subunit rDNA or small-subunit rDNA) with conserved common base sequence regions between bacteria are used. The conserved target region enables amplification of a large group of microorganisms. The microorganisms are then identified by utilising the regions within the amplification product (signature sequences) (Relman and Persing 1996).

Using ss rDNA-based consensus PCR, it is possible to demonstrate many novel, difficult to grow or unculturable bacterial pathogens (e.g., *Tropheryma whippelii, Bartonella henselae, Ehrlichia equi*) directly in infected tissue or blood. One of the bacterial identification applications based on this method is the rapid and accurate identification of 52 species within the genus *Mycobacterium* using genus-specific mycobacterial 16S rDNA PCR products (Relman and Persing 1996).

In the broad-range PCR technique used to investigate bacterial pathogens in meningitis, it was possible to differentiate between contaminant bacteria and true pathogens by using three groups of probes. In the first group, broad-range probes, in the second group, probes that can detect seven major pathogens (*N. meningitidis, H. influenzae, S. pneumoniae, S. agalactiae, E. coli, L. monocytogenes and S. aureus*) and in the third group, probes that distinguish contaminants (*Bacillus, Corynebacterium, Propionibacterium* and coagulase negative *Staphylococci*) were used. The most important problem arising in the application of this method in clinical diagnosis is that there are difficulties in the evaluation in the presence of commensal bacteria or normal flora members contaminated from the environment in the sample (Relman and Persing 1996).

7.3. Nested and semi-nested PCR

It involves two steps of amplification methods. In nested PCR, sensitivity is increased due to the detection of small amounts of target by using two sets of primers involving a double amplification process. The first set of primers allows for initial amplification. The product of this PCR is subjected to a second PCR using the second set of primers. These primers used in the second PCR are specific for the internal amplified sequence in the first PCR. The specificity of the first PCR product is therefore confirmed by the second PCR. The disadvantage of this technique is the possibility of contamination during the transfer of the first amplified product to the tube in which the second

amplification will be performed (Viljoen et al. 2005, Broll 2010, Hernández-Rodríguez and Ramirez 2012).

In the first step, two outer primers are used to amplify a long region of the target molecule (15-30 cycles). In subsequent steps, a shorter section of DNA from the sample taken from the amplification tube is amplified with inner primers (15-30 cycles). The inner primers bind to their specific sites, which are inside the binding sites of the outer primers (Fig. 27). In Semi-Nested PCR, one of the primers used in the second step starts from the same place as the inner and the other starts from the same place as the outer primer (Fig. 28).

Even if only one molecule of target DNA is present in the sample, it is possible to obtain a large number of DNA with two-step amplifications. Thus, the excess amount of target molecules formed as a result of amplification can be easily observed without any false results. Since the probability of positive results increases with these methods, the sensitivity of the test also increases. An important disadvantage of the high sensitivity is that during the transfer of the DNA from the tube to the second amplification tube after the first amplification, even a small amount of the product can cause airborne contamination in subsequent trials (Persing 1993a, Broll 2010). By applying broad-range PCR in the first step and multiplex PCR in the second step of nested PCR, it is possible to detect multiple microorganisms that may be present in clinical samples at the same time in a sensitive manner (Messmer et al. 1997, Viljoen et al. 2005, Broll 2010).

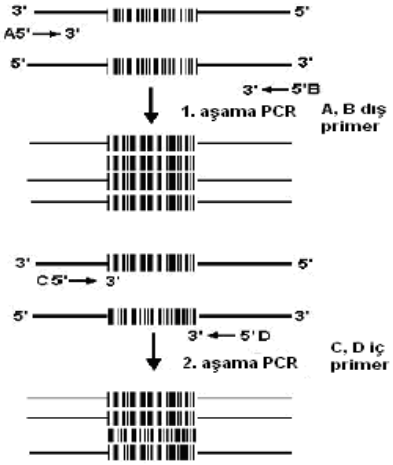


Figure 27. Nested-PCR

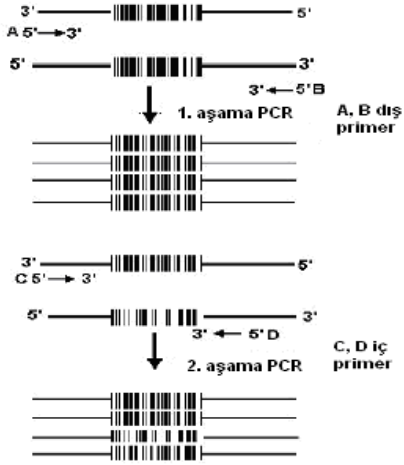


Figure 28. Semi-Nested PCR

7.4. In situ PCR

An important step in PCR studies is the amplification of the target DNA of the microorganism in the infected cell detected on the slide and the demonstration of the result by in situ hybridisation. After the tissue or cells are placed on the slide, they are air dried and kept at 105 ºC for 30 seconds. Processed with phosphate buffered saline (2%) with paraformaldehyde for one hour. Washed once with 3×0.1 M PBS and three times with 1×0.1 M PBS. After treatment with Proteinase K enzyme (5µg/ml) at 55 ºC for two hours, Proteinase K is inactivated by keeping at 96 ºC for two minutes. The slide is washed with water and air dried. Then PCR solution is added to the slide and covered with a plastic cap. The plastic cover is covered with nail polish. The slide is placed on the thermocycler. After covering with aluminium paper, amplification is started. After amplification, the slide is kept in absolute ethyl alcohol for 3-5 minutes. Then it is denaturated at 92 ºC for 30 seconds and washed with 2×SSC (sodium chloride + sodium citrate) buffer. Following this, in situ hybridisation is performed with specific probes and the amplification result is observed. The

most important advantage of this method is the absence of contamination due to amplicons since amplification takes place inside the cell (Bagasra et al. 1993).

7.5. Real time PCR

Real-time PCR, also known as quantitative PCR (qPCR), is a method that enables rapid generation of quantitative results by monitoring fluorescence signals that increase concurrently with nucleic acid amplification. Among the commercially available platforms, notable brands include LightCycler (Roche), TaqMan (Thermo Fisher Scientific), and CFX Real-Time PCR Detection Systems (Bio-Rad).

In the application of the LightCycler system, using dyes that fluoresce only when bound to double-stranded DNA (sybr green 1), amplification-induced DNA amplification is measured by the amount of fluorescent emitted. During the elongation phase following primer binding, the amount of sybr green 1 binding to DNA increases as the target DNA becomes double stranded and the amount of fluorescence emitted increases accordingly. The steps in the method are shown in Figures 29, 30 and 31, respectively (Kubista et al. 2006, Williams 2010).

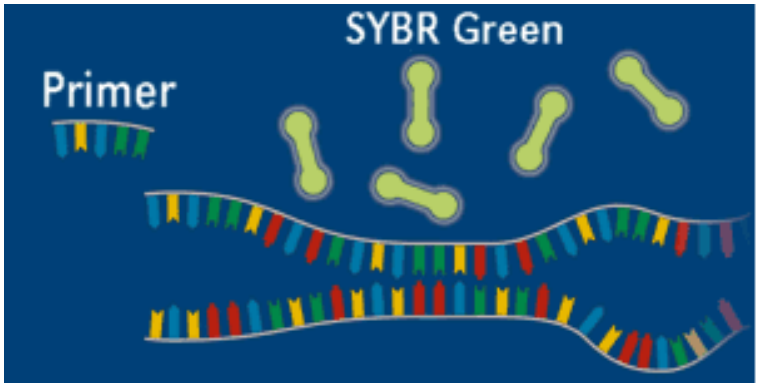


Figure 29. Initial stage of Real Time PCR amplification

As shown in Fig. 29, at the start of amplification the reaction mixture contains denatured DNA, primers and sybr green 1 dye. Unbound dye molecules fluorescent weakly, producing a minimal background fluorescence signal in computer analyses.

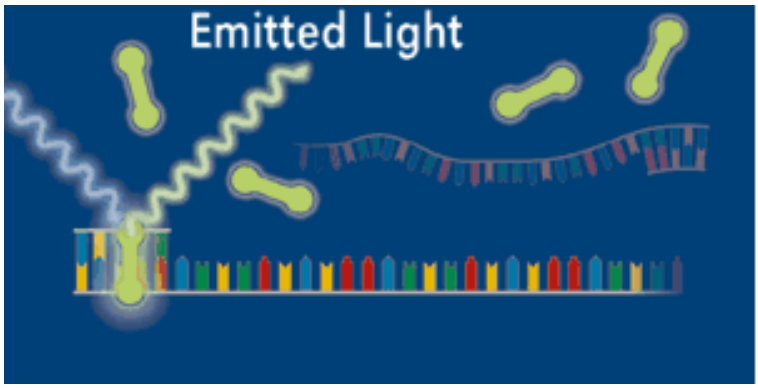


Figure 30. Real Time PCR primer binding to target molecule

As shown in Fig. 30, following the binding of primers to the target molecule, a small amount of sybr green 1 is incorporated into the double helix structure and as a result, the amount of fluorescence emitted is also low (Holland et al. 1991, Livak et al. 1995).

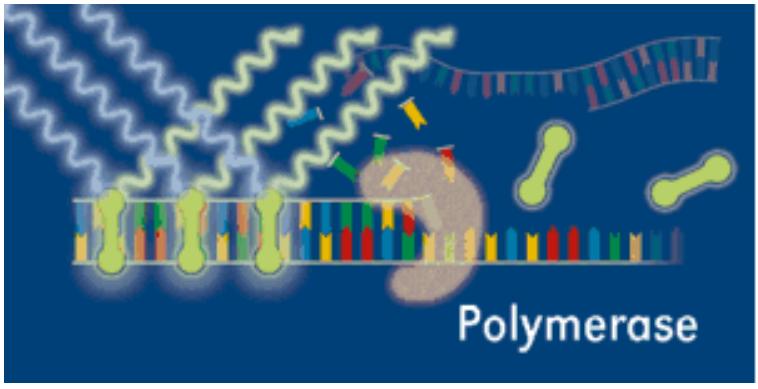


Figure 31. Real Time PCR primer extension step

As shown in Fig. 31, during primer elongation, the incorporation of additional dye into the structure of the newly synthesized double-stranded DNA results in a gradual increase in fluorescence over time. If the reaction is monitored continuously, the increase in fluorescence can be visualised in real time (Holland et al. 1991, Livak et al. 1995).

In this application, fluorescence increase may not always indicate specific amplification. Because, sybr green 1, which is integrated into the double helix DNA, can cause fluorescence formation by joining the structure as a result of the binding of primers (primer dimer) when there are no target molecules in the environment. In order to eliminate this negative factor, melting curve analysis of amplification products is performed. Each double-stranded DNA has its own melting temperature (Tm, the temperature required for 50% of the double-stranded DNA to become single-stranded). After PCR amplification, the temperature is gradually increased and the amount of fluorescence in the tube is recorded at regular intervals. When the double stranded DNA chains start to separate from each other, sybr green 1 dye is released and the amount of fluorescence decreases. When denaturation occurs, the fluorescence signal drops rapidly. The melting curve can be used to determine the Tm of the amplicon. By comparing the Tm of the analysed sample with the Tm of the positive control processed under the same conditions, it is decided whether the PCR result is correct or incorrect (Holland et al. 1991, Livak et al. 1995, Hernández-Rodríguez and Ramirez 2012).

Another application of the LightCycler is to use target-specific probes. Here the specificity of the test is increased with probes. One probe is labelled with a fluorescent dye (marker) at the 3' end and the other with an acceptor dye at the 5' end. The probes are bound close to each other (1-5 nucleotides apart) on the target amplicons and the labelled ends are juxtaposed. The energy released by the juxtaposition of the two dyes affects the acceptor dye on the second probe, leading to fluorescence formation. The amount of fluorescence generated as a result of this energy transfer, called FRET (Fluorescence Resonance Energy Transfer), increases

depending on the degree of hybridisation in the medium, in other words, the amount of amplicons formed during the PCR cycle (Holland et al. 1991, Livak et al. 1995).

In the TaqMan system, a probe labelled with fluorochrome (fluorescent) substances at the 5' and 3' ends is used. The 5' end of the probe contains reporter fluorochrome (6-carboxyfluorescein : 6-FAM) and the 3' end contains quencher fluorochrome (6-carboxytetramethylrhodamine : TAMRA). The probe binds to the single-stranded target molecule between the binding site of the primers. As long as hybridisation between the probe and the target molecule continues, the signal generation of the reporter fluorochrome is blocked by the suppressor fluorochrome at the 3' end. When the primer elongation initiated following the binding of the primers to the target nucleic acid reaches the point where the probe binds, the *Taq* DNA polymerase enzyme 5' → 3' starts to degrade the probe at the 5' end using its nuclease activity so that synthesis can continue. This releases the reporter fluorochrome and generates the signal as shown in Fig. 32. The signal intensity increases in parallel with the amount of amplicon produced in each cycle (Holland et al. 1991, Livak et al. 1995).

Real-time PCR can give quantitative results in a short time. Since the diagnosis is made without opening the tubes, the risk of contamination is low. Results can be obtained during amplification without the need for electrophoresis. In addition, mutations in the target nucleic acid can be detected by using fluorescent probes (Morris et al. 1996).

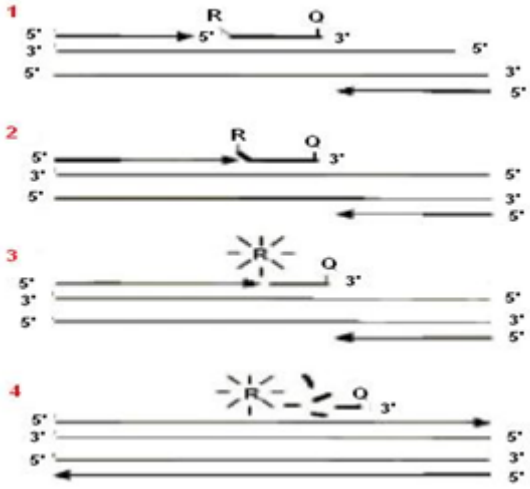


Figure 32. TaqMan Real Time PCR

7.6. Hot-start PCR

Hot-start activation is increasingly used. The main purpose is to increase PCR performance. In a standard PCR protocol, non-specific binding between the target DNA and primers may occur during the preparation of PCR solution, and many non-specific bands may occur as a result of the extension of these bindings with polymerase enzyme. In the Hot-Start PCR technique developed to prevent this reaction, one of the basic substances (polymerase enzyme, nucleotides, Mg^{+2} or primers) is not initially added to the amplification tube. After initial denaturation of the mixture, the missing substance is added and the temperature is cooled down to normal binding. This ensures specific binding of the primers. Amplification after this step results in clearer DNA bands (Persing 1993b, Paul et al. 2010).

7.7. Touchdown PCR

Touchdown PCR is a method developed to determine the optimal degree of primer binding. In this method, the binding temperature is reduced by 1-2 ºC between cycles to determine the appropriate degree of binding temperature. In

the first cycle of amplification, the binding temperature starts at approximately 15 ºC above the calculated Tm temperature and is decreased by 1-2 degrees during the following cycles to approximately 5 ºC above the Tm temperature. Hence, specific amplicons from the target region are obtained (Don et al. 1991, Viljoen et al. 2005).

7.8. PCR-ELISA

It is a method in which DNA sequence products obtained by PCR reaction are determined by combining with specific ELISA method. It was developed as an alternative to real time PCR method. In PCR-ELISA method, DNA molecules are labelled with digoxigenin during or after amplification.

Digoxigenin-labelled target DNA molecules are hybridised with specific biotin-labelled probes. In the next step, the hybridised DNA molecules and probes are transferred to streptavidin-coated ELISA microplate wells. Enzyme-labelled antidigoxigenin antibodies (horse radish peroxidase) and enzyme-specific substrates are added to the microplate wells and the colour reaction is observed.

The results are evaluated by reading at 450 nm wavelength in the spectrophotometer. The PCR-ELISA method prevents the formation of weak bands that may occur in PCR reactions and eliminates the need for the use of ethidium bromide (Hahn et al. 1995).

7.9. Inverse PCR

This method of PCR aims to amplify DNA segments adjacent to known sequences where the bases are unknown. Since the unknown sequences are inverted, this method is called inverted PCR. When this method is applied, the segments with unknown base sequences at both ends of the known sequence are cut with restriction endonuclease at a certain distance and sticky ends are formed on both sides. This cleavage results in the linearization of the molecule. By joining the two sticky ends, the molecule is transformed into a circular shape. The DNA segment in the middle with known sequences is cleaved in the middle with another restriction endonuclease and the molecule is converted back into a linear form. This molecule has known sequences at both ends. Two separate primers complementary to the known sequences at these two ends are prepared and added to the reaction medium. Taq polymerase and dNTPs required for polymerisation are also added. The rest of the procedure is carried out according to the standard PCR procedure (Ochman et al. 1988, Bej et al. 1991).

7.10. Asymmetric PCR

It is a PCR method for the amplification of only one strand of the target DNA using different amounts of primers. Extra PCR cycles are required due to slow amplification in later stages of the reaction after using the limting primer. Generally, after the double-stranded DNA is amplified by standard PCR, the target DNA is re-amplified by asymmetric PCR and the single-stranded product is produced in sufficient quantity. In asymmetric PCR, one of the primers used is much more abundant than the other. As a result, one of the DNA strands will be replicated more than the other (Innis et al. 1988).

7.11. PCR with homopolymer

This method of PCR is applied when only one sequence is known as the primer binding site. In this method, the mRNA molecule is converted into cDNA by reverse transcriptase enzyme. Poly guanines (poly G) are bound to the 3' ends of the single strand DNA molecule obtained. To bind to these ends, primers with poly-cytosines (poly C) at the 5' end are prepared and added to the reaction. These primers are used to synthesize a complementary DNA strand to the single-stranded cDNA. The double-stranded DNA is then separated, and each strand is amplified using specific primers for each. It has been effectively applied in *Vibrio cholerae* and *Streptococcus pneumoniae* genome research (Lazinski and Camilli 2013).

7.12. Overlap-extension PCR

Overlap-extension PCR or Splicing by overlap extension is a genetic engineering technique. It is used to combine two or more pieces of DNA with complementary sequences. This method is often applied to splice genes, joining sections of DNA that contain regulatory sequences or mutation sites. In this way, it allows the creation of specific and long DNA structures (Moller 2006).

7.13. Booster PCR

One of the main problems encountered in PCR amplification is primer-dimer formation and the other is the amplification of non-specific regions When these issues occur, the primers and enzyme in the PCR solution are consumed inefficiently. Booster PCR addresses these problems. This method was developed to ensure efficient amplification of target sequences present in low quantities. Initial PCR cycles are performed using a low concentration of primers. After 20 cycles, primer concentrations are increased to 50 or additional PCR cycles are performed (Bej et al. 1991).

7.14. Anchored PCR (A-PCR)

Anchored PCR is used in sequence analyses with different terminations. A-PCR can be used to clone a gene segment. It can also be used to clone a complete gene from the genome when the amino acid sequence of the amino (NH_2) or carboxyl (-COOH) terminal end is known (Bej et al. 1991, Viljoen et al. 2005).

7.15. Membrane-bound PCR

This method is used when the amount of target DNA is limited or the DNA is contaminated. It can be applied by purifying the DNA by electrophoresis and then labelling it, or it can be applied by first labelling and then purifying it. It is a technique with low efficiency and sensitivity. Membrane-bound PCR amplification requires 40 cycles (Bej et al. 1991).

7.16. Reverse-transcriptase PCR (amplification of RNA)

Reverse-transcriptase PCR was designed to amplify RNA sequences (especially mRNA) by cDNA synthesis with reverse transcriptase (RT). It is especially widely used in tests related to genetic diseases and in the characterization of gene expression. It is useful for further use with molecular biology techniques by cloning cDNAs. It has been useful in the diagnosis of RNA viruses as well as in the evaluation of antimicrobial therapy. Since the resulting cDNA preserves the original RNA sequence, it has also been used to study gene expression *in vitro* (Hernández-Rodríguez and Ramirez 2012, Bachman 2013). However, obtaining intact RNA from tissues is very difficult and problematic due to the active ribonucleases present in the tissues and the change-prone structure of RNA (Bej et al. 1991, Lion 1996, Viljoen et al. 2005, Bachman 2013).

7.17. Immuno magnetic PCR

Immuno magnetic separation (IMS) technique was developed in the early 1990s. The essence of the IMS technique is that substances such as monoclonal or polyclonal antibodies, lectins, agglutinins detected on magnetic carriers hold the target microorganism cells and thus ensure their separation from their environment. The greatest advantage of the IMS method is that target microorganisms can be distinguished even amidst significant contamination by other microorganisms present in the samples. In addition, the ability to separate a small number of target microorganisms from large quantities of samples sufficiently for analyses is also important. The IMS technique is used for the efficient isolation of eukaryotic cells from liquid media such as blood or for the detection of prokaryotic organisms such as bacteria and viruses (Duncanson 2004).

In standard PCR applications, sample amounts are generally between 1 and 20 µl. In some microbiological applications, such as the detection of *Salmonella* spp. and other pathogens in foods, the presence of one culturable microorganism in 25 grams of samples is interpreted. Such situations limit the sensitivity of PCR applications where sample quantities between 1 and 20 µl are used. The sensitivity of PCR applications is also affected by the presence of 5.000-10.000 microorganisms per ml of samples and/or the presence of Taq polymerase inhibitors. In immuno magnetic PCR technique, immuno magnetic separation is performed before PCR applications. With IMS application, the sensitivity of PCR processes is increased as a result of the separation of microorganisms present in small amounts in the samples and the removal of Taq polymerase inhibitors (Duncanson 2004).

7.18. Enterobacterial repetitive intergenic consensus PCR

ERIC-PCR is performed to determine Enterobacterial repetitive intergenic consensus (ERIC) sequences, also defined as Intergenic repetitive units. These sequences differ from other bacterial sequence repeats because they show a wide species distribution. The sequences were identified and demonstrated for the first time in *Vibrio cholerae*, *Escherichia coli*, *Salmonella typhimurium*, Salmonella enterica serovar Typhimurium and other members of *Enterobacteriaceae*. It represents a defective palindrome of 127 bp. The fact that ERIC sequences were detected only in intergenic regions and only in transcribed areas is important in terms of specificity. However, the observed differences in copy numbers between species also indicate that orthologous intergenic regions may contain an ERIC sequence in one species but not in another. By investigating these features with the ERIC-PCR method, it is aimed to reveal the clonal relationships of the isolates (Wilson and Sharp 2006).

7.19. Digital PCR

Digital PCR (dPCR) is a new technique developed for the determination, quantification and analysis of nucleic acids. It can be used to amplify nucleic acids, including DNA, cDNA or RNA, directly or clonally. dPCR also performs a single reaction in a sample, but the sample portions are divided into multiple fractions and the reaction is performed within each chamber. This separation allows for more reliable collection and precise measurement of nucleic acid quantities. The basis of the dPCR workflow is the random distribution of the PCR mixture (containing target nucleic acid, primers, probe, and master mix) into multiple uniformly sized aliquots. Thus, some segments contain no nucleic acid template and others contain one or more template copies. Advances in dPCR technology have enabled this technology to be used in many areas such as food safety, monitoring food quality, detection of pathogens, and determination of microbial epidemiology and ecology. Its high sensitivity feature allows detecting even very small differences in the amounts of nucleic

acid molecules. The development and detection of clinical-level biomarkers are used in studies such as genome editing, genetic screening, detection of genome amplification status and detection of genetic variants in cancer (Pinheiro and Emslie 2018).

7.20. Allele-specific PCR

This technique is based on nucleotide variations as a diagnostic or cloning method. If the mutation features include a single base change, all mutations can be detected by this system. The system requires prior knowledge of a DNA sequence. Primers are used that cover single nucleotide variations at the 3' ends (Gaudet et al. 2009).

7.21. Assembly PCR

It is a method for the synthesis of long DNA sequences from a large number of oligodeoxyribonucleotides. The Polymerase Cycling Assembly technique is based on the artificial synthesis of long DNA sequences by performing PCR on a pool of oligonucleotides with short overlapping segments (Stemmer et al. 1995).

7.22. Convective PCR

Convective PCR is a pseudo-isothermal method of performing PCR, where the solution is subjected to thermal gradients rather than repeated heating and cooling cycles. This method relies on the thermal instability created within the solution to drive convective flow. The PCR reagents move between hot and cold zones, enabling repeated activation of the PCR process. This technique eliminates the need for traditional thermal cycling, facilitating efficient DNA amplification. PCR is repeatedly activated by separating PCR reagents from cold and hot zones. Such convective flow PCR setup significantly reduces device power requirement and operation time (Krishnan et al. 2002, Priye et al. 2013).

7.23. Methylation-specific PCR

Methylation-specific PCR is used to determine the methylation of CpG islands in genomic DNA. It is a PCR method that can obtain qualitative data about methylation. A variation in which quantitative data is obtained through combination with qPCR can also be used (Ku et al. 2011).

7.24. Miniprimer PCR

Miniprimer PCR method that uses a thermostable polymerase that can be extended from very short primers (about 9 or 10 nucleotides). This method allows targeting of small primer binding sites. It is used to amplify conserved DNA sequences such as the 16S (or eukaryotic 18S) rRNA gene (Isenbarger et al. 2008).

7.25. Nanoparticle-assisted PCR

Nanoparticle-assisted PCR covers applications to increase PCR specificity and efficiency through the use of some nanoparticles such as carbon nanopowder, zinc oxide, titanium dioxide and silver, and carbon nanotubes (Shen et al. 2009).

7.26. Dial-out PCR

The dial-out PCR method is based on creating the correct DNA molecules for gene synthesis. The pool of complex DNA molecules is replaced with unique tags, and the primers for these tags are aimed to create molecules with the desired sequences (Schwartz et al. 2012).

7.27. Intersequence-specific PCR

Intersequence-specific PCR method is applied to produce a unique fingerprint. DNA fingerprint is created by amplifying regions between simple sequence repeats (Zietkiewicz et al. 1994).

8. SCIENTIFIC ADVANCEMENTS OF PCR APPLICATIONS

With the rapid advances following the discovery of molecular techniques, especially PCR and its different variations have become the most widely used techniques in laboratories and research institutes. These applications have contributed to the identification and characterisation of various organisms and to the understanding of the physiopathology of various diseases in humans, animals and plants. Important data have been provided for research on topics that have an impact on public health, such as the genetics and biochemistry of antimicrobial resistance (Hernández-Rodríguez and Ramirez 2012).

8.1. PCR Applications in Medical Science

The versatility of molecular biology techniques has enabled advances and changes in every field of medicine. In infectious diseases researches, PCR applications have made very important contributions especially in clinical microbiology. It has enabled the development of clinical microbiology because it is possible to identify microorganisms that are difficult to culture, live but cannot be cultured, have high laboratory requirements or are pathogenic to laboratory personnel. These problems have been reduced with the introduction of molecular diagnostic techniques that provide high sensitivity, specificity, accuracy and speed. Rapid diagnosis of microorganisms has become possible with PCR applications. It has made the work of biochemists, immunologists, microbiologists and other health professionals more effective, especially in determining multiple factors associated with antibiotic resistance. Genetic level microbial epidemiology and surveillance data are used as reliable methods for obtaining data (Dreier et al. 2007, Hutchins and Grabsch 2009, Weile and Knabbe 2009).

In the specific use of blood obtained from blood banks for patients with different pathologies, PCR technique is important in the criteria of meeting the selection and assurance elements (Dreier et al. 2007). The fact that it is a rapid, sensitive and effective molecular diagnostic method in the identification and

characterisation of many viruses, including influenza, contributes to public health. In fact, besides its benefits for treating individual patients, its ability to enable early treatment to control infections is crucial for public health (Wyczałkowska-Tomasik and Zegarska 2009, Dale 2010).

The application of molecular tools has also contributed to the development of pathological studies. Today, these techniques are frequently used in the diagnosis and treatment of diseases of complex character requiring a multidisciplinary clinical team and in the histopathological evaluation of tissues in morphological examinations. The ability to identify disease-related molecular changes to clarify diagnosis and therapeutic guidance has become inevitable in today's medical practice (Wyczałkowska-Tomasik and Zegarska 2009). Molecular biology applications in the field of pathology allow the identification of mutations and transporters in diabetes, obesity, neurology, muscle and heart diseases, ophthalmic problems, metabolic and congenital disorders, pathologies associated with sensory organs (Hernández-Rodríguez 2003, Hernández-Rodríguez and Ramirez 2012). With the development of molecular technology, it has become possible to understand the symptoms and genetic characteristics of diseases such as retinitis pigmentosa, microphthalmia, retinoblastoma, open-angle glaucoma, ocular diseases due to changes in mitochondrial DNA and various types of corneal dystrophy. In particular, the findings obtained with PCR have been important in understanding genetic physiopathology, early treatment, preventive medicine, and the creation of a new concept of clinical practice (Hernández-Rodríguez and Ramirez 2012).

Molecular tools have also enabled the application of preimplantation genetic diagnostics for the genetic analysis of embryos prior to transfer. PCR for preimplantation genetic diagnosis was first introduced in the UK in 1990 as part of developments in reproductive medicine, genetics and molecular biology. In this way, early detection of genetic abnormalities enables the use of unaffected

embryos and the birth of healthy children (Hernández-Rodríguez and Ramirez 2012).

8.2. PCR Applications in Forensic Science

In forensic pathology, classical morphological examinations remain the basic procedure for determining the cause of death. However, molecular biology is a very useful tool to investigate systemic changes involved in the pathophysiological process of death that cannot be detected morphologically. The genetic basis of diseases resulting in sudden death can also be investigated by molecular methods. Recent studies using variations of conventional PCR suggest that the relative quantification of RNA transcripts can be applied in molecular pathology for the purpose of molecular autopsy. Broadly speaking, forensic molecular pathology involves the application of molecular biology in medical science to investigate the genetic basis of the pathophysiology of diseases leading to death. Therefore, molecular tools support and strengthen morphological and physiological evidence in unexplained death investigations (Maeda et al. 2010). In forensic medicine, molecular methods are used to determine a person's ancestry (e.g., paternity testing) or to obtain evidence from tissue remains (Hernández-Rodríguez and Ramirez 2012).

8.3. PCR Applications in Agriculture and Environmental Sciences

The applications of molecular techniques in agricultural sciences and environmental research are numerous and diverse. One of the most important contributions of the application of some molecular techniques, such as PCR, is likely to be the identification and characterisation of multiple infectious agents that have a major impact on human and animal health (Hernández-Rodríguez and Ramirez 2012).

Today, the genome of domestic animals and major infectious agents are known through the use of molecular tools that facilitate the study of disease-associated mutations. Numerous examples can be given in this regard.

Identification of polymorphisms in the ABCB1 gene in phenobarbital-sensitive and phenobarbital-resistant idiopathic epileptic Border Collies is possible by molecular detection methods. Similarly, identification of an EDA gene mutation associated with anhidrotic ectodermal dysplasia in Holstein cattle, deletion of the Meq gene that significantly reduces immune suppression caused by Marek's disease virus in chickens, MTM1 mutation associated with X-linked myotubular myopathy in Labrador Retrievers, and an insertion mutation in ABCB4 associated with gallbladder mucocele formation in dogs are exemplary developments (Hernández-Rodríguez and Ramirez 2012).

Molecular techniques, such as conventional PCR or qPCR, have also enabled advances in research for the detection of pathogens in plants, animals and the environment, the identification and characterisation of specific pathogens, the understanding of their epidemiology, the development of new diagnostic tests, the determination of treatment procedures or the production of vaccines (Hernández-Rodríguez and Ramirez 2012).

9. PCR-BASED TECHNIQUES for TYPING INFECTIOUS AGENTS

Although they show similar clinical findings, there may be different subtypes of infectious agents with different antigenic property and pathogenicity. The typing and subtyping of infectious agents is important in terms of taking effective preventive and curative measures and establishing eradication programmes. PCR-based methods of typing infectious agents can be divided into three general classes as PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), PCR-Random Amplified Polymorphic DNA (PCR-RAPD) and DNA Macrorestriction Analysis by Pulsed Field Gel Electrophoresis (PFGE) methods.

9.1. PCR-Restriction Fragment Length Polymorphism

Restriction enzymes specifically cut DNA at specific sites, usually forming fragments of 1.000 to 20.000 base pairs. Restriction Fragment Length Polymorphism (RFLP) is the diversity obtained by comparing the location and number of DNA bands formes on the gel stained with ethidium bromide and then subjected to agarose gel electrophoresis after the DNA is cut with restriction enzymes. RFLP analysis is used to determine the restriction profiles of bacterial chromosome and extra chromosomal DNA or viral genome. The method is carried out in four basic stages; isolation of DNA, cutting of DNA with restriction enzymes, electrophoresis of the cut DNA and visualisation of DNA fragments in the gel. In PCR-RFLP method, the target sequences of various strains amplified by PCR and the bands formed by restriction fragments are compared (Persing et al. 1993a).

9.2. PCR-Random Amplified Polymorphic DNA

In this method, instead of amplifying a known specific DNA region, multiple regions of DNA are amplified with one or more randomly selected primers. Differences in the distance between the binding sites of the primers result in the formation of bands of different numbers and lengths that can be detected in the

agarose gel. The primers used are usually short primers of 9-10 bases and are rich in G-C. Since the primers were rich in G-C but short, the binding temperature is reduced to 40-50 ºC. In the binding step carried out at low temperature, the primers selected bind to both specific and non-specific regions on the chromosome. In different strains of the same species, the number of primer binding sites and their distances from each other will vary, so the number and size of the fragments amplified in agarose electrophoresis will also vary. Described in 1990 by Welsh and McCleland, the method has been cited by many researchers and is used in the analysis of all types of microorganisms. As a result of amplification, the band profiles of each isolate observed in gel electrophoresis are compared with each other. Isolates showing the same band profile can be interpreted as epidemiologically related (van Belkum 1994).

9.3. DNA Macrorestriction Analysis by Pulsed Field Gel Electrophoresis

DNA Macrorestriction Analysis by Pulsed Field Gel Electrophoresis (PFGE) analysis is the process of separating and recognizing DNA fragments. PFGE is aimed to embed bacterial cells containing chromosomal DNA, which is enzymatically degraded and whose cellular proteins are digested, in agarose gel by subjecting them to variable field gel electrophoresis and to separate the DNA fragments. This method allows the recognition of DNA fragments larger than 40 kb. Generally, linear double-stranded DNA molecules larger than 1 mb migrate at the same speed because the pore size of the gel is not sufficient for linear DNA to migrate in the gel. This causes DNA fragments to exhibit similar band profiles. Therefore, it prevents the differences in fragment lengths from being displayed. The pore size can be increased by using agarose with a concentration of 0.1%, but in this case the gel becomes unstable and becomes brittle, making it difficult to use. This problem was solved with the PFGE technique developed in 1984. PFGE, developed by Schwartz and Cantor, allows the direction of the electric field on the gel to be changed in agarose gel electrophoresis used in restriction enzyme analysis (Graves et al. 2007).

10. PCR APPLICATIONS IN FOOD MICROBIOLOGY

PCR has become a cornerstone technique in studies within the field of food microbiology. The isolation and identification of microorganisms from foods by classical culture techniques are time consuming and causes significant problems, especially in the foods with short shelf life. In addition, isolation of bacteria such as *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes* by classical cultural methods is not always possible due to the effects of stress factors caused by technological processes applied at different stages of food production. Some pathogenic bacteria that can be found alive in foods but cannot be cultured by classical methods (Viable But Non Culturable, VBNC) can be detected more reliably by *in vitro* DNA amplification with high specificity.

The PCR technique for detecting microorganisms that can be found in foods is based on *in vitro* amplification of DNA fragments of existing microorganisms and can give results within 1-2 days. It is possible to identify a single bacterial colony that can be isolated from food by PCR technique. In food microbiology, DNA analyses with PCR technique based on nucleic acid amplification are mainly performed in 3 stages. These are

- Sample preparation phase,
- Target DNA amplification stage,
- Analysis stage of amplification products.

Sample preparation is a crucial step in the detection of microorganisms by PCR as it involves a wide range of factors. It reduces the sample volume and increases the volume available for PCR. The first challenge in selecting an effective sample preparation protocol is to estimate whether the agent is highly contaminating the food. This is important for determining whether it will be necessary to propagate the microorganism by an enrichment culture prior to processing by PCR. For example, a relatively small amount of meat product

may contain very few *Listeria* microorganisms. However, even in small numbers, these organisms are known to cause serious infections in pregnant women. In fact, this situation is also a discrimination criterion for the presence/absence analyses, which are accepted as zero tolerance and which cause an infection table when a certain number reaches a certain number in terms of clinical microbiology in the evaluation of the effects. It is almost impossible to obtain a small number of microorganism cells directly and detect them by PCR technique. Although of a different nature, the same applies to VBNC microorganisms. In such cases, an agent-specific enrichment culture is very useful to multiply the microorganism cells. With enrichment, the microorganism cells can be detected by PCR faster than they can be identified using standard bacteriological methods. In such cases, the PCR protocol includes sample preparation, enrichment culture, collection of microorganisms from the enrichment liquid, extraction of DNA from the microorganism, followed by PCR. In the PCR investigation of microorganism in food samples, DNA extraction methods should be optimised specifically (Table 5) according to food matrix and agent characteristics (Lee and Fairchild 2006).

Table 5. Extraction Methods Used in Different Foods

Food Category -Difficulties	Example	Method of concentrating the pathogen	DNA (RNA) Extraction method	Pathogen
Dairy products -PCR inhibitors (fat, protein, calcium, chelators) -Dead cells, low number of pathogenic cells, -Other bacteria	Skimmed milk, pasteurised milk, dry milk, hard and soft cheese, reconstituted whey powder.	Differential centrifugation or none.	Solvent-based nucleic acid extraction or guanidinium isothiocyanate extraction.	*E. oli O157, Listeria, Staphylococcus, Yersinia, Campylobacter*
	Raw milk	Centrifugation	Cells treated with boiling by removal of Chelex-100 inhibitors; enhancement of *Tth* polymerase sensitivity	*Staphylococcus*
	Raw milk	Enrichment and centrifugation	Commercial kits	*Salmonella*
	Soft cheese	None of them	Detergent lysis of DNA by NaI extraction	*Listeria*
Vegetable-Fruit -PCR inhibitors (chelators) - Small number of bacteria	Raspberry	Column filtration and centrifugation	Commercial kit	Protozoa
	Lettuce	Homogenisation, centrifugation and precipitation with polyethylene glycol	Commercial kit	Hepatitis A virus, Norwalk-like virus

Table 5 Continued. Extraction Methods Used in Different Foods

Food Category -Difficulties	Example	Method of concentrating the pathogen	DNA (RNA) Extraction method	Pathogen
Meat and poultry -PCR inhibitors (fat, protein, collagen, blood) -Small number of bacteria	Chicken carcass rinses, red meat	Enrichment and centrifugation	Commercial kits	*Listeria, Salmonella, E. coli*
	Chicken skin, whole chicken legs, chicken sausages, turkey legs, minced meat, meatballs, veal, pork homogenates	High density centrifuge	Guanidinium isothiocyanate and detergent extraction	*Campylobacter*
	Raw whole chicken rinses	High-density centrifugation and enrichment culture	Cells subjected to boiling	*Campylobacter*
	Chicken and turkey muscle, skin, internal organs, raw carcasses	Enrichment	Multiple methods including boiled cells, alkaline lysis and commercial kits	*Salmonella*
	Beef mince	High-density centrifugation, immunomagnetic separation, enrichment	Boiling treated cells or Chelex extraction	*E. coli*
	Ham	Immunomagnetic separation	Lysozyme and detergent extraction	*Listeria*
	minced pork, raw whole pork leg	High-density centrifugation and enrichment	Commercial extraction and heat	*Yersinia*
	Pork mince	Enrichment	Chelex resin-based commercial kit	*Yersinia*

Table 5 Continued. Extraction Methods Used in Different Foods

Food Category -Difficulties	Example	Method of concentrating the pathogen	DNA (RNA) Extraction method	Pathogen
	Sausage and meatballs	Homogenisation of the food, followed by filtration and centrifugation	Commercially available kits	*Clostridium*
	Deli meats: ham, turkey, roast beef	None of them	Commercial extraction	Norwalk-like virus, Hepatitis A virus
	Smoked salmon	Homogenisation	Detergent extraction and Tween 20 facilitator; PCR inhibitors removed by solvent extraction or column purification	*Listeria*
Seafood -PCR inhibitors (phenolics, cresols, aldehydes, proteins, fats), -Small number of bacteria	Fish cakes, fish pudding, peeled frozen prawns, salted herring, marinated and sliced charcoal fish in oil	Enrichment	Detergent and decoction extraction	*Listeria*
	Shellfish: mussels and oysters	Homogenisation and high-speed centrifugation	Guanidinium isothiocyanate and silica purification	Norwalk-like virus, *Adenovirus*, *Enterovirus* Hepatitis A virus
	Raw oysters	Homogenisation and high density centrifugation	Commercial kit	*Norovirus*

The use of methods based on PCR techniques in the field of food microbiology is becoming increasingly widespread due to their high sensitivity in detecting of microorganisms and their quick turnaround time for results. Different PCR methods have been used in the diagnosis of pathogenic microorganisms in foods (Table 6).

Table 6. PCR Applications for Pathogenic Microorganisms in Foods

Microorganism	Target Gen	Sources
Listeria monocytogenes	Listeriolysin O	Oravcova et al. 2007, Blais et al. 1993, Fitter et al. 1992, Fluit et al. 1993a, Furrer et al. 1991, Niederhauser et al. 1993, Niederhauser et al. 1992, Rossen et al. 1991, Thomas et al. 1991, Wernars et al. 1991
	prfA gene	Rossmanith et al. 2006
	Listeria surface cell protein	Wang et al. 1992
***Salmonella* spp.**	OriC, chromosomal origin of replication	Fluit et al. 1993b
***Campylobacter* spp.**	16S rRNA	Giesendorf et al. 1992, Neubauer and Hess 2006
Yersinia enterocolitica	yadA gene	Estrada et al. 2007
Vibrio cholerae	CtxAB	Koch et al. 1993
Vibrio vulnificus	Cytotoxin haemolysin	Hill et al. 1991
Shigella flexneri	Plasmid invasion gene	Lampel et al. 1990
Campylobacter jejuni, Campylobacter coli	flaA-flaB genes	Wegmuller et al. 1993

Hepatitis A virus, Norwalk virus	Polymerase gene	Atmar et al. 1993, Goswami et al. 1993
Escherichia coli	malB, LT1, ST1	Candrian et al. 1991,
	LT	Wernars et al. 1991
	SLTI, SLTII	Gannon et al. 1992
	VT1, VT2 and VTE	Read et al. 1992
	Plasmid invasion gene	Andersen et al. 1992
***Arcobacter* spp, *Helicobacter* spp.**	16S rRNA	Neubauer and Hess 2006
Bacillus sporothermodurans	16S rRNA	Herman et al. 1997
***Bifidobacter* spp.**	hsp60 gene	Delcenserie et al. 2005
Mycobacterium avium subsp. paratuberculosis	16S rRNA	Kaufmann et al. 1997
	16S rRNA	Sharma et al. 2008
Clostridium botulinum	Neurotoxin gene	Lindström et al. 2001
Brucella melitensis	omp31 gene	Gupta et al. 2006
Clostridium perfringens	Phospholipase C gene	Fach and Popoff 1997
Coxiella burnetii	16S rRNA	Muramatsu et al. 1997
Cryptosporidium parvum	Oocyte protein gene	Laberge et al. 1996

10.1. PCR for Microbiological Water Analysis

One of the most important health problems that may arise from drinking and utility water is the occurrence of epidemics with the effect of pathogenic microorganisms in water. For this reason, microbiological control of drinking and utility water is one of the most important fields of public health and

environmental microbiology. PCR technique entered the of water and environmental microbiology in the early 1990s and has been widely used especially for the detection of microorganisms that are difficult to produce and isolate in culture media. In standard bacteriological water analysis methods, the results are obtained in 48 hours and a single microorganism group is investigated, but with the multiplex PCR method, the analysis time is reduced to an average of four hours and more than one microorganism group can be examined in one analysis. Thermotolerant coliform, *E. coli*, *Shigella* and *Salmonella* bacteria in water samples have been detected using multiplex PCR method (Hasde et al. 2002).

In the microbiological examination of foods by PCR, primers targeting DNA regions of the microorganism are first determined. Primer selection can be carried out by the previous studies on the subject or by the procedures to be performed for primer design. The base sequences and fragment sizes of the primers (Operon Technologies Inc., USA) for certain microorganisms reported above are given in Table 7 (Hasde et al. 2002).

Table 7. Primers Used in Multiplex PCR

Primer	Base Sequence	Part Size
ZL-1675 Coliforms	5'-ATGAAAGCTGGCTACAGGAAGGAAGGCC-3'(24mer)	326 bp
ZR-2025 Coliforms	5'-GGTTTTATGCAGCAACGAGACGTCA-3'(24mer)	326 bp
ual 754 ***E. coli***	5'-AAAACGGCAAGAAAAAGCAG-3'(20mer)	147 bp
uar 900 ***E. coli***	5'-ACGCGTGGTTACAGTCTTGCG-3'(21mer)	147 bp
SalA 1144 ***Salmonella***	5'-ACGGTTTGTTTAGCCTGATAC-3'(20mer)	526 bp
SalB 1650 ***Salmonella***	5'-CTGGATGAGATGGAAGAATG-3'(20mer)	526 bp
ShigA ***Shigella***	5'-TTGACCGCCTTTCCGATAC-3'(19mer)	408 bp
ShigB ***Shigella***	5'-ACTCCCGACACACGCCATAGA-3'(19mer)	408 bp

10.1.1. Collection and preparation of water samples

Two samples of 100 ml each are taken from the waters to be investigated microbiologically. The samples are delivered to the laboratory within two hours and firstly the coarse particles are removed by passing through a filter, then the microorganisms that may be present are transferred to the filter by passing through 45 nm nylon microfilters. These filters are transferred to 10 ml sterile tubes and 1 ml sterile distilled water is added. After the tubes are shaken with a vortex device for 30 seconds, the water in the tubes is transferred to 1.5 ml sterile microcentrifuge tubes using a micropipette. At this stage, the bacterial

stocks to be used as positive control are reconstituted with distilled water and the volume is completed to 1 ml. The tubes are kept in a cooler (4 ºC) until use (Hasde et al. 2002).

10.1.2. DNA extraction

The freeze-thaw method is used to isolate the DNA of bacteria. All microcentrifuge tubes are placed in a deep freezer (-20 ºC) for five minutes and then placed in a hot water bath (+60 ºC) for ten minutes. Meanwhile, the tubes are placed in special chambers that can be compressed from the top to prevent the caps of the tubes from loosening and absorbing water. This freezing-thawing process is repeated five times. After the freezing-thawing process is completed, the microcentrifuge tubes are centrifuged at 7500 rpm and +4 ºC for 10 minutes to precipitate particles other than nucleic acid and substances that may inhibit the PCR process. The liquid remaining on top is used as DNA source in the subsequent PCR step (Hasde et al. 2002).

10.1.3. Preparation of master mix and multiplex PCR

Immediately before PCR, PCR master mix is prepared. This solution contains mM 10 Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 200 mM dNTP, 0.5 mM of each primer and 2.5 IU Taq DNA polymerase. The final volume of the solution with the DNA from water samples is 50 µL. Multiplex PCR is performed in a thermocycler under specific conditions (Table 8). After the final extension, the tubes are kept at 4 ºC until visualisation (Hasde et al. 2002).

Table 8. Multiplex PCR Conditions

Operation	Temperature (ºC)	Duration
Initial denaturation	95	5 min
Denaturation	95	25 sec
Annealing	55	30 sec
Extension	72	55 sec
Final extension	72	10 min

10.1.4. Visualisation of PCR products

In order to visualise PCR products, a 2% (can vary depending on the product size) agarose gel is prepared by adding 0.4 g agarose into 20 ml 0.5X TBE solution. This mixture is heated to boil in a microwave and is provided to cool down. In the following 5 µl ethidium bromide is added. Then it is poured into the gel chamber and is waited for 15 minutes for cooling and solidification. The wells in the agarose gel placed in a horizontal mini gel electrophoresis containing 0.5X TBE are loaded with PCR products mixed with gel loading dye as shown in Fig. 33. One of the wells (often the left well) is loaded with the marker used as a scale in the visualisation phase (Hasde et al. 2002).

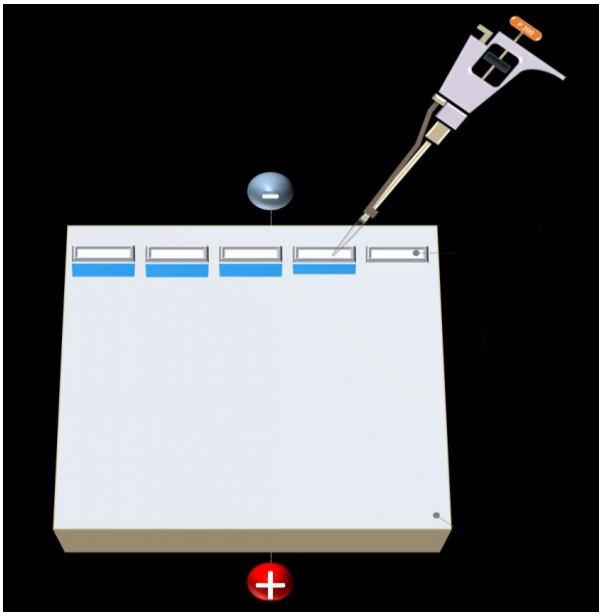


Figure 33. Loading of PCR products in agarose gel

After loading, electrophoresis is performed under constant voltage (80-120 V) for 45 min-2h (Fig. 34). Positive and negative controls are used for each sample (Hasde et al. 2002).

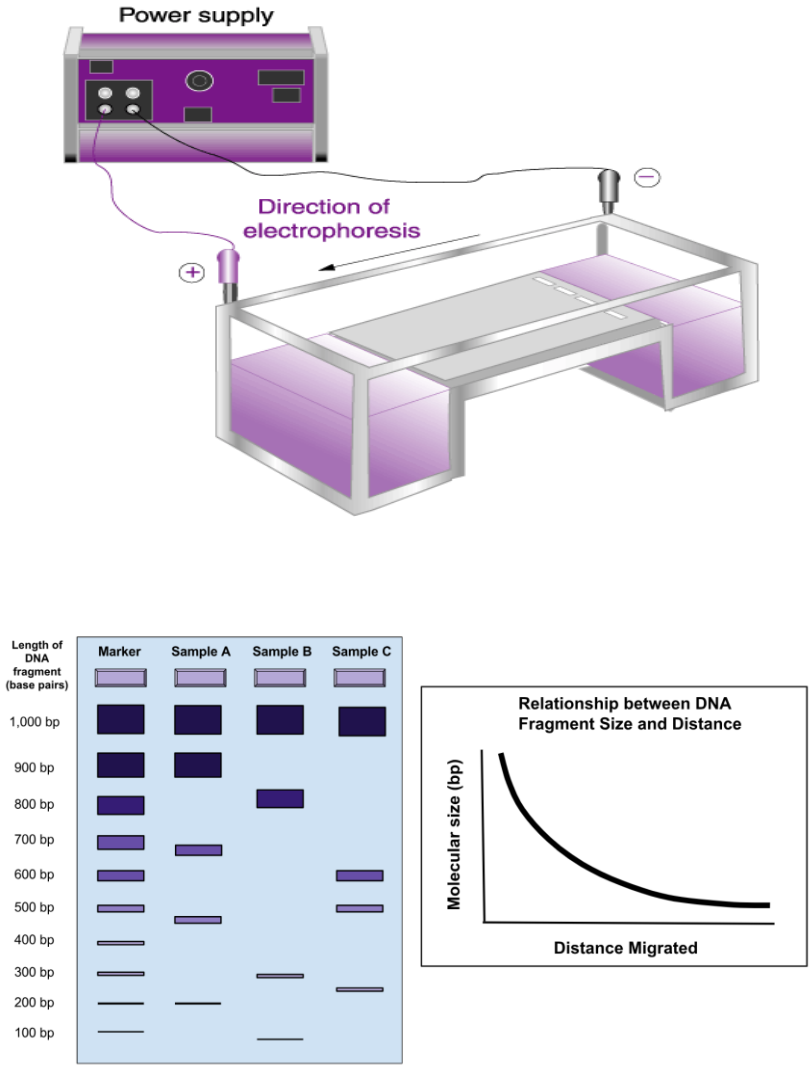


Figure 34. Electrophoresis process

At the end of the electrophoresis process, the gel is removed and visualised and evaluated using the UV transilluminator shown in Fig. 35 (Hasde et al. 2002).

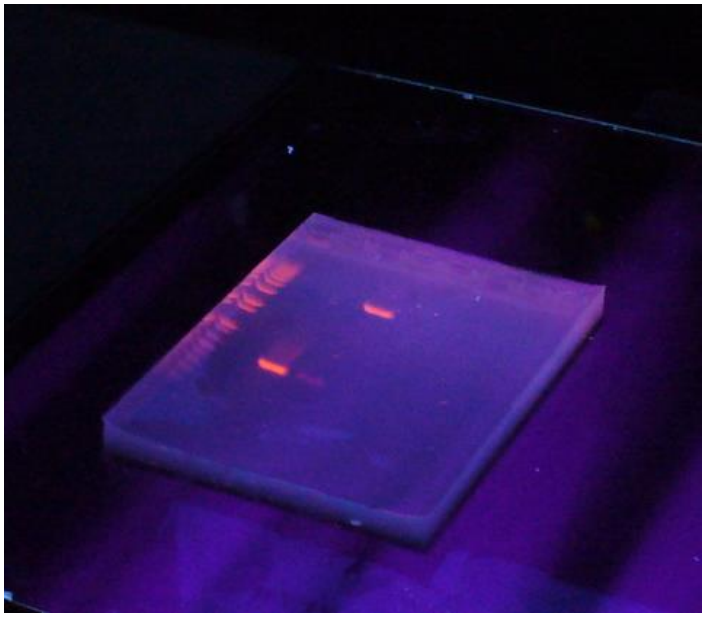


Figure 35. UV transilluminator

10.1.5. Evaluation of results

The positive controls used for each of the microorganisms visualised in the UV transilluminator should appear in the corresponding bp regions as expected (Fig. 36). The positivity of the wells of each sample for the investigated

microorganism is interpreted according to the bp areas detected by the marker. Fig. 37 shows an example UV transilluminator image and the bp areas where the samples show migration. The well with the marker (on the left) acts as a kind of ruler (Hasde et al. 2002).

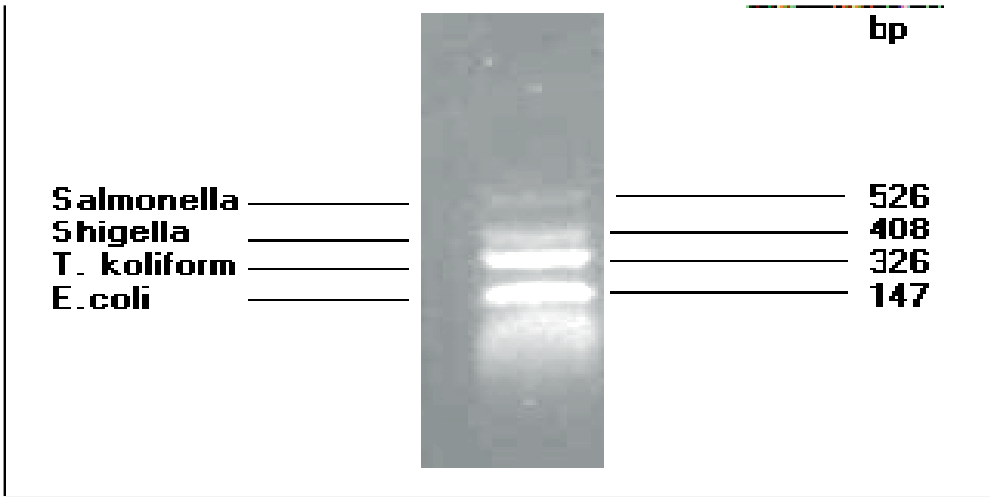


Figure 36. Visualization of positive controls

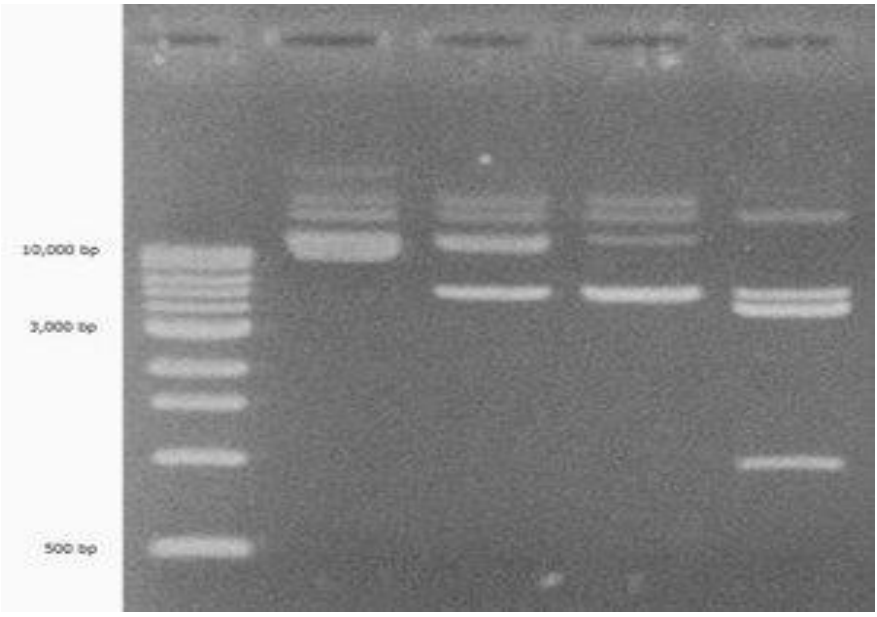


Figure 37. Image of the band patterns

11. APPLICATION of MULTIPLEX PCR for DETECTING FOODBORNE PATHOGENS

Detection of foodborne pathogens is performed for each pathogen individually using colonies of culturable microorganisms on selective or differential media. These classical methods are time-consuming and can take up to five days for definitive confirmation of some specific pathogens. This poses problems for the implementation of treatment and preventive measures when factors such as shelf life of food, severity of clinical picture, and sustainability of food supply. However, rapid/effective detection of nine different foodborne pathogens can be possible through a single multiplex PCR reaction and can directly contribute to public health (Villamizar-Rodríguez and Lombó 2017).

11.1. Material

Foodborne pathogens such as *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Cronobacter sakazakii*, *E. coli* O157: H7, *Enterobacteriaceae*, *L. monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* can cause infection and/or intoxication in consumers and are important for public health. The materials required are given below in the multiplex PCR reaction for the detection of the relevant microorganisms in food matrices.

11.1.1. Primer Design

The resources that can be utilised for the determination of specific primer regions for the microorganisms to be detected are as follows;

- gDNA database such as GenBank (NCBI).
- Blastn/Blastp online tools.
- Primer3 online primer design tool.
- FastPCR Software.

11.1.2. Multiplex Primer Optimisation and PCR Reagents

- Amplitaq Gold 360 Master Mix.
- Multiplex Primer set for nine selected pathogens.
- 200 μL PCR reaction tubes, strips or 96-well plates.
- Double distilled, nuclease-free or Milli-Q H_2 O.
- Thermal cycler.
- Vortex mixer.
- 0.5-10, 10-100 and 100-1000 μL micropipette set and tips.
- Genomic DNA (gDNA) extracted from pure cultures of each microorganism.

11.1.3. Requirements for Sampling and Pretreatment

- Sampling representative quantities of food samples.
- Sterile spatulas, spoons and forks.
- Laboratory analytical balance.
- Homogeniser.
- Sterile bags with filter membrane for stomacher.
- Microorganism specific pre-enrichment media; 20 g/L buffered peptone water, 50 mL/L haemolysed and defibrinated horse blood, 20 g/L Mannitol, 4 mL/L *Campylobacter* Growth Supplement (e.g. OXOID SR0232), Distilled water.
- Incubator

11.1.4. Genomic DNA Extraction Requirements

- Extraction kit for gDNA extraction from pure cultures (e.g., Qiagen DNeasy Blood & Tissue Kit-Qiagen).
- Extraction kit for gDNA extraction from food samples (e.g., PrepMan® Ultra Reagent, Thermo Fisher Scientific).
- Microcentrifuge tubes (e.g., 1.5 mL Eppendorf® Safe-Lock).
- Hot water bath, double boiler or thermal block.
- Microcentrifuge
- 0.5-10, 10-100 and 100-1000 µL micropipette set and tips.

11.1.5. Requirements for Agarose Gel Electrophoresis of DNA

- Electrophoresis Buffer Tris-Borate-EDTA (TBE) 1×: 108 g/L Tris-HCl, 20 mL 0.5 M EDTA, 55 g/L Boric Acid, Purified water.
- High purity molecular grade agarose (low electroendosmosis level).
- Laboratory analytical balance.
- Microwave.
- Glass bottles for liquid media.
- RedSafe™ 20,000× DNA stain (e.g., Chembio) or non-toxic DNA-Dye (e.g., PanReac AppliChem).
- 0.5-10, 10-100 and 100-1000 µL micropipette set and tips.
- Electrophoresis Kit (trays, combs, chamber and power supply).
- UV Transilluminator/ Gel documentation system.

11.1.6. Requirements for Capillary Electrophoresis of DNA

- 2100 Bioanalyzer® (e.g., Agilent).
- DNA 1000® chips (e.g., Agilent).
- DNA 1000® reagents.
- 2100 Computer with expert software (e.g., Agilent).

Vortex with chip adapter (part of the 2100 Bioanalyzer kit) (Villamizar-Rodríguez and Lombó 2017).

11.2. Method

The methods to be applied for the multiplex PCR reaction are described below.

11.2.1. Primer Design and Synthesis

Specific primer design is carried out in 3 stages: selection of DNA targets, design of PCR primers and synthesis of PCR primers.

- *Selection of DNA targets*

For primer design, it is necessary to select DNA targets that should be specific for each pathogen. For this purpose, obtaining the genome of each pathogen from DNA databases such as GenBank constitutes the first stage of the process.

In the selection process, it is important to avoid genes with high variation or genes related to virulence/resistance properties that can only be found in some strains of the particular species. The selected target gene should have a well conserved sequence or be common to all strains of the target pathogen.

- *Design of PCR primers*

After the identification of the gene sequence target, different programmes available on-line or installed software versions can be used to design PCR

primers. For example, Primer3 and Fast PCR are useful tools for determining the final size of the amplicons.

This step is necessary to obtain amplicons of different sizes, which will be visualised later. Relevant software additionally allows the addition of GC clusters at the end of the selected sequences. Fast PCR also allows primer evaluation to avoid primer-dimer and cross-amplification.

- *Synthesis of PCR primers*

The designed oligonucleotides are synthesised by the manufacturers as lyophilised or dissolved in specific buffer solutions. The synthesised products should be stored in appropriate conditions in laboratories until they are used as stock solutions (Villamizar-Rodríguez and Lombó 2017).

11.2.2. Multiplex Primer Optimisation

- The individual performance of each primer pair is tested with the corresponding gDNA target. It is recommended to use a concentration of 1 μM for each primer. During the primer design process, the melting temperature (Tm) is fixed by following the data obtained from the software.
- The concentration of each primer is set within a certain Tm range. This Tm range is tested starting at 2 °C below the theoretical Tm and increasing by 2 °C in each trial.
- Primer concentrations of 0.4, 0.6, 0.8 and 1 μM for each oligonucleotide are tested.
- The PCR process is performed and the best combination of two concentrations and Tm temperature is selected to provide the most efficient PCR product yield.

- A second primer pair is selected. It is tested in a duplex reaction. The amplification result is evaluated with this combination of two Tm and concentration.
- The combination considered to be more efficient than the previous duplex reaction is selected. The addition of another primer pair should be continued, and the Tm/concentration combination should be retested.
- New primer pairs are added sequentially using all primers and gDNA targets in the same reaction until the desired amplifications are obtained.

11.3. Sampling of Food Matrices

Samples should be taken from foods for microbiological and molecular analyses in accordance with standards outlined by regulatory authorities (e.g., FDA) for sampling procedures. If samples are not to be processed immediately, they should be stored under appropriate conditions until use (Villamizar-Rodríguez and Lombó 2017).

11.4. Pre-enrichment

Pre-enrichment process is carried out to ensure adaptation and development, especially considering the possibility of destruction of the microorganism cells. The procedures carried out for this purpose are given below.

The medium for pre-enrichment (e.g. Germán Villamizar Universal Medium-GVUM) is weighed according to the manufacturer's instructions and supplemented with distilled water to achieve the desired volume. Sterilisation should be carried outin an autoclave at 121 °C and 1.1 atm pressure for 15 minutes.

Transfer 25 g of the food matrix should be transferred to sterile bags with filter membranes using sterile sampling devices. The pre-prepared medium is directly added from the sterile bottle into the bags until a total weight of 100 g

is reached, considering the sample and the pre-enrichment medium. The homogenization should be carried out in a stomacher and should be incubated for 24 hours at an appropriate temperature according to the microorganism type (Villamizar-Rodríguez and Lombó 2017).

11.5. Genomic DNA Extraction

After incubation, 1 mL samples are collected from each sample bag using a sterile serological pipette and transferred to 1.5 mL centrifuge tubes. The tubes are centrifuged at 13.523 × g for 1 min. The supernatant is discarded and 180 µL of PrepMan® Ultra reagent is added. The thorough mixing can be accompanished by vortexing or pipetting. Then, the tubes are placed in a thermal block or water bath at 100 °C for 10 min. At the end of the incubation period, the tubes are removed and allowed to cool down to room temperature. The sample is centrifuged at 12.000 rpm for 2 minutes. Then 100 µL of the süpernatant is taken and placed in another sterile tube. It is stored at 4 °C until use.

11.6. PCR Setup

The Master Mix is prepared following the manufacturer's instructions and considering the optimised concentration for each primer pair. To compensate for possible pipetting errors during preparation, at least one or two reactions more than the number of samples to be tested are calculated. 1-2 µL of the previously prepared genomic DNA samples are added in to the PCR tubes. The desired volume of the relevant Master Mix is added to the main mixture. The tubes are placed in the thermal cycle and the amplification programme is started. At the end of the process, the samples are stored at 4 °C for the next step (Villamizar-Rodríguez and Lombó 2017).

11.7. Agarose Gel Electrophoresis of DNA

To prepare 5% agarose, add 5 g agarose is added to 100 mL TBE buffer and mixed well. It is heated in the microwave until dissolved (~ 3 minutes). After allowing it to cool for a few minutes and then add 5 μL/100 mL DNA dye is added and mixed thoroughly avoiding excessive bubble formation. An electrophoresis tray and is prepared, and the agarose is transferred into it. Agarose is dispersed homogeneously and a comb is placed. When the gel solidifies, the comb is removed, taking care not to break the gel. Pipette the corresponding volume of loading buffer is pipetted into each sample tube containing PCR product. Each sample is placed in a well and a DNA Ladder is usually placed in the first well.

The electrophoresis is started by programming the power unit to the correct voltage and time. After completion, the gel is removed and transferred to a UV transilluminator/gel documentation system.

11.8. Capillary Electrophoresis of DNA

Today, traditional capillary electrophoresis methods have been replaced by techniques based on the Lab-on-chip (LOC) technology approach. These techniques are described as new miniaturised devices that allow for cleaner, more accurate and faster electrophoresis studies. LOC devices have a network of microchannels and wells printed on the inner glass, silica or polymeric surface of the cartridge. Pressure or electrokinetic forces cause displacement of the sample along the channel and allow separation of the different DNA fragment or molecule to be detected by fluorescence in the analyser. For LOC DNA chips and kits of the 2100 Bioanalyzer System, the following instructions can be followed.

The Gel-Paint mixture is allowed to equilibrate to room temperature for 30 minutes. The syringe plunger is positioned at 1 mL and placed in the DNA 1000 chip preparation station. After the gel-dye temperature has equilibrated, pipette 9 μL into the well marked with a round "G", taking care to avoid bubbles in the chip. Close the filling station and press the plunger until a "click" is heard. The plunger is released after waiting for 60 seconds. The plunger is then slowly withdrawn to the 1 mL position and the priming station is opened. 9 μL is pipetted into each of the other two wells marked with a square "G". Load 5 μL of gel-dye mixture is loaded into the 12 sample wells, taking care to ensure that no wells are left empty and no bubbles are created in the wells. The chip is placed in the vortex adaptor and mixed at 28.97 × g for 1 min. The chip is placed the chip in the 2100 Bioanalyzer, the lid is closed and checked that the chip is recognised by the software. The reaction is started. The results can be viewed, analysed and edited in the data window of the 2100 Expert software (Villamizar-Rodríguez and Lombó 2017).

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