MOLECULAR DIAGNOSTIC PCR HANDBOOK

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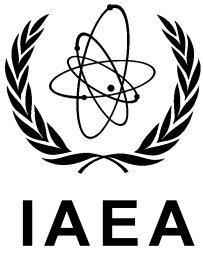

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PREFACE

The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture is involved in agricultural research and development and assists Member States of FAO and IAEA in improving strategies to ensure food security through the use of nuclear techniques and related biotechnologies, where such techniques have a valuable and often unique role. In particular, molecular diagnostic methods have rapidly evolved in the past twenty years, since the advent of the Polymerase Chain Reaction (PCR). They are used in a wide range of agricultural areas such as, improving soil and water management; producing better crop varieties; diagnosing plant and animal diseases; controlling insect pests and improving food quality and safety.

The uses of nucleic acid-directed methods have increased significantly in the past five years and have made important contributions to disease control country programmes for improving national and international trade. These developments include the more routine use of PCR as a diagnostic tool in veterinary diagnostic laboratories. However, there are many problems associated with the transfer and particularly, the application of this technology. These include lack of consideration of: the establishment of quality-assured procedures, the required set-up of the laboratory and the proper training of staff. This can lead to a situation where results are not assured.

This book gives a comprehensive account of the practical aspects of PCR and strong consideration is given to ensure its optimal use in a laboratory environment. This includes the setting-up of a PCR laboratory; Good Laboratory Practice and standardised of PCR protocols. Examples of Standard Operating Procedures as used in individual specialist laboratories and an outline of training materials necessary for PCR technology transfer are presented. The difficulties, advantages and disadvantages in PCR applications are explained and placed in context with other test systems.

Emphasis is placed on the use of PCR for detection of pathogens, with a particular focus on diagnosticians and scientists from the developing world. It is hoped that this book will enable readers from various disciplines and levels of expertise to better judge the merits of PCR and to increase their skills and knowledge in order to assist in a more logical, efficient and assured use of this technology.

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Chapter 1

CHAPTER ONE
BACKGROUND

1. Aims of this book

The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture is involved in research and development in the fields of agriculture with particular responsibility to Member States. Nuclear techniques play an increasingly valuable and often unique role in agricultural research and development. They are used for a wide range of purposes such as improving soil and water management, producing better crop varieties, diagnosing animal diseases, controlling insect pests and improving food quality and safety. As such the Polymerase Chain Reaction (PCR), with its massive potential and already proven value, is at the forefront of debate and interest to a wide range of scientists in developing countries.

The transfer of PCR[1] technology, although challenging, is often made without adequate consideration of the way it is set up, for the training of staff and for exactly how it is to be used. Implementation of this modern technology that appears to promise all, can potentially hold many dangers and conventional techniques should never be ignored at the expense of the PCR alone. A well-considered argument as to why PCR should be set up, along with considerations of the cost benefit in the short and long term, is necessary. This book is intended to give a concise overview of the practical aspects of PCR; to consider its best use in terms of laboratory practice; setting up of laboratories to perform PCR; GLP and standardisation of PCR protocols; to highlight difficulties as well as advantages in its application; to deal with more recent advances in the PCR methods and to place the PCR into context of other tests. It is hoped that readers of all levels and disciplines can be in a better position to judge the merits of the techniques and to develop the technology in a logical and efficient way. The book will be detailed in many areas as a direct help to those involved in everyday PCR. Thus, specific examples of protocols as they are being used as standard operating procedures (SOP's) in individual laboratories; examples of routine uses; latest developments and potential of PCR technologies and an outline of training material necessary for PCR technology transfer, are given. Specific emphasis is placed on the use of PCR for diagnosis of infectious diseases, aimed at diagnosticians and scientists of the developing world.

2. What is PCR?

Molecular biology has been revolutionised by PCR, a method that efficiently increases the number of DNA molecules in a logarithmic and controlled fashion. The concept of DNA amplification by PCR is simple and its impact has been extraordinary. Kary Mullis conceived PCR in 1983 and the first PCR publication appeared in 1985. Every year thereafter, the number of papers on PCR has risen exponentially

In 1989, Science magazine selected PCR as the "major scientific development" and Taq polymerase, the enzyme essential to PCR's success, as "molecule of the year". In 1993, Kary Mullis received the Nobel prize for chemistry. The advent of PCR meant that insufficiencies in the quantity of DNA were no longer a limitation in molecular biology research or diagnostic procedures. It is indeed difficult to find publications in the biological sciences that do not describe the application of PCR in some or other way. The chemistry involved in PCR depends on the complementarity (matching) of the nucleotide bases in the double-stranded DNA helix. When a molecule of DNA is sufficiently heated, the hydrogen bonds holding together the double helix are disrupted and the molecule separates or denatures into single strands.

[1] The PCR process and 5' nuclease process are covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman-la Roche, Ltd.

If the DNA solution is allowed to cool, the complementary base pairs can reform to restore the original double helix. In order to use PCR, the exact sequence of nucleotides that flank (lay on either side of) the area of interest (the target area that needs to be amplified), must be known. This is the absolute minimum data necessary before a typical PCR reaction can be used.

This data is necessary for the design of PCR primers that are 5'-3' oligonucleotides of about 20 nucleotides in length. These are designed to be complementary to the flanking sequences of the target area, as mentioned previously. Thus, the researcher has to either use previous data (known information of sequences) or, if this is unavailable, determine the sequence of these regions experimentally. The two primers (primer pair) can then be synthesized chemically and will then serve as leaders or initiators of the replication step.

The key to the replication reaction is that it is driven by a heat-stable polymerase molecule that reads a template DNA in the 3'-5' direction and synthesises a new complementary template in the 5'-3' direction, using free dideoxy nucleoside triphosphates (dNTP's = nucleotide bases) as building blocks.

2.1. PCR principles

The principles of the PCR are shown in **Figures 1-5.** These figures illustrate the fundamental aspects of the steps leading to the amplification of DNA in a stepwise PCR and introduce terms and some practical features. These can be summarised as:

2.1.1. Denaturation

When a double stranded DNA (dsDNA) molecule is heated to 94^{o}C, the paired strands will separate (denature). This allows the primers access to the single stranded DNA (ssDNA) templates.

2.1.2. Annealing

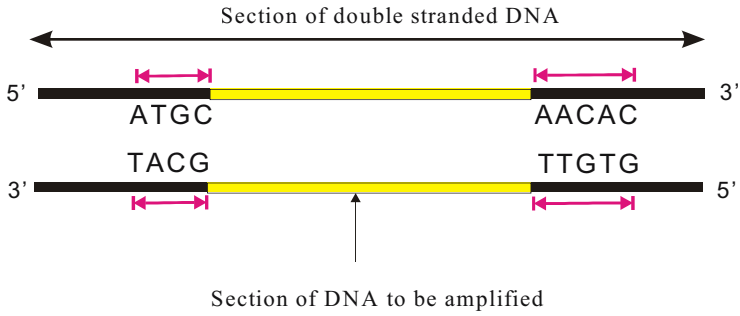
The reaction mixture is cooled (about 50^{o}C) to allow primers to select and bind (hybridize) to their complementary positions on the ssDNA template molecules.

2.1.3. Elongation

The ssDNA/primer solution is heated to 72^{o}C. In the presence of the heat stable polymerase, PCR buffer, dNTP's and magnesium (Mg^{2+}) molecules, the replication procedure begins.

With each repetition of this cycle, the target is doubled and soon, after about 30 cycles, the reaction will yield in excess of 1 million copies of the target DNA fragment.

1a. Amplification target



1b. PCR components

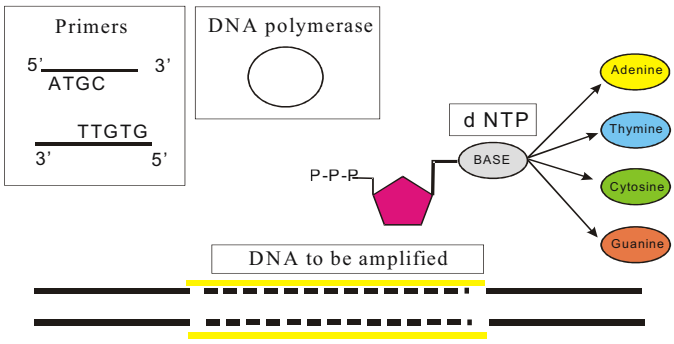


Figure 1a. Part of a double stranded DNA molecule is indicated schematically, with some bases highlighted on either side of the region to be amplified. These are the primer docking sites. Note the anti-parallel 5' to 3' orientation of the two strands.
Figure 1b. The components needed for PCR, including primers, polymerase enzyme and a mixture of bases.

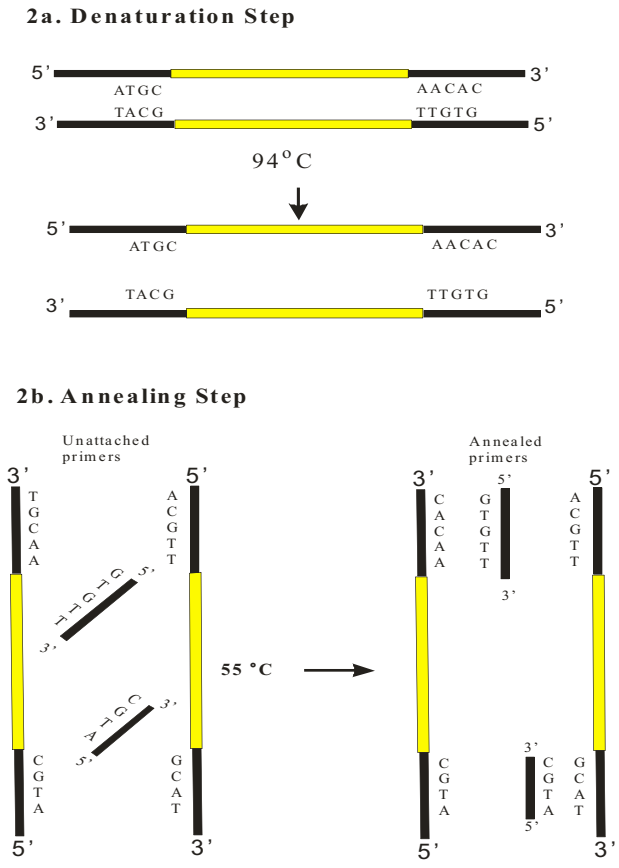


Figure 2a. The effect of heating dsDNA at 94^{0}C. Hydrogen bonds are broken and the complementary strands of dsDNA separate.
Figure 2b. Specific primers with complementary bases to ssDNA template are added. At 55^{0}C the primers anneal with the complementary ssDNA bases.

Figure 3a. First PCR cycle Elongation step at 72°

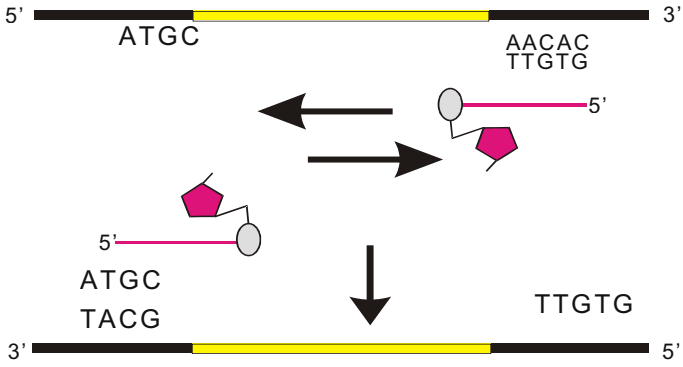


Figure 3b. Amplification

Figure 3a. Two ssDNA strands with annealed primers are indicated. The addition of a polymerase enzyme and a mixture of dNTPs allows for the production of a new strand through the addition of complementary bases to this growing strand. The DNA produced (amplicon) is derived from both template DNA strands.
Figure 3b. The target DNA segment is duplicated following a sequence of events initiated by the binding of primer pairs to the flanking regions.

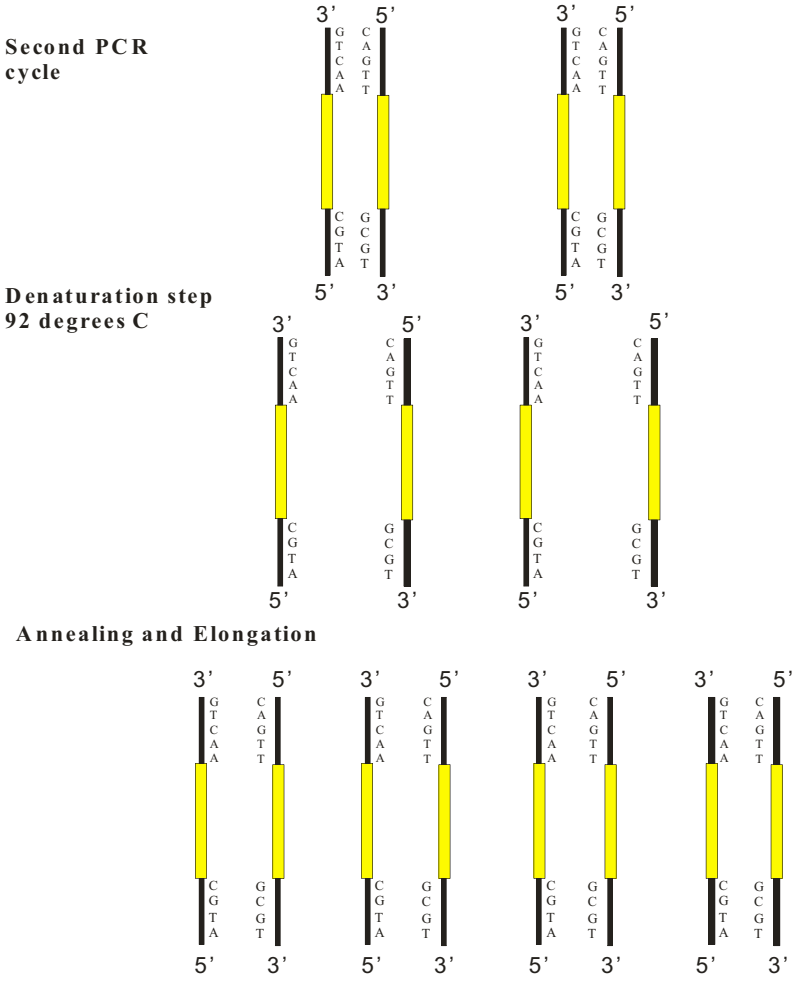


Figure 4. The second cycle of a PCR reaction: The two double strands of DNA produced (amplicon) in the first cycle are denatured to give 4 single strands. Addition of primers, enzyme and bases allows these to be copied as in the first cycle, to produce 4 double stranded amplicons.

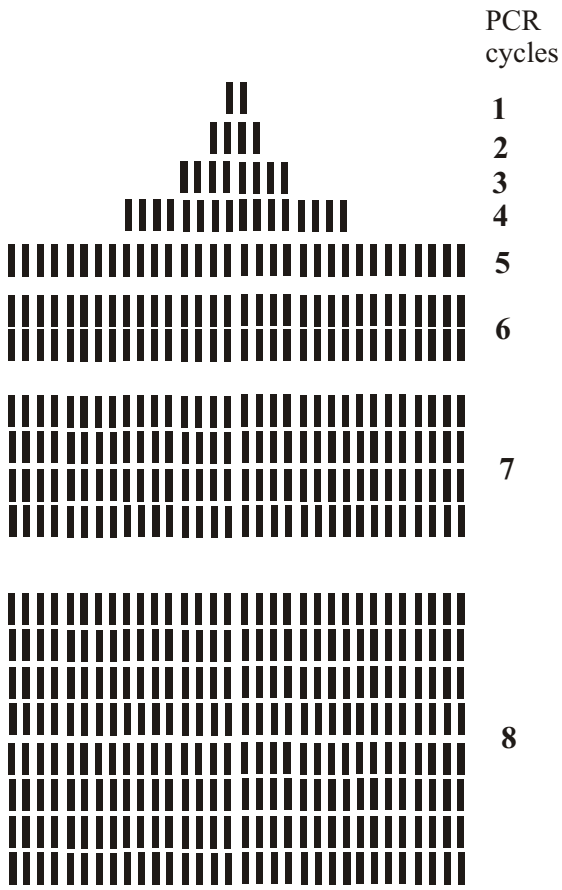


Figure 5. The increase in DNA with increased number of PCR cycles (denaturation, annealing and extension). In can be seen graphically that the number of molecules increase rapidly in a short time. In 30 cycles of PCR, a single target DNA molecule is increased to billions of copies (2^{30}).

3. What is the use of PCR?

PCR is primarily a method to spectacularly amplify a desired DNA fragment (piece of DNA) in order to increase the target DNA to detectable levels. This has had a profound effect on all molecular studies including those in the diagnostic area. It suddenly changed the way sensitivity was defined, as we are now able to detect very low numbers of pathogens with great accuracy. We are also able to detect carrier animals more easily, detect mixed populations of pathogens in an infection and to determine pathogen load. The method has found numerous related applications in molecular biology and now forms the fundamental basis of most studies involving genetic material.

As illustration of this uniqueness, PCR can be used very effectively to modify DNA. Such modification may include the addition of restriction enzyme sites (in order to facilitate cloning requirements) or regulatory elements (e.g., the addition of promoter sequences to a DNA cistron). A further type of modification can be the generation of desired site-directed mutations in a gene, inclusive of sequence alterations, additions or deletions.

Cycle-sequencing, a modification of the classical di-deoxy sequencing method pioneered by Fred Sanger in the early 1980's, uses the principles of PCR to rapidly perform sequence reactions in a thermal cycler.

The way in that PCR has dramatically impacted on diagnosis of genetic and infectious disease is one of the focus areas of this book.

For PCR-directed diagnostics, it is for example, possible to work with crude samples and minute amounts of material, that may include degraded templates, blood, sperm, tissue, individual hairs, etc.

In related applications, PCR today plays a central role in genetic typing of organisms or individuals and molecular epidemiology.

Some examples of the general application of PCR in molecular biology are given in **Table 1** and specific focus is given to the diagnostic uses of this technique in further sections of the book.

Table 1. General Applications of PCR

1. Diagnosis of pathogens	a) PCR b) Nested PCR c) Quantitative PCR d) Multiplex PCR e) Differential on-line and real time PCR
2. Typing genetic markers	a) RFLPs b) AFLPs c) Short tandem repeat polymorphisms
3. DNA template for	a) Genomic mutation screening intron-specific primers flanking exons b) RT-PCR cDNA used as templates for pairs of exon-specific primers to generate overlapping fragments
4. Detecting point mutations	a) Restriction site polymorphisms b) Allele specific amplification
5. cDNA cloning	a) DOP-PCR b) RACE
6. Genomic DNA cloning	New members of a DNA family a) DOP-PCR. Whole genome or subgenomic amplification b) DOP-PCR c) Linker-primed PCR
7. Genome walking	a) Inverse PCR b) Bubble linker (vectored) PCR c) IRE-PCR
8. DNA templates for DNA sequencing	a) ssDNA by asymmetric PCR b) dsDNA for direct sequencing or for cloning followed by sequencing
9. *In vitro* mutagenesis	a) 5' add-on mutagenesis to create a recombinant PCR product b) Mispaired primers to change a single predetermined nucleotide

3.1. PCR and infectious diseases-the veterinary picture

Infectious diseases can be caused by microbial pathogens, include agents of fungal, protozoan, bacterial, clamydial, rickettsia and viral nature. Despite many advances in diagnostics and vaccinology, infectious diseases still have devastating consequences for agricultural, economies, worldwide.

Three examples of such devastation since the 1990s with regard to animal husbandry, include the emergence of the prion, bovine spongiform encephalopathy (BSE); the huge outbreaks of foot-and-mouth disease (FMD) in Europe and avian influenza (AI) in Asia and elsewhere.

A great many of these infectious diseases can be transmitted from vertebrate animal to man (called zoonoses) and in fact, more than 200 such zoonotic diseases are known. Infectious diseases are typically transmitted through the skin or eyes (direct contact, insect vectors, bite wounds, sexual contact). In other cases, the agents are airborne and infect the epithelial cells lining the respiratory tract from where further systemic infection may proceed. Additional sources of infectious microorganisms are contaminated food and water with a route of infection through the mouth and alimentary tract, or through the respiratory system.

Many infectious diseases can be considered new, emerging, re-emerging or resurgent. These are diseases that have been described in the last 10-30 years, or they are caused by specific modifications of agents that are already present in the environment (e.g., in a different host reservoir). These agents then evolve, mutate or are otherwise epidemiologically affected by changing conditions or other selective advantage.

Typically, re-emerging diseases are those diseases that have persisted at a subdued level in the population and recur as a result of antimicrobial drug resistance or any other changes that might favor dramatic increases in disease incidence.

Re-emerging diseases can also be described as resurgent, pertinently referring to an abrupt increase in incidence or geographic distribution of the particular disease. The emergence and re-emergence of diseases are clearly related to changes in the infectious pathogen, the vector or transmission system and the host population.

Apart from drug resistance already mentioned, some other epidemiologically important changes are: mutations that lead to increased virulence; changes in the distribution or activity of vectors; globalization and increased travel; war, population explosions; climatic and ecological changes; geographical displacement of species; movement into previously uninhabited areas; poverty and breakdown of animal or healthcare systems; changes in agriculture and industrialisation.