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# Early, Rapid and Sensitive Veterinary Molecular Diagnostics - Real Time PCR Applications

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Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

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# Foreword

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, nuclear and nuclear related molecular diagnostic methods have rapidly evolved in the past 20 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 5 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, Real-Time PCR and PCR-Sequencing as diagnostic and characterization tools 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 real-time PCR and strong consideration is given to ensure its optimal use in a diagnostic laboratory environment. This includes the basic principles, setting-up of a Real-Time PCR laboratory; Good Laboratory Practice and Standard Operating Procedures; Diagnostic Implementation, Execution and Interpretation, Analysis and Problem Solving. Examples of Standard Operating Procedures as used in individual specialist laboratories and an outline of training materials necessary for Real-Time PCR technology transfer are presented. The difficulties, advantages and disadvantages in PCR and Real-Time PCR applications are explained and placed in context with other test systems.

Emphasis is placed on the use of Real-Time 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 early and rapid nuclear and nuclear related molecular diagnostic approaches and to increase their skills and knowledge in order to assist in a more logical, efficient and assured use of these technologies.

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# Chapter 1
# Background

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## 1.1 Aims of This Book

*The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (NAFA) and Nuclear Sciences and Applications Laboratories (NAAL) are jointly involved in research and development in the fields of agriculture with particular responsibility to the diagnostic laboratories of their Member States. Nuclear related techniques play an increasingly valuable and often unique role in agricultural research and development. They have been applied in a wide range of disciplines including improving soil and water management; producing better crop varieties; diagnosing animal diseases; controlling insect pests and improving food quality and safety. The temperature template amplification technologies - Polymerase Chain Reaction (PCR), with their already proven value and massive potential, are at the forefront of debate and interest to a wide range of scientists in developing and developed countries alike. It is relevant to note that real-time PCR (rt-PCR) and quantitative PCR (qPCR) are derivatives of the polymerase chain reaction. This book extends the information of a previous publication by the IAEA (Molecular Diagnostics PCR Handbook, Viljoen, G.J., Nel, L.H., and Crowther, J.R., Springer, 2005) and deals with the basic principles of both PCR and real-time PCR with a view to their use for the early and rapid diagnosis of harmful pathogens of animals and those of zoonotic nature origin to form the basis of real-time PCR technologies and applications.*

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The transfer of PCR technology is challenging and often made without adequate consideration of the way it is set up; the training of staff and as to exactly how it is to be used. Implementation of this modern technology, that promises so much, holds 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 considering its best use in terms of laboratory practice; the setting up of laboratories to perform PCR; and GLP and standardisation of PCR protocols. Difficulties, as well as advantages in PCR application, are highlighted dealing with more recent advances in methods and putting the PCR in context of other tests. It is hoped that the book will appeal to readers of all disciplines and levels so that they can better judge the merits of the techniques and develop the technology in a more logical and efficient way. The book is detailed in many areas as a direct help to those involved in everyday PCR. Specific examples are given of protocols used as standard operating procedures (SOP's) in individual laboratories; routine uses; latest developments and potential of PCR technologies and training material necessary for PCR technology transfer. Specific emphasis is placed on the use of PCR for diagnosis of infectious diseases, aimed at diagnosticians and scientists of the developing world. All patents and licences, directly or indirectly related to the technologies and processes addressed in this book should be respected.

## 1.2 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 complementarities (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. 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.

## 1.3 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 1980s, uses the principles of PCR to rapidly perform sequence reactions in a thermal cycler. Equally often used is the sensitive protein-DNA or protein-RNA interaction analysis (e.g., for the activation of Polymerase type II promoters) approach of the Maxim and Gilbert sequence reactions, using nuclear applications to study protein and nucleic acid interactions on a molecule by molecule basis. The way in that PCR has dramatically impacted on diagnosis of genetic and infectious disease is one of the foci of this book. For PCR-directed diagnostics it is 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 plays a central role in genetic typing and molecular characterization of organisms or individuals and molecular epidemiology. Some examples of the general application of PCR in molecular biology are given in **Table 1.1** and specific focus is given to the diagnostic uses of this technique in further sections of the book.