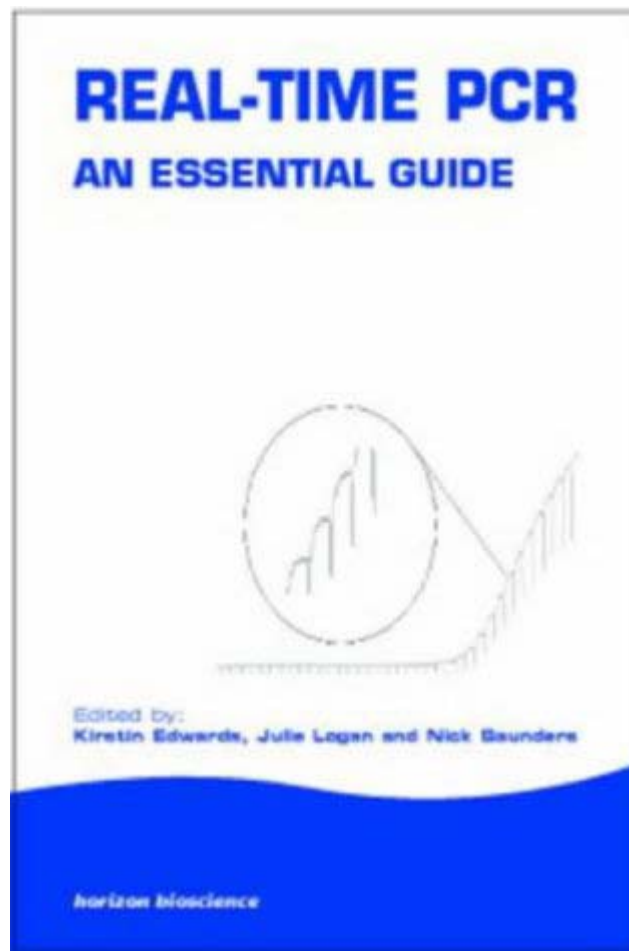


Real-Time PCR: An Essential Guide



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Real-time PCR (RT-PCR) is a powerful and rapid technique for nucleic acid amplification. The accumulation of specific products in a reaction is monitored continuously during cycling. This is usually achieved by monitoring changes in fluorescence within the PCR tube. This essential manual presents a comprehensive guide to the most appropriate and up-to-date technologies and applications as well as providing an overview of the theory of this important technique. Written by recognized experts in the field this timely and authoritative volume is an essential requirement for all laboratories using PCR. Topics covered include: Real-time PCR instruments and probe chemistries, set-up, controls and validation, quantitative real-time PCR, analysis of mRNA expression, mutation detection, NASBA, application in clinical microbiology and diagnosis of infection.

Key Features:

- Completely up-to-date
- Internationally renowned authors
- Fully illustrated throughout
- Comprehensive coverage of the latest RT-PCR technology
- Explains key principles and concepts
- Includes a broad range of applications
- Useful index

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Real-Time PCR: An Essential Guide

Chapter Abstracts

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Chapter 1

An Introduction to Real-Time PCR

N.A. Saunders

The development of instruments that allowed real-time monitoring of fluorescence within PCR reaction vessels was a very significant advance. The technology is very flexible and many alternative instruments and fluorescent probe systems have been developed and are currently available. Real-time PCR assays can be completed very rapidly since no manipulations are required post-amplification. Identification of the amplification products by probe detection in real-time is highly accurate compared with size analysis on gels. Analysis of the progress of the reaction allows accurate quantification of the target sequence over a very wide dynamic range, provided suitable standards are available. Further investigation of the real-time PCR products within the original reaction mixture using probes and melting analysis can detect sequence variants including single base mutations. Since the first practical demonstration of the concept real-time PCR has found applications in many branches of biological science. Applications include gene expression analysis, the diagnosis of infectious disease and human genetic testing. Due to their capability in fluorimetry the real-time machines are also compatible with alternative amplification methods such as NASBA provided a fluorescence end-point is available.

Chapter 2

An Overview of Real-Time PCR Platforms

J.M.J. Logan and K.J. Edwards

Real-time PCR continues to have a major impact across many disciplines of the biological sciences and this has been a driver to develop and improve existing instruments. From the first two commercial platforms introduced in the mid 1990s, there is now a choice in excess of a dozen instruments, which continues to increase. Advances include faster thermocycling times, higher throughput, flexibility, expanded optical systems, increased multiplexing and more user-friendly software. In this chapter the main features of each instrument are compared and factors important to weigh up when deciding on a platform are highlighted.

Chapter 3

Homogeneous Fluorescent Chemistries for Real-Time PCR

M.A. Lee, D.J. Squirrell, D.L. Leslie, and T. Brown

The development of fluorescent methods for a closed tube polymerase chain reaction has greatly simplified the process of quantification. Current approaches use fluorescent probes that interact with the amplification products during the PCR to allow kinetic measurements of product accumulation. These probe methods include generic approaches to DNA quantification such as fluorescent DNA binding dyes. There are also a number of strand-specific probes that use the phenomenon of Fluorescent Resonance Energy Transfer (FRET). In this chapter we describe these methods in detail, outline the principles of each process, and describe published examples. This text has been written to provide an impartial overview of the utility of different assays and to show how they may be used on various commercially available thermal cyclers.

Chapter 4

Performing Real-time PCR

K.J. Edwards

Optimisation of the reagents used to perform PCR is critical for reliable and reproducible results. As with any PCR initial time spent on optimisation of a real-time assay will be beneficial in the long run. Specificity, sensitivity, efficiency and reproducibility are the important criteria to consider when optimising an assay and these can be altered by changes in the primer concentration, probe concentration, cycling conditions and buffer composition. An optimised real-time PCR assay will display no test-to-test variation in the crossing

threshold or crossing point and only minimal variation in the amount of fluorescence. The analysis of the real-time PCR results is also an important consideration and this differs from the analysis of conventional block-based thermal cycling. Real-time PCR provides information on the cycle at which amplification occurs and on some platforms the melting temperature of the amplicon or probe can be determined.

Chapter 5

Internal and External Controls for Reagent Validation

M.A. Lee, D.L. Leslie and D.J. Squirrell

PCR applications that require a high confidence in the result should be designed to control for the occurrence of false negatives. False negatives can occur from inhibition of one or more of the reaction components by a range of factors. While an external, or batch control is often used, the ideal control is one that is included in the reaction cocktail in a multiplex format. Early approaches used different sized amplicons combined with end-point analysis. Fluorescent homogenous real-time PCR methods have a number of advantages for implementing internal controls. Here we discuss the application and development of molecular mimics for use as controls in real-time PCR, and explain a number of concepts and experimental considerations that will aid in the optimisation of the controlled multiplexed assay.

Chapter 6

Quantitative Real-Time PCR

N.A. Saunders

Unlike classical end-point analysis PCR, real-time PCR provides the data required for quantification of the target nucleic acid. The results can be expressed in absolute terms by reference to external quantified standards or in relative terms compared to another target sequence present within the sample. Absolute quantification requires that the efficiency of the amplification reaction is the same in all samples and in the external quantified standards. Consequently, it is important that the efficiency of the PCR does not vary greatly due to minor differences between samples. Careful optimisation of the PCR conditions is therefore required. The use of probes in quantitative real-time PCR improves its performance and a range of suitable systems is now available. Generally quantitative real-time assays have excellent performance characteristics including a wide dynamic range, high sensitivity and accuracy. This has led to their use in a wide range of applications and two examples are presented. Viral quantification is now an important factor in the control of infection. The problems associated with virus quantification in cytomegalovirus (HCMV) infection are similar to those presented by other viruses. Quantitative PCR is finding an increasing role in the diagnosis of cancer. The assessment of *c-erbB2/Her2/neu* gene duplication is useful in predicting the disease prognosis in breast cancer. Several different real-time quantitative PCR protocols are available for these applications and have been applied successfully to their respective diseases.

Chapter 7

Analysis of mRNA Expression by Real-Time PCR

Stephen A Bustin and Tania Nolan

The last few years have seen the transformation of the fluorescence-based real-time reverse transcription polymerase chain reaction (RT-PCR) from an experimental tool into a mainstream scientific technology. Assays are simple to perform, capable of high throughput, and combine high sensitivity with exquisite specificity. The technology is evolving rapidly with the introduction of new enzymes, chemistries and instrumentation and has become the "Gold Standard" for a huge range of applications in basic research, molecular medicine, and biotechnology. Nevertheless, there are considerable pitfalls associated with this technique and successful quantification of mRNA levels depends on a clear understanding of the practical problems and careful experimental design, application and validation.

Chapter 8

Mutation Detection by Real-Time PCR

K.J. Edwards and J.M.J Logan

Real-time PCR is ideally suited for analysis of single nucleotide polymorphisms (SNPs) and has been increasingly used for this purpose since the advent of real-time PCR and as whole genome sequences have

become available. It requires methods that are rapid, sensitive, specific and inexpensive, and several real-time methods have evolved which fulfil these requirements. Additionally real-time PCR is a technique that is readily amenable to automation and no post-PCR handling is required. Different formats have been applied including hybridisation probes with melting curve analysis, hydrolysis probes, molecular beacons and scorpion primers. SNP detection by real-time PCR has found applications in diagnosis of human disease, pharmacogenetics, clinical microbiology and drug development, and has replaced techniques such as sequencing, single strand conformation polymorphism and restriction enzyme digestion.

Chapter 9

The Quantitative Amplification Refractory Mutation System

Parvinder Punia and N.A. Saunders

The amplification refractory mutation system (ARMS), which has also been described as allele-specific PCR (ASP) and PCR amplification of specific alleles (PASA), is a PCR-based method of detecting single base mutations. ARMS has been applied successfully to the analysis of a wide range of polymorphisms, germ-line mutations and somatic mutations. The technique has the ability to discriminate low-levels of the mutant sequence in a high background of wild-type DNA. In an ARMS PCR the terminal 3' nucleotide of one of the PCR primers coincides with the target mutation. Most applications of the method rely on 'end-point' analysis, utilising the classic gel-electrophoresis method. However, end-point analysis can only assess the presence or absence of mutant or wild-type sequences and does not give an indication of the ratio of mutant to wild-type in a mixed population of DNA. Here we describe a real-time PCR adaptation of ARMS, quantitative ARMS, that allows measurement of the size of the population of each variant in a mixture. A method for the detection of human hepatitis B virus mutations that confer resistance to the antiviral lamivudine is described as an example.

Chapter 10

Real-Time NASBA

Sam Hibbitts and Julie D Fox

NASBA is an isothermal nucleic acid amplification method that is particularly suited to detection and quantification of genomic, ribosomal or messenger RNA. The product of NASBA is single-stranded RNA of opposite sense to the original target. The first developed NASBA methods relied on liquid or gel-based probe-hybridisation for post-amplification detection of products. More recently, real-time procedures incorporating amplification and detection in a single step have been reported and applied to a wide range of targets. Thus real-time NASBA has proved to be the basis of sensitive and specific assays for detection, quantification and analysis of RNA (and in one case DNA) targets. Molecular beacons have been utilised for detection of NASBA products in all published real-time procedures whether for commercially-available kits or for in-house diagnostic assays. As experience in design of such fluorescent-labelled probes increases and fluorimeters suitable for their detection become widely available, real-time NASBA methodology will be confirmed as a suitable alternative to other real-time amplification methods such as reverse transcriptase PCR (RT-PCR).

Chapter 11

Applications of Real-time PCR in Clinical Microbiology

Andrew David Sails

The introduction of real-time PCR assays to the clinical microbiology laboratory has led to significant improvements in the diagnosis of infectious disease. There has been an explosion of interest in this technique since its introduction and several hundred reports have been published describing applications in clinical bacteriology, parasitology and virology. There are few areas of clinical microbiology which remain unaffected by this new method. It has been particularly useful to detect slow growing or difficult to grow infectious agents. However, its greatest impact is probably its use for the quantitation of target organisms in samples. The ability to monitor the PCR reaction in real-time allows accurate quantitation of target sequence over at least six orders of magnitude. The closed-tube format which removes the need for post-amplification manipulation of the PCR products also reduces the likelihood of amplicon carryover to subsequent reactions reducing the risk of false-positives. As more laboratories begin to utilise these methods standardisation of assay protocols for use in diagnostic clinical microbiology is needed, plus participation in external quality control schemes is required to ensure quality of testing.

Chapter 12**Application of Real-Time PCR to the Diagnosis of Invasive Fungal Infection**

N. Isik and N.A. Saunders

The management of invasive fungal infections has been hampered by the inability to make a diagnosis at an early stage of the disease. Molecular diagnosis by PCR appears very promising since fungal DNA can be detected in the blood of infected patients earlier than when using conventional methods. Recently, interest in the diagnosis of invasive fungal infections by real-time PCR has increased. Real-time methods also have quantitative properties and are useful both for initial diagnosis and to assess the response to treatment. Many recent studies have combined serological tests with measurement of fungal DNA by using real-time PCR. Real-time PCR helps early diagnosis and arrangement of treatment protocols for patients with high risk of fungal infection. Here real-time PCR methods for diagnosis of invasive fungal infections are described and discussed.

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