

Quantitative Real-Time PCR

N. A. Saunders

Abstract

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.

Introduction

The early years of the PCR were not without difficulty. One of the greatest barriers to successful exploitation was the problem of contamination that becomes evident due to the high sensitivity of the method. It was also quickly realised that the term ‘quantitative PCR’ could almost be considered an oxymoron due to the non-linearity of the relationship between amplicon yield and starting copy number. The combined effect of these factors was that even a single amplicon contaminating a reaction mixture could give a false positive result. The practical uses of PCR were therefore restricted and there remained many applications requiring highly sensitive and quantitative nucleic acid detection assays that could not be addressed. This led to the development of fixes that rendered PCR at least semi-quantitative. This was generally achieved by limiting the number of temperature cycles to ensure that reactions containing template in the desired quantitative range did not pass the linear phase of product accumulation. Truly quantitative but relatively inconvenient methods were also developed. These were based on either the competitive target or limiting dilution approaches. These methods are used for RNA and DNA copy number measurement in applications such as viral load estimation and gene expression studies. The widespread use of quantitative PCR was therefore already well established by the time real-time PCR machines were introduced.

The problem with using PCR quantitatively stems from the variable kinetics of product accumulation after different numbers of cycles. Although the process is essentially exponential during the early cycles this rate cannot be maintained when nanogram/ μ l quantities of product are present. The main reasons for this are the increasing level of amplicon reannealing during the priming step and the overstretching of reaction resources including polymerase, primers and nucleotides as the quantity of DNA to be synthesized during the elongation phase

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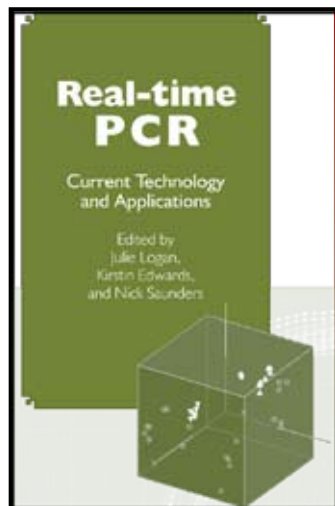
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Real-time PCR (RT-PCR) technology is highly flexible and many alternative instruments and fluorescent probe systems have been developed recently. The decreased hands-on time, increased reliability and improved quantitative accuracy of RT PCR methods have contributed to the adoption of RT PCR for a wide range of new applications.

This essential manual presents a comprehensive guide to the most up-to-date technologies and applications as well as providing an overview of the theory of this increasingly important technique. Renowned experts in the field describe and discuss the latest PCR platforms, fluorescent chemistries, validation software, data analysis, and internal and external controls. This timely and authoritative volume also discusses a wide range of RT-PCR applications including: clinical diagnostics, biodefense, RNA expression studies, validation of array data, mutation detection, food authenticity and legislation, NASBA, molecular halotyping, and much more.

An essential book for all laboratories using PCR.



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