Quantitative PCR Protocols

ETHODS IN MOLECULAR MEDICINE™

Quantitative PCR Protocols

Edited by

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Preface

Since the polymerase chain reaction (PCR) was first developed in 1985, an enormous number of research reports have documented the versatility of this brilliant technique for in vitro amplification of nucleic acids. Although PCR has had a profound impact in many areas of research, contrary to expectation its routine application to the quantitation of nucleic acids has proven problematic in several aspects. The shortcomings are principally caused by the exponential nature of PCR, whereby small variations in amplification efficiency may dramatically affect the yield of amplification product. Even minimal temperature deviations that occur between adjacent wells of a thermocycler or day-to-day variations in the efficiency of nucleic acid preparation can lead to significant differences in the extent of amplification between otherwise identical samples.

However, knowing more about the intrinsic limitations of PCR is the first step towards surmounting the shortcomings associated with this promising methodology. With the introduction of appropriate standards of known amount, which are co-amplified with the sample using the same primers, it is increasingly feasible to address biological or diagnostic questions that are difficult or impossible to answer using any other experimental approach.

The techniques and experimental strategies described in *Quantitative PCR Protocols* are representative of those most generally applicable to routine work at present. Apart from a brief description of the principles of quantitative PCR, the book describes both established and novel strategies, each of which has been applied successfully to such problems as the analysis of eukaryotic gene expression, the quantitation of viral loads in clinical specimens, reporter gene expression, and quantitative oncogene analysis.

Particular emphasis is placed on the underlying principles of the design of competitive or noncompetitive standards, as well as the optimization of the amplification process; these are crucial in any successful quantitative application. Basic problems with the interpretation of the results are addressed as well. Some duplication of important topics has been introduced purposely to offer the reader several approaches to the same problem. It is hoped that this collection of detailed protocols, providing comprehensive and up-to-date information, will be especially useful to researchers and to students needing

to become familiar with the principles of quantitative PCR, and guiding them to set up test systems tailored to their specific practical needs. Since approaches to the amplification of nucleic acids in a quantitative manner and to the technology involved in product detection are subject to continual improvement, *Quantitative PCR Protocols* does not attempt the impossible task of treating every variety of experimental approach in the field. Rather, it depicts a kind of cross-section of realistic possibilities for the user's conception of still more refined assays.

Quantitative PCR—myth or reality? At the present moment the truth lies somewhere in-between and, since the usefulness of quantitation mainly depends on the particular application, only the future will show which assays will prove most useful in individual diagnostic situations.

We are especially indebted to Prof. Hans Wolf and Prof. Wolfgang Jilg for giving us the opportunity to gain substantial experience in the field. Without their confidence and continuous support many things would not have been possible. We also thank Prof. John Walker for his encouragement and Humana Press for their excellent assistance during the assembly of this volume. Finally, we are grateful to all of the contributing authors for their constantly high level of motivation and enthusiasm and, last but not least, for providing such good manuscripts.

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Contents

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Reviews

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Quantitative PCR

A Survey of the Present Technology

Udo Reischl and Bernd Kochanowski

1. Introduction

The polymerase chain reaction (PCR) is a powerful tool for the amplification of trace amounts of nucleic acids, and has rapidly become an essential analytical tool for virtually all aspects of biological research in experimental biology and medicine. Because the application of this technique provides unprecedented sensitivity, it has facilitated the development of a variety of nucleic acid-based systems for diagnostic purposes, such as the detection of viral *(1)* or bacterial pathogens *(2)*, as well as genetic disorders *(3)*, cancer *(4)*, and forensic analysis *(5)*. These recently developed systems open up the possibility of performing reliable diagnosis even before any symptoms of the disease appear, thus considerably improving the chances of success with treatment. For many routine applications, particularly in the diagnosis of viral infections, the required answer is the presence or the absence of a given sequence in a given sample. Therefore, PCR is in able for the early diagnosis of HCV infection *(6)*, HSV encephalitis *(7)*, or HIV infection of babies of HIV-positive mothers *(8)*. On the other hand, since even minute amounts of DNA are detected, the medical interpretation of positive results for widespread infectious agents like CMV *(9)* or HHV6 *(10)* turned out to be rather difficult.

Nevertheless, with the continuous development of PCR technology, there is now a growing need, especially in areas, such as therapeutic monitoring *(11–13)*, quality control, disease diagnosis *(14)*, and regulation of gene expression *(15)*, for the quantitation of PCR products, and thereby deducing the number of template molecules present in a sample prior to amplification.

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In contrast to a simple positive/negative determination, inherent features of the amplification process may constrain the use of PCR in cases where an accurate quantitation of the input nucleic acids is required. Although the theoretical relationship between the amount of starting template nucleic acid and the amount of PCR product can be demonstrated under ideal conditions, this does not always apply for most typical biological or clinical specimens. Dealing with PCR-based quantification of nucleic acids, one has always to keep in mind that any parameter that is capable of interfering with the exponential nature of the in vitro amplification process might ruin the in sic quantitative ability of the entire procedure. Even very small differences in the kinetic and efficiency of individual amplification steps will have a large effect on the amount of product accumulated after a limited number of cycles.

Inherent factors that will lead to tube-to-tube or sample-to-sample variability are, for example, thermocycler-dependent temperature deviations, the presence of individual DNA polymerase inhibitors in clinical samples, pipeting variations, or the abundance of the target sequence in the specimen of interest *(16,17)*. Various approaches have been developed in the last few years to circumvent these problems, but the extremely desirable goal of truly quantitative PCR has still proven elusive.

Here we would like to present an overview on the current methodology and to address the advantages as well as the limitations of individual protocols. Since the number of applications is increasing with the volumes of relevant journals, this article should provide a knowledge base for investigators to become familiar with quantitative PCR-based assays and even guide them in setting up their own assay systems. For ease of presentation, a brief summary of statistical aspects of the amplification reaction will be given, followed by a more detailed overview of detection strategies and procedures, and an appraisal of their value in the quantitation of PCR products.

2. Strategies to Obtain a Quantitative Course of Amplification: How to Make an Exponential Reaction Calculable

2.1. Theoretical Framework of PCR

It is well known that the PCR educt is amplified during the PCR procedure in an exponential manner. (Note: throughout the text, we will use the term "PCR educt" for the target of interest prior to amplification, whereas the term "PCR product" refers to the corresponding amplification products.) A mathematical description for the product accumulation within each cycle is:

$$Y_n = Y_{n-1} \cdot (1 + E_v) \text{ with } 0 \leq E_v \leq 1. \quad (1)$$

E_v represents the efficiency of the amplification, Y_n the number of molecules of the PCR product after cycle n, and Y_{n-1} the number of molecules of the PCR

product after cycle $n-1$. To calculate the number of molecules of the PCR product after a given number of cycles from the starting amount of PCR educt, this recursive equation has to be solved. Since E_v stays constant for a limited number of cycles during the exponential phase of the amplification reaction, this is only possible within this particular period. Therefore, the accumulation of the PCR product can be approximately described by **Eq. 2**:

$$Y = X \cdot (1 + E_e)^n \quad (2)$$

Y represents the number of molecules of the PCR product, X the PCR educt molecules, n the number of cycles, and E_e the efficiency with a value between 0 and 1. **Equation 2** is valid only for a restricted number of cycles, usually up to 20 or 30. Then the amplification process slows down to constant amplification rates, and finally it reaches a plateau where the target is not amplified any more. For **Eq. 1** this would result in a steady decline of E_v, until the value reaches 0. The over all efficiency (E) of the amplification process is dependent on the primer/target hybridization, the relative amount of the reactants, especially the DNA polymerase/target quotient, and it may vary with the position of the sample in the thermocycler or the presence of coisolated DNA polymerase inhibitors in different clinical samples. The number of cycles for which **Eq. 2** holds true is partly determined by the amount of PCR educt. Target strand reannealing and enzyme saturation events are leading to a decline of E_v ***(16,17)***.

As described later, is it easy to quantitate the PCR product, but because of varying efficiencies (E_e) and varying numbers of cycles (n) for which **Eq. 2** is valid, the result does not necessarily represent the amount of PCR educt. As already mentioned, inherent tube-to-tube and sample-to-sample variations are potential causes. At least three procedures of a PCR setup are described in the following paragraphs that have been devised to rule out those variabilities. The measures that have to be carried out are dependent on the desired precision. In general, it is much easier to determine relative changes than to quantitate absolute numbers of the PCR educt. For measuring RNA copy numbers, the varying efficiencies of the reverse transcription process have to be normalized, and for low copy numbers of the PCR educt, stochastical problems have to be taken into account ***(18)***.

2.2. PCR-Based Quantification with External Standards

A serial dilution of a known amount of standard, often a plasmid, can be amplified in parallel with the samples of interest. Provided that a linear PCR product/PCR educt relation for the standard dilution series is observed, the relative amount of PCR educt for samples in the same PCR run can be deduced. A typical example is shown in **Fig. 1**. Using replicates, this method may provide fairly accurate results and even rule out tube-to-tube variations, but it is

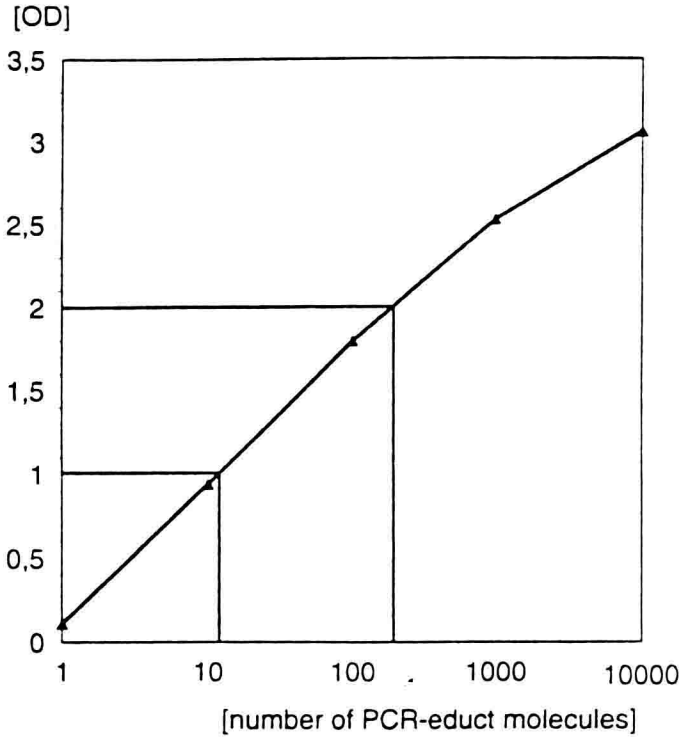

Fig. 1. ELOSA-based PCR quantification of HBV amplification products according to the external standard procedure. As a reference, a standard plasmid dilution series was subjected to PCR amplification. The biotin-labeled PCR product was hybridizised with a digoxigenin-labeled probe, bound to streptavidin-coated microtiter plates and subsequently quantitated using <DIG>:HRP conjugate and 2.2'-azino-di {2-ethyl-benzthiazolin-sulfonat] ***(6)***. An examplary curve is shown—with the variation that the ELOSA-derived value for 1 molecule of PCR educt is not positive in every experiment (for statistical reasons). It is shown that two samples with OD values of 1.0 and 2.0 would correspond to 15 and 200 mol of PCR educt/vol, respectively.

not capable to rule out sample-to-sample variations. A potential and always lurking drawback to this simple procedure is the sensitivity of the PCR for small variations in the setup. Because of resulting differences in the efficiency, they may devastate precision and reproducibility. Therefore, if a quantification with external standard is established, precision (replicates in the same PCR run) and reproducibility (replicates in separate PCR runs) has to be analyzed to understand the limitations within a given application.

Keeping **Eq. 2** in mind, it is clear that quantification with this procedure must be done in the exponential phase, which is also dependent on the relative