

Quantitative PCR

A Survey of the Present Technology

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Abstract

PCR-based amplification of nucleic acids has had a major impact in almost every field of basic research and has already found extensive applications in the area of clinical diagnosis. For many of these applications, quantitative data are sought to relate the quantity of amplified product to the amount of original target nucleic acid present in the sample. Since the PCR methodology with its exponential nature can be adapted for this purpose, a lot of different strategies have emerged in the last few years for sensitive and specific PCR product detection and quantification. Basic strategies, including the use of external and internal standards, are presented with respect to statistical aspects, and the advantages as well as the limitations of individual protocols are discussed. Furthermore the suitability of conventional laboratory techniques, such as gel systems or HPLC, nonradioactive labeling procedures, and the principles of advanced solid-phase-mediated strategies for the precise determination of amplification products, are outlined with the help of selected examples.

Index Entries: Quantitative PCR; internal standards; external standards; nonradioactive labeling; solid-phase capture; ELOSA; biotin; digoxigenin.

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 indispensable 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.

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

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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 intrinsic 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 overall 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, stochastic 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 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 amount of the PCR educt. Rigorous analyses have to be performed to demonstrate that with increasing number of cycles, the results do not change.

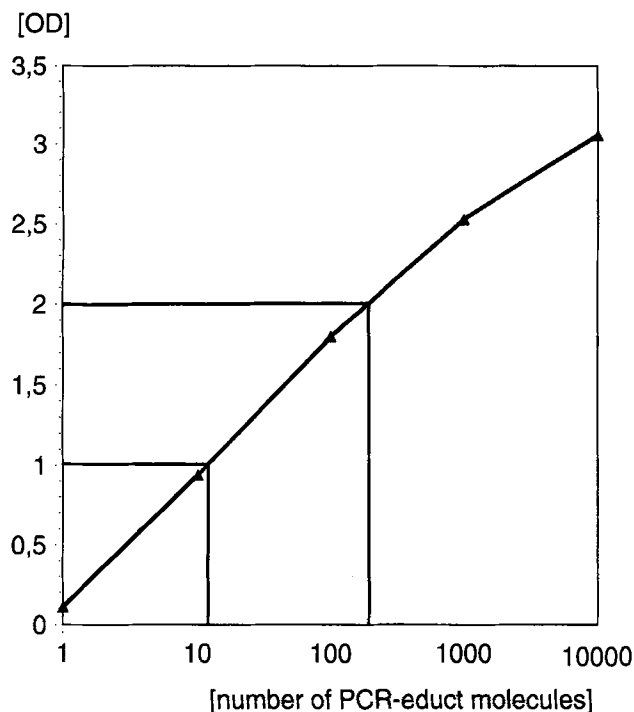


Fig. 1. ELOSA-based PCR quantification of HBV amplification products according to the external standard procedure. As a reference, a standard plasmid dilution series was subjected to PCR amplification. The biotin-labeled PCR product was hybridized with an digoxigenin-labeled probe, bound to streptavidin-coated microtiter plates and subsequently quantitated using <DIG>:HRP conjugate and 2,2'-azino-di [3-ethyl-benzthiazolin-sulfonate] (6). An exemplary 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.

A more sophisticated application for PCR quantification is the determination of the amount of PCR product molecules with increasing number of cycles. After the transformation of Eq. (2) to:

$$\log(Y) = \log(X) + \log(1 + E_e) \cdot n \quad (3)$$

a linear relationship between the PCR product $\log(Y)$ and n can be drawn, provided E_e remains constant. Then the PCR educt $\log(X)$ can be tentatively determined as the y-intercept, which can be extrapolated from the slope $\log(1 + E_e)$ as shown

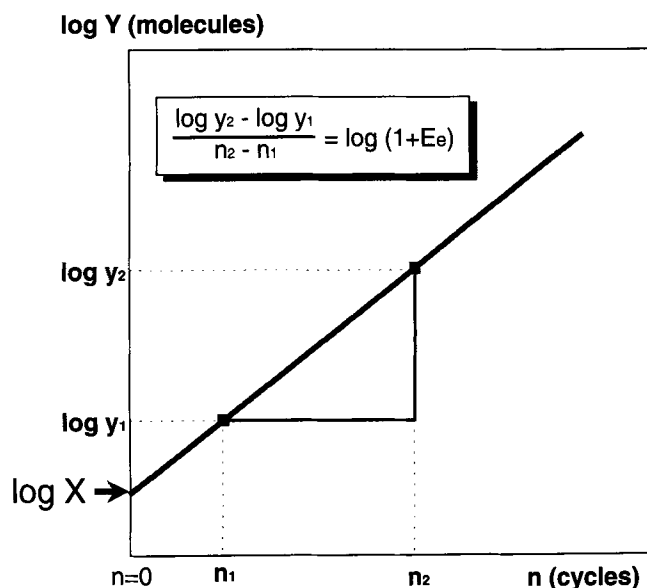


Fig. 2. Determination of the number of molecules of the PCR educt (X) from the amount of PCR product after cycle number n_1 and n_2 (Y_1 and Y_2 , respectively) (30). X can be calculated according to Eq. (3).

in Fig. 2. In this case, no external standards are needed, although well-defined positive controls seem essential. A possible problem with this procedure is the fact that within the first few cycles of the PCR, the efficiency (E) is much lower than between cycles 10 and 30 (18). In spite of this theoretical problem, it seems nevertheless possible to gain realistic results (19).

This procedure has the advantage that different amplification efficiencies (E_e) of the samples will be detected, if the absolute number of PCR product molecules can be determined. In our hands, quantification with external standards proved to be sufficient to gain primarily quantitative results of DNA targets isolated from acellular clinical samples. The isolated DNA is then subjected to competitive PCR, where less competitors are necessary (see Section 2.4.).

Because of higher sensitivity, PCR-based quantification with an external standard has been recently used in connection with nested PCR, but since the major problem of nested PCR is contamination, there is a greatest risk if no internal control is used. If one of the recently developed

highly sensitive detection methods (see below) is applied for the detection of the first-round products, nested PCR can be avoided at most of the common applications.

A variation of this procedure is the limited dilution analysis of the PCR educt. The PCR analysis is performed with a dilution series of the educt (10,20–22). The least positive sample is thought to contain the same amount of PCR educt as the last positive sample of a dilution series of a known standard. This procedure also has been used in conjunction with nested PCR.

Limited dilution analysis has the disadvantage that efficiencies of different PCR runs may vary, so that the reproducibility could be low. Another problem that emerges is the Gaussian distribution of a low number of PCR educts within a sample. Therefore, each dilution has to be analyzed repeatedly for a correct identification of the least positive sample.

2.3. Quantification with Noncompetitive Internal Standards

Depending on the extraction procedure applied, nucleic acids isolated from cellular material usually contain a lot of nontarget DNA or RNA. The presence of cellular nucleic acids facilitates the coamplification of one of these cellular targets with the target of interest within the same PCR tube (multiplex PCR). This second cellular target shares neither the primer binding sites nor the region in between with the target of interest. For DNA-PCR, almost any gene would do. Typical targets, for example, are pyruvate dehydrogenase (23), proenkephalin (24), or β -actin (25). For RNA-PCR, the task turns out to be more difficult. Here a cellular mRNA has to be selected that has an even level of transcription and is independent of different degrees of cellular activation. A lot of mRNAs have been evaluated for this purpose. First attempts had been performed with mRNA for HLA, β -actin, DHFR, or GPDH (26–29). More recently, the mRNA of histone H3.3 or the 14S rRNA has been used as a cellular target (30,31).

To our knowledge, no comparison of the different internal standards has been published so

far, and it is still unknown if all of them fulfill the criteria of an even and undisturbed transcription. Since this is the crucial point of the entire procedure, more attention should be paid to it. Since, for example, HLA-antigens, and thus the corresponding mRNA, are downregulated by Epstein-Barr virus (EBV) (32), they should not be used as internal standards for the quantification of EBV mRNA. It is also known that β -actin mRNA levels are increasing with the malignant transformation of cells (33).

The main advantage of this procedure is its simplicity and the fact that no profound molecular biology is needed. Replicates rule out tube-to-tube and to some extent sample-to-sample variations, although individual inhibitors of the polymerase may be missed. On the other hand, this method bears some pitfalls that should be kept in mind. The efficiency of the reverse transcription for the internal standard and the target of interest may vary, and more disturbing, it may even vary dramatically for the same target (34). Therefore, it seems to be very cumbersome to use this procedure for RNA-PCR. Quantitation during the exponential phase of the amplification process makes it possible to determine relative changes of the primary target, but if it is not checked that both targets are showing the same amplification efficiency (E) within a given number of cycles, absolute quantification is not possible.

Quantification with a noncompetitive internal standard has been reviewed in detail by Ferre (34). He demonstrated, as reasoned above, that the procedure is useful for monitoring relative changes of nucleic acid targets. He stated, nevertheless, that several replicates have to be applied and that, owing to a given precision, at least a twofold change of the PCR educt is required to detect a relative change. Therefore, each new setup of the assay requires a complete reevaluation of the parameters discussed above.

2.4. Competitive PCR

For competitive PCR, an internal standard has to be constructed that competes with the primary target for enzyme, nucleotides, and primer molecules. The competitor bears the same primer

binding region, but the sequence in between is modified in such a way that amplification products derived from the competitor and the target of interest can be differentiated, for example, by gel-electrophoreses, enzyme-linked oligonucleotide sorbent assay (ELOSAs), or HPLC. As long as the number of molecules of both PCR educts are equal, it is theoretically possible to use a competitor within a nested PCR assay (35). *In praxi*, for each application, it has to be demonstrated that it really works in conjunction with nested PCR. We observed, for example, that a reduction of the cycle number within the second PCR did increase the capability power of the nested PCR procedure for quantification purposes.

For initial attempts, competitors were used that differ from the wild-type target only by a point mutation. In most cases, these point mutations are introduced in such a way that an additional restriction enzyme recognition site is created within the competitor nucleic acid (36,37). Following restriction enzyme cleavage, the resulting products of competitor and primary target can be easily separated by electrophoresis on an agarose gel and quantitated by hybridization with a labeled probe or with the help of a labeled PCR primer. Although these competitors are showing a very high degree of similarity to the wild-type product, this procedure is no longer regarded as a quantitative one. This is owing to the fact that the amplification products have to be diluted and that a second enzymatic step is necessary. In particular, if the amplification products of the competitor are not cut completely by the restriction enzyme, a false quantification results.

More recently, deletions of a part of the wild-type sequence or insertions of foreign sequences are used for the *de novo* construction of competitors, which are analyzed by gel electrophoresis (38). Reviewing the literature, it seems obvious that there are no general rules or strategies for the construction of these modifications (39–43). Often a critical analysis of precision and reproducibility is found, but a more detailed evaluation of the amplification efficiencies (E_e) of the wild-type target and the competitor has, to our knowledge, in most cases not been performed. Usually

it is demonstrated that these applications allow a relative quantification, and it is assumed that an absolute quantification can also be performed. Computer simulations confirmed recently that different amplification efficiencies (E_c) of the wild-type target and the competitor may allow a very precise relative quantification, although an absolute quantification is out of reach (44). For absolute quantification, it is therefore most important to demonstrate that E_c of the wild-type target and the competitor are equal. It may be also very helpful to evaluate the competitor on samples with a known amount of wild-type target molecules.

Competitors for microtiter plate-based assays do not need to have a different length, since they are differentiated from wild-type amplification products by sequence. Therefore, specific sequences may be deleted or inserted, and both targets can be detected separately by hybridization procedures. Again, the amplification efficiencies of both target and competitor have to be equal to allow absolute quantification; otherwise, only relative quantification is possible.

For quantitating single clinical samples, one has to perform several competitive PCR assays with a constant amount of the target of interest and varying amounts of competitor. That is owing to the fact that only equimolar amounts of competitor and the target of interest result in a reliable quantification. It is likely that the number of competitive PCR assays needed is reduced by the application of ELOSA-based assays (B. K., unpublished results and 41).

In general, since competitive PCR is capable of ruling out tube-to-tube and sample-to-sample variations, it seems to be the method of choice for accurate PCR quantification. If the criteria mentioned above are taken into account, we consider this procedure appropriate for absolute quantification and for quantification of low copy targets.

3. Detection and Quantitative Measurement of PCR Products

3.1. Labeling of PCR Products

By itself, the amplification of a target nucleic acid is not an analytical procedure. To detect the presence and specificity of amplified DNA and, if

necessary, to quantitate the amount of specific PCR products present in the reaction mixture, the amplification system has to be linked to an appropriate detection system. For this purpose, the amplification products have to be equipped with any kind of label that can be detected subsequently either in a direct or indirect way. For many years, the most commonly used methods for the detection of PCR-amplified DNA were based on radioactive labels. Because of the difficulties encountered in the handling of such radioactive isotopes, a variety of highly sensitive nonradioactive indicator systems have been developed. Suitable nonradioactive labels include helix-intercalating dyes, like ethidium bromide or bis-benzimide (45), covalent bound dyes (e.g., fluorescein) or enzymes (e.g., horseradish peroxidase [HRP]) (46), and alkaline phosphatase (47) as well as distinct reporter molecules, such as digoxigenin or biotin. For detailed reviews on the variety of direct and indirect nonradioactive bioanalytical indicator systems, see (48,49).

Since the PCR is based on the oligonucleotide-primed *de novo* synthesis of template-complementary DNA by the enzymatic action of a DNA polymerase, nonradioactive reporter molecules can be easily incorporated into the amplification products either in the presence of labeled deoxyribonucleotide (dNTP) analogs and/or labeled primer oligonucleotides present in the amplification mixture (50,51). Labeled deoxyribonucleotides are commercially available in the form of digoxigenin- or biotin-dUTP (e.g., Boehringer Mannheim GmbH, Mannheim, Germany). Primer oligonucleotides can be precisely labeled at their 5'-end during their chemical synthesis using digoxigenin-, biotin- or fluorescein-phosphoramidite components. Labeling with photodigoxigenin, a photoreactive compound that binds covalent to amino groups upon UV irradiation (52), results in a statistical distribution of digoxigenin molecules along the oligonucleotide.

Bifunctional conjugates, like antidigoxigenin antibody fragments (<DIG>) or streptavidin (SA), covalently linked to the customary enzymes HRP or alkaline phosphatase (AP) were commonly

used for the detection of labeled PCR products in an ELISA-type reaction. The high stability of these enzymes, their wide application in diagnostic assays, and the development of appropriate detection systems are factors that have contributed to their suitability as reporter enzymes. Once a digoxigenin-labeled amplification product is fixed on a solid phase, incubation with <DIG>:AP conjugate, for example, resulted in a tight attachment of the antibody portion to the digoxigenin residues, and the enzyme portion of the bifunctional conjugate is capable of catalyzing subsequent color reactions that yield optical, luminescing (53) or fluorescing signals (54), depending on the substrate used. Since the resulting signal can be precisely quantified by appropriate instrumentation, this strategy has recently become well established in the field of quantitating PCR products. The use of enzymes for signal generation can also be considered an amplification method, since many product molecules are produced per enzyme molecule.

Detection strategies for amplification products can generally be divided in two parts. On the one hand, there are assay systems that are capable of detecting the presence or the absence of amplification products, and on the other hand, there are assay systems that are specific for amplification products with a given sequence. Although the border between these assay formats is vague, for ease of presentation, we decided to divide this chapter into nonsequence-specific and sequence-specific detection systems, and to outline the individual principles with the help of selected examples.

3.2. Nonsequence-Specific Detection Systems

A lot of PCR applications are already optimized with regard to the buffer $MgCl_2$ concentration, temperature profile, and so forth, and are leading to well-defined amplification products without the formation of any byproducts that are different in size. Under suitable conditions, the relative amount of amplification products in these cases is strictly dependent on the amount of starting material present in the amplification mixture. Therefore, quantification of the PCR

products by physical or enzymatic means is almost sufficient for a rough determination of the amount of the PCR educt (*see* Section 2.1.).

3.2.1. Gel Systems

Applicable formats include well-established laboratory techniques, like agarose or polyacrylamide gel electrophoresis, and subsequent quantitative detection of ethidium bromide-stained amplification products using gel scanners or suitable computer-assisted video equipment. A quantitative detection of radioactive labeled amplification products can be accomplished either by autoradiography or by Cherenkov counting of excised gel pieces. A recent development in the field is the application of automated DNA sequencers for the quantification of fluorescence-labeled nucleic acids (e.g., Applied Biosystems 373A DNA sequencer in combination with the GeneScan software [Applied Biosystems, a division of Perkin-Elmer, Foster City, CA]). With the help of these instruments, the gel-associated lack of sequence specificity can be nearly overcome by an accurate size determination in the basepair range and ultimate detection sensitivities in the femtomole range of individual dye-labeled amplification products. Since these instruments can differentiate up to four distinct fluorescence dyes, internal or external standards can be applied and analyzed in parallel within the same gel lane as the amplification products, thus reducing the possibility of lane-to-lane artifacts. Since automated DNA sequencers and fluorescence-labeled primers are still expensive, at present, this promising technique is mainly restricted to research applications.

3.2.2. HPLC

Direct HPLC of PCR products using, for example, a 2.5- μm nonporous polymer-based anion-exchange column, a 12- to 25-min gradient cycle time and UV absorbance detection have been shown to meet the analytical criteria for practical PCR product quantitation (55). PCR samples can be injected onto the column directly after amplification without further purification, and the sensitivity is adequate to provide the detection of

unlabeled amplification products in the femto-gram range (this corresponds to around 10^3 molecules of PCR educt). The detection limit of labeled amplification products may be lower and will depend on the availability of suitable detector systems (e.g., the use of fluorescence-labeled primers in conjunction with a fluorescence detection device [56]). The size-differentiating features of HPLC even allow the use of internal standards different in size to align variations in amplification efficiency more precisely. If the amplification parameters are well adjusted, the linear form of the graph of PCR product output vs log (template input) leads to a calibration curve that compresses up to four decades of target concentration into one decade of HPLC-quantitated PCR product concentration.

3.2.3. Solid-Phase Assays

In general, the attachment of amplification products to a solid phase is advantageous to carrying out several measures in parallel and under comparable conditions. The most widely used and convenient solid-phase plastic support medium for this kind of bioanalytical assay is the 96-well microtiter plate. These plates lend themselves to some degree of automation, such as the use of plate washers and, for colorimetric enzyme assays, the use of multichannel spectrophotometric plate readers. Since many proteins adsorb passively to polystyrene by hydrophobic interaction, it is possible to coat microtiter plates with molecules like streptavidin. This results in a solid-phase medium that is capable of the specific capture of biotin or, in practice, biotinylated molecules. Streptavidin-precoated plates are already available from different manufacturers and are well suited for setting up quantitative assays for biotinylated PCR products. Double-labeling of PCR products with biotin and reporter molecules like digoxigenin can be employed for a subsequent quantification in microtiter plate-based assay formats. The simultaneous incorporation of biotin and digoxigenin into the amplification products can either be achieved in the presence of both digoxigenin- and biotin-deoxyribonucleotide analogs (DIG-/

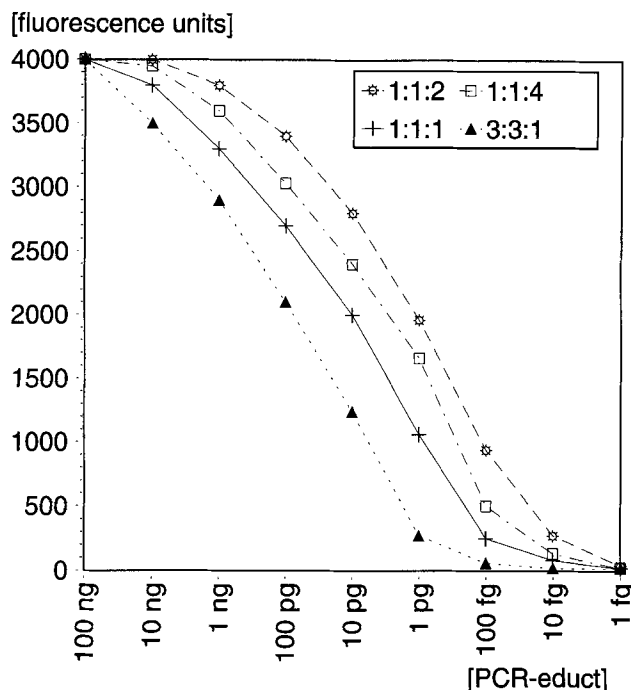


Fig. 3. Quantification of biotin/digoxigenin double-labeled HBV amplification products (543 bp) in streptavidin-coated microtiter plates using <DIG>:AP conjugate and 4-methyl-umbelliferyl-phosphate as fluorescing substrate (plot is based on average values). The molar ratio of DIG-dUTP:bio-dUTP:dTTP in the reaction mixture is indicated.

bio-dUTP) in the amplification mixture or, in a more specific manner, in the presence of a biotinylated primer 1 and DIG-dUTP or a biotinylated primer 1 and a digoxigeninylated primer 2. Since the absolute concentration of labeled deoxyribonucleotide analogs in the reaction mixture has a significant influence on the *Taq* DNA polymerase activity (reduced elongation rate), the optimal concentration of DIG-/bio-dUTP has to be determined individually (*see* Fig. 3).

In a typical assay format, double-labeled amplification products were ethanol-precipitated (to remove unincorporated label) and subsequently incubated in streptavidin-coated microtiter plates for at least 2 h at room temperature with occasional shaking. Following several wash steps, incubating with <DIG>:AP conjugate and a substrate solution results in the generation of a quan-

titative color or fluorescence signal, depending on the substrate used. The solid-phase capture of labeled amplification products mediated by the streptavidin–biotin interaction allows for the accomplishment of the indicator reaction in solution; this is essential for quantitatively determining the concentration of target molecules. Similar to standard ELISA, the colorimetric detection of the PCR products makes this quantification procedure suitable for screening a large number of samples. Taken into account that this convenient assay format is not sequence-specific and the signal measured is assembled from all amplification products, whether specific or not, present in the amplification mixture, its use is limited to PCR reactions that lead to a well-defined product. On the other hand, these intrinsic limitations can be easily overcome by the sequence-specific hybridization of biotin-labeled probes to digoxigenin-labeled amplification products and a subsequent detection in form of double-labeled hybrids according to the ELOSA principle (*see* Section 3.3.2.).

In general, streptavidin-mediated solid-phase capture of biotin-labeled target molecules in solution turned out to be an effective, versatile, and easy to handle assay format and will certainly evolve into a key technology in the field of quantitative PCR.

3.2.4. SPA Assay

The scintillation proximity assay (SPA) is based on a similar concept. This assay relies on the use of fluomicrospheres as the solid phase, coated with acceptor molecules that are capable of binding labeled ligands in solution (57). In a typical application of this technique, one of the PCR primers is labeled with biotin, and tritiated nucleotides are incorporated during the amplification reaction. Once the amplification procedure is complete, streptavidin-coated SPA beads (Amersham International, UK) are added to capture the biotinylated PCR products. This capture event brings the tritium close enough to the microsphere so that the fluor incorporated within it is excited to emit a pulse of light that is measurable in a conventional scintillation counter. On the

other hand, the majority of unincorporated tritium molecules are too far away from the SPA beads to enable the transfer of energy. Compared to color-developing assays, the SPA format has a broader linear detection range. Using unlabeled primers in combination with a postamplification hybridization with biotinylated probes complementary to an internal sequence of the amplicon, this quantitative assay format can also be configured to be sequence-specific. For example, this system has been successfully applied to the quantification of cytomegalovirus DNA in blood specimens and was capable of detecting changes in the level of viral DNA within a three-log dynamic range and a detection limit of 4×10^4 molecules of PCR educt (22).

3.2.5. Transcription-Mediated Detection

Quantitative measurement of specific mRNA species can be achieved by a combination of RT-PCR and a subsequent *in vitro* transcription reaction. In the course of this straightforward method, a T7 RNA polymerase promoter sequence is incorporated during the PCR reaction at the 5'-end of the amplification products, and following the amplification reaction, an *in vitro* transcription reaction is carried out in the presence of labeled ribonucleotides. The linear transcription reaction greatly increases the amount of amplified product and, thereby, gains an additional dimension of sensitivity for the detection of low-abundance mRNA. Using the expression of an endogenous gene as a denominator for normalization of the quantitative data, an internal control is provided for the amount of intact RNA successfully isolated and converted to cDNA. This method is aimed at measuring the relative rather than the absolute levels of gene expression by determining a ratio between PCR products of the desired target gene and an endogenous internal standard gene in separate reactions, and then comparing it with the same ratio in another sample. Using serial dilutions of the cDNA samples, a less than twofold difference in gene expression can be discriminated even if the absolute amount of input mRNA or cDNA is not known (58).

3.3. Sequence-Specific Detection Systems

Although the theory of PCR is straightforward, the primers are frequently annealing to nontarget sequences, especially in complex template mixtures, and this so-called mispriming significantly lowers the purity of the amplified target portion in the final product. Therefore, probe-based methods remain a key feature of current detection systems primarily because of the additional information and sequence specificity they provide. Probes have been adjusted to nonisotopic colorimetric systems by labeling them with reporter molecules, such as digoxigenin, biotin, or distinct enzymes, or with dye molecules capable of emitting light (chemiluminescence). In the field of quantitative PCR, probes were mainly bound to the wells of microtiter plates since this format has certain advantages for reproducible results and automation.

3.3.1. Dot-Blot Procedures

Classically, hybridization assays are carried out by dot-blot or Southern blot procedures, in which the amplified target is denatured, immobilized on a nitrocellulose or a nylon membrane surface, and then hybridized with an appropriate labeled DNA probe. Even if a laboratory is not equipped with an ELISA plate reader, sequence-specific detection and quantification of amplification products can be carried out within a simple dot-blot format. After spotting and immobilization of the PCR products on a nylon membrane, the dot-blot methodology utilizes the sequence-specific hybridization of labeled oligonucleotides to indicate the presence or absence of specific amplified sequences. The reverse dot-blot procedure is based on sequence-specific oligonucleotide probes immobilized on a nylon membrane via linkage of poly-T tails and subsequent hybridization with denatured labeled amplification products that are in solution. Since the target is not directly bound to the membrane surface, the reaction kinetics in this assay essentially approach a liquid phase, which allows a rapid hybridization reaction. If biotinylated probes or biotinylated amplification products (in the case of the reverse dot-blot) are used, the nonradioactive detection is usually car-

ried out with streptavidin-AP conjugates producing a colored dot. Under ideal conditions and in comparison with samples with known concentration, the color intensity represents the relative amount of specific amplification products. In the case of reverse dot-blot procedures, a more accurate quantification of amplification products can be achieved by providing membrane strips with a series of dots containing a shading amount of probe. Apart from quantitative applications, this format offers the practical advantage of detecting multiple alleles within a given amplification product simultaneously (HLA-DQA genotyping [59]) or different pathogens in a single hybridization reaction.

3.3.2. Solid-Phase Capture

The adaption of the solid-phase capture technique to microtiter plates or paramagnetic beads results in the most convenient assay formats for the sequence-specific detection and quantification of PCR products in routine practice. With respect to the basic principle, they were recently named ELOSA. Although individual strategies have been developed, these assay formats share a common principle: molecules that support the solid-phase capture and molecules that mediate the subsequent detection are located on different strands of nucleic acids. In contrast to the double-labeling of PCR products mentioned above, double-labeled molecules are formed within these assays exclusively on the hybridization of labeled probes to labeled PCR products.

Providing the sequence-specific detection of distinct amplification products in a complex mixture, this post-PCR hybridization event is also crucial for most of the quantitative procedures. In principle, there are two different hybridization-based concepts for the capture and subsequent detection of amplification products on a solid phase.

3.3.2.1. IMMOBILIZED CAPTURE PROBE

Oligonucleotides representing a characteristic part of the amplified sequence, so-called capture probes, are attached either covalently or via biotin:streptavidin linkage to a solid phase, and labeled PCR products are hybridized using strin-

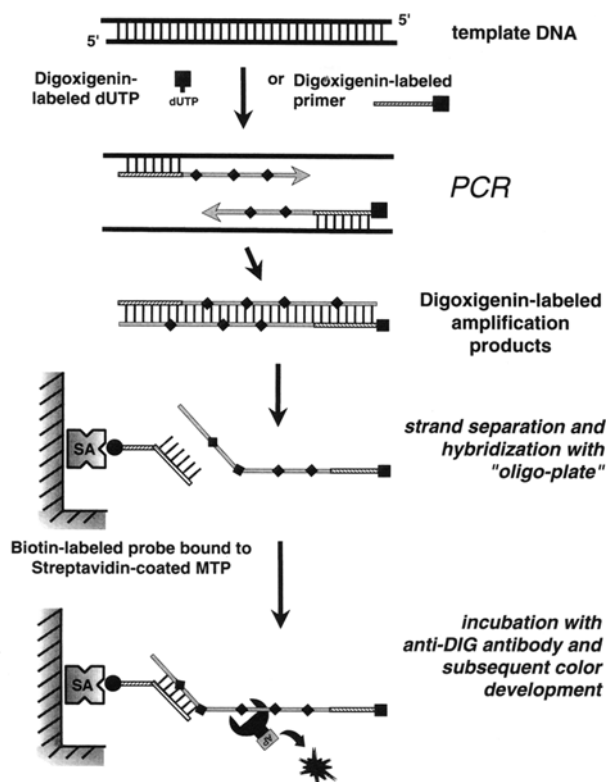


Fig. 4. Immobilized capture probe. Following strand separation, a sequence-specific detection of digoxigenin-labeled amplification products is carried out by hybridization to immobilized probes.

gent conditions. Following several wash steps, the amount of specific amplification products can be determined by a label-mediated detection reaction (see Fig. 4).

3.3.2.2. IMMOBILIZED AMPLIFICATION PRODUCT

Biotin-labeled PCR products are attached to a streptavidin-coated solid phase and subsequently hybridized with a labeled probe complementary to internal sequences of the specific amplification product (see Fig. 5). Another possibility is the covalent binding of aminated amplification products to carboxylated wells of microtiter plates (60).

Although ingenious protocols have been developed (e.g., 61), for reliable results, it is advisable to denature the double-stranded PCR products via heat or alkali treatment before hybridization with a specific probe. As an in-solution assay, there is

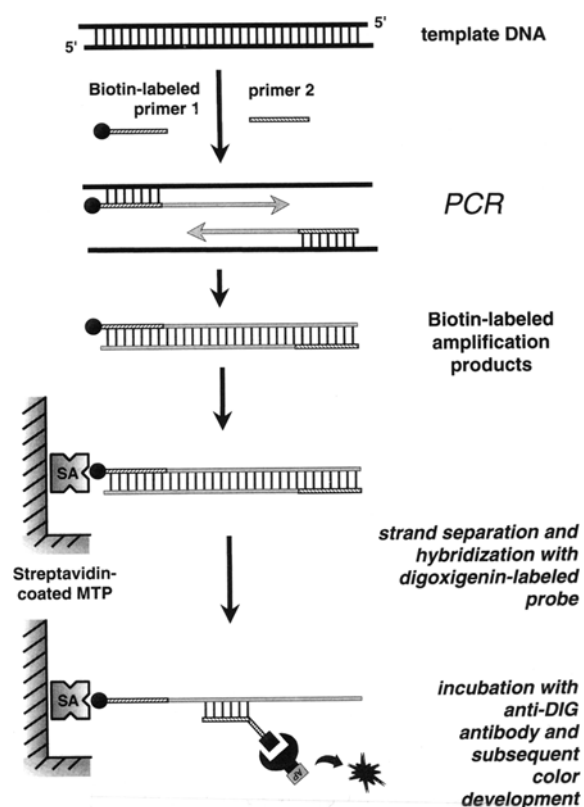


Fig. 5. Immobilized amplification product. 5' biotin-labeled amplification products are immobilized on a streptavidin-coated microtiter plate. Following strand separation, a sequence-specific detection is carried out with the help of digoxigenin-labeled probes.

a constant diffusion of target and probe that speeds up the reaction kinetics and allows for a rapid hybridization reaction. The sensitivity mainly depends on the label used for the subsequent detection of the hybrids. Within these kinds of experiments, the use of digoxigenin labels and <DIG>:AP or <DIG>:HRP conjugate is recommended in combination with substrates yielding an optical, luminescing, or fluorescing signal. The detection can be automated using ELISA readers, and usually sensitivities in the attomole range of PCR educt are obtained. Although this detection format does not offer the utmost sensitivity levels, in our hands, it proved to be sufficient for the majority of quantitative applications (see Fig. 1). Furthermore, this hybridization format opens up the simultaneous quantitation of amplification products and inter-

nal standards that are equal in length, but differ in distinct nucleotide sequences. For example, target molecules and internal standards are coamplified using a set of biotinylated primers, attached to a streptavidin-coated solid phase, and subsequently hybridized to specific probes bearing different labels. After separate quantitation of the amount of each label, the initial concentration of the target molecules can be determined precisely in comparison to the internal standard. Apart from reporter molecules, like digoxigenin, chemiluminescent probes or distinct antibodies can be used as well for the hybridization-mediated detection of specific amplification products.

3.3.3. Electrochemiluminescence

A recently developed assay, the QPCR System from Perkin Elmer Instruments (Foster City, CA), utilizes the analytical capabilities of an electrically initiated chemiluminescent reaction (electrochemiluminescence) to provide sensitive and reproducible DNA quantitation at the attomole level. Again, the convenient assay format of streptavidin:biotin-mediated solid-phase capture of the amplification products to magnetic beads is applied in combination with a sequence-specific oligonucleotide probe labeled with Tris (2,2'-bipyridine) ruthenium (II) chelate (TBR). In contrast to commonly applied acridinium esters (62), the high stability of ruthenium bipyridyl labels allows their incorporation during oligonucleotide synthesis (63). Following hybridization, the bead-bound sample is supplemented with a tripropylamine solution (TPA) and is delivered to the detection cell of the electrochemiluminescence device. As the increasing voltage of the electrode reaches a specific level, a simultaneous oxidation of both the TPA and TBR occurs. The oxidized TPA is converted to an unstable highly reducing intermediate that reacts with oxidized TBR converting it to the excited state form. The excited-state species relaxes back to the ground state with the emission of light at 620 nm. Since the intensity of the emitted light is directly proportional to the amount of TBP labels present in the detection cell, the initial amount of spe-

cific amplification products can be quantitatively determined by measuring and integrating the light intensity at 620 nm. This system provides linear responses over more than three orders of magnitude (which corresponds to a dynamic range of at least four logs of initial PCR-product copy numbers), sensitivities down to 70 attomoles (64), and can be easily automated. In comparison to ELOSA techniques, no error-prone enzymatic steps are involved in these electrochemiluminescence procedures. Nevertheless, the impact of this theoretical advantage in practice has to be determined.

3.3.4. DNA Immunoassay

The availability of a monoclonal antibody (MAb) recognizing selectively double-stranded DNA has permitted the development of a novel enzyme immunoassay capable of detecting specific hybridization events. This methodology was adapted to the "immobilized capture probe format" mentioned above and has been termed "DNA Enzyme Immuno Assay" (DEIA; Sorin Biomedica, S.p.A. Saluggia, Italia) (65). When DNA:DNA hybrids are formed between the capture probe and specific amplification products, the monoclonal anti-dsDNA antibody is added and, as in conventional diagnostic ELISA systems, the presence and amount of DNA-antibody complexes are indicated subsequently by a colorimetric reaction developed with the help of a secondary enzyme-conjugated antibody (murine anti-IgG:POD). A comparable assay format is based on the hybridization of biotinylated PCR products with unlabeled RNA probes and a subsequent detection of the resulting hybrids with the help of an enzyme-labeled antibody specific for DNA:RNA hybrids. These immunoassays can be used for the detection of any type of amplified DNA and eliminate the need for labeling DNA or primers. The DEIA assay has already been successfully applied to detect the presence of the gene coding for HBV core antigen and HLA typing. The possibility of crossreactions and the cost of these MAb are limiting the assays potential large-scale application at present.

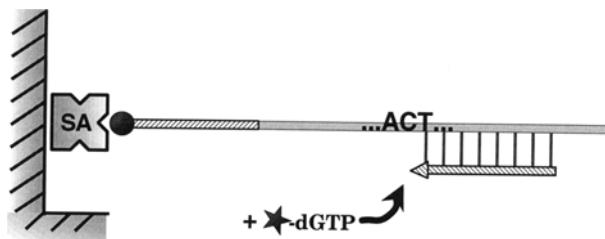


Fig. 6. Primer elongation assay. A distinct oligonucleotide primer is hybridized with its 3'-end immediately next to the base of interest within a denatured amplification product and subsequently elongated in the presence of a corresponding labeled deoxyribonucleotide by the enzymatic action of a DNA polymerase.

3.3.5. Primer Elongation Assay

The single nucleotide primer extension assay (SNUPE) represents one of the most practicable assay formats for the identification and quantification of point mutations (e.g., allelic variants in DNA or RNA) and the measurement of specific mRNA levels. This post-PCR assay consists of the enzymatic extension by one base of an oligonucleotide primer hybridized just 5' to the position of mismatch in the presence of only one labeled dNTP specific for either the wild-type or a variant sequence (*see* Fig. 6). Here a previous solid-phase capture of amplification products is not absolutely required, since the introduction of the label by the template-dependent elongation of a perfect matching primer is specific for a given sequence within the amplification products. Nevertheless, a selective ethanol precipitation and agarose gel purification of the PCR products should be carried out prior to the assay, since the complete removal of dNTPs present in the initial amplification mixture is an essential prerequisite to obtaining quantitative results. A major advantage of the method is its usefulness for quantitative measurement over a wide range. Furthermore, a given transcript can be detected in up to 1000-fold excess of RNA from other alleles, depending on which nucleotides differ. This method can be easily adapted for quantitation of absolute amounts of a specific transcript by the addition of an internal standard (66). Under

optimal conditions, the background is below 1%, but varies significantly with the different kind of mismatches (67).

4. Future Prospects

Techniques allowing for a precise quantification of minute amounts of nucleic acids derived from *in vitro* amplification techniques will undoubtedly have a substantial future impact on the practice of molecular biology and laboratory medicine. Especially in the field of medical diagnosis, techniques are desirable that are capable of providing the absolute amount of distinct nucleic acids rather than providing relative amounts. In the case of HIV infection, for example, absolute measurements of particular RNA levels will provide a means for following the progression of viral infection and monitoring the efficacy of therapeutic intervention (11).

In the last few years, much effort has been spent in the development of detection systems with ultimate sensitivity. Since the overall performance of an analytical system is mainly dependent on its weakest part, some still unpredictable features of the real amplification procedure should be investigated in more detail. These investigations will provide further insight into the complex kinetics and may result in more robust amplification systems showing improved reliability. In contrast to the original purpose of PCR (the detection of as few target molecules as possible), for quantitative aspects, more stress should be placed on novel strategies that could improve the performance (e.g., linearization or enlargement of the exponential phase of the amplification procedure) rather than improving the overall sensitivity. For quantitative aspects, it is more important to differentiate between 100 and 500 molecules of PCR educt rather than to detect single PCR educt molecules. Emphasis should also be placed on the identification of suitable noncompetitive internal standards, which are not dependent on cell-cycle or cell-activation events. Another important aspect is the design of competitors, that are as similar as possible to the target of interest. This object can be achieved by the application of hybridization-based detection formats.

A promising application in the field of basic research and medical diagnostics is the quantification of distinct mRNA levels with the aim of elucidating gene regulation, virus replication, or immunological responses. Since this knowledge is an essential prerequisite for causative therapy and therapy monitoring, quantitative RT-PCR will evolve as a key technology in this field. The introduction of a thermostable DNA polymerase from *Thermus thermophilus* (rTth), which has both reverse transcription and DNA polymerase activities under certain reaction conditions, may eliminate the need for reopening the reaction tubes in the course of a RT-PCR and therefore reducing carryover contaminations.

Similar to techniques for the in vitro amplification of nucleic acids, the spread and acceptance of individual assays for the quantification of amplification products will ultimately be limited by cost, sensitivity, and specificity.

5. General Considerations

For standard PCR conditions, quantification should be carried out during the exponential phase of amplification. For this reason, it is important to optimize individual parameters of the entire amplification process carefully, so that the overall amplification can be controlled and the "plateau" phase avoided.

A quantitative PCR assay consists of three elements. Therefore, potential variations in the performance of the initial sample preparation should also be ruled out carefully, in addition to refining the amplification and detection procedures.

Standards used for the quantification of the sample should be chosen carefully to ensure reliable and accurate results. Here we recommend the use of recombinant plasmids, which can be easily created from individual amplification products with the help of the TA cloning kit (Invitrogen BV, NV Leek, The Netherlands).

For absolute quantification, the amplification efficiencies of the target of interest and the internal standard, whether competitive or noncompetitive, have to be determined. Internal standards should coamplify with the target of interest in equal efficiency.

To enhance the statistical validity of the data, it is recommended to carry out the assays several times.

Streptavidin-precoated microtiter plates from different manufacturers vary significantly in their ability to bind biotin-labeled amplification products, and there are no rules governing the choice of plate. Generally, a precise comparison should be carried out on sample plates using a well-defined dilution series of biotin-labeled amplification products.

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