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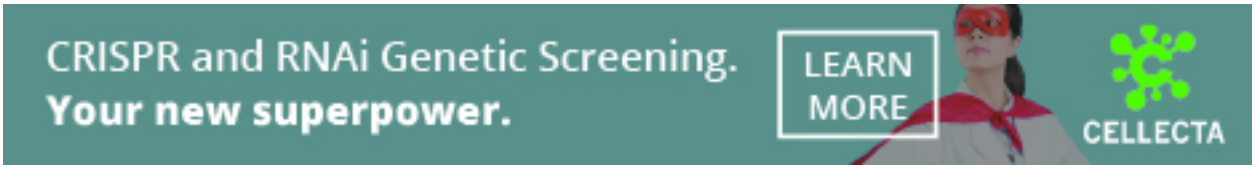

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A Commentary on the Practical Applications of Competitive PCR

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One of the applications of PCR is the quantitative analysis of specific nucleotide sequences. The main advantage of this method, as compared with other methods based on probe hybridization, is its extreme sensitivity and specificity. However, PCR by itself is not an accurate quantitative assay. Because of the many amplification steps, small differences in amplification efficiency result in dramatic differences in product yield. Furthermore, the exponential phase of the reaction is of limited duration because of the accumulation of product. If the PCR is run beyond the exponential phase into the saturation phase, initial differences in the amount of template will be obscured. To compensate for these intrinsic difficulties, a number of controls have been introduced, as will be summarized below. Recently, however, based on theoretical considerations and practical experience, we and others have issued warnings of specific pitfalls in quantitative PCR, particularly in one of its variants which uses an external standard sequence, known as competitive PCR.[1–3]

One of the prerequisites for reliable quantification in competitive PCR is the proper shape of the standard curve, a fact that has been overlooked in the original description of the method. Although an amendment to the method of competitive PCR has been published,[1] a survey of the literature reveals that in many cases this necessary condition has not been met as yet. The purpose of this commentary is to discuss these and other problems and possibilities of quantitative PCR.

CONTROLS USED IN QUANTITATIVE PCR

Two different types of controls have been used for the standardization of PCR results. One form of control consists of performing preliminary experiments to gain information on the amplification efficiency and on the duration of the exponential phase. For valid comparison of the amount of product in different PCR tubes, the amplification factor should be the same in the whole range of conditions used in the assays. Therefore, it is obligatory that the amount of template in the preliminary control experiment is chosen at the extreme upper end of the range that will be used in the assays. The reason for this is that the amplification efficiency starts to decrease when the amount of product exceeds a certain level. This stage will be reached sooner in PCR tubes containing a greater initial amount of template.

The second type of control is the use of an internal standard. The amount of PCR product obtained from a specific mRNA species is compared with that amplified from a reference sequence in the same PCR tube. The internal standard is either an mRNA species, which is constitutively expressed and the amount of which is considered to be invariant (e.g., housekeeping genes such as actins or glyceraldehyde phosphate dehydrogenase; for reviews, see Volkenandt et al. and Sugimoto et al.),[4,5] or an exogeneously added standard sequence (for review, see Siebert and Larrick).[6] Such coamplification may allow compensation for tube-to-tube variation in amplification efficiency. In the case of the coamplification of sufficiently similar templates, it may allow extension of the PCR beyond the exponential phase.

The coamplification of the target sequence with housekeeping genes imposes several constraints on the PCR conditions in which reliable quantification can be achieved: It requires the addition of two primer pairs that should be compatible; in addition, the difference in the initial amount between target and standard should not be too great. The difference should be sufficiently small such that the amount of product of both templates will exceed the detection limit before the end of the exponential phase is reached. If the initial amounts differ too much, it is likely that the efficiency of amplification of the product present in the largest amount will start to decrease faster than that of the other one, resulting in an apparent change of the ratio of both templates.

Instead of housekeeping genes, many investigators have used exogenously added standards. The conditions for reliable quantification are much less stringent if the standard and the target sequences possess identical primer binding sites. The use of an exogeneously added standard that contains the same sequences for primer binding as the target was first described by Wang et al.,[7] by Becker-André and Hahlbrock[8] (who named the method PATTY PCR-aided transcript titration assay), and by Gilliland et al.,[9] who introduced the term "competitive PCR." This latter name has prevailed in the litera-

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ture. Because only one primer pair is needed in this approach, it can be assumed that the amplification efficiency is always identical for both sequences if the region in between the primer-binding sites also is very similar. Therefore, the PCR can be extended into the later phase where a decrease of the efficiency occurs, if this decrease occurs with an identical time course for both sequences. (The possibility of extending the PCR into the nonexponential phase is very useful in practice as it avoids many additional controls and allows accurate quantification of the PCR products.) However, as will be explained in the next section, there are many examples in the literature of a nonparallel decrease of the amplification efficiency that apparently have escaped notice.

A related quantitative application of PCR coamplification is the determination of the relative mRNA levels of members of gene families. A proper name for this method could be "ratio PCR" (see below).

COMPETITIVE PCR

The name "competitive PCR" refers to the fact that the PCR is allowed to proceed into the saturation phase in which competition occurs between target and standard templates for available substrates: Because the sum of the masses of both products cannot exceed some maximum value, the amount of product formed from one template will decrease with the increasing quantity of the other template.

The products of target and standard sequences are discriminated either by a difference in length or by a specific restriction site in the region between the primer templates. In practice, a series of PCR tubes containing the same but unknown amount of target sequence is spiked with a dilution series of defined quantities of the standard. If the amplification factor is the same for both sequences, their ratio will remain constant during the amplification and the amount of the unknown template can be quantitated from the ratio of the two products. In the practical application of the method, it was recommended that a curve be constructed relating the logarithm of the ratio of PCR products standard/target to the logarithm of the initial amount of standard cDNA added.[6,9] The amount of initial target template can be read easily from the point on the curve where the amounts of target (T) and standard (S) are equal [the equivalence point where $S/T = 1$, or $\log(S/T) = 0$].

Although competitive PCR has been used in several examples for reliable quantification, also many incorrect results have been published also, as is apparent from the shape of the log–log calibration curve, which in many cases is not as predicted from the basic assumption that the amplification factor should be identical for *T* and *S*. Theory predicts that this calibration curve should be linear and have a slope of 1.[1] Whereas it is mathematically obvious why this should be so, it is less straightforward to explain in a few words the causes of standard curves that are either curved or rectilinear but do not have a slope of 1. However, it can be easily seen from the prerequisite that the ratio S/T should be the same at the start and at the end of the PCR, that an invalid standard curve must be caused by a difference in the amplification factor between *T* and *S*, and by the fact that this difference was not identical in all of the PCR tubes of the dilution series. A computer simulation of such a situation has been made.[1] It has been shown that

1. The part of the calibration curve used for quantification should be rectilinear and have a slope equal to 1.

2. A significant deviation from the linearity and/or the slope of the curve cannot be explained by a difference in the amplification efficiency between standard and target if this difference is identical in all of the PCR tubes of the dilution series. If there is a difference in the amplification factor and this difference is the same in all of the PCR tubes, theory predicts a parallel shift of the standard curve. This shift results in a displacement of the point of equivalence and in a faulty quantification. In practice, this displacement cannot be detected because a reference point is not available. Therefore, the slope=1 characteristic is in theory no proof that the amplification efficiency is the same for target and standard. The equal amplification of target and standard can be assumed for very similar sequences or otherwise it has to be demonstrated in separate experiments. In practice, however, correct standard curves can be considered as a good indication for equal amplification (see point 3).

Standard curves of slope=1 obtained for templates with a different amplification factor can in theory be used for relative quantification, that is, the comparison of the amount of target template among different PCR tubes. In practice, however, such a situation may seldom or never occur.

3. To obtain incorrect standard curves it is necessary to postulate (a) that the amplification factor for target and template was not the same, and in addition, (b) that the differences among the amplification factors are not identical in all the PCR tubes of the dilution series. It is easy to imagine how such a situation might originate, considering that any difference in the amplification efficiency between target and standard will more likely show up if there is competition between both templates for substrates, that is, when the PCR reaches the saturation phase. Because the total quantity of template at the start of the PCR varies among the tubes of the dilution series, the saturation phase of the PCR will be reached sooner in the tubes containing more template. The difference in amplification efficiency will then be greater in PCR tubes spiked with more standard, causing a greater deviation of the ratio S/T in these tubes, resulting in a change of the slope. A consequence of this conclusion is that a rectilinear log–log calibration curve of slope=1, which includes points obtained from PCR tubes that have reached the saturation phase as well as points obtained from the exponential phase, represents a strong argument for equal amplification. Discrepant amplification will be most prone to show up in the later phases of the PCR when the concentration of PCR components decreases below the saturation level required for optimal amplification. Therefore, if a calibration curve of invalid format is obtained, a possible remedy is to reduce the number of PCR cycles and/or the initial amount of template.

The fact that incorrect calibration curves may be obtained despite the fact that the target and the standard templates possess the same sequences in their primer-binding sites indicates that the chances of different kinetics of product accumulation in the nonexponen-

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tial phase, even for some of the sequences containing the same primer templates, may be greater than often assumed.

It should be noted that acrylamide gels have been shown to be superior to agarose gels for the quantification of ethidium bromide-stained DNA products.[10] Possibly, some apparently anomalous standard curves are not caused by unequal amplification but by the use of agarose. An underestimation or overestimation of faint gel bands could cause a tilting of the standard curve without shifting the point of equivalence, in which case the quantification would be valid if, and only if, the point of equivalence is used for quantification. In our experiments, preferably, we use labeled deoxynucleotides, and quantification of the bands on dried polyacrylamide gels with a PhosphorImager scanner and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RATIO PCR

For some types of problems it is not necessary to measure absolute levels of mRNA. Especially in the case of multigene families, useful information can be obtained from the comparison of the relative expression level of the members of a gene family in different cells or tissues. Obtaining this kind of information is much easier, in principle, than obtaining absolute quantitative data. In this approach, all members of the gene family are amplified simultaneously and with equal efficiency using primers in conserved regions. Because in principle, the PCR can be run into the saturation phase, it can be considered a form of competitive PCR. This method was used first by Buck et al.[11] to study the expression of the $GABA_A$ receptor family. However, from our own experience, several improvements to this method can be suggested. The first concerns the choice of the primer templates. Whereas Buck and colleagues chose conserved regions for primer annealing without stressing the importance of complete identity of the sequences of the primer templates, it is clear from the considerations of competitive PCR that all possible prerequisites for equal amplification efficiency should be fulfilled. The sequences for primer annealing should therefore be identical in all members of the gene family to assure equal amplification efficiency.

Another improvement concerns the strategy of discrimination among the different sequences in the PCR product if the sequences do not differ in length (which is the case most of the time). Buck et al.[11] used Southern blotting and isoform-specific probes to discriminate and quantitate the PCR products. In our applications, a more convenient way to achieve this discrimination proved to be the use of specific restriction sites in the region between the common primer sequences. Using restriction analysis to discriminate both products has several advantages over isoform-specific probes. It is technically simpler, and it allows the discrimination of highly conserved sequences that cannot be discriminated by hybridizing probes. As examples of ratio PCR, Figure 1 shows the relative expression level of the highly conserved actin gene family in smooth muscle cells and the expression of the multidrug resistance genes *MDR1* and *MDR2* in human lymphocytes. The fact that the discrimination of the PCR products is still possible even if they are very similar in sequence has additional advantages. There is a better chance of finding primer templates of identical sequence in all the targets to be amplified, a prerequisite for equal amplification efficiency; also the similarity of the region in between the primer sequences increases the probability of equal amplification efficiency.

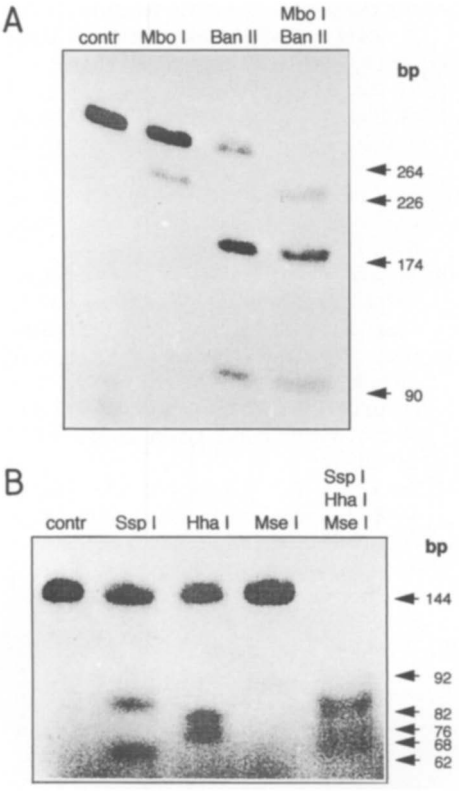

FIGURE 1 Digitized images (using the PhosphorImager) of ^{32}P-labeled PCR products and their restriction fragments, separated on a 6% polyacrylamide gel. (*A*) The ratio of P-glycoprotein messengers *MDR1* and *MDR2/3* in MM (from multiple myeloma patients) lymphocytes in culture. A 265-bp fragment was amplified from reverse-transcribed poly(A)$^+$ mRNA using the sense primer TGCCTATGGAGACAACAGCCGGGT and the antisense primer GTCCAGGGCTTCTTGGACAACCTT, corresponding to the 100% conserved regions in both P-glycoprotein isoforms expressed in humans. The 5′ base in the sense primer corresponds to nucleotide 3817 in the *MDR1* messenger (GenBank accession no. M14758), 3422 in *MDR2/3* (GenBank accession no. M23234). The temperature of the annealing step was 60°C. (Control lane) The combined undigested *MDR1+MDR2/3* amplification product. Digestion with *Mbo*I yields 39- and 226-bp fragments in *MDR1*; digestion with *Ban*II gives rise to 175- and 90-bp fragments in *MDR2/3*. The comparison of the radioactivity in the largest fragments of each digest, and correcting for the difference in the GC content of each band, yielded relative values of 15% for *MDR1* and 85% for *MDR2/3*. The same result was obtained by comparing the amount of the largest fragment with that of the undigested band within each digest. (*B*) The ratio of actin messengers in rat aorta. A 144-bp fragment was amplified from reverse-transcribed poly(A)$^+$ mRNA using the sense primer GCTGACAGGATGCAGAAGGAG and the antisense primer GGAAGGTCGTCTACACCTAG, corresponding to the 100% conserved regions in the three actin isoforms expressed in rat aorta. The 5′ base in the sense primer corresponds to nucleotide 976 in α-vascular actin (GenBank accession no. X06801), 2810 in the β-actin genomic sequence (V01217), and 951 in γ-smooth muscle actin (GenBank accession no. X52815). The temperature of the annealing step was 54°C. (Control lane) The combined undigested α- (vascular), β-, and γ (cytoplasmic)-actin amplification product. The other lanes show the specific digestion of α-, β-, and γ-actin respectively, using, *Ssp*I (yielding 82- and 62-bp fragments), *Hha*I (76- and 68-bp fragments), or *Mse*I (52- and 92-bp fragments), and the combined digestion with all three restriction enzymes. The comparison of the radioactivity in the largest fragments of each digest, and correcting for the difference in the GC content of each band, yielded relative values α-, β-, and γ-actin of, respectively 47%, 44%, and 8.5%. The same result was obtained by comparing the amount of the largest fragment with that of the undigested band within each digest.

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We found this approach by restriction analysis to be applicable in all cases examined. In addition to the examples shown in Figure 1, the method described here has been applied to the sarcoendoplasmic reticulum Ca^{2+} transport ATPases SERCA2/3,[12] the IP_3 receptor family,[13] to the three MDR genes from mouse, and to ClC-1 and ClC-2 chloride channels of the rat.[14] For accurate quantification, routinely we carried out at least three additional PCR cycles in the presence of [α-^{32}P]dCTP. The PCR products and their restriction fragments were separated on polyacrylamide gels and quantitated using a PhosphorImager scanner and correcting for the GC content of each band.

As a control, it was necessary in these experiments to check for complete digestion of each isoform. The best control was to carry out a digestion of the PCR fragment with all of the isoform-specific restriction enzymes, either combined in one tube, as in Figure 1, or consecutively. Alternatively, the amount of the fragments of each isoform-specific digest was summed up. A value ≅100% of the undigested band should be obtained. The latter procedure can be used only if the volume of the PCR mixture used in each digest and in each corresponding lane of the gel is accurately determined. Incomplete digestion may be caused by formation of heterodimers, as discussed by Siebert and Larrick[6] and by Becker-André and Hahlbrock,[8] by mutation of restriction sites by mistakes of the DNA polymerase, or by the presence of unknown members of the gene family.

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REFERENCES

1. Raeymaekers, L. 1993. Quantitative PCR: Theoretical considerations with practical implications. *Anal. Biochem.* **214:** 582–585.
2. Ferre, F. 1992. Quantitative or semi-quantitaive PCR: Reality versus myth. *PCR Methods Applic.* **2:** 1–9.
3. Weisner, R.J., B. Beinbrech, and J.C. Rüegg. 1993. Quantitative PCR. *Nature* **366:** 416.
4. Volkenandt, M., A.P. Dicker, D. Banerjee, R. Fanin, B. Schweitzer, T. Horikoshi, K. Danenberg, P. Danenberg, and J.R. Bertino. 1992. Quantitation of gene copy number and mRNA using the polymerase chain reaction. *Proc. Soc. Exp. Biol. Med.* **200:** 1–6.
5. Sugimoto, T., M. Fujita, T. Taguchi, and T. Morita. 1993. Quantitative determination of DNA by coamplification polymerase chain reaction: A wide detectable range controlled by the thermodynamic stability of primer template duplexes. *Anal. Biochem.* **211:** 170–172.
6. Siebert, P.D. and J.W. Larrick. 1992. Competitive PCR. *Nature* **359:** 557–558.
7. Wang, A.M., M.V. Doyle, and D.F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci.* **86:** 9717–9721.
8. Becker-André, M. and K. Hahlbrock. 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res.* **17:** 9437–9446.
9. Gilliland, G., G.G. Perrin, K. Blanchard, and H.F. Bunn. 1990. Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci.* **87:** 2725–2729.
10. Bouaboula, M., P. Legoux, B. Pességué, B. Delpech, X. Dumont, M. Piechaczyk, P. Casellas, and D. Shire. 1992. Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control. *J. Biol. Chem.* **267:** 21830–21838.
11. Buck, K.J., R.A. Harris, and J.M. Sikela. 1991. A general method for quantitative PCR analysis of mRNA levels for members of gene families: Application to $GABA_A$ receptor subunits. *BioTechniques* **11:** 636–641.
12. Wuytack, F., B. Papp, H. Verboomen, L. Raeymaekers, L. Dode, R. Bobe, J. Enouf, S. Bokkala, K.S. Authi, and R. Casteels. 1994. A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. *J. Biol. Chem.* **269:** 1410–1416.
13. De Smedt, H., L. Missiaen, J.-B. Parys, M.D. Bootman, L. Mertens, L. Van Den Bosch, and R. Casteels. 1994. Determination of relative amounts of inositol trisphosphate receptor mRNA isoforms by ratio polymerase chain reaction. *J. Biol. Chem.* **269:** 21691–21698.
14. Raeymaekers, L. (unpubl.)

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