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Bias in Estimations of DNA Content by Competitive Polymerase Chain Reaction[1]

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Competitive PCR is a highly sensitive method for specific DNA quantification. Despite the lack of studies related to the accuracy of the method it has been widely used. Here we present a simulation model for competitive PCR, which takes into account the efficiency decay as a linear relationship of the total product yield. The model helped us to study the kind and magnitude of errors that arise from quantitative and semiquantitative competitive PCR protocols and to find ways to minimize them. The simulation data suggest that differences in amplification efficiency between target and standard templates induce stronger biases in quantitative than in semiquantitative competitive PCR. Quantitative competitive PCR can only be used when both efficiencies are equal. In contrast, semiquantitative competitive PCR can be used even when the target is amplified with a higher efficiency than the standard, since under such conditions the method tends to underestimate the differences in initial DNA content. These predictions have been confirmed with experimental data and show that the estimation of the amplification efficiencies is a prerequisite for the use of quantitative and semiquantitative competitive PCR. A simple method for this estimation is also presented. © 2000 Academic Press

Key Words: **competitive PCR; amplification efficiency; quantitative; errors; model.**

Understanding biological system responses to environmental stimuli or changes in developmental programs usually requires the quantification of mRNA. A highly sensitive method, based on reverse transcription followed by polymerase chain reaction, has been developed (1–3). This method makes use of an exogenously added standard, which differs in internal sequence but shares the primer-binding sequences with the target template. The standard is used to compensate for tube-to-tube variations in amplification efficiency and allows, at least in principle, to extend the PCR beyond the exponential phase into the saturation phase (4). Despite the lack of studies related to the accuracy of the method, it has been widely used for absolute and relative quantification of specific nucleic acid sequences (5–7). The technical aspects for reliable quantification have been reviewed (8), but our knowledge about the theoretical aspects of the competitive PCR process is still very poor. Some authors have already issued warnings about specific pitfalls in competitive PCR (9, 10), but these considerations lack a theoretical framework, are restricted to absolute quantitative PCR protocols (11–13), or assume that the target and standard templates amplification efficiencies are the same (14, 15). However, the use of a common primer set does not guarantee equal amplification efficiencies among DNAs sharing the same primer sequences (16). Moreover, most competitive PCR protocols described to date suggest the use of 30–50 PCR cycles (8), which in most cases drives the PCR beyond the exponential phase. Thus, an analysis of the biases during absolute and relative quantification under such conditions becomes of outstanding relevance.

We present a simulation model for competitive PCR and its utility for the estimation of errors committed during quantitative and semiquantitative PCR. In addition, this model provides a theoretical tool to evaluate the effect of some experimental parameters on the

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quantification accuracy, such as the ratio between the standard and target amplification efficiencies or the ratio between the standard and target product yields used for semiquantitation. In such a way, simulation data allowed us to develop guidelines to optimize the competitive PCR protocol. Practical examples and applications of this model are also presented.

MATERIALS AND METHODS

The Model

Because of the discrete nature inherent to the PCR process, the amount of product for the target template (T) and standard sequence (S) at cycle n will be

$$T_n = T_{n-1}(1 + E^T_{n-1}) \quad [1]$$

$$S_n = S_{n-1}(1 + E^S_{n-1}), \quad [2]$$

where E^T and E^S represent the amplification efficiency of target and standard templates, respectively.

If E^T and E^S are constants, which is only true during the exponential phase of the PCR, Eqs. [1] and [2] can be expressed as functions of the cycle number,

$$T_n = T_0(1 + E^T)^n \quad [3]$$

$$S_n = S_0(1 + E^S)^n, \quad [4]$$

where T_0 and S_0 are the initial amounts of target and standard templates, respectively. Then, making a ratio of Eqs. [3] and [4] and taking the logarithm we obtain

$$\log \frac{T_n}{S_n} = \log T_0 - \log S_0 + n \log \frac{1 + E^T}{1 + E^S}. \quad [5]$$

According to Gilliland *et al.* (1), a curve is constructed relating log (T_n/S_n) to log S_0. If $E^T = E^S$, a basic assumption for absolute quantification (1, 17), Eq. [5] simplifies to

$$\log \frac{T_n}{S_n} = \log T_0 - \log S_0 \quad [6]$$

which describes a straight line with slope equal to -1 and displacement from origin equal to log T_0. However, if $E^T \neq E^S$, but both remain constant during the assay, the curve obeying Eq. [5] will be shifted in parallel with an amplitude proportional to the number of cycles. Under these conditions, the error in the absolute quantification of T_0 will increase with the number of cycles, but its relative quantification will be fairly accurate and independent of *n.* Experimental data have revealed calibration curves that cannot be explained by Eq. [5], such as curves with slopes different from -1 (1) or nonlinear curves (17). As we have mentioned, the assumption of constant efficiency during the assay is only valid for the exponential phase. If the PCR is extended into the saturation phase, the thermal inactivation of the DNA polymerase and the increasing competition between primer–template duplexes for the available polymerase, among other factors, will determine a decrease in amplification efficiency during the assay. Therefore, E^T and E^S will not be constant, and thus Eqs. [3] and [4] will not be valid anymore. To further study the origin and magnitude of the errors that arise when the PCR is extended into the linear or saturation phase, it becomes necessary to express the amplification efficiency as a function of product yield.

Assuming that there is not any secondary structure involving both the primer-binding sequences and the rest of the template, the primer–template duplex formation rate will be the same for both templates, since both target and standard share primer-binding sequences. Then, assuming that primer–template duplexes compete for a limiting amount of DNA polymerase, and the rate of standard–target heteroduplex formation is proportional to the amount of template, the simplest way to describe the amplification efficiency is by a linear relationship inversely proportional to the total amount of templates,

$$E^T_n = E^T_{\mathrm{i}}\left(1 - \frac{T_n + S_n}{P_{\max}}\right) \quad [7]$$

$$E^S_n = E^S_{\mathrm{i}}\left(1 - \frac{T_n + S_n}{P_{\max}}\right), \quad [8]$$

where E_{i} (intrinsic amplification efficiency) is the putative amplification efficiency for $T_n + S_n = 0$ and $P_{\max}$ is the maximum amount of product that can be formed. E_{i} must lie in the range $0 < E_{\mathrm{i}} < 1$ and depends on conditions inherent to the PCR itself and to the primers used. Among such conditions are the length and base composition of templates; the initial concentration of DNA polymerase, dNTPs and primers; the denaturing, annealing, and synthesis temperatures; the length of cycles; and the presence of inhibitors in the sample. Equations [7] and [8] are similar to a particular form of Raeymaekers' equations, in which the constants α and β are equal to E^T_0 and E^S_0 (11). It should be noted that E_0 is not equivalent to E_{i}, because Raeymaekers defines E_0 as the initial amplification efficiency (i.e., the efficiency of the first PCR cycle) (11); and in the present model E_{i} is the intrinsic amplification efficiency.

Since there is no obvious analytical solution for the system that arises from the combination of Eqs. [1], [2], [7], and [8], the amount of product was calculated for

each cycle with a numerically iteration method. The values of the parameters were chosen in a way that the overall simulation resembles a typical PCR assay.

RNA Extraction and Reverse Transcription

The RNA from rat spleen was extracted using the TRIzol reagent (Gibco BRL, Grand Island, NY) and reverse transcribed according to the manufacturer's protocol (Superscript II RT kit, Gibco BRL).

Competitive PCR

The multicompetitor standard pRat6 (18) was linearized with *Eco*RI and coamplified with the total cDNA from rat spleen in a 50-μl reaction mixture containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each of the rat β_2-microglobulin sense (5′-TCTTTCTGGTGCTTGTCTC-3′) and antisense (5′-AGTGTGAGCCAGGATGTAG-3′) primers, 200 μM each dNTP, and 2.5 units of *Taq* polymerase (Gibco BRL). PCR was performed in a PTC-200 thermocycler (MJ Research, Watertown, MA) as follows: 2.5 min denaturation at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 3 min at 72°C. The expected sizes for target and standard amplification products are 241 and 480 bp, respectively.

Amplicons were separated by 1.5% agarose gel electrophoresis containing 5 μg/ml ethidium bromide for 1 h at 7.5 V/cm. Bands were visualized by excitation at 316 nm and digitalized with a frame grabber (White/UV transilluminator, UVP, Cambridge, UK). The bands were quantified with the software Gelworks 1D Intermediate version 3.01 (UVP). Alternatively, radiolabeled amplification products were separated in 10% polyacrylamide gel electrophoresis and quantified measuring either the optical density of ethidium bromide-stained bands or the radioactivity of bands excised from the gel with a β-counter (Beckman, Palo Alto, CA).

Estimation of Intrinsic Amplification Efficiencies

Kinetic analysis (parallelism test) (19) was carried out at various cycle numbers (26 to 42) and the products yield for each cycle was measured. The intrinsic target and standard amplification efficiencies were estimated from the kinetic analysis data using the software "Amplification Efficiency," which is available at http://www.iib.uba.ar/Lab103/. Briefly, the amplification efficiency of target and standard templates for each cycle was estimated from the kinetic analysis data as follows:

$$E_n^T = \left(\frac{T_{n+a}}{T_n}\right)^{1/a} - 1 \quad [9]$$

$$S_n^T = \left(\frac{S_{n+a}}{S_n}\right)^{1/a} - 1, \quad [10]$$

where a is the number of cycles between two consecutive data points. Equations [9] and [10] are derived from Eqs. [3] and [4] assuming that the amplification efficiencies are constant through the few cycles that lie between two data points. Equations [7] and [8] were fitted to these estimated efficiencies with nonlinear regression in order to estimate the parameters E_i^T and E_i^S. The sum of squared residuals was minimized with the Solver module of Microsoft Excel 2000 software (Microsoft Corp.).

RESULTS AND DISCUSSION

By the use of a simulation model, we have studied the origin and magnitude of the biases introduced by the competitive PCR method in absolute and relative quantifications of DNA. The classical model for PCR amplification (Eqs. [3] and [4]) is restricted to the exponential phase of the amplification process, since it is derived from Eqs. [1] and [2] assuming that the amplification efficiency remains constant over the entire PCR assay. If this condition is not fulfilled, as is the case when the PCR is extended beyond the exponential phase, an alternative model that considers the efficiency decay must be used. The simplest way to describe the amplification efficiency is by a linear relationship inversely proportional to the amount of template (Eqs. [7] and [8]). This empirical function seems particularly valid for competitive PCR, because both target and standard templates share primer-binding sequences and hence the primer–template duplex formation rate will be the same for both templates. Then, when template concentration rises, the primer–template duplexes can compete for a limiting amount of DNA polymerase, leading to a proportional decrease in amplification efficiency for both target and standard templates. A similar assumption has been used to formulate a theoretical description of the amplification efficiency based on the enzymatic kinetic formalism (20). However, that model considers that the amplification efficiency for target and standard templates are always the same. The results obtained with an alternative model derived from the hyperbolic description of Schnell and Mendoza (20) (but considering that the intrinsic amplification efficiency of target and standard templates (E_i) could be different) were similar to those obtained with the linear empirical approximation (data not shown).

The competition between QC-PCR[3] products and primer dimmers has been recently analyzed (21), and it

[3] Abbreviations used: QC-PCR, quantitative competitive PCR; SQC-PCR, semiquantitative competitive PCR.

has been postulated as responsible for an inherent quantitative capacity of RT-PCR. Furthermore, we found primer dimmer products in our experiments. Then, we reformulate the model to account for primer dimmer amplification. However, we did not find any qualitative differences between the simulation data from both models (data not shown), so here we describe the results obtained with the simplest one, which does not involve primer dimmers.

Calibration Curve and Quantitative Competitive PCR

In the description of the method (1), a calibration curve was constructed relating log (T_n/S_n) to log S_0, and notice was taken of the linearity and slope –1 of that curve as an index for equal amplification efficiency of target and standard templates (19). In spite of this, calibration curves with slope different from −1 (1, 22) and nonlinear curves (17) have been used for absolute quantification.

If amplification efficiencies for both standard and target templates are the same and remain constant during the PCR assay, theory predicts a linear calibration curve with slope −1 (11) and a displacement from origin equal to the logarithm of the initial amount of target template (Eq. [6]). Moreover, if the amplification efficiencies of standard and target templates are not the same, but both remain constant during the assay, the calibration curve will be shifted in parallel with an amplitude proportional to the number of PCR cycles (n). Under these conditions, the bias in the absolute quantification of the initial amount of target template will increase with the number of cycles, but its relative quantification will be fairly accurate and independent of n (11). The above considerations are strictly true for the exponential phase of the PCR, but we have not any theoretical or empirical background to assume that the extrapolation of this pattern to the nonexponential phase will be valid.

The model presented here predicted a calibration curve equivalent to the one described by Eq. [6] only if the amplification efficiencies of target and standard templates are the same along the entire PCR, even when the simulation was run beyond the exponential phase into the saturation phase (Fig. 1). Thus, the initial target template amount can be accurately determined at any value within the dilution series of standard template and regardless of the number of PCR cycles. On the contrary, if the amplification efficiencies of target and standard templates are different, the referred pattern for the exponential phase cannot be extrapolated to the nonexponential phase, since the model predicted a shift from origin, change in slope, and a slight deviation from linearity for the calibration curve (Fig. 1). Then, two different scenarios could be envisaged:

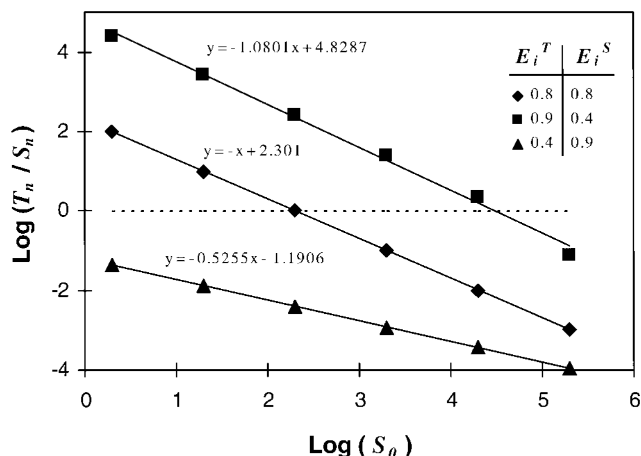

FIG. 1. Simulated calibration curves for the coamplification of a target sequence T at an initial amount T_0 = 200, with a dilution series of standard template S at initial amount (S_0) of 2, 20, 200, 2000, 2×10^4, and 2×10^5. The ratio of the products (T_n/S_n) after 30 cycles (n = 30) was calculated for each set of initial conditions. P_{max} was set to 2×10^7 and the values for the parameters E_i^T and E_i^S are indicated in the graph.

1. For the target template being amplified with a greater efficiency than the standard sequence, the model predicted a parallel shift of the calibration curve, whereas the slope was very little affected. These results are in agreement with the work of Raeymaekers (11), indicating that a calibration curve with slope −1 is not a sufficient condition to assume that target and standard templates are amplified with equal efficiency.

2. For the target sequence being amplified with a lower efficiency than the standard template, the model predicted a shift about the origin and a significant decrease in slope (Fig. 1). This result is in contrast to a previously published model (11), but it is in agreement with previous experimental results (1, 17). Moreover, simulation data showed that the slope of the calibration curve depends on the initial template amount T_0 (Fig. 2A). To confirm these predictions experimentally, rat spleen cDNA and the nonhomologous standard pRat6 (18) were coamplified using a β_2-microglobulin-specific primers set (Fig. 2C). Kinetic analysis of this system showed that the intrinsic amplification efficiencies E_i^T and E_i^S were 0.35 and 0.68, respectively (R^2 = 0.92). As is shown in Fig. 2B, the experimental calibration curve was equivalent to that obtained with the model (Fig. 2A). The slope of the calibration curve below one was not due to faint ethidium bromide-stained bands analyzed on agarose gels as has been suggested (9, 19), since we obtained similar results with radiolabeled PCR products resolved on polyacrylamide gel electrophoresis (data not shown). Then, the

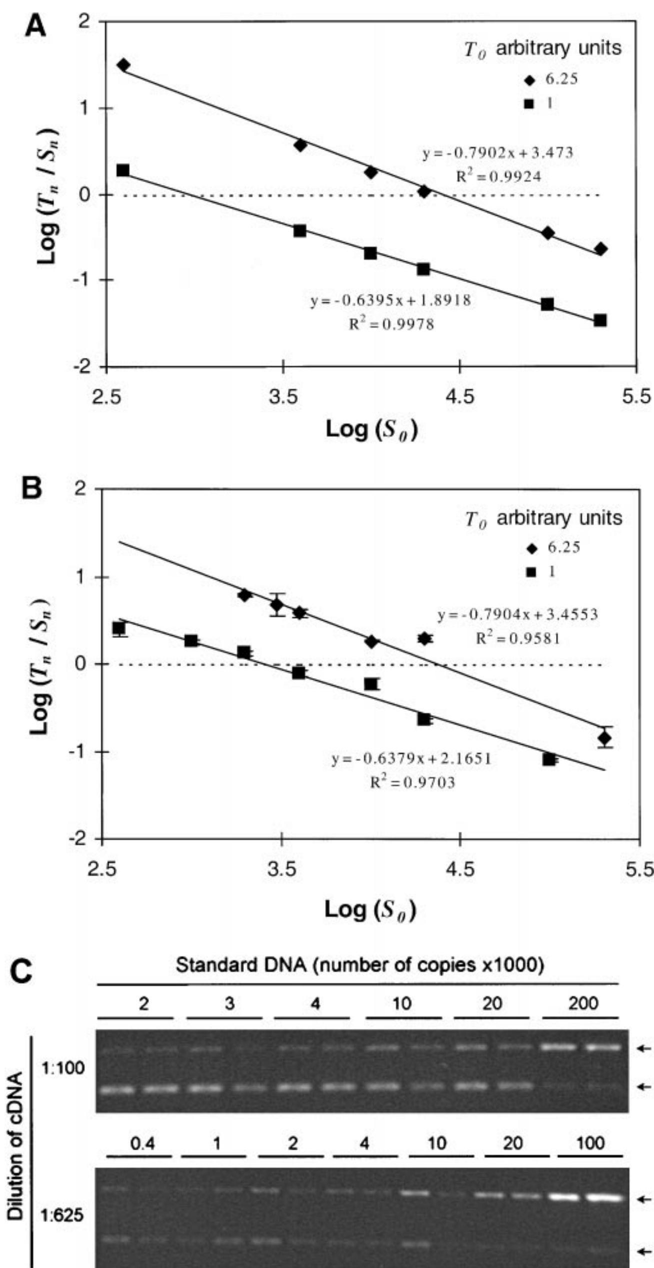

FIG. 2. Effect of the initial target amount (T_0) on the calibration curve for $E_i^T < E_i^S$. (A) Simulated calibration curves for the coamplification of target sequence T at initial amount (T_0) of 10^4 and 6.25×10^4, with a dilution series of standard template S at initial amount (S_0) of 400, 4000, 10^4, 2×10^4, 10^5, and 2×10^5. The parameters E_i^T, E_i^S, and P_{max} were set to 0.35, 0.68, and 1.1×10^8, respectively. The ratio of the products (T_n/S_n) after 30 cycles ($n = 30$) was calculated for each set of initial conditions. (B) Experimental calibration curves and (C) ethidium bromide-stained agarose gels corresponding to the coamplification of 1:100 and 1:625 dilutions of cDNA from rat spleen (T) and a dilution series of the nonhomologous standard pRat6 (S) (18), using a β_2-microglobulin-specific primer set.

overall results demonstrate that the change in slope is inherent to the PCR amplification process itself and is not due to experimental manipulation artifacts.

If $E_i^T \neq E_i^S$, the use of Eq. (6) introduces severe biases in the estimation of T_0. The model predicted a 420% overestimation of T_0 for E_i^T being only 1.25-fold higher than E_i^S (Fig. 3). Thus, the absolute quantification will be accurate only if both target and standard templates are amplified with the same efficiency. Since a linear calibration curve with slope −1 is not a sufficient condition for the amplification efficiency being the same, other kinds of analysis must be done. A frequently used procedure, called parallelism test, is based on a kinetic analysis for the simultaneous amplification of target and standard templates (5, 19). Moreover, the intrinsic amplification efficiencies, E_i^T and E_i^S can be estimated from parallelism test data.

Slight deviations from the equivalent efficiencies assumption induce strong deviations in absolute quantification derived from the use of the calibration curve (Fig. 3) (12). The biases in QC-PCR are due to the displacement from origin and the change in slope of the calibration curve. Since SQC-PCR is only dependent on the slope of the calibration curve, this method could be an alternative of choice in order to diminish the error when T and S are amplified with different efficiencies.

Semiquantitative Competitive PCR

The SQC-PCR method assumes that T and S are amplified with the same efficiency, thus the ratio of their products T_n/S_n will remain constant throughout the PCR and the value of T_n/S_n will be identical to the

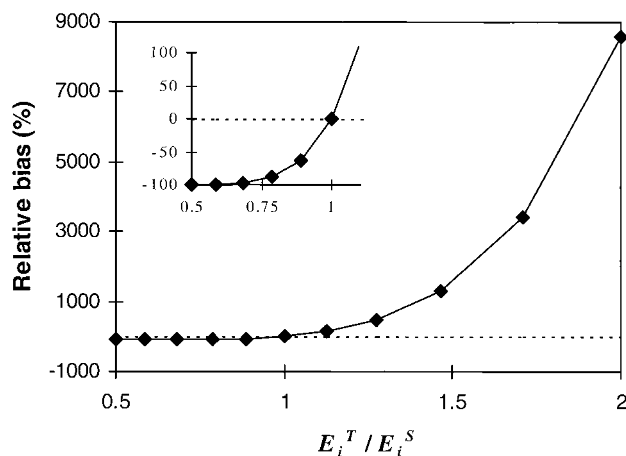

FIG. 3. Effect of the intrinsic amplification efficiency ratio of target and standard templates (E_i^T/E_i^S) over the relative bias in initial target template amount (T_0) quantified by QC-PCR (T_0^*). Simulated calibration curves for the coamplification of a target sequence T at an initial amount $T_0 = 200$, with a dilution series of standard template S at initial amount (S_0) of 2, 20, 200, 2000, 2×10^4, and 2×10^5 where used for the estimation of T_0. The relative bias after 30 cycles ($n = 30$) of simulated coamplification at different amplification efficiency ratios (E_i^T/E_i^S) was calculated as $RB = (T_0^* - T_0)/T_0$. T_0^* was estimated at the equivalence point $T_n = S_n$ for each calibration curve.

initial ratio T_0/S_0. Then, the mixed ratio $T^2_nS^1_n/T^1_nS^2_n$, where T^k_n is the PCR product of target k and S^k_n is the PCR product of standard from reaction k, will be equal to the initial ratio of target templates $T^2_0S^1_0/T^1_0S^2_0$. In practice, this condition is not always achieved; if E^T differs from E^S, the SQC-PCR method introduces a bias in the initial ratio estimation, whose nature and magnitude has not been evaluated yet. The model was used to study the effect of differences in amplification efficiency on the outcome of the semiquantitation, with the aim of clarifying the kind and magnitude of the errors and finding possible ways to minimize them.

When the intrinsic amplification efficiencies of target and standard templates were not the same, the relative error of semiquantitative estimations resulted exponentially related to the cycle number during the exponential phase and became constant when the PCR simulation was extended into the saturation phase (data not shown). Since the relative error approached to maximum values when the PCR reached the saturation phase (i.e., $n > 25$), a simulation of 30 cycles was used hereafter. As is shown in Fig. 4, the relative bias in semiquantification depended on the difference between E^T_i and E^S_i, and also on differences in template amounts to be compared. Results from simulations showed that when the target template was amplified with a lesser efficiency than the standard sequence, differences in the initial amount of target template were overestimated. On the contrary, when the target template was amplified with the greatest efficiency, differences in the initial amount of target template were underestimated. This is also reflected in the experimental data present in Fig. 2C. For the calculations we used the results of the coamplification of 1:100 cDNA dilution with 20,000 copies of standard template and 1:625 cDNA dilution with 4000 copies of standard, because they are less prone to densitometric errors (1). In that way we estimated the 1:100 dilution as 15-fold more concentrated than the 1:625 dilution, which means an overestimation of 140%. Conversely, the 1:625 dilution resulted 58% underestimated. The overall experimental results were in agreement with the model's predictions. It should be noted that the bias was always smaller for SQC-PCR than for QC-PCR (see Figs. 3 and 4), showing that SQC-PCR is a more robust method than QC-PCR.

The results suggest that the PCR must be performed under conditions that ensure a more efficient amplification for the target, because under such conditions differences in DNA content estimated by SQC-PCR could not be due to the bias introduced by the method. Since an inverse exponential relationship between template size and amplification efficiency has been observed (16, 23), the easiest way to obtain a more efficient amplification of target than standard could be the use of longer standard templates. They can be obtained by insertions (24) or by the use of nonspecific spacer DNA (25, 26). The last one, called nonhomologous competitor, has the further advantage of avoiding heteroduplex formation (16). Nonspecific spacer sequences have been also frequently used to construct multicompetitor standards containing priming sites for various genes of interest (3, 5, 18, 27). Unfortunately, the use of nonhomologous competitors longer than the target template does not ensure a more efficient amplification of the target template, as is the case for our system, possibly due to differences in secondary structure among templates.

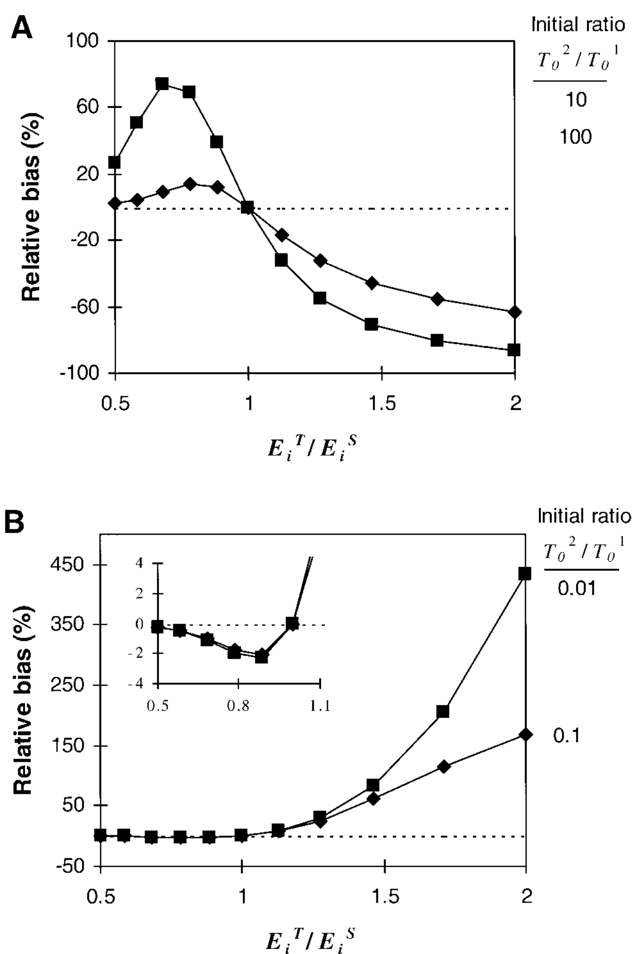

FIG. 4. Effect of the intrinsic amplification efficiency ratio of target and standard templates (E^T_i/E^S_i) over the relative bias in the initial templates ratio (T^2_0/T^1_0) estimated by SQC-PCR. The simulated relative bias after 30 cycles ($n = 30$) was calculated for each initial target ratio as $RB = (ET_0 - T^2_0/T^1_0)/(T^2_0/T^1_0)$, where ET_0 is the SQC-PCR estimation of the initial target templates ratio T^2_0/T^1_0. The values for the initial target ratio T^2_0/T^1_0 are indicated in the graph. (A) $T^2_0 > T^1_0$. (B) $T^2_0 < T^1_0$.

For competitive PCR it has been stated that the initial target amount (T_0) should be calculated at the point of equivalence, $\log(T_n/S_n) = 0$. This statement is based on practical issues, such as the precision of den-

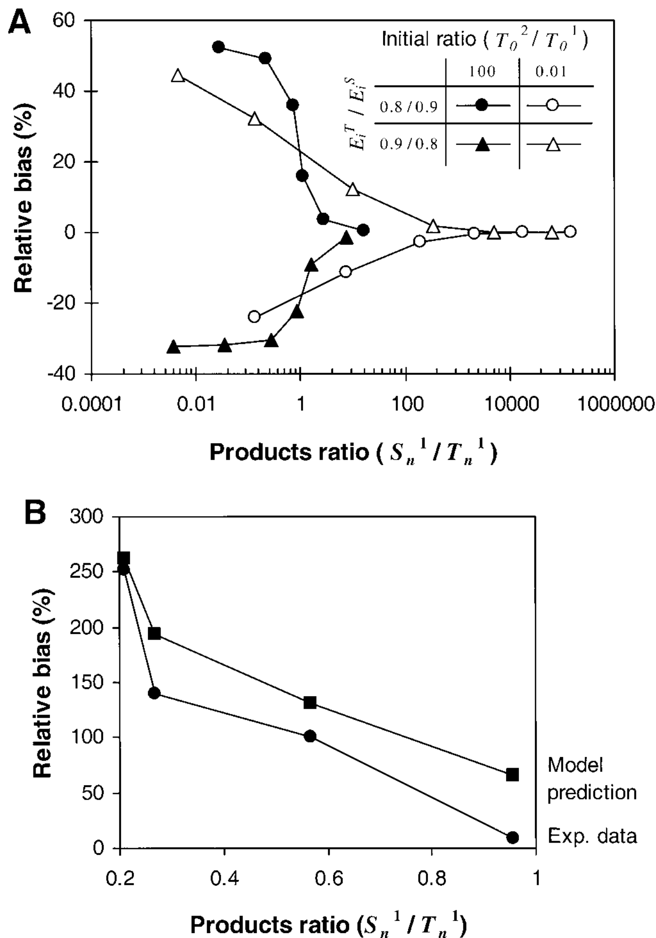

FIG. 5. Effect of the standard to target products ratio (S_n^1/T_n^1) on the relative bias in the initial templates ratio (T_0^2/T_0^1) estimated by SQC-PCR. (A) The simulated relative bias after 30 cycles ($n = 30$) was calculated for a target initial amount $T_0^1 = 200$ and for each standard initial amount (S_0), 2, 20, 200, 2000, 2×10^4, and 2×10^5 in combination with target initial amounts (T_0^2), 2 and 2×10^4 as $RB = (ET_0 - T_0^2/T_0^1)/(T_0^2/T_0^1)$, where ET_0 is the SQC-PCR estimation of the initial target templates ratio T_0^2/T_0^1. The values for the parameters E_i^T and E_i^S are indicated in the graph. (B) Relative bias in the semiquantitation of cDNA from rat spleen (Exp. data). 1:100 and 1:625 dilutions of cDNA were coamplified with a dilution series of the nonhomologous standard pRat6 and β_2 microglobulin-specific primers. The bias in the semiquantitation of the 1:100 dilution from the 1:625 dilution was estimated for different values of standard template. The predicted bias (Model prediction) was calculated from simulation data with intrinsic efficiencies equal to the experimental ones (i.e., $E_i^T = 0.35$ and $E_i^S = 0.68$).

sitometric determinations of gel bands (1, 17) and compensation of heteroduplex formation when homologous competitors are used (28). It should be noted, however, that there is not any theoretical background to choose the point of equivalence for relative quantifications. The model showed that the semiquantitation results are accurate despite the standard template amount (S_n) used when the target and competitor are amplified with the same efficiency. On the contrary, if target and standard efficiencies are not the same, the bias introduced by the method results inversely related to S_n in such a way that when S_n rises, the relative error decreases tending to zero (Fig. 5A). The same pattern was observed when we experimentally estimated the initial target amount of a 1:100 dilution of rat spleen cDNA compared to a 1:625 dilution. We estimated the bias in the semiquantitation of a 1:100 dilution of cDNA from the results of the coamplification of 1:100 cDNA dilution with 20,000 copies of standard template and 1:625 cDNA dilution with different initial standard amounts. As is shown in Fig. 5B, the experimental relative bias decreased when we used higher template amounts. These results indicate that the use of a high standard concentration could be a strategy to minimize the bias in semiquantitative determination of initial target amount.

Guidelines for Competitive PCR

Simulation data confirmed by experimental results allowed us to suggest the following guidelines in order to minimize the errors introduced by competitive PCR:

- The QC-PCR will be reliable only if the target and standard are amplified with the same efficiency. This condition must be checked with a kinetic analysis (parallelism test) (19).
- If the equal amplification efficiency is not fulfilled, the SQC-PCR could be an alternative of choice. The last method is more robust and the errors can be controlled.
- To avoid false increments or decrements estimations by SQC-PCR, the target template must be amplified with a higher efficiency than the standard. The initial amplification efficiencies could be easily obtained from kinetic analysis data (see materials and methods).
- The bias in SQC-PCR could be further reduced using an initial standard template amount as high as possible and extending the PCR just the number of cycles necessary to detect the products.

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