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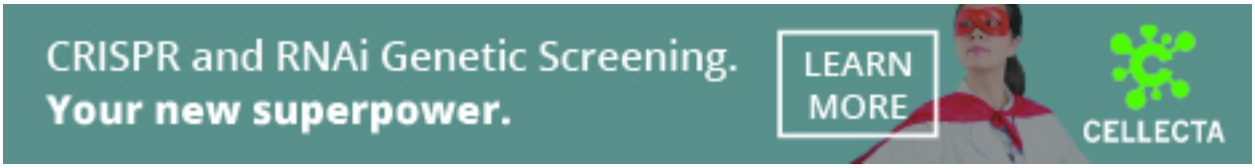

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Quantitative PCR of Bacteriophage λ DNA Using a Second-Generation Thermocycler

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In many applications in molecular biology and biomedical research, it may be necessary to measure the concentration of a target DNA sequence in native DNA. PCR methodology can be employed to measure the concentration of a target DNA sequence in native DNA quantitatively. General aspects of PCR quantitation have recently been described.[1] Because PCR results in an exponential accumulation of target DNA during the amplification process, the following relationship is employed to relate the initial concentration of target DNA to the concentration of the amplified DNA:

$$Y = X(1 + E)^n \quad (1)$$

where Y is the concentration of PCR-amplified DNA, X is the concentration of target DNA prior to PCR, E is the average reaction efficiency for each cycle, and n is the number of amplification cycles.

In practice, this equation is utilized in its logarithmic form:

$$\log(Y) = \log(X) + n \times \log(1 + E) \quad (2)$$

By plotting the logarithmic value of the PCR product yield, log (Y), against the number of amplification cycles, n, a linear curve can be generated with the intercept equal to the logarithmic value of target DNA, log (X), and the slope equal to log ($1+E$).

When the logarithmic values of the PCR product yield are plotted against the logarithmic values of known quantities of target DNA, again a linear curve is obtained. According to equation 2 the slope of this linear curve has to be 1. It is possible that in some cases the slope is not equal to 1. This fact can change equation 2 to the following expression:

$$\log(Y) = k \times \log(X) + n \times \log(1 + E) \quad (3)$$

where k is the slope of the linear curve.

The aim of the present study was to explore the potential of ion-exchange chromatography for PCR quantitation using a second generation thermocycler for amplification. A sequence (+ strand) of a 500-nucleotide segment of the 48.5 kb bacteriophage λ genome was chosen as target DNA.[2]

Most of the work on quantitative PCR, for example, the method by Wang et al.,[3] uses internal standards to quantitate initial target concentrations. These methods are often connected to complicate working procedures such as the preparation of the internal standard. Our method overcomes the use of an internal standard and therefore is very simple to perform, which also makes it useful for laboratories where most of the equipment used for molecular biology is not available.

MATERIALS AND METHODS

PCR

PCR mixture (100 μl) consisted of sterile water, buffer (10 mM Tris-HCl at pH 9.0, 50 mM KCl, 0.01% (wt/vol) gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100), dNTPs (each 200 μM), primers (each 1 μM), *Taq* DNA polymerase (0.38 unit), and bacteriophage λ DNA (20pg–1.5 ng).

Primer sequences were from Perkin-Elmer Cetus and are listed in Table 1. Primers were synthesized by Oligos ETC. INC., dNTPs and bacteriophage λ DNA were part of the GeneAmp PCR Reagent Kit (Perkin-Elmer Cetus). The Vienna Lab HiTaq Polymerase including 10× buffer was used for all experiments. The PCR mixture was prepared with positive displacement pipets (Vienna Lab), with autoclaved tips in autoclaved tubes. Amplification was carried out in the GeneAmp PCR System 9600 by Perkin-Elmer Cetus. For PCR, MicroAmp Tubes were used. Therefore, it was not necessary to overlay the PCR mixture with mineral oil.

Samples were cycled in the following manner: Two-step PCR—1 min at 94°C; 20 cycles: 90 sec at 68°C, 5 sec at 94°C; 5 min at 68°C.

Detection

The amplification product was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The length of the product was estimated by comparing it with a molecular weight standard (100-bp DNA ladder by GIBCO BRL) separated on the same gel.

HPLC was carried out according to a protocol of Perkin-Elmer Cetus on the System Gold HPLC system (Beckman). Columns were supplied by Perkin-Elmer:

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TABLE 1 Primer Sequences

Primer	Nucleotides	Sequence	Strand
1	7131–7155	5′-GATGAGTTCGTGTCCGTACAACTGG-3′	(complement of − sequence)
2	7606–7630	5′-GGTTATCGAAATCAGCCACAGCGCC-3′	(complement of + sequence)

Column: TSK DEAE–NPR column, 3.5 cm×4.6 mm ID, 2.5 μm
Guard column: TSK DEAE–NPR column, 0.5 cm×4.6 mm ID, 5 μm
Detector: Uvikon 730 LC (Kontron)
Detection wavelength: 260 nm

Twenty microliters of the amplification product was injected directly into the HPLC system without any further purification. Samples were separated in the following nonisocratic manner (flow rate 1 ml/min):

Eluents
Buffer A: 25 mM Tris-HCl, 1 M NaCl (pH 9.0)
Buffer B: 25 mM Tris-HCl (pH 9.0)

Starting composition: 30% A in B

Gradient:
In 0.1 min convex (curve 2) from 30% to 40% A
In 2.9 min linear from 40% to 52% A
In 7 min linear from 52% to 60% A
In 0.5 min linear from 60% to 100% A
5 min 100% A
In 0.5 min linear from 100% to 30% A
10–15 min 30% A

Quantitation

1. Peak areas of the 500-bp amplification product were converted into absolute DNA concentrations by the following procedure: Four different concentrations of the 100-bp DNA ladder (GIBCO BRL), 22.2–111.1 μg/ml, were separated by HPLC under the same conditions as the amplification product. Using the total peak areas of the four concentrations, a factor for converting the peak area of any DNA sample into absolute DNA concentration was calculated. The linearity of the HPLC system was then tested by injecting a serial dilution (seven different concentrations) of the 500-bp amplification product achieved from 1.5 ng of λ DNA by 25 PCR cycles and measuring the corresponding peak areas.

2. The influence of the cycle number on the amplification product was investigated by amplifying 1 ng of bacteriophage λ DNA per 100 μl of PCR mixture and measuring the amplification product concentration by HPLC after cycling the sample for 20, 25, 30, 35, and 40 cycles, respectively.

3. Evaluation of equations 2 and 3, respectively, was done by amplifying target concentrations of 20–900 pg of λ DNA per 100 μl of PCR mixture and measuring the concentration of the amplification product by HPLC as described.

4. Reproducibility of the method was checked by running the same samples (100–900 pg of bacteriophage λ DNA per 100 μl of PCR mixture) under identical conditions on two different days.

RESULTS

A factor (f) for converting the peak area of any DNA sample into absolute DNA concentration (in μg/ml) was calculated from the separation of four different concentrations of the 100-bp DNA ladder ($f = 1.69$) and used in all of the following experiments. We found good agreement between the peak areas of the four different concentrations. A bacteriophage λ 500-bp fragment was used to establish a standard curve for the quantitative evaluation of the ion-exchange chromatograms after amplification. A linear curve (coefficient of correlation $r = 0.999$) was obtained when peak areas of the serial dilution of the amplification product were plotted against DNA concentrations up to a concentration of 11 μg per 100 μl of PCR mixture (Fig. 1). The influence of the number of cycles on the amplification process is shown in Figure 2. Using a constant target DNA concentration of 1 ng per 100 μl of PCR mixture, the number of amplification cycles was varied from 20 to 40. When the logarithmic values of the amplified DNA were plotted against the number of cycles, a linear curve was obtained from 20 to 30 cycles; a plateau was then reached. Quantitation has to be done outside of this plateau in the linear range of the curve, which corresponds to the exponential phase of the PCR reaction. Therefore, we used 20 PCR cycles in all further experiments. The efficiency of

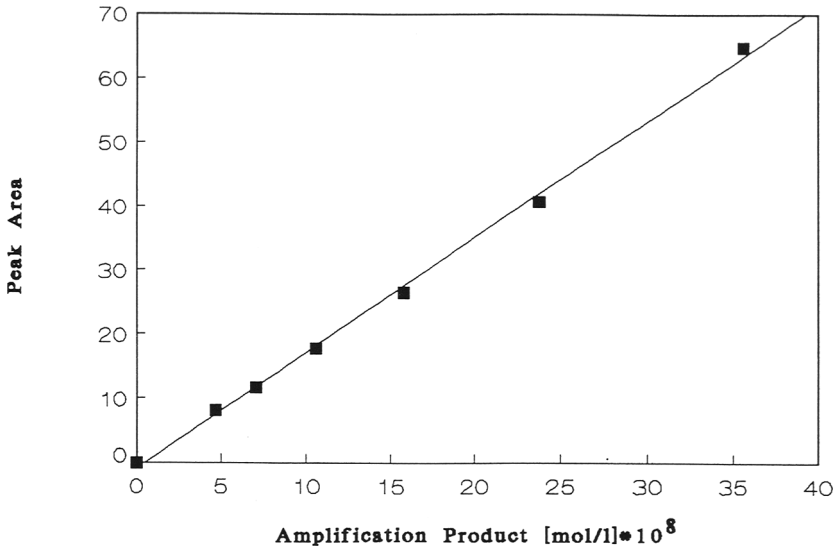

FIGURE 1 Standard curve of the 500-bp amplification product of bacteriophage λ DNA detected by HPLC. The peak areas were plotted against a serial dilution of the amplification product.

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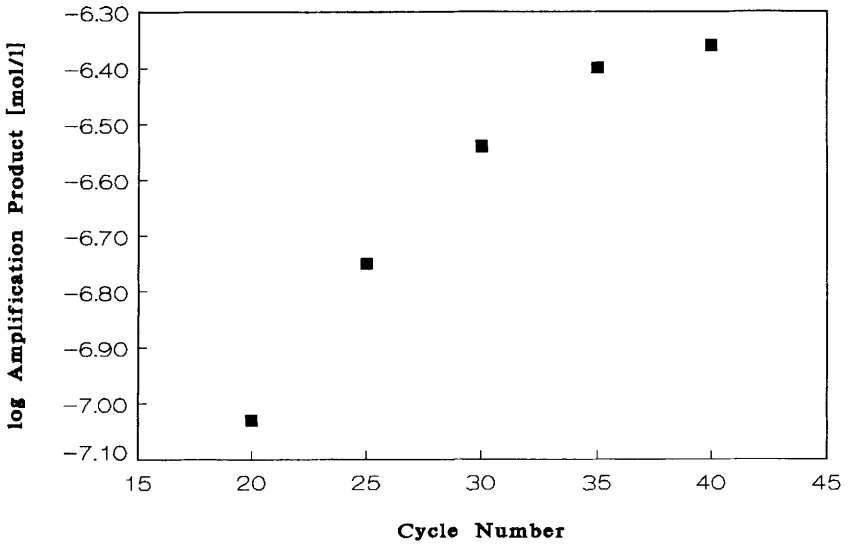

FIGURE 2 Dependence of the amplification product on the cycle number. According to equation 3 the logarithm of amplification product concentration was plotted against the cycle number. The linear region of the curve is the exponential phase of amplification.

amplification was tested over a concentration range from 20 to 900 pg per 100 µl of PCR mixture target DNA (3.7×10^5 to 1.7×10^7 DNA copies per sample). A linear relationship (coefficient of correlation $r=0.994$) was found when the logarithmic values of the amplified DNA were plotted against the logarithmic values of the target DNA. The slope was not 1 but 0.66 (Fig. 3), which makes equation 3 suitable for this reaction. An efficiency of the process, $E=0.17$, was calculated from the intercept of the curve, which resulted from plotting all values in moles per liter. This unit was used, because the value of the intercept depends on the concentration units applied, if the slope is not equal to 1. Reproducibility was found to be good. Running the same samples under the same conditions on two different days, the average deviation from the mean was 5.4%.

DISCUSSION

The second generation of thermocyclers is reported to provide excellent well-to-well temperature uniformity, fast amplification times, and high sample throughput.[1] On the basis of these characteristics of the amplification method and the high reproducibility of the quantitation of DNA by ion-exchange chromatography with UV-spectroscopic detection, an excellent agreement was found when the theoretical relationship between the concentration of the initial target DNA and the concentration of amplified DNA (equations 2 and 3) was tested for a 500-bp segment of the bacteriophage λ DNA (Fig. 3).

Use of equations 2 and 3 is valid if PCR is performed in the exponential phase, that is, for a given number of cycles, E is a constant between 0 and 1. The efficiency of the PCR process is very dependent on reaction conditions. Factors that control the efficiency of amplification and, therefore, the yield of PCR product have been discussed by Katz et al.[1] By performing 20 PCR cycles, we obtained an efficiency of $E=0.17$ for our experiments. Under the conditions of this study, the exponential phase was observed up to 30 cycles.

In agreement with Katz et al.,[1] we conclude from our results that with the recent developments in PCR instrumentation and PCR chemistry the PCR process has become very reliable and reproducible.

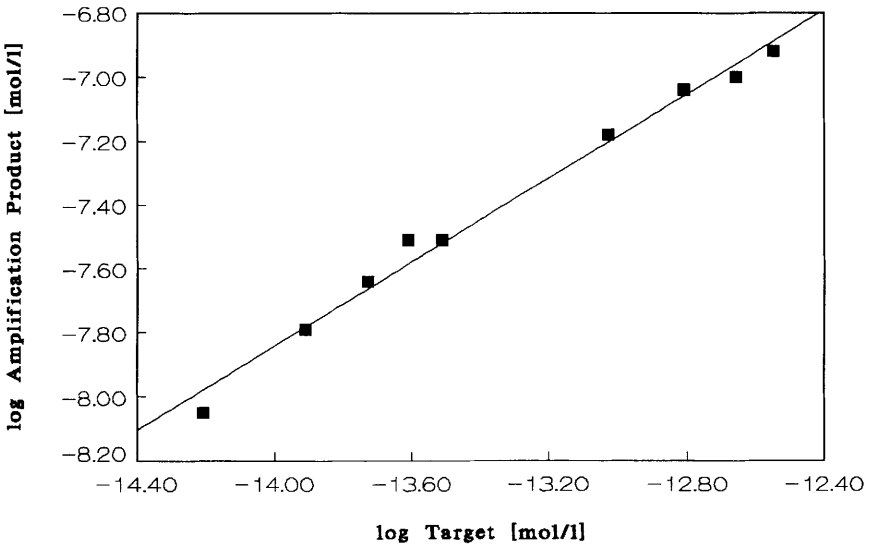

FIGURE 3 Dependence of the amplification product on the target DNA concentration. According to equation 3 the logarithm of amplification product concentration was plotted against the logarithm of target DNA concentration. A linear relationship and, therefore, an excellent agreement with theory was found.

ACKNOWLEDGMENTS

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