Note

Suppressed-Priming PCR, a Novel Concept of DNA Quantification Based on PCR Kinetics

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The concept of an unbiased DNA quantification method is proposed based on PCR kinetics. A set of PCRs was performed with a dilution series of primers, which differently limited DNA amplification. The difference in amplification efficiency between the DNA templates reflect the estimates of their relative amounts. This concept might serve as a theoretical base for further development of accurate and robust PCR-based quantification methods.

Key words: abundance estimation; community analysis; image analysis; PCR-DGGE; relative DNA amount

The community structure of micro-organisms and other small organisms and its development are important characteristics to understand better the biological and biochemical processes in fermentation, food degradation, plant-microbe interactions in the rhizosphere, etc. However, community analysis of such organisms typically requires a special knowledge of taxonomy and skills in preparing specimens. Only experts can conduct it on a regular basis, and this poses a major obstacle to community studies.

For non-experts, PCR-based methods are increasingly powerful tools to estimate the abundances of bacteria, fungi, and other small organisms. Among these methods, real-time PCR is preferably applied to estimate accurately the abundance of the corresponding species, e.g., a nematode, based on the kinetics of PCR. However, it is still hard to quantify many species in a mixture simultaneously. It also requires sequence data of target species to design specific primers before experimentation.

On the other hand, PCR-DGGE (denaturing gradient gel electrophoresis) is preferably applied in profiling community structure. DGGE can separate one DNA fragment from another depending on GC content, even if two fragments are of the same length. PCR-DGGE with universal primers is thus useful in estimating the diversity of DNA templates in environmental samples or any other assemblage of small organisms, e.g., bacteria.

Despite these advantages of PCR-DGGE, biases in PCR amplification can lead to inaccurate estimation. To correct for PCR biases, standard curves must be drawn before estimation, as done for PCR-RFLP image analysis. This, however, is often unrealistic for environmental samples composed of various unidentified species. Few attempts have been made at quantitative analysis, and DGGE banding data are considered to be qualitative, and, in fact, are often dealt this way in community profiling. All other PCR-based methods also have the same problem of accuracy of quantification, unless amplification biases in PCR can be corrected. Practical methods to correct PCR biases are needed to increase the usefulness of PCR-based quantification methods for community analysis. In this study, a concept of unbiased quantification of DNA template was developed based on PCR kinetics. The reliability of the concept was briefly tested by applying it to DNA quantification using PCR-DGGE.

Let us consider a PCR in which more than two kinds of DNA templates are amplified competitively with respect to the same primer set. Assuming that the amount of newly synthesized DNA template in a PCR cycle is proportional to the amounts of the template and primer, and is affected by the inhibitory effect of template-template re-annealing, we approximated the increment of a template DNA to a differential equation written as a function of unit time $t$:

$$\frac{dM_i(t)}{dt} = k_i \cdot M_i(t) \cdot P(t) - \frac{P(t)}{S(t) + P(t)}$$

where $M_i(t)$ is the concentration of the $i$-th DNA template, $P(t)$ is that of the primer, and $S(t)$ is that of total DNA templates at time $t$. $k_i$ is a coefficient of amplification. The relationship between the concentration of the $i$-th and $j$-th DNA templates is simply described as

$$\frac{dM_i(t)}{dM_j(t)} = \frac{k_j \cdot M_j(t)}{k_i \cdot M_i(t)} \quad (k_i \neq 0)$$

Thus,

$$\Rightarrow \int \frac{dM_i}{k_i \cdot M_i} = \int \frac{dM_j}{k_j \cdot M_j}$$

$$\Rightarrow \frac{\ln(M_j)}{k_j} = \frac{\ln(M_i)}{k_i} + \text{Const.}$$

Given $t = 0$, the equation is solved for $\text{Const.}$ as

$$\text{Const.} = \frac{\ln(M_j(0))}{k_j} - \frac{\ln(M_i(0))}{k_i}$$

Thus $M_i$ is linear-regressed on $M_j$ after log-transformed, as follows:

$$\ln(M_i) = \frac{k_j}{k_i} \cdot \ln(M_j) + \ln(M_j(0)) - \frac{k_j}{k_i} \cdot \ln(M_j(0))$$

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where \( M_i \) and \( M_j \) are the initial concentration of the \( i \)-th and the \( j \)-th DNA templates respectively. Considering \( M_i \) and \( M_j \) to be the concentration of an internal standard and a target DNA template respectively, the initial concentration of the target \( M_i \) is calculable with the slope and \( y \)-interception of the regression line if the initial concentration of the internal standard \( M_j \) is given. The linear relationship as shown in eq. (6) is always maintained if the \( k_i \) and \( k_j \) values are constant or always proportional, even when the PCRs reach plateau phase. Equation (1) implies that the final concentration of the DNA templates varies depending on the initial concentration of the primers. Thus a regression line can be determined by the data set obtained at the endpoint of a series of PCRs performed with primers of different initial concentrations, instead of monitoring the concentration of DNA templates in realtime. We denote this novel concept of DNA quantification suppressed-priming PCR, referring to this mode of data collection.

Two nematode species, Acrobeloides sp. (Acro) and Tylencholaimus parvus (Tyl), maintained in laboratory culture were chosen for experimental use. Acro was regarded as an internal standard against Tyl, the target. Nematode DNA samples were prepared following Okada and Oba, except that 300 nematodes were used per sample. Fragments of 18S rDNA were amplified from the DNA with primers SSU9R-GC (5'-GC GCG CCG CGC CCC GCG GCC GCG GCC CGG CCC GCG CTG GAA TTA CCG CGG CTG-3') and SSU18A (5'-AAA GAT TAA GCC ATG CAT G-3'), originally designed by Blaxter et al. and modified with a GC clamp. The products were quantified using a spectrophotometer (model ND-1000; NanoDrop Technologies, Wilmington, DE) and diluted to 10^-6 ng/μl with nuclease-free water after purification using the Wizard SV PCR product purification System (Promega, Madison, WI) according to the manufacturers’ instructions, and used as templates in the subsequent PCR-DGGE.

PCR-DGGE was performed following Okada and Oba, except that a dilution series of primer sets was used to limit PCR amplification differently. A set of reaction mixtures contained 0.6 units of Prime Star Polymerase HS (Takara, Otsu, Japan), 1× PCR buffer, 0.2 mM dNTPs, and 0.40 × 10^-7 ng/μl the each of the template DNAs and each of the primers at 0.096, 0.144, 0.192, 0.240, and 0.288 μM. PCR was carried out using the following thermal cycles: 98 °C for 3 min, 26 cycles at 98 °C for 10 s, 52 °C for 15 s, 72 °C for 40 s, and 72 °C for 10 min. DGGE was performed using the DCode™ System (Bio-Rad Laboratories, Hercules, CA). PCR products were immediately loaded onto 6% acrylamide gels (acrylamide:bisacrylamide = 37.5:1; denaturant gradient ranging from 20 to 50%) at 10μl/well in 1× Tris-acetate-EDTA (TAE) buffer and dissolved by electrophoresis at 75 V and 60 °C for 16 h. Gels stained with SYBR® Green I (Cambrex, Rockland, ME) were scanned and analyzed using a software Molecular Imager FX (Bio-Rad). Typical DGGE banding patterns are shown in Fig. 1. The “trace” values (integrated density of bands) acquired by the software were corrected so that the values would be proportional to the DNA amounts (data not shown). The DNA amounts of the target and the internal standard, \( M_i \) and \( M_j \), were estimated by comparing their trace values to that of the molecular marker (DGGE Marker V; Nippon Gene, Tokyo). Data were log-transformed, and regression analysis was done using eq. (6) to estimate the initial concentration of the DNA template of the target.

Log-transformed data sets obtained from suppressed-priming PCR-DGGE were well regressed linearly (Fig. 2) with highly significant coefficients of determination (\( r^2 = 0.985–1.000 \), \( p = 0.0004–0.0155 \)). The average of estimates, 0.93 × 10^-7 ng/μl, was comparable to the true value, 0.40 × 10^-7 ng/μl, although it was a slight overestimation. Hence, the model of suppressed-priming PCR appears to be a reasonable approximation of the fact. The estimates of target DNA amount varied...
widely across trials (95% confidence limit 0.32–2.75 × 10⁻⁷ ng/μl; CV 131%). This might be assigned to errors in post-PCR processes.

The accuracy of estimation depends largely on the sensitivity of the detector for PCR products. If the detector has only low sensitivity, the amount of target DNA template must be estimated inevitably by an extreme extrapolation from the regression line, which can enhance estimation errors. The sensitivity of the detector can also affect the dynamic range of estimation significantly. Further studies are needed to reduce background noise and to combine suppressed-priming PCR with sensitive detection methods, e.g., T-RFLP.

To control estimation errors, competitive PCR is often applied. In the conventional concept of competitive PCR, the internal standard serves to control errors that can occur during DNA extraction, purification, PCR preparation, signal detection, etc. among samples. The internal standard itself does not serve to compensate amplification biases between the internal standard and the target DNA template and among the targets in single PCRs. Previous studies based on conventional competitive PCRs overcame this problem by designing an internal standard that was amplified at a rate equal to the target or by correcting biases with standard curves. In contrast to these palliative treatments, our present study suggests a novel and fundamental concept to correct PCR biases among DNA templates based on PCR kinetics.

Suppressed-priming PCR is theoretically robust to the numbers of PCR cycles even if the targets and internal standard are amplified at different rates. In practice, however, it is recommended that one reduce the number of PCR cycles in order not to enlarge the deviation of the relative amount of the templates from the original state so much that it exceeds the dynamic range of the detection system. Suppressed-priming PCR disadvantageously needs a set of several PCRs per sample. Hence, it can turn to be of inferior cost performance when applied to quantify a single DNA template, but suppressed-priming PCR is applicable to simultaneous quantification of multiple DNA templates even if there is no information on their amplification efficiency, and it does not require standard curves for the estimation of relative DNA amounts. We think, then, that further studies should be done to apply suppressed-priming PCR advantageously to community samples of microorganisms.

The concept of suppressed-priming PCR is applicable to any PCR-based quantification method. We emphasize that suppressed-priming PCR is a universe concept to achieve accurate and robust DNA quantification, especially when combined with methods that sensitively detect traces of PCR products, e.g., PCR T-RFLP.

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**References**