Quantitative PCR: the determination of template copy numbers by temperature gradient gel electrophoresis (TGGE)

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Low copy numbers of DNA or RNA sequences can be quantified easily by combining PCR (Polymerase Chain Reaction) and TGGE (Temperature Gradient Gel Electrophoresis). This combination allows to overcome the problems of quantitative PCR caused by plateau effects, uneven priming or, variable cycle efficiencies.

We have developed a general method using an internal standard identical, except for a single nucleotide, with the authentic template. After PCR, the amplified nucleic acids, although differing only in one single base position, can be separated by TGGE (1, 2). Fig. 1 shows a generally applicable protocol. Because of identical priming sites and almost identical sequences in between, the ratio between template and standard remains constant during extensive PCR cycling. Subsequently, a small amount of labelled standard (approx. 1 ng) is added to the 0.1 - 1 µg of amplified DNA. After denaturation and renaturation, the labelled standard has formed homoduplexes with amplified standard and heteroduplexes with amplified template. The ratio is identical with that of standard and template before amplification. An aliquot of this mixture is analyzed in the TGGE gel with the temperature gradient being superimposed on the electrical field in parallel. The amplified standard as a homoduplex exerts the highest thermal stability and, therefore, migrates to the highest temperature in the T-gradient. The heteroduplex denatures partially at lower temperatures due to the mismatch formation. Thus, it is retarded drastically in the gradient at lower temperature in comparison to the homoduplex.

The initial template copy number is easily calculated according to the following formula:

\[
\text{template copy number} = \frac{\text{intensity heteroduplex}}{\text{intensity homoduplex}} \times \text{number of initial standard copies.}
\]

Our method is based on the quantitative evaluation of heteroduplexes. This is a clear advantage. In other approaches it is just heteroduplex formation that generates erroneous results, e.g. by destruction of a restriction site used as an internal marker. Fig. 2 shows the template quantification of a human β-globin gene. The accuracy of the method is clearly demonstrated by comparing the experimental data with the theoretical values (straight line). The experimental error is below 15%.

Nearly any segment can be used as template for amplification and for quantitative determination of copy numbers. As shown in the insert of Fig. 1, the mutation in the standard may be introduced by a primer p3 carrying a mismatching base in the additional sequence as opposed to the primer p1 for template amplification. An amplified segment most suitable for quantitative TGGE analysis should contain 100 - 300 bp and an AT-rich part on the side of p1. In order to increase the difference in stability between the lower melting AT- and the higher melting GC-rich part, a GC-clamp may be added to the GC-rich side (3). For easy labelling and cloning of the standard, a sticky end restriction site is added to one side and a blunt end restriction site to the other. Details of such constructions were described elsewhere (2). For RNA quantification, 'run-off' RNA transcripts are used as the standard in order to include the first reverse transcription step for calibration (manuscript in preparation).

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Figure 1. Schematic protocol for PCR/TGGE-template quantification. The insert shows the construction of the primer for template and standard amplification, respectively.
Figure 2. Experimental data for the template quantification of a human gene (β-globin). Chromosomal DNA was prepared from 1 ml of blood by the QIAGEN procedure which yields DNA fragments of approx. 1000-2000 bp (manuscript in preparation). $10^3$ copies of a linearized plasmid covering the IVS-1-6 mutant (2) were added as internal standard to a dilution series of a sample aliquot. The amplified segment (126 bp) contains a 18 bp G:C clamp. The labelled standard is added after PCR (1 ng, $10^3$ cpm $^{32}$P, one strand labelled by filling in an EcoRI site). After denaturation (2 min, 98°C) and renaturation (30', 50°C, Tm approx. 40°C), the samples were analyzed in a TGGE gel using a gradient between $T_1 = 25°C$ and $T_2 = 60°C$, 0.1 × TBE, 4M urea (DIAGEN-TGGE-System, Düsseldorf, Germany). The measured intensities follow the theoretical values within more than two orders of magnitude with an accuracy of +/-15%. The high molecular weight band refers to labelled vector DNA. The deviation at dilutions $>1:100$ results from the labelled probe which contributes to the total amount of standard.

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REFERENCES