Absolute quantification of target DNA: a simple competitive PCR for efficient analysis of multiple samples

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PCR is an exquisitely sensitive method for detection of minute amounts of target DNA. The exponential nature of DNA amplification, however, is prone to burden the experimental data with significant standard error due to the tube-to-tube inherent variations in amplification efficiency. Therefore the reliable quantitation of DNA or RNA templates by PCR necessitates introduction of an internal standard. Typically, in a series of replicates of analyzed sample the defined range of such standards is coamplified together with constant unknown amount of wild-type template (1, 2) or alternatively both templates are subjected to endpoint dilution (3). Obviously, each of the methods is based on multiple PCR reactions which are to be performed simultaneously with each of the analyzed samples. Such procedure, nevertheless, may become cumbersome when large numbers of samples are to be analyzed.

We describe a modification of competitive PCR which is based on standardization of interpolation from a calibration curve. This approach enables accurate analysis of multiple samples by comparison with a single set of standard samples containing known amounts of the wild-type template of interest. Effective compensation for intra-assay variability is achieved using a constant amount of internal standard to spike all samples being analyzed as well as the samples used as standards. The comprehensive equation — $\log(N_{n1}/N_{n2}) = \log(N_{01}/N_{02}) + n\log(\text{eff}_1/\text{eff}_2)$ defines the yield of the two products in any competitive PCR in terms of the ratio of the two original templates and their relative efficiencies of amplification, and simplifies when the products of both the endogenous wild-type template and the exogenous internal standard are generated with the same efficiency. Provided that this requirement ($\text{eff}_1 = \text{eff}_2$) is

Figure. A) Amplification of standard amounts of wild-type template DNA using competitive PCR. Log dilutions from 5 ng to 0.5 pg of a wild-type plasmid containing U3 and R regions of HIV-1 3B LTR were prepared using a lysate as diluent equivalent to 500 cells of the type used as a transfection target; this first plasmid would result in an amplification product of 429 bp. Each reaction was then spiked with 0.25 ng of the $\Delta 207$ bp internal standard giving rise to 222 bp band. Reaction components (10 mM Tris-HCl, pH = 8.5; 50 mM KCl, 2.5 mM MgCl_2 , 0.1% Triton X-100, 4 pmol of each primer, 200 μM dNTPs and 0.75 U Taq DNA polymerase) were mixed with 5 μl of sample at 72°C (hot start) to yield a final volume of 25 μl , and subjected to 30 cycles of amplification (94°C for 15 sec, 55°C for 45 sec, and 72°C for 45 sec) in the TempCycler II (Coy Lab Products, Ann Arbor, MI). Aliquots of 10 μl were electrophoresed in 1.5% agarose in $1 \times$ TAE buffer and optically analyzed in order to construct the calibration curve (see B). A faint image of 429 bp product from 0.5 pg of standard wild-type template was discernible during the digital video processing; however, it became blurred in the final print due to reprographic process. B) Calibration curve constructed from the data obtained by quantitative analysis of the electrophoretic pattern in A). The logarithm of the ratios of the masses of DNA in the wild-type and deleted bands separated by electrophoresis as determined from the photographic print by using the digital image processing system (AMBIS, San Diego, CA) was plotted (ordinate) against the log of the input of wild-type standards (abscissa). This standard curve calculated using a least squares analysis ($y = -1.229 + 0.606x$) was used to interpolate the amount of full length template (429 bp) in unknown samples by using the ratio of 429 bp to 222 bp PCR products.

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fulfilled, the ratio of products (N_{n1}/N_{n2}) during any cycle (n) of amplification depends solely on the ratio (molar or mass) of the initial templates (N_{01}/N_{02}). Under these conditions, direct comparison of the mass ratios of the products in unknown samples with the corresponding ratios determined from the known standard samples can be accurately performed regardless of the phase of amplification; thus, the quantity of wild-type template originally present in any sample can be estimated from the calibration curve. Moreover, if the use of the assay is restricted to the exponential phase of amplification, the quantitation will remain valid even when $eff_1 \neq eff_2$, assuming that the eff_1/eff_2 ratio is a constant value. In such a case the equivalency of both efficiencies of amplification does not have to be verified *a priori*, which may prove advantageous from a practical point of view.

In our PCR assay, the analyzed wild-type template (a recombinant plasmid containing the LTR of HIV-1) was exponentially diluted to set up a series of standards. In order to achieve minimal discrepancies in amplification efficiencies (4), an internal standard was derived from wild-type template by deleting a 207 bp internal segment (Figure A). After coamplification of both templates, the standard curve was constructed as a plot of the log of the ratios of the PCR products amplified from wild-type and deleted internal standard templates (ordinate) against the log of the known amounts of wild-type template standards (abscissa) (Figure B).

The method described here was introduced in our laboratory routinely with the aim to normalize between transient expression assays for the efficiencies of transfection using reporter gene constructs which contained the HIV promoter/enhancer. The principle of the assay suggests, nevertheless, general applicability to other specific tasks.

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