

Quantitative PCR: validation of the use of a multispecific internal control

Françoise Cottrez*, Claude Auriault, André Capron¹ and Hervé Groux

Unite CNRS-URA 1854 and ¹INSERM 167, Institut Pasteur de Lille, 1 rue du Pr. A. Calmette, 59019 Lille, France

Received April 15, 1994; Revised and Accepted May 25, 1994

Although RT-PCR has many advantages over RNA blotting methods, it can be difficult to obtain quantitative information, due to the enzymatic nature of the PCR amplification, where small variations in amplification conditions result in drastic changes in product yield. In addition, due to the consumption of the reaction components and generation of inhibitors, the amount of products generated plateaus during later stages of the reaction. These problems can be overcome by the use of internal controls in the PCR. In competitive PCR, a DNA fragment containing the same primer template sequences as the target is used to compete for primers annealing and amplification. PCR products are then distinguished by size, restriction sites or Southern blot analysis. Competitive PCR methods require that the target gene and competitive fragment amplify with equal efficiency; this is the case when the internal standard is as similar as possible to the sequence of interest. It had been postulated that the length or primary sequence differences between the two templates could have an effect on relative amplification efficiency. However, results obtained by different laboratories using a multispecific internal control template show that this is not the case, at least in the exponential phase of the amplification (1,2). In those reports, the analysis was limited to cycles 4 to 5 of the late exponential phase, hence further analysis to define relative amplification efficiency, at other stages of the reaction, are necessary. This is the major drawback to a routine application of the original Wang *et al.* technique (1).

In our report we have examined whether measurements using a multispecific internal control can also be performed beyond the exponential phase of reaction, with the aim of rendering the technique independent of the number of cycles used. This possibility has already been suggested for methods employing an internal standard with the same sequence as the cellular template, apart from the presence of either a small intron (3), a small deletion (4) or a mutated restriction site (5). To address this question, we have used for our multispecific internal control two templates both different in size and nucleotides composition from the cellular template. One pair of primers, specific for mouse IFN γ cDNA and multispecific internal control, was used to amplify a 280 bp and a 250 bp fragment, respectively, and the second pair of primers, specific for mouse IL10, to amplify two fragments of 400 and 250 bp, respectively.

For quantitative analysis, fluorescent labeled primers were used for the PCR and individual products were separated on

sequencing gels and quantified using a fluorescent automated DNA analyzer (4,6). In order to determine the quantity of products before PCR, we have taken advantage of this labeling and purified fluorescent amplified PCR products. Standard and cellular specific PCR products were mixed at different ratios (1/1 and 1/10 approximately for IL10 and IFN γ) and then analyzed with the automated DNA analyzer to calculate the real ratio obtained (stars on right panels). Mixes were then diluted up to 10^6 or 10^7 times and reamplified with the same primers for different cycles numbers. Subsequently, the fluorescent intensity obtained was quantified by computer analysis and then plotted on a log scale against the number of cycles. As Fig. 1 clearly shows the amplification proceeded with the same efficiency for both templates, not only during the exponential phase, but also during the non-exponential phase just up to the plateau. Moreover, the ratios obtained over the entire amplification period, up to the plateau phase, were always identical to the initial ratios (stars on right panels). This showed that despite a difference in primary sequence, a difference in size of 150 bp for IL10 and a difference in initial quantities (up to 12 times), the two templates were amplified in an identical manner for up to 40 cycles. Thus, the amount of a specific cDNA, in the total cDNA, could be calculated by comparison with the internal standard at any cycle number. Our results support the validity of a multispecific internal control, coupled to the quantification of PCR products with fluorescent primers, for the quantification of a variety of mRNA molecules.

ACKNOWLEDGEMENT

We thank S.Gianini for reviewing the manuscript.

REFERENCES

1. Wang, A.M., Doyle, M.V. and Mark, D.F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9717–9721.
2. Funk, C.D. and FitzGerald, G.A. (1989) *J. Biol. Chem.* **266**, 12508–12513.
3. Gilliland, G., Perrin, S., Blanchard, K. and Bunn, H.F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2725–2729.
4. Porcher, C., Malinge, M.C., Picat, C. and Grandchamp, B. (1992) *BioTechniques* **13**, 106–113.
5. Becker-Andre, M. and Hahlbrock, K. (1989) *Nucleic Acids Res.* **17**, 9437–9446.
6. Cottrez, F., Auriault, C., Capron, A. and Groux, H. (1994) *J. Immunol. Methods* (in press).

*To whom correspondence should be addressed

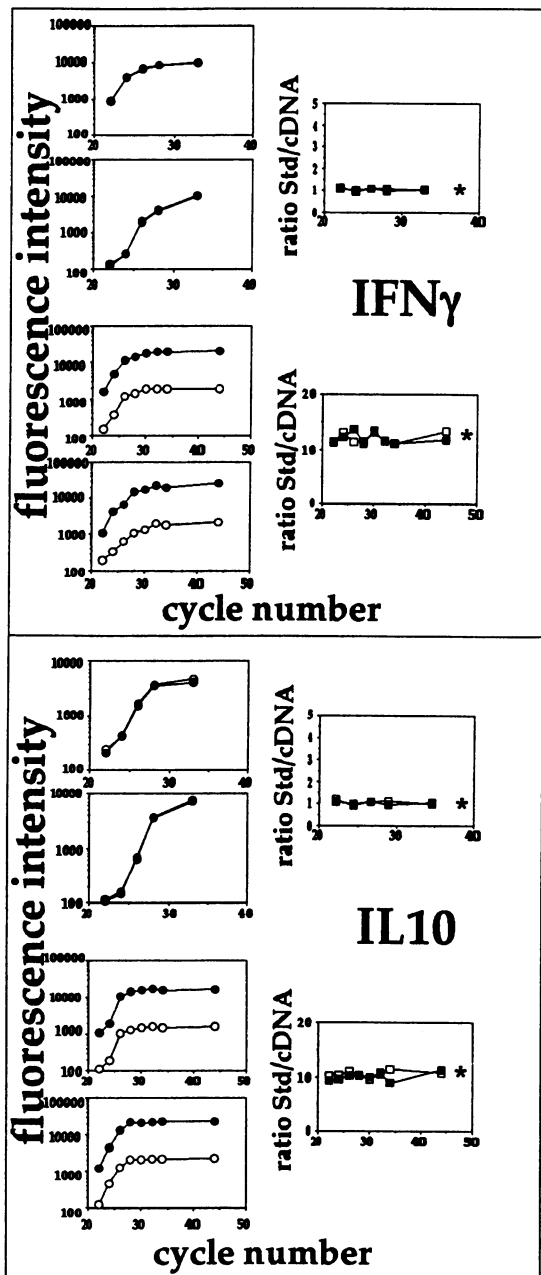


Figure 1. Simultaneous amplification of IFN γ or IL10 from cellular cDNA or multispecific standard cDNA: kinetic and ratios analysis. cDNA from mouse lymphocytes and from the multispecific standard were amplified separately in the presence of fluorescently labeled primers specific for either IFN γ or IL10, and combined at different ratios. An aliquot from each sample was analyzed using a DNA analyzer and the ratio of the two products was determined (the star on the right panels). Two dilutions (10^{-6} and 10^{-7} , respectively for upper and lower panel for each ratio) from each sample were re-amplified. The relative fluorescent intensity for each PCR product (open circle for cellular cDNA, close circle for standard cDNA) was determined with the fragment manager softwareTM (Pharmacia) and the values were plotted on a log scale against the number of amplification cycles (left panels). Ratios of standard/cDNA (open square for 10^{-6} dilution, close square for 10^{-7} dilution) were also determined and plotted against the number of amplification cycles and compared with initial ratios (stars) (right panels).