Benchmarks

Transfer RNA Reduces the Formation of Primer Artifacts During Quantitative PCR

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Recent advances have facilitated the real-time monitoring of polymerase chain reaction (PCR) by the inclusion of fluorescent labels, such as SYBR® Green I (FMC BioProducts, Rockland, ME, USA), and have triggered the widespread use of quantitative PCR of cDNA targets (5–7). Nevertheless, because SYBR Green I binds nonspecifically to double-stranded (ds)DNA, it is essential to assess the specificity of product formation. Primer artifacts are commonly encountered when target concentrations are low, where mRNA quantities available can be precious or limited (3), difficult to extract (2) or might even constitute a rare component of the sample (4). Finding compounds that effectively reduce or eliminate the formation of primer artifacts is a major challenge in

the field of fully quantitative PCR.

Current methodology allows the identification of product differentiation through the analysis of DNA melting curves by plotting fluorescence as a function of temperature over the dissociation temperature of amplified products (5). The outcome of the analysis can result in several possible types of fluorescent signal, of which, only one is

permissible for inclusion of quantitative PCR. Analysis is only permissible where exclusively the true product amplifies and thus results in the detection of a single sharp peak at the melting point specific to the amplified product (which is dependent on G/C content and product length). At lower concentrations, the signal derived from the artifact competes, in relative terms,

Figure 1. The outcome of three extreme melting curve analyses performed after fluorescent-based quantitative PCR. Note that the specific amplification of 100 pg target cDNA results in one sharp peak, which climaxes at the particular melting point of this product. However, with increasing template dilution, the fluorescence specific to the true product competes, in relative terms, with the fluorescent signal obtained from primer artifacts, which characteristically have lower melting points. This interference might cause a partial or total inhibition of true product formation, as seen with 10 and 1 fg target concentration, respectively.

Figure 2. The addition of tRNA reduces the formation of primer artifacts during quantitative PCR. The term fluorescence or F, used to describe the y-axis is shortened for reasons of simplicity and should read $[F(\text{measured}) - F(\text{min})] / [F(\text{max}) - F(\text{min})] \times 100$. As seen in Panels B and E, the addition of tRNA markedly inhibits the formation of primer artifacts. Resolving the true product from the primer artifacts, by TAE/agarose gel (2%) electrophoresis, substantiates that the addition of picogram amounts of tRNA suffices to significantly reduce the formation of primer artifacts (C) and (F). Control PCRs in the absence of cDNA template and nucleic acid molecular weight markers are represented by O and M, respectively.

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significantly and in a dose-dependent manner with the fluorescent reading obtained from the amplification of low-template concentrations (Figure 1).

The controlled experiment described in this short report shows that the addition of picogram amount of transfer RNA (tRNA, *Escherichia coli*, Strain W; Sigma, St. Louis, MO, USA) can delay the appearance of primer artifacts by at least two orders in magnitude (Figure 2, A–F), which in turn significantly increases the sensitivity of quantitative PCR. Quantitative amplification of actin was performed over 40 cycles using the LightCycler™ (Idaho Technology, Idaho Falls, ID, USA) essentially as described previously (6) with the exception of the template origin (in this case, actin cDNA amplified and cloned from reverse-transcribed mRNA isolated from the nematode *Plectus acuminatus*), the template concentrations (100 pg to 1 fg actin), the primer dilution (0.3 pM) and the presence or absence of tRNA. Subsequent melting-curve analysis was performed as previously described (5) by continuous fluorescent monitoring during the stepwise increase in temperature (0.1°C/s) from 72°–95°C.

Figure 2, A and D, confirms that the addition of tRNA shows no visual effect on amplification characteristics of corresponding serial dilutions (in which 10⁻¹⁰ equals 100 pg target cDNA). The concentration for optimal primer artifact inhibition was shown to range from 5–50 pg tRNA per µL final PCR, whereby at higher concentrations (100, 250, 500, 750 pg and 1 ng), the described effect notably diminishes (data not shown). tRNA loosely binds through hydrogen bonds to excess primer that is available during the early cycles of the amplification process and thus reduces the possibility of self-priming, a fact reflected by the routine use of tRNA during northern blotting protocols (1). Presumably, this weak association is disrupted during the denaturing step of each PCR cycle, resulting in a sufficient release of primers to ensure successful commencement of exponential amplification of the target. In summary, this work shows that the addition of tRNA is equally relevant to quantitative PCR as it is to northern blotting. Its effect should facilitate or at

least ease the process of quantitative analysis of rare transcripts.

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