Short Technical Note

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Improved mRNA Quantitation in LightCycler RT-PCR

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Key Words
Real-time PCR · LightCycler · SYBR Green · Primer dimer · RT-PCR

Abstract

Background: Real-time polymerase chain reaction (PCR) utilizing the LightCycler and similar systems is an increasingly used technique for quantitative reverse transcription (RT)-PCR of mRNA levels from genes of immunologic interest. A commonly encountered limitation with these systems is that the fluorescence induced by SYBR® Green (a fluorophore that binds double-stranded DNA) can result from primer dimers (PDs) as well as the PCR product of interest, thus interfering with the ability to reproducibly quantitate mRNA levels. ***Methods:*** We use a modification of the LightCycler PCR strategy to overcome this problem by altering the PCR strategy to take advantage of the LightCycler's ability to measure fluorescence at a temperature greater than the melting point of PDs. The resulting measurements determine fluorescence of only the desired PCR product. ***Results:*** We demonstrate that by using this modified PCR strategy, one can eliminate the fluorescence induced by PDs and obtain accurate product quantitation. ***Conclusions:*** This simple modification allows more precise quantitation of sample mRNA levels by eliminating the contaminating fluorescence induced by the formation of PCR PDs. This modification obviates the need to redesign PCR primers in RT-PCR experiments where this is impractical or impossible.

Introduction

The reverse transcription polymerase chain reaction (RT-PCR) has become a commonly used method for analyzing mRNA levels from genes of immunologic interest [1, 2]. Accurate quantification of PCR products is extremely problematic due to differences in PCR efficiency and altered amplification parameters of large and small quantities of starting template. This led to the development of methodologies such as semiquantitative and competitive RT-PCR [3]. However, such procedures are reagent and labor intensive, and also error prone due to the large number of sample manipulations involved [4]. With the advent of real-time PCR, it became possible to directly measure amplicon production during PCR, eliminating most of these technical concerns and providing a more reliable estimate of mRNA transcript levels.

Real-time PCR systems such as the LightCycler™ (Roche Diagnostics, Mannheim, Germany) and the iCycler Thermal Cycler (BioRad, Hercules, Calif., USA) use fluorescent dyes like SYBR® Green that bind double-stranded (ds) DNA. Thus, it is possible to determine the

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quantity of PCR amplicons present after each round of amplification. Since the cycle at which PCR enters log linear amplification is directly proportional to the amount of starting template, one determines the concentration of an unknown sample by comparing it to a standard curve generated by dilutions of known amounts of product [5–7].

However, an important limitation of SYBR Green is that it binds equally well to nonspecific products like primer dimers (PDs) as well as the desired PCR product. Fluorescence induced by SYBR Green binding reflects total dsDNA, including both the desired amplicon as well as PDs. This interferes with quantitation of the desired PCR product. PDs are the product of nonspecific elongation of PCR primers. Their formation can be reduced by careful primer design, optimization of he PCR with 'hot-start' PCR and amplification under stringent conditions [8]. However, PDs can occur even under optimally determined conditions regardless of primer design and irrespective of primer complementarity [9]. Thus, redesigning primers is not always an acceptable solution as it is sometimes impossible or impractical.

In developing a quantitative LightCycler RT-PCR system for a 398-nucleotide fragment of the murine IL-12 receptor β2 (IL-12R β2), PDs were generated under reaction conditions that produced little or no PDs using traditional PCR techniques. We were unable to eliminate these PDs even under the most stringent conditions. We report an approach that can be used to eliminate these products, thereby improving the efficiency of quantitation. This approach has previously proved useful in quantifying mRNA levels from constitutively expressed genes such as metallothionein and glyceraldehyde-3-phosphate dehydrogenase in rodents, as well as the *rec A* gene in bacteria [10–12]. Here, we report the utility of this strategy for quantifying more immunologically relevant genes with restricted expression patterns such as the murine IL-12R β2.

Materials and Methods

C57B16 mice were obtained from Charles River Canada (St. Constant, P.Q., Canada). All mice were used in strict accordance with the guidelines of the Canadian Council on Animal Care. Naïve mice were sacrificed and spleenocytes were cultured at 7.5×10^6 cells/ml alone or in the presence of 125 ng/ml anti-CD3 monoclonal antibody (BD Pharmingen, Mississauga, Ont., Canada) and 50 pg/ml recombinant IL-12 (BD Pharmingen) for 24 h. RNA was prepared using an RNeasy® Mini kit (Qiagen, Mississauga, Ont., Canada) as described by the manufacturer. cDNA was synthesized from 0.5–1 μg of RNA in a total volume of 20 μl using oligo $dT_{(12-18)}$ (Invitrogen, Burlington, Ont., Canada) as primer and SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR was carried out using a LightCycler (Roche, Laval, P.Q., Canada). Reactions were set up in microcapillary tubes using the following final concentrations: 0.4 μ*M* each of IL-12R β2 sense and IL-12R β2 antisense primers, as described by Yamane et al. [13], 2.5 μ*M* $MgCl_2$, 1 × SYBR Green master mix and 1 μl of cDNA. Cycling conditions were as follows: denaturation (95°C for 10 min), amplification and quantitation (95°C for 15 s, 57°C for 5 s and 72°C for 32 s, with a single fluorescence measurement at the end of the 72°C for 32 s segment) repeated 35 times, a melting curve program (60–95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) and a cooling step to 40°C. The modified cycling conditions were as follows: denaturation (95°C for 10 min), amplification and quantitation repeated 35 times (95°C for 15 s, 57°C for 5 s, 72°C for 32 s and then 84°C for 5 s, with a single fluorescence measurement at the end of the 84°C segment), a melting curve program (60–95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) and a cooling step to 40°C. Data were analyzed using LightCycler analysis software.

Results and Discussion

The effects of PDs are shown in a quantitative RT-PCR on serial dilutions of a positive control for the murine IL-12R β2 (fig. 1a). The negative control sample lacks PCR template, and PDs are the only source of fluorescence (confirmed by ethidium bromide-stained agarose gel electrophoresis, data not shown). As observed in figure 1a, fluorescence from the negative control increases parallel to that of the positive control series, making determination of the log linear amplification phase difficult and subjective. Importantly, it is impossible to determine the proportion of the fluorescence resulting from PDs and the proportion that is attributable to the relevant PCR product. This makes precise and reproducible quantitation of IL-12R β2 expression impossible.

A LightCycler melting curve analysis distinguishes between PCR products such as PDs and the desired product. Amplicons are heated to determine the melting temperature (Tm), i.e. the point at which dsDNA melts to single-stranded DNA. PDs exhibit a lower Tm than the desired amplicon as they are considerably shorter. Figure 1b shows a melting curve analysis of the same IL-12R β2 PCR product dilution series depicted in figure 1a. The two melting peaks observed in the positive controls indicate that these samples contain PDs as well as the desired IL-12R β2 amplicon. This was confirmed by ethidium bromide-stained agarose gel electrophoresis (data not shown). The negative control products (PDs only) have a lower Tm than the IL-12R β2 product (83 vs. 87°C).

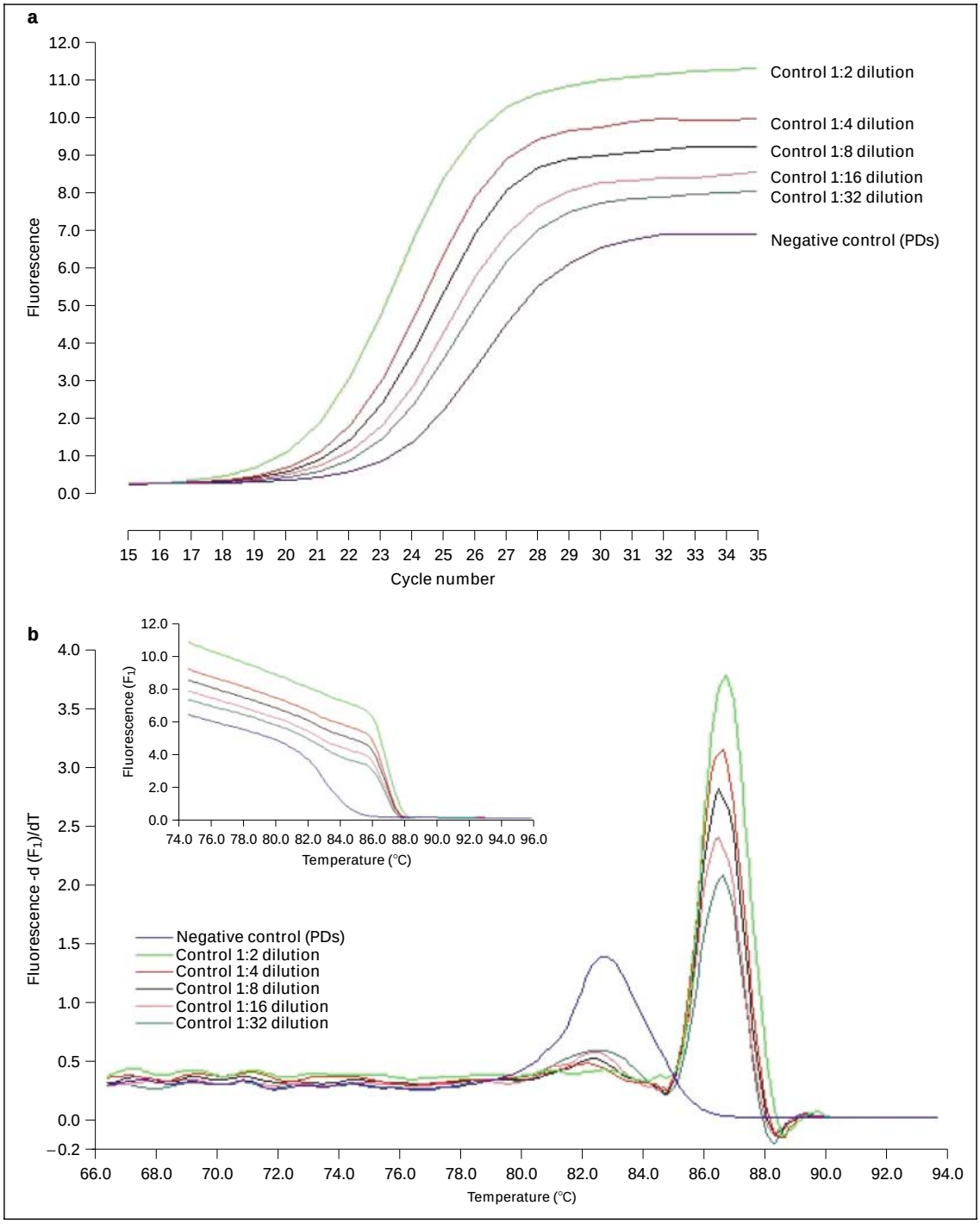

Fig. 1. LightCycler PCR of a serial dilution of a positive control for IL-12R β2 using SYBR Green dye and a melting curve analysis of the resulting products. a A representative experiment in which the total fluorescence was measured at the end of each cycle. The negative control contains no input cDNA. Fluorescence in this sample is the result of PDs (determined by an ethidium bromide-stained agarose gel). All samples were amplified simultaneously for 35 cycles and the fluorescence was measured at the end of each extension step. b A melting curve analysis in which melting peaks were determined by plotting the negative derivative of fluorescence emitted by each sample during a melting curve analysis (inset) in which PCR products were slowly melted and fluorescence was determined continuously. The negative control sample containing only PDs had a Tm of 83°C, while the positive control dilution series containing the product of interest had a Tm of 87°C. The presence of PDs in the positive control dilution series can be confirmed by observing the presence of melting peaks at 83°C. dF = decrease fluorescence (inset); dT = decrease temperature.

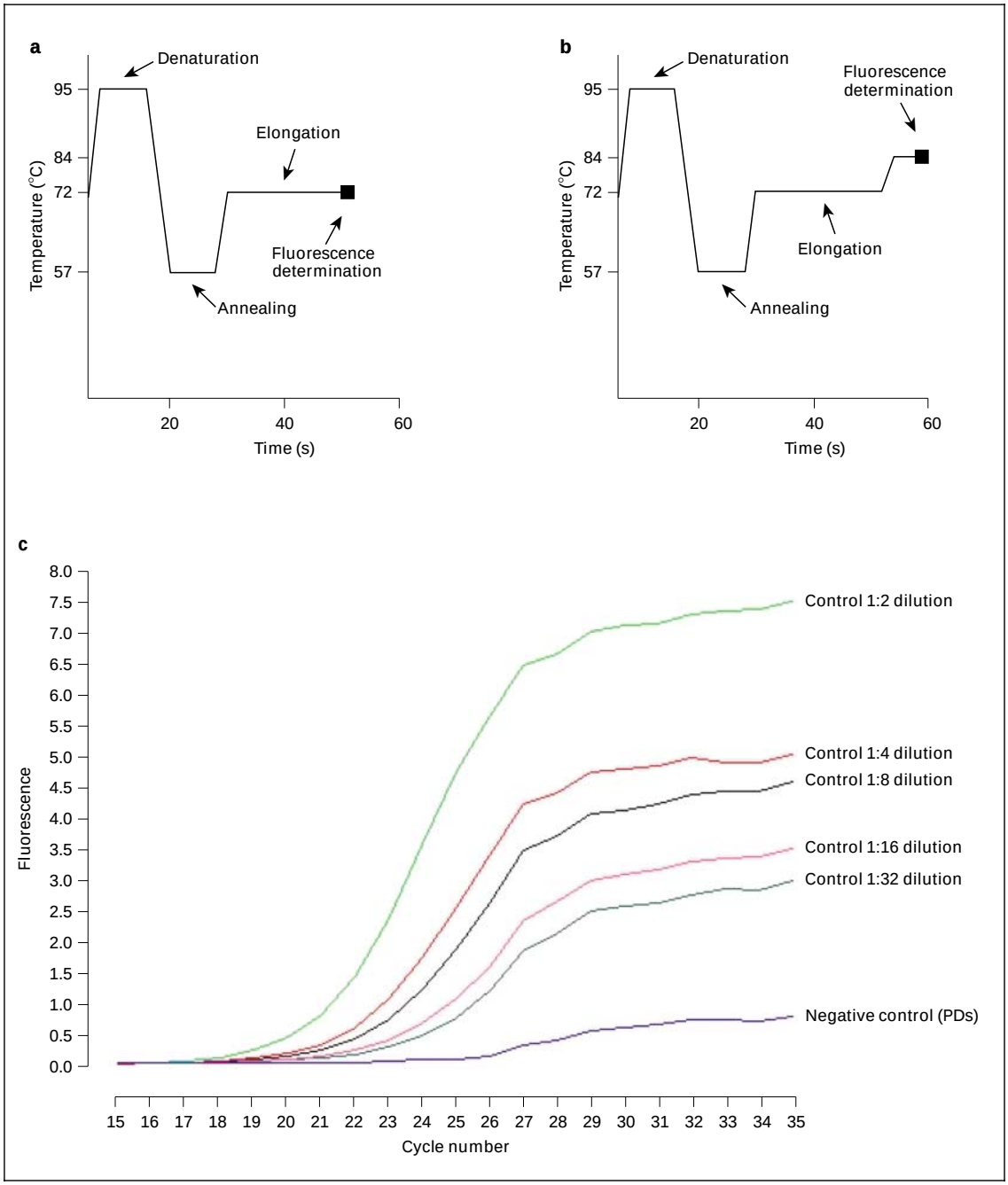

Fig. 2. Model of the PCR protocols used to amplify the murine IL-12R β2 cDNA and the result of using the modified PCR protocol. a The standard PCR protocol, which is as follows: 35 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 4 s and extension at 72°C for 32 s. Fluorescence is measured at the end of the elongation phase. b The modified protocol used to eliminate the fluorescence obtained from PDs. The PCR cycle is essentially the same: 35 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 4 s and extension at 72°C for 32 s. However, after the extension phase, the temperature is raised to 84°C to denature PDs for 5 s and the fluorescence is determined at this time point. c A representative experiment (1 of 4) showing total fluorescence measured at the end of each PCR cycle using the LightCycler and SYBR Green dye using the modified PCR protocol. The serial control dilution series is of murine IL-12R β2 cDNA. All samples were amplified simultaneously for 35 cycles and the fluorescence was measured at the end of each modified PD denaturation step.

The standard protocol for quantitative LightCycler PCR (fig. 2a) utilizes 35 cycles of denaturation, annealing and extension, with florescence measured at the end of each extension segment. Taking advantage of our knowledge of the Tm of the PDs (83°C) and the ability to read fluorescence at any point during the cycle, we added an additional segment in order to circumvent PD interference. After elongation, the temperature was increased to 84°C, one degree less than the Tm of our desired product (87°C), but greater than the Tm of the PDs (83°C). Fluorescence was measured at this point, ensuring that only the fluorescence of the desired amplicon is detected, as at this temperature PDs are single stranded and do not bind SYBR Green. Figure 2b depicts this modified protocol.

Quantitative RT-PCR of the positive control series using this modified protocol demonstrated virtually complete elimination of the PD contribution to the total fluorescence (fig. 2c). Thus, compared to figure 1a, a clear dose response florescence profile for the positive control serial dilutions was obtained. We noted a 35–40% decrease in florescence of the positive controls compared to reactions not employing this strategy (fig. 1a); we believe this is due to loss of fluorescence from PDs. The results obtained thus reflect fluorescence from IL-12R β2 products uncontaminated by PD fluorescence.

In summary, this method takes advantage of the LightCycler's ability to determine the Tm of the PDs and the desired PCR amplicon. By modifying the PCR protocol to include a PD melting step, the contaminating effects of PD fluorescence are largely eliminated, without a requirement to redesign primers and reoptimize the protocol. By ensuring that only the fluorescence of the product of interest is measured, the amount of template in the initial sample can be more accurately determined. This yields a more accurate measurement of mRNA levels for genes of immunologic interest, including those that previously have been difficult to quantify and that are relevant to allergic disease and other immunologic disorders, such as the IL-12 receptor, IL-4 and IL-15. This strategy provides an enhanced capacity to quantitate differential expression of these molecules in allergic versus nonatopic or resistant versus susceptible populations.

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