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Influence of reagents formulation on real-time PCR parameters

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Real-time polymerase chain reaction (PCR) techniques are increasingly used to quantify target sequences for diagnostic and research purposes. Due to its 'quantitative' character, it is very important to determine the variability of this technique correlating with several experimental conditions. The objective of this study was to analyse the effect of manufacturing lots of PCR reagents on two main PCR parameters, specificity and sensitivity. For this study, we used four different amplicons, using either mouse genomic DNA or viral DNA. Although a PCR product could be obtained in any of the conditions, we observed that there are relevant variations in sensitivity depending on the reagents formulation. We conclude that different lots of reagents may determine the analytical performance of PCR assays indicating that reagents testing are of special importance when the PCR protocol is used for quantitative purposes.

KEYWORDS: real-time PCR, SYBR Green I dye, β-actin, DNA polymerase, glycoprotein B, thimidine kinase.

INTRODUCTION

The recently introduced real-time fluorescence polymerase chain reaction (PCR) technologies (LightCycler™) offer the quick and reliable quantification of any target sequence.[1] While there are many methods available for quantification of nucleic acids, quantitative PCR is becoming the method of choice.[2] However, now it is essential to evaluate the potential variations inherents to this technique, with the aim to profit the highest possibilities that this methodology offers.

In our approach, the amount of DNA at each cycle is quantified by staining with a double strand specific DNA binding dye such as SYBR® Green I Dye, following standard methods. The fluorescence generated is proportional to the amount of product present. The cycle number at which the level of fluorescence rises above a background threshold value is inversely proportional to the log of the initial number of template copies.[3] The potential benefits of using SYBR Green I dye to continuously monitor PCR product formation have been extensively demonstrated.[1,4]

The aim of this work was to test different lots of reagents, differing only in 'minor' characteristics, in order to analyse the variation of parameters in the real-time PCR assays. Here, we have analysed two parameters; the sensitivity by determination of the detection threshold (ST), and the specificity by the melting temperature (Tm) analysis, using four different PCRs previously established in our laboratory.

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MATERIALS AND METHODS

Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche Diagnostics Ltd, Lewes, UK). Reactions were performed in a 10 µl volume reaction mixture containing 1 µM primers and 2 mM $MgCl_2$. Nucleotides, Taq polymerase and buffer were included in the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK). To this study we have been used six kits from two lots of the Master SYBR Green I mix. Eight oligonucleotides were used for the four PCR protocols: β-Actin (5′-AACCCTAAGGCCAACCGTGAAAAGATGACC-3′ and 5′-CCAGGGAGGAAGAGGATGCGGC-3′), HSV-1 glycoprotein B (gB) (5′-ACGGGGGCCCTGCCACTC-3′ and 5′-GACGGTCGCGTTGTCGCC-3′), HSV-1 DNA polymerase (pol) (5′-GGTGAACGTCTTTTCGCACT-3′ and 5′-GTGTTGTGCCGCGGTCTCAC-3′) and HSV-1 thimidine kinase (TK) (5′-ATACCGACGATCTGCACCT-3′ and 5′-TTATTGCCGTGCGG-3′). The PCR protocol included a 10 min denaturation step followed by 40–50 cycles with 95°C between 15 and 30 s, annealing temperature optimised were 55°C (for β-actin and TK) or 60°C (for gB and pol) between 5 and 30 s and 72°C between 10 and 40 s. Detection of the fluorescent product was carried out either at the end of the 72°C extension period. After the PCR, these samples were heated from 65–99°C. When the temperature reaches the Tm of each fragment, there is a steep decrease in fluorescence as the product denatures to single strands that no longer bind SYBR Green I Dye. PCR amplification was performed with template dilutions ranging from 10^{-2}–10^{6} pfu (plaque formation units) for the HSV-1 DNA and 0·08–20 ng for the murine genomic DNA, both extracted by conventional methods (NucleoSpin®, Cat. K3053-2, ClonTech, USA). In each case melting curve analysis, agarose gel electrophoresis and restriction analysis confirmed the specificity of the amplification products. Each experiment was performed in triplicates.

RESULTS AND DISCUSSION

Figure 1 shows the melting temperature (A) and the corresponding agarose gel analysis (B) for the amplified fragments of the different PCRs. As observed, all the PCRs were highly specific, with at least 95% of the product corresponding to the specific one; this was observed for both lots of reagents (not shown). Figure 2 shows a comparison between two PCR protocols (pol and TK) that illustrates the two types of results obtained in this study: The pol PCR was insensitive to the lot of reagents used, showing the same slope and y axis crossing point for both lots of reagents, whilst that for the TK PCR the y axis crossing point varied with the reagent lot, indicating that the sensitivity of this PCR depends on the reagent lot.

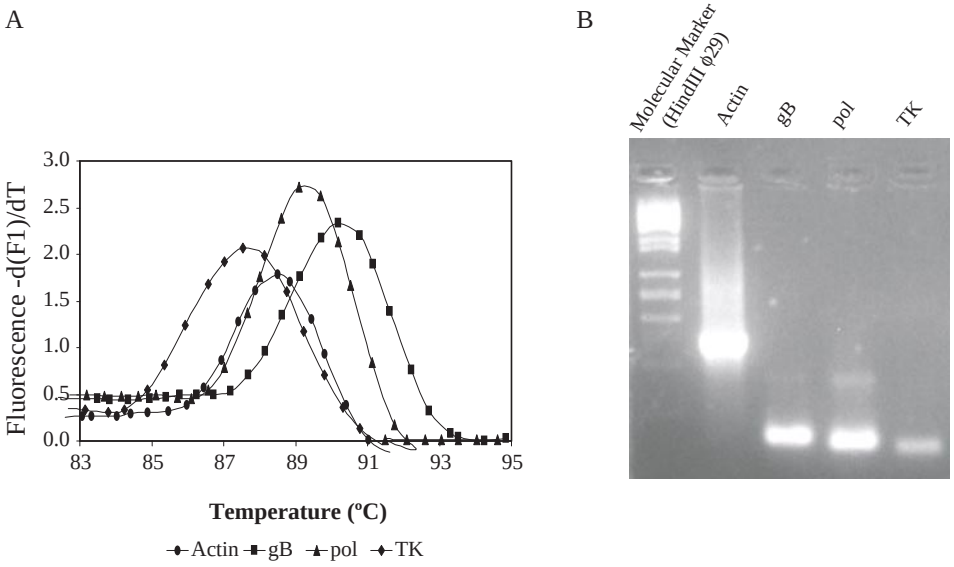

Fig. 1. (A) Melting analysis showing the specific melting temperatures for the four PCRs tested. (B) Gel electrophoresis of amplified products after the real-time quantitative PCR. The amplified fragment sizes were 379 bp for the β-actin, 128 for the glycoprotein B (gB), 120 bp for the DNA polymerase (pol) and 87 bp for the thimidine kinase (TK).

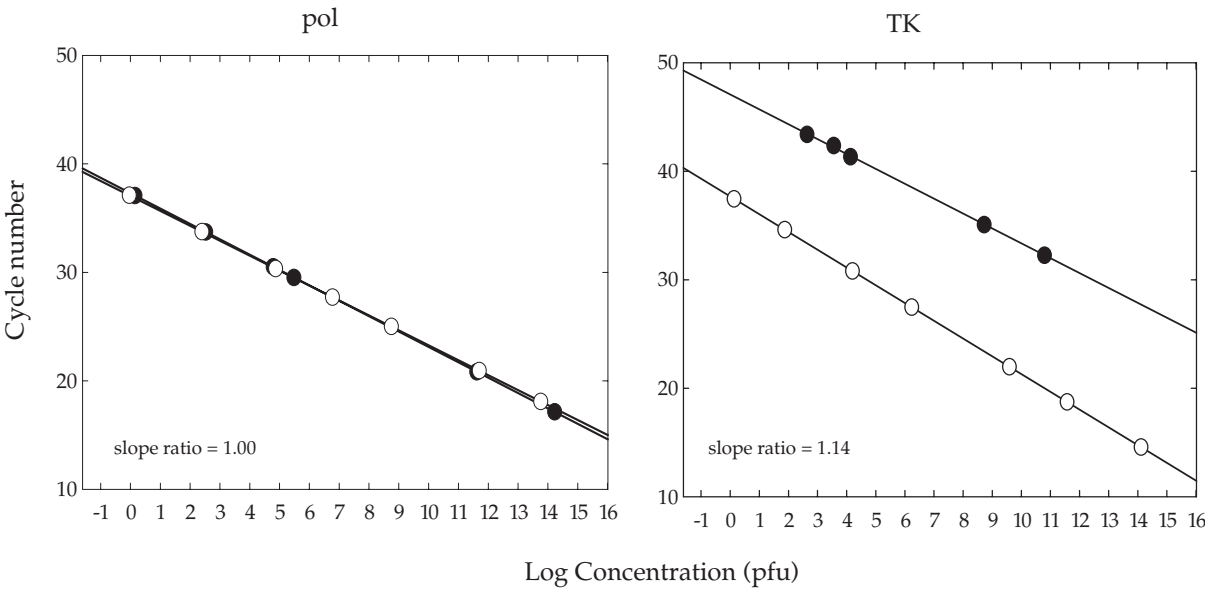

Fig. 2. Comparison of the HSV-1 DNA polymerase (pol) PCR and thimidine kinase (TK) PCR with both reagent lots (the symbols ● and ○ correspond to the lots A and B respectively). The slope ratios (○/●) reveal the similarities and differences in these PCRs.

Table 1. Tm and sensitivity threshold (ST) values corresponding to several PCR protocols correlated with two different lots of the manufacturing reagents. The amplified fragments correspond to mouse β-actin gene obtained from murine genomic DNA and glycoprotein B (gB), thimidine kinase (TK) and DNA polymerase (pol) HSV-1 genes. [ST ratio = ST (lot A)/ST (lot B); ΔTm = Tm (lot A) − Tm (lot B)]

Gene	Lot A	Lot B	ST ratio	ΔTm
β-Actin				
Tm	89·52 ± 0·48°C	88·70 ± 0·31°C		0·82°C
ST	0·4 ng	0·08 ng	5	
gB				
Tm	90·83 ± 0·11°C	89·87 ± 0·28°C		0·96°C
ST	1 pfu	1 pfu	1	
pol				
Tm	90·30 ± 0·53°C	89·63 ± 0·43°C		0·67°C
ST	1 pfu	1 pfu	1	
TK				
Tm	88·39 ± 0·50°C	87·50 ± 0·18°C		0·89°C
ST	10 pfu	1 pfu	10	

All these data are summarised in Table 1, that shows the amplification effectiveness according to the manufacturing reagent lots for the studied PCR protocols. The specificity (number of melting peaks) and quantitative capacity were not significantly altered by the reagent lot, although according to manufacturer specifications, we observed a decrease in the product Tm for lot B compared to A (0·84 ± 0·11°C). However, the sensitivity threshold varied considerably for two of the studied PCRs, the mouse β-actin PCR and the viral TK PCRs being 5 and 10 times, respectively, more sensitive for lot B than for lot A.

CONCLUSIONS

We tested four PCRs used in a routine way in our laboratory, to evaluate the possible variations in PCR qualitative and quantitative parameters due to minor

differences in manufacturing reagent lots, and found a nice lot to lot reproducibility in PCR specificity and in quantitative ability of the reactions for relatively high template copy numbers (above 100 copies). By contrast, and depending on the specific PCR, sensitivity varied until 10 times, for viral TK. This observation is especially relevant when, as frequently occurs in clinical samples, the template DNA is in low copy numbers. In summary, this work shows that some particular real-time PCR protocols could vary considerably in their parameters depending on different lots of reagents supplied by the manufacturer, and this fact needs to be taken into account for a reproducible quantification of the DNA being studied.

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