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Influence of DNA polymerases on quantitative PCR results using TaqMan™ probe format in the LightCycler™ instrument

K-A. Kreuzer, A. Bohn, U. Lass, U. R. Peters and C. A. Schmidt*

Abteilung für Innere Medizin und Poliklinik m.S. Hämatologie und Onkologie, Campus Virchow-Klinikum, Medizinische Fakultät Charité der Humboldt-Universität zu Berlin, Berlin, Germany

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Real-time fluorescence polymerase chain reaction (PCR) techniques are increasingly used to quantitate target sequences for diagnostic and research purposes. Currently, the so called TaqMan™ probe chemistry is mostly used as fluorogenic system. This probe format is strictly dependent on the 5′-exonuclease activity of DNA polymerase as fragmentation of the probe during the reaction is essential for this assay. Based on our experience that dramatic differences in quantitative PCR results may be due to different DNA polymerases we performed a detailed comparison of 15 enzymes. We found that clear differences exist between polymerases of different manufacturers. Thus, three out of seven polymerases which were declared to posses 5′-exonuclease activity appeared to be completely unsuitable for this method while the remaining had significantly different reaction efficiencies. We conclude that different DNA polymerases may determine the entire analytical performance of TaqMan™ assays suggesting that DNA polymerase testing is of special importance when this probe format is used.

KEYWORDS: real-time PCR, DNA polymerases, 5′-exonuclease activity, fluorescent probes.

INTRODUCTION

The recently introduced real-time fluorescence polymerase chain reaction (PCR) technologies (ABI PRISM 7700 SDS™ and LightCycler™) offer the quick and reliable quantitation of any target sequence.[1,2] Thus, these new technologies appear to be very promising and may provide new insights in various pathological changes. However, conventional PCR conditions have to be modified for these methods such that fluorescence detection during the reaction is optimized. Among others, magnesium concentrations are thought to be critical for secondary structures of the fluorescent probes and additional oligonucleotides must be considered in the reaction.[3] In our opinion the TaqMan™ probe chemistry is superior to other probe formats (e.g. HybProbes™) as it is highly flexible and convenient to handle. The principle of this assay is a target specific probe which is labeled with a reporter and a quencher dye in a distance of a few base pairs within the probe sequence. As the DNA polymerase extends the primers the probe is hydrolysed through the 5′-exonuclease activity of the enzyme.[4] Thus, reporter and quencher dyes are separated leading to a measurable increase of reporter fluorescence which is measured during the reaction.

* Address to whom all correspondence should be addressed at: Abteilung für Innere Medizin und Poliklinik m.S. Hämatologie und Onkologie, Campus Virchow-Klinikum, Medizinische Fakultät Charité der Humboldt-Universität zu Berlin, Forum 4, 1.0310, Augustenburger Platz 1, 13353 Berlin, Germany. Tel: +49 30 450 53663; Fax: +49 30 450 53929; E-mail: christian.schmidt@charite.de

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Considering this enzymatic probe fragmentation is the central event which leads to a fluorescence signal in this assay.

Currently, a great number of commercial DNA polymerases are available of which several are claimed to possess 5′-exonuclease activity. However, these enzymes may differ with regard to their individual polymerase and exonuclease efficiency. While it is well known that different enzymes have varying polymerase characteristics their 5′-exonuclease properties are not well studied. The aim of this work was to find an enzyme with both maximum polymerase and maximum 5′-exonuclease activity in order to reach maximum sensitivity in our real-time PCR assays.

MATERIALS AND METHODS

As target sequence we used chimeric bcr/abl fusion transcripts. Briefly, 10^4 copies of a DNA standard preparation ranging from 10^7 to 10^0 was amplified in the LightCycler™ (Boehringer Mannheim, Mannheim, Germany) and fluorescence measured using a target specific fluorescent probe (5′-AGCGGCTTCACTGACCATGG). The probe was labelled with 6-carboxy-fluorescein phosphoramidite (FAM) at the 5′ end and 5-carboxy-tetramethyl-rhodamine (TAMRA) was incorporated at nucleotide nine of the oligonucleotide. Initial denaturation (94°C, 10 min) was followed by 50 cycles of 94°C for 10 s 65°C 30 s.

A total of 15 different DNA polymerases were used: AmpliTaq™ and AmpliTaq Gold™ (Perkin Elmer, Foster City, USA), Gibco *Taq* DNA polymerase and Gibco Platinum™ (Gibco BRL, Karlsruhe, Germany), Vent™ and Deep Vent™ (New England Biolabs, Beverley, USA), *Taq* DNA polymerase and *Tfu* DNA polymerase (Appligene Oncor, Ilkirch, France), Qiagen *Taq* DNA polymerase (Qiagen, Hilden, Germany) InViTAQ™, DeltaPol™ and CombiPol™ DNA polymerases (InViTek, Berlin, Germany), Biotherm™ and KlenTherm™ DNA polymerases (Rapidozym, Luckenwalde, Germany) and *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The 20 µl reaction mix contained 10 × PCR buffer, 4·5 mM $MgCl_2$, 0·8 mM dNTP (Gibco BRL, Karlsruhe, Germany), 30 µg bovine serum albumine (BSA), 0·5 mM of each primer, 1 µM TaqMan™ probe and 1·25 u DNA polymerase. For each master mix corresponding reagents (buffer, magnesium, additives) of the respective manufacturer were used. Each experiment was performed in triplicates.

RESULTS

In Table 1 investigated polymerases are listed with regard to the 5′-exonuclease activity according to the manufacturers information. Fig. 1a shows reporter fluorescence curves measured by the LightCycler™ instrument using seven different DNA polymerases with suggested 5′-exonuclease activity (DNA polymerases coded as A-O). As can be seen from the diagram fluorescence intensity generated by different polymerases varied substantially. TaqMan™ PCR is defined to become positive when fluorescence of a given sample exceeds significantly those of the background. The time point of the reaction when this is the case is marked as threshold cycle (Ct). Once a calibration curve is generated by correlation of known template concentrations against the respective Ct values individual template number is calculated by considering the Ct of an unknown sample. Thus, the Ct is critical for the determination of individual quantitative PCR results. As illustrated in the figure, Ct values differed also greatly from enzyme to enzyme. In Fig. 1b corresponding agarose gel analysis is shown with a target amplicon size of 127 bp. While all polymerases yielded comparable band intensities when fluorescent probes were omitted, as expected intensities of the bands reflect exactly the results obtained with the LightCycler™. Inversely, primer dimerization increased when amplification was reduced or absent.

DISCUSSION

We could clearly show that different commercially available DNA polymerases yield varying fluorescence in real-time PCR using the TaqMan™ probe format. It is of interest that some enzymes for which 5′-exonuclease activity was claimed did not produce any fluorescence signal in our assay while all enzymes without this declared activity did not produce any fluorescence. It is surprising that only one polymerase (D) showed a sigmoid amplification curve as expected for the exponential reaction kinetics. In contrast, four other enzymes exhibited nearby linear amplification curves suggesting that reactions using these DNA polymerases do not follow theoretic PCR kinetics. Gel analysis confirmed that not only fluorescence generation is affected by different polymerases but amplification may also vary dependent on the used enzyme with maximum amplification by using polymerase D. As without fluorescent probes all polymerases yielded comparable amplification bands this could be explained by incomplete primer extension when the probe is not sufficiently cleaved from the

Table 1. Fifteen DNA polymerases tested with regard to their 5′-exonuclease activity using TaqMan™ chemistry in the LightCycler™

DNA polymerase	5′-exonuclease activity (manufacturers declarations)	
AmpliTaq®, Perkin Elmer	Yes	
AmpliTaq Gold®, Perkin Elmer	Yes	
Taq DNA Polymerase, Gibco BRL	Yes	
Platinum® Taq DNA Polymerase, Gibco BRL	Yes	
$Vent_R$®, New England Biolabs		No
Deep $Vent_R$®, New England Biolabs		No
Taq DNA Polymerase, Appligene Oncor		No
Tfu DNA Polymerase, Appligene Oncor		No
Qiagen Taq DNA Polymerase, Qiagen	Yes	
InVITAQ™, InViTek		No
DeltaPol™ (Exo-) DNA Polymerase, InViTek		No
CombiPol™, InViTek		No
KlenTherm, Rapidozym	Yes	
BioTherm, Rapidozym	Yes	
Taq DNA Polymerase, Boehringer Mannheim		No

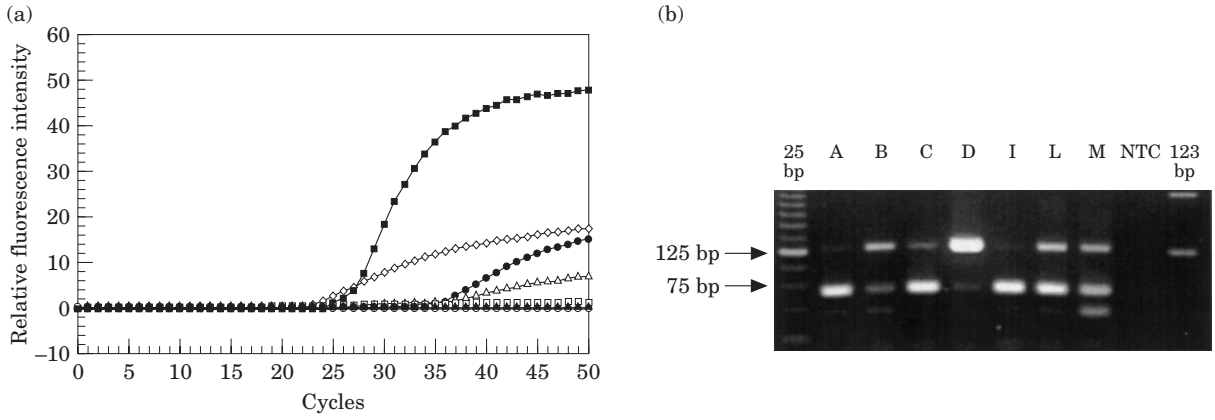

Fig. 1. (a) Fluorescence plot of the bcr/abl target sequence obtained by the LightCycler when using TaqMan™ probe format and seven DNA polymerases with suggested 5′-exonuclease activity. (b) Conventional agarose gel electrophoresis of the PCR products after fluorescence PCR. DNA polymerase A, (—▲—); DNA polymerase B, (—●—); DNA polymerase C, (—△—); DNA polymerase D, (—■—); DNA Polymerase I, (—□—); DNA polymerase L, (—◇—); DNA polymerase M, (—○—).

target strand by the exonuclease activity of the DNA polymerase. These altered reactions may lead to the different Ct values which again directly influence the sensitivity of the assay. In summary, we found that only one DNA polymerase fulfilled equally polymerase and 5′-exonuclease characteristics necessary for real time PCR using the TaqMan™ probe format in our hands.

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