Comparison between *Taq* DNA Polymerase and Its Stoffel Fragment for Quantitative Real-Time PCR with Hybridization Probes

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ABSTRACT

In quantitative real-time PCR assays, fluorophor-labeled oligonucleotide probes are employed to generate sequence-specific signals for the quantitative evaluation. Whereas TaqMan® probes have to be hydrolyzed during PCR by the endonucleolytic activity of Taq DNA polymerase to generate a signal, the hybridization probes in LightCycler® assays must not be hydrolyzed. In this study, we demonstrate for four different targets that the probes are degraded during PCR by Taq DNA polymerase. Signal yield, quality of amplification curves, and accuracy of quantitative measurements can be improved using the Stoffel fragment lacking an endonucleolytic activity and TaqStart® antibody suppressing the formation of nonspecific products, without laborious efforts to optimize the amplification protocol.

INTRODUCTION

Real-time quantitative PCR is becoming an increasingly important method for determining copy numbers of nucleic acid sequences (1,2,7). For sequence-specific signal generation, fluorescent probes are usually employed that change their fluorescence intensity when they bind to the amplified DNA. Therefore, the fluorescence signal, measured once per cycle, will increase with the product accumulation during the PCR. The change in fluorescence intensity is either due to a loss of fluorescence quench [e.g., TaqMan® probes (4) or Molecular Beacons™ (6)] or due to a gain of fluorescence resonance energy transfer (FRET) from a donor to an acceptor fluorophor [hybridization probes format (9)]. For signal generation, the double-labeled TaqMan probes have to be hydrolyzed by the endonucleolytic activity of the *Taq* DNA polymerase to unlink the quencher and the fluorophor. Initially, *Taq* DNA polymerase partially removes hybridized oligonucleotides on the template strand, obstructing the passage for polymerization, forming a Y-shaped DNA structure. Then, the unpaired 5'-arm of the bifurcated duplex is removed by the structure-specific endonuclease activity of the *Taq* DNA polymerase (5). In contrast, the single-labeled oligonucleotides of the hybridization probe pairs have to be reused in each PCR cycle and, therefore, should not be hydrolyzed to avoid a decrease of the effective probe concentration. There are no data in the literature that would demonstrate whether and to what extent probe hydrolysis in-

terferes with quantification using the LightCycler® system (Roche Diagnostics, Penzberg, Germany).

Here, we demonstrate on four different targets that hybridization probes are hydrolyzed endonucleolytically by this *Taq* endonuclease activity. The signal yield might be reduced because of the successively reduced amount of intact probes. Therefore, the signal-to-noise ratio becomes smaller, which finally may affect accuracy and sensitivity. These potential disadvantages in LightCycler (8) quantitative PCR assays, employing hybridization probes, can be avoided using the Stoffel fragment of *Taq* DNA polymerase (3) lacking endonucleolytic activity.

MATERIALS AND METHODS

DNA Isolation

Human genomic RNA-free DNA was isolated from lymphocytes of fresh EDTA-treated blood using the QIAamp® DNA Blood Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality and concentration were evaluated by absorption spectroscopy between 220 and 320 nm using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan).

Oligonucleotides

Primers (HPSF®-purified; MWG, Ebersberg, Germany) and hybridization probes (TIB Molbiol, Berlin, Germany) (Table 1) were checked with the program Oligo® 5.0 (National Biosciences, Plymouth, MN, USA) for the

Table 1. Oligonucleotides Used in This Study

Target	Name	Sequence (5'→3')	Genome Localization ^a
<i>p53</i>	p53-F ^b	TTCCTAGCACTGCCCAACA	14674–14692
<i>p53</i>	p53-R	GACTGGAAACTTTCCACTTG	14779–14798
<i>p53</i>	p53-up	CCCCAGCCAAAGAAGAAACCACTGGATGGAGAAT- FAM ^c	14707–14740
<i>p53</i>	p53-down	Red640 -TTTCACCCTTCAGGTACTAAGTCTTGGGACCTCTT- p	14742–14776
<i>p16</i>	p16-F	AAGCCATTGCGAGAACTT	146–163
<i>p16</i>	p16-R	CAGAGGGCAGAAAGAAAA	253–270
<i>p16</i>	p16-up	CGGTAGGGACGGCAAGAGAGGAGGGCGGGA- FAM	185–214
<i>p16</i>	p16-down	Red640 -TGTGCCACACATCTTTGACCTCAGTTTCTAACG- p	215–248
<i>IGF-1</i>	igf-F	AGCTCGGCATAGTCTT	1135–1150
<i>IGF-1</i>	igf-R	CCAAGTGAGGGGTGTGA	1241–1257
<i>IGF-1</i>	igf-up	ATGAGACAGTGCCCTAAAGGGACCAATCCAATG- FAM	1174–1206
<i>IGF-1</i>	igf-Down	Red640 -CTGCCTGCCCTCCATAGTTCTAGGAAATGAG- p	1207–1239
<i>HBB</i>	hbb-F	ACACAACTGTGTTCACTAGC	143–162
<i>HBB</i>	hbb-R	CAACTTCATCCACGTTACC	233–252
<i>HBB</i>	hbb-up	CAAACAGACACCATGGTGCACCTGACTCCTGAGGA- FAM	168–202
<i>HBB</i>	hbb-down	Red640 -GAAGTCTGCCGTTACTGCCCTGTGGGGCAA- p	203–232

^aNucleotide positions are based on sequence of the human genes *TP53* (GenBank[®] accession no. X54156), *p16* (*INK4A*) (GenBank accession no. U12820), *IGF-1* (GenBank accession no. M12659), *HBB* (*Homo sapiens* β -globin gene) (GenBank accession no. AF007546).

^bF, forward primer; R, reverse primer; up, donor probe; down, acceptor probe.

^c**FAM**, 5,6-carboxyfluorescein attached to 3'-O-ribose; **Red640**, LightCycler Red 640 attached to 5' terminus; **p**, phosphate group attached to the 3' terminus.

absence of false priming sites, formation of primer dimers, primer/probe hybrids, and secondary structures. The probe pairs hybridize between the primer binding sites such that donor (fluorescein) and acceptor (Red 640) fluorophore are in sufficient proximity for an efficient FRET process.

Real-Time PCR

A LightCycler system (Roche Diagnostics) was used for amplification and data collection (software version 3). Reactions were carried out in a total volume of 10 μ L per capillary. Each reaction mixture contained 0.5 mg/mL BSA, 6 mM MgCl₂, 0.5 μ M each primer, 0.2 μ M each probe, 0.2 mM dATP, dCTP, dGTP, and dTTP, 0.5 U *Taq* DNA polymerase (Roche Diagnostics) or 0.5 U Stoffel fragment (Applied Biosystems, Weiterstadt, Germany), and 15 ng (unless otherwise stated) hu-

man genomic template DNA in 1 $\times$ PCR buffer (for *Taq* DNA polymerase: 10 mM Tris-HCl, pH 8.3, 50 mM KCl; for Stoffel fragment: 10 mM Tris-HCl, pH 8.3, 10 mM KCl). All other biochemicals were from Roche Diagnostics. The reaction conditions were optimized to obtain the highest signal-to-noise ratio.

The reaction mixture, produced at 4°C to prevent the formation of nonspecific products, was distributed into pre-chilled capillaries. For reactions containing TaqStart[®] antibody (Clontech Laboratories, Heidelberg, Germany), DNA polymerase and antibody were premixed (molar ratio 1:28) in TaqStart dilution buffer (50 mM KCl, 10 mM Tris-HCl, pH 7.0) and incubated for 5 min at room temperature.

For analysis of the kinetics of probe hydrolysis during PCR, the 6-fold amount of reaction mixture was prepared for each target and kept on ice. Five reactions with an increasing num-

ber of amplification cycles (0, 20, 30, 40, and 50 cycles) were carried out sequentially.

Each target was amplified in four different assays: either with *Taq* DNA polymerase or Stoffel fragment, in the presence or absence of TaqStart antibody. To determine the reproducibility of quantifications, these four assays were carried out two times in quadruplicate. The threshold value was adjusted separately for each set of quadruplicate measurements to minimize the standard deviation of the resulting C_T values.

Amplification Protocol

Initial denaturation for 30 s at 95°C was followed by 20–50 amplification cycles with 0 s at 95°C, 5 s at 55°C, and 10 s at 72°C (temperature ramp always 20°C/s). The measurement was done at the end of the annealing phase at 55°C. The PCR raw data were evaluated with

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the LightCycler software, version 3. The signal of channel 2 and the ratio channel 2:channel 1 were used to calculate the C_T values (fit points method using three points per curve within the detected exponential phase of amplification; arithmetic baseline adjustment).

Analysis of the Donor Probes by Capillary Electrophoresis

The FAM-labeled donor probes were quantitatively analyzed for hydrolysis by capillary electrophoresis using an ABI PRISM™ 310 Genetic Analyzer (instrument, reagents, and consumables from Applied Biosystems). Immediately after PCR, each reaction mixture was 10-fold diluted with water. Two microliters of each dilution were mixed with 12 μ L template suppression reagent, heated for 3 min at 95°C, and instantaneously chilled on ice.

These samples were injected electrokinetically for 5 s at 15 kV and separated at 15 kV and 60°C in a 47 cm $\times$ 50 μ m capillary of uncoated fused silica filled with polymer POP4 using the module "GS STR POP4 (1 mL) C". Laser-induced fluorescence signals were recorded by the collection software version 1.0.4 and evaluated with the GeneScan® software, version 2.1 (Applied Biosystems).

The fraction of hydrolyzed probes p was calculated using Equation 1:

$$p = \frac{A_H}{A_T} - \frac{A_{H_0}}{A_{T_0}} \quad [\text{Eq. 1}]$$

with A_H : sum of peak areas corresponding to hydrolyzed donor probes; A_T : sum of all peak areas of the donor probe; A_{H_0} : A_H before PCR; A_{T_0} : A_T before PCR. The ratio A_{H_0}/A_{T_0} corresponds to the initial fraction of non-full-length donor probe, which is determined from an aliquot of the pre-PCR reaction mixture. The error of these calculations is approximately 2%.

RESULTS

We studied the influence of the endonucleolytic activity of *Taq* DNA polymerase on the sensitivity and accuracy in quantitative PCR experiments using the LightCycler employing

hybridization probes. Our goal was to determine the optimum conditions for very precise gene copy number quantification, as it is required for the detection of oncogene amplifications or tumor suppressor gene deletions in clinical samples.

Quantifications of the *IGF-1* copy number in a sample containing 15 ng human genomic DNA were carried out with *Taq* DNA polymerase or its Stoffel fragment (3). The Stoffel fragment showed several nonspecific amplification products (data not shown), which were not formed when TaqStart antibody was added. Figure 1 shows the cycle-dependent signal for the external standards used for these quantifications. Generally, the fluorescence signals of the PCRs carried out with Stoffel fragment (Figure 1, c and d) reach higher values than those using *Taq* DNA polymerase (Figure 1, a and b) (in the example presented, approximately 2.5-fold higher intensity). Because of the higher signal intensities in the case of the Stoffel fragment, the signal-to-noise ratio of these curves is lower and the curves appear to be smoother in shape (similar results were found for amplifications of *p16*, *p53*, and *HBB* (human β -globin gene), data not shown). In addition, the curves of the standards are more parallel and equidistant. Therefore, the error of the standard curves obtained is lower, which is indicative of a higher accuracy. The lowest error of standard curves, and therefore the highest accuracy, is obtained with the combination of Stoffel fragment plus antibody (Figure 1d). This is confirmed by the lower standard deviations of the C_T values of measurements carried out in quadruplicate. The mean standard deviations of the C_T values were approximately 30% smaller when Stoffel fragment was used instead of *Taq* DNA polymerase (mean SD over all targets: *Taq* DNA polymerase: 0.11; Stoffel fragment: 0.07). The reproducibility of the measurements is always better if the ratio of the acceptor and donor fluorescence is used for evaluation rather than the acceptor fluorescence only: the SD of the measurements is 2–3 times higher when the acceptor fluorescence value only is used for evaluation (*Taq* DNA polymerase: $\overline{SD} = 0.27$; Stoffel fragment: $\overline{SD} = 0.17$).

To verify whether the better suitability of the Stoffel fragment for precise quantifications is due to the absence of probe hydrolysis, we analyzed the probes and their degradation products by ABI PRISM 310 capillary electrophoresis (CE) (Figure 2). Because this CE system uses an argon ion laser for excitation, only the fluorescein of the donor probes is excited, while the Red 640-labeled acceptor probes cannot be detected. Unhydrolyzed probes show two peaks in the electropherograms corresponding to the isomers 5- and 6-carboxy-fluorescein, which exhibit a slightly different mobility. As shown for *IGF-1* (Figure 2a), there is no change in the electropherogram even after 50 cycles of amplification when the Stoffel fragment is used. In contrast, the amount of shorter fragments is increased after 50 cycles when using *Taq* DNA polymerase, as demonstrated for *IGF-1* (Figure 2b), *p16* (Figure 2c), *p53*, and *HBB* (data not shown). The probes are not sequentially degraded but hydrolyzed at certain preferred internal positions, mostly around 10 nucleotides away from the 5'-end (cf. Figure 2).

Because the situation after 50 PCR cycles (very late plateau phase) does not reflect the situation during the exponential phase that is used for evaluation of real-time quantifications, we also analyzed the cycle-dependent degradation of probes: after every 10 cycles, one reaction of a series of identical PCRs was stopped and analyzed by CE. The fraction of hydrolyzed probes increases with the amplification signal (Figure 3): at the end of the time segment, used for calculation of the C_T values, up to approximately 15% of the donor probes are degraded (e.g., *HBB* and *p16*). The amount of donor probe hydrolysis for the various targets is given in Table 2.

It may be argued that, by lowering the Mg^{2+} concentration in the reaction, the amount of probe hydrolysis by *Taq* DNA polymerase could be effectively decreased. This is indeed the case but only to a minor degree (Table 2) and accompanied by a substantial loss and decrease in quality of the signal (Figure 4a). The Stoffel fragment does not appear to hydrolyze the probe at any Mg^{2+} concentration tested; however,

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Table 2. Dependence of Donor Probe Hydrolysis on Mg^{2+} Concentration

Mg^{2+}	Degradation after 30/50 cycles [%]			
	<i>HBB</i>	<i>IGF-1</i>	<i>p53</i>	<i>p16</i>
3 mM	8/21	< 1/10	12/27	3/24
4 mM	26/78	7/19	12/32	6/41
5 mM	31/44	3/12	12/55	10/32
6 mM ^a	23 ± 8/39 ± 18	3 ± 2/14 ± 9	13 ± 6/40 ± 11	10 ± 4/39 ± 1

^aThese experiments were carried out in duplicate.

only at 6 mM Mg^{2+} is a useful signal obtained (Figure 4b).

DISCUSSION

The *Taq* DNA polymerase, commonly used for real-time quantitative PCR assays (1,2,7,8), has two different enzymatic activities: the DNA polymerase activity, localized in the C-terminal domain, and a structure-specific endonuclease activity in the N-terminal domain (3,5). Whereas the double-labeled TaqMan probes have to be hy-

Figure 1. *IGF-1* amplification profiles of a dilution series of human genomic DNA. The DNA was diluted with water in steps of 1:4 from 8000 down to approximately 2 genomic equivalents (3 ng were assumed to be 1000 haploide genome equivalents). The negative control without DNA is indicated by "0". Amplifications were done with (a) *Taq* DNA polymerase, (b) *Taq* DNA polymerase and TaqStart antibody, (c) *Taq* Stoffel fragment, and (d) *Taq* Stoffel fragment and TaqStart antibody. The standard curve, obtained by plotting log copy number versus C_T and given in the form $y = ax + b$, was calculated using the fit points method with arithmetic background correction. The error value of the standard curve is the error calculated by the LightCycler software; the coefficient of correlation was greater than 0.98 in all cases.

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drolyzed for the generation of fluorescence signal by the nucleolytic activity of the *Taq* DNA polymerase (1), ideally, the single-labeled hybridization probes used for LightCycler PCRs must not be hydrolyzed to avoid a decrease of the effective probe concentration during the PCR.

In a LightCycler PCR, *Taq* DNA polymerase leads to probe hydrolysis, the extent to which this occurs is dependent on the sequence of the probe and on the concentration of Mg^{2+} ions (Table 2). The hybridization probes are degraded by the endonucleolytic activity of the *Taq* DNA polymerase (5). This was shown by the capillary electrophoretic analysis of the PCR mixtures. Fresh preparations of hybridization probes already contain shorter products (Figure 2) caused by incomplete synthesis. The amount of shorter products increases during PCR, when *Taq* DNA polymerase is used but not when Stoffel fragment is used. This proves that the accumulation of degraded probes is specifically due to the endonucleolytic activity of the *Taq* DNA polymerase. The degradation of the probes is nonsequential because the relative proportions of individual hydrolyzed probe fragments remain constant during the PCR. The *Taq* DNA polymerase mainly cleaves the donor probes around 5 and 12 nucleotides from their 5'-terminus as shown by Lyamichev et al. (5). The exponential phase of product accumulation of the PCR is used for the evaluation of quantifications. During this phase, up to approximately 15% of the probes are degraded. The amount depends not only on the sequence. Even in identical reactions, the amounts of degraded probes,

Figure 2. Hydrolysis of donor probes analyzed by denaturing capillary electrophoresis. Aliquots of the reaction mixture before PCR (0 cycles) and after 50 amplification cycles are compared. The two peaks indicated by arrows correspond to the non-hydrolyzed probes with the isomers 5- and 6-carboxy-fluorescein. The peaks left from the full-length probes correspond to degraded probes labeled with fluorescein. (a) igf-up probe before and after PCR with *Taq* Stoffel fragment, (b) like (a) with *Taq* DNA polymerase, (c) p16-up probe before and after PCR with *Taq* DNA polymerase. The lengths of the non-hydrolyzed probes are 33 (igf-up) and 30 nucleotides (p16-up). The traces shown correspond to the separation time between 10 and 12 min after injection.

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accumulated within the exponential phase, differ by $\pm 10\%$, perhaps due to minor temperature fluctuations (7). Different degrees of probe degradation are likely to be the reason for a decreased accuracy because identical reactions can yield different C_T values.

The accuracy of quantifications is increased by the application of DNA polymerase without endonucleolytic activities, which does not degrade hybridization probes in LightCycler real-time quantitative PCR. Nevertheless, it might well be that, by careful optimiza-

tion of the *Taq* DNA polymerase catalyzed reaction, similar results as with the Stoffel fragment can be obtained.

The problem of probe hydrolysis does not arise with Stoffel fragment, whose amplification efficiency is significantly more sensitive to the Mg^{2+} concentration than that of the *Taq* DNA polymerase. With less than 6 mM Mg^{2+} , the amplification curves for *Taq* DNA polymerase but in particular for Stoffel fragment become flat and the C_T values higher, indicating a lower efficiency (Figure 4). On the other hand,

with these high Mg^{2+} concentrations, the Stoffel fragment tends to form substantial amounts of nonspecific products, which is correlated with low accuracy of quantification (unpublished data). When TaqStart antibody is added before amplification, the formation of nonspecific products is completely prevented and high accuracy is achieved. At 6 mM Mg^{2+} , reactions with Stoffel fragment always yielded higher amplification signal curves than obtained in corresponding experiments with *Taq* DNA polymerase. For both enzymes,

Figure 3. Accumulation of donor probe hydrolysis products during PCR. *Taq* DNA polymerase was used for amplification. The fractions of hydrolyzed donor probes were calculated from the peak areas of the corresponding electropherograms as described in Materials and Methods. Data points within the detectable exponential phase of each reaction are labeled with rhombic symbols. These data points are also shown in semi-logarithmic plots (see the small inserted diagrams).

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Figure 4. Dependence of cycle-dependent signal development on Mg^{2+} concentration. PCRs containing 30 ng genomic template DNA were carried out with different Mg^{2+} concentrations under the same conditions. Higher Mg^{2+} concentrations led to an earlier increase of signal (lower C_T values), higher signals, higher signal-to-noise ratio, and a better defined segment of the exponential phase required for determination of the C_T values. (a) refers to Taq DNA polymerase and p53, and (b) refers to Stoffel fragment and p53.

the efficiencies calculated from several independent standard curves ($\epsilon = 10^m$; m: slope) varied between 1.84 and 1.92. Within this range, there was no correlation between efficiencies and maximum signal intensities. These higher signal curves can be due to the absence of probe hydrolysis of the Stoffel fragment during the PCR. This results in two effects influencing the accuracy of the quantification: a higher signal yield reduces the signal-to-noise ratio and measuring points of earlier cycles can be assigned to the exponential phase of the amplification used for data evaluation. Therefore, more points can be used to calculate the C_T values, resulting in a more robust filtering of noise. We do not imply that there is a strict causal correlation between probe hydrolysis and signal quality but that a poor signal may be due in part to probe degradation.

Generally, when using the acceptor:donor fluorescence ratio for evaluation instead of the acceptor fluorescence, more accurate results are obtained. This ratio eliminates stochastic effects that contribute similarly to the signal intensities of both fluorophores. This reduces noise and errors caused by differences in fluorophore concentrations because of pipetting errors.

The number of LightCycler users is continuously growing because this system is known to allow for the fast, but nevertheless accurate, quantifications of initial target copy numbers. Details of

the assay conditions described in this paper will be useful for the analysis of very minor differences in initial copy numbers by rapid-cycle, real-time quantitative PCR.

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