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Amplification efficiency of thermostable DNA polymerases

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Abstract

The amplification efficiencies of several polymerase chain reaction (PCR) enzymes were compared using real-time quantitative PCR with SYBR Green I detection. Amplification data collected during the exponential phase of PCR are highly reproducible, and PCR enzyme performance comparisons based upon efficiency measurements are considerably more accurate than those based on endpoint analysis. DNA polymerase efficiencies were determined under identical conditions using five different amplicon templates that varied in length or percentage GC content. *Pfu*- and *Taq*-based formulations showed similar efficiencies when amplifying shorter targets (<900 bp) with 45 to 56% GC content. However, when amplicon length or GC content was increased, *Pfu* formulations with dUTPase exhibited significantly higher efficiencies than *Taq*, *Pfu*, and other archaeal DNA polymerases. We discuss the implications of these results.

Keywords: PCR enzymes; Real-time PCR; *Pfu* DNA polymerase; *Taq* DNA polymerase

PCR is one of the most powerful techniques in molecular biology used for in vitro amplification of DNA [1]. The efficacy of PCR is determined by its specificity, efficiency, and fidelity. An ideal PCR results in one specific product that is generated in high yield, with minimal cycles containing the fewest number of polymerase-induced errors. Amplification efficiency, or fold amplification per cycle, is typically the most important parameter in routine applications, as it determines PCR product yield. Amplification efficiency is influenced by a number of factors including target length and sequence, primer sequence, buffer conditions, sample impurities, cycling conditions, and PCR enzyme. Since PCR consists of many amplification steps, adjusting reaction conditions to achieve even slight improvements in amplification efficiency can lead to dramatic increases in PCR product yield. Moreover, once determined for a particular PCR system, amplification efficiency can be used to predict PCR product yield and minimum amplification cycles required.

Historically, researchers have used endpoint methods, such as quantifying PCR product bands on agarose gels, to compare PCR enzymes or adjust reaction conditions, in an effort to optimize yield and therefore efficiency. However, endpoint methods can be misleading because although high-efficiency reactions reach saturation (a point at which the rate of amplification plateaus) at earlier cycle numbers than low-efficiency reactions, product yields often appear similar when examined on gels after 30–40 cycles. Amplification efficiency can be more accurately determined by real-time PCR methods, which are routinely used for the quantification of specific DNA molecules from biological samples [2]. Amplification efficiency is determined from the threshold cycle (C_T) value obtained prior to the reaction reaching saturation, when none of the reaction components are limiting. Real-time methods provide highly reproducible C_T values for reactions with the same starting copy number and reaction stoichiometry, which can be used to quantify amplification efficiency as follows.

Theoretically, the amount of product doubles during each PCR cycle; in other words, $N = N_0 2^n$, where N is the number of amplified molecules, N_0 is the initial number of molecules, and n is the number of amplification cycles [3–5]. Experimentally, amplification efficiency (E) is less than perfect, ranging from 0 to 1, and therefore the real PCR equation is $N = N_0(1 + E)^n$.

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At threshold cycle, where the emission intensity of the amplification product measured by a real-time PCR instrument (such as the Mx4000 Multiplex Quantitative PCR System; Stratagene, La Jolla, CA) is recorded as statistically significant above the background noise, the PCR equation transforms into $N = N_0(1+E)^{C_T}$. This equation can also be written as $\log N = \log N_0 + C_T \log(1+E)$, and therefore C_T is proportional to the negative of the log of the initial target copy number. Thus, the plot of C_T versus the log of initial target copy number is a straight line, with a slope of $-[1/\log(1+E)]$ corresponding to amplification efficiency via the equation $E = 10^{[-1/\text{slope}]} - 1$.

The amplification efficiency of *Taq* DNA polymerase has been measured in several different studies and was shown to vary between 36% (''nonoptimized conditions;'' 0.5–1.5 mM dNTPs, pH 8.0, 5 mM Mg^{2+}; [6]) and 88% (''optimized conditions''; 16.6 mM dNTPs, pH 8.8, 10 mM Mg^{2+}; [7–9]). Although *Taq* is suitable for a number of PCR applications, it lacks proofreading activity, and as a result exhibits relatively low fidelity [10–13]. Moreover, product yields generally decrease with increasing amplicon size above 1 kb. Over the past decade, a number of DNA polymerases have been introduced that tout improved specificity, fidelity, yield, and/or amplification of larger then *Taq*. Although several studies comparing PCR enzyme fidelity have been reported, amplification efficiency comparisons have not been performed.

PCR enyzme formulations developed for improved specificity are typically hot start versions, in which the DNA polymerase has been heat-reversibly inactivated with neutralizing monoclonal antibodies or chemical modification (requires 95 °C preactivation step) [14–16]. Higher-fidelity amplification is performed using archaeal DNA polymerases (i.e., *Pfu*, *KOD*), which possess an associated 3′-5′ exonuclease-dependent proofreading activity. Unlike *Taq*, archaeal DNA polymerases also possess a ''read-ahead'' function that detects uracil (dU) residues in the template strand and stalls synthesis [17]. Uracil detection can limit the performance of proofreading DNA polymerases when dUTP, arising from dCTP deamination during PCR, is incorporated but dU-containing products fail to replicate. Newer versions of *Pfu* (e.g., *PfuTurbo* DNA polymerase) are formulated with a thermostable dUTPase to minimize dU-DNA synthesis and to improve PCR product yield and amplification of long targets [18]. In addition to uracil sensitivity, processivity and elongation rate are also thought to influence the relative performance of archaeal DNA polymerases [19].

Improved yield and amplification of long targets has also been achieved with DNA polymerase blends. Commercial DNA polymerase blends typically consist of *Taq* plus a lesser amount of a proofreading DNA polymerase to enhance PCR product yield, amplification of long targets, and fidelity compared to *Taq* alone [12]. Adding a proofreading enzyme to *Taq* is thought to improve long PCR by excising misincorporated bases that would otherwise stall *Taq* [12]. One unique blend (Herculase DNA polymerase) consists predominantly of *Pfu* DNA polymerase, a lesser proportion of *Taq* DNA polymerase, and thermostable dUTPase to minimize dU-DNA formation and enhance *Pfu*'s contribution to blend performance and fidelity [18].

To date, the relative performance of DNA polymerase blends, proofreading PCR enzymes, and hot start enzyme formulations has been documented largely with endpoint methods. In this study, we measure the amplification efficiencies of a variety of commercially available DNA polymerases using real-time Q-PCR with SYBR Green I detection. The optimal SYBR Green I concentration is determined for each PCR enzyme, and amplification efficiencies are measured under identical conditions using a set of templates designed to address the influence of amplicon size, DNA sequence, and GC content. Such comparisons allow us to quantify the effects of hot start antibodies, uracil sensitivity, and blending with *Taq* on the performance of *Pfu* DNA polymerase and to accurately compare the performance of *Pfu*-based formulations to *Taq* and other proofreading enzymes.

Materials and methods

Cloned *Pfu*, *PfuTurbo*, *PfuTurbo* Hotstart, *PfuUltra*, Herculase, Herculase Hotstart, and SureStart *Taq* DNA polymerases were from Stratagene. Platinum *Taq* and Platinum *Pfx* were purchased from Invitrogen. *Tgo* DNA polymerase was purchased from Roche Molecular Biochemicals (Indianapolis, IN). SYBR Green I, ROX, and DMSO[1] were from Stratagene (La Jolla, CA). Human genomic DNA was obtained from BD Biosciences Clontech (La Jolla, CA).

Preparation of templates for efficiency comparisons

We used amplicons as templates for efficiency comparisons. Three of the amplicons were amplified from human genomic DNA using *PfuTurbo* DNA polymerase and primer pairs Fu-outF and Fu-outR, AT-outF and AT-R1, AT-outF, and AT-outR (Table 1), generating fragments of 622, 923, and 3983 bp, respectively. The amplicons were gel-purified using StrataPrep DNA Gel Extraction Kit (Stratagene) according to manufacturer's instructions. The purified amplicons were quantified by absorbance at 260 nm. After calculating the approximate copy number of each purified PCR amplicon, they

[1] *Abbreviation used:* DMSO, dimethyl sulfoxide.

Table 1
Primer sequences

Primer pairs	Gene/fragment length	Oligo sequence (5′-3′)
Fu-outF	α-L-Fucosidase/622 bp	TGGGTGGAGAAGAGAAGTTCGTTGA
Fu-outR		AGTTGTATATCAGGATAACAAGTCAGGT
Fu-F	α-L-Fucosidase/545 bp	CAGGAAAACAGTGAGCAGCGCCTC
Fu-R		CAAGTCAGGTATCTTTGACAGTGTTGT
AT-outF	α-1-Antitrypsin/3983 bp	CCTGCCAGAAGAGACAGAGCTTGA
AT-outR		AGCACTAGCTTCATAGCTGTACAATGGA
AT-outF	α-1-Antitrypsin/923 bp	CCTGCCAGAAGAGACAGAGCTTGA
AT-R1		GAGGTACAGGGTTGAGGCTAGTG
AT-F	α-1-Antitrypsin/890 bp[a]	GAGGAGAGCAGGAAAGGTGGAAC
AT-R1		GAGGTACAGGGTTGAGGCTAGTG
AT-F	α-1-Antitrypsin/2598 bp[a]	GAGGAGAGCAGGAAAGGTGGAAC
AT-R2		TGCAGAGCGATTATTCAGGAATGC
AT-F	α-1-Antitrypsin/3915 bp[a]	GAGGAGAGCAGGAAAGGTGGAAC
AT-R3		CCTTTGGACAGGGATGAGGAATAAC
GC-For	Fragile-X/494 bp	CACGCCACTGAGTGCACCTCTGCAGAA
GC-Rev		TTCAGCCCTGCTAGCGCCGGGAGCCCG
GCFF-in		CAGGAAAACAGTGAGCAGCGCCTCGCGACCTGTCACCGCCCTTCAG
GCRF-in	545 bp	CAAGTCAGGTATCTTTGACAGTGTTGTTTCAGCCCTGCTAGCGCCGGGAGCCCG
GCFF-out		GTGACCAAGTTCATGCTCAGGAAAACAGTGAGCAGCGCCTC
GCRF-out	579 bp	GTCGGAGTCAACGGATTCAAGTCAGGTATCTTTGACAGTGTTGT

[a] To simplify, the sizes of these fragments are referred to as 0.9, 2.6, and 3.9 kb in the text.

were adjusted to 2.5×10^9 molecules/μl, and a series of 10-fold dilutions were prepared with deionized distilled water. Generally, 10-fold dilutions from 5×10^7 to 5×10^2 amplicon molecules were used in real-time amplifications. In all efficiency experiments, nested primers were used for amplification.

The GC-rich amplicon was amplified from human genomic DNA using *PfuTurbo* DNA polymerase and primers GC-For and GC-Rev (Table 1) in the presence of 7% DMSO under the following conditions: 5 min initial denaturation at 98 °C; 35 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, followed by final extension of 10 min at 72 °C. Annealing sites for primers Fu-F and Fu-R were added to the 5′ ends of the fragment using primers GCFF-in and GCRF-in. Finally, 17 random nucleotides were added to the 5′ ends of the amplicon using primers GCFF-out and GCRF-out to create nested annealing sites for Fu-F and Fu-R. The final product was purified with the StrataPrep DNA Gel Extraction Kit and quantified, and the copy number was calculated as described above. Generally, 10-fold dilutions from 5×10^7 to 5×10^2 molecules were used.

Real-time PCR conditions

All real-time PCRs were carried out using the Mx4000 Multiplex Quantitative PCR System (Stratagene). All PCR amplifications were performed using the recommended buffer supplied by the manufacturer. Except where indicated, PCRs (50 μl) consisted of 2 μl of standard template (2.5×10^2 to 2.5×10^7 molecules/μl), 200 μM each dNTP, 7.5 pmol each primer, 2.5 U DNA polymerase, 2.5 μl of SYBR Green I (diluted to 1:10,000 to 1:240,000 (v/v) as indicated), and 0.75 μl of ROX internal reference dye (2 μM stock). To amplify the 0.9-, 2.6-, or 3.9-kb fragment, primer AT-F was used in combination with primer AT-R1 (0.9-kb amplicon template), AT-R2 (2.6-kb amplicon template), or AT-R3 (3.9-kb amplicon template), respectively, while the 545-bp target was amplified with primers Fu-F and Fu-R from either the 45% GC amplicon (Fu-outF/Fu-outR product) or the 78% GC amplicon (GCFF-out/GCRF-out product) (Table 1). Enzyme comparisons employed identical concentrations of PCR primers and template; however, DNA polymerase amount, dNTPs, and Mg^{2+} concentration were adjusted according to manufacturers' recommendations as follows: 1.25 U Platinum *Pfx* (2.5 U was inhibitory) with 1 mM $Mg^{2+}$and 300 μM dNTPs, 0.5 U *Tgo* (generated higher yields in comparisons to 1 U). Triplicate or quadruplicate reactions were performed for each template amount. DNA standard template was replaced with water in all no-template controls.

PCR cycling conditions were as follows: initial denaturation at 95 °C for 5–10 min according to manufacturers' recommendations, followed by 40 cycles of 95 °C for 30 s, 1 min annealing (annealing temperature adapted for specific primer set used), and 1 min/kb extension at 72 °C except for Platinum *Pfx* DNA polymerase where 68 °C (supplier's recommended temperature) was used. The identities of the PCR products were examined by melting curve analysis on the Mx4000 and by agarose gel electrophoresis.

Data analysis was performed using the Mx4000 software. C_Ts were determined using the signal/noise ratio set to 20 standard deviations above background subtracted mean fluorescence values (dRn) for cycles 3–8. The slope was calculated from the plot of log initial target copy number versus C_T, and amplification efficiency was determined from the equation $E = 10^{[-1/\text{slope}]} - 1$.

Results

Sensitivity of DNA polymerases to SYBR green I

We opted to use SYBR Green I, rather than sequence-specific probes, to allow detection of both long (up to 3.9 kb) and low-copy-number (10^2) PCR amplicons. DNA polymerases are normally inhibited at high concentrations of SYBR Green I. Therefore, for accurate analysis of amplification efficiency, we first determined the optimal SYBR Green I concentration for each DNA polymerase and PCR system examined (Table 2). A typical amplification plot is shown in Fig. 1, where delayed C_Ts indicate inhibition of *PfuTurbo* Hotstart DNA polymerase at relatively high concentrations of SYBR Green I (1:10–40,000 dilution).

All DNA polymerases examined were inhibited by SYBR Green I at 1:10,000 dilution, while only SureStart *Taq* (chemically modified hot start), Platinum *Taq* (antibody-neutralized hot start), Platinum *Pfx* (antibody-neutralized *KOD* DNA polymerase), and the Herculase blends showed some inhibition with longer targets in the presence of SYBR Green I at 1:60,000 dilution (Table 2). Overall, relative resistance to SYBR Green I was determined to be as follows: (most resistant) *Pfu*-only formulations (*Pfu*, *PfuTurbo*, *PfuTurbo* Hotstart) > *PfuUltra* ⩾ *Pfu*/*Taq* blends (Herculase, Herculase Hotstart) > *Taq*-only formations (Platinum *Taq*, SureStart *Taq*) (least resistant). Although some DNA polymerases (e.g., *Pfu*) were insensitive to higher concentrations of SYBR Green I, we used either 1:60,000 or 1:120,000 dilutions of SYBR Green I (final in PCR: 4.2- or 8.3×10^{-7} (v/v)) in Q-PCRs. These SYBR Green I concentrations generated sufficient signal intensity for detection and analysis and resulted in similar amplification efficiencies when amplifying the same target (data not shown).

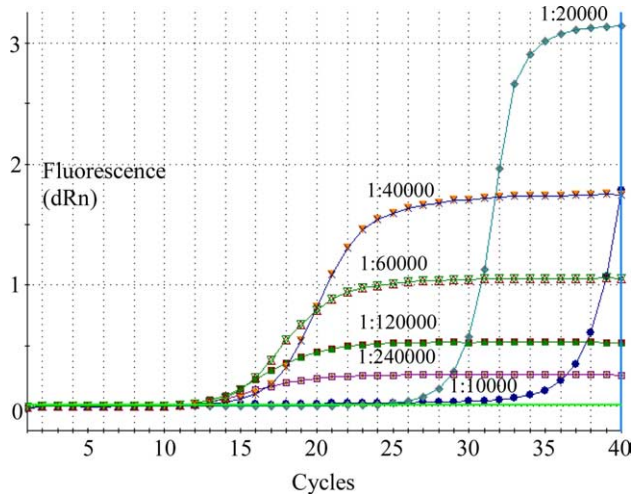

Fig. 1. Inhibitory effects of high SYBR Green I concentrations on DNA polymerases. *PfuTurbo* Hotstart DNA polymerase was used to amplify the 0.9-kb target, as described under Materials and methods. Reactions at each SYBR dilution were performed in duplicate.

Amplification efficiency comparisons for different target lengths

Efficiency was quantified in amplification reactions employing PCR amplicons as template. Using a wide range of amplicon template amounts (10^2–10^7 copies) allowed us to obtain a strong linear correlation between C_Ts and initial copy number (a high regression coefficient) and therefore a high degree of reproducibility.

To examine the effect of target length on the amplification efficiency of DNA polymerases, primer AT-F was used in combination with primers AT-R1, AT-R2, and AT-R3 to amplify 0.9-, 2.6-, and 3.9-kb fragments, respectively. PCR amplifications were performed with nested primers using each DNA polymerase in its optimal PCR buffer. All PCR parameters were identical, except that PCR enzyme amount, Mg^{2+} concentration, and PCR cycling parameters were adjusted according to the manufacturers' recommendations (see Materials and methods). Fig. 2 shows an example of an amplification plot and standard curve for Herculase Hotstart DNA polymerase. Amplification efficiencies were calculated from the slope of standard curves as $E = 10^{[-1/\text{slope}]} - 1$.

Table 3 summarizes the amplification efficiencies of various PCR enzymes as a function of amplicon size. The two hot start versions of *Taq* exhibited similar amplification efficiencies (82–83%, 0.9 kb; 62–66%, 2.6 kb), even though reversible inactivation was achieved by very different means (chemical modification, SureStart *Taq*; antibody neutralization, Platinum *Taq*). Despite the use of longer extension times (1 min/kb), amplification efficiency decreased with increasing amplicon size above 1–2 kb. In fact, amplification efficiency could not be accurately determined for the 3.9-kb target as both *Taq* formulations produced smears and multiple bands.

Like *Taq*, the amplification efficiency of *Pfu* DNA polymerase decreased with increasing template size above 1–2 kb (78%, 0.9 kb; 71%, 2.6 kb; 49%, 3.9 kb). In contrast, the amplification efficiencies of *Pfu* formulations with dUTPase (*PfuTurbo*, *PfuUltra*, Herculase) were significantly higher for PCRs employing the 2.6-kb

Table 2
SYBR Green I sensitivity of DNA polymerases

Polymerase	Amplicon size (GC content)	Dilutions (× 1/1000)					
		1:10	1:20	1:40	1:60	1:120	1:240
Pfu	545 bp (78%)	−−	−−	++	++	++	++
	545 bp (45%)	−−	−−	++	++	++	++
	0.9 kb (56%)	−−	−/+	−/+	++	++	++
	2.6 kb (56%)	−−	++	++	++	++	++
	3.9 kb (53%)	−−	++	++	++	++	++
PfuTurbo	545 bp (78%)	−−	−−	++	++	++	++
	545 bp (45%)	−−	−−	++	++	++	++
	0.9 kb (56%)	−−	−−	−/+	++	++	++
	2.6 kb (56%)	−−	−/+	++	++	++	++
	3.9 kb (53%)	−−	−/+	++	++	++	++
PfuTurbo Hotstart	545 bp (78%)	−−	−−	−/+	++	++	++
	545 bp (45%)	−−	−−	−/+	++	++	++
	0.9 kb (56%)	−−	−−	−/+	++	++	++
	2.6 kb (56%)	−−	−/+	++	++	++	++
	3.9 kb (53%)	−−	−/+	++	++	++	++
PfuUltra	545 bp (78%)	−−	−−	−−	++	++	++
	545 bp (45%)	−−	−−	−/+	++	++	++
	0.9 kb (56%)	−−	−−	−−	−/+	++	++
	2.6 kb (56%)	−−	−−	++	++	++	++
	3.9 kb (53%)	−−	−−	++	++	++	++
Platinum *Pfx*	545 bp (78%)	−−	−−	++	++	++	++
	545 bp (45%)	−−	−−	−−	−/+	++	++
	0.9 kb (56%)	−−	−−	−−	−/+	++	++
	2.6 kb (56%)	N/A	N/A	N/A	N/A	N/A	N/A
Tgo	545 bp (78%)	N/A	N/A	N/A	N/A	N/A	N/A
	545 bp (45%)	−−	−−	−/+	++	++	++
	0.9 kb (56%)	N/A	N/A	N/A	N/A	N/A	N/A
SureStart *Taq*	545 bp (78%)	−−	−−	−−	++	++	++
	545 bp (45%)	−−	−−	−/+	++	++	++
	0.9 kb (56%)	−−	−−	−−	−/+	++	++
	2.6 kb (56%)	−−	−−	−−	−/+	++	++
	3.9 kb (53%)	N/A	N/A	N/A	N/A	N/A	N/A
Platinum *Taq*	545 bp (78%)	−−	−−	−−	−/+	++	++
	545 bp (45%)	−−	−−	−−	−/+	++	++
	0.9 kb (56%)	−−	−−	−−	−−	−/+	++
	2.6 kb (56%)	−−	−−	−−	−−	−/+	++
	3.9 kb (53%)	N/A	N/A	N/A	N/A	N/A	N/A
Herculase	545 bp (78%)	−−	−−	−/+	++	++	++
	545 bp (45%)	−−	−−	−/+	++	++	++
	0.9 kb (56%)	−−	−−	−−	−/+	++	++
	2.6 kb (56%)	−−	−−	++	++	++	++
	3.9 kb (53%)	−−	−−	−/+	++	++	++
Herculase Hotstart	545 bp (78%)	−−	−−	−/+	++	++	++
	545 bp (45%)	−−	−−	−/+	++	++	++
	0.9 kb (56%)	−−	−−	−/+	++	++	++
	2.6 kb (56%)	−−	−−	−−	−/+	++	++
	3.9 kb (53%)	−−	−−	−−	−/+	++	++

−−, Inhibition shown as >2 C_T delay and weaker or no band on the gel; −/+, slight inhibition shown as >0.5 C_T and ⩽2 C_T delay; ++, no inhibition (optimal amplification); N/A, no data available (no specific product or the presence of smear or multiple bands).

(80–84% vs 71% for *Pfu* and 62–66% for *Taq*) and 3.9-kb (66–74% vs 49% for *Pfu*) amplicons. *PfuUltra*, which is formulated with *Pfu* mutant and possesses higher proofreading activity [20], demonstrates somewhat lower amplification efficiencies (2.3 to 8% lower) than *PfuTurbo*. Neutralizing monoclonal antibodies had minimal effects on the amplification efficiency of hot start *Pfu* formulations (varied within 0.9–4.8%).

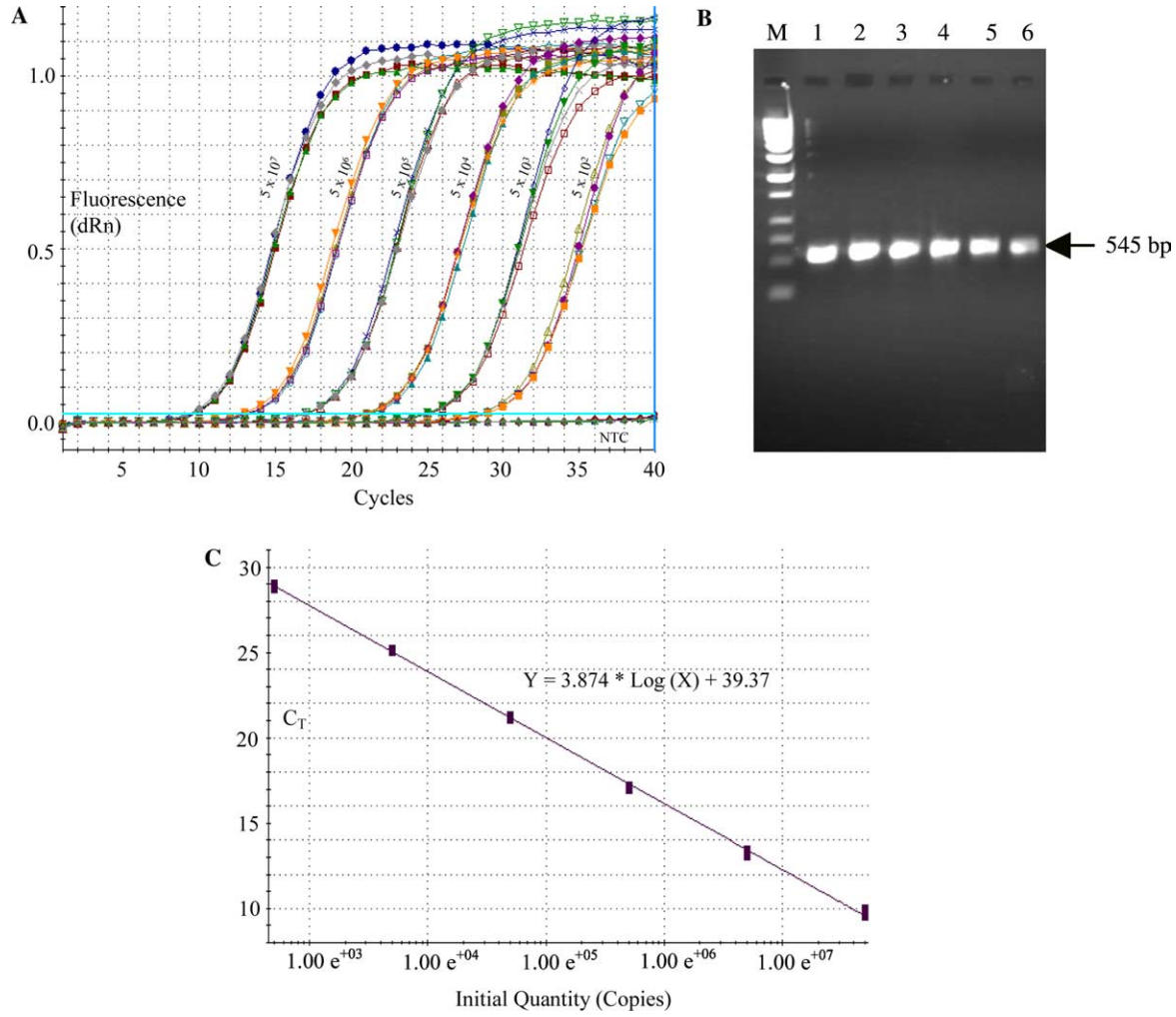

Fig. 2. Real-time PCR amplification of 10-fold serial dilutions of the 545-bp amplicon. Herculase Hotstart DNA polymerase was used to amplify the 545-bp amplicon with 45% CG content, as described under Materials and methods. (A) Real-time PCR amplification plot; (B) PCR products amplified from 5×10^7 to 5×10^2 (10-fold serial dilutions, lanes 1–6) molecules/μl by gel electrophoresis; (C) standard curve with (R^2) value and regression fit equation indicated. Each PCR was performed in quadruplicate. NTC, no-template control.

Table 3
Amplification efficiencies (%) as a function of target length

Polymerase	Target length (kb)		
	0.9	2.6	3.9
Pfu	78.8 ± 1.7	71.2 ± 1.6	49.1 ± 1.7
PfuTurbo	83.2 ± 0.6	83.7 ± 1.1	74.4 ± 0.6
PfuTurbo Hotstart	82.1 ± 1.9	81.6 ± 1.0	70.8 ± 1.0
PfuUltra	80.9 ± 1.31	80.4 ± 1.56	66.4 ± 1.21
Platinum *Pfx*	66.1 ± 1.6	N/A	N/A
Tgo	N/A	N/A	N/A
SureStart *Taq*	82.6 ± 1.2	62.3 ± 1.2	N/A
Platinum *Taq*	81.9 ± 0.4	65.7 ± 2.0	N/A
Herculase	89.7 ± 1.2	81.7 ± 2.0	71.6 ± 1.8
Herculase Hotstart	90.7 ± 1.0	80.8 ± 1.8	73.5 ± 0.3

Amplification efficiencies are the averages obtained from at least three independent experiments with the standard deviations indicated. Between four and six serial template dilutions were used in each experiment (each dilution was prepared in triplicate or quadruplicate). N/A, no data available (no specific PCR product or generation of smear or multiple products).

We also examined other archaeal DNA polymerases such as *KOD* (Platinum *Pfx*) and *Tgo* DNA polymerases. Platinum *Pfx* DNA polymerase amplified the 0.9-kb fragment with 66% amplification efficiency, which is significantly lower than the efficiency of *Pfu* alone (78%) or with dUTPase (83%). Efficiencies could not be

determined for longer targets using Platinum *Pfx* (>0.9-kb fragments) or *Tgo* (>0.6-kb fragments) DNA polymerase due to failure to amplify.

Compared to *PfuTurbo*, Herculase contains a minor percentage of *Taq* and a unique PCR buffer, which have been shown previously to enhance the target-length capability of *Pfu* in the presence of dUTPase (increases from 19 to 37 kb for genomic targets) [20]. With one exception (the 0.9-kb system), Herculase and *PfuTurbo* DNA polymerases exhibited similar amplification efficiencies over the range of targets sizes examined. Presumably, differences in amplification efficiency would be apparent in comparisons employing longer amplicons.

Variation of amplification efficiency with percentage GC content

To address the contribution of GC content on amplification efficiency, we employed two 545-bp amplicons with identical PCR primer annealing sequences, but either 45 or 78% GC content (see Materials and methods). To enhance amplification of the GC-rich target, DMSO was added (1 to 15% (v/v); 1% increments), and the optimal concentration that generated the lowest C_Ts was determined for each DNA polymerase examined. The results were as follows: *Pfu* (6–10%), *PfuTurbo* (Hotstart) (7–11%), *PfuUltra* (6–9%), SureStart *Taq* (8–10%), Platinum *Taq* (7–8%), and Herculase (Hotstart) (6–10%). As an example, the DMSO titration for *PfuTurbo* is shown in Fig. 3. All DNA polymerases amplified the 78% GC target optimally at 8% DMSO, although each exhibited a unique DMSO sensitivity profile. With the exception of Platinum *Pfx*, amplification efficiency was determined from PCRs employing either 8% DMSO (78% GC amplicon) or 0% DMSO (45% GC amplicon). DMSO did not affect

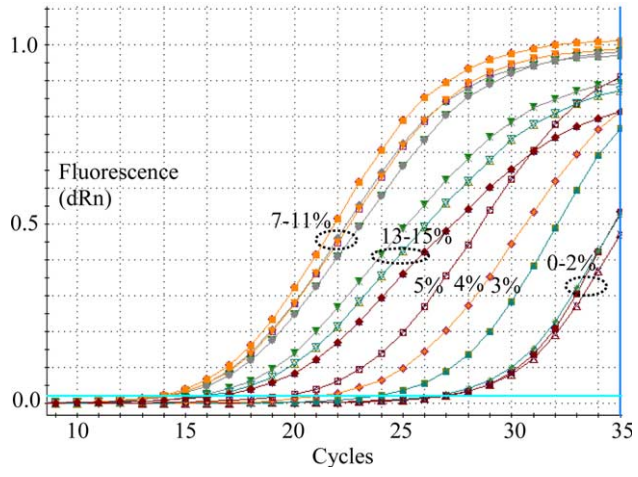

Fig. 3. Real-time PCR amplification in the presence of varying DMSO amounts. *PfuTurbo* DNA polymerase was used to amplify the 545-bp amplicon with 78% CG content in the presence of different amounts of DMSO (0–15% (v/v), in 1% increments) (see Materials and methods).

Table 4
Amplification efficiencies (%) as a function of GC content

Polymerase	GC content	
	45%	78%[a]
Pfu	77.2 ± 1.8	51.3 ± 1.6
PfuTurbo	79.9 ± 1.6	55.2 ± 1.8
PfuTurbo Hotstart	75.1 ± 1.1	54.5 ± 1.9
PfuUltra	70.8 ± 1.9	44.1 ± 1.8
Platinum *Pfx*	61.2 ± 1.1	28.7 ± 0.7[b]
Tgo	56.9 ± 1.9	N/A
SureStart *Taq*	78.7 ± 1.5	42.1 ± 0.4
Platinum *Taq*	78.5 ± 0.5	43.3 ± 0.5
Herculase	79.7 ± 1.1	54.7 ± 1.3
Herculase Hotstart	80.4 ± 1.2	54.5 ± 0.8

Amplification efficiencies are the averages obtained from at least three independent experiments with the standard deviations indicated. Between four and six serial template dilutions were used in each experiment (each dilution was prepared in triplicate or quadruplicate). N/A, no data available (generation of smear or multiple products).

[a] 8% DMSO was used in the amplification of this target.

[b] PCRx solution was added according to the manufacturer's recommendation.

efficiency measurements for the 45% GC target (data not shown). In our hands, Platinum *Pfx* generated weak and multiple PCR products even when DMSO was added. Therefore, the proprietary PCRx solution recommended by the manufacturer was used instead to successfully amplify the GC-rich amplicon (2.5 × final concentration).

As shown in Table 4, all DNA polymerases amplified the 78% GC amplicon with significantly lower efficiency compared to the 45% GC amplicon. *Pfu* exhibited an efficiency of 77.2% when amplifying the 45% GC target. However, when GC content increased to 78%, the amplification efficiency dropped to 51.3%. Likewise, SureStart *Taq*, Platinum *Taq*, *PfuTurbo*, and Herculase exhibited similar efficiencies when amplifying the 45% GC target (75 to 80%). However, with the GC-rich amplicon, amplification efficiency was significantly lower and varied as follows: (highest efficiency): 54–55%; *PfuTurbo* (Hotstart), Herculase (Hotstart) > 51%; *Pfu* > 42–44%; *PfuUltra*, SureStart *Taq*, Platinum *Taq* > 29%; *Pfx* (lowest efficiency). Even in the presence of DMSO (up to 15% (v/v)), *Tgo* DNA polymerase generated smears while amplifying the 78% GC target, and thus amplification efficiency could not be determined (data not shown).

Discussion

Unlike most enzymatic reactions, PCR is an exponential process and therefore very small changes in amplification efficiency, E, can result in dramatic differences in the amount of final product, even if the initial number of target molecules is the same. For example, if $E = 74.7\%$ (e.g., *PfuTurbo*, 3.9-kb

fragment) and $n = 30$, then $N = N_0(1 + 0.747)^{30}$ or $1.86 \times 10^7 N_0$. In other words, after 30 cycles, this PCR would theoretically produce a 1.86×10^7-fold increase in the amount of target molecules. However, if $E = 49.1\%$ (e.g., *Pfu*, 3.9-kb fragment), after 30 cycles, the target would be amplified only 1.6×10^5 times by PCR. Thus a 25.6% difference in amplification efficiency leads to a 116-fold difference in the amount of final product.

PCR product yield is generally the most important parameter considered when selecting a PCR enzyme for amplification. Despite its importance, very little comparative information exists with regard to amplification efficiencies of commercial PCR enzymes. In this study, we determined the amplification efficiency of 10 different DNA polymerase formulations under optimal conditions (enzyme amount, PCR buffer, extension temperature) using identical reaction parameters (primer and template concentrations, cycling regimen). Significant differences in the PCR enzyme efficiency were apparent in carefully controlled comparisons employing five templates of varying length and GC content.

All DNA polymerases examined exhibited roughly similar efficiencies when amplifying smaller fragments (<900 bp) of 45–56% GC content. However, as amplicon size or GC content increased, amplification efficiency decreased to varying extents with different DNA polymerases. *PfuTurbo*, *PfuUltra*, and Herculase were shown to be less sensitive to amplicon size than *Pfu* alone, due to the presence of dUTPase (Table 3). It has been previously shown that adding *Pyrococcus furiosus* dUTPase enhances the performance of *Pfu* and other archaeal DNA polymerases by eliminating dUTP (dUTP → dUMP), which acts as a potent inhibitor and accumulates during PCR due to hydrolytic deamination of dCTP [18]. In this study, we demonstrate that dUTPase significantly increases the efficiency of amplifying long targets (12.5 and 25.3% increases for 2.6- and 3.9-kb amplicon, respectively). Moreover, the amplification efficiency of *Pfu*, in the absence of dUTP poisoning (presence of dUTPase), is significantly higher than that of *Taq* for amplicons greater than 1–2 kb in length.

Although processivity is thought to contribute to PCR yield and rate, exhibiting higher processivity does not necessarily guarantee higher amplification efficiency for longer targets as illustrated by the results obtained with Platinum *Pfx*, SureStart *Taq*, and Platinum *Taq* DNA polymerases. *KOD* and *Taq* have been shown to exhibit 10- to 15-fold [19] and 4-fold [21] higher processivity, respectively, than *Pfu* DNA polymerase. However, the amplification efficiency of Platinum *Pfx* (*KOD*) was significantly lower (13–23% lower for 3/3 systems of 0.5–0.9 kb) than that of *Pfu* alone (no dUTPase), suggesting that factors other than processivity or uracil sensitivity contribute to efficiency. In the case of *KOD*, lower efficiency may be related to the enzyme's relatively high level of 3′-5′ exonuclease activity [19], which could degrade PCR primers and/or template. The somewhat lower amplification efficiency exhibited by *PfuUltra* (a *Pfu* mutant formulation with 3-fold higher fidelity) may also be attributed to the higher exonuclease activity of *PfuUltra* compared to that of *PfuTurbo*.

Although *Taq* lacks 3′-5′ exonuclease activity and uracil sensitivity and is more processive than *Pfu*, both SureStart and Platinum *Taq* show lower amplification efficiency for longer targets than *PfuTurbo* (18–21% lower for 2.6-kb amplicon) (Table 2). In the case of *Taq*, lower amplification efficiency for longer targets may be related to the enzyme's lack of proofreading activity. As amplicon size increases, so does the probability that *Taq* will incorporate an incorrect nucleotide and either stall or slow due to inefficient extension from mispaired primers, as compared to correctly paired termini. However, for Platinum *Pfx*, Platinum *Taq*, and SureStart *Taq*, we cannot rule out the possibility that impurities in the PCR enzyme or reaction buffer, or the use of suboptimal (manufacturer supplied) buffer conditions may also contribute to lower efficiency compared to *Pfu*.

In addition to amplicon length, GC content was shown to significantly influence amplification efficiency. Table 4 indicates that *Pfu*, *PfuTurbo*, *PfuUltra*, and Herculase exhibit less of a decrease in PCR efficiency than SureStart *Taq*, Platinum *Taq* and Platinum *Pfx* (25–27% vs 32–36% decrease) when amplifying a 78% GC amplicon compared to a 45% GC target. Therefore, the same *Pfu*-based formulations that demonstrated higher efficiencies for longer targets also showed higher efficiencies when amplifying a GC-rich amplicon.

Hot start modifications did not appear to greatly influence amplification efficiency. Antibody-neutralized and chemically inactivated versions of *Taq* exhibited similar efficiencies, irrespective of amplicon size or GC content. Direct comparisons between *PfuTurbo* and Herculase formulations, lacking or containing polymerase-specific antibody, showed that only the hot start version of *PfuTurbo* exhibited slightly lower amplification efficiency (0.7–4.8% lower) compared to the nonhot start enzyme. Abu Al-soud and Radstrom [22] had previously reported that heating purified plasma IgG in the presence of DNA template can inhibit amplification through the formation of IgG–DNA complexes, and thus any uncomplexed IgG in commercial hot start enzymes could potentially interfere with amplification.

To ensure maximum yield in the shortest period of time, amplification efficiency should be considered when selecting a PCR enzyme, particularly when amplifying long (> 1–2 kb) or GC-rich amplicons. For example, Table 5 shows the number of cycles required to achieve 10^6-fold amplification using the efficiency values deter-

Table 5
Variation of cycle number and mutation frequency with DNA polymerase

DNA polymerase	Error rate (mutation per bp per duplication)[a]	545-bp amplicon			2.6-kb amplicon	
		PCR-induced mutant fraction (%)[b]	Number of cycles required[c]		PCR-induced mutant fraction (%)[b]	Number of cycles required[c]
			45% GC	78% GC + additives		
Pfu	1.3×10^{-6}	1	24	33	7	26
PfuTurbo	1.3×10^{-6}	1	24	31	7	23
PfuUltra	4.3×10^{-7}	0.5	26	38	2	23
Pfx	3.5×10^{-6}	4	29	55	18	NA
Herculase	2.8×10^{-6}	3	24	32	15	23
SureStart *Taq*	8.0×10^{-6}	9	24	39	42	29

[a] From Hogrefe and Borns [20].
[b] Fraction of error-containing products following 10^6-fold amplification of the indicated target sequence given the DNA polymerase error rate. Mutation frequencies were calculated using the equation $mf = ER \times bp \times d$, where mf is the mutation frequency, ER is the error rate, bp is the length of the target, and d is the number of template doublings [13].
[c] Number of cycles required to obtain 10^6-fold amplification given the efficiency per cycle in Tables 3 and 5.

mined for the 545- (Table 4) and 2.6-kb (Table 3) amplicons. Although all DNA polymerases can amplify the low %-GC amplicons 10^6-fold within 30 cycles, the desired level of amplification is achieved 1 to 7 cycles earlier, depending on the DNA polymerase employed. For example, the 2.6-kb amplicon can be amplified 10^6-fold in 23 cycles using *PfuTurbo*, *PfuUltra*, and Herculase DNA polymerases, compared to 29 cycles using SureStart *Taq* DNA polymerase. However, depending on the target DNA sequence, the minimum number of cycles required to achieve 10^6-fold amplification may greatly exceed 30 cycles. For example, depending on the DNA polymerase employed, an additional 7 to 26 cycles is required to amplify the 78% GC amplicon compared to the 45% GC amplicon (Table 5). The higher the number of PCR cycles, the higher the chances of amplifying undesired products, such as primer dimers. In addition to efficiency, PCR enzyme fidelity is another important consideration when amplifying long targets (>1 kb), since the percentage of clones containing errors increases proportionally with increasing amplicon size. As shown in Table 5, *Pfu* + dUTPase formulations (*PfuUltra*, *PfuTurbo*) are expected to amplify longer targets with both the fewest number of cycles and the fewest polymerase-induced errors.

A number of modifications to the basic PCR format including additives such as formamide [23], DMSO [24,25], betaine [26], etc. have been published in an attempt to increase amplification efficiency and specificity, regardless of amplicon length or composition. Real-time PCR methods, such as those described in this report, represent a powerful tool for monitoring efforts to optimize amplification efficiency. The data generated by this protocol are collected at the exponential phase of PCR and therefore demonstrate high reproducibility compared to endpoint analysis by gel electrophoresis. Quantitative methods show promise in the development and quality control of PCR enzyme/buffer formulations to ensure consistency and maximal performance.

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