Brazilian Journal of Medical and Biological Research (1998) 31: 1239-1242
ISSN 0100-879X Short Communication

Accuracy of replication in the polymerase chain reaction. Comparison between *Thermotoga maritima* DNA polymerase and *Thermus aquaticus* DNA polymerase

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Abstract

For certain applications of the polymerase chain reaction (PCR), it may be necessary to consider the accuracy of replication. The breakthrough that made PCR user friendly was the commercialization of *Thermus aquaticus* (*Taq*) DNA polymerase, an enzyme that would survive the high temperatures needed for DNA denaturation. The development of enzymes with an inherent 3' to 5' exonuclease proofreading activity, lacking in *Taq* polymerase, would be an improvement when higher fidelity is needed. We used the forward mutation assay to compare the fidelity of *Taq* polymerase and *Thermotoga maritima* (*ULTMA*™) DNA polymerase, an enzyme that does have proofreading activity. We did not find significant differences in the fidelity of either enzyme, even when using optimal buffer conditions, thermal cycling parameters, and number of cycles (0.2% and 0.13% error rates for ULTMA™ and *Taq*, respectively, after reading about 3,000 bases each). We conclude that for sequencing purposes there is no difference in using a DNA polymerase that contains an inherent 3' to 5' exonuclease activity for DNA amplification. Perhaps the specificity and fidelity of PCR are complex issues influenced by the nature of the target sequence, as well as by each PCR component.

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Publication supported by FAPESP.

Received December 10, 1997
Accepted July 28, 1998

Key words
- Polymerase chain reaction fidelity
- *Thermus aquaticus* (*Taq*) DNA polymerase
- *Thermotoga maritima* DNA polymerase

The polymerase chain reaction (PCR) is utilized for rapid *in vitro* amplification of a specific fragment of genomic DNA or RNA. The ideal PCR is one with high specificity, efficiency, and fidelity. Differences in fidelity during amplification can lead to differences in the fraction of PCR products with a sequence identical to the original target. Some studies have demonstrated that primers containing mismatches at the 3' end were not extended as efficiently as the perfectly matched primers (1,2). Theoretically, enzymes with exonuclease activities recognize the 3' mismatch, which would be the first to be repaired and then extended. For PCR applications in which a relatively homogeneous DNA population is analyzed (i.e., direct sequencing or restriction endonuclease digestion), the polymerase-induced mutations during PCR are of little concern. In general, polymerase-induced mutations are distributed randomly over the sequence of interest, and an accurate consensus sequence is usually obtained. However, for sequencing us-

ing cloning procedures or for studies of rare molecules in a heterogeneous population, such as studies of allele polymorphism in individual mRNA transcripts (3), characterization of the allelic stages of single sperm cells (4) or single DNA molecules (5) and characterization of rare mutations in tissue (1), it is vital that the polymerase-induced mutant sequences do not mask the rare DNA sequences. Each polymerase-induced error, once introduced, will be amplified exponentially along with the original wild type sequences during subsequent cycles. This will result in an overall increase in the fraction of polymerase-induced mutant sequences as a function of number of amplification cycles.

Studies have shown that the thermostable DNA polymerases have distinct characteristics which affect the efficacy of PCR. For example, purified 94-kDa *Thermus aquaticus* (*Taq*) polymerase (Perkin Elmer, Norwalk, CT) does not contain an inherent 3' to 5' exonuclease activity. Biochemical fidelity measurements of single nucleotide incorporation/misincorporation have indicated that the ability of "nonproofreading" DNA polymerases (AMV reverse transcriptase and *D. megalanogaster* DNA polymerase) to misincorporate a deoxynucleotide triphosphate is critically determined by the concentration of the triphosphate (6). Similar data have been obtained with regard to extension of a mismatched primer template (7). It is not known if *T. aquaticus* also contains a separate 3' to 5' exonuclease activity that may be associated with the polymerase *in vivo*. Other conditions known to reduce the fidelity of *Taq* polymerase *in vivo* are high $MgCl_2$ in the presence of $MnCl_2$, and high number of PCR cycles starting with low target input (8). These conditions result in a cumulative error frequency of 2% and a mutant yield (for targets over 300 bp) greater than 90%.

Some newly isolated thermostable enzymes for PCR, such as *Vent*™, *Pfu*™, and more recently *ULTMA*™ (Perkin Elmer), do have the editing function and are expected to be more accurate than *Taq* polymerase (reviewed in Ref. 9). Purified *Thermotoga maritima* (*ULTMA*™) DNA polymerase is a 70-kDa recombinant DNA polymerase that does have inherent 3' to 5' exonuclease proofreading activity with no associated 5' to 3' nuclease activity. This should improve the fidelity of *ULTMA*™ by making the enzyme less likely to misinsert a base or to extend a mismatched primer.

In the present study we compared the accuracy of replication in PCR between *Thermus aquaticus* (*Taq*) polymerase and *Thermotoga maritima* (*ULTMA*™) DNA polymerase by DNA sequencing. There are at least three different methods for measuring the fidelity of PCR: 1) the forward mutation assay (10); 2) the reversion mutation assay (11), and 3) denaturant gradient gel electrophoresis (DGGE)-analysis (12). We utilized the forward mutation assay consisting of cloning individual DNA molecules from an amplified population and determining the number of DNA sequence changes by the fraction of the cloned population that displays mutations determined by DNA sequence analysis. The analysis of cloned gene-amplified products is a rapid method that allows a quantitative evaluation of the specificity and fidelity of the amplification method.

The reagent mix preparation for each PCR is described in Table 1. The conditions for each enzyme described above are the optimal conditions suggested by the manufacturer in their package insert.

Cloned HIV DNA (HXB2) inserted into a plasmid was amplified by nested PCR. DNA preparations were serially diluted so that when they were used as targets for nested PCR, only fewer than one in five reactions yielded PCR products. This technique of endpoint PCR was used so that the PCR product would mostly be derived from targets consisting of only single DNA molecules (13). Using this procedure we confirmed that both *Taq* and *ULTMA*™ PCRs were equally single copy sensitive. The V3-

through-V5 region of the gene envelope was amplified with nested primers that had the map positions in the HIV-1 HXB2 clone indicated in parentheses: 5' TACAATGTA CACATGGAAT 3' (sense, positions 6957 to 6976), 5' GCAGTCTAGCGAAGAAGA 3' (sense, positions 7009 to 7027), 5' CTTCTC CAATTGTCCCTCATA 3' (antisense, positions 7644 to 7665), and 5' CGCCATAGTG CTTCCTGCTGCT 3' (antisense, positions 7792 to 7814). Nested PCR employed two sequential amplification rounds each of 35 cycles. For the second round of nested PCR, 5-µl aliquots of the first round were added to 50-µl aliquots of the second round reaction mix. Primers were designed not to have mismatches with the template. The second round inner primers were modified to contain uracil instead of thymidine and to have additional nucleotides at the 5' prime ends for subsequent cloning with the Clone-Amp system (Gibco BRL, Gaithersburg, MD). Thermal cycling conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles, with a final extension at 72°C for 10 min. Bands were visualized by ethidium bromide fluorescence and the same amount of one band of the expected size for both the *Taq* and *ULTMA*™ PCR reactions was seen, suggesting comparable amplification efficiency in both systems. The PCR product of modified primers as described above was annealed with the pAM vector of the Clone-Amp system (Gibco BRL). Appropriate competent bacteria were transformed, and DNA mini-preparations from individual bacterial colonies were prepared using Qiagen-tip 20 columns (Qiagen, Chatsworth, CA). Sequencing of 10 clones for each *Taq* or *ULTMA*™ was performed using Sequenase version 2.0 (United States Biochemical, Cleveland, OH), and autoradiographs were read using the Millipore BioImage Electrophoresis Analyzer (Millipore, Ann Arbor, MI). About 300 base pairs of the same region of each sequence were read.

There was no statistically significant difference in the error rates of the enzymes after 35 cycles for each round of nested PCR, after reading about 3,000 bases for each enzyme (Table 2).

Table 1 - Reaction conditions used for *Taq* and *ULTMA*™ polymerases. The conditions are those recommended by each manufacturer using 10X reagent buffer supplied by each manufacturer.

Data are reported as final concentrations except for the enzymes which are 3 U/100 µl reaction volume.

Taq	*ULTMA*™
10 mM Tris-HCl (pH 8.3)	10 mM Tris-HCl (pH 8.8)
50 mM KCl	10 mM KCl
2.5 mM $MgCl_2$	2.5 mM $MgCl_2$
200 µM each dNTP	40 µM each dNTP
0.2 µM each primer	0.2 µM each primer
3 U *Taq* polymerase	3 U *ULTMA*™ polymerase

Table 2 - Comparison of the error frequencies obtained with *ULTMA*™ and *Taq* polymerases.

Fisher's exact test (P = 0.4) shows that the differences were not statistically significant. Errors were differences from the known HXB2-HIV-1 sequence after 35 cycles for each round of nested PCR.

	Bases read	Error	Rate	%
ULTMA™	2952	6	1/492	0.20
Taq	3000	2	1/750	0.13

PCR fidelity is a result of a complex process which is affected by many factors, including the enzyme, buffer conditions, thermal cycling parameters, and number of cycles. High dNTP concentrations increase error rate by driving the reaction in the direction of DNA synthesis and by decreasing error discrimination at the extension step. A reduction in dNTP and Mg^{2+} concentration leads to improvements in fidelity (8). In addition, post-replication DNA damage-induced errors at elevated temperatures can contribute to infidelity. Various groups have

estimated the fidelity of *Taq* DNA polymerase. Saiki and Gelfand (14) observed a cumulative error frequency of about 0.25% (17/6,692) after 30 cycles of PCR (1.5 mM each dNTP, 10 mM $MgCl_2$). Kunkel (11) calculated a frameshift of about 1/30,000 and a substitution frequency of about 1/8,000 per single cycle extension (1 mM each dNTP and 10 mM $MgCl_2$). Goodenow et al. (15) did not detect mutations among 5,400 nucleotides sequenced of 34 *env* and *gag* region-clones from 30-cycle PCR-amplified HIV-1 plasmid sequences.

In the present study, we did not detect any significant difference in the error rate between *Taq* polymerase and *ULTMA*™ DNA polymerase as determined by the forward mutation assay, even when a reduction in dNTP and Mg^{2+} concentration was used with *ULTMA*™ and not with *Taq*. This result allows us to conclude that for sequencing purposes there is no difference in using a DNA polymerase that contains an inherent 3' to 5' exonuclease activity for DNA amplification. Perhaps the specificity and fidelity of PCR are complex issues influenced by the nature of the target sequence, as well as by each PCR component.

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