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SUBSTITUTION OF Ile(707) FOR Leu IN KLENTAQ DNA POLYMERASE REDUCES THE AMPLIFICATION CAPACITY OF THE ENZYME

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A b s t r a c t: The high yield and specificity of PCR amplifications are affected by DNA polymerase activity at room temperature. One way of preventing this unwanted activity is by genetic modifications of the DNA polymerase. For *Taq* DNA polymerase, mutations in the gene (Glu626Lys, Trp706Arg, Ile707Leu and Glu708Asp), when introduced individually or in certain combinations, were found to contribute to a significant decrease of the enzyme activity at room temperature. The aim of this study was to evaluate the usefulness of the Ile707Leu cold-sensitive mutation in the N-terminal deletional variant of *Taq* DNA polymerase in PCR reaction. The Ile(707) to Leu substitution was introduced to Klentaq278 by site-directed mutagenesis. Normal and mutant DNA polymerases were expressed under a *tac* promoter and purified to homogeneity. The mutant polymerase showed reduced polymerase activity at room temperature by up to 12 times and no significant change in thermostability, compared to Klentaq278 DNA polymerase. The major effect of the amino acid substitution was the reduction of the amplification capacity of the polymerase. Mutant polymerase could not amplify fragments over 1 kb.

In conclusion, the substitution of Ile707Leu in Klentaq278 DNA polymerase reduces the overall processivity of the enzyme and therefore limits the application of this DNA polymerase in PCR.

Key words: Klentaq278, site-directed mutagenesis, hot start, cold sensitive DNA polymerase, PCR.

Introduction

Thermostable DNA polymerases have been used for amplification of DNA fragments since the invention of PCR [1]. The success, yield and specificity of the PCR, particularly for amplifications involving a high cycle number, high CG content of the template and multiple primer pairs are affected by the DNA polymerase activity at room temperature. This activity of DNA polymerases sometimes causes the amplification of non-target oligonucleotides during the PCR set up, due to mispriming of primers on non-target DNA, RNA or the primers themselves and presents a significant problem.

The most simple but laborious method of prevention of unwanted DNA synthesis during PCR is the addition of thermostable DNA polymerase in the reaction mixture at an elevated temperature (72^{o}C or higher) which is known as a "hot start". Several other methods of achieving a hot start have been invented and they are based on either physical separation of the polymerase from the reaction mixtures by wax [2], or reversible polymerase inactivation with antibodies [3] or chemicals [4]. All these methods have limitations and shortcomings. Another way of preventing the DNA polymerase activity at room temperature is by genetic modification of thermostable DNA polymerases, in order to reduce the polymerase activity at room temperature without significantly affecting the optimal temperature activity and the thermostability of the enzyme. In the study of Kermekchiev *et al*. [5], four amino acid changes in the N-terminal deletional variant of *Taq* DNA polymerase (Glu626Lys, Trp706Arg, Ile707Leu and Glu708Asp), when introduced individually into the enzyme, were found to contribute to a significant decrease in the DNA polymerase activity at room temperature or the "cold-sensitive" phenotype of the enzyme. On the other hand, the reported processivity of these mutant enzymes at the optimal temperature (72^{o}C) and thermostability at 95^{o}C was not significantly affected by the cold-sensitive mutation. Some of those cold-sensitive mutants, especially the double mutant (Glu626Lys/Ile707Leu) and single mutant (Ile707Leu), were found to outperform the parent enzyme in various PCR amplifications when added to the reaction mixture at room temperature. Also, these cold-sensitive mutants in an unbalanced mixture with 2% (v/v) Deep Vent DNA polymerase as a proofreader were found to amplify long DNA fragments efficiently by up to 9.8 kb.

The aim of this study was to introduce Ile707->Leu cold-sensitive mutation in codon 707 of the N-terminal deletional variant of *Taq* DNA polymerase in order to test the practical benefit from this cold-sensitive DNA polymerase.

Materials and Methods

Cloning and expression of Klentaq278 polymerase

Klentaq278 is an N-terminal deletional variant of *Taq* polymerase, which lacks the 278 amino acids from the N-terminus of the full-length (823 amino acids) wild-type enzyme. A fragment of 1668 bp containing the Klentaq278 gene was obtained by PCR using 15 pmol of each forward Klentaq278-F (5'- CAC GAA TTC GGC AGC CTC CTC CAC GAG TTC GGC CTT-3') and reverse primer Taq-R (5'-GTG GCG GCC GCA TCA CTC CTT GGC GGA GAG CCA GTC-3'), 200 ng genomic DNA from *Thermus aquaticus* YT-1 and Expand Long template PCR system (Roche). The PCR programme was under enzyme manufacturer's recommendations. The forward primer contained the recognition sequence of EcoRI (underlined) and part of the sequence of the *Taq* polymerase gene starting from codon 278, while the reverse primer contained the 3' end of the gene together with the stop codon (TGA) and recognition sequence of NotI (underlined). The amplification fragment was digested with *Eco*RI and *Not*I, purified from agarose gel with Qiaex II Gel Extraction Kit (Qiagen) and ligated into expression vector pGex-6P-1 (Amersham), previously digested with the same restriction enzymes. Correct plasmid constructions were identified by restriction digestion of plasmid minipreps and confirmed by induction of 2 ml cultures of *E.coli*BL21/pGex-6P-1/Klentaq278 with 1 mM IPTG for 2 hours. Klentaq278 was expressed under a *tac* promoter, as a fusion protein with glutathione S-transferase (GST) from *Schistosoma japonicum* [6]. The presence/absence of the 90 kDa fusion protein from different colonies was monitored by 10% SDS-PAGE.

Site-directed mutagenesis of Klentaq278

The nucleotide substitution ATT->CTT in codon 707 of the native *Taq* DNA polymerase gene was introduced into the Klentaq278 gene by the method of overlap mutant primers [**7**]. The sequences of the mutant primers used were M 707-F (5'-GCC TGG CTT GAG AAG ACC CTG-3') and M707-R (5'-CTT CTC AAG CCA GGC CCG CAC-3'). The presence of the inserted mutation in the PCR-generated mutant fragment was confirmed by sequencing (BigDye Terminator v1.1 cycle sequencing kit, Applied Biosystems). The mutant Klentaq278(Ile707Leu) gene was cloned in pGex-6P-1 (Amersham) and the nucleotide sequence of the mutant plasmids was verified by sequencing.

Purification of Klentaq278 and Klentaq278(Ile707Leu)

Large scale purification was done from 1l induced cultures in 2 × YTA medium (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) supplemented with 100 μg/ml ampicilin, under optimal conditions (t_{growth} = 30°C;

A_{600nm}=1; 0.1 mM IPTG for 4 hours). Bacterial cells were harvested by centrifugation (8500 rpm/5 min), resuspended in 50 ml of 1 × PBS (140 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and lysed by sonication (4 × 30 sec) in an ice bath. The fusion protein was purified to homogeneity by affinity chromatography on Glutathione Sepharose 4 Fast Flow (Amersham). The cleared supernatant was loaded onto a column equilibrated with 1 × PBS and the fusion protein was eluted with $1V_{bed}$ of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). GST protein was removed from the fusion protein by PreScission protease (Amersham) cleavage in a protease buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM Dithiotreitol, pH 7.5) and the digestion mixture was loaded onto a new Glutathione Sepharose 4 Fast Flow column. The eluate contained the pure enzyme. The enzyme was dialyzed against a storage buffer (20 mM Tris-HCl, 50 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 0.5% (v/v) TritonX-100, 0.5% (v/v) Tween 20, 50% (v/v) glycerol, pH 8.55) and stored at -20°C. The resulting Klentaq278 and Klentaq278(Ile707Leu) polymerases had 8 amino acids coded by the vector (N-Gly-Pro-Leu-Gly-Ser-Pro-Glu-Phe), covalently attached to the N-terminus. The thermostable polymerases had a specific activity of 20,000 U/mg.

DNA polymerase activity assay

DNA polymerase activity of the purified recombinant enzymes was assayed by incubation of a range of enzyme dilutions with 5 μg activated calf thymus DNA (Sigma) in a PCR buffer (50 mM Tris-HCl, 2.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 0.1% Tween 20, pH 9.2), in the presence of 0.2 mM d(A, T, G)TP, 0.1 mM dCTP and 1 μCi [1,2,5-^{3}H] dCTP (Amersham) at 75°C for 30 min. Aliquots of 5 μl of the samples were dotted on Whatman GF/C glass-fibre filters (Whatman International, Ltd) followed by an acid precipitation assay [8]. The whole and incorporated radioactivity was measured in a TriathlerTM multilabel tester (Hidex). Polymerase units were determined according to the standard definition of polymerase units (incorporation of 10 nmol of nucleotides in 30 min at 75°C). The Klentaq278 and Klentaq278(Ile707Leu) polymerases had a working concentration of 2 U/μl and 1U/μl, respectively.

Polymerase activity at room temperature and thermostability test

DNA polymerase activity at room temperature was assayed by a standard DNA polymerase activity assay at 25°C and 75°C and the activity at room temperature was calculated as a fraction of the polymerase activity at 75°C. The thermostability of the polymerases was assayed by incubation of 2U of polymerase in 20 μl of PCR buffer for 1 hour at 95°C and the residual activity was determined by a DNA polymerase activity assay.

DNA primers

The sequences of DNA primers used for amplification of various DNA fragments with Klentaq278 and Klentaq278(Ile707Leu) polymerases are given in Table 1 and the primer combinations are given in Table 2. For amplification of the 1.8 kb fragment from plasmid DNA (pGex-6P-1/Taq construct), primers for cloning the Klentaq278 gene were used, while for amplification of the 2.5 kb fragment, primer Taq-F (5'-CAC GAA TTC ATG AGG GGG ATG CTG CCC CTC TTT GA-3') in combination with primer Taq-R was used. Primers were purchased from MWG (Eurofins MWG GmbH, Anzinger Strasse 7a, Ebersberg, Germany).

Table 1 – Табела 1

Primer sequences used for amplification of DNA fragments with Klentaq278 and Klentaq278(Ile707Leu) polymerase
Секвенци на прајмерите кои се користени за амплификација на ДНК фрагменти со Klentaq278 и Klentaq278(Ile707Leu) полимеразите

Primer	*Gene*	*Sequence*
beta gl-1-F	human β-globin gene	5'-TGAGGAGAAGTCTGCCGTT - 3'
beta gl-6-R		5'-GAGCACTTTCTTGCCATGAG - 3'
beta gl-16-F		5'-GCAGGTTGGTATCAAGGTT - 3'
beta gl-45-R		5'- AAATTGGACAGCAAGAAAGC- 3'
beta gl-108-F		5'- GCCAAGGACAGGTACGGCTGTCATC- 3'
beta gl-109-R		5'-CCCTTCCTATGACATGAACTTAACCAT- 3'
beta gl-229-F		5'-ATACAATGTATCATGCCTCTTTG CACC-3'
beta gl-230-R		5'- GTATTTTCCCAAGGTTTGAACTAGCTC- 3'
FVIII-Ex14.3-F	Human factor VIII	5'-TTCATCAGACAATTTGGCAG -3'
FVIII-Ex14.4-R		5'-GATGTAAAACGGATCACGAG -3'
FVIII-Ex14.6-R		5'-TCCAGGAAGACTATTTACACT-3'
FVIII-Ex14.8-R		5'-CAACTATCCACTCCAACCTGA -3'

Table 2 – Табела 2

Primer combinations and size of resulting DNA fragments
Комбинација на прајмери и големина на амплифицираните ДНК фрагменти

Forvard primer	*Reverse primer*	*Product size (bp)*
beta gl-1-F	beta gl-6-R	317
beta gl-229-F	beta gl-230-R	631
beta gl-108-F	beta gl-109-R	712
beta gl-16-F	beta gl-45-R	1360
beta gl-1-F	beta gl-230-R	1677
FVIII-Ex14.3-F	FVIII-Ex14.4-R	1043
FVIII-Ex14.3-F	FVIII-Ex14.6-R	1848
FVIII-Ex14.3-F	FVIII-Ex14.8-R	2713

PCR assay and incubation conditions

The PCR mixture (50 μl) contained PCR buffer (given above), 3.5 mM $MgCl_2$, 200 μM dNTP, 20 pmol of forward and reverse primers and 100 ng human DNA. DNA polymerases (2 U) were added to the PCR mixture either by cold start during PCR set up or by hot start at 80°C. The influence of Na, K and Mg ions on the amplification capacities of the tested polymerases was assayed by PCR performed with primers beta gl-229-F and beta gl-230-R (Table 1). NaCl and KCl were added to PCR mixture to the final concentrations of 0, 5, 10, 20, 40, 60, 80 and 100 mM. $MgCl_2$ was added to the PCR mixture to the final concentrations of 0, 1, 2, 5, 10, 20 and 30 mM, not counting the Mg^{2+} present in the reaction buffer. The PCR programme consisted of initial denaturation at 94°C for 2 min, followed by 35 amplification cycles of denaturation at 94°C for 30s, primer aniling at 56°C for 30s and DNA extension at 72°C for 1–4 min depending on fragment length. Incubation was carried out in a model 2720 thermal cycler (Applied Biosystems). The products of PCR amplifications were analyzed by agarose gel electrophoresis on 1–1.5% gels in 1 × TBE buffer. All agarose gels were stained with ethidium bromide, visualized under UV light and photographed.

Results

The thermostability of Klentaq278(Ile707Leu) polymerase compared to Klentaq278 was in the range of the previously reported thermostability of the cold-sensitive mutant polymerases [5]. The polymerase activity of the mutant polymerase at room temperature (25°C) was 0.7% of the activity at 75°C. Compared to this, Klentaq278 had shown 8.6% of the optimal temperature activity at room temperature. The influence of Na, K and Mg ions on the amplification capacities of the Klentaq278 and mutant polymerase is shown in Table 3. The Mg tolerance of Klentaq278(Ile707Leu) is slightly lower than that of Klentaq278, while the tolerance of K and Na ions are 10 and 20 times less, respectively. When we compared the PCR amplification product in four multiplex reactions routinely used in our laboratory, in cases where both enzymes were added by cold and hot start, we noticed that mutant polymerase has a slightly better performance in cold start as compared to Klentaq278 polymerase (data not shown). However, in one multiplex reaction we noticed that mutant polymerase cannot amplify two fragments of 349 and 375 bp that Klentaq278 polymerase readily amplifies, regardless of whether the enzyme is added to the reaction mixture by hot or cold start. Then we tested the two DNA polymerases for the amplification of various length DNA fragments from the human β-globin gene which, in our experience, is a relatively easy region for amplification (Figure 1). Klentaq278 polymerase amplified all the fragment lengths, but the performance of this enzyme clearly benefited from a hot start especially for fragments over 1 kb. On the other hand, Klentaq278(Ile707Leu) polymerase had a limited performance, and could not amplify fragments of 1360 bp and 1677 bp either by cold or hot start. When we tested the amplification capacities of Klentaq278(Ile707Leu) polymerase with plasmid DNA used as a template, we noticed that 4 U of the enzyme cannot amplify fragments of 1800 bp and 2500 bp that 2 U of Klentaq278 amplified with a high yield. The same result was obtained even when 6 U of the mutant polymerase were used (Figure 2). Mutant polymerase was also tested for the amplification of DNA fragments of various length (from 1043 bp to 2713 bp) in a mixture with recombinant DNA polymerase *Tne* from *Thermotoga neapolitana* DSM 5068 (unpublished results) as a proofreader. Fragments of 1040 bp and 1861 bp were not amplified by Klentaq278(Ile707Leu) polymerase, but were faintly amplified with *Tne* polymerase and polymerase mixtures of Klentaq278(Ile707Leu)/*Tne* in the ratio 1 : 1 (data not shown). These results were the opposite of the results obtained with Klentaq278/*Tne* mixtures where the highest yield of the amplification products were obtained with polymerase mixtures in ratio 2 : 1, 10 : 1 and 20 : 1. The PCR amplifications of 2713 bp fragment with separate polymerases Klentaq278, Klentaq278(Ile707Leu), *Tne* and polymerase mixtures of Klentaq278/*Tne* and Klentaq278(Ile707Leu)/*Tne* are shown in Figure 3. Neither single polymerases or Klentaq278(Ile707Leu)/*Tne* mixtures in the tested ratios were able to amplify the DNA fragment. On the other

hand, Klentaq278/*Tne* mixtures in all tested ratios amplified the 2713 bp fragment successfully and with high yield.

Table 3 – Табела 3

Effect of different concentrations of Na^+, K^+ and Mg^{2+} ions on amplification capacities of Klentaq278 and Klentaq278(Ile707Leu) thermostabile DNA polymerases*
Ефект на различните концентрации на Na^+, K^+ и Mg^{2+} јони на амлификационите способности на Klentaq278 и Klentaq278(Ile707Leu) термостабилни ДНК полимерази*

	mM	*Klentaq278*	*Klentaq278 (Ile707Leu)*
NaCl	0	+	+
	5	+	-
	10	+	-
	20	+	-
	40	-	-
	60	-	-
	80	-	-
	100	-	-
KCl	0	+	+
	5	+	+
	10	+	+/-
	20	+	-
	40	-	-
	60	-	-
	80	-	-
	100	-	-
$MgCl_2$	3,5	+	+
	4,5	+	+
	5,5	+	+
	8,5	+	+/-
	13,5	+/-	-
	23,5	-	-
	33,5	-	-

*PCR amplification of 631 bp fragment of human β-globin gene. The results were scored as follows: (+) strong intensity of PCR product on agarose gel; (±) weak intensity of PCR product on agarose gel; (-) no visible PCR product on agarose gel.

* PCR амплификација на фрагмент од 631 bp од хуманиот β-глобин ген. Резултатите од амплификацијата се оценуваа по следниот клуч: (+) јак интензитет на PCR продуктот; (±) слаб интензитет на PCR продуктот; (-) отсуство на видлив PCR продукт на агарозен гел.

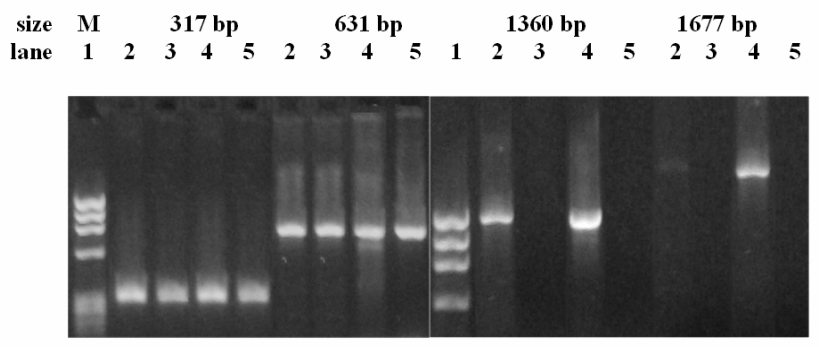

Figure 1 – PCR amplifications of human β-globin gene fragments with Klentaq278 and Klentaq278(Ile707Leu) polymerases, respectively (2 and 4 – with cold start; 3 and 5 – with hot start). Lane 1 – marker IX (Amersham)
Слика 1 – PCR амплификација на фрагменти од хуманиот β-глобин ген со Klentaq278 и Klentaq278(Ile707Leu) полимеразите, соодветно (2 и 4 – со ладен старт; 3 и 5 со топол старт). 1 – маркер IX (Amersham)

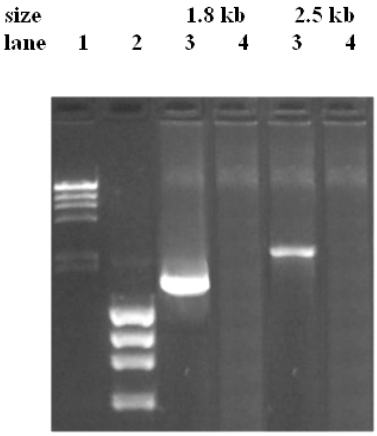

Figure 2 – PCR amplification of 1.8 and 2.5 kb plasmid DNA with Klentaq278 polymerase (lane 3) and Klentaq278(Ile707Leu) polymerase (lane 4). Target product size (kb) is indicated above each lane. 1 – λ/HindIII digestion; 2 – marker IX (Amersham)
Слика 2 – PCR амплификација на 1.8 и 2.5 kb плазмидска ДНК со Klentaq278 полимераза (ред број 3) и Klentaq278(Ile707Leu) полимеразата (ред број 4). Големината на продуктот на PCR амплификацијата е дадена во килобази (kb) над секој ред. 1 – λ/HindIII дигестија; 2 – маркер IX (Amersham)

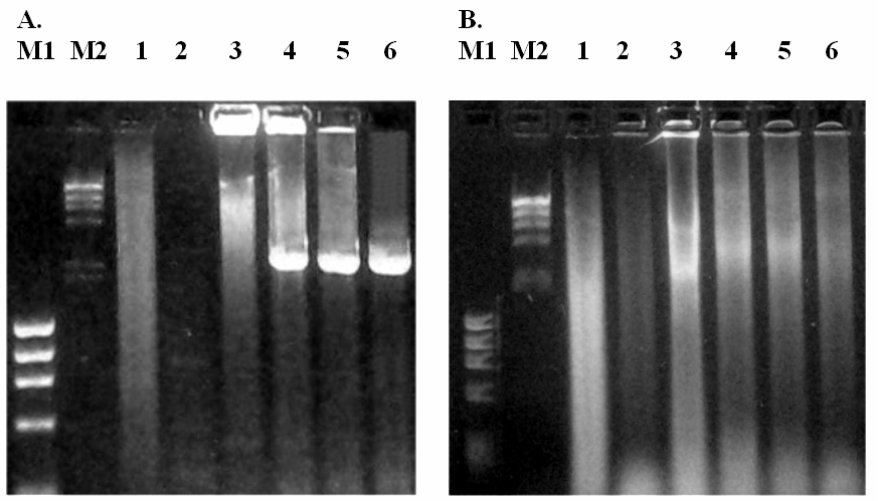

*Figure 3 – PCR amplification of 2713 bp fragment of exon 14 of human factor VIII gene by using mixtures of Klentaq278 (**A**) or Klentaq278(Ile707Leu) (**B**) with Tne polymerase in ratio 1 : 1 (lane 4), 2 : 1 (lane 5) and 10 : 1 (lane 6). PCR amplification with: AmpliTaq Gold (lane 1), Klentaq278 (lane A-2), Klentaq278(Ile707Leu) and Tne polymerase (A-3 and B-3). M1-marker IX (Amersham); M2- λ/HindIII digestion*
*Слика 3 – PCR амплификација на 2713 bp фрагмент од егзон 14 на хуманиот ген за фактор VIII со користење на смеси од Klentaq278 (**A** или Klentaq278(Ile707Leu) (**B**) со Tne полимеразата во однос 1 : 1 (4), 2 : 1 (5 и 10 : 1 (6). Како контрола, PCR амплификации со AmpliTaq Gold (1), Klentaq278 (A-2), Klentaq278(Ile707Leu) (B-2) и Tne полимеразата (A-3 и B-3). M1-маркер IX (Amersham); M2- λ/HindIII дигестија*

Discussion

In this work, we studied the effect of Ile(707) to Leu substitution on the overall characteristics of the Klentaq278 polymerase. This substitution has no significant effect on the thermostability of the enzyme, while it has an effect on the reduction of the polymerase activity at room temperature. Also this substitution greatly reduces the polymerase tolerance of monovalent ions (K and Na). The most significant effect of this substitution was the reduction of the amplification capacity of the polymerase. When we attempted to amplify DNA fragments of over 700 bp from human genomic DNA, we could not obtain a PCR product with the mutant polymerase. The presented results from Kermekchiev *et al*. [5] only showed PCR amplification with single polymerases for up to 323 bp. On the other hand, they stated that a mixture of cold-sensitive mutant with Ile(707) to Leu substitution and Deep Vent DNA polymerase as a proofreader

can, efficiently and without a hot start, amplify long DNA fragments up to 9.8 kb. Although we did not use the same polymerase as a proofreader, the comparison of PCR performance of Klentaq278/*Tne* vs Klentaq278(Ile707Leu)/*Tne* mixtures clearly showed that the processivity of the mutant polymerase is greatly reduced. Klentaq278/*Tne* polymerase mixture in ratio 10:1 was able to amplify a 7.72 kb fragment (unpublished results), while the same ratio of polymerase mixture of Klentaq278(Ile707Leu)/*Tne* failed to amplify a 1.04 kb fragment.

The most probable explanation of these differences is the use of betaine (N,N,N-trimethylglycine) in almost all PCR with mutant polymerases and their mixtures with Deep Vent DNA polymerase by Kermekchiev *et al*. [5]. The proposed mechanism of betaine action is that it suppresses DNA polymerases pauses and stops during synthesis and therefore improves PCR yield and specificity. The use of betaine and greatly prolonged extension times in PCR might compensate for the reduction of the amplification capacity of the Klentaq278(Ile707Leu) DNA polymerase.

However, the mutation Ile707Leu in Klentaq278 DNA polymerase clearly has an impact on the overall processivity of the enzyme. According to a model proposed by Barnes [9], the reduced processivity of the Klentaq DNA polymerase is due to poorer binding of Klentaq to the primer/template, because of the loss of protein domain necessary for normal processivity and therefore an increased likelihood of dissociation from the DNA template. This reduced processivity is accompanied by increased fidelity, because Klentaq enzyme has greater discrimination of misincorporated nucleotides than wild type *Taq* DNA polymerase [9]. According to Kermekchiev [5], Klentaq278(Ile707Leu) DNA polymerase has a reduced error rate and therefore increased fidelity by 2–3 times compared to Klentaq278 DNA polymerase. By this logic, mutation Ile707Leu in Klentaq278 DNA polymerase reduces the processivity of the enzyme which is in concordance with our findings.

In conclusion, Klentaq278(Ile707Leu) DNA polymerase has limited application in PCR, and can be used only for amplification of DNA fragments of less than 1 kb.

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REFERENCES

1. Saiki RK., Gelfand DH., Stoffel S., Scharf SJ., Higuchi R., Horn GT., Mullis KB. and Erlich HA. (1988): Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science;* 239: 487–491.

2. Hebert B., Bergeron J., Potworowski EF. And Tijssen P. (1993): Increased PCR sensitivity by using paraffin wax as a reaction mix overlay. *Mol Cell Probes;* 7: 249–252.

3. Scalice ER., Sharkey DJ. And Daiss JL. (1994): Monoclonal antibodies prepared against the DNA polymerase from *Thermus aquaticus* are potent inhibitors of enzyme activity. *J Immunol Methods;* 172: 147–163.

4. Moretti T., Koons B. and Budowle B. (1998): Enhancement of PCR amplifycation yield and specificity using AmpliTaq Gold DNA Polymerase. *Biotechniques;* 25: 716–722.

5. Kermekchiev MB., Tzekov A. and Barnes WM. (2003): Cold-sensitive mutants of *Taq* DNA polymerase provide a hot start for PCR. *Nucleic Acids Res*; 31(21): 6139–6147.

6. Smith DB. And Johnson KS. (1988): Single-step purification of polypeptides expressed in *Escherichia coli* as a fusions with glutathione S-transferase. *Gene;* 67(1): 31–40.

7. Ho SN., Hunt HD., Horton RM., Pullen JK. And Pease LR. (1989): Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene;* **77** (1): 51–59.

8. Sambrook J., Fritisch EF. And Maniatis T. (1989): Molecular cloning: A Labaratory Manual, 2nd edn., Cold Spring Harbor, NY.

9. Barnes WM. (1992): The fidelity of *Taq* polymerase catalyzing PCR is improved by an N-terminal deletion. *Gene;* 112: 29–35.

Резиме

ЗАМЕНАТА НА Ile(707) ЗА Leu ВО KLENTAQ ДНК ПОЛИМЕРАЗАТА ЈА НАМАЛУВА АМПЛИФИКАЦИОНАТА СПОСОБНОСТ НА ЕНЗИМОТ

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Апстракт: Високиот принос и специфичноста на PCR амплификациите е условена од активноста на ДНК полимеразите на собна температура. Еден од начините за спречување на оваа несакана активност е генетската модификација на ДНК полимеразите. За *Taq* ДНК полимеразата, мутации во генот (Glu626Lys, Trp706Arg, Ile707Leu и Glu708Asp) внесени поединечно или во одредени комбинации, придонесуваат за значително намалување на ензимската активност на собна температура. Целта на ова истражу-

вање беше да се оцени корисноста на ладно-сензитивната замена Ile707Leu во N-терминалната делециона варијанта на *Taq* ДНК полимеразата за примена во PCR. Замената Ile(707) во Leu е внесена во Klentaq278 ДНК полимеразата со насочена мутагенеза. Нормалната и мутантната ДНК полимераза се експримирани со помош на *tac* промотер и прочистени до хомогеност. Мутантната полимераза покажа намалена полимеразна активност на собна температура за 12 пати и незначителна промена во термостабилноста, споредена со Klentaq278 ДНК полимеразата. Најважниот ефект на аминокиселинската замена е намалувањето на амплификационата способност на полимеразата. Мутантната полимераза не може да амплифицира фрагменти поголеми од 1 kb. Замената Ile707Leu во Klentaq278 ДНК полимеразата ја намалува вкупната процесивност на ензимот и ја ограничува нејзината примена.

Клучни зборови: Klentaq278, насочена мутагенеза, топол старт, сензитивна на ладно ДНК полимераза, PCR.

Running Title: Reduction of Klentaq polymerase efficiency by Ile(707) to Leu substitution.

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