

INFLUENCE OF SALTS AND PCR INHIBITORS ON THE AMPLIFICATION CAPACITY OF THREE THERMOSTABLE DNA POLYMERASES

Katarina Davalieva, Georgi D. Efremov

Research Centre for Genetic Engineering and Biotechnology, Macedonian Academy of Sciences and Arts,
Krstе Misirkov 2, MK-1000 Skopje, Republic of Macedonia
gde@manu.edu.mk

The full potential of PCR as a rapid DNA detection method, is limited by inhibition of thermostable DNA polymerases by components in biological samples and substances used for purification of template DNA. We compared the inhibition effect of blood, phenol and various ions on *Taq*, *Tth* and *Tne* thermostable DNA polymerases prepared by us. The amplification capacity of DNA polymerases was tested on the amplification of a 631 bp fragment of a β -globin gene from a 100 ng DNA template under the optimal PCR buffer. Blood above 1%(v/v) and phenol above 0.1%(v/v) inhibited *Taq*, while *Tne* and *Tth* tolerated 10 to 30 times more. The inhibitory effect of ions is lowest for the *Tth* DNA polymerase, followed by *Taq* and *Tne* DNA polymerase. We conclude that the PCR inhibiting effect of some substances to *Taq* DNA polymerase can be eliminated by the use of a more resistant thermostable DNA polymerase, such as *Tth* or *Tne* DNA polymerases.

Key words: thermostable DNA polymerase; PCR; PCR-inhibitors

ВЛИЈАНИЕ НА СОЛИ И РСР-ИНХИБИТОРИ ВРЗ АМПЛИФИКАЦИОНАТА СПОСОБНОСТ НА ТРИ ТЕРМОСТАБИЛНИ ПОЛИМЕРАЗИ НА ДНК

Целосниот потенцијал на РСР како брз ДНК-базиран метод е лимитиран од инхибицијата на термостабилните ДНК полимерази со компоненти од биолошките примероци и супстанции кои се користат за прочистување на ДНК. Целта на ова истражување беше споредбата на инхибиторниот ефект на крв, фенол и различни јони врз термостабилните *Taq*, *Tth* и *Tne* ДНК полимерази. Амплификационата способност на ДНК полимеразите беше тестирана врз амплификација на фрагмент од 631 bp од β -глобинскиот ген од 100 ng ДНК, со користење на оптимален РСР-пуфер. Крв над 1%(v/v) и фенол над 0,1%(v/v) ја инхибираат *Taq* ДНК полимеразата, додека *Tne* и *Tth* толерираат 10 до 30 пати повеќе, соодветно. Инхибиторниот ефект на јоните е најмал кај *Tth* ДНК полимеразата, по која следат *Taq* и *Tne* ДНК полимеразите. РСР инхибирачкиот ефект на некои супстанции врз *Taq* ДНК полимеразата може да се елиминира со користење на поотпорни термостабилни ДНК полимерази, како што се *Tth* и *Tne* ДНК полимеразите.

Клучни зборови: термостабилни ДНК полимерази; РСР; РСР-инхибитори

1. INTRODUCTION

Factors that inhibit the amplification of nucleic acids in the PCR are often present with the target DNAs. The inhibitors generally act at one or more of three essential points in the reaction in the following ways: they interfere with the cell lysis necessary for extraction of DNA, they interfere by nucleic acid degradation and they inhibit polymerase activity for the amplification of the target

DNA [1]. The usefulness of PCR detection methods is limited in part by the presence of substances that inhibit the PCR or reduce the amplification efficiency. The inhibition can be total or partial, and can be manifested as a complete reaction failure or as reduced sensitivity of detection. In some cases the inhibition can be the cause for false-negative reactions.

PCR inhibitors are commonly present in the insufficiently purified target DNA. A number of

components have been reported to be PCR inhibitors, like salts [2], heme in blood [3], phenolic compounds [4], etc.

The most important target site of PCR inhibiting substances are the thermostable DNA polymerases. *Taq* DNA polymerase is inhibited by numerous substances such as proteinases [5], phenol [6], detergents [7] and blood [3]. However, *Tth* polymerase is more resistant to phenol [6] and to aqueous and vitreous fluids than polymerases *Taq*, *Tli* and *Stoffel* fragment [8]. Commercially available thermostable polymerases under standard conditions (recommended by the manufacturer) are inhibited by complex biological samples in various ranges [9]. Determination of the inhibition range of some well defined complex biological samples that are known as PCR inhibitors on different thermostable polymerases under standard conditions, allows elimination of the PCR – inhibiting effect simply by choosing the appropriate thermostable polymerase.

In this study we investigated the inhibition effect of different concentrations of blood, phenol and certain ions on the DNA amplification capacity of the recombinant thermostable DNA polymerases *Taq*, *Tth* and *Tne*. While *Taq* and *Tth* are well described and widely used thermostable DNA polymerases, *Tne* DNA polymerase is relatively a new polymerase and its inhibitory range has not been reported yet. The aim of this study was to determine the range of inhibition of these polymerases under the same conditions by various inhibitors and salts in order to compare between them and with commercially available polymerases from the same bacterial sources.

2. EXPERIMENTAL

Preparation of DNA polymerases

Taq polymerase from *Thermus aquaticus* YM-1, *Tth* polymerase from *Thermus thermophilus* HB8 and *Tne* polymerase from *Thermotoga neapolitana* DSM 5068 were cloned in the expression vector pTTQ18 (Amersham) and expressed by induction with IPTG in *E. coli* BL21 [10]. Large scale purification was done from 1 l induced culture in the LB medium with 100 µg/ml ampicillin, according to a previously described method [11]. The enzyme fraction was dialyzed against the storage buffer (20 mM Tris-HCl, 50 mM (NH₄)₂SO₄, 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 optimal buffer for activity of DNA polymerases was determined from the PCR assay with two different buffers and with enzyme dilutions from 0.1 – 3 µl. Commercial buffer of AmpliTaq Gold (AppliedBiosystems) and Jump Start polymerase – Rb (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, pH 8,3) and KLA buffer (50 mM Tris-HCl, 2.5 mM MgCl₂, 16 mM (NH₄)₂SO₄, 0.1% Tween 20, pH 9,2) [12] were tested. All three recombinant polymerases showed significantly higher polymerase activity in KLA buffer. 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 KLA buffer in the presence of 0.2 mM d(A, T, G)TP, 0.1 mM dCTP and 1 µCi [1,2,5-³H] dCTP (Amersham) at 75°C for 30 min. Polymerase units were determined with the acid precipitation assay [13] according to the standard definition of polymerase units (incorporation of 10 nmol of nucleotides in 30 min at 72°C). Recombinant polymerases had the concentrations of 3 U/µl (*Taq*), 3.6 U/µl (*Tth*) and 2U/µl (*Tne*). Recombinant DNA polymerases were not purified to homogeneity (specific activity of *Taq* – 3300 U/mg; *Tth* – 6000 U/mg; *Tne* – 2,500 U/mg) but the enzymes were with adequate purity for the PCR application.

PCR-inhibitory samples

The blood sample used, was drawn from a healthy person into 10 ml blood collecting tube containing 0.12 ml of 15% EDTA (BD Vacutainer Systems, Plymouth, UK). The phenol used, was equilibrated at pH 8.0 (Ultrapure MB Grade, USB, Cleveland, OH, USA). Different blood concentrations (0, 0.001, 0.005, 0.01, 0.05, 0.1, 1.0, 10, 15, 20, 25 and 30 % (v/v)) and different phenol concentrations (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10, 15, 20 and 30 % (v/v)) were added to the PCR mixtures of three polymerases.

Ion solutions

Sterile solutions of 500 mM NaCl, 500 mM KCl and 100 mM MgCl₂ were prepared. Na⁺ and K⁺ ions were added to the PCR mixture to the final concentrations of 0, 5, 10, 20, 40, 60, 80, 100, 150 and 200 mM. Mg²⁺ ions were 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.

PCR assay and incubation conditions

The PCR assay was performed with primers #229 (5'ATACAAATGTATCATGC CTCTTTGC-ACC-3') complementary to nucleotides 1085->1112 and #230 (5'-GTATTTT CCCAAGGTTT-GAACTAGCTC-3') complementary to nucleotides 1716->1690 of the human β -globin gene. The PCR mixture for each PCR amplification contained the KLA buffer (50 mM Tris-HCl, 2.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 0.1% Tween 20, pH 9.2), 3.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol of forward and reverse primers, 100 ng human DNA, 1.5 U of the thermostable DNA polymerase and the tested inhibitor or ion solution with defined concentration. The template DNA was isolated from the whole blood using the QIAamp Blood Mini Kit (Qiagen) and was with good purity ($A_{260}/A_{280} = 1.8$). The volume of the PCR mixture was 50 μ l. Positive controls (standard PCR mixture without inhibitor or ion solution) and negative controls (standard PCR mixture without DNA, inhibitor or ion solution) were also carried out. The PCR assays were carried out in triplicate. The reaction mixtures were subjected to the initial denaturation at 94°C for 2 min, followed by 33 amplification cycles consisting of denaturation at 94°C for 20 s, primer aniling at 56°C for 30 s and DNA extension at 72°C for 2 min. Finally, the mixtures were maintained at 72°C for 7 min for the final extension of the DNA. Incubation was carried out in a model 2720 thermal cycler (Applied Biosystems). The 631-bp PCR product was visualized by 1.5% agarose gel electrophoresis containing ethidium bromide. The results were scored when

minimum two out of three assays had the same intensity, as follows: (+) strong intensity of the PCR product on agarose gel; (\pm) weak intensity of the PCR product on agarose gel; (-) no visible PCR product on agarose gel.

3. RESULTS AND DISCUSSION

Effect of blood on DNA polymerases

Tth polymerase efficiently amplified the selected fragment for all tested concentrations of blood (up to 30% (v/v)), whereas concentrations of more than 1%(v/v) and 10%(v/v) inhibited *Taq* and *Tne* polymerases (Table 1 and Figure 1). *Taq* polymerase was the most susceptible to inhibition by blood, compared to other tested polymerases. Our results for *Taq* polymerase are in concordance with the previously published results of Mercier et al. [14] that *Taq* polymerase can amplify in the presence of 1–2 %(v/v) of blood, although different buffers, $MgCl_2$ concentrations and PCR amplification conditions were used in these studies. The results for *Tth* polymerase outranked the previously reported 8 %(v/v) of blood concentrations by Panaccio and Lew [15] in which *Tth* polymerase can amplify target DNA. On the other hand, our results on *Tth* inhibition with blood are more comparable with the previously reported 20 % by Al-Soud and P. Rådström [9], although they used different buffer and 100-times less DNA for PCR amplification than in our assay. A possible explanation for these different ranges of blood inhibition by *Tth* polymerase may be the use of different template DNA for amplification, different $MgCl_2$ concentrations, different DNA concentrations and PCR buffers which are factors that greatly affect the activity of DNA polymerases.

Table 1

Inhibitory effect of different concentrations of blood in PCR mixture on the amplification capacities of Taq, Tth and Tne thermostable DNA polymerases

DNA polymerase	PCR results with blood concentration % (v/v) of:											
	0	0.001	0.005	0.01	0.05	0.1	1	10	15	20	25	30
<i>Taq</i>	+	+	+	+	+	+	+/-	-	-	-	-	-
<i>Tth</i>	+	+	+	+	+	+	+	+	+	+	+	+/-
<i>Tne</i>	+	+	+	+	+	+	+	+/-	-	-	-	-

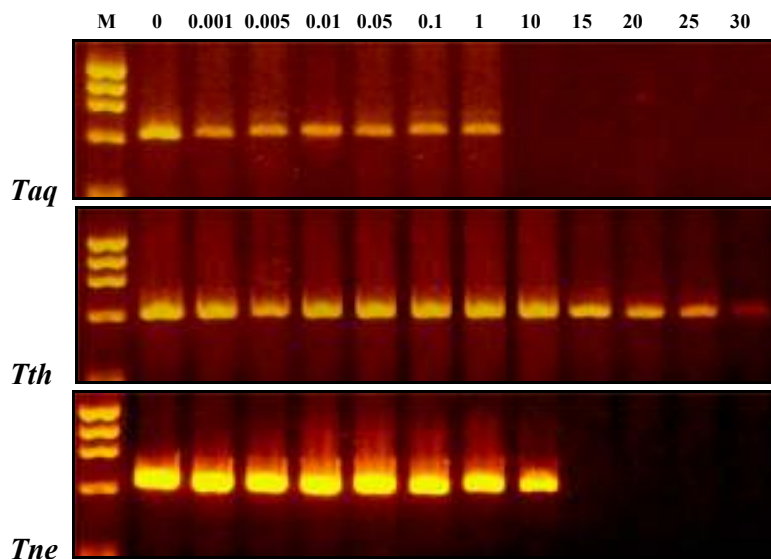


Fig. 1. PCR amplification of a 631 bp fragment of the human β -globin gene by three thermostable DNA polymerases in the presence of different concentration of blood % (v/v) in the PCR mixture. M-marker IX (Amersham)

Effect of phenol on DNA polymerases

Taq polymerase amplified the selected fragment in the presence of up to 0.1% (v/v) phenol, while concentrations of 0.5% (v/v) were totally inhibitory (Table 2). *Tth* and *Tne* polymerases had greater phenol tolerance and could amplify the selected fragment in the presence of 1% (v/v) phenol. This phenol tolerance of *Tth* polymerase is less than the reported tolerance of 2–5% (v/v) [6],

and is probably due to different reaction buffers and conditions used. However, *Tth* and *Tne* polymerases showed 10 times greater resistance to phenol than *Taq* polymerase. Since phenol inhibits the PCR reaction by denaturing the DNA polymerase, one explanation of the different resistance of the tested polymerases is that *Tth* and *Tne* polymerases have protein structure more resistant to denaturation than *Taq* polymerase.

Table 2

Inhibitory effect of different phenol concentrations on the amplification capacities of Taq, Tth and Tne thermostable DNA polymerases

DNA polymerase	PCR results with phenol concentration % (v/v) of:													
	0	0.001	0.005	0.01	0.05	0.1	0.5	1	2	5	10	15	20	30
<i>Taq</i>	+	+	+	+	+	+	–	–	–	–	–	–	–	–
<i>Tth</i>	+	+	+	+	+	+	+	+	–	–	–	–	–	–
<i>Tne</i>	+	+	+	+	+	+	+	+	–	–	–	–	–	–

Effect of Na⁺, K⁺ and Mg²⁺ ions on the DNA polymerases

The inhibitory effect of Na⁺ and K⁺ ions were present over 100 mM for *Tth* and 20 mM for *Tne* (Table 3). For *Taq* polymerase, Na⁺ ions were

more inhibitory (over 40 mM) than K⁺ ions (over 80 mM). The inhibitory effect of divalent ions (Mg²⁺) was more pronounced than that of monovalent ions (Na⁺ and K⁺). MgCl₂ showed the inhibitory effect when present in concentrations more than 13.5 mM for *Taq*, 23.5 mM for *Tth* and 8.5

mM for *Tne* (Table 3). *Tth* polymerase had the greatest tolerance of ions, followed by that of *Taq* and of *Tne*. The range of ion tolerance of our recombinant DNA polymerases – *Taq* and *Tth* is comparable with the previously reported tolerance of commercially available *Taq* and *Tth* polymerases by Al-Soud and Rådström [9].

Table 3

Effect of different concentrations of Na⁺, K⁺ and Mg²⁺ ions on the amplification capacities of Taq, Tth and Tne thermostable DNA polymerases

Ion concentration (mM)	PCR result with concentration (mM) of:			
	Taq	Tth	Tne	
NaCl	0	+	+	+
	5	+	+	+
	10	+	+	+
	20	+	+	+
	40	+/-	+	-
	60	-	+	-
	80	-	+	-
	100	-	+/-	-
KCl	0	+	+	+
	5	+	+	+
	10	+	+	+
	20	+	+	+
	40	+	+	-
	60	+	+	-
	80	+/-	+	-
	100	-	+	-
MgCl ₂	150	-	-	-
	3.5	+	+	+
	4.5	+	+	+
	5.5	+	+	+
	8.5	+	+	+/-
	13.5	+/-	+	-
	23.5	-	+/-	-
	33.5	-	-	-

4. CONCLUSION

Taq polymerase, as the most used thermostable DNA polymerase in PCR at present, can be inhibited by different biological or organical substances and salts. These inhibitions can be reduced or eliminated by the use of another, more resistant

thermostable DNA polymerase. With the selection of an appropriate polymerase, it is possible to amplify DNA more efficiently in the presence of inhibitors without the need for extensive sample processing prior to PCR. This approach can also be very cost effective, since it allows partially purified DNA, obtained by rapid and inexpensive procedures to be suitable for PCR amplification. Such one DNA polymerase is *Tth* polymerase, which is commercially available from different manufacturers and has superior tolerance to blood, phenol and ions than *Taq* polymerase. We confirm these findings by comparing the inhibition and ions tolerance ranges of our recombinant *Tth* and *Taq* DNA polymerases under our conditions. But we found that *Tne* polymerase also exhibits greater resistance to blood and phenol than *Taq* polymerase, although, less tolerance to salts. By our findings, the inhibition range of *Tne* DNA polymerase is for the first time described in this study. Recombinant *Tne* DNA polymerase is a useful addition to the family of known thermostable DNA polymerases.

Acknowledgments: This work was supported by the Funds for Science of the Macedonian Academy of Sciences and Arts (No.09-40/12), Skopje, Republic of Macedonia.

REFERENCES

- [1] I. G. Wilson, Inhibition and facilitation of nucleic acid amplification, *Applied and Environmental Microbiology*, **63** (10), 3741–3751 (1997).
- [2] N. Favre, W. Rudin, Salt-dependant performance variation of DNA polymerases in co-amplification PCR, *BioTechniques* **21**, 28–30 (1996).
- [3] A. Akane, K. Matsubara, H. Nakamura, S. Takahashi, K. Kimura, Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from blood strains, a major inhibitor of polymerase chain reaction (PCR) amplification, *J. Forensic Sci.* **39**, 362–372 (1994).
- [4] C. Young, R. L. Burghoff, L. G. Keim, V. Minak-Bernero, J. R. Lute, S. M. Hinton, Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soil, *Appl. Environ. Microbiol.* **59**, 1972–1974 (1993).
- [5] H. A. Powell, C. M. Gooding, S. D. Garrett, B. M. Lund, R. A. McKee, Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction, *Lett. Appl. Microbiol.* **18**, 59–61 (1994).
- [6] H. L. Katcher, I. Schwartz, A distinctive property of *Tth* DNA polymerase: enzymatic amplification in the presence of phenol, *BioTechniques* **16**, 84–92 (1994).
- [7] L. Rossen, P. Norskov, K. Holmstromet, O. F. Rasmussen, Inhibition of PCR by components of food samples, mi-

- crobial diagnostic assays and DNA-extraction solutions, *Int. J. Food. Microbiol.* **17**, 37–45 (1992).
- [8] D. L. Wiedbrauk, J. C. Werner, A. M. Drevon, Inhibition of PCR by aqueous and vitreous fluids, *J. Clin. Microbiol.* **33**, 2643–2646 (1995).
- [9] W.A. Al-Soud and P. Rådström, Capacity of Nine Thermostable DNA Polymerases to Mediate DNA Amplification in the Presence of PCR-Inhibiting Samples, *Applied and Environmental Microbiology*, **64** (10), 3748–3753 (1998).
- [10] K. Davalieva, *Cloning and expression of thermostable DNA polymerase genes and construction of modified forms with improved characteristics*, PhD thesis, 2009. Faculty of Technology and Metallurgy, University “St. Cyril and Methodius” in Skopje, Republic of Macedonia.
- [11] Grimm, E. and P. Arbuthnot, Rapid purification of recombinant *Taq* DNA polymerase by freezing and high temperature thawing of bacterial expression cultures. *Nucleic Acids Res.* **23** (21) 4518–4519 (1995).
- [12] Barnes W. M. PCR amplification of up to 35 kb DNA with high fidelity and high yield from λ bacteriophage templates. *PNAS*, **91**: 2216–2220 (1994).
- [13] J. Sambrook, E. E. Fritsch, T. Maniatis, *Molecular cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor, N.Y.: Cold Spring Harbor Lab. Press, 1989.
- [14] B. Mercier, C. Gaucher, O. Feugeas, C. Mazurier, Direct PCR from whole blood, without DNA extraction. *Nucleic Acids Res.*, **18** (19), 5908 (1990).
- [15] M. Panaccio, A. Lew, PCR based diagnosis in the presence of 8% (v/v) blood. *Nucleic Acids Res.*, **19** (5), 1151 (1991).