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Original Research Article

Identifying stability of polymerase in master mixes used in PCR and repair possibilities for the degraded reagents

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ABSTRACT

Introduction: The component of commercial available master mix most sensitive to unfavorable conditions is a polymerase. Available commercial polymerase chain reaction (PCR) master mixes are generally recommended for storage at −20°C, otherwise they may lose their activity.

Aim: The aim of the experiment was to verify if storing mixes in adverse and extreme conditions may influence the quality of a PCR product. In the second phase of the research, it was to indicate if inactive PCR reagents that have lost their activity, may recover their enzymatic properties.

Material and methods: Five different commercially available master mixes were incubated in unfavorable conditions. After the PCR, an electrophoresis was carried out and the obtained product was an evidence of a proper PCR reaction.

Results and discussion: Total degradation of mixes was caused by their incubation at room temperature for 28 days and incubation at 100°C for 60 minutes. Addition of polymerases to the degraded mixes (incubation at room temperature for 28 days) resulted in a regeneration of all of five mixes. In the case of polymerases incubated at 100°C for 60 minutes, regeneration was effective only in two of the five mixes.

Conclusions: Our research confirms that PCR master mix is characterized by high resistance to varied conditions as well as in some cases can be repaired after degradation.

1. Introduction

Polymerase was first isolated from *Escherichia coli* in the 1950s.[1] Since then, a number of different polymerases were isolated and they are used in special molecular biology techniques. One of the techniques is polymerase chain reaction (PCR), developed by Kary Mullis[2] and has become a fundamental technique in molecular biology.[3] It allows specific amplification of DNA

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fragment in the laboratory conditions. The reaction mixture includes primarily:

- DNA – which is a template to reproduce,
- two primers – short nucleotide sequences designating the amplified nucleic acid fragment,
- polymerase – an enzyme involved in the synthesis of newly synthesized DNA,
- deoxyribonucleotides triphosphates – the building blocks for the synthesis of the PCR product and a buffer with magnesium ions, which provides a suitable chemical environment.

These components (except DNA and primers) may be included in the so-called master mixes. Available commercial PCR master mixes are generally recommended for storage at −20°C, otherwise they might lose their activity. Component of the mix, the most sensitive to unfavorable conditions is the polymerase. Frequently used is Taq polymerase from bacteria *Thermus aquaticus* first isolated and characterized by Chien.[4] It is one of the best-characterized polymerases: the gene was isolated, cloned and characterized,[5–7] with a crystal structure,[8] active-site[9] and molecular diverstity presented.[10] This polymerase is a DNA-dependent deoxynucleotidyl transferase.[11] The thermostable enzyme enables the amplification reaction to be carried out at higher temperatures without enzyme inactivation.[12]

2. Aim

The aim of the experiment was to determine whether the storage of master mixes at different temperatures than those recommended by the manufacturer may affect the quality of the PCR. Furthermore, it was examined whether other extreme conditions (repeated freezing and thawing, UV, boiling or addition of alcohol) will affect performance of mix in the reaction. Also the aim of the experiment was to demonstrate if mixes used in PCR, which lost their activity under the influence of unfavorable external factors, may recover its enzymatic properties.

3. Material and methods

3.1. *Determination of mixes stability*

In the experiment five different commercially available and popular in sale master mixes (names coded) were tested. All mixes included Taq polymerase. In the first step control PCR were performed with mixes stored at −20°C (including an active enzyme).

DNA was isolated from human peripheral blood using a DNA isolation kit GeneJet Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific). Purity and quality of isolated nucleic acid was measured using a NanoDrop spectrophotometer. The PCR amplified fragment was 235 bp. Primers were designed by Primer3 program and had the following sequences: IF: ACAAGCCAAGCATTCAGGAC, IR: ATGCAGACGTTTTTGTGCAG. PCR conditions were as follows: 0.2 μL of the primers IF and IR, each in concentrations of 50 pmol/μL, ca. 150 ng of genomic DNA, 10 μL of test mix and H_2O ad 25 μL. The samples were amplified in Applied Biosystems thermocycler under following conditions: initial denaturation of DNA (95°C, in 3 minutes), denaturation (95°C in 30 s), annealing of primers (61°C in 30 s), elongation (72°C in 30 s), ending elongation (72°C in 10 minutes). After PCR, electrophoresis in 1.5% agarose gel was carried out with ethidium bromide and visualized on transilluminator. In all control samples a band of size 235 bp was obtained.

In order to check the thermal stability of the reagent five mixes were incubated for 12, 24, 48, 72 hours and 7, 13, 18, 19, 23 and 28 days at two temperatures: at 4°C (refrigerator) and at 22°C (room temperature). Incubation conditions were chosen in such a way as to determine the point to which the storage is secure. There were also tested extreme conditions as:

Table 1 – Results of the PCR mixes undergoing incubation.

Incubation conditions	Mix 1		Mix 2		Mix 3		Mix 4		Mix 5	
Control −20°C, 0 day	+		+		+		+		+	
Time	Mix 1		Mix 2		Mix 3		Mix 4		Mix 5	
	Temperature									
	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C
12 h	+	+	+	+	+	+	+	+	+	±
24 h	+	+	+	+	+	+	+	+	±	–
48 h	+	+	+	+	+	+	+	+	±	–
72 h	+	+	+	+	+	+	+	+	±	–
7 days	+	+	+	+	+	+	+	+	±	–
13 days	+	+	+	+	+	+	+	+	–	–
18 days	+	+	+	+	+	+	+	+	–	–
19 days	+	+	+	+	+	+	+	+	–	–
23 days	+	–	±	–	+	–	+	–	–	–
28 days	+	–	±	–	+	–	+	–	–	–

Comments: + standard PCR product; ± PCR product was present, but low quality; – no PCR product.

Table 2 – Results of PCR mixes undergoing incubation at extreme conditions.

Incubation conditions	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5
Control (−20°C, 0 day)	+	+	+	+	+
UV irradiation, 30 min	+	+	+	+	+
UV irradiation, 60 min	+	+	+	+	+
Addition of 96% alcohol	+	+	+	+	+
Multiple freezing (50×)	+	+	+	+	−
Multiple freezing (100×)	+	+	+	±	−
100°C, 30 min	±	±	±	±	±
100°C, 60 min	−	−	−	−	−

Comments: + standard PCR product; ± PCR product was present, but low quality; − no PCR product.

(1) multiple (approximately 50 and 100 times) freezing (−20°C) and thawing,
(2) incubation at 100°C for 30 and 60 minutes,
(3) UV irradiation for 30 and 60 minutes,
(4) addition of 96% alcohol (1:10).

After the incubation, PCR was carried out differing only in the test mix.

3.2. *Examination of mixes regeneration*

In the second part of experiment, mixes that lost their activity were used in the PCR according to previously established procedures, using the same reagents (primers, autoclaved water) and DNA. The difference was in the use of mixes that have been degraded, but after the addition of polymerase stored according to the producers' recommendations (−20°C). Polymerase was added to the mix in the amount of 1 U and 1 μL of buffer (recommended by the manufacturer) for 25 μL PCR mixture. Each polymerase used in experiment was produced by the same manufacturer as the mix.

After the PCR, an electrophoresis was carried out and the obtained product was an evidence of a proper PCR – an evidence of mix regeneration by the addition of the polymerase. In the cases of no product occurrence, it was concluded that the addition of the polymerase does not affect the repair of a degraded mix.

4. Results and discussion

The results of this study confirmed the results of earlier studies on the thermal stability of polymerases isolated from different bacterial species[1,12–14] in different temperature ranges. The mixes we analyzed did not lose their activity after 30 minutes of incubation at 100°C, but the product was lower than the control (mix held at −20°C). This is not surprising considering that *T. aquaticus* is a bacteria that lives in hot springs, and is an enzyme used in PCR, with temperatures from 50°C to 95°C.

Our examination has also shown that the test reagents for PCR (despite the recommendations of manufacturers who recommend storing at −20°C) are stable at room temperature for some time. At 4°C, some of test mixes did not lose its activity even after 28 days. We also noted that even prolonged incubation at room temperature (22°C) does not affect the catalytic activity of the enzyme for 72 hours. But after 7 days of

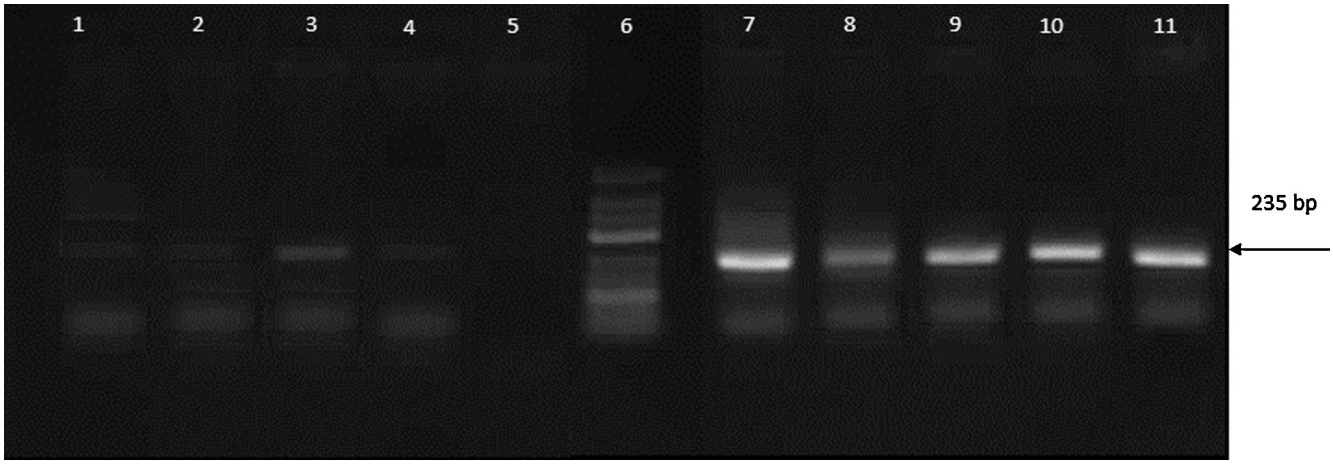

Fig. 1 – The electrophoregram of PCR reactions using mixes 1, 2, 3, 4, 5 incubated in room temperature (22°C) for 28 days and with polymerases stored in −20°C. Paths 1, 2, 3, 4, 5 – mixes 1, 2, 3, 4 and 5, respectively – without polymerase added. Path 7 – mix 1 and polymerase 1. Path 8 – mix 2 and polymerase 2. Path 9 – mix 3 and polymerase 3. Path 10 – mix 4 and polymerase 4. Path 11 – mix 5 and polymerase 5. Path 6 – DNA size marker.

incubation in 22°C some of the mixes had lost their functionality.

This experience, in addition to resistance to high temperature, also showed significant resistance to repeated freezing mixes, UV light or the addition of 10% alcohol. To summarize this part of the experience should be noted that it is relatively difficult to degrade PCR mix. Total degradation of mixes was caused by their incubation at room temperature for 28 days and incubation at 100°C for 60 minutes. PCR results obtained from the incubation mix at different temperatures at different times and other extreme conditions are shown in Tables 1 and 2.

In the second part of the experiment we indicated that some of the degraded mixes can be regenerated by addition of the polymerase. Addition of polymerases to the degraded mixes stored under conditions recommended by the manufacturer (−20°C) and in unfavorable conditions (incubation at room temperature for 28 days) resulted in a regeneration of all of five mixes. In the case of polymerases incubated at 100°C for 60 minutes, regeneration was effective only in two of the five cases. Probably high temperature treatment led to irreversible changes in the physico-chemical properties of mixes. In the cases of no product occurrence, it was concluded that the addition of the polymerase does not affect the repair of a degraded mix. An exemplary electrophoregram (Fig. 1) shows the results of PCR.

5. Conclusions

Our research confirms that PCR master mix is characterized by high resistance to varied conditions as well as in some cases can be repaired after degradation.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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