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EXPRESSION AND PURIFICATION OF DNA POLYMERASE FROM *THERMUS THERMOPHILUS*INE.*COLI* EXPRESSION SYSTEM

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ABSTRACT

Isolation of thermostable DNA polymerase from *Thermusaquaticus* was a significant stage in molecular biology and laboratory performance. After *Taq* DNA polymerase was discovered PCR method became widespread among laboratories around the world. *Taq* DNA polymerase was the first tool for fast and highly specific amplification of targeted nucleotide sequences. Thus, thermostable polymerases became vital for laboratory performance.

We managed to express and purify recombinant *Tth* DNA polymerase that has both polymerase and reverse transcriptase activities. At first we cultivated *Thermusthermophilus* strain HB8 and isolated genomic DNA. The gene was amplified and cloned into expression plasmid vector pET-28c(+) under T7 promoter. *E.coli* cells BL-21(DE3) were transformed with obtained recombinant vector and cultivated with kanamycin antibiotic in LB-broth. Induction was started with IPTG. Cells were disrupted by lysozyme and ultrasonication. Liquid fraction was loaded into sepharose column.

Obtained purified enzyme is highly thermostable which was tested in a condition of high temperature and are able to preserve polymerase activity even after heating at 95°C during 30 minutes. Recombinant Tth polymerase has 95% SDS-PAGE purity. Also we have managed to made different storage and reaction buffers (with various concentrations of salts, stabilizers and detergents) in order to determine the best combination.

Keywords: Tth, *Thermusthermophilus*, recombinant protein, *E.coli*, polymerase, enzyme.

INTRODUCTION

After the first polymerase from *Escherichia coli* (Pol I) was characterized polymerase class of enzyme was becoming more and more in-demand in laboratory researches. Development and further researches of different polymerases made them necessary tool in science routine as well as in diagnostics researches.

During this period of time such polymerases as *Taq* DNA polymerase and modified version of T7 DNA polymerase was obtained and introduced in such performance like dideoxy sequencing of DNAs with retained secondary structure [1,2].

Today thermostability is the one of the most necessary features of any polymerase and it mostly depends on original organism's environment. A number of thermophilic polymerases were isolated and characterized from both mesophilic bacteria and archaea sources. As expected, these enzymes have elevated temperature optima and thermal stabilities that roughly correspond to the thermal extremes of environmentfrom which they were isolated. Despite the fact that different enzymes have different level of thermostability they all have common temperature of polymerization which is about 70-80°C [3-5].

In this paper, we describe another thermostable polymerase that was isolated from *Thermusthermophilus* that is an extremely thermophilic Gram negative eubacterium, with an optimal growth temperature of about 65°C. *T. thermophilus* was originally isolated from a thermal vent within a hot spring in Izu, Japan by TairoOshima and KazutomoImahori [6].

T.thermophilus DNA polymerase is a thermostable Mg^{2+} depended enzyme. The polymerase has 5'-3' polymerase and 5'-3' exonuclease activity, but lack of 3'-5' exonuclease activity.

MATERIALS AND METHODS

DNA source

Strain HB8 *T.thermophilus* was selected as host of*Tth*DNA polymerase gene. Cells were cultivated in selective broth based on mineral water Vittel (pH 7.2, TDS 403 mg/l, Ca^{2+} 91 mg/l, Mg^{2+} 19.9 mg/l, Na^{+} 7.3 mg/l, HCO_3^{-} 258 mg/l, Cl^{-} 3.7 mg/l, SO_4^{2-} 105 mg/l, K^{+} 4.9 mg/l, F^{-} 0.6 mg/l). Other components for 1 L of medium are 8g tryptone, 2.5 yeast extract, 3g NaCl and 0.5 mM of each $MgCl_2$and $CaCl_2$before inoculating the cell culture.

Cells were incubated at +65°C during 24 hours and centrifuged at 6000×g for 7 min at +4°C. Genomic DNA was isolated with Promega DNA isolation kit. We designed couple primers intentionally with restriction sites for Tth gene amplification and further restriction:

Forward 5'-gggttt**catATG**GAGGCGATGCTTCC-3'(*Nde*I)

Reverse 5'-ccg**gaattc**cggCTAACCCTTGGCGGAAAG-3'(*EcoR*I)

Tth DNA polymerase gene was amplified from *T. thermophlus* genomic DNA with 1 U Phusion Hot Star II DNA polymerase (Thermo Scientific). Assay reactions (50 µl) contained: 10 µl Reaction Buffer, 4 µl 2.5 mM dNTPs (each), 1 µl (702 ng/µl template DNA), 10 µM primers NdeITthpol and EcoRTthpol (each). Thermocycling conditions were: preheating 98°C for 2 min, followed by 30 cycles of 98°C (15 sec), 55°C (30 sec), 72°C (2 min). Reaction was finished at 72°C for 10 min.

Cloning

Obtained PCR amplicon was purified with chloroform extraction method[7]**.** Amplified sequence's length was 2526 bp. Obtained amplificate and plasmid vector pET-28c(+) were treated with restriction enzymes in order to perform subsequent ligation.

The fragment was digested by *Nde*I and *EcoR*I restriction enzymes in 2x Orange buffer at 37°C for 2 hours as well as vector pET-28c(+). Ligation was performed with T4 DNA ligase at 4°C overnight.

Competent cells JM105 were transformed with ligation mix by heat shock method. Transformed cells were inoculated on solid LB-agar 1.5% with kanamycin (50 µg/ml). PCR screening was carried out with T7-primers and Taq/Pfu DNA polymerases mix [8, 9]. Positive colonies were inoculated in liquid LB-medium in presence of kanamycin. Isolation of plasmids was performed using a Quick Plasmid Miniprep Kit (Invitrogen, USA), thereby another competent cells was transformed with obtained modified vector to make producing strain of Tth pol. Transformed cells were inoculated on solid LB-agar 1.5% with kanamicin (50 µg/ml).

Expression and purification of Tthpol

Single colony was inoculated in 50 ml LB broth with 50 µg/ml of kanamycin. Inductor IPTG was added to final concentration 0.5 mM after optical density at wavelength 600 nm had reached 0.6. To estimate level of expression and possible stages of protein denaturation 4 fractions (10 ml each) for 0, 2, 4 and 16 hours of expression were collected by centrifugation at 6000×g 4°C during 7 minutes.

Collected fractions were resuspended in buffer (20 mMNaCl, 40 mMTris-HCl, pH 7.5) and processed with ultrasonic disintegrator (50% amplitude, 0.5 cycling). Fractions were divided in 2 parts soluble and insoluble by centrifugation at 16000×g 4°C during 30 minutes. Expression level of target protein was assessed by SDS-PAGE.

Protein expression was repeated in the larger scale – 500 ml LB broth with kanamycin. This time culture was centrifuged in 4 hours after expression induction, since observed maximum of protein was in supernatant from 4 hour fraction.

Supernatant was heated at 75°C during 1hour and centrifuged at 16000×g 4°C. Purification was performed by elution with imidazole containing buffers A, B and C (20, 100 and 500 mM respectively). Preheated supernatant was loaded into equilibrated 1ml-HisTrap Chelating FF column charged with Ni^{2+}. 3 buffers with imidazole concentration: 20, 100 and 500 mM were prepared. Each buffer includes 500 mMNaCl, 40 mMTris-HCl, pH 7.5. After loading supernatant onto column elution of bound proteins was performed by subsequent addition to the column all three buffers with increasing imidazole concentration.

Eluted fractions with target protein were loaded onto 1ml-HiTrap Heparin HP column. 5 buffers with concentration of KCl: 40 mM (Buffer A), 100 mM (Buffer B), 150 mM (Buffer C), 200 mM (Buffer D) and 500 mM (Buffer E) were prepared. Each buffer includes 40 mMTris-HCl, pH 7.5.

Quantitative determination of the protein concentration in the fractions was determined by the Bradford assay using bovine serum albumin as a standard [10]. Electrophoretic separation of proteins was performed by Laemmli method [11] in 15% polyacrylamide gel under denaturing conditions. Samples were pre-denatured prior to their application to the gel at +95°C for 5 minutes. Electrophoresis was performed at 90 V for 2 hours. Staining of gel was performed using 0.2% Coomassie Brilliant Blue R - 250 (Sigma) in 50% ethanol, 10% acetic acid. Nonspecific binding of dye was removed with 25% ethanol and 7,5% acetic acid.

PCR with recombinant Tth DNA polymerase

There are a few different reaction and storage buffers for recombinant*Tth*DNA polymerase which led to testing their different combinations.We varied concentration of DTT, Tris-HCl, NaCl, KCl, EDTA, BSA, Triton X-100, $(NH_4)_2SO_4$ until it was optimal.

Polymerase activity was evaluated via PCR with obtained enzyme. Also we have tested protocol with betaine to preserve enzyme from overheating and decrease non specific annealing. 2 groups of samples were tested: with and without betaine. Each group was consisted of 11 samples with different anneling temperature (from 40 to 55°C), to determine feature of betaine to lower annealing temperature.

RESULTS AND DISCUSSION

We decided to use original genomic DNA from *Thermusthermophilus* as matrix for gene amplification. Thus *T.thermophilus* cells were cultivated in three types of media based on Vittel mineral water, «Asu» mineral water and distilled water. The best cell grow was in Vittel based broth. Temperature was set to 65°C since it is optimal for *T.thermophilus*. Gram-staining was performed to visualize and determine *Thermusthermophilus* cells (fig. 1).

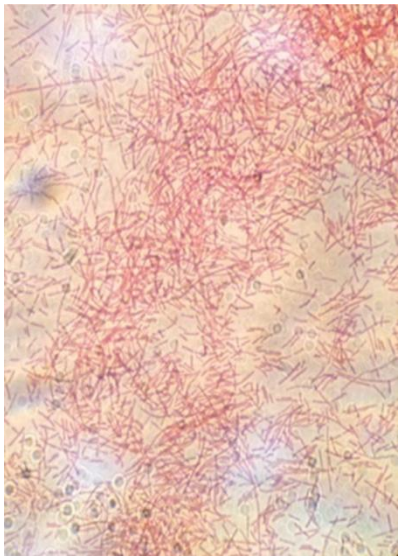
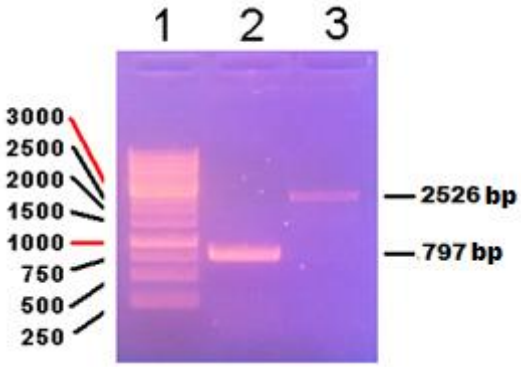


Fig. 1. Gram-staining of *Thermusthermophilus* HB8 strain

Next step was to extract genomic DNA and amplify *Tth*DNA polymerase gene. We carried out PCR with specific primers to amplify targeted gene. Amplification products were analyzed with agarose gel electrophoresis (fig. 2).



1 –marker Thermo SM1163; 2 –amplified 16S region;3 –*Tth* DNA polymerase gene 2526 bp

Fig. 2.*Tth* DNA polymerase full length gene amplification

PCR product was treated by endonuclease enzymes *Nde*I and *Eco*RI as well as expression vector pET-28c(+). As a result we have obtain modified vector pET-28c(+)/Tth (fig. 3).

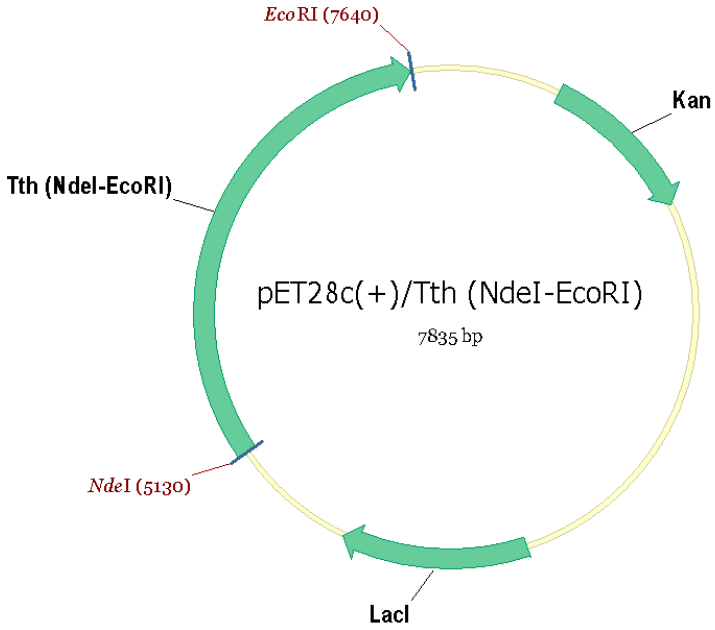
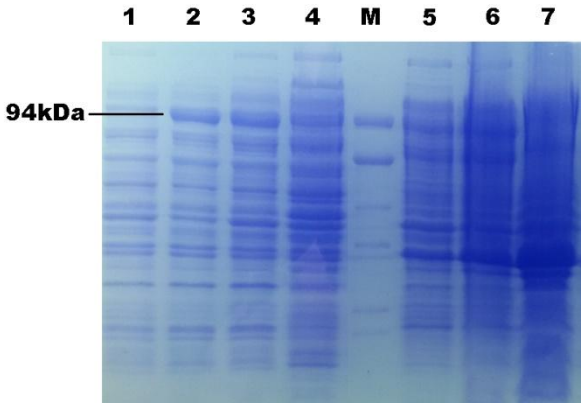


Fig. 3. pET-28c(+) modified with *Tth* DNA polymerase gene

Test of expression levels was performed in volume of 50 ml. Timeline points were 0, 2, 4 and 16 hours after induction. After cell denaturation solution was divided in two fractions by centrifugation. Targeted protein was detected at point 2h and 4h in soluble fraction. At point 16h was no significant band which is explained by protein degradation during long incubation (fig. 4).

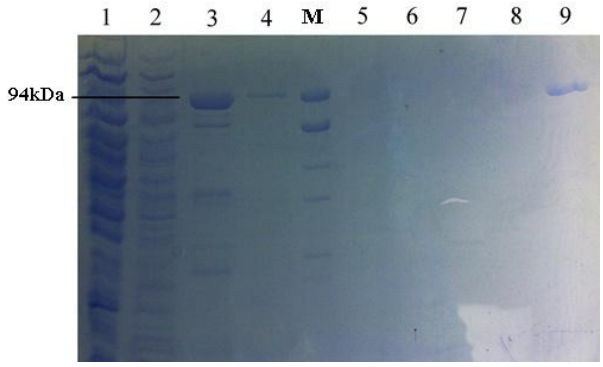


1 – no induction; 2 – supernatant 2h; 3 – supernatant 4h; 4 – supernatant 16h; 5 – pellet 2h; 6 – pellet 4h; 7 – pellet 16h

Fig. 4. Expression of recombinant *Tth* DNA polymerase

Purification of recombinant *Tth* DNA polymerase was carried out in 3 steps: heating, metal-affinity chromatography and affinity heparin chromatography.

Sample was heated at 75°C during 1 hour and centrifuged 18000×g 1 hour at 4°C. Liquid fraction was loaded into chromatographic column charged with Ni^{2+} to elute it with imidazole gradient buffers. Purified protein was loaded in heparin column with subsequent elution with KCl gradient buffers(fig. 5).



1 – flow through; 2 – wash (buffer A imidazole); 3 – fraction 1 (buffer B imidazole); 4 – fraction 2 (buffer C imidazole); 5 – wash 2 (buffer A heparin); 6 – fraction 1 (buffer B heparin); 7 – fraction 2 (buffer C heparin); 8 – fraction 3 (buffer D heparin); 9 – fraction 4 (buffer E heparin)

Fig. 5. Chromatographic purification of *Tth* DNA polymerase

Purified protein was divided and stored in different buffers. We used different combinations of storage and reaction buffer to figure out the best ones (fig. 6). The best combination were storage buffer 20 mMTris-HCl (pH 8.5), 1 mM DTT, 0.1 mM EDTA, 100 mMKCl, 50% Glycerol and reaction buffer x10 100 mMTris-HCl (pH 8.8), 1 mM $MgSO_4$, 800 mM $(NH_4)_2SO_4$, 0.5 mM BSA, 0.5% Tween 20.

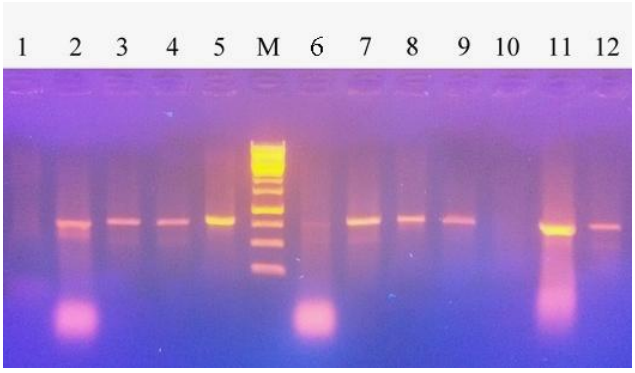
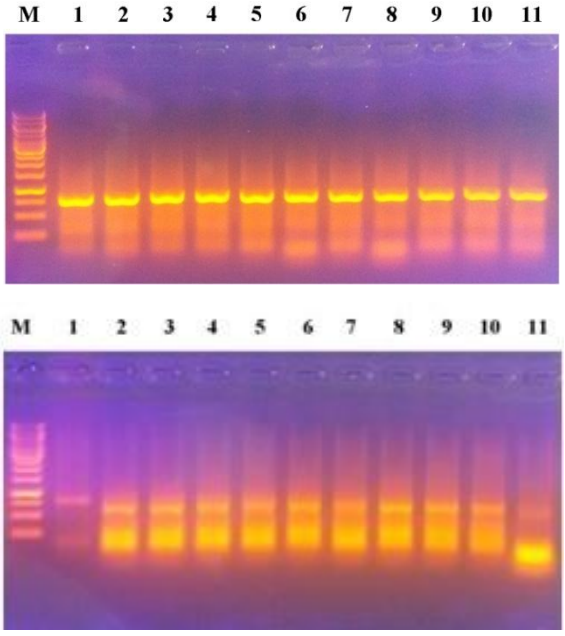


Fig. 6. Different combination of storage and reaction buffers

In many protocols it is suggested to add betaine to reaction mix. We tested both properties of betaine. 22 exact samples were preheated at 97°C during 7 minutes before PCR cycling started, half of these reactions was proceed with betaine half of them without. Annealing temperature was varying from 40 to 55°C(fig. 7).

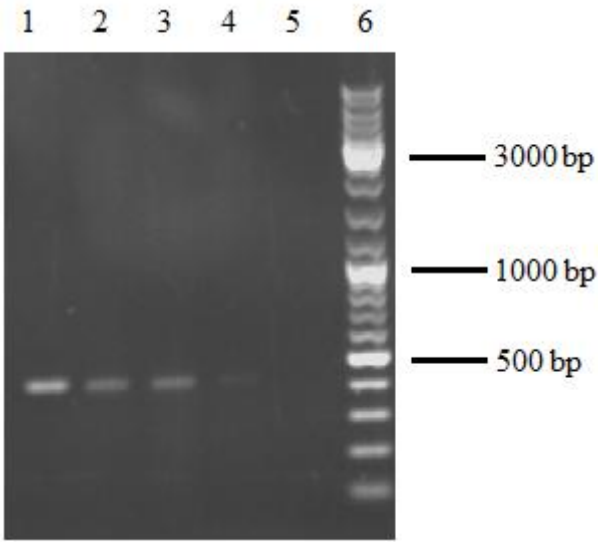


A – Amplification in presence of betaine B – Amplification without betaine. Temperature of annealing step for the lines was 1 – 40°C, 2 – 41.4°C, 3 – 42.9°C, 4 – 43.4°C, 5 – 44.8°C, 6 – 46.3°C, 7 – 48.8°C, 8 – 50.3°C, 9 – 51.8°C, 10 – 53.2°C, 11 – 55°C

Fig. 7. Amplification with *Tth* DNA polymerase after 97°C heating during 7 minutes, temperature gradient of annealing step from 40 to 55°C

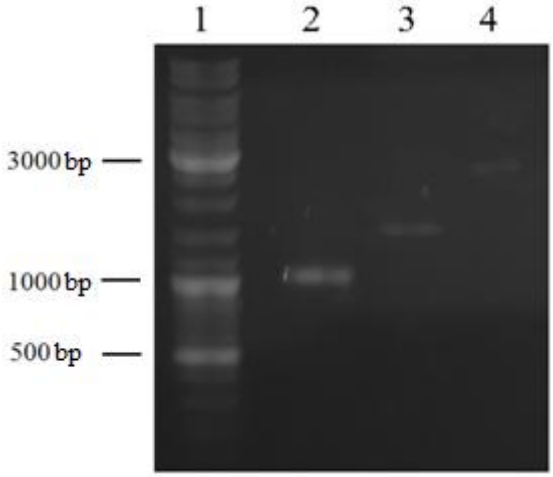
Also we tested enzyme's temperature endurance and capacity. *Tth* DNA polymerase was heated at 95°C during different period of time (15, 20, 25, 30 and 40 minutes) and then used in PCR to amplify 400 bpfragment. According to obtained data after 40 minutes there is no significant polymerase activity (fig. 8).

Enzyme's capacity was measured by amplification of fragments with different length. Results showed that *Tth*DNA polymerase is able to amplify fragment of 2800 bp, but quantity of product is very low, whereas in case of 1100 bp fragment amplification was significantly more efficient (fig. 9).



1 – 15 minutes, 2 – 20 minutes, 3 – 25 minutes, 4 – 30 minutes, 5 – 40 minutes

Fig. 8. Temperature endurance of *Tth* DNApolymerase depending on time. Heating was performed at 95°C



1 – marker SM1173, 2 – 1100 bp fragment, 3 – 1500 bp fragment, 4 – 2800 bp fragment

Fig. 9. Capacity of *Tth*DNA polymerase

CONCLUSION

The article describes a procedure for obtaining the recombinant *Tth* DNA polymerase, including the steps of cloning a gene, its expression in a heterologous environment, and purification. *E.coli*BL21(DE3) strain was transformed with modified pET-28c(+) vector with inserted gene of *Tth* DNA polymerase from the thermophilic

bacterium *Thermusthermophilus* under the control of bacteriophage T7 promoter. Adding an additional 20 amino acids coding a hexahistidine tag did not affect the DNA polymerase activity and thermostability of the enzyme. The expression system and purification method allows to purify a sufficient amount of the recombinant enzyme.

Obtained enzyme with 95% purity (SDS-PAGE) has polymerase activity. Level of capacity allows to amplify fragments up to 2800 bp. Optimized storage and reaction buffers with betaine provides PCR at high denaturation and low annealing temperature. Temperature endurance of original recombinant polymerase is 25 minutes at 95°C.

Acknowledgements

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E.COLI ЭКСПРЕССИЯ ЖҮЙЕСІНДЕ *THERMUS THERMOPHILUS*-ТАН АЛЫНҒАН ДНҚ ПОЛИМЕРАЗАНЫҢ БӨЛІНІП АЛЫНУЫ ЖӘНЕ ЭКСПРЕССИЯСЫ

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ТҮЙІН

***Thermus aquaticus-ң* термотұрақты ДНҚ полимеразасының бөлініп алуы молекулалық биологияда және зертханалық зерттеулер үшін маңызды қадам болды. *Taq* ДНҚ полимеразасы ашылғаннан кейін ПТР әдісі әлемдегі зертханалардың арасында кең тарады. *Taq* ДНҚ полимеразасы нысана нуклеотидті тізбегін тез және жоғары спецификалы түрде амплификациялау үшін арналған бірінші құрал болды. Сол себепті термотұрақты полимераза зертханалық зерттеулер үшін өте маңызды болып табылады.**

Біз қос полимеразды және кері транскриптазалық белсендігі бар, рекомбинантты *Tth* рекомбинантты ДНҚ полимеразасын экспрессиялап, тазаладық. Ең

біріншіден *Thermus thermophilus* HB8 штаммы дақылдандырылып, геномдық ДНҚ бөлініп алынды. Ген амплификацияланып, Т7 бақылауындағы pET-28c(+) экспрессиялық плазмидті векторына клондалды. *E.coli* BL-21(DE3) жасушалары алынған рекомбинантты вектормен трансформацияланып, канамицин антибиотигі қосылған ЛБ қоректік ортасында дақылдандырылды. Индукция ИПТГ көмегімен жүргізілді. Жасушалардың бұзылуы лизоцим және ультрадыбыстау көмегімен іске асырылды. Сұйық фракция сефарозды бағанаға енгізілді.

Алынған тазаланған фермент термотұрақты, ал ол өз кезегінде жоғары температура жағдайында тексерілді. Және де бұл фермент 30 минут бойы 95 °C -та қыздырылғаннан кейін де полимеразалық белсенділігін сақтауға қабілетті. Рекомбинантты Tth полимеразаның SDS-PAGE тазалығы 95%-ды құрайды. Ең жақсы тіркесімдерді анықтау үшін біз реакциялық буферлер мен сақтау буферлерінің (тұздар, стабилизаторлар мен детергенттердің әртүрлі концентрацияларымен) әртүрлі нұсқаларын тандадық.

Негізгі сөздер: Tth, *Thermus thermophilus*, рекомбинантты ақуыз, *E.coli*, полимераза, фермент.