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PURIFICATION OF RECOMBINANT *PFU* DNA POLYMERASE BY DOUBLE STEP AFFINITY CHROMATOGRAPHY

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ABSTRACT

In Kazakhstan, the production of enzymes for molecular biology is not established. This explains the absence of any high-tech production and low demand for this specific product. However, PCR diagnostics and genomic technologies in Kazakhstan is already a reality, and the need for thermostable polymerases in the country is growing up.

Recombinant *Pfu* DNA polymerase was purified from *Escherichia coli.* Expression vector with cloned gene coding *Pfu* DNA polymerase protein was made by standard genetic engineering methods. Recombinant protein has 20 additional amino acids at N-end and has a molecular mass of 92,3 kDa. Results of this work showed that PCR made with recombinant analog of *Pfu* DNA polymerase yields amplification products of different DNA sequence length. In addition, the experiment for thermostability was done and enzyme showed polymerase activity after several hours of heating.

Keywords: *Pfu* DNA polymerase, polymerase chain reaction, *Pyrococcus furiosus*, recombinant protein.

INTRODUCTION

Polymerases – enzymes that synthesize polymers of nucleic acids (DNA and RNA) using complementary duplication of the original matrix. These proteins play a key role in all processes that are involved in DNA and RNA, including: replication, recombination and transcription. Consequently, the structure and properties of polymerases evolutionarily demonstrate high conservatism. Study of polymerases could lead to better understanding of the diversity and evolution of living organisms [1, 2].

Application importance of polymerases is their ability to conduct duplication deoxyribonucleic acid molecules in the extracellular environment, thus obtaining a synthetic DNA molecule and being a major enzyme in the polymerase chain reaction [3]. Initially, it was used in the reaction polymerase of bacterium *Escherichia coli* PolI, however, the disadvantage was the lowest temperature of protein denaturation. In this respect, the thermophilic polymerase and hyperthermophilic organisms were preferable as the temperature was kept around 90°C-95°C. This circumstance provided thermally stable polymerases applicability in molecular biology [4]. Evolutionary DNA polymerase emerged at an early stage of the cells , which is confirmed by their conservative structure [5].

The best solution for polymerases in the polymerase chain reaction is the use of thermostable DNA polymerases derived from thermophilic microorganisms that live in hot

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springs: *Thermus aquaticus, Thermus thermophilus, Thermococcus litoralis, Pyrococcus furiosus, Sulfolobus acidocaldarius, Pyrococcus kodakaraensis, Thermococcus gorgonarius* et al [6-14].

In contrast to the well-known and most widely used *Taq*-polymerase of *Thermus aquaticus*, making mistakes in the polymerization [15, 16], *Pfu*-polymerase from *Pyrococcus furiosus* archaea strain is more accurate due to the 3'-5' exonuclease activity of the enzyme. *Pyrococcus furiosus* strain was isolated and characterized Gerhard Fiala and Karl Stette in 1986 from hyperthermal sediments on the island of Vulcano in the Tyrrhenian Sea[17]. *Pfu* polymerase was isolated and described in 1991 by Lundberg et al [18]. The crystal structure of the protein *Pfu*-pol has been thoroughly studied in detail in 2008 [19-21]. *Pfu*- polymerase is the most famous and popular as "accurate" polymerase and is used independently and in a mixture with *Taq*- polymerase. Moreover, in the mixture, both polymerases complement each other: *Taq*-polymerase ensures a high polymerization rate, and *Pfu*- polymerase - a high copying precision.

In the literature there are quite detailed methods for producing recombinant Pfu-polymerase in the form of articles [22, 23] and patents [24-27] .

The aim of this work is to obtain recombinant *Pfu* DNA polymerase for use in research and diagnostic practice.

MATERIALS AND METHODS

Bacterial strains , DNA, plasmids and reagents

Escherichia coli JM109 and DH5α strains were used for production of vectors and cloning. For the expression of heterologous protein following strains of *Escherichia coli* were used: BL21(DE3), ArcticExpress(DE3)RP, Rosetta(DE3). DNA of *Pyrococcus furiosus* was used as a source of DNA. The plasmid pET-28c(+) (Novagen, UK) was used to construct the expression vector.

Escherichia coli cells with the plasmids were cultured at +37°C in LB medium containing kanamycin at a concentration of 50 μg/ml. Enzyme Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA) was used for the amplification of the target gene. Restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific (USA).

For protein purification were used HiTrap Chelating HP 1 ml reactivated with ions Ni^{2+} and HiTrap Heparin HP 1 ml columns (GE Healthcare, USA).

Oligonucleotides

In this paper we used oligonucleotides presented in table 1.

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Table 1. List of oligonucleotides that were used in this work

Oligonucleotide	Sequence
Pfu-pol-fr	5’-ATGATTTTAGATGTGGATTAC-3’
Pfu-pol-rv	5’-GGATTTTTTAATGTTAAGCC-3’
5’-NdeI-Pfu-pol	5’-GGGAATTCCATATGATTTTAGATGTGGATTAC-3’
3’-NdeI-Pfu-pol	5’-GGAATTCCATATGTTAGGATTTTTTAATGTTAAGC-3’
Pfu(400-421)	5’-GAGCTAAAGATTCTTGCCTTCG-3’
Pfu(431-451)	5’-CTTCTCCTTCGTGATAGAGGG-3’
Pfu((780-800)	5’-GTATCATGTAATAACAAGGAC-3’
Pfu(812-832)	5’-CAGCCTCTAGTGTGTATGTTG-3’
Pfu(1199-1220)	5’-GTTGTGGGAAAACATAGTATAC-3’
Pfu(1231-1251)	5’-GTGGGTAATTATAATCGAGGG-3’
Pfu(1601-1621)	5’-GATTTAAAGTCCTCTACATTG-3’
Pfu(1623-1643)	5’-GTTGCATAGAGACCATCAGTG-3’
Pfu(1995-2014)	5’-GCAGATAACAAGACCATTAC-3’
Pfu(2035-2055)	5’-CTTTGCAACAGCTACGTGAGG-3’
M13fr	5`-GTAAAACGACGGCCAG-3`
M13rv	5`-CAGGAAACAGCTATGAC-3`
8F	5'-AGAGTTTGATCCTGGCTCAG-3'
806R	5'-GGACTACCAGGGTATCTAAT-3'
5’-gfpPM	5’-CCGGAATTCCGGATGGTGAGCAAGGGCGAGG-3’
3’- gfpPM	5’-CGCGGATCCGCTTGTACAGCTCGTCC-3’
5’-BamHI-alkA	5’-CGCGGATCCCGATGTATACCCTGAACTGGCAG-3’
3’-EcoRI-alkA	5’-CCGGAATTCCGGTCATGCTTCGTCTGGTTGC-3’
appA-fr	5’-ATGAAAGCGATCTTAATCCC-3’
appA-rv	5’-CAAACTGCACGCCGG-3’
T7Promoter	5’- TAA TAC GAC TCA CTA TAG GG -3`
T7Terminator	5’- GCT AGT TAT TGC TCA GCG G -3'

Media

Different versions of Luria Bertani broth were used: normal saline for cell culturing *E. coli* DH5α/pPfh (1% tryptone, 0,5% yeast extract, 1% NaCl) and low-salt for cells ArcticExpress(DE3)RP/pPfh (1% tryptone, 0,5% yeast extract, 0,5% NaCl). For incubation of the transformed cells, SOC medium was used (2% tryptone, 0,5% yeast extract, 0,05% NaCl, 2,5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose, pH 7,5). Preparation of media was carried out according to the protocol of Maniatis [28].

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Cloning of a *pfu -pol* gene into expression vector

PCR amplification of target *pfu-pol* gene was performed with genomic DNA of *Pyrococcus furiosus* (100 ng), 1 µl (10 µM) of each oligonucleotide (5'-NdeI-Pfu-pol primer contains a restriction site *Nde*I, and 3'-NdeI-Pfu-pol primer contains the same site *Nde*I), 4 µl (10 mM total) dNTPs, 10 µl of 5x PCR buffer (containing 7,5 mM $MgCl_2$) and 0.5 µl of Phusion Hot Start II DNA Polymerase (2 U/µl) in thermocycler Mastercycler nexus gradient (Eppendorf, Germany). The amplificated product was about the expected size (~ 2352 bp). The amplified gene was ligated into the expression vector pET-28c(+) digested by enzyme *Nde*I. Ligation was performed with T4 DNA ligase at +4°C for 16 hours. Isolation of plasmids from the positive clones was performed using a kit MiniPrep (Promega, USA) according to the manufacturer's protocol. Check of plasmids for the presence of *Pfu* DNA polymerase gene was performed using restriction analysis. Genetic constructs were confirmed by DNA sequencing with list of primers: T7Promoter, T7Terminator, Pfu(400-421), Pfu(431-451), Pfu(780-800), Pfu(812-832), Pfu(1199-1220), Pfu(1231-1251), Pfu(1601-1621), Pfu(1623-1643), Pfu(1995-2014), Pfu(2035-2055) and tested for compliance with the required sequence of genomic data bank (GenBank: D12983.1, *Pyrococcus furiosus* pol gene for DNA polymerase, complete cds) using Vector NTI software Advance (TM) 11,0 (Invitrogen, USA).

Constructed genetic expression vector pET-28c (+)/pfu-pol, which later received the name pPfh, was accumulated in the cells of *E. coli*.

Strain producing recombinant *Pfu*-polymerase and parameter optimization of cultivation and protein induction

E. coli expression strains were characterized with different ability to express heterologous proteins. In order to determine the optimal strain three strains of *E.coli* with different genotypes: BL21(DE3), ArcticExpress(DE3)RP and Rosetta(DE3) were tested. Competent cells of these strains were transformed by resulting vector pPfh.

Expression of *Pfu* and purification of enzyme

Transformation of electrocompetent cells by a plasmid vector pPfh performed by electroporation using MicroPulser (BioRad, USA) under the following conditions: 100 ng of plasmid per 50 µl cell, voltage – 2,5 kV, electric capacity – 25 µF, resistance – 200 Ohms. Transformed cells were incubated in 950 µl of SOC at +37°C for 1 hour with vigorous shaking. Then 50 µl of cells were plated on LB agar with kanamycin and grown at +37°C for 16 hours. Single colonies-transformants were cultured in LB broth with kanamycin in a volume of 400 ml. In the middle of logarithmic growth phase of the bacterial mass (OD_{600} = 0,6) inductor – isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0,1 mM and

incubated for 16 hours. Collection of cells was performed at +4°C by centrifugation, 6,000 rcf for 7 minutes. Further, SDS-PAGE electrophoresis was performed to determine the recombinant protein expression level. As a marker commercial ladder of Thermo Scientific, SM1811 was used.

Lysate was resuspended in buffer 20 mM NaCl, 20 mM Tris-HCl (pH 7,5). Then suspension was frozen at –20°C, thawed and re-frozen for the efficiency of lysis followed by sonication with ultrasonic disintegrator UP200S at 24 kHz pulsing regime (10 pulses, 10 seconds/pulse) on ice and after the lysate was incubated at +70°C for 1 hour. The lysate was clarified by centrifugation for 1 hour at +4°C, 40000 rcf. The resulting supernatant was subjected to a Ni^{2+} HiTrap Chelating HP column 1 ml, pre- equilibrated with Buffer Low Imidasol (500 mM NaCl; 50 mM Tris-HCl (pH 7,5), 20 mM Imidazole).

Protein was eluted with a linear gradient of imidazole using Buffer Low Imidazole (500 mM NaCl; 50 mM Tris-HCl (pH 7,5), 20 mM Imidazole) and High Imidazole (500 mM NaCl; 50 mM Tris-HCl (pH 7,5), 500 mM Imidazole) on liquid chromatograph for rapid protein purification FPLC (Fast protein liquid chromatography) AKTA model Purifier 10. Fractions containing the recombinant protein Pfu-pol were combined and in the combined fractions salt concentration was reduced to 50 mM NaCl. Prepared combined fractions were loaded on a HiTrap Heparin HP column 1 ml, equilibrated with 3 volumes of buffer Heparin Low Salt (50 mM KCl; 50 mM Tris-HCl pH 8,0). Further purification of the protein was carried out using a linear salt gradient starting with KCl buffer Heparin Low Salt Buffer and ending Heparin High Salt (1050 mM KCl; 50 mM Tris-HCl (pH 8,0). Fractions containing the recombinant protein Pfu-pol, were selected for analysis by SDS-PAGE to check purity. Electrophoretic separation of proteins was performed by the method of Laemmli [29] in 12% polyacrylamide gel under denaturing conditions.

RESULTS AND DISCUSSION

Target gene, *pfu-pol* was cloned in pET-28c(+)vector under the control of a strong promoter of T7 bacteriophage RNA polymerase, which provides high expression. *Pfu-pol* gene has long length combined with an additional domain which contain 20-peptide hexahistidine tag. Amplified Pfu-polymerase gene was detected by 0,8% agarose gel electrophoresis. The expected size of the PCR product was 2353 bp. Purified PCR amplificate *pfu -pol* and plasmid pET-28c (+) were hydrolyzed in buffer O (Thermo Scientific) using the restriction enzyme NdeI and then ligated at +4^{o}C for 16 hours. After transformation by ligation mixture total number of colonies was about 300 colonies. PCR screening of DH5α colonies-transformants was performed for presence of vector pET-28c with the *pfu-pol* gene insert by T7 primers. Samples of the plasmids

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were used in restriction analysis with BamHI enzyme to determine the orientation of the inserted *pfu-pol* gene. Selection of the restriction enzyme BamHI is caused by that sequence GGATCC, a restriction enzyme recognizable site, occurs in gene once in position 614-619. Preliminary calculations have shown that, depending on the orientation of the inserted *pfu-pol* gene into the vector pET-28c(+) by NdeI site to form two pairs of fragments after hydrolysis of the resulting vector with endonuclease BamHI 1753 bp and 5935 bp (correct version), 642 bp and 7056 bp (inverted version). From the results of restriction analysis only in clone 23 *pfu-pol* gene was integrated properly. In addition to restriction analysis, DNA of clones 21-24 were used as DNA template in a polymerase chain reaction using primers T7 Promoter and 3'-NdeI-Pfu-pol. As in the case of restriction analysis, only the clone 23 shows a positive result.

Sequencing of plasmid from clone 23 confirmed the absence of mutations in the open reading frame. The complete amino acid sequence of the recombinant protein Pfu-pol has 795 amino acid residues with a calculated mass of 92,3 kDa.

Optimization for cultivation

It was optimized different conditions (screening for strains producing capacity, optimal cell concentration, IPTG concentration, optimum temperature mode and optimization of time incubation) for cultivation of cells for producing of *Pfu*-pol.

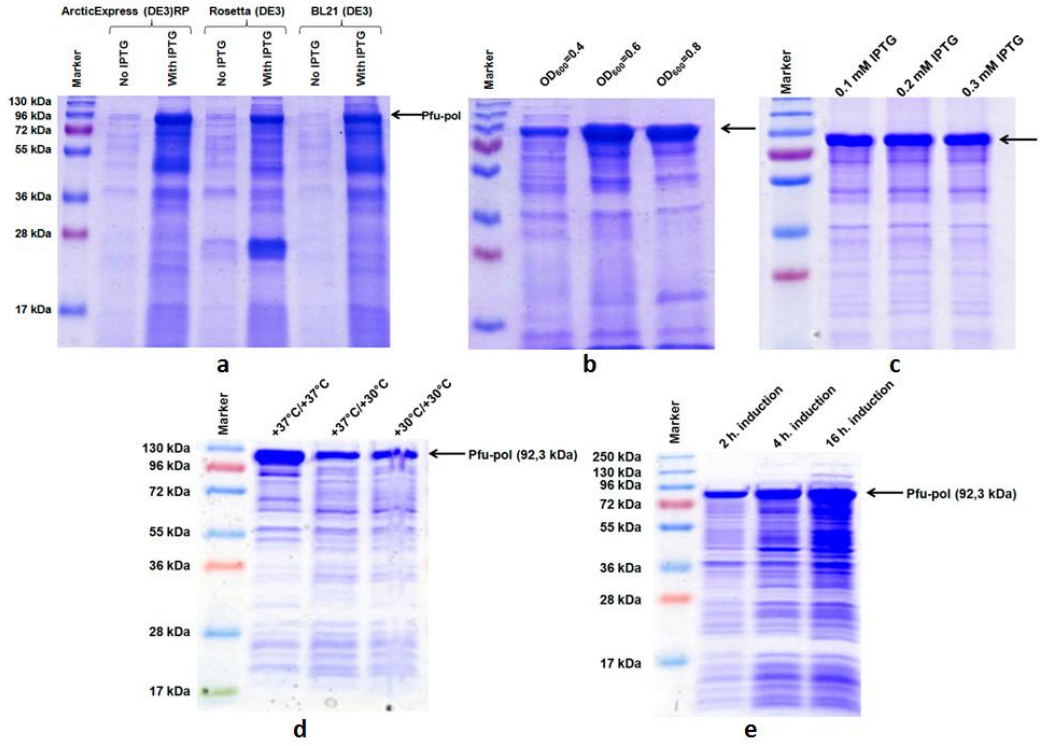

Fig 1. Parameters for optimization: a – results of screening for strains producing capacity; b – results of optimization of optical density; c – the accumulation of Pfu-pol protein in ArcticExpress(DE3)RP/pPfh cells depending on the concentration of inducer IPTG; d – the accumulation of Pfu-pol protein in ArcticExpress(DE3)RP/pPfh cells depending on the incubation temperature; e – the accumulation of Pfu-pol protein in ArcticExpress(DE3)RP/pPfh cells depending on the incubation time

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Three strains of *E.coli* (BL21(DE3), ArcticExpress(DE3)RP, Rosetta(DE3) were used for screening of strains producing capacity. As follows from the results of electrophoresis from figure 1a, all three transformed strains are capable of accumulating in the cells the recombinant protein Pfu-pol. ArcticExpress(DE3)RP strain has slightly higher producing ability compared to other, and this strain was used as a producer strain of recombinant Pfu-polymerase. It was determined that the most promising is the induction of culture when the optical density of OD_{600}=0,6-0,8 (fig. 1b). As seen from figure 1c, there are no significant differences in bandwidth corresponding to expected mass of the recombinant Pfu-pol protein. From this it follows that a sufficient concentration of IPTG in the culture is 0,1 mM.

As can be seen from the presented data, it is clearly noticeable greater accumulation of heterological protein Pfu-pol in cells that were cultured and induced at +37°C. These results suggest non-toxic or low toxicity Pfu-pol proteins for the *Escherichia coli*.

It was carried out to optimize the incubation time of cultivation with IPTG induction activator of 0,1 mM concentration and at temperature of +37°C. Selection of culture was carried out at 2 hours, 4 hours and 16 hours induction. Figure 1e shows the results of this experiment. As seen from the experiment results, the accumulation of the protein occurs already after 2 hours of induction with IPTG and continues throughout the period of incubation.

Isolation and purification of recombinant Pfu-polymerase

It was optimized the conditions of lysis and purification of recombinant Pfu-polymerase. The cells of the recombinant strain ArcticExpress(DE3)RP, transformed by vector pPfh, after induction of protein, were centrifuged by high-speed centrifugation. Optimization of enzymatic methods of lysis were tested based on the use of bacteriophage T7 lysozyme, muramidase and physical method of destruction of bacterial cell wall by ultrasound (figure 2).

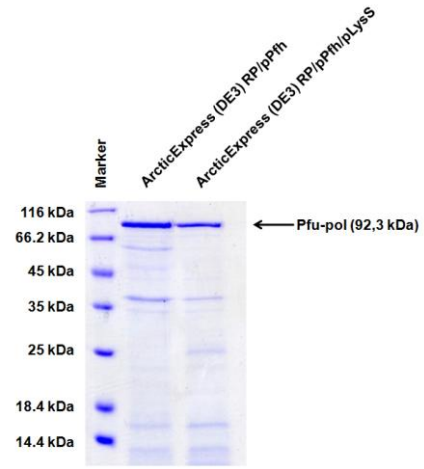

Fig. 2. Protein electrophoresis after cell lysis ArcticExpress(DE3)RP/pPfh using T7 lysozyme (pLysS) or without it

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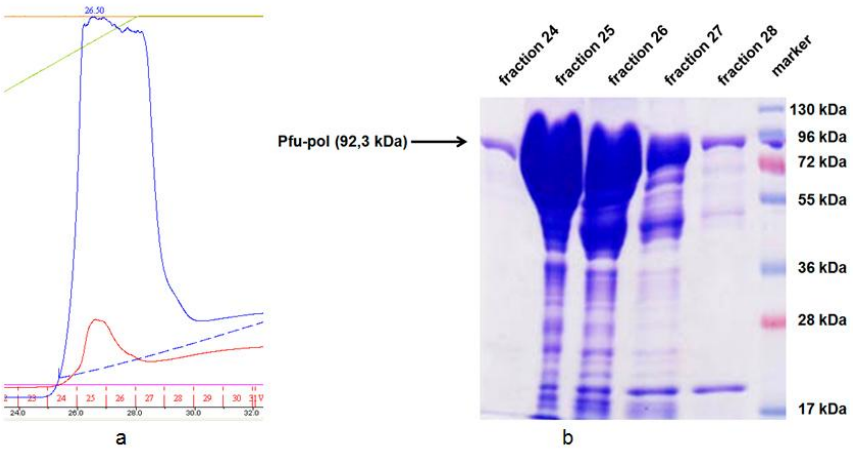
Comparison of total bacterial lysates showed that the most effective is combination of incubation of the cells with muramidase followed by sonication. Experiments showed poor effectiveness of bacteriophage T7 lysozyme.

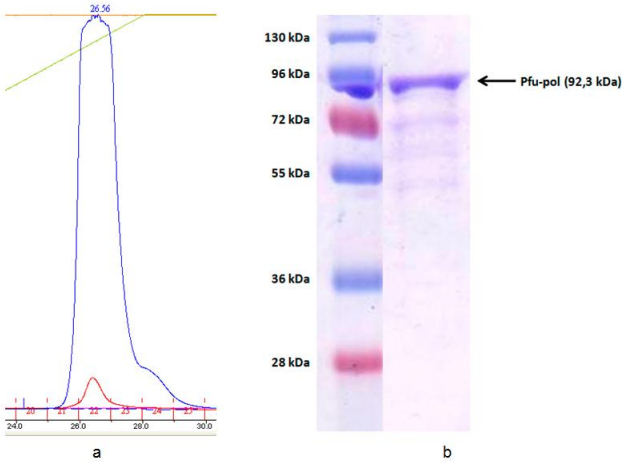
Isolation of Pfu-pol was performed in several steps, using the features of the recombinant protein: its thermal stability, the presence of polyhistidine tag and the DNA-binding domain. Figure 3a shows a chromatogram of this purification step using imidazole gradient from 20 mM to 500 mM. The purity of the five fractions (24-28) was tested by protein electrophoresis (fig. 3b).



a – chromatogram of purification step using imidazole gradient; b – electrophoresis of fractions 24-28 after metal affinity chromatography

Fig. 3. Purification from ArcticExpress(DE3)RP/pPfh on Ni^{2+}HiTrap HP 1ml column

Protein electrophoresis showed that the quantity of protein in the fractions is enough, however, together with Pfu-pol there are another proteins present. Therefore, re-purification was performed by affinity chromatography. Figure 4 shows a purification on heparin column.



a – chromatogram of purification step using salt gradient; b – electrophoresis of fractions 22 after affinity chromatography

Fig. 4. *Pfu*-pol protein purification of combined fractions 24-28 after affinity chromatography on a HiTrap Heparin HP 1ml column

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As can be seen from the figure4a, the DNA binding domain of Pfu-pol polymerase protein provides high affinity for heparin. The recombinant Pfu-pol protein gone one peak in fractions 21-23. The purity of 22 fraction was tested by protein electrophoresis (fig. 4b).

Measurement of the protein concentration were performed by Bradford assay [30] showed that the concentration of 22 fraction was 2,3 mg/ml, which corresponds to a yield of Pfu-pol protein with 10 mg from 1 liter of induced culture.

Test of polymerase activity

Activity test was conducted with purified recombinant Pfu-polymerase in the polymerase chain reaction with different genes as DNA matrix (table 2).

Table 2. Genes and corresponding primers for testing Pfu-polymerase

№	Gene	Length, bp	Matrix DNA	Primers
1	*gfp*	750	pGEM-T/gfp	5'-gfpPM, 3'- gfpPM
2	*appA*	1299	pET-28c/appA	appA-fr, appA-rv
3	*alkA*	872	pET-28c/alkA	5'-BamHI-alkA, 3'-EcoRI-alkA

To verify the absence of plasmid and genomic DNA in a laboratory sample of recombinant *Pfu*-polymerase it was performed PCR with negative control (K^-) on the primers M13fr – M13rv, T7 Promoter – T7 Terminator, 8F – 806R, focused on the M13 and T7 region on the expressed vector and 16S rRNA chromosomal (genomic) DNA. Positive control (K^+) was the *gfp* gene using commercial Pfu-polymerase (Thermo Scientific).

Test results showed that the purified *in vitro* recombinant *Pfu*-polymerase, consistent with the expected characteristics and biochemical activity is not inferior to commercial counterparts. Figure 5 shows the results of testing.

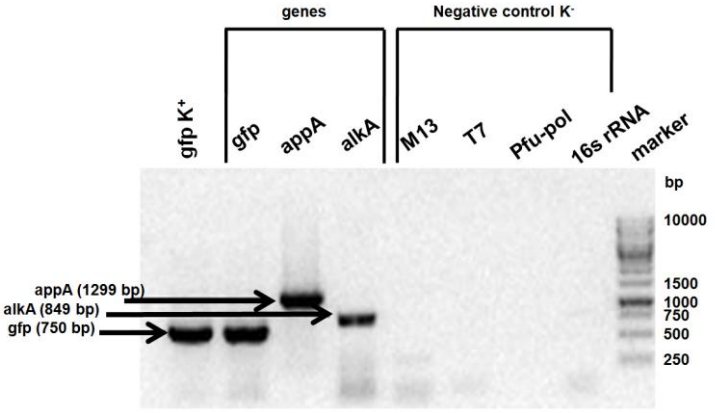

Fig. 5. Checking the polymerase activity of purified *Pfu*-pol

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As can be seen from the figure, the resulting recombinant Pfu-polymerase demonstrates inherent polymerase activity and is not inferior to commercial counterparts.

The reaction buffer for *Pfu*-pol in a concentration of 1X includes the following components: 20 mM Tris-HCl (pH 8,8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0,1% (v/v) Triton-X100, 0,1 mg/ml BSA.

It was selected the storage buffer composition for Pfu-polymerase enzyme at –20°C without loss of polymerase activity. The reaction buffer comprises the following components: 20 mM Tris-HCl (pH 8,2), 1 mM DTT, 0,1 mM EDTA, 100 mM KCl, 0,1% (v/v) Nonidet P – 40, 0,1% (v/v) Tween 20 and 50% glycerol by volume.

Storage of recombinant Pfu-polymerase in the storage buffer for two months at a temperature of –20°C didn't reduced polymerase activity. PCR screening demonstrated polymerase activity using 5 different plasmid vectors as a template DNA, and T7 primers (fig. 6).

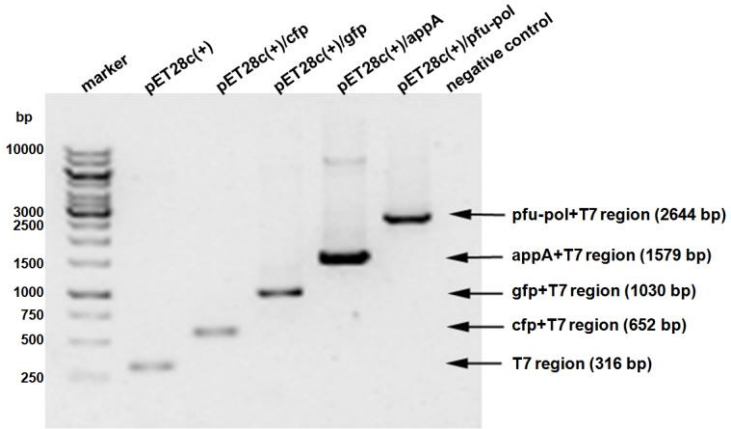

Fig. 6. Test results of *Pfu*-polymerase after 2 months of storage

As seen from the results of electrophoresis of PCR screening storage buffer is safe to recombinant Pfu-polymerase. Experiments to determine the thermostability of the recombinant Pfu-polymerase showed that recombinant Pfu-pol withstand eight hours of incubation at +95°C without significant loss of biochemical activity (fig. 7).

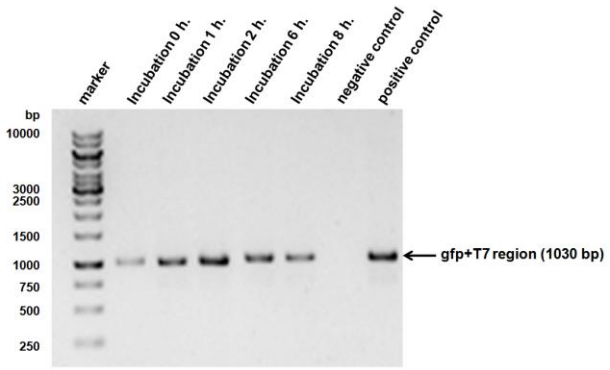

Fig. 7. Test results of *Pfu*-polymerase activity after incubation at +95°C

CONCLUSION

As a result of genetic engineering and purification recombinant Pfu DNA polymerase was obtained on the basis of the bacterial expression vector pET-28c(+) with integrated *pfu-pol* gene. Recombinant Pfu DNA polymerase was purified by double step affinity chromatography methods. At the first step purification was performed by metal-affinity chromatography. The second step was used on a heparin affinity chromatography, DNA mimic sorbent.

It was optimized different conditions for the cultivation of the strain producer ArcticExpress(DE3)RP/pPfh and induction for maximum accumulation of Pfu-polymerase. During experimental studies it was varied the following parameters: temperature cultivation, aeration, concentration of induction activator and time of incubation. The data obtained allowed to choose the most optimal conditions for maximum accumulation of the Pfu protein in *Escherichia coli* cells.

Storage of recombinant Pfu-polymerase in the buffer storage for two months at a temperature of -20°C didn't reduced polymerase activity. Thermostability of recombinant Pfu-polymerase provides several hours of heating at a temperature of +95°C without loss of enzymatic activity.

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ТҮЙІН

Қазақстанда молекулалық биологияға арналған ферменттердің өндірісі қолға алынбаған. Бұл осы ерекше өнімнің жоғары технологиялық өндірісінің жоқтығымен және оған деген сұраныстың төмендігімен түсіндіріледі. Дегенмен, ПТР диагностикасы мен геномдық технологиялар Қазақстан үшін жаңалық емес, сол себепті термотұрақты полимеразаларға деген сұраныс өсуде.

Рекомбинантты Pfu ДНҚ полимераза *E. coli* ішек таяқшасы бактериясынан тазартылып алынды. Pfu ДНҚ полимераза ақуызын кодтайтын гені клондалған экспрессиялық вектор стандартты генетикалық инженерия әдістері арқылы жасалды. Рекомбинантты ақуыз N-ұшынан қосымша 20 амин қышқылына және 92.3 кДа молекулалық салмаққа ие. Осы жұмыстың нәтижесі рекомбинантты Pfu ДНҚ полимераза аналогымен жасалған ПТР арқасында, әртүрлі ұзындықтағы ДНҚ тізбектері өнім ретінде амплификацияланатынын көрсетті. Бұған қоса, термотұрақтылықты тексеруге арналған тәжірбиелер жасалды, фермент өзінің полимеразды белсендігін бірнеше сағат қыздырудан кейін де сақтап қалды.

Кілтті сөздер: Pfu ДНҚ полимераза, полимеразды тізбекті реакция, *Pyrococcus furiosus,* рекомбинантты ақуыз.