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(54) Title: FUSION SINGLE-STRANDED DNA POLYMERASE BST, NUCLEIC ACID MOLECULE ENCODING FUSION DNA POLYMERASE NEQSSB-BST, METHOD OF PREPARATION AND UTILISATION THEREOF

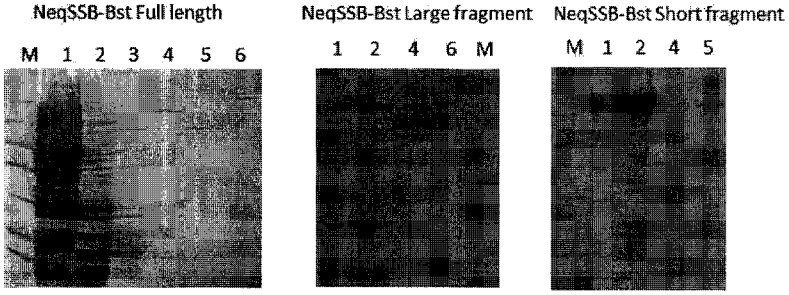

Fig.1

(57) Abstract: The subject of the invention is the fusion single-stranded DNA polymerase Bst linked with NeqSSB protein at the N-end of the polymerase using the linker consisting of six amino acids with the amino acid sequence Gly-Ser-Gly-Gly-Val-Asp, wherein the given polymerase is present in three different variants, and the preparation method thereof. Moreover, the subject of the invention is the nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst Full Length, Large Fragment, Short Fragment and their utilisation.

WO 2020/005084 A1

Fusion single-stranded DNA polymerase Bst, nucleic acid molecule encoding fusion DNA polymerase NeqSSB-Bst, method of preparation and utilisation thereof

The subject of the present invention are the fusion single-stranded DNA polymerases Bst and the method of preparation thereof. The subject of the invention is also the nucleic acid molecule encoding the fusion polymerase NeqSSB-Bst according to the one of three variants of *Bst* polymerase: *Full Length, Large Fragment, Short Fragment*, and utilisation of the fusion DNA polymerases for isothermal amplification reactions.

DNA polymerases are the enzymes which play an essential role in the process of DNA replication and repair. They are widely used in various fields of science and are successfully utilised in sequencing or various PCR (Polymerase Chain Reaction) variants, where they catalyse the DNA synthesis processes *in vitro*, and this reaction is conducted in cycles with precisely defined thermal stages. Another and increasingly popular approach is the utilisation of the DNA polymerases in the isothermal techniques of DNA amplification which are not based on thermocycles and the reaction is conducted under constant temperature of elongation. Up to date, many such techniques have been developed for both DNA amplification and RNA amplification. The selection of the appropriate polymerase for a given technique depends mainly on its properties. Besides the basic polymerisation capabilities, the polymerases can also show the ability to hydrolyse DNA molecules thanks to the presence of the exonucleolytic domain or the reverse transcriptase activity. These characteristics are determined by the presence of the respective domains. The basic domains present in these enzymes are the polymerisation domain and 3'-5' and 5'-3' exonucleolytic domain. There are polymerases present where the deletion of the exonucleolytic domain leads to the obtaining of a functional protein with partially altered characteristics comparing with the native enzyme. The most popular polymerase of this type is *Taq* polymerase isolated from *Thermus aquaticus* bacterium, the discovery of which diametrically transformed the molecular biology. Without the 5'-3' exonuclease activity the *Taq*289 polymerase displays the increased thermostability, while it requires more Mg^{2+} ions and newly formed DNA strand contains fewer errors. The *Bst* polymerase is used for the isothermal amplification techniques. Its native form contains the non-active 3'-5' exonucleolytic domain and active 5'-3' exonucleolytic domain which activity can be disabled with a point mutation in position 73 ($Tyr^{73} \rightarrow Phe^{73}$ and $Tyr^{73} \rightarrow Ala^{73}$). This polymerase as well as *Taq* polymerase is the part of family A and is isolated from bacterium *Bacillus stearothermophilus*. Its optimum activity is about 60°C and without the exonuclease

activity the polymerase exhibits the strand displacement activity which is highly useful in Loop Mediated Isothermal Amplification (LAMP) reaction. The polymerase has an increased tolerance to clinical or environmental inhibitors comparing with other polymerases of this family, but still, taking into account the applications of this polymerase, it is important to seek the solutions which would lead mainly to the improvement of its processivity and the resistance to inhibitors.

NeqSSB protein is the member of Single-Stranded DNA Binding (SSB) protein family. The SSB proteins have various amino acid sequences and structures. However, they still contain one characteristic highly conserved Oligonucleotide/Oligosaccharide Binding (OB) Fold Domain consisting of about 100 amino acids. This domain is widely present in proteins exhibiting ssDNA-binding capability, and thus determines its basic characteristic common for all SSB proteins – non-specific binding of single-stranded DNA and, discovered far later, RNA binding capability. The SSB proteins play a key role in the processes closely associated with ssDNA. They are crucial in replication, recombination, and DNA repair. These proteins are responsible for the interactions with the single-stranded DNA, prevent the generation of secondary structures and protect against the degradation by nucleases.

The discovery of SSB proteins is dated for the first half of 1960s. The first SSB protein to be discovered are SSB proteins of T4 phage and *E. coli*. During the discovery, their high capabilities to interact with ssDNA interaction and to elute the protein using ssDNA-cellulose beads with high salt concentration (2 M NaCl) were elucidated. Additionally, the very high selectivity of this protein for the single-stranded DNA was also found out. The confirmation of the fundamental role of SSB proteins in the processes related to ssDNA is the fact that these proteins are present in all living organisms as well as the viruses.

The binding of SSB proteins with ssDNA is based on the packing of the aromatic amino acid residues between the residues of the oligonucleotide chain. Moreover, the positively-charged amino acid residues interact with the phosphate backbone of the ssDNA molecule.

Despite the fact that the NeqSSB protein belongs to the SSB family of proteins, it deviates with its characteristics from classical SSB proteins, hence it is referred to as NeqSSB-like protein. The protein originates from the hyperthermophilic archaeon *Nanoarchaeum equitans,* the parasite of craenarchaeon *Ignicoccus hospitalis*. The optimum growth conditions for this microorganisms require strictly anaerobic conditions and temperature of 90°C. Interestingly, *Nanoarchaeum equitans* contains the smallest known genome consisting of 490,885 base pairs. In contrast with most of known organisms with reduced genomes, this

microorganism contains full set of enzymes taking part in replication, repair, and DNA recombination, including SSB protein.

The *Neq*SSB protein, as well as other proteins of this family, has the natural ability to bind DNA. It consists of 243 amino acid residues, and contains one OB domain in its structure and is biologically active as a monomer, similarly as in case of some viral SSB proteins. The reports show that comparing with other SSB proteins *Neq*SSB protein exhibits unusual capabilities concerning binding of all DNA forms (ssDNA, dsDNA), and mRNA without structural preferences. Moreover, the protein shows high thermostability. The half-life while maintaining the biological activity is 5 min in 100°C, while the melting temperature is 100.2°C.

To meet the requirements posed by modern diagnostics, molecular biology, or genetic engineering, it is necessary to improve the DNA polymerases to provide useful features in these fields of science. The modification introduced so far focus mainly on the introduction of the improved buffers, amplification reaction enhancers, or mutation of the DNA polymerases. The mutations lead to the obtaining of the enzymes with the increased thermostability and resistance to the inhibitors present in the clinical or environmental samples.

The DNA polymerase mechanism of action includes several significant steps. The first one consists of the attachment of the enzyme to the DNA matrix. Obtained DNA-DNA complex associates the respective dNTPs (deoxyribonucleotide triphosphates) as the result of the nucleophilic attack of the 3' OH end on the nucleotide phosphorus atom. The last step leads to the generation of the phosphodiester bond and the release of the pyrophosphate.

One of the important stages of the polymerising actions of these enzymes, which is responsible for their final efficiency, is the initiation process related with the biding to the matrix DNA. Due to that reason the modification of the known polymerases is justified to facilitate the binding to the DNA strand subjected to polymerisation. An example of such an modification can be the generation of fusion DNA polymerases with proteins which exhibit a natural ability to bind single- and/or double-stranded DNA. The literature presents only several examples of such fusion DNA polymerases with the majority of them being fusions with thermostable enzymes used mainly for the Polymerase Chain Reactions.

The studies suggest that the fusion of the *Taq, Pfu, Tpa,* or KOD DNA polymerase with the DNA-binding protein *Sso*7d from hyperthermophilic archaeon *Sulfolobus solfataricus,* lead to the 5 to 17 times increase in the polymerase processivity. Similarly, the increase in the processivity and fidelity of the DNA polymerase of *RB69* bacteriophage was observed after fusion with its indigenous SSB protein (*RB69*SSB) which binds single-stranded DNA.

The European Patent EP 1 934 372 B1 discloses the DNA polymerase of *Thermococcus zilligi* fused with *Sso*SSB protein of archaeon *Sulfolobus solfataricus* indicating the increase in the efficiency and the processivity of the modified enzyme.

Additionally, the fusion of *TaqStoffel* polymerase with *Neq*SSB protein capable of binding to the all types of DNA and with DBD domain of *P. furiosus* ligase was recently reported. Both fusions lead to the improvement in the functional features of enzymes, especially improved the processivity and thermostability of the native enzyme and significantly increased its tolerance to the clinical inhibitors (lactoferrin, heparin, whole blood). Small number of fusions of polymerases such as *Bst* and□□29 used in the isothermal reactions was also conducted. They are connected via HhH (Helix-hairpin-helix) domain of the topoisomerase V of *Methanopyrus kandleri,* what increased the affinity of the polymerase to DNA without a negative influence on the strand displacement activity (in fusion polymerases Bst and □29). Moreover, higher fidelity and amplification efficiency using plasmid and genome DNA was observed (in case of □29).

The literature presents also the fusion of *Bst*-like polymerase isolated from Geobacillus sp. 777. The chimers of the polymerase with DBD domain of the ligase *Pyrococcus abyssi* and Sto7d protein were generated and exhibited an increase in the processivity and the resistance to inhibitors (urea, whole blood, heparin, EDTA, NaCl, and ethanol) in comparison with the native polymerase.

The purpose of the present invention is to provide a fusion DNA polymerase *Bst* with *Neq*SSB protein binding to all types of DNA and RNA. Surprisingly, the problem was solved to a high extent in the present invention.

The subject of the present invention is a fusion DNA polymerase *Bst* with *Neq*SSB protein binding all types of DNA and RNA. Three *Bst* polymerase variants were subjected to the modifications: Full length – whole amino acid sequence of DNA I *Bst* polymerase with the disabled 5'-3' activity thanks to the point mutation; Large Fragment – DNA I *Bst* polymerase without 5'-3' domain and Short Fragment – short version with the deletion of both exonucleolytic domains. All variants of the *Bst* polymerase were fused with the *Neq*SSB protein by the polymerase N-end using a linker consisting of six amino acids.

The essence of the invention is the fusion polymerase of single-stranded DNA polymerase Bst or another polymerase of this class of DNA polymerases connected with

*Neq*SSB protein or a protein with a sequence similar to NeqSSB in the degree not higher than 50% at N-end of the polymerase using a linker of an exemplary amino acid sequence Gly-Ser-Gly-Gly-Val-Asp or fused directly without a linker, wherein the mentioned polymerase is present in three different variants.

Fusion DNA polymerase NeqSSB-Bst containing one of the three *Bst* polymerase variants:

- Full length – whole amino acid sequence of DNA polymerase I *Bst* with disabled 5'-3' activity thanks to the point mutation;

- Large Fragment – DNA polymerase I *Bst* without 5'-3' domain;

- Short Fragment – short version with the deletion of both exonucleolytic domains

Fusion DNA polymerase NeqSSB-Bst which binds to all types of DNA and RNA.

Fusion DNA polymerase NeqSSB-Bst with the sequence presented in SEQ1.

Fusion DNA polymerase NeqSSB-Bst with the sequence presented in SEQ2.

Fusion DNA polymerase NeqSSB-Bst with the sequence presented in SEQ3.

Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst Full Length presented in SEQ 4.

Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst Large Fragment presented in SEQ 5.

Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst Short Fragment presented in SEQ 6.

Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst defined above.

The preparation method of fusion DNA polymerase NeqSSB-Bst defined above, where:

- first step includes the expression of the gene encoding the enzyme in the optimised conditions in microbiological shaker: growth temperature 28-37°C, incubation time of the medium after induction – 3-20 h, inductor concentration – 0.1-1 mM IPTG,
- obtained cell lysate is subjected to the disintegration using ultrasound and elimination of the DNA genomic contamination using dsDNase.

- second purification step utilises the metal affinity chromatography with His-Trap beads,
- next steps cover triple dialysis (10 mM Tris-HCl pH 7.1, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 0.1% Triton X-100), gel filtration, and concentration of the preparation.
- all processes were conducted in 4°C,
- the purity of the obtained proteins was tested using SDS-PAGE electrophoresis, and the number of units for the obtained preparation was determined using EvaEZ Fluorometric Polymerase Activity Assay Kit.

in vitro utilisation of the fusion single-stranded DNA polymerase Bst defined above for the isothermal amplification reactions.

Description of the sequences and Figures:

Seq. 1 – presents the amino acid sequence of the fusion polymerase NeqSSB-Bst Full length

Seq. 2 – presents the amino acid sequence of the fusion polymerase NeqSSB-Bst Large Fragment

Seq. 3 – presents the amino acid sequence of the fusion polymerase NeqSSB-Bst Short Fragment

Seq. 4 – presents the sequence of the gene encoding fusion DNA polymerase NeqSSB-Bst Full Length

Seq. 5 – presents the sequence of the gene encoding fusion DNA polymerase NeqSSB-Bst Large Fragment

Seq. 6 – presents the sequence of the gene encoding fusion DNA polymerase NeqSSB-Bst Short Fragment

Fig. 1 – presents the electrophoretic 10% polyacrylamide gel separation of proteins from individual stages of the fusion DNA polymerase purification

M – Protein mass marker (Thermo-Fischer Scientific) with the masses of the standard proteins: 116; 66,2; 45; 35; 25; 18.4; 14.4 kDa;

1 – whole cell-free extract of the recombinant *Escherichia coli* TOP10F'-pETNeqSSB-Bst strain;

2 – whole cell-free extract subjected to the preliminary thermal denaturation

3 – fraction not bound with His-Trap column;

4 – wash fraction of His-Trap beads containing 40 mM imidazole

5 – wash fraction of His-Trap beads containing 100 mM imidazole

6 – collected fraction containing fusion DNA polymerase after elution using 500 mM imidazole

Fig. 2 – presents the charts concerning dependence of the EvaGreen dye fluorescence on time starting from the DNA amplification for the fusion DNA polymerases which enables the calculation of the number DNA polymerase units. The legend assigns the amounts of used DNA polymerase for the reaction in microliters to the curves.

Fig. 3 – presents the electrophoretic 10% polyacrylamide gel separation of lysates for various expression conditions

M – Protein mass marker (Thermo-Fischer Scientific) with the masses of the standard proteins: 116; 66,2; 45; 35; 25; 18.4; 14.4 kDa;

1 – whole cell-free extract of the recombinant *Escherichia coli* TOP10F'-pETNeqSSB-Bst strain before induction;

2 – whole cell-free extract 3 h after induction with 1 mM IPTG, expression conducted in 28°C;

3 – whole cell-free extract 4 h after induction with 1 mM IPTG, expression conducted in 28°C

4 – whole cell-free extract 5 h after induction with 1 mM IPTG, expression conducted in 28°C

5 – whole cell-free extract 6 h after induction with 1 mM IPTG, expression conducted in 28°C

6 – whole cell-free extract 20 h after induction with 1 mM IPTG, expression conducted in 28°C

7 – whole cell-free extract 3 h after induction with 0.1 mM IPTG, expression conducted in 28°C;

8 – whole cell-free extract 4 h after induction with 0.1 mM IPTG, expression conducted in 28°C

9 – whole cell-free extract 5 h after induction with 0.1 mM IPTG, expression conducted in 28°C

10 – whole cell-free extract 6 h after induction with 0.1 mM IPTG, expression conducted in 28°C

11 – whole cell-free extract 20 h after induction with 0.1 mM IPTG, expression conducted in 28°C

12 – whole cell-free extract of the recombinant *Escherichia coli* TOP10F'-pETNeqSSB-Bst strain before induction;

13 – whole cell-free extract 3 h after induction with 1 mM IPTG, expression conducted in 37°C;

14 – whole cell-free extract 4 h after induction with 1 mM IPTG, expression conducted in 37°C

15 – whole cell-free extract 5 h after induction with 1 mM IPTG, expression conducted in 37°C

16 – whole cell-free extract 6 h after induction with 1 mM IPTG, expression conducted in 37°C

17 – whole cell-free extract 20 h after induction with 1 mM IPTG, expression conducted in 37°C

18 – whole cell-free extract of the recombinant *Escherichia coli* TOP10F'-pETNeqSSB-Bst strain before induction;

19 – whole cell-free extract 3 h after induction with 0.1 mM IPTG, expression conducted in 37°C;

20 – whole cell-free extract 4 h after induction with 0.1 mM IPTG, expression conducted in 37°C

21 – whole cell-free extract 5 h after induction with 0.1 mM IPTG, expression conducted in 37°C

22 – whole cell-free extract 6 h after induction with 0.1 mM IPTG, expression conducted in 37°C

23 – whole cell-free extract 20 h after induction with 0.1 mM IPTG, expression conducted in 37°C

Fig. 4 – shows the graphs presenting the change in the activity of the fusion DNA polymerases with the increase in temperature comparing with the reference DNA polymerase I Bst. The blue line presents the result of DNA polymerase I Bst, the red line for fusion DNA polymerase Bst Full length, violet line for fusion DNA polymerase Bst Large Fragment, and the green line

for the fusion DNA polymerase Bst Short Fragment. The activity is described using the GelAnalyzer program based on the intensity of the obtained PCR products in an agarose gel.

Fig. 5 – shows the electrophoretic separation in 1.5% agarose gel with ethidium bromide presenting the comparison of the processivity of DNA polymerases defined as the amplification rate during isothermal PCR. The reactions were conducted in various periods of time what is indicated over the lines.

Fig. 6 – shows the electrophoretic separation in 1.5% agarose gel presenting the comparison of the DNA polymerase resistance to the inhibitors: blood lactoferrin (A), soil polyphenols (B).

A:

A: 1 – the reaction product generated as the result of the DNA amplification with added 6 μg of lactoferrin

2 – the reaction product generated as the result of the DNA amplification with added 0.6 μg of lactoferrin

3 – the reaction product generated as the result of the DNA amplification with added 0.06 μg of lactoferrin

4 – the reaction product generated as the result of the DNA amplification with added 6 ng of lactoferrin

K+ reaction product generated during DNA amplification without the addition of an inhibitor.

B:

1 – the reaction product generated as the result of the DNA amplification with added 100 μg of polyphenols

2 – the reaction product generated as the result of the DNA amplification with added 10 μg of polyphenols

3 – the reaction product generated as the result of the DNA amplification with added 1 μg of polyphenols

4 – the reaction product generated as the result of the DNA amplification with added 0.1 μg of polyphenols

5 – the reaction product generated as the result of the DNA amplification with added 0.01 μg of polyphenols

K+ reaction product generated during DNA amplification without the addition of an inhibitor.

Fig. 7 – shows the electrophoretic separation in 2% agarose gel with ethidium bromide presenting the results of the DNA electrophoretic mobility shift assay in the presence of the fusion DNA polymerases. The reaction mixture contained 10 pmol (dT_{76}) of fluorescein-labelled (green) and 2.5 pmol PCR product of 100 bp (orange)

1 – $d(T)_{76}$

2 – 100 bp

3 – $d(T)_{76}$+ 100 bp + 3.3 pmol of the fusion DNA polymerase

4 – $d(T)_{76}$+ 100 bp + 6.6 pmol of the fusion DNA polymerase

5 – $d(T)_{76}$+ 100 bp + 13.2 pmol of the fusion DNA polymerase

6 – $d(T)_{76}$+ 100 bp + 26.4 pmol of the fusion DNA polymerase

7 – $d(T)_{76}$+ 100 bp + 52.8 pmol of the fusion DNA polymerase

8 – $d(T)_{76}$+ 100 bp + 105.6 pmol of the fusion DNA polymerase

9 – $d(T)_{76}$+ 100 bp + 211.2 pmol of the fusion DNA polymerase

The invention is illustrated by the embodiment, including but not limited to.

Example:

Fusion DNA polymerase NeqSSB-Bst

Fusion DNA polymerases *Neq*SSB-*Bst* were obtained by fusion of three various *Bst* polymerases with *NeqSSB* protein at polymerase N-end using the linker consisting of six amino acids of the following sequence: Gly-Ser-Gly-Gly-Val-Asp. The sequences of the three variants of fusion DNA polymerase are presented in the figure SEQ. 1-3 (amino acid sequences) and SEQ. 4-6 (nucleotide sequences). DNA polymerases were obtained in the laboratory scale in the prokaryotic system based on *Escherichia coli* bacteria.

Preparation – example 1

First step of the DNA polymerase preparation includes the expression of the gene encoding the enzyme in the optimised conditions in microbiological shaker: growth temperature – 30°C, incubation time of the medium after induction – 3 to 20 h, inductor concentration – 0.1 to 1 mM IPTG. In the protein purification process the obtained cell lysate is

subjected to the disintegration using ultrasound and removal of the DNA genomic contamination using dsDNase. Thanks to the presence of the oligohistidine domain, the second purification step utilises the metal affinity chromatography with His-Trap beads (Fig. 1). The next steps cover triple dialysis until obtaining the conditions which provide the stability for DNA polymerase (10 mM Tris-HCl pH 7.1, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 0.1% Triton X-100), gel filtration, and densification of the preparation. All the processes were conducted in 4°C. The purity of the obtained proteins was tested using SDS-PAGE electrophoresis, and the number of units for the obtained preparation was determined using EvaEZ Fluorometric Polymerase Activity Assay Kit Biotium (USA) according to the definition of unit: 1 activity unit [1 U] is the amount of DNA polymerase which can incorporate 10 nmol of nucleotides in 30 min in its optimum operation temperature 65°C (Fig. 2). 1 litre of the laboratory scale culture provides approx. 5 mg of purified preparation with the activity of approx. 10 000 U, what enables the respective number of amplification reactions.

Preparation – example 2

Expression of the gene encoding the fusion DNA polymerase was conducted in the conditions providing appropriate oxygenation of the liquid culture in the temperature of 28°C. Logarithmic-phase cultures were induced using IPTG with the amount providing the protein expression – IPTG in the range of 1 mM to 0.1 mM and incubation of 3 to 20 hours (Fig. 3). After that the cell lysate was mechanically disintegrated and purified using metal affinity chromatography and ion-exchange chromatography. The obtained fusion DNA polymerases were subjected to the dialysis for the storage conditions (10 mM Tris-HCl pH 7.1, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 0.1% Triton X-100) and provided in the concentration of 1 U/µL based on the commercial EvaEZ Fluorometric Polymerase Activity Assay Kit of Biotium (USA) according to the definition of unit.

Preparation – example 3

An efficient expression of the gene encoding the polymerase Bst fused with NeqSSB protein was obtained in culture in 37°C and induction of IPTG in range of 1 mM to 0.1 mM for 3 to 20 hours (Fig. 3). Centrifuged and mechanically disrupted cell lysate was purified using chromatographic techniques (metal affinity chromatography and ion-exchange chromatography), suspended in the formulation buffer (10 mM Tris-HCl pH 7.1, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 0.1% Triton X-100) and provided in the concentration of

1 U/µL. The amount of DNA units was defined based on the definition of unit using the EvaEZ Fluorometric Polymerase Activity Assay Kit of Biotium (USA).

The comparative analysis of the properties of the enzymes being the subject of the invention with the reference DNA polymerase *Bst* have shown that the presence of an additional DNA-binding NeqSSB protein has a positive impact on the DNA polymerase properties. The thermostability of the all obtained fusion variants of the DNA polymerases in comparison with the reference DNA polymerase *Bst* was increased by approx. 20% (Fig. 4). Moreover, the DNA polymerases in fusion with *Neq*SSB protein shown the threefold increase of processivity (Fig. 5). The fusion DNA polymerases tolerate the concentration of clinical (lactoferrin, heparin) and environmental (humic acid, soil, polyphenols) inhibitors in the reaction mixtures increased even by several dozen of times comparing with the reference polymerase (Fig. 6). The fusion DNA polymerases exhibited increased sensitivity by several times and thus increased affinity to the DNA matrix comparing with the reference DNA polymerase *Bst*.

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Claims:

1. Fusion polymerase of single-stranded DNA polymerase Bst or another polymerase of this class of DNA polymerases connected with *Neq*SSB protein or a protein with a sequence similar to NeqSSB in the degree not higher than 50% at N-end of the polymerase using a linker of an exemplary amino acid sequence Gly-Ser-Gly-Gly-Val-Asp or fused directly without a linker, wherein the mentioned polymerase is present in three different variants.
2. Fusion DNA polymerase NeqSSB-Bst according to the claim 1 **characterised in that** it contains one of the three variants of *Bst* polymerase:
   - Full length – whole amino acid sequence of DNA polymerase I *Bst* with disabled 5'-3' activity thanks to the point mutation;
   - Large Fragment – DNA polymerase I *Bst* without 5'-3' domain;
   - Short Fragment – short version with the deletion of both exonucleolytic domains.
3. Fusion DNA polymerase NeqSSB-Bst according to the claims 1 to 2 **characterised in that** it binds to all types of DNA and RNA.
4. Fusion DNA polymerase NeqSSB-Bst according to the claims 1 to 3 **characterised in that** it contains the sequence presented in SEQ1.
5. Fusion DNA polymerase NeqSSB-Bst according to the claims 1 to 3 **characterised in that** it contains the sequence presented in SEQ2.
6. Fusion DNA polymerase NeqSSB-Bst according to the claims 1 to 3 **characterised in that** it contains the sequence presented in SEQ3.
7. Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst Full Length presented in SEQ 4.
8. Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst Large Fragment presented in SEQ 5.
9. Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst Short Fragment presented in SEQ 6.
10. Nucleic acid molecule encoding the fusion DNA polymerase NeqSSB-Bst according to any of the claims 7 to 9.
11. The preparation method of the fusion DNA polymerase NeqSSB-Bst defined in the claim 1 characterised in that:

- first step includes the expression of the gene encoding the enzyme in the optimised conditions in microbiological shaker: growth temperature 28-37°C, incubation time of the medium after induction – 3-20 h, inductor concentration – 0.1-1 mM IPTG,
- obtained cell lysate is subjected to the disintegration using ultrasound and elimination of the DNA genomic contamination using dsDNase.
- second purification step utilises the metal affinity chromatography with His-Trap beads,
- next steps cover triple dialysis (10 mM Tris-HCl pH 7.1, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 0,1% Triton X-100), gel filtration, and concentration of the preparation.
- all processes were conducted in 4°C,
- the purity of the obtained proteins was tested using SDS-PAGE electrophoresis, and the number of units for the obtained preparation was determined using EvaEZ Fluorometric Polymerase Activity Assay Kit.

12. In vitro utilisation of the fusion single-stranded DNA polymerase defined in the paragraphs 1 to 6 in the isothermal amplification reactions.

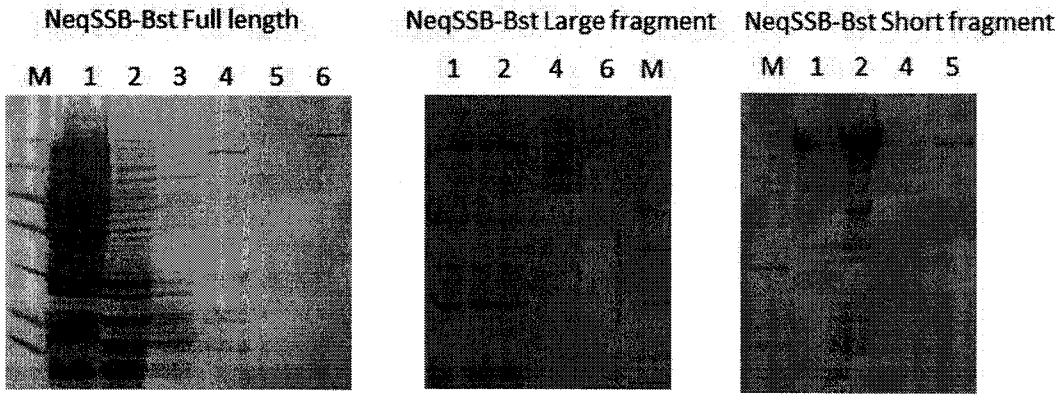

Fig.1

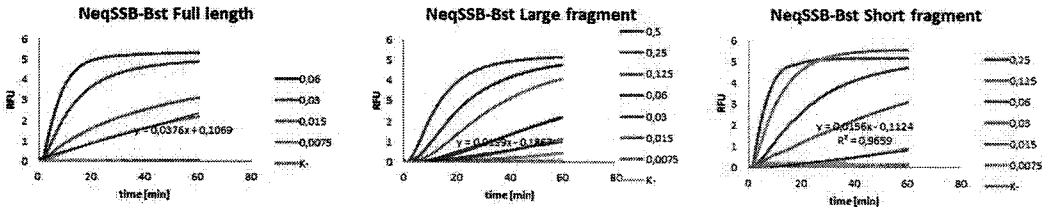

Fig.2

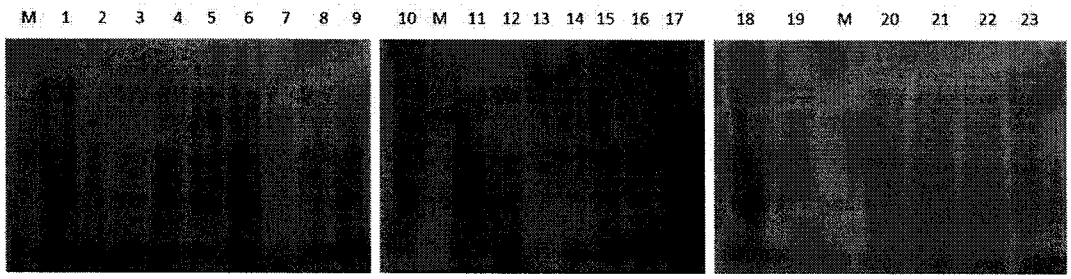

Fig.3

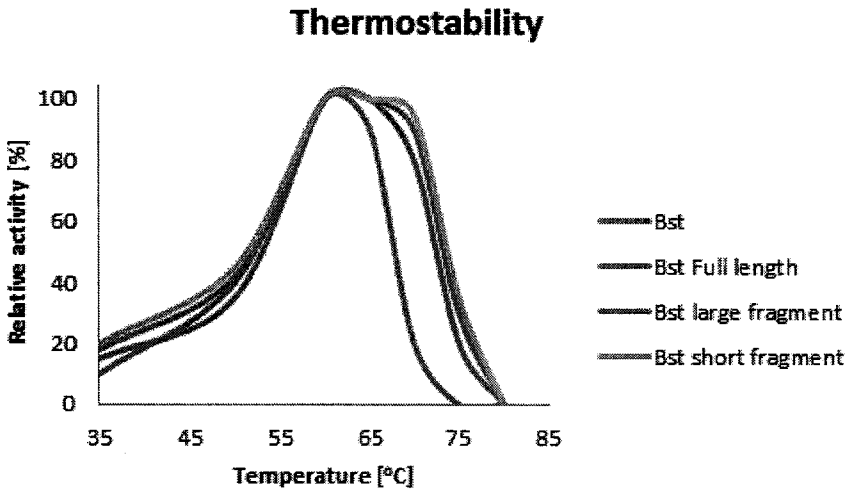

Fig.4

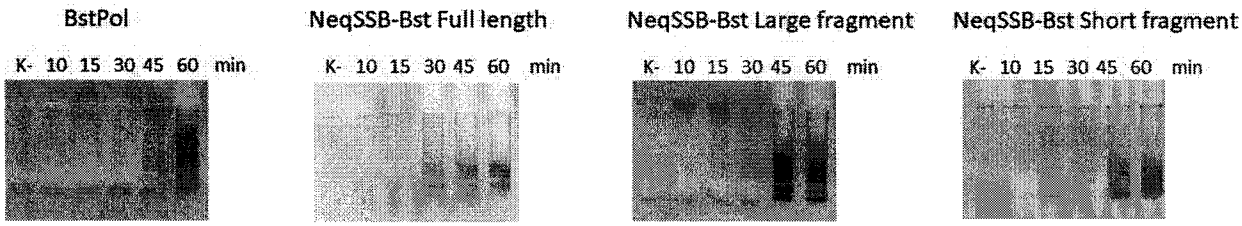

Fig.5

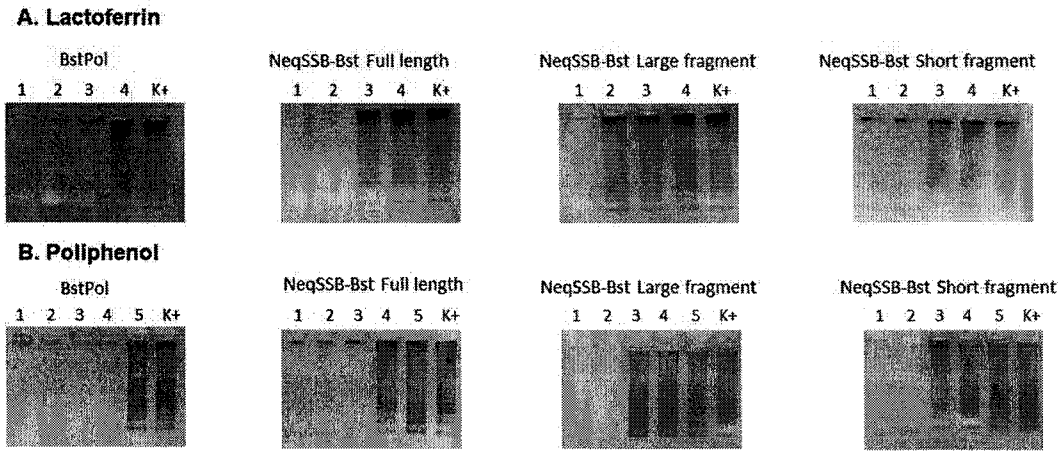

Fig.6

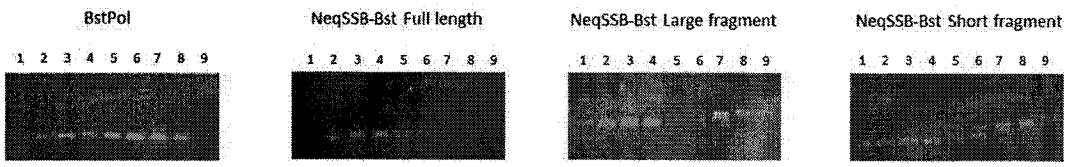
BstPol
1 2 3 4 5 6 7 8 9
NeqSSB-Bst Full length
1 2 3 4 5 6 7 8 9
NeqSSB-Bst Large fragment
1 2 3 4 5 6 7 8 9
NeqSSB-Bst Short fragment
1 2 3 4 5 6 7 8 9

Fig.7

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2019/000046

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/195 C12N9/12 C12N15/62 C12Q1/6844
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, Sequence Search, EMBASE, FSTA, INSPEC, IBM-TDB, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARCIN OLSZEWSKI ET AL: "Fusion of Taq DNA polymerase with single-stranded DNA binding-like protein of Nanoarchaeum equitans-Expression and characterization", PLOS ONE, vol. 12, no. 9, 1 September 2017 (2017-09-01), page e0184162, XP055626163, DOI: 10.1371/journal.pone.0184162 cited in the application	1,3,12
Y	the whole document pages 3, 13 page 2, paragraph 3 page 14, paragraph 2 page 4, paragraph 1 pages 4,5	2,4-11

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 2019	17/10/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Madruga, Jaime

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2019/000046

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 1 934 372 A2 (INVITROGEN CORP [US]) 25 June 2008 (2008-06-25) cited in the application the whole document paragraphs [[0004]], [[0018]], [[0027]]; claims; example 1-; table 1	1,3,12 2,4-11
Y	SPIBIDA MARTA ET AL: "Modified DNA polymerases for PCR troubleshooting", JOURNAL OF APPLIED GENETICS: AN INTERNATIONAL JOURNAL OF GENETICS AND BREEDING, SPRINGER, GERMANY, vol. 58, no. 1, 28 October 2016 (2016-10-28), pages 133-142, XP036137678, ISSN: 1234-1983, DOI: 10.1007/S13353-016-0371-4 [retrieved on 2016-10-28] the whole document	1-12
Y	WO 2010/091203 A2 (LUCIGEN CORP [US]; NELSON R M [US] ET AL.) 12 August 2010 (2010-08-12) the whole document pages 9-10; examples 2, 5	1-12
Y	SUN SIYANG ET AL: "Structure and enzymatic properties of a chimeric bacteriophage RB69 DNA polymerase and single-stranded DNA binding protein with increased processivity", PROTEINS: STRUCTURE, FUNCTION, AND BIOINFORMATICS, JOHN WILEY & SONS, INC, US, vol. 65, no. 1, 31 July 2006 (2006-07-31), pages 231-238, XP002543925, ISSN: 1097-0134, DOI: 10.1002/PROT.21088 the whole document	1-12
Y	WO 2007/050125 A2 (UNIV RICE WILLIAM M [US]; SHAMOO YOUSIF [US]; SUN SIYANG [US]) 3 May 2007 (2007-05-03) the whole document figure 1; examples	1-12

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INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2019/000046

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IGOR P. OSCORBIN ET AL: "Derivatives of Bst-like Gss-polymerase with improved processivity and inhibitor tolerance", NUCLEIC ACIDS RESEARCH, vol. 45, no. 16, 26 July 2017 (2017-07-26) , pages 9595-9610, XP055626293, ISSN: 0305-1048, DOI: 10.1093/nar/gkx645 cited in the application the whole document Results; figure 1	1-12
Y	Rebecca B. Kucera ET AL: "DNA-Dependent DNA Polymerases" In: "Current Protocols in Molecular Biology", 1 October 2008 (2008-10-01), John Wiley & Sons, Inc., US, XP055626429, ISSN: 1934-3639 DOI: 10.1002/0471142727.mb0305s84, the whole document page 3.5.16; table 3.5.1,	1-12
Y	T. J. KELLY ET AL: "Identification and characterization of a single-stranded DNA-binding protein from the archaeon Methanococcus jannaschii", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (PNAS), vol. 95, no. 25, 8 December 1998 (1998-12-08), pages 14634-14639, XP055626557, US ISSN: 0027-8424, DOI: 10.1073/pnas.95.25.14634 the whole document figure 1	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/PL2019/000046

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2010075382 A1	25-03-2010
		US 2014377810 A1	25-12-2014
		WO 2007029200 A2	15-03-2007
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		US 2013022980 A1	24-01-2013
		WO 2010091203 A2	12-08-2010
WO 2007050125 A2	03-05-2007	US 2011217737 A1	08-09-2011
		WO 2007050125 A2	03-05-2007