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Production of Bst polymerase for diagnosis of different infections using loop-mediated isothermal amplification

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Abstract

Introduction. The large fragment of DNA polymerase I from *Geobacillus stearothermophilus* GIM1.543 (Bst DNA polymerase) possesses 5'-3' DNA polymerase activity, 5'-3' displacement activity and high processivity. These properties make it possible to use Bst DNA polymerase in loop-mediated isothermal amplification (LAMP), which provides highly specific amplification of the target sequence and is used for rapid detection of agents causing human infectious diseases.

The **purpose** of the study was to produce a recombinant Bst polymerase enzyme in the bacterial expression system and to assess its properties for LAMP-based diagnostics of infectious diseases.

Materials and methods. Expression constructs carrying the Bst polymerase gene were obtained using genetic engineering techniques. Different *Escherichia coli* strains were used for protein expression. Metal-chelate and gel filtration chromatography techniques were used for protein purification. Catalytic characteristics of the enzyme were assessed in loop-mediated isothermal amplification reactions using AmpliSens® SARS-CoV-2-IT, AmpliSens® IAV-IT and AmpliSens® IBV-IT diagnostic systems designed for high-quality detection of SARS-CoV-2, influenza A virus (IAV) and influenza B virus (IBV) RNA, respectively.

Results. The offered protocol for production, extraction and purification of recombinant Bst polymerase makes it possible to produce the enzyme in the bacterial expression system using *E. coli* cells in a soluble form and reaching the yield up to 20% of the total cell mass. In LAMP reactions, the obtained enzyme demonstrates activity comparable with that of the commercial enzyme Bst 2.0 (NEB).

Conclusion. Considering the fast purification and production of the enzyme, the obtained recombinant Bst polymerase can be used in LAMP-based diagnostic kits.

Keywords: *polymerase, isothermal amplification, recombinant enzyme*

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Оригинальное исследование
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Получение Bst-полимеразы для диагностики различных инфекций методом петлевой изотермической амплификации

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Аннотация

Введение. Большой фрагмент ДНК-полимеразы I из *Geobacillus stearothermophilus* GIM1.543 (ДНК-полимераза Bst) обладает 5'-3'-ДНК-полимеразной активностью, 5'-3'-вытесняющей активностью и высокой процессивностью. Благодаря этим свойствам ДНК-полимераза Bst используется в петлевой изотермической амплификации (LAMP), которая обеспечивает амплификацию целевой последовательности с высокой специфичностью и применяется для быстрого обнаружения возбудителей инфекционных заболеваний человека.

Цель работы — получение рекомбинантного фермента Bst-полимеразы в бактериальной системе экспрессии и оценка его свойств в условиях LAMP для диагностики инфекционных заболеваний.

Материалы и методы. Методами генетической инженерии получали экспрессионные конструкции, несущие ген Bst-полимеразы. Экспрессию белка проводили в различных штаммах клеток *Escherichia coli*. Для получения очищенных препаратов белка использовали методы металл-хелатной и гель-фильтрационной хроматографии. Оценку каталитических свойств фермента проводили в реакциях петлевой изотермической амплификации в диагностических системах «АмплиСенс® SARS-CoV-2-IT», «АмплиСенс® IAV-IT» и «АмплиСенс® IBV-IT», предназначенных для качественного определения РНК SARS-CoV-2, вируса гриппа A (IAV) и вируса гриппа B (IBV) соответственно.

Результаты. Разработанный протокол наработки, выделения и очистки рекомбинантной Bst-полимеразы позволяет получать фермент в бактериальной системе экспрессии на основе клеток *E. coli* в растворимой форме с выходом до 20% от собранной клеточной массы. В реакциях LAMP полученный фермент демонстрирует активность, сопоставимую с коммерческим ферментом Bst 2.0 («NEB»).

Заключение. Полученная рекомбинантная Bst-полимераза, учитывая быстрый способ очистки и получения фермента, пригодна для применения в диагностических наборах на основе LAMP.

Ключевые слова: *полимераза, изотермическая амплификация, рекомбинантный фермент*

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Nucleic acid amplification methods are widely used for diagnostics of infectious diseases, for detection of single nucleotide substitutions in genotyping and for other clinical purposes. However, the conventional polymerase chain reaction (PCR) is a time-consuming process and requires expensive specialized equipment. The comparatively recent technique developed by Notomi et al. for rapid detection of nucleic acid bases is loop-mediated isothermal amplification (LAMP) demonstrating high sensitivity and specificity in amplification of the target DNA sequence at constant temperatures in any temperature-controlled equipment, requiring no thermal cycles and speeding up the process [1–4]. The LAMP technique is widely used for detection of agents of infectious diseases of viral etiology such as SARS-CoV-2 [5], influenza viruses and respiratory syncytial virus [6], hepatitis C virus [7], dengue virus DENV [8], human immunodeficiency virus HIV [9], and Ebola virus EVD [10].

Bst polymerase has been extensively used in isothermal amplification and has played a key role in the development of rapid clinical diagnosis of viral infections. DNA polymerase I from *Geobacillus stearothermophilus* [11] was first isolated in 1972 [12] and purified in 1982 [13]. The three-dimensional structure of the

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enzyme led to the conclusion that DNA polymerase I is comprised of 3 distinct domains [14]. Domain I is responsible for 5'-3' exonuclease activity; domain II is responsible for 5'-3' DNA polymerase activity; domain III resembles domains responsible for 3'-5' exonuclease activity in other polymerases [14]. Domains II and III are located at the C terminus of DNA polymerase I and are referred to as the large fragment (LF) [15]. The full-length DNA polymerase I gene from *Geobacillus stearothermophilus* consists of 2,628 base pairs and encodes a protein composed of 876 amino acids, with the weight of 98 kDa. The truncated Bst polymerase LF gene, which lacks the 5' terminus of 930 base pairs, encodes a protein of 587 aa, with weight of 64 kDa, and can be cloned with the C-terminal polyhistidine tag, with an estimated weight of 65 kDa. In this case, due to the presence of the hexahistidine tag at the C terminus of the gene, Bst polymerase lacks 5'-3' exonuclease activity and was demonstrated to be efficient in dNTP polymerization in PCR [16, 17]. In the meantime, there are works reporting cloning of Bst polymerase LF gene without a fusion tag to avoid misfolding of the recombinant protein [18].

Since Bst DNA polymerase has high processivity, polymerase and displacement activity, is able to remain active at 65ºC [17] for a long time, and is widely used in isothermal amplification for rapid detection of infectious diseases, the **purpose** of this study is to offer an efficient method of production and purification of Bst polymerase suitable for diagnostic application in LAMP.

Materials and methods

The study was performed using NdeI and XhoI restriction endonucleases (Thermo Scientific), pET16b+ (Merck Millipore), *E. coli* strains XL2, ER2566, BL21 (DE3) pLys, Rosetta (DE3) (Merck Millipore); the growth media included bacto tryptone, bacto agar, and yeast extract (Helicon); for protein extraction – tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), NaCl, imidazole, ethylenediaminetetraacetic acid (EDTA) disodium salt, glycerol (Panreac); PageRuler™ Prestained Protein Ladder, 10–250 kDa (Thermo Scientific).

Obtaining a plasmid expression vector containing a Bst polymerase gene

Oligonucleotide primers were used for amplification of the gene encoding Bst polymerase:
Bst-for 5'-GGTGGT**CATATG**CCGTCTTCTGAGGAAGAAAAGCCGCTG-3`;
Bst-rev 5'-GGTGGTTAA**CTCGAG**TTTCGCTCATACCAAGTAGAACCGTAGT-3',
containing recognition sites of NdeI and XhoI restriction enzymes, respectively (underlined).

The resulting amplicons were treated with NdeI and XhoI restriction endonucleases and cloned into plasmid vector pET16b+, which was pretreated with the same restriction endonucleases. As a result, we were able to construct the pET16-Bst-NHm4 expression vector. The correctness of the nucleotide sequence of the cloned gene was confirmed by sequencing.

Selection of E. coli strains for expression of the Bst-NHm4 gene

E. coli strains ER2566, BL21 (DE3) pLys, and Rosetta (DE3) were used as carrier strains for the constructed pET16-Bst-NHm4 vector. Transformed cells were seeded in the LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl) with agar containing 100 μg/ml of ampicillin for ER2566 cells and 20 μg/ml of chloramphenicol for BL21 (DE3) pLys and Rosetta (DE3) cells, and were grown for 14 hours at 37ºC to obtain colonies. Then, several colonies were transferred to 100 ml of the LB medium supplemented with 100 μg/ml of ampicillin and grown for 16 hours at 37ºC in a shaker at 180 rpm. The overnight culture of *E. coli* producer strains BL21 (DE3)pLys/pET16-Bst-NHm4, ER2566/pET16-Bst-NHm4 and Rosetta (DE3)/pET16- Bst-NHm4 was seeded in the LB medium supplemented with 100 μg/ml of ampicillin in Erlenmeyer flasks (the cell confluency was 2%) and grown at 37ºC at 160 rpm. When the optical density reached 0.8 AU, isopropyl-β-D1-thiogalactopyranoside was added to a concentration of 0.4 mM and the culture was grown at 23ºC and 37ºC for 4 hours and 24 hours. The optical density was measured spectrophotometrically at a wavelenght of 595 nm on a BioPhotometer Plus (Eppendorf). Cells were collected by centrifugation at 4,000 rpm for 20 min at 4°C using the Avanti JXN-30 centrifuge (Beckman Coulter).

Extraction of Bst-NHm4

The cell pellets (2 g) of the *E. coli* producer strain BL21 (DE3)pLys/pET16-Bst-NHm4 were resuspended in buffer containing 50 mM Tris-HCl, pH 8.5 with 1 mM PMSF at a ratio of 1:10 (w/v) and lysed by sonication using the Branson sonifier 250 (Branson Ultrasonics) for 20 min at 4ºC (the cycle – 0.5 sec, the amplitude – 50%). Then, they were centrifuged at 8,000 rpm for 30 min in the Allegra X-30R centrifuge (Beckman Coulter). After the centrifugation, the supernatant was diluted 2-fold with buffer containing 50 mM Tris-HCl, pH 8.5 (buffer A) and applied to the IMAC FF chromatography sorbent pre-equilibrated with the same buffer. Buffer A was used for removal of ballast proteins. The target protein was eluted with a liner gradient of buffer A with 500 mM imidazole.

Following the metal-chelate chromatography, the eluate was applied to the HiTrap column containing 5 ml of G-25 sorbent in buffer of 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 20% glycerol, pH 8.5.

Assessment of Bst-NHm4 activity

To assess the activity of the Bst polymerase obtained in LAMP, we used reagents of the AmpliSens®

SARS-CoV-2-IT kit (RU No. RZN 2021/14599); plasmid DNA containing the target fragment of the SARS-CoV-2 genome was used as a sample. The reaction mixture in a volume of 10 µl contained 5 µl of SARS-CoV-2 IT-mix, 4.2 µl of glycerol, thioglycerol, and fluorescent intercalating dye mixture, and 0.8 µl of tested Bst polymerase. Bst 2.0 DNA Polymerase enzyme (NEB; hereinafter referred to as Bst 2.0) was used as a reference enzyme; the reaction mixture contained 5 µl of IT-mix SARS-CoV-2, 4 µl of glycerol, thioglycerol, and fluorescent intercalating dye mixture, and 1 µl of Bst 2.0 polymerase. The prepared reaction mixtures were supplemented with 10 µl of plasmid DNA (at a concentration of 10^4 and 10^6 copies/ml). Each sample was represented in 2 repeats. The reaction was performed in the CFX 96 thermocycler (Bio-Rad Laboratories). The amplification program consisted of 50 cycles lasting 30 sec at 65ºC for 25 min with signal detection in the FAM channel.

Analytical methods

The protein concentration was measured using Qubit™ (Thermo Scientific); the purity of a protein was estimated using denaturating SDS-PAGE electrophoresis [19].

Reverse transcription loop-mediated isothermal amplification

To assess the possibility of using the received Bst polymerase in diagnostic testing systems, we performed real-time reverse transcription LAMP using reagents from AmpliSens® SARS-CoV-2-IT, AmpliSens® IAV-IT, and AmpliSens® IBV-IT kits designed for qualitative detection of SARS-CoV-2, IAV, and IBV RNA, respectively. Samples were represented by dilutions of viral RNA specimens from biological materials (nasopharyngeal and oropharyngeal swabs) at a concentration of 10^4–10^7 copies/ml.

The reaction mixture in a volume of 10 µl contained 5 µl of the pathogen-specific reagent of IT-mix, 4.2 µl of glycerol, thioglycerol, and fluorescent intercalating dye mixture, and 0.8 µl of Bst polymerase. The reference enzyme was represented by Bst 2.0 polymerase diluted to the concentration identical to that of the tested enzyme, 320 U/ml (1 unit of the enzyme catalyzes the incorporation of 25 nmol of dNTP into the product in 30 min at 65ºC). The reaction mixtures were supplemented with 10 µl of RNA. The reaction was performed in the DTprime thermocycler (DNA-Technology). The amplification program included preliminary incubation for 5 min at 37ºC and isothermal amplification for 25 min (50 cycles) at 65ºC with detection of a fluorescent signal every 30 sec in the FAM channel.

Licensed PCR-based reagent kits, AmpliSens® COVID-19-FL (RU No. RZN 2021/14599) and AmpliSens® Influenza virus A/B-FL (RU No. FSR 2009/05010) manufactured by the Central Research Institute of Epidemiology were used for the reference analysis of RNA samples.

Results

The unique synthetic sequence of the *Bst-pol* gene was developed to produce recombinant Bst polymerase. For this purpose, we initially performed reverse translation of the fragment of the amino acid sequence of DNA polymerase I from *Geobacillus stearothermophilus* (AAB52611) from 290 to 876 aa. Then, we optimized the nucleotide composition of the sequence with consideration for the frequency of codon occurrence for the bacterial expression system based on *E. coli* cells. The final gene sequence was obtained by assembly of long overlapping primers [20]. The gene encoding Bst-pol was cloned into the plasmid vector pET16b+ for prokaryotic expression in *E. coli.* During the selection of carrier strains for expression of the *Bst-NHm4* gene, we analyzed the changes in accumulation of the Bst-NHm4 enzyme at different temperatures (23ºC and 37ºC) and depending on the protein biosynthesis induction time (4 and 24 hours). Generally, the protein was effectively produced in *E. coli* carrier strains BL21 (DE3) pLys and Rosetta (DE3) in a soluble form; however, it was not produced in the carrier strain ER2566. Slightly higher biomass yields were achieved when the producer strain BL21 (DE3)pLys/ pET16- Bst-NHm4 was cultured at 23ºC for 4 hours (**Table 1)**.

During the first stage, metal-chelate affinity chromatography with an imidazole gradient was used to purify the enzyme from cellular proteins (**Fig. 1**). Fractions having purity of more than 90% (**Fig. 2**) were pooled and gel-filtration chromatography was performed. The resulting Bst-NHm4 enzyme was characterized using SDS-PAGE electrophoresis. The electrophoretic analysis showed that the purity of the enzyme was at least 95% (**Fig. 3**) at a concentration of 0.85 mg/ml.

The activity of the extracted Bst-NHm4 enzyme was verified by real-time LAMP using samples of plasmid DNA containing a fragment of the SARS-CoV-2 genome.

The test was performed using enzymes at a concentration of 320 U/ml. There were also two concentrations of DNA template: 10^2 and 10^4 copies (**Table 2**). **Fig. 4** clearly shows that the produced Bst_NHm4 polymerase demonstrates higher activity (lower Ct values) in the LAMP reaction with DNA samples compared to the reference enzyme Bst 2.0.

The activity of the Bst-NHm4 enzyme was verified by the analysis of SARS-CoV-2, IAV, and IBV RNA samples isolated from biological material (nasopharyngeal and oropharyngeal swabs) using reverse transcription LAMP and reagents from AmpliSens® SARS-CoV-2-IT, AmpliSens® IAV-IT, and AmpliSens® IBV-IT kits compared to the Bst 2.0 enzyme. For control purposes, RNA samples were analyzed using reverse transcription PCR with AmpliSens® COVID-19-

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Table 1. Content of the Bst-NHm4 enzyme in producer strains compared to total cell protein

E. coli carrier strain	Culture conditions	Protein content, %	Cell biomass yield, g/L
ER2566	23ºC, 4 h	Bst-NHm4 enzyme is not accumulated	
	23ºC, 24 h		
	37ºC, 4 h		
	37ºC, 24 h		
BL21 (De3) pLys	23ºC, 4 h	20,2	4,8
	23ºC, 24 h	19,8	4,5
	37ºC, 4 h	19,7	4,2
	37ºC, 24 h	18,9	4,9
Rosetta (De3)	23ºC, 4 h	19,5	5,1
	23ºC, 24 h	19,3	4,9
	37ºC, 4 h	18,8	4,5
	37ºC, 24 h	18,7	4,7

Note. The data were obtained using the Image Lab v. 5.2.1 software (Bio-Rad).

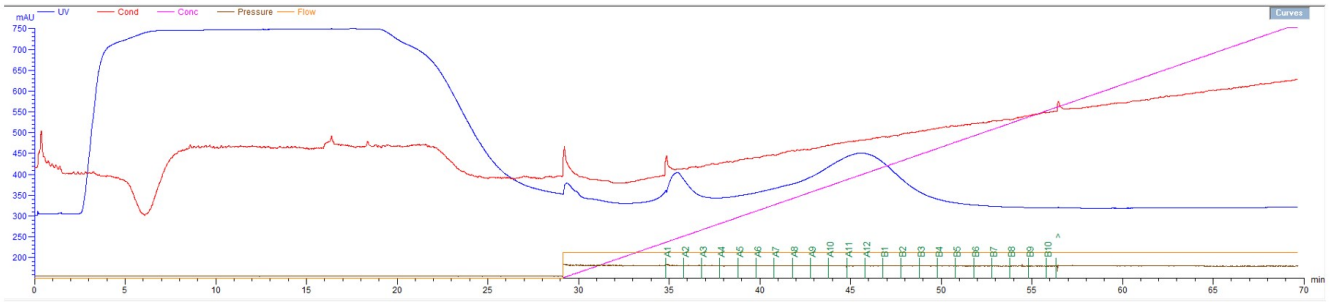

Fig. 1. Chromatographic profile of purification of the recombinant Bst-NHm4 enzyme using IMAC FF sorbent.

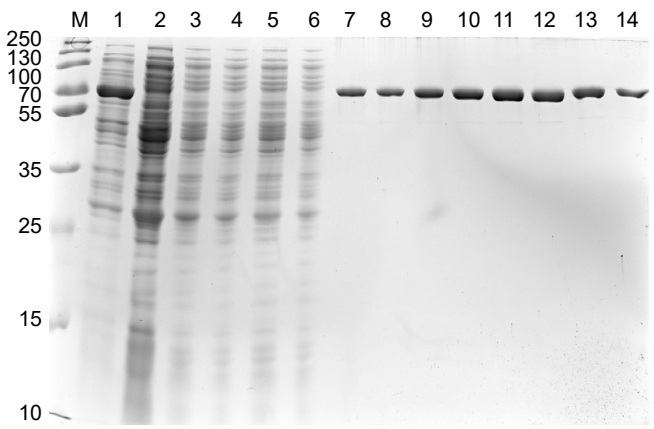

Fig. 2. Chromatographic purification of Bst-pol using IMAC FF sorbent.

M — molecular weight marker; *1* — clarified cell lysate of the producer strain BL21 (DE3)pLys/pET16-Bst-NHm4; *2* — ballast proteins; *3–14* — Bst-NHm4 fractions.

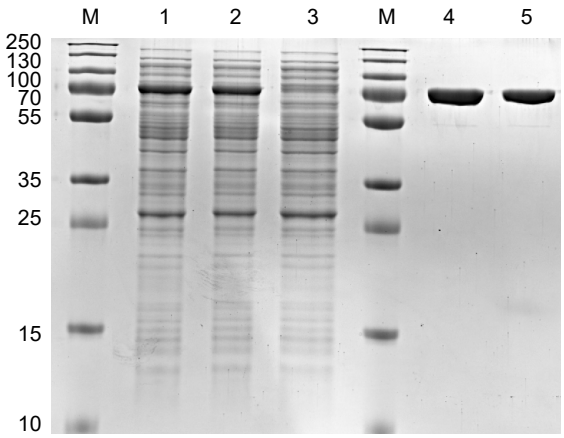

Fig. 3. Production and purification of Bst-NHm4.

M — molecular weight marker; *1* — total cell lysate; *2* — clarified cell lysate; *3* — cell sediment; *4* — total fraction of Bst-NHm4 after metal-chelate affinity chromatography; *5* — total fraction of Bst-NHm4 after gel-filtration chromatography.

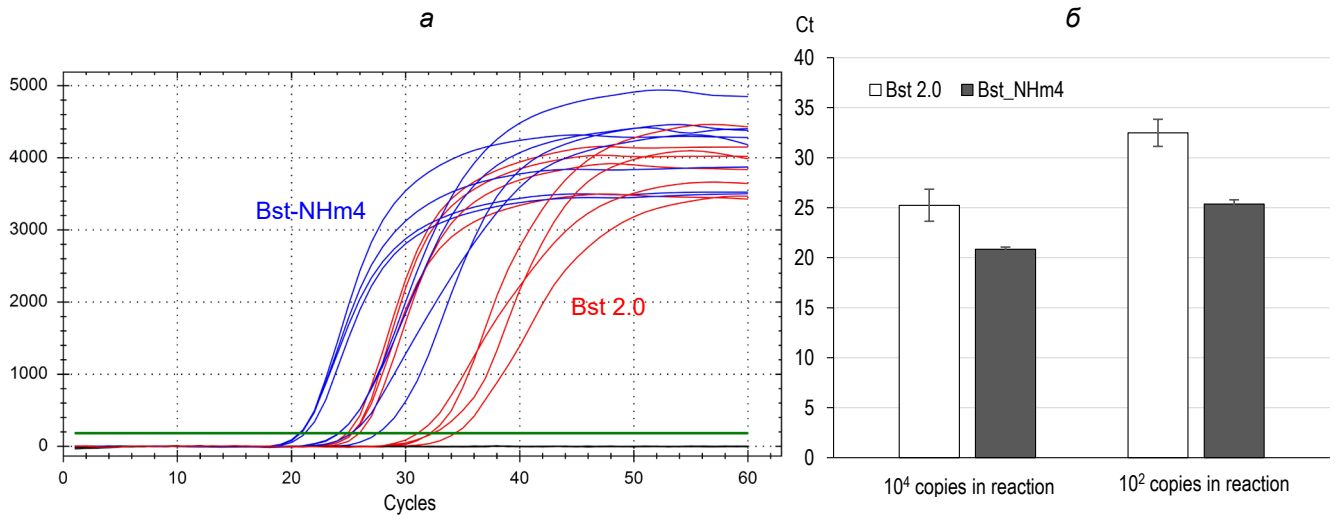

Fig. 4. Assessment of Bst-NHm4 polymerase activity in the model system.
a — product accumulation curves; *b* — mean cycle threshold value.

FL reagent kit or PCR with AmpliSens® Influenza virus A/B-FL reagent kit and cDNA that had been obtained using REVERTA-L reagent kit.

The Bst_NHm4 polymerase demonstrates the activity comparable to that of the Bst 2.0 polymerase in the LAMP reaction with SARS-CoV-2 RNA samples; however, its activity is lower (higher Ct values) with IAV and IBV RNA samples using respective reagent kits (**Fig. 5**). Despite the delay in passing of IBV RNA samples in reaction with the Bst-NHm4 polymerase

Table 2. Cycle threshold values using Bst-NHm4 in the LAMP reaction

Sample No.	Sample type	Bst polymerase	Ct values (FAM)
1	Control	Bst 2.0	–
2	Control	Bst-NHm4	–
	Number of copies		
3	10^2	Bst 2.0	30,1
4	10^2	Bst 2.0	32,3
5	10^2	Bst 2.0	31,0
6	10^2	Bst 2.0	33,4
7	10^2	Bst-NHm4	22,0
8	10^2	Bst-NHm4	22,4
9	10^2	Bst-NHm4	24,1
10	10^2	Bst-NHm4	24,9
11	10^4	Bst 2.0	22,2
12	10^4	Bst 2.0	22,8
13	10^4	Bst 2.0	24,0
14	10^4	Bst 2.0	24,3
15	10^4	Bst-NHm4	20,2
16	10^4	Bst-NHm4	19,8
17	10^4	Bst-NHm4	20,9
18	10^4	Bst-NHm4	22,1

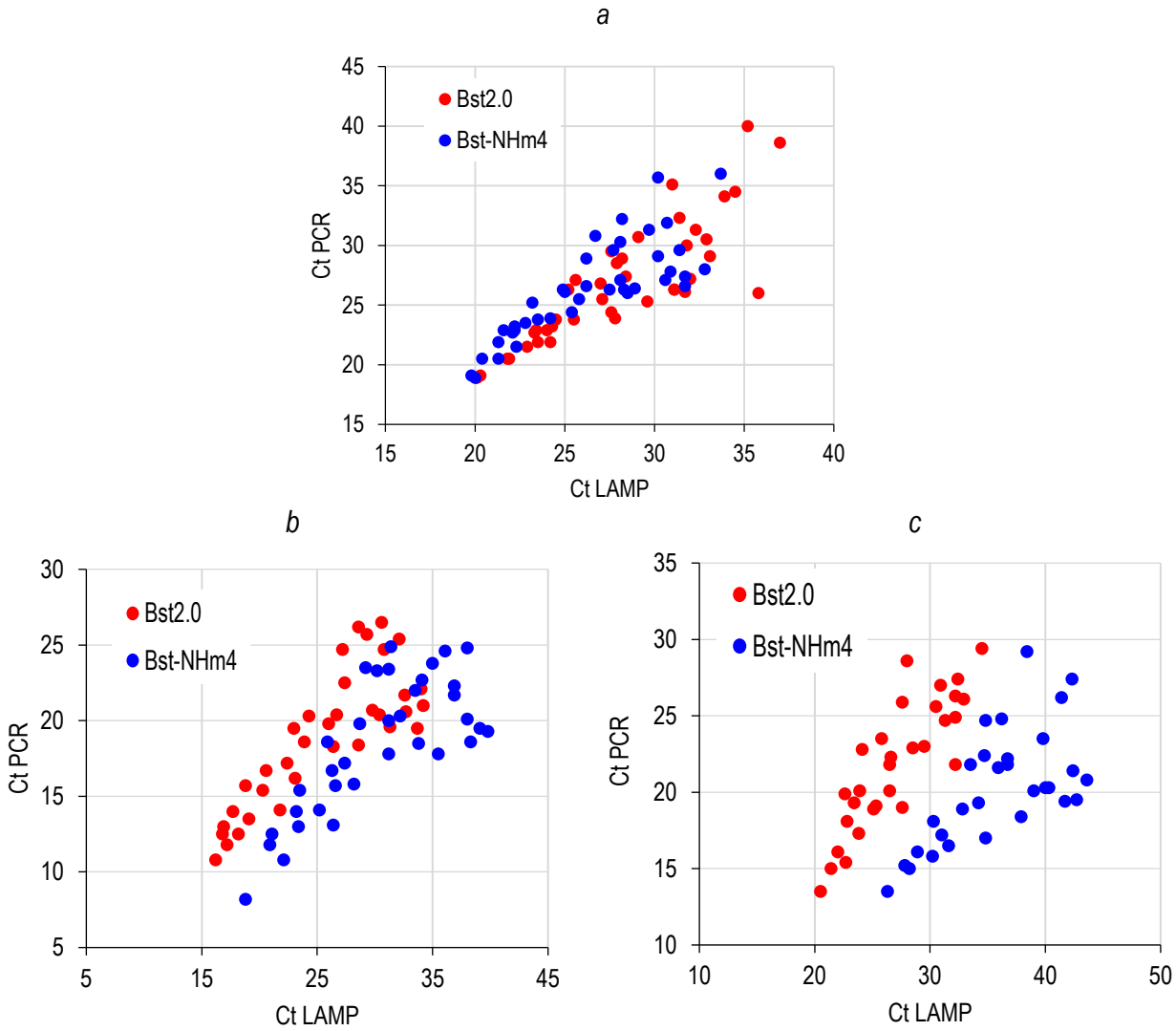

Fig. 5. Analysis of SARS-CoV-2 (*a*), IAV (*b*), and IBV (*c*) RNA samples isolated from biological material (nasopharyngeal and oropharyngeal swabs) using reverse transcription LAMP.

compared to Bst 2.0, the tested RNA samples were identified as positive, and it was sufficient for diagnostic systems with qualitative detection.

According to the user guides for AmpliSens® SARS-CoV-2-IT, AmpliSens® IAV-IT, and AmpliSens® IBV-IT reagent kits, they have a detection limit of max 10^4 copies/ml when used for RNA extraction from biological material with reagents from the RIBO-prep kit and high specificity provided by 4–6 diagnostic primers that are complementary to 6–8 regions of the target DNA.

The obtained results confirm that LAMP-based reagent kits have high diagnostic specificity (100%) compared to PCR test systems when used for analysis of RNA samples at a concentration of minimum 10^4 copies/ml. A major advantage of LAMP-based reagent kits is a significant reduction in the testing time (31 min) compared to PCR using AmpliSens® COVID-19-FL (103 min) and AmpliSens® Influenza virus A/B-FL (150 min) reagent kits due to the shorter time required for the amplification program.

The findings demonstrate that the developed construct and the protocol for extraction and purification of Bst-NHm4 polymerase enzyme have promising prospects and need to be further studied to be used in new and existing reagent kits for LAMP-based diagnostics of different infections.

Conclusion

The offered protocol for extraction and purification of the enzyme provides an efficient method for obtaining recombinant Bst polymerase in a soluble form, with the protein yield reaching up to 19–20% of the total cell mass. Thus, considering the fast and efficient method of purification and production of the enzyme, Bst-NHm4 is suitable for further application in LAMP for diagnostic purposes.

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