Invitrogen

Characterization Of The Enhanced Enzyme For Isothermal Amplification – Invitrogen™ Lyo-ready Bst DNA Polymerase

J Zujienė, U Krukonytė, A Klimentov, L Gabartienė, V Dubovskaja, A Alminaitė, R Sukackaitė
Thermo Fisher Scientific Baltics, Vilnius, Lithuania

Abstract

Note: the abstract has been revised since submission to expand LR Bst active temperatures and exclude Xylan for readability.

Purpose: Characterization of the Invitrogen™ Lyo-ready Bst DNA Polymerase, in-vitro enhanced enzyme, Large Fragment of the Bst DNA polymerase (LR Bst).

Methods: Polymerase activity was tested using radiolabelled dNTPs at 65°C. This temperature was varied during active temperature range testing. Functional testing was carried out using loop-mediated isothermal amplification (LAMP). The method was also used to test polymerase inhibitor resistance by spiking the reaction with inhibitors.

Results: Activity testing revealed that LR Bst active temperature range is 15-75°C. At 37°C, LR Bst has retained >45% of its activity compared to the activity at 65°C. The increase in activity was linear with regards to increasing temperature. LAMP testing also showed that the Invitrogen Lyo-ready Bst DNA Polymerase has high salt tolerance. The addition of ≤100 mM KCl should not impact reaction time significantly and, whereas the addition of 125 mM KCl to most LAMP reactions using competitor Bst polymerases inhibited DNA amplification, our LR Bst was able to withstand up to 200 mM additional KCl to the optimised buffer. Inhibitor tolerance was tested further by adding various common amplification inhibitors. Adding up to 5% urea to the reaction failed to significantly increase time to result when using the LR Bst. In addition, the Invitrogen Lyo-ready Bst DNA Polymerase showed consistently faster amplification speed with titres of other inhibitors, such as humic acid, or ethanol; than competitor Bst polymerases. Overall, the Invitrogen Lyo-ready Bst DNA Polymerase's ammonium salt and glycerol-free composition, wide active temperature range, and high speed brings an extreme advantage to diagnostic assay developers designing assays for field or clinical applications.

Introduction

Most pathogens that infect humans are of animal origin[1]. While a number of new zoonotic diseases have been recorded in the past century, the ongoing anthropogenic changes continue to increase the risk of novel zoonoses emerging[2,3]. The COVID-19 pandemic has shown that, when it comes to rapidly spreading novel diseases, preventative measures through quick and reliable diagnostics are key if therapeutic options are unavailable[4].

The current gold standard to carry out nucleic acid amplification in molecular diagnostics is the polymerase chain reaction (PCR)[5]. Though it shows great specificity and sensitivity, the time and resources needed for the reaction may not be sustainable when a rapid response is required. During the COVID-19 pandemic, where the first-line defense was largely based on diagnosis, the world has seen a significant increase to attempt adapting isothermal nucleic acid amplification techniques (iNAATs) to molecular diagnostics[5]. In fact, multiple SARS-CoV-2 LAMP tests have been since approved by the U.S. Food & Drug Administration, some of which may be used at home[6]. Isothermal nucleic acid amplification allows for sensitive detection at constant temperatures, which can be applicable in a wider range of settings than PCR. In addition, whole assays can be lyophilised to provide room temperature stability and easy use at reconstitution[7]. Bst polymerases are some of the most versatile polymerases for LAMP; their strand-displacement feature combined with thermostability allows for applicability in a range of iNAATs. Thus, to address demands of molecular diagnostic assays developers using iNAATs, Thermo Fisher Scientific has developed an evolved LR Bst: the Invitrogen Lyo-ready Bst DNA Polymerase.

Materials and methods

Polymerase activity testing

Polymerase activity testing for the Invitrogen Lyo-ready Bst DNA Polymerase (Thermo Fisher Scientific) was conducted using an in-house test with radiolabelled nucleotides. One unit is the amount of enzyme that will incorporate 10 nmol of dNTP into a polynucleotide fraction at 65°C in 30 minutes. Incubation temperatures were varied for temperature activity testing.

LAMP

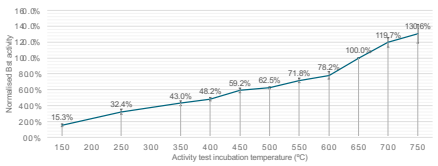
All LAMP and RT-LAMP reactions were assembled according to vendor recommendations (Thermo Fisher Scientific for Invitrogen Lyo-ready Bst DNA Polymerase, New England BioLabs for Bst2.0 Warmstart and Bst 3.0 DNA Polymerase, Optigene for GspSSD2.0. 3 U of Bst, LF were used per (RT-)LAMP reaction. Reaction inhibitors were mixed in-house. They were mixed with sample before addition to the reaction. Targets: Adenovirus41 (AdV41) DNA (Vircell); *Mycoplasma pneumoniae* FH strain of Eaton Agent [NCTC 10119] (ATCC); Synthetic SARS-CoV-2 RNA control 1, Measles virus RNA control (Twist Bioscience). Primers were ordered from Metabion. AdV41-specific hexon targeting primers designed based on Ziros et al. (2015)[8]. *M. pneumoniae* primers from Arfaatabar et al. (2019)[9]; Measles primers designed based on Fujino et al. (2005)[10]; SARS-CoV-2 primers targeting designed based on Alekseenko et al. (2021)[11].

Results

Invitrogen Lyo-ready Bst DNA Polymerase activity testing

Bst DNA Polymerases may be used in a number of isothermal amplification reactions at various reaction temperatures, based on primers and the thermostability of other enzymes. Thus, Invitrogen Lyo-ready Bst DNA Polymerase activity was first measured at temperatures 35-75°C to reflect its applicability in whole genome amplification (WGA), rolling circle amplification (RCA), and LAMP[12, 13, 14]. Then, down to 15 °C due to promising activity results and possible applicability in low temperature reactions. This was achieved by measuring the LR Bst activity at different temperatures and normalising the results to that of activity at 65°C (*Figure 1*). Overall, we observed a steep increase in activity from 60 to 65°C (22%) suggesting an optimal temperature range for the LR Bst of 65 to 70°C (20%). A further 11% increase in activity from 70 to 75°C was also observed, which could indicate entering suboptimal conditions for LR Bst. Activity at even higher temperatures was not measured. When polymerase activity was measured at temperatures ≤60 ° C, the activity decreased by a mean of 6.7% every 5°C to 35°C. That being said, at temperatures 25 and 15°C the LR Bst is not as effective. However, the possibility remains for it to be used in NA amplification protocols optimised for such temperatures.

Figure 1. Normalised Invitrogen Lyo-ready Bst DNA Polymerase activity at different temperatures



Invitrogen Lyo-ready Bst DNA Polymerase (40 U/µL) activity testing at different incubation temperatures normalised to the activity of that at 65.0° C. 3 repeats per test, results averaged from 2 tests. Activity for 65°C was normalised from each measurement thus the error bar is not shown.

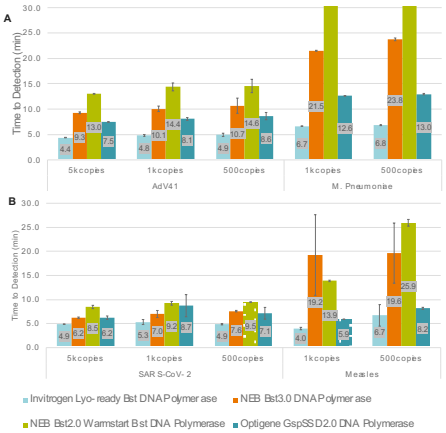
Overall, the LR Bst performed best at higher temperatures further demonstrating its suitability for reactions that require thermostable strand-displacing polymerases. LR Bst also maintained about half of its activity at 35-45°C which, considering its effective use in (RT-)LAMP at 3 U/rxn, shows promise in low temperature reactions. Finally, some activity was still observed at <30°C that, with some optimisation, could be applied for diagnostics at room temperature.

Results

Invitrogen Lyo-ready Bst DNA Polymerase in LAMP: Speed

Bst polymerases are most commonly used in LAMP reactions, thus LAMP was chosen as the model iNAAT to evaluate Bst polymerases functionality. Reaction speed was tested before any inhibitor testing was carried out. In all cases the Invitrogen Lyo-ready Bst DNA Polymerase exibited the fastest reaction speed, allowing to go forward with inhibitor testing in LAMP (*Figure 2*).

Figure 2. Target detection time in LAMP and RT-LAMP

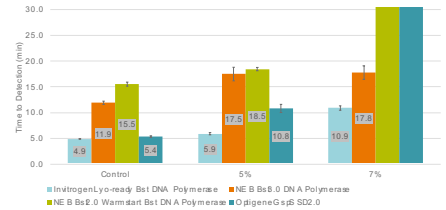


Real-time LAMP (**A**) and RT-LAMP (**B**) results for 30 min reactions at 65°C. 4 replicates per reaction. Dotted bars ±75% sensitivity. Error bars based on relative standard deviation (RSD), specificity of results on melt curve. **A)** DNA targets, M. pneumoniae using 4 primers only, no detection over 30 min = no time of detection shown; **B)** RNA targets with suggested RT

LAMP in the presence of inhibitors

Loop-mediated isothermal amplification is one of the most popular current uses for Bst DNA polymerases in diagnostics. However, samples and their preparation can introduce a variety of inhibitors into a reaction. Thus, it is crucial that the LAMP reaction withstands a considerable amount of inhibiting material and still provides reliable results. Here, we selected a number of inhibitors that may be introduced through different means: ethanol (sample prep); KCl (sample or sample prep); humic acid, urea (sample) (*Figure 3, 4, 5*). The concentrations of inhibitors were chosen by probably to be introduced into the reaction.

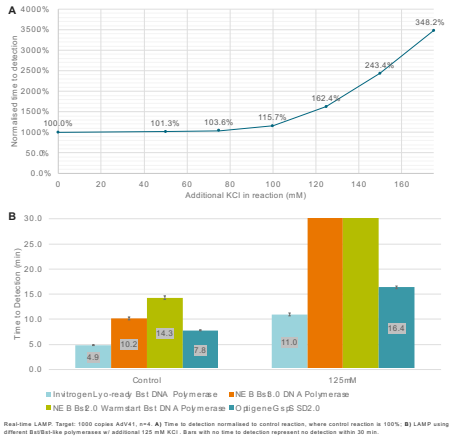
Figure 3. LAMP results for samples containing ethanol.



Real-time LAMP. Target: 1000 copies AdV41, n=4. 5% and 7% represent the total concentration of ethanol/rxn. Bars with no time to detection represent no detection within 30 min.

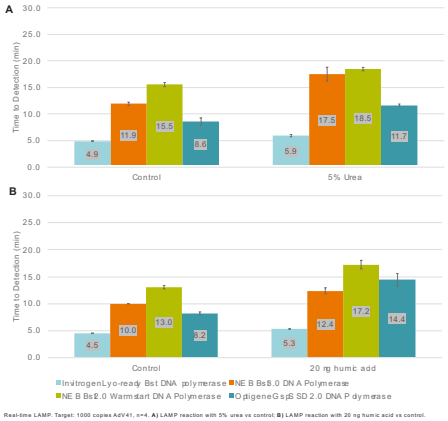
Results

Figure 4. LAMP results for samples containing higher KCl concentrations.



Real-time LAMP. Target: 1000 copies AdV41, n=4. **A)** Time to detection normalised to control reaction, where control reaction is 100%; **B)** LAMP using different Bst/Bst-like polymerases w/ additional 125 mM KCl . Bars with no time to detection represent no detection within 30 min.

Figure 5. LAMP results for samples containing urea and humic acid.



Real-time LAMP. Target: 1000 copies AdV41, n=4. **A)** LAMP reaction with 5% urea vs control; **B)** LAMP reaction with 20 ng humic acid vs control.

Overall, The Invitrogen Lyo-ready Bst DNA Polymerase, when compared to other commercially available Bst DNA polymerases, had faster target detection speed both with and without inhibitors in the reactions. Most importantly, Invitrogen Lyo-ready Bst DNA polymerase showed great resistance to salt concentration even at 175 mM additional KCl. High salt resistance can be correlated to higher processivity[15] and help in reactions where high salt concentrations are present.

Conclusions

Invitrogen Lyo-ready Bst DNA Polymerase shows great promise in future diagnostic applications. Its wide active temperature range, salt tolerance, along with the exceptional functionality in LAMP reactions and lyophilisation compatible formulation allows for a variety of diagnostic uses and applications.

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