Improved Reagents for Isothermal DNA Amplification

Amplification of a specific nucleic acid sequence or target is a fundamental modern laboratory technique that is used worldwide for both molecular biology research and diagnostic purposes. While the Polymerase Chain Reaction (PCR) has long been the standard sequence-specific amplification technique, recent years have seen a proliferation of non-thermal cycling (isothermal) techniques that offer certain advantages over PCR.

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Introduction:

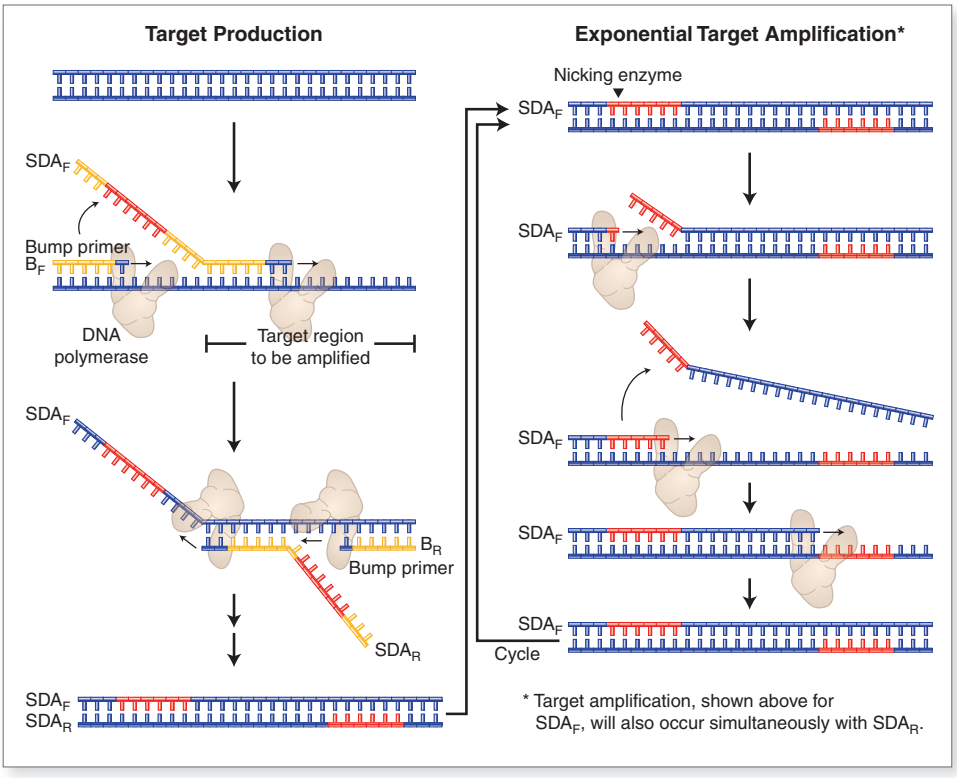
Isothermal techniques, in which amplification reactions are performed at a single temperature, do not require expensive thermal cycling equipment. Reactions can be performed using a water bath, heat block or even ambient temperature. Quantitative detection of amplification is achieved using fluorescence instruments, measuring intercalating dyes, specific probes or cation sensitive dyes. Additionally, isothermal methods provide some unique alternative detection methods. For example, due to the high amounts of DNA synthesized in some techniques (up to 50X PCR yield), precipitation of magnesium pyrophosphate (MgP_2O_7) occurs and can be measured either by simple visual inspection or quantitatively using a turbidimeter (1,2). The potential for simplified instrumentation and range of detection options has spurred the adoption of isothermal amplification methods for field and point-of-care testing, enabling nucleic acid diagnostics without expensive equipment, or even electricity (2-4).

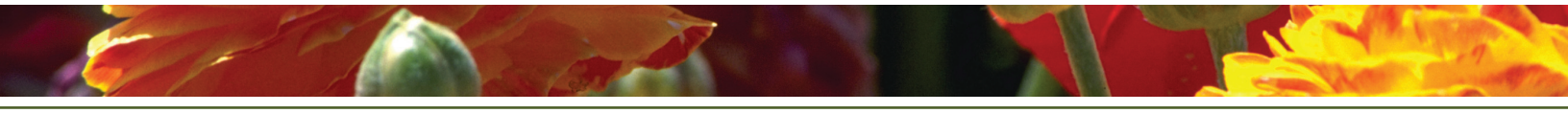
In lieu of a heat denaturation step, most isothermal techniques rely on the strand displacement activity of a DNA polymerase for strand separation of dsDNA. Typically, the large fragment of *Bst* DNA Polymerase (*Bst* DNAP, LF, NEB #M0275) is used, due to its high degree of strand displacement and its optimal temperature range (50–65°C), which facilitates primer annealing. Some isothermal sequence-specific amplification (SSA) methods involve the DNA polymerase alone (e.g., loop-mediated isothermal amplification, LAMP; smart amplification process; SmartAmp), while others use the polymerase in combination with other enzymes (e.g., strand displacement amplification, SDA; nicking enzyme amplification reaction, NEAR; helicase-dependent amplification, HDA; recombinase polymerase amplification, RPA) (5,6). Isothermal techniques are remarkably fast and sensitive, detecting femtograms or very low copy numbers (< 100) of DNA, in as little as five minutes.

While *Bst* DNA Polymerase is quite robust and suitable for most basic applications, it has some limitations when used in isothermal SSA. For example, activity is inhibited at temperatures above 65°C, preventing its use at higher temperatures. In addition, *Bst* DNA Polymerase does not efficiently incorporate dUTP and is severely inhibited when > 50% of dTTP is replaced by dUTP. Non-specific amplification can also be observed at lower temperatures (25°C) (7,8). However, *Bst* DNA Polymerase does exhibit greater tolerance to inhibitors typically found in diagnostic specimens (e.g., blood, humic acid), which is an advantage as compared to PCR polymerases (9). This feature makes *Bst* DNA Polymerase useful for diagnostic testing, although it can be inhibited at low amounts of certain compounds (e.g., 70 mM monovalent salt).

To address some of the limitations described above, NEB has engineered two new DNA polymerases: *Bst* 2.0, which displays improved amplification speed, yield, salt tolerance and thermostability, and *Bst* 2.0 WarmStart, which is designed to enable room temperature reaction setup (for more information, see page 6). Performance improvements, with respect to *Bst* DNAP, LF, can be highlighted using the isothermal diagnostic technique known as strand displacement amplification (SDA). In SDA (Figure 1), internal primers (red), containing 5′ extensions that recognize a target sequence, are extended and then displaced by synthesis that initiates at additional external primers (Bump primers). This initial target generation step results in a short fragment of dsDNA ending with the 5′ extensions of the initial primers. These extensions contain a recognition site for the restriction endonuclease BsoBI, which will cut the target DNA at each end.

Figure 1. Overview of strand displacement amplification (SDA)





However, SDA reactions are performed with a modified deoxyribonucleoside triphosphate, 2´-deoxycytidine-5´-O-(1-thiotriphosphate), or dCTPαS. Incorporation of dCTPαS leaves the top strand of the BsoBI site (C/TCGGG) cleavable, but the bottom strand (GAGCC/C) not cleavable due to created phosphorothioate linkages in the product dsDNA. Thus, as a nick is generated, *Bst* DNAP, LF initiates synthesis and displaces the forward strand, and the amplification reaction will proceed exponentially (10,11).

Results:

Improved reaction performance of *Bst* 2.0

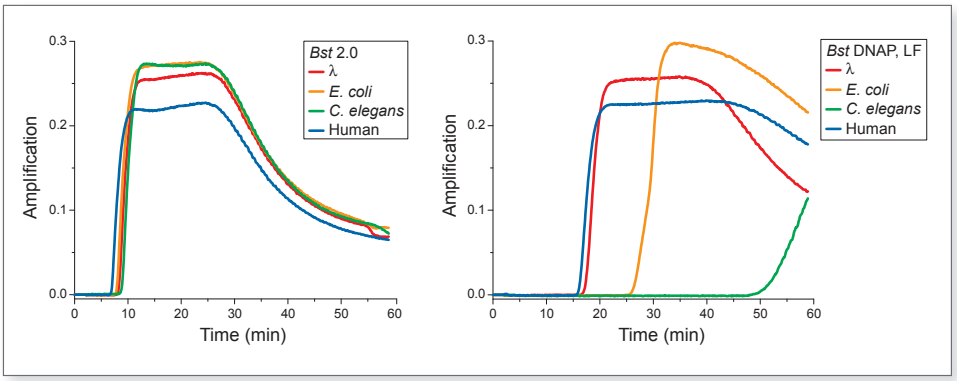
Amplification results from SDA reactions, as measured by turbidity, are presented in Figures 2 and 3. In Figure 2, SDA reactions are graphed as increasing turbidity (amplification) over time. These reactions were performed using various targets, from simple (λ, 48.5 kbp) to increasingly complex (*E. coli, C. elegans* and human) genomic DNA, with equal units of *Bst* DNAP, LF and *Bst* 2.0. In all cases, *Bst* 2.0 provided robust amplification in under 10 minutes, whereas *Bst* DNAP, LF amplification was variable, ranging from 15–45 minutes. Speed is, of course, a major consideration of any amplification technique, and Figure 2 demonstrates the speed improvements conferred in using *Bst* 2.0. However, the consistency of amplification across target and genome is of equal or greater significance, and *Bst* 2.0 displayed little variability in reaction speed with the various targets.

Improvements in speed, sensitivity and salt tolerance are also illustrated in Figure 3. Figure 3A is a standard curve measured across varying amounts of human genomic DNA with BRCA1-specific SDA primers. Linear dose response curves were generated when using either *Bst* DNAP, LF or *Bst* 2.0 highlighting the usability of SDA and these polymerases for reliable target sequence quantification. *Bst* 2.0 reached the detected threshold faster at each concentration of genomic DNA tested, further demonstrating its speed versus *Bst* DNAP, LF.

Bst 2.0 also showed increased resistance to inhibitors, as demonstrated in Figure 3B. *Bst* DNAP, LF was significantly inhibited by salt concentrations above 50 mM, while *Bst* 2.0 proved much more robust, with no inhibition in SDA at salt concentrations up to 150 mM. This property enables a much broader range of reaction conditions, and is especially accommodating of lower purity DNA samples which can contain high amounts of salt.

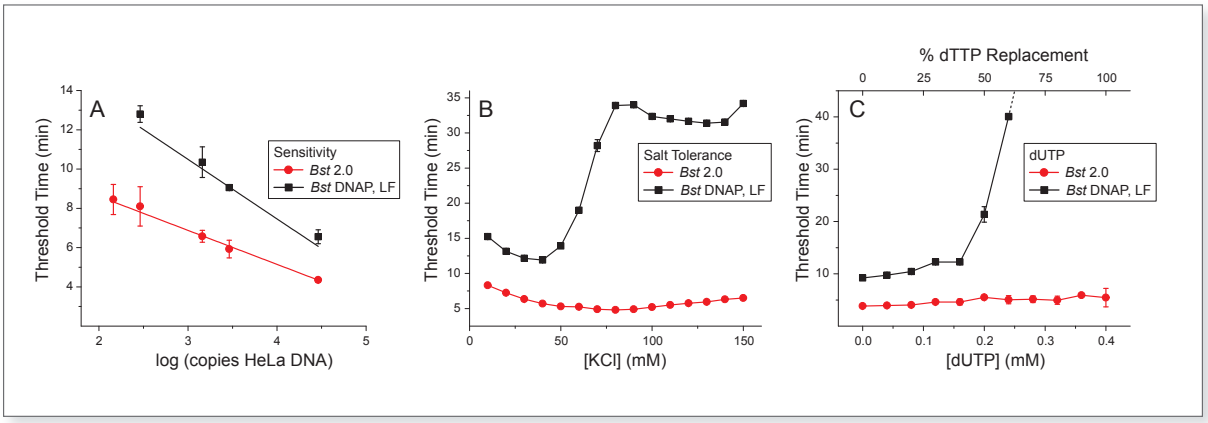
Another consideration for DNA polymerases is incorporation and tolerance of dUTP. The incorporation of dUTP during amplification is commonly used for the prevention of carry-over contamination (7). Figure 3C shows data for dUTP replacement of dTTP in SDA. *Bst* DNAP, LF was completely inhibited above 50% replacement of dTTP with dUTP, while *Bst* 2.0 displayed no inhibition up to 100% replacement. Although various isothermal techniques may be differentially sensitive to dUTP use, *Bst* 2.0 is much more tolerant to dUTP in the reaction than *Bst* DNAP, LF.

Figure 2. *Bst* 2.0 displays improved speed and consistency, as compared to Bst DNA Polymerase, Large Fragment



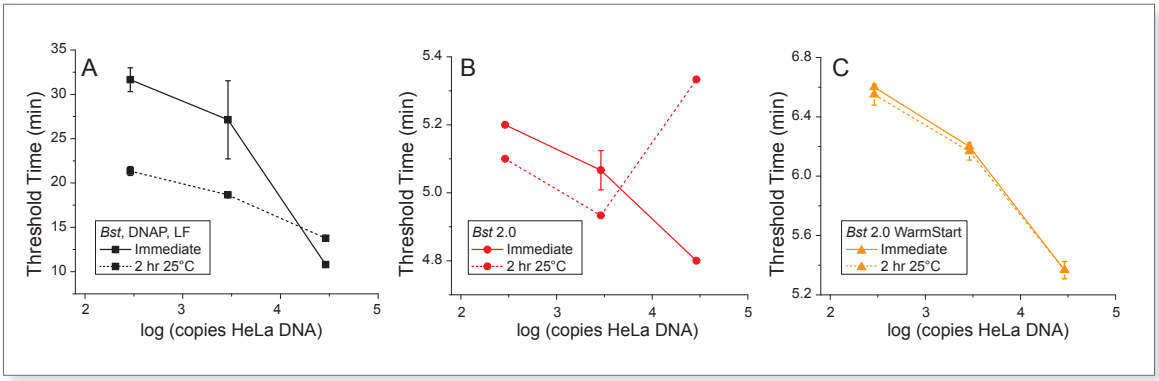
Amplification curves from strand displacement amplification (SDA) reactions using various targets and genomes show consistent, rapid performance using Bst 2.0. All amplicons were detected in less than 10 minutes with Bst 2.0 while Bst DNAP, LF detection required 15–60 minutes. Amplification was followed by real-time turbidity measurements.

Figure 3. *Bst* 2.0 offers increased sensitivity, salt tolerance and tolerance of dUTP



Various SDA reaction parameters show the improved performance of Bst 2.0. Faster, more reliable detection of lower target copy number is shown in Panel A. Panel B shows salt tolerance of the two polymerases in SDA, with significant inhibition of Bst DNAP, LF above 50 mM KCl, while Bst 2.0 provided robust amplification through 150 mM. dUTP incorporation was shown to be inhibitory to Bst DNAP, LF (Panel C), with > 50% dTTP replacement completely inhibiting SDA reactions (dashed line). In contrast, Bst 2.0 performed equally well from 0–100% dUTP in SDA reactions.

Figure 4. *Bst* 2.0 WarmStart offers consistent, reliable amplification, even after a room temperature pre-incubation step



Setting up an SDA reaction on ice and immediately starting the reaction gave consistently accurate results with all three DNA polymerases tested: Bst DNAP, LF; Bst 2.0; and Bst 2.0 WarmStart (Panels A, B, and C, respectively). However, if the amplification reactions were set up at room temperature and then left at room temperature (pre-incubated) for 2 hours before transferring the reactions to a real-time instrument, both Bst DNAP, LF and Bst 2.0 showed inconsistency and inaccuracy (Panels A and B). Only Bst 2.0 WarmStart gave consistent and accurate results even under the extreme case of a 2 hour preincubation (Panel C).

Increased specificity with *Bst* 2.0 WarmStart

As mentioned above, DNA polymerases often display undesired amplification at low temperature, a result of extension of DNA primers or nonspecific annealing of primers to template. This amplification produces nonspecific bands in PCR, with the potential to give high backgrounds and/or false positives in nucleic acid diagnostics. Any reaction or test must be set up on ice and transferred directly to the detection instrument to avoid spurious amplification and inconsistent results. This property imposes a technical challenge to high throughput diagnostic applications, where consistency and reproducibility are of extreme importance. To address this problem, various approaches have been successfully applied to making "Hot Start" DNA polymerases, which are inactive until the reaction temperature is raised above a certain permissive temperature. Most of these methods, whether chemical modification or antibody-based, work well for PCR, where the temperature is raised to approximately 95°C and the inactivating component is heat labile. However, isothermal reaction temperatures do not typically rise above 65–70°C, thus requiring the temperature sensitive inhibitor to undergo thermal inactivation at a much lower temperature than in PCR. *Bst* 2.0 WarmStart employs a specifically-selected and modified DNA aptamer that tightly binds to *Bst* 2.0 at low temperatures, and is released from the polymerase above 45°C, permitting amplification reactions at temperatures typically used in isothermal amplification techniques. We termed the resulting DNA polymerase-aptamer complex *Bst* 2.0 WarmStart, as temperatures are significantly below those of PCR hot start enzymes. This temperature range was chosen to accommodate isothermal techniques currently using *Bst* DNAP, LF, while enabling room-temperature setup and consistent reaction performance.

Data from SDA using *Bst* DNAP, LF, *Bst* 2.0, and *Bst* 2.0 WarmStart is shown in Figure 4. Three concentrations of target DNA were used, and reactions performed in two sets: one immediately after setup, and another after pre-incubation for two hours at room temperature. For both *Bst* DNAP, LF and *Bst* 2.0, the two conditions produced strikingly dissimilar results, with substantial variability in the detection time at each template concentration (data plots have different scales for the y-axis to accommodate the faster reaction times of *Bst* 2.0). However, reactions with *Bst* 2.0 WarmStart produced identical results with or without a two hour pre-incubation. This property enables room temperature setup (temperatures up to 40°C) and, equally important, provides consistent, reliable results in diagnostic applications.

Conclusion

The performance enhancements obtained with the engineered *Bst* 2.0 and *Bst* 2.0 WarmStart DNA Polymerases bring much-needed flexibility to isothermal amplification techniques. Tolerance of a broader range of reaction conditions enables improvements to the different assay technologies to be realized. Improving the capabilities of the essential reagents will also expand the role of isothermal methods and facilitate additional progress as these techniques become an even more significant part of molecular diagnostics and molecular biology research.

References

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Bst 2.0 and *Bst* 2.0 WarmStart DNA Polymerases

Bst 2.0 DNA Polymerase is an *in silico* designed homologue of *Bacillus stearothermophilus* DNA Polymerase I, Large Fragment (*Bst* DNA Polymerase, Large Fragment). *Bst* 2.0 DNA Polymerase possesses 5´→3´ DNA polymerase activity and strong strand-displacement activity, but lacks 5´→3´ exonuclease activity. *Bst* 2.0 DNA Polymerase displays improved amplification speed, yield, salt tolerance and thermostability compared to wild-type *Bst* DNA Polymerase, Large Fragment.

Bst 2.0 is tolerant of inhibitors, such as salt, and is able to incorporate dUTP during amplification. *Bst* 2.0 is active at salt concentrations up to 150 mM allowing flexibility in buffer choice. This is potentially useful in techniques that require multiple enzymes, such as Helicase Dependent Amplification or Strand Displacement Amplification. Furthermore, the ability to incorporate dUTP makes *Bst* 2.0 useful in methodologies for prevention of carryover contamination.

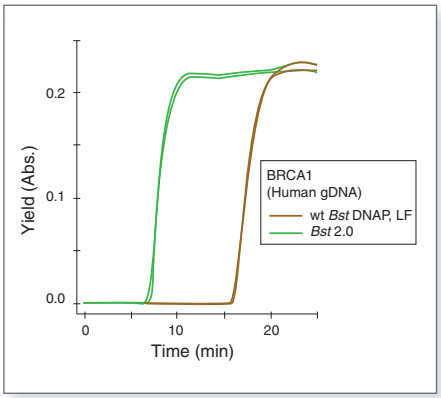
The WarmStart feature of *Bst* 2.0 WarmStart DNA Polymerase is unique among isothermal polymerases. Like "Hot Start" PCR polymerases, this feature prevents activity at temperatures below the optimal reaction temperature. This enables room temperature reaction set up and increases the reproducibility of the results.

In contrast to chemical modifications or antibodies commonly used with hot start PCR polymerases, NEB's *Bst* 2.0 WarmStart DNA Polymerase utilizes aptamer technology. Aptamers are extensively modified, unique oligonucleotides which bind to the polymerase through non-covalent interactions, inhibiting activity at non-permissive temperatures (< 50°C). Additionally, no separate activation step is required for *Bst* 2.0 WarmStart DNA Polymerase.

Advantages

- Fast polymerization
- Robust reactions with a broad range of conditions and primer sets
- Flexible reaction conditions, including a higher salt tolerance than *Bst* DNA Polymerase, LF
- Optimal reaction performance from 60-72°C
- Minimal effect of substitution of dTTP with dUTP
- Highly pure product with minimal lot-to-lot variation
- WarmStart feature enables room temperature reaction set-up

Bst 2.0 DNA Polymerase improves amplification speed



Strand Displacement Amplification (SDA) of BRCA1 from HeLa genomic DNA under optimal SDA conditions for each enzyme. Data shows that the reaction reaches threshold faster with Bst 2.0 than with wild-type (wt) Bst DNA Polymerase, Large Fragment.

Ordering Information

PRODUKT	NEB #	GRÖSSE	PREIS
Bst 2.0 DNA Polymerase	M0537 S/L	1.600 u / 8.000 u	63 € / 252 €
Bst 2.0 DNA Polymerase, high conc.	M0537 M	8.000 u	252 €
Bst 2.0 WarmStart™ DNA Polymerase	M0538 S	1.600 u / 8.000 u	68 € / 272 €
Bst 2.0 WarmStart DNA™ Polymerase, high conc.	M0538 M	8.000 u	272 €
Isothermal Amplification Buffer Pack	B0537 S	6 ml	20 €

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