Novel Thermostable DNA Polymerase with a Strong Strand Displacement Activity

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Abstract: In this paper we describe properties of a Novel Thermostable DNA Polymerase (SD polymerase) with a strong strand displacement activity, which is suitable for both LAMP and PCR methods of DNA amplification. SD polymerase is an artificial variant of Taq DNA polymerase. It has strong strand-displacement activity similar to Bst DNA polymerase and high thermostability similar to Taq DNA polymerase. Thus it is an unique enzyme, which is suitable for both isothermal (LAMP, SDA & so on) and PCR-based methods of DNA amplification. Additionally, SD polymerase demonstrates extraordinary properties for long PCR and highly sensitive PCR, and may be used for creation and performing new methods of DNA amplification.

Introduction: DNA Polymerases with Strong Strand Displacement Activity (SSD-activity) and their applications.

At present, three types of DNA Polymerases with SSD-activity are well known: RTs (like MMLV), enzymes from the B-family of DNA polymerases (such as φ29-pol), and DNA polymerases from the A-family (such as Bst-pol). It should be mentioned that SSD-activity is not a common property of all enzymes from these polymerase families. It is found in a limited circle of the enzymes in the each family.

The φ29 and Bst DNA polymerases are employed in practical work more often than the other enzymes capable of strand displacement. The polymerase of phage φ29 is a B family polymerase with an unusual structure. It has extraordinary processivity and very strong strand displacement. Structural and modeling studies of *φ*29 DNA polymerase suggest that the intrinsic strand displacement and processivity of this enzyme can be explained through specific topological

mechanisms. The structure of its complex with DNA indicates that the downstream template DNA bound by φ29 DNA polymerase passes through a tunnel before entering the polymerase active site. Since this tunnel is only large enough to allow the passage of a single strand of DNA, it suggests a structural basis for both the intrinsic strand displacement and processivity of this polymerase [1]. Usually, this enzyme is applied for isothermal Whole-Genome Amplification. Low temperature optimum of the enzyme (about 37C) does not allow using it for sequence-specific amplification.

Another DNA polymerase with strong strand displacement activity is the Bst polymerase. It is a typical member of polymerase A-family and its structure is similar to that of Taq polymerase or other members of the family [2]. *Bacillus stearothermophilus* DNA Polymerase I or Bst polymerase is one of the most popular enzyme having strand displacement because its optimum is about 63C and it is suitable for sequence-specific amplifications like LAMP. Unfortunately, the mechanism of strand displacement used by Bst and other family A polymerases is unclear.

Strand displacement activity allows DNA polymerase to use DNA templates with complex secondary structure. Thus, polymerases with strand displacement are suitable for DNA synthesis or sequencing through “difficult” regions. Another application of these enzymes is Isothermal DNA Amplification. A variety of isothermal amplification methods have been developed, for example:

- Whole-Genome Amplification (WGA)
- Rolling Circle Amplification (RCA)
- Strand Displacement Amplification (SDA)
- Loop mediated AMPlification (LAMP)

All these methods require a strong strand displacement activity of polymerase.

Loop mediated AMPlification (LAMP) [3] is one of the most interesting and promising methods of isothermal sequence-specific amplification which can be used in clinical diagnostics [4].

The short animation available in the link below illustrates key steps of LAMP: http://loopamp.eiken.co.jp/e/lamp/anim.html .

Sequence-specific amplification like LAMP needs high temperature for specific annealing of primers and therefore only the Bst polymerase was suitable for these methods.

A relative thermostability and strong strand displacement activity of Bst make it helpful for other applications as well; for example, in Illumina's Next Generation Sequencing technology for cluster amplification of DNA molecules. Figure 1 illustrates the process of cluster amplification on a chip surface. One can see that each cluster consists of complementary single-stranded DNA molecules, so for successful amplification a polymerase with strong strand displacement activity is needed.

Unfortunately, the Bst polymerase is useful for temperatures only up to 66-68^{o}C. At 68^{o}C and higher temperatures it becomes inactivated. To overcome this limitation, a numerous attempts have been made in different labs but without much success. Recently scientists from the New England Biolabs have created a new version of the enzyme which was named Bst 2.0. This new version displays improved amplification speed and salt tolerance, but its thermostability is similar to those of the wild-type *Bst* Polymerase.

Another group of scientists from *Lucigen,* Joint Genome Institute and Montana State University attempted to find a suitable polymerase for both PCR and isothermal amplification. They used the Metagenomic Sequencing technology. Unfortunately, the polymerases found so far do not have enough strand displacement activity.

Results and Discussion

New Polymerase (*SD DNA polymerase*)

Taking into the account the unsuccessful attempts of our colleagues we have chosen another way for creating a new polymerase and spent a few years studying

the mechanism of strand displacement activity of the Bst polymerase. As a result, we have found sites in the protein molecule that are essential for strand displacement of Bst and similar polymerases.

Figure 2 illustrates four sites of the enzyme which are essential for this activity and shows their role in strand displacement.

One site (marked in red) destabilizes complementary base pairs in the fork of the template and displaces the DNA strand. Another (green) site binds to the single-stranded template and prevents reassociation of the DNA duplex. The two sites marked in yellow create an ion bridge which stabilizes the correct conformation of the enzyme. It should be mentioned that these sites have common patterns of amino acid sequences in the A family of DNA polymerases possessing the SSD-activity.

Based on our hypothesis, we have modified the corresponding sequences in the Taq polymerase and obtained a new variant of Taq polymerase with a strong strand displacement activity. The new enzyme was named SD-polymerase.

The following results illustrate and disclose some properties and applications of *SD DNA polymerase.*

LAMP amplification with SD polymerase

Figure 3 illustrates the results of LAMP amplification performed with Bst and SD-polymerases. The KlenTaq polymerase (a truncated form of Taq) was used as a negative control, i.e. an enzyme lacking strand displacement activity. The reactions were carried out with and without preheating at 94^{o}C for 2 min. Preheated samples are indicated by the plus mark.

It's obvious that SD-polymerase is able to perform LAMP with and without preheating, Bst-pol is totally inactivated at 94^{o}C, and the KlenTaq-pol is unable to carry out LAMP reaction. Thus, in contrast to Bst, the SD-pol has a much higher thermostability, and in contrast to Taq, it has a strong strand displacement activity which allows it to perform LAMP.

Figure 4 demonstrates virtually the same results. In addition, one can see that the denaturation step at temperatures over 90°C can increase the efficiency of LAMP. The reaction was carried out with SD-polymerase, with or without the denaturation step at 92°C. This finding is in agreement with results described in the literature. Earlier, a number of researchers described a significant increase of sensitivity and efficiency of LAMP achieved by pre-denaturation of the DNA template. For example, a number of studies [5-8] have reported a high sensitivity in the detection of *Mycobacterium tuberculosis* in clinical specimens obtained following an initial heat-denaturation of template DNA. In the absence of such step, a 200-fold reduction of the sensitivity of the assay was observed [5, 8].

PCR amplification with SD polymerase

The high thermostability of the SD polymerase allowed us to use it for PCR amplification. We reasoned that the strong strand displacement activity could ensure better amplification of complex templates. We compared the SD- and Taq-polymerases in a PCR with a 64% GC-rich template. A 1.3 kb fragment of *Mycobacterium tuberculosis* genome was amplified (Fig. 5). Admittedly, in this experiment the SD polymerase worked much better than Taq, and increasing the Taq amount did not improve the situation.

Figure 6 shows the results of a fast PCR amplification of a 5 kb phage λ DNA fragment. The reactions were performed with various amounts of Taq and SD polymerases using short cycles. The elongation time was 100 seconds in each cycle or 20 seconds per 1 kb. Under these conditions SD-polymerase again demonstrated much higher efficiency than Taq polymerase.

Figure 7 shows our results of amplification of a 8-kb DNA fragment. We tested *SD polymerase* vs. *GoTaq polymerase* in the GoTaq buffer (Promega, USA) with 2 mM, 3 mM or 4 mM $MgCl_2$. An 8-kb fragment of λ DNA was amplified with 2, 4, 6 or 8 units of *SD or Taq polymerase*. The reaction mixture (50 μl)

contained: 5 ng λ DNA as template, 0.25 mM dNTP (each), 10 pmol (0.2 μM) of each primer. PCR was carried out for 25 cycles: preheating 92 C -2 min; cycling 92 C, 30 sec; 60 C, 30 sec; 68 C, 2 min 40 sec (20 sec/kb). Figure 7 shows that *SD polymerase* offers much higher efficiency of PCR than *Taq.*

SD-pol vs. Taq polymerase from different suppliers (*GoTaq*, Promega USA; *MyTaq*, Bioline UK; *TaqPol*, Evrogen Russia)

We have compared a few Polymerase/PCR-Buffer systems from different suppliers in PCR amplification of a 8-kb λ DNA fragment: *SD*-pol/GoTaq-buf; *GoTaq*-pol/GoTaq-buf; *MyTaq*-pol/GoTaq-buf; *Taq*-pol/GoTaq-buf; *MyTaq*-pol/MyTaq-buf; *Taq*-pol/Encyclo-buf.

Figure 8 demonstrates that the *SD polymerase* provides much higher efficiency of PCR than *Taq* from different suppliers.

PCR sensitivity (SD polymerase vs. Taq polymerase)

We have compared SD polymerase and GoTaq polymerase (Promega, USA) in PCR amplification of a 364-bp fragment of murine G3PDH cDNA. Ten-fold dilutions of the murine cDNA library (100 pg, 10 pg, 1 pg, 0.1 pg, 0.01 pg) were used as a template. The reaction mixtures (50 mkl) contained: 1x GoTaq buffer; 3 mM $MgCl_2$; 0.25 mM dNTPs (each); primers 15 pmol (each). PCRs were performed for 28 cycles: 92 C, 30 sec; 65 C, 40 sec; preheating 92 C,1 min 30sec.

Figure 9 demonstrates that *SD polymerase* provides more than a ten-fold (about 50-100 times) higher sensitivity of PCR than *Taq.*

Real-Time PCR (SD polymerase vs. Taq polymerase)

Figure 10 shows our results of RT-PCR with ***Eva Green*** of a 500 bp λ DNA fragment. We tested *SD polymerase* vs. *Taq polymerase* in RT-PCR with different template concentrations. The reaction mixtures (15 μl) contain: 1x Encyclo buffer (Evrogen) with 3.5 mM $MgCl_2$; dNTP 0.2 mM (each); primers 0.2 mkM (each); 1x

Eva Green; DNA polymerase (*Taq* or *SD*) 1.5 U; and different amounts of λ DNA as a template (500 pg, 50 pg, 5 pg, 0.5 pg).

The results of RT-PCR with SD polymerase are indicated by Red color. The results of RT-PCR with Taq polymerase are indicated by Green color.

Figure 10 demonstrates that SD polymerase can provide better Ct in RT-PCR than Taq polymerase.

Hot Start PCR

We have successfully tested antiTaq antibodies and *SD polymerase* for performing Hot Start PCR. We used MAbs kindly provided by Dr. Rybalkin: http://www.ncbi.nlm.nih.gov/pubmed/8074881

A 230-bp DNA fragment was amplified from Human gDNA using GoTaq buffer and Taq-pol (with/without MAbs) or SD-pol (with/without MAbs). The reaction mixture (50 µl) contained: 25 ng gDNA as template, 0.25 mM dNTP (each), 10 pmol (0.2 µM) of each primer, 1x GoTaq buffer, 3 mM $MgCl_2$, and 2.5 units of Taq or SD polymerase.

PCR was carried out for 30 cycles: preheating 92 C, 2 min; cycling 92 C, 20 sec, 60 C, 15 sec, 68 C, 15 sec.

Figure 11 demonstrates that *AntiTaq* antibodies enable Hot Start PCR with *SD* DNA polymerase.

Long PCR

Amplification of long DNA fragments (over 5-8 kb) by means of PCR (long-distance or long PCR) is an important and widely used tool in molecular biology. Unfortunately, the most popular and robust enzymes for use in PCR such as Taq, Tth or other DNA polymerases alone are unable of performing long PCR.

In 1994 Barnes published his groundbreaking method for carrying out long PCR, which helped to resolve the problem [9, 10]. This method consisted of adding to DNA polymerases lacking 3'→5' exonuclease (proofreading) activity, such as Taq or Tth DNA polymerase, a DNA polymerase which does possess the

proofreading activity, e.g. Pfu DNA polymerase. The proofreading activity of the added polymerase eliminates misincorporated nucleotides and enhances PCR efficiency, especially in long PCR [9, 10]. Since the Barnes' publications in 1994, his method of carrying out long PCR with enzymes mixture has been the main approach up to present time

Theoretically, a strand-displacement activity can solve problems with secondary structures of templates and improves DNA amplification, especially in long PCR. We have compared SD polymerase in long-distance amplification of 8–20 kb DNA fragments versus commercial available Encyclo polymerase mixture for long PCR (Evrogen; http://evrogen.com/products/Encyclo-PCR-kit/Encyclo-PCR-kit.shtml) (fig. 12, 13) and Taq polymerase (fig. 13). We found that SD polymerase can amplify a 20 kb λ DNA fragment alone without any proofreading exonuclease activity and does it much better than specially designed "Barnes's" mixture for long PCR. It should be noted that up to now only special enzyme mixtures have been able to amplify such long (20 kb) DNA fragments.

In (Fig. 14) we added DeepVent DNA polymerase (an enzyme with proofreading activity) to SD polymerase. We could not detect any significant improvement of long PCR by addition of polymerase with proofreading activity to SD-pol. These data show that SD-polymerase does not need extra enzymes to efficiently perform long PCR.

In (Fig. 15) we increased amount of SD-polymerase for amplification of the 20 kb λ DNA fragment. We found that increasing amounts of the enzyme could enhance the yield of long PCR by many times.

Conclusion

The results that were present here bring us closer to understanding of the strand-displacement mechanism employed by the A family DNA polymerases.

Based on our hypothesis, we have modified the Taq polymerase and obtained a new enzyme (the SD-polymerase) which has both high thermostability and a strong strand-displacement activity.

The new polymerase can be useful in various applications including LAMP and PCR amplifications. We would like to emphasize here that our enzyme is the first of its kind that can be used in both LAMP and PCR. And the preliminary data of the SD-polymerase performance looks very promising indeed.

Our data also indicates that SD polymerase provides much higher PCR efficiency and sensitivity than Taq and can carry out long PCR with high efficiency without addition of other enzymes.

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FIGURES

Fig. 1

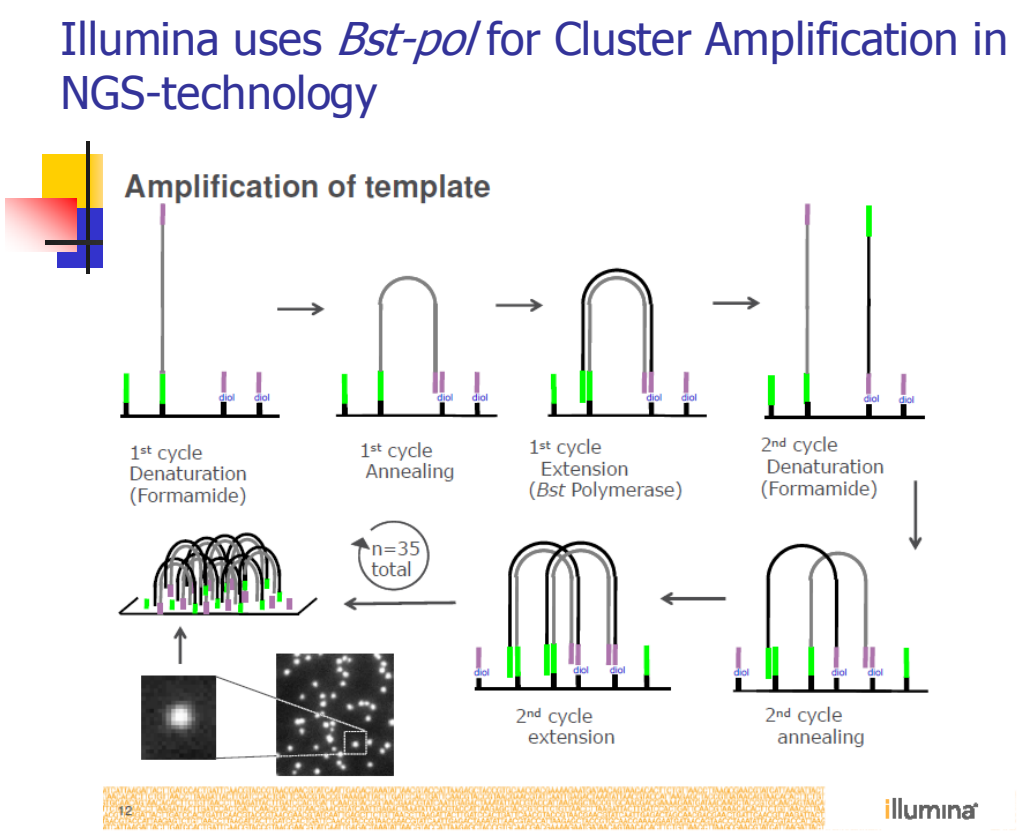
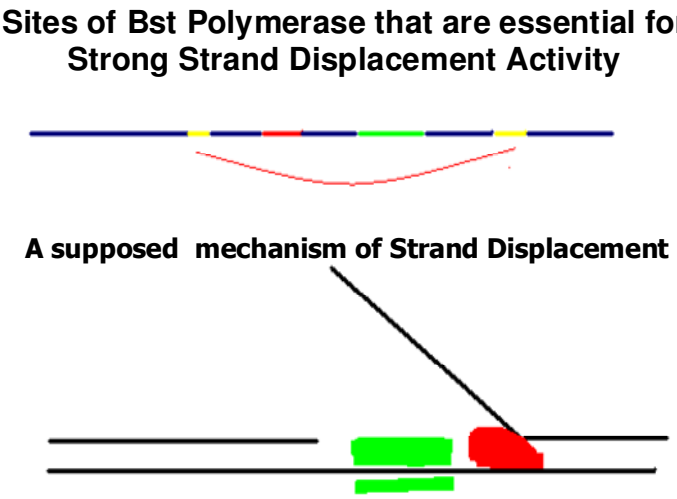


Fig. 2

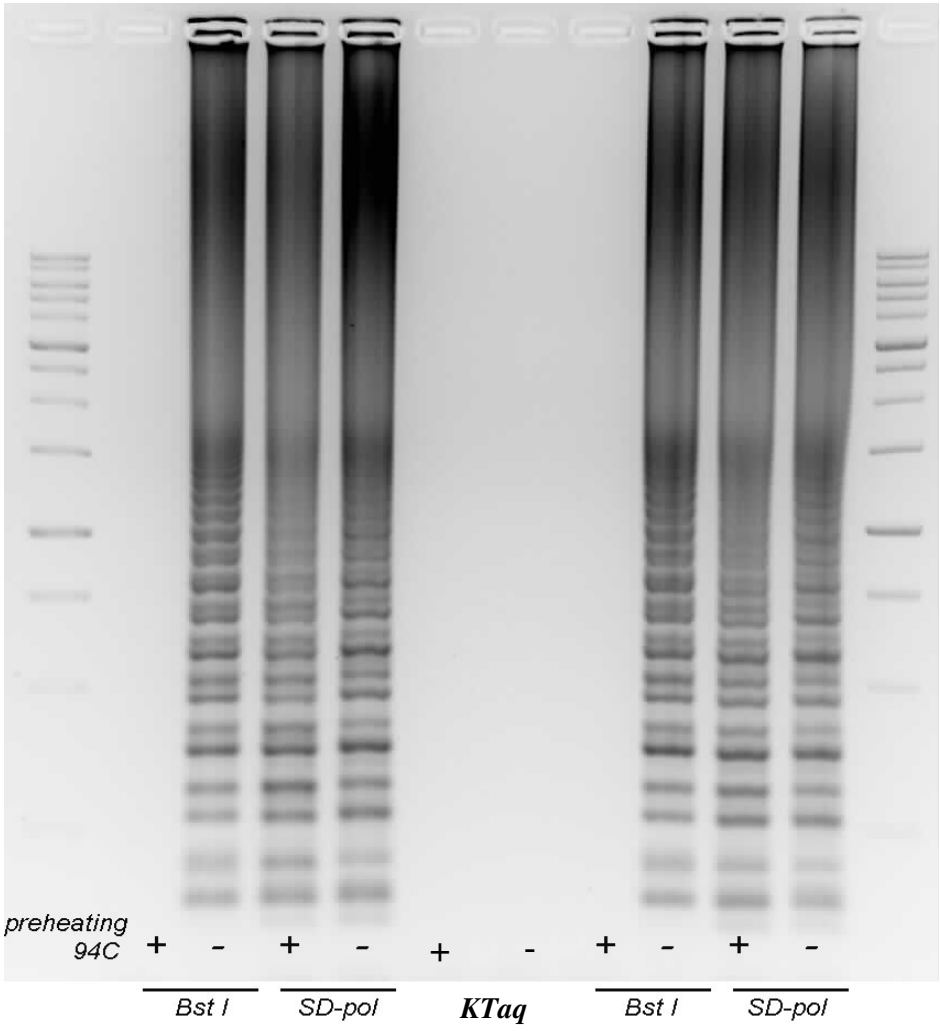


Sites that are essential for strand displacement activity of Bst DNA polymerase. One site (marked in red) destabilizes the complementary base pairs in the DNA fork of the template and displaces the DNA strand. Another (green) site binds to the single strand template and prevents reassociation of the DNA duplex. The two sites marked in yellow create an ion bridge which stabilizes the correct conformation of the enzyme.

The following figures illustrate our results:

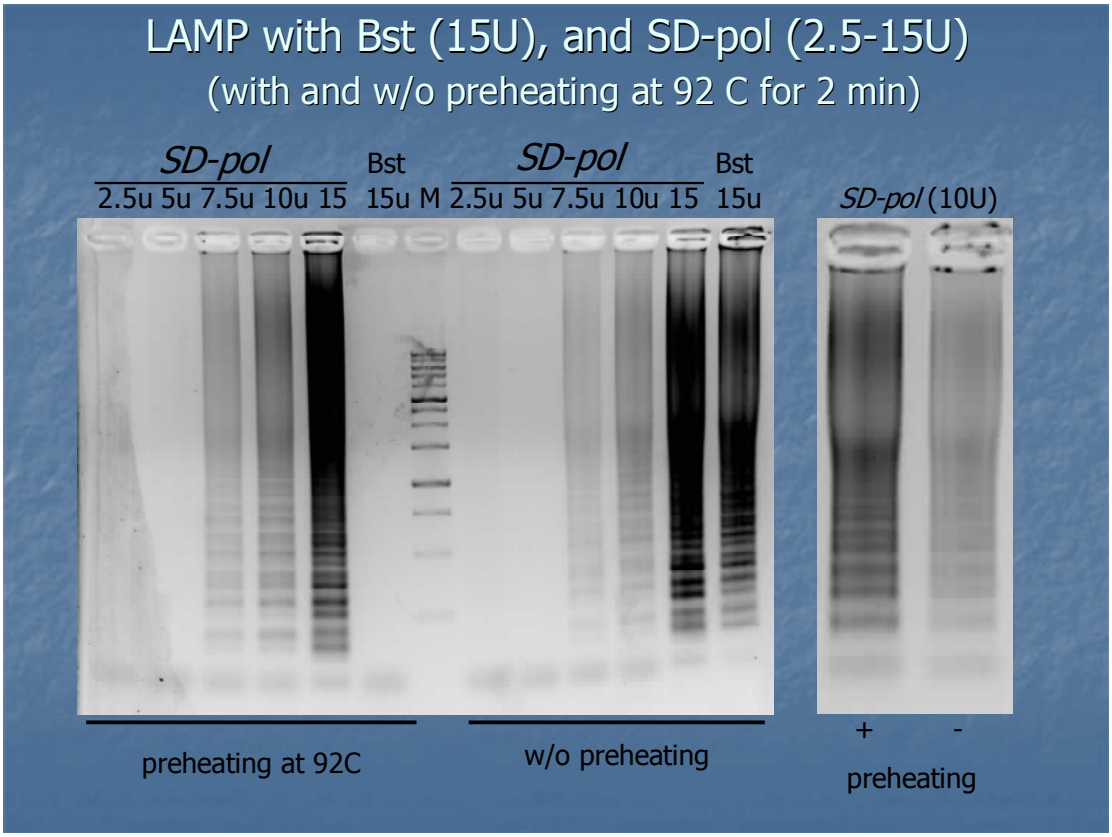
Fig. 3

LAMP with Bst, Taq and SD-pol
(with and w/o preheating at 94 C for 2 min)



LAMP amplification performed with Bst and SD-polymerases. KlenTaq polymerase (a truncated form of Taq) was used as a negative control as an enzyme lacking the SD activity. The reactions were carried out with or without preheating at 94 C for 2 min. The preheated samples are indicated by the plus sign.

Fig. 4



Denaturation step at temperatures over 90 C can increase the efficiency of LAMP. The reactions were carried out with SD-polymerase, with and without the denaturation step at 92 C.

Fig. 5

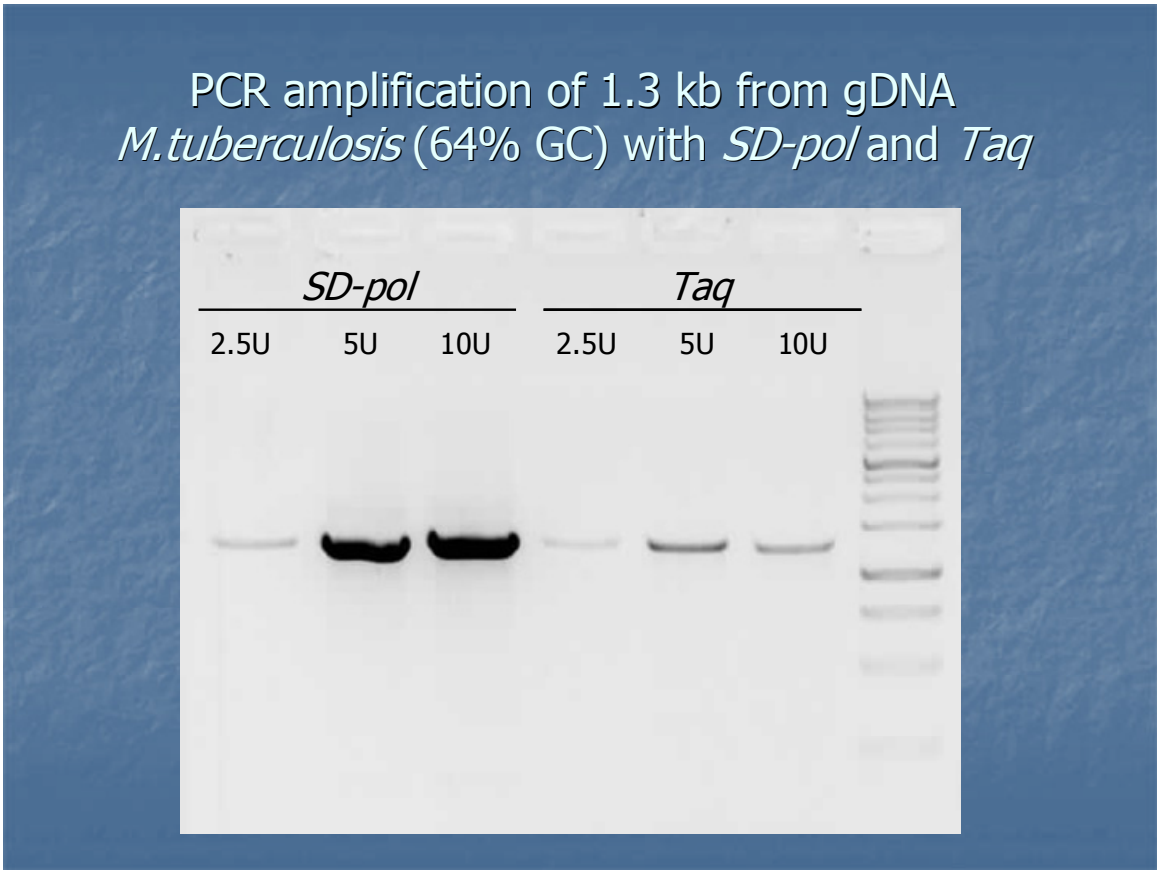

PCR amplification of 1.3 kb from gDNA
M.tuberculosis (64% GC) with SD-pol and Taq
SD-pol
Taq
2.5U
5U
10U
2.5U
5U
10U

Fig. 6

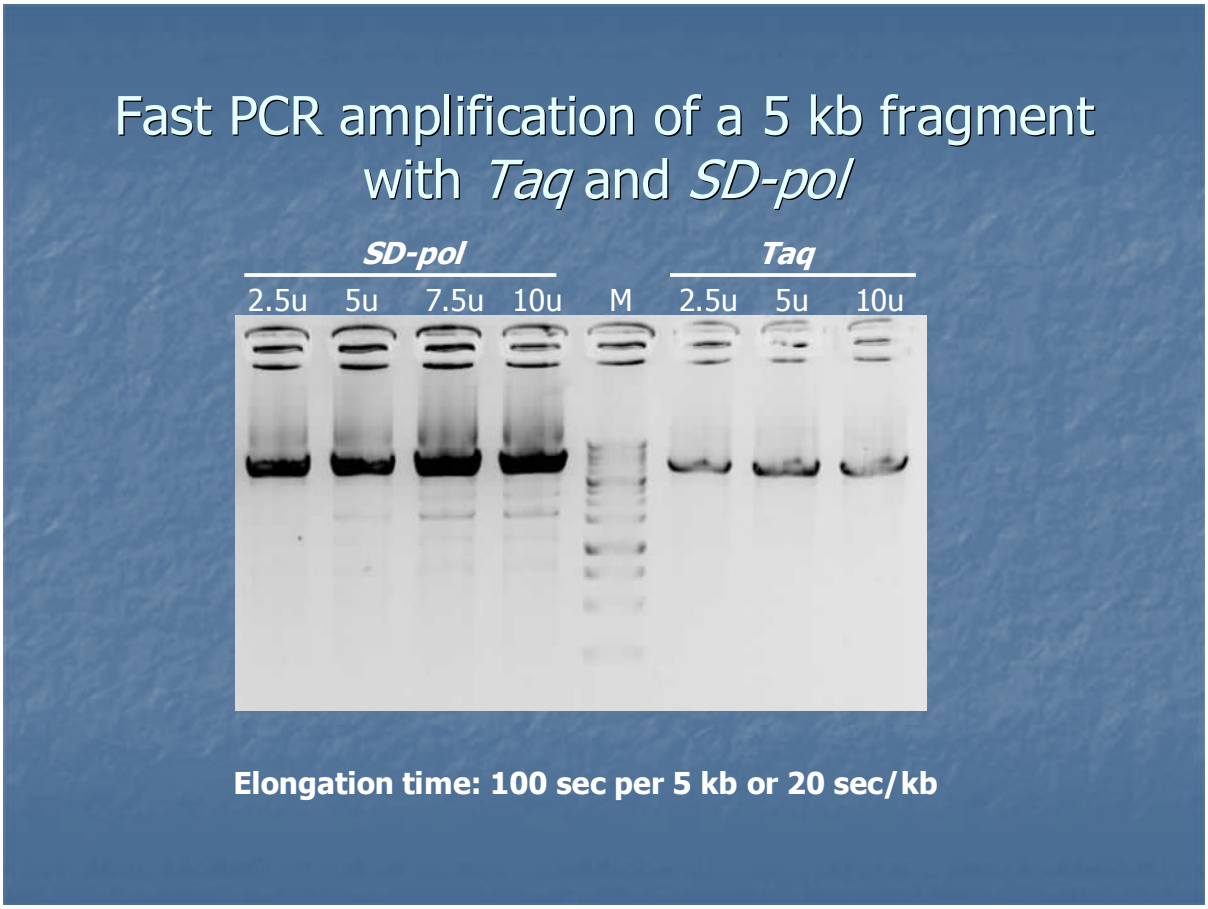
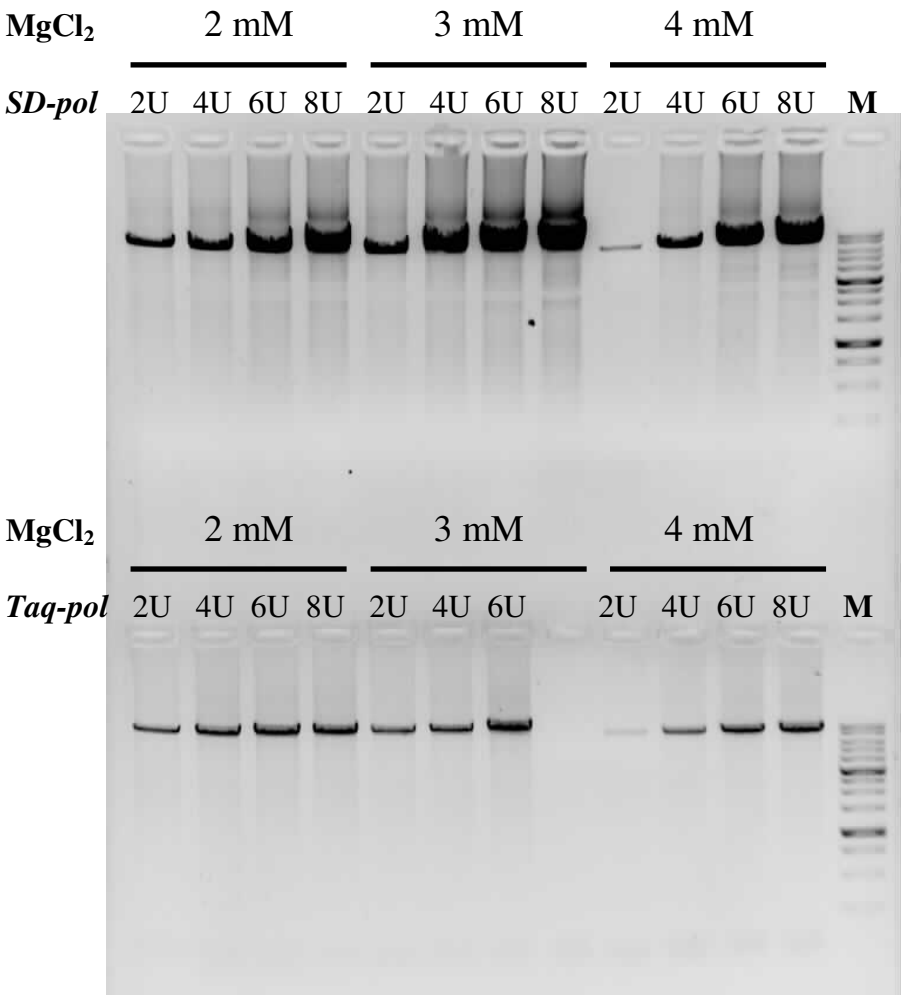

Fast PCR amplification of a 5 kb fragment with *Taq* and *SD-pol*
SD-pol
Taq
2.5u
5u
7.5u
10u
M
2.5u
5u
10u
Elongation time: 100 sec per 5 kb or 20 sec/kb

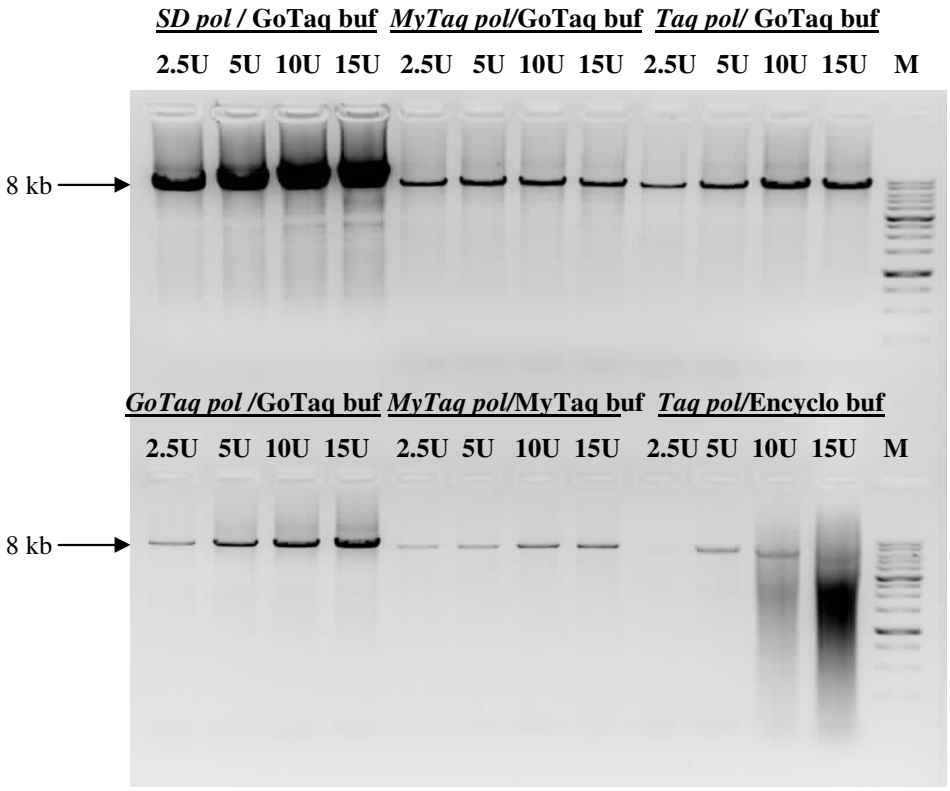
Fig. 7



SD polymerase* vs *GoTaq polymerase in GoTaq buffer (Promega, USA) with 2 mM, 3 mM and 4 mM $MgCl_2$. An 8-kb fragment of λ DNA was amplified with 2, 4, 6 or 8 units of *SD or Taq polymerase*. The reaction mixture (50 μl) contained: 5 ng λ DNA as template, 0.25 mM dNTP (each), 10 pmol (0.2 μM) of each primer. PCR was carried out for 25 cycles: preheating 92 C, 2 min; cycling 92 C, 30 sec, 60 C, 30 sec, 68 C, 2 min 40 sec (20 sec/kb).

Figure 7 demonstrates that *SD polymerase* provides much higher efficiency of PCR than *Taq*.

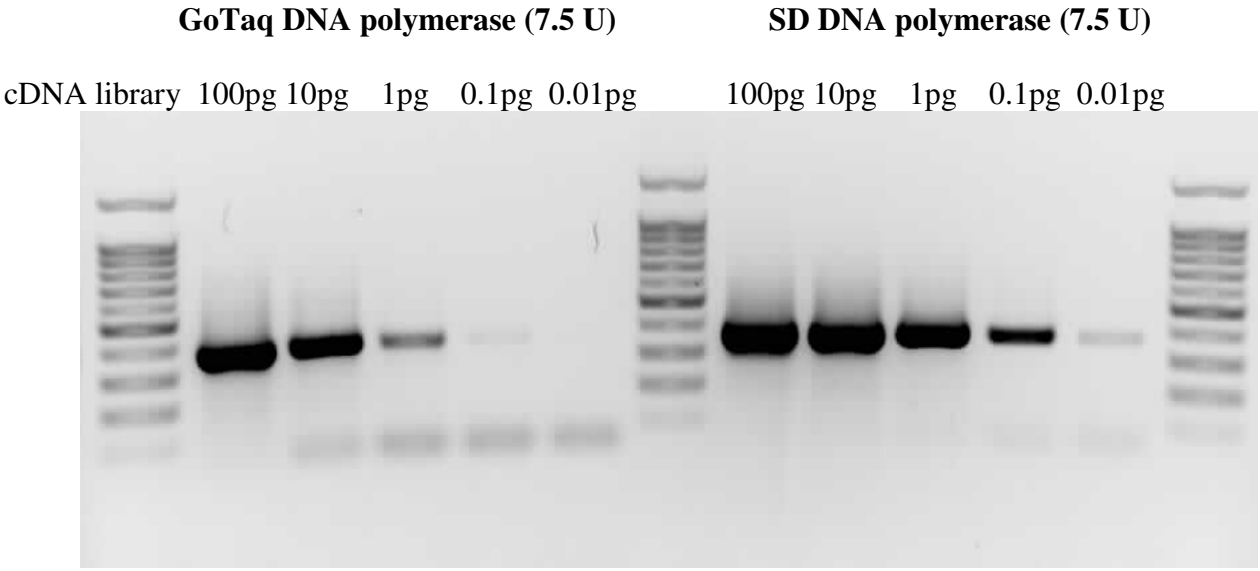
Fig. 8



An 8-kb fragment of λ DNA was amplified with 2.5, 5, 10 or 15 units of indicated DNA polymerase. Reaction mixture (50 µl) contained: 5 ng λ DNA as template, 0.25 mM dNTP (each), 10 pmol (0.2 µM) of each primer, 1x PCR buffer, and 3 mM MgCl2 (GoTaq and MyTaq buffers) or 3.5 mM MgCl2 (Encyclo buffer).

PCR was carried out for 25 cycles: preheating 92 C, 2 min; cycling 92 C, 30 sec, 60 C, 30 sec, 68 C, 2 min 40 sec (20 sec/kb).

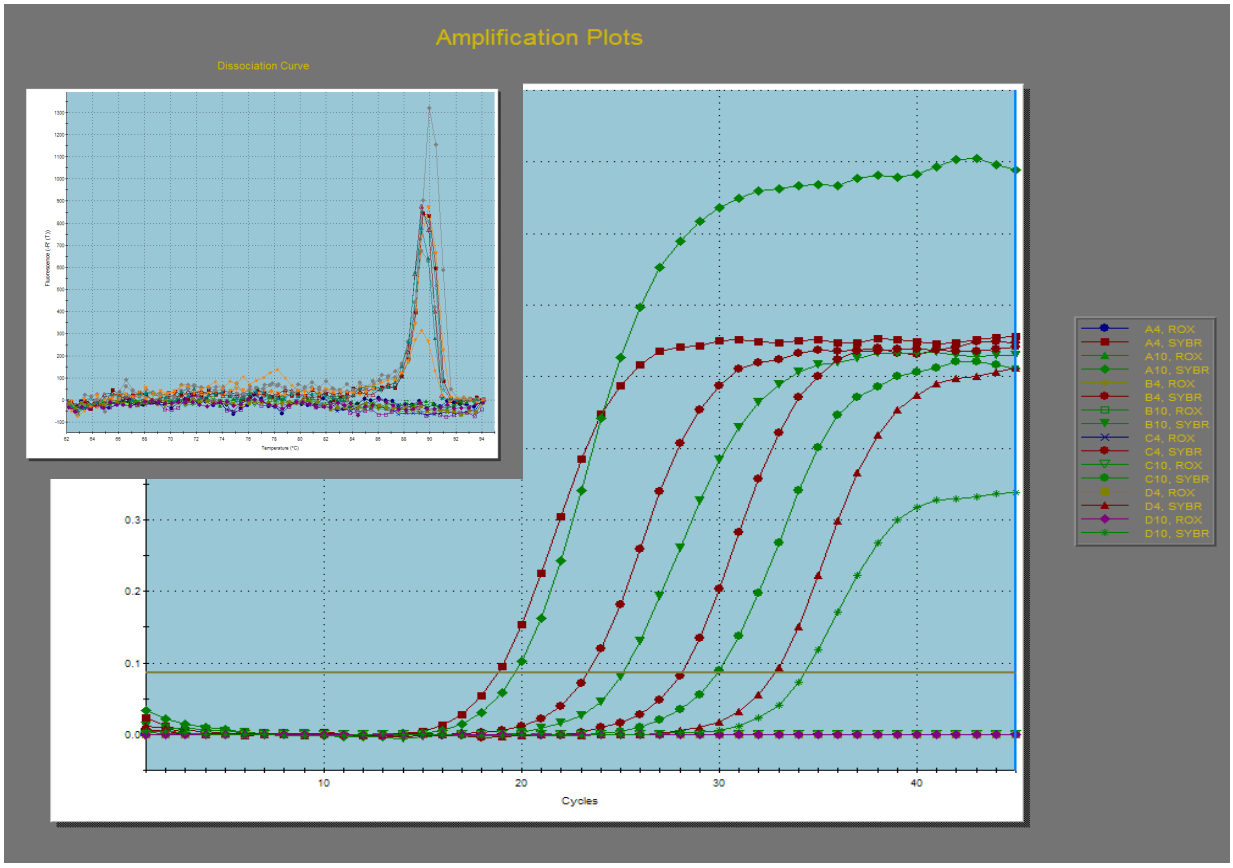
Fig. 9



A 364-bp fragment of murine G3PDH cDNA was amplified with 7.5 U of GoTaq (Promega) or SD DNA polymerase. Ten-fold dilutions of murine cDNA library (100 pg, 10 pg, 1 pg, 0.1 pg, 0.01 pg) were used as template. Reaction mixtures (50 µl) contained: 1x GoTaq buffer; 3 mM $MgCl_2$; 0.25 mM dNTPs (each); primers 15 pmol (each). PCRs were performed for 28 cycles: 92 C, 30 sec, 65 C, 40 sec; preheating 92 C, 1min 30sec.

Figure 9 demonstrates that *SD polymerase* provides more than ten-fold (about 50-100 times) higher sensitivity of PCR than *Taq.*

Fig. 10

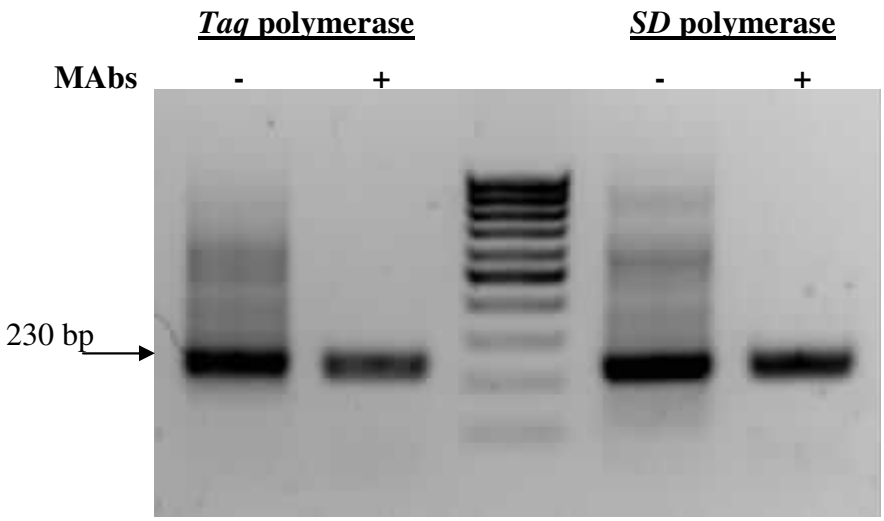


***SD polymerase* vs. *Taq polymerase* in RT-PCR with different concentrations of λ DNA template**. A 500 bp λ DNA fragment was amplified in RT-PCR with *Eva Green*. The reaction mixtures (15 mkL) contain: 1x Encyclo buffer (Evrogen) with 3.5 mM $MgCl_2$; dNTP 0.2 mM (each); primers 0.2 mkM (each); 1x Eva Green; DNA polymerase (*Taq* or *SD*) 1.5 U; and different amounts of λ DNA as a template (500 pg, 50 pg, 5 pg, 0.5 pg).

The results of RT-PCR with SD polymerase are indicated by Red color. The results of RT-PCR with Taq polymerase are indicated by Green color.

Figure 10 demonstrates that SD polymerase can provide better Ct in RT-PCR than Taq polymerase.

Fig. 11



A 230-bp DNA fragment was amplified from Human gDNA using GoTaq buffer and Taq-pol (with/without MAbs) or SD-pol (with/without MAbs). Reaction mixture (50 µl) contained: 25 ng gDNA as template, 0.25 mM dNTP (each), 10 pmol (0.2 µM) of each primer, 1x GoTaq buffer, 3 mM $MgCl_2$, and 2.5 U of Taq or SD polymerase.

PCR was carried out for 30 cycles: preheating 92 C, 2 min; cycling 92 C, 20 sec, 60 C, 15 sec, 68 C, 15 sec.

Figure 11 demonstrates that *AntiTaq* antibodies enable Hot Start PCR with the *SD* DNA polymerase.

Fig. 12

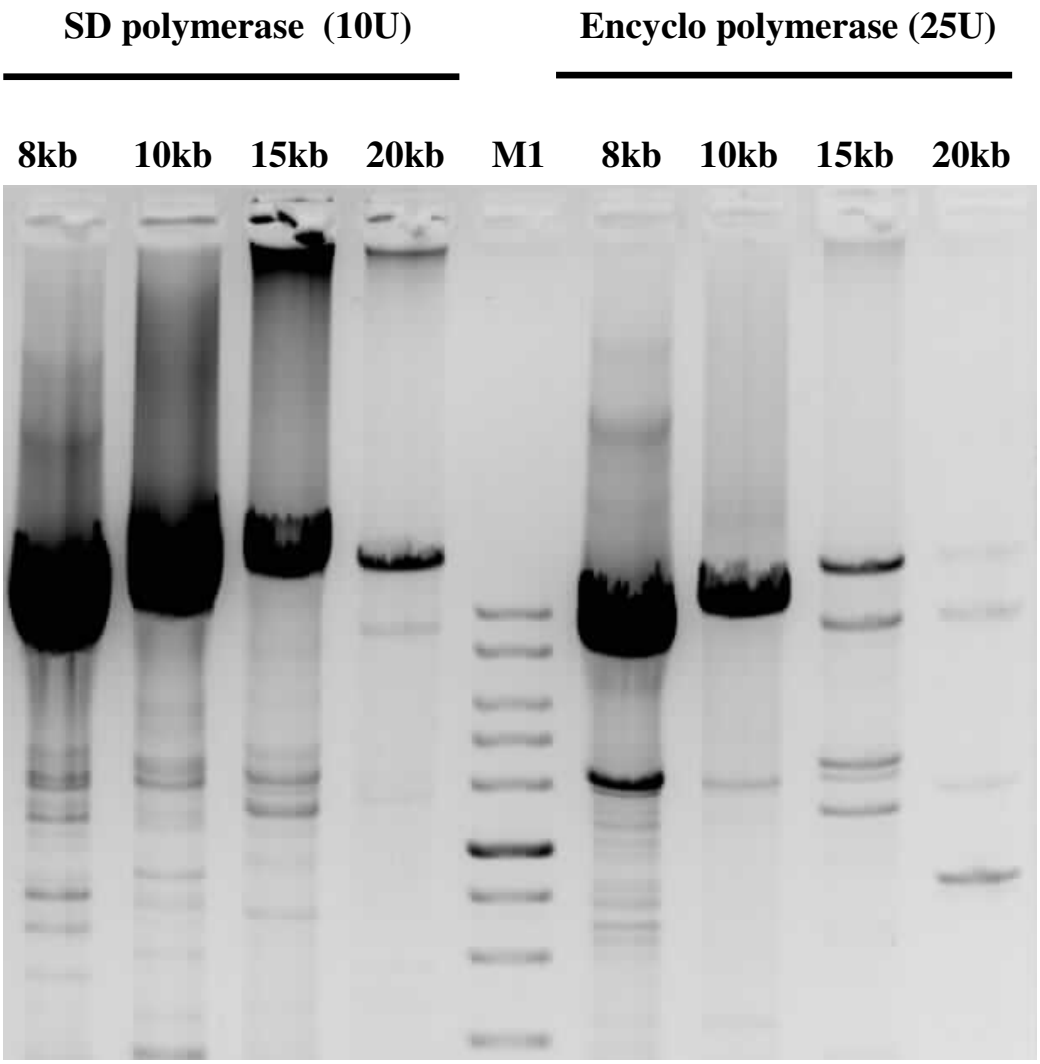


Fig. 12. Long PCR (fragments from 8 to 20 kb) was carried out with 10 units of SD polymerase and 25 units of Encyclo polymerase for 25 cycles: 92 C, 30 sec; 60 C, 30 sec; 68 C, 8 min. The reaction mixtures (50 μL) contained: 20 ng λ DNA as template; 10 pmol of each primer; 1x Encyclo buffer; and 0.2 mM of each dNTP.

Lane M1 was loaded with a 1-kb DNA ladder.

The electrophoresis was performed in a 1% gel, and 4 μL of each sample were loaded.

Fig. 13

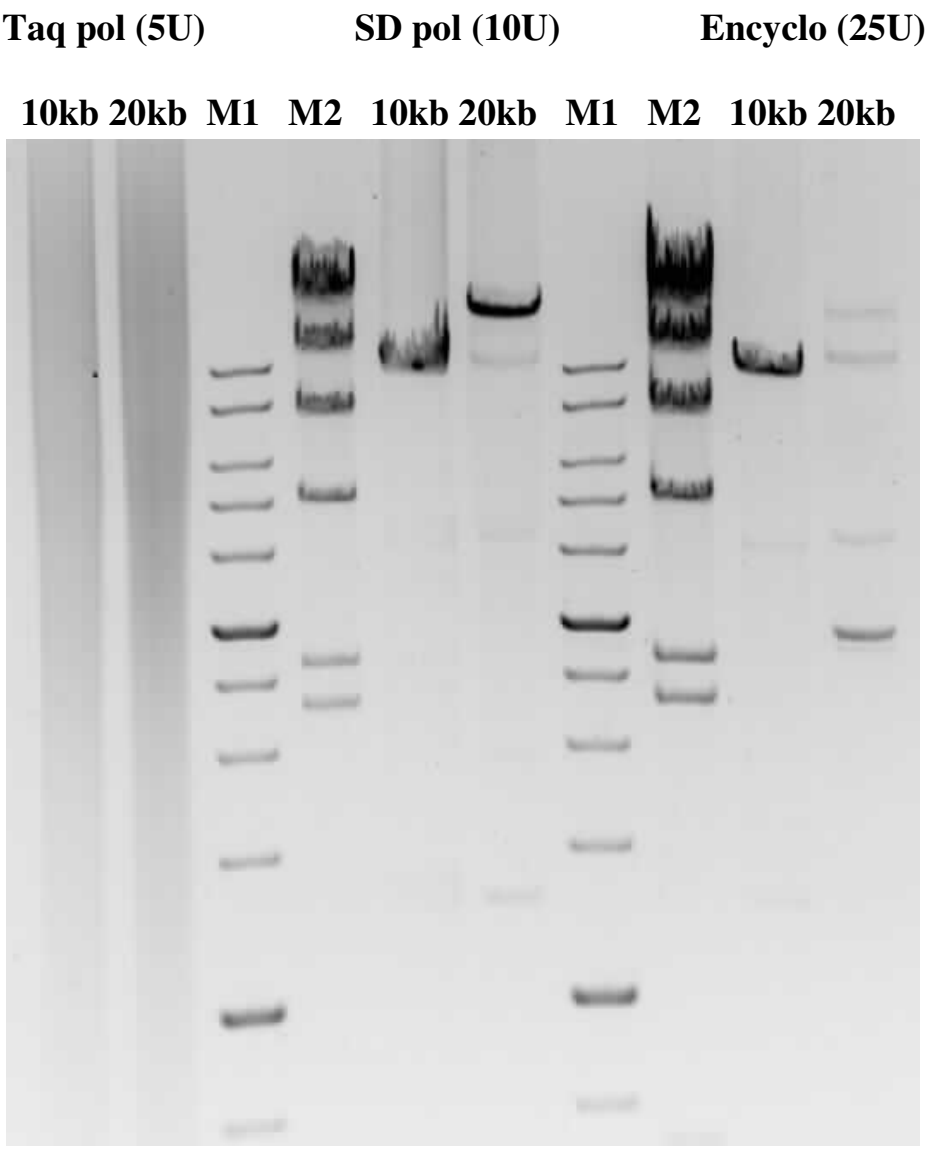


Fig. 13. Long PCRs (10 kb and 20 kb fragments) were carried out with indicated amounts of SD, Encyclo and Taq polymerases for 25 cycles: 92 C , 30 sec; 60 C, 30 sec; 68 C, 8 min. Reaction mixtures (50 mkL) contained: 20 ng λ DNA as template; primers – 10 pmol each; 1x Encyclo buffer; and 0.2 mM dNTPs each.

DNA size markers: M1, 1 kb marker; M2, λ/Hind III digest.

The electrophoresis was performed in 0.8% gels; 2 μL of 10 kb samples and 6 μL of 20kb samples were loaded.

Fig. 14

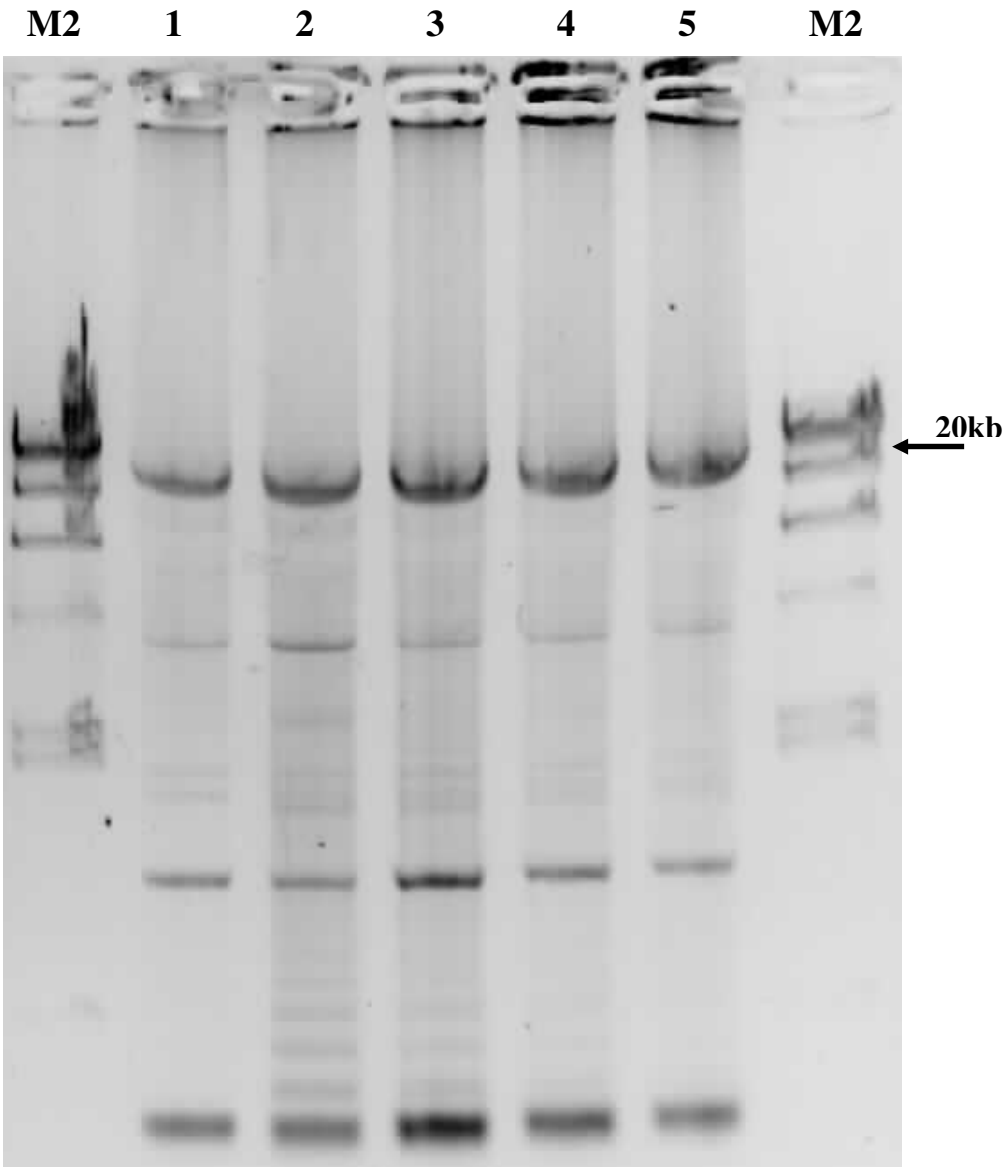


Fig. 14. Long PCR (20 kb fragment) were carried out with 5 units of SD polymerase as described above and with or without addition of DeepVent polymerase. The reaction mixtures (50 μL) contained DeepVent: 0 U, lane 1; 0.0025 U, lane 2; 0.0017 U, lane 3; 0.00125 U, lane 4; 0.001 U, lane 5.

The DNA size marker: M2, λ/Hind III digest.

The electrophoresis was performed in 0.75% gel, 5 μL of the samples were loaded.

Fig. 15

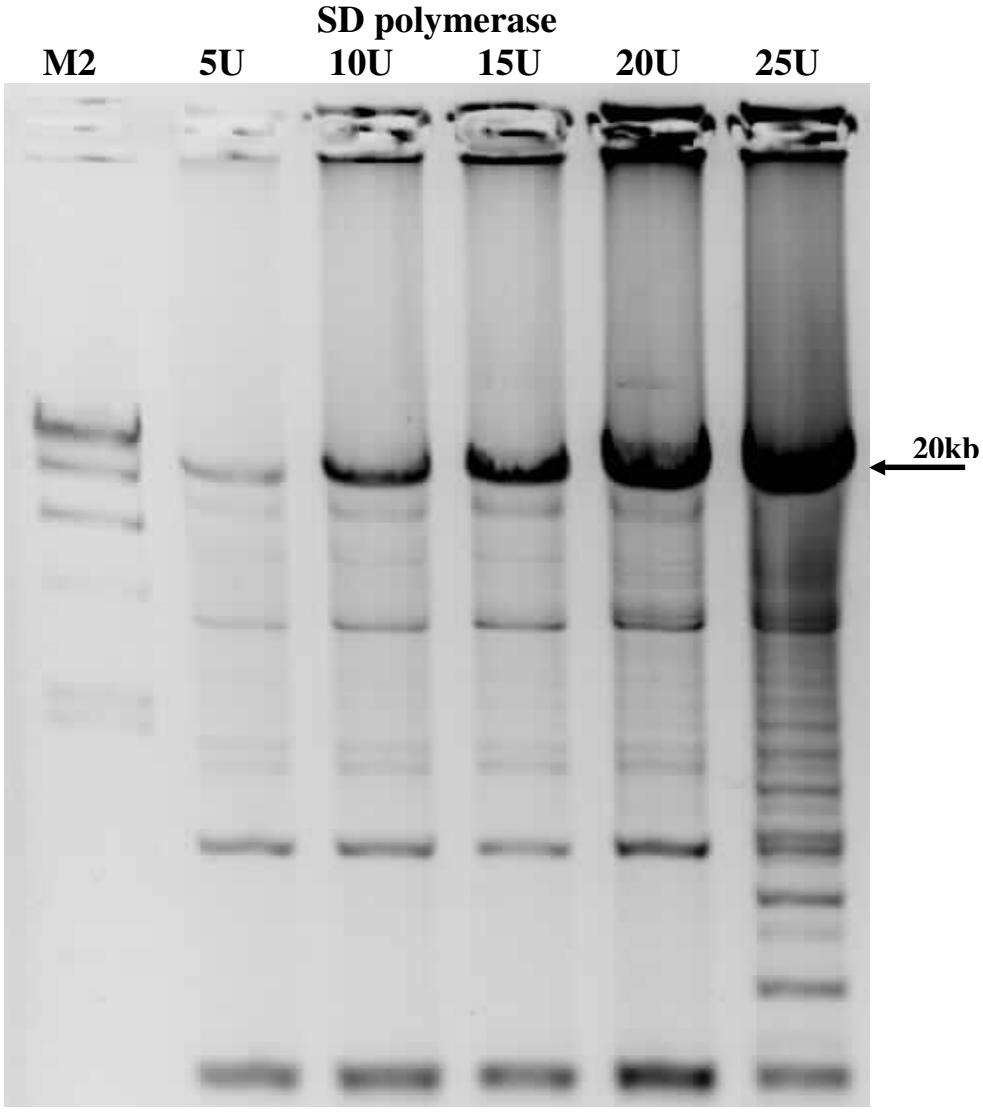


Fig. 15. Long PCR (20 kb fragment) were carried out as described above with indicated amounts of SD polymerase (5 -25 units per 50 μL).
The size marker: M2, λ/Hind III digest.

The electrophoresis was performed in 0.75% gel, 5 μL of the samples were loaded to the gel.