

# Improvement of $\phi$ 29 DNA polymerase amplification performance by fusion of DNA binding motifs

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**Bacteriophage  $\phi$ 29 DNA polymerase is a unique enzyme endowed with two distinctive properties, high processivity and faithful polymerization coupled to strand displacement, that have led to the development of protocols to achieve isothermal amplification of limiting amounts of both circular plasmids and genomic DNA. To enhance the amplification efficiency of  $\phi$ 29 DNA polymerase, we have constructed chimerical DNA polymerases by fusing DNA binding domains to the C terminus of the polymerase. The results show that the addition of Helix-hairpin-Helix [(HhH)<sub>2</sub>] domains increases DNA binding of the hybrid polymerases without hindering their replication rate. In addition, the chimerical DNA polymerases display an improved and faithful multiply primed DNA amplification proficiency on both circular plasmids and genomic DNA and are unique  $\phi$ 29 DNA polymerase variants with enhanced amplification performance. The reported chimerical DNA polymerases will contribute to make  $\phi$ 29 DNA polymerase-based amplification technologies one of the most powerful tools for genomics.**

DNA amplification | processivity | protein engineering | strand displacement | helix-hairpin-helix domain

A cornerstone of molecular biology, as genomic studies and phylogenetic and epidemiological analysis, is the amplification of limited amounts of DNA (1).

Presently, the polymerase chain reaction (PCR) (2) is still the most widely used methodology for DNA amplification. However, PCR depends on a previous knowledge of the sequence to be amplified, and it yields relatively short amplicons. Whole genome amplification technologies have been recently developed to generate large amounts of high quality DNA for genomic studies. Although several of the approaches are still based on PCR cycling, as primer extension preamplification (PEP) (3) or degenerated oligonucleotide primed PCR (DOP-PCR) (4, 5), isothermal multiple displacement amplification (MDA) by bacteriophage  $\phi$ 29 DNA polymerase has successfully gained ground in the DNA amplification area (6, 7). Due to the use of random hexamer primers, no previous sequence information is required, thus, any DNA is susceptible to be amplified; on the other hand, amplification performed by the  $\phi$ 29 DNA polymerase can generate large DNAs of hundreds of Kbs. In addition, multiply primed rolling circle amplification (RCA) has been shown to be the most robust technology to amplify circular templates of variable size required for genome sequencing (7), for the efficient amplification and detection of known and unknown circular viral genomes, for genotyping of single nucleotide polymorphisms (8), for whole genome analysis of noncultivable viruses (9), for detection and identification of circular plasmids in zoonotic pathogens (10), and very recently also for the description of new metagenomes (11). The combination of plasmid amplification and further direct sequencing represents a simple, fast, and low-cost method to get results comparable to those obtained with the use of other methods available (12). The success of MDA relies on the intrinsic and distinctive features of  $\phi$ 29 DNA polymerase: outstanding processivity and ability to couple polymerization to strand displacement (13).

Crystallographic and biochemical studies gave insights into the molecular mechanism by which  $\phi$ 29 DNA polymerase is singular in coupling processive polymerization to strand displacement (14, 15). Thus, an amino acid insertion specifically conserved in the polymerization domain of the protein-primed subgroup of DNA polymerases, named TPR2 (16, 17), contributes to form a narrow tunnel around the template strand, forcing melting of the duplex ahead of the polymerase (strand displacement) to gain access to the polymerase active site (see Fig. 1) (15). Additionally, in the polymerization domain, the TPR2 insertion along with the palm and thumb subdomains form a closed doughnut-shaped structure that encircles the upstream duplex product, in contrast to the open polymerization domain of most DNA polymerases, endowing  $\phi$ 29 DNA polymerase with high processivity in a manner analogous to sliding-clamp factors (14, 15, 18).

These structural data also unraveled the specific and delicate spatial arrangement of the different polymerization subdomains and the 3′–5′ exonuclease domain of  $\phi$ 29 DNA polymerase, allowing the efficient coupling of processive DNA polymerization to strand displacement, neither impairing the polymerization catalytic rate nor the proofreading of the insertion errors.

Due to their structural complexity and the multiple interactions with the substrates, acquisition of new properties by DNA polymerases is likely to require large structural modifications, which is difficult to tackle with point mutations. In this work we show that fusion of specific DNA binding domains to the C terminus of  $\phi$ 29 DNA polymerase provides the enzyme with a better DNA binding that leads to an improvement in its amplification efficiency.

## Results and Discussion

**Design of Chimerical  $\phi$ 29 DNA Polymerases.** The most promising strategies to confer new properties to a DNA polymerase are domain swapping and domain tagging (19). In this sense, efforts have been focused to increase the low processivity of the thermostable enzymes used in PCR, as *Taq* and *Pfu* DNA polymerases. Thus, Motz et al. (20) fused the PCNA binding domain of *Archaeoglobus fulgidus* DNA polymerase B to the C terminus of *Taq* DNA polymerase. The hybrid polymerase was stimulated in the presence of PCNA, although it was less active than the original enzyme in PCR. Davidson et al. (21) inserted the thioredoxin-binding domain of T3 DNA polymerase at an analogous position of *Taq* DNA polymerase. Despite the fact that addition of the processivity factor thioredoxin increased the processivity of the hybrid DNA polymerase from 80 nt to 300 nt, it was very inefficient to carry out PCR of fragments larger than 5 kb. An alternative and encouraging approach to improve PCR perfor-

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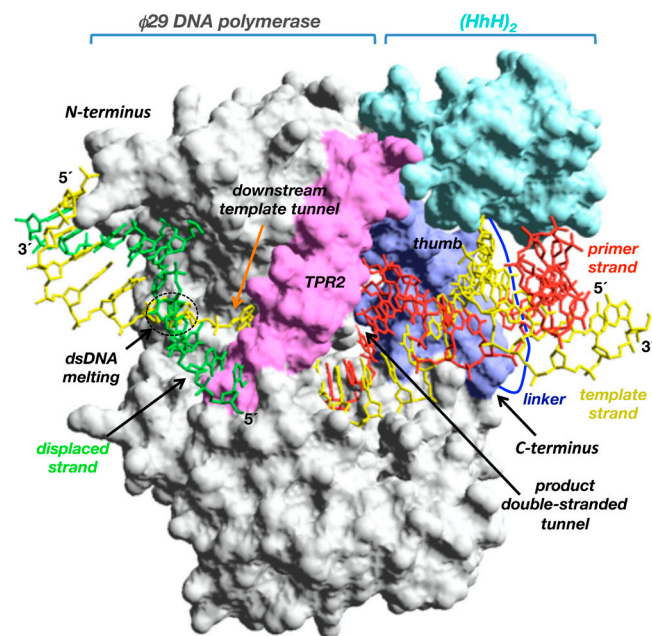
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mance consists in the fusion of DNA binding proteins to the amplification polymerase. Thus, Wang et al. (22) were successful in conferring a higher processivity to *Taq* (from 22 nt to 104 nt) and *Pfu* (from 6 nt to 55 nt) DNA polymerases by linking the polymerase domain to the dsDNA binding protein of *Sulfolobus solfataricus*. Additionally, it has been reported that the fusion of (HhH)<sub>2</sub> domains of *Methanopyrus kandleri* Topoisomerase V to the C or N terminus of *Taq* and *Pfu* DNA polymerases produced hybrid enzymes that retained the intrinsic low processivity at high levels of salt and other inhibitors of DNA synthesis (19, 23).

Because isothermal MDA using  $\phi$ 29 DNA polymerase is the most promising alternative to PCR, a major goal is the construction of  $\phi$ 29 DNA polymerase variants with improved amplification efficiency.  $\phi$ 29 DNA polymerase is provided with a nearly unlimited processivity (13). Thus, our efforts have been focused to get new  $\phi$ 29-based DNA polymerases with an enhanced DNA binding to increase the DNA amplification performance exhibited by this enzyme. In this sense, *M. kandleri* Topo V (HhH)<sub>2</sub> domain H (residues 696–751) or H and I (residues 696–802) (24–27) have been fused to the C terminus of  $\phi$ 29 DNA polymerase because it lies just at the exit of the upstream dsDNA product (see Fig. 1) (14, 18). Fusion of a (HhH)<sub>2</sub> domain at the N terminus of  $\phi$ 29 DNA polymerase could hinder its intrinsic strand displacement capacity, because biochemical and structural data demonstrated that unwinding of parental DNA takes place close to it (Fig. 1) (14, 15). Either one or two (HhH)<sub>2</sub> domains were fused to the polymerase through the flexible linker Gly-Thr-Gly-Ser-Gly-Ala (28) to preserve the structural folding of the enzyme and the DNA binding domains, rendering the chimerical polymerases  $\phi$ 29-H and  $\phi$ 29-HI (H and I stand for Topo V domains H and I, respectively; see *Materials and Methods*). A modeling of a



**Fig. 1.** Modeling of the chimerical DNA polymerase. The figure represents the structural model of a (HhH)<sub>2</sub> domain (colored in cyan) joint through a linker peptide (in dark blue) to the C terminus of  $\phi$ 29 DNA polymerase (colored in gray).  $\phi$ 29 DNA polymerase TPR2 insertion and thumb subdomain are colored in pink and dark blue, respectively. The modeled primer, template, and displaced strands are colored in red, yellow, and green, respectively. The upstream tunnel that encircles the newly synthesized dsDNA, the downstream template tunnel, as well as the N and C termini of  $\phi$ 29 DNA polymerase are indicated. Crystallographic data are from Protein Data Bank ID codes 2PZX ( $\phi$ 29 DNA polymerase binary complex) (18) and 1BPX [(HhH)<sub>2</sub> domain of DNA polymerase  $\beta$  (residues 91–148) (39)]. Figure was made by using the Swiss-PdbViewer software ([www.expasy.org/spdbv](http://www.expasy.org/spdbv)).

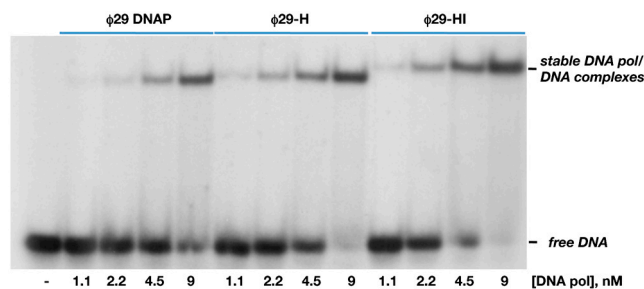
(HhH)<sub>2</sub> domain linked to the C-terminus of  $\phi$ 29 DNA polymerase is shown in Fig. 1.

**Chimerical DNA Polymerases Show a Higher DNA Binding.** To analyze whether fusion of the (HhH)<sub>2</sub> domains to  $\phi$ 29 DNA polymerase enhances its DNA binding, DNA gel retardation assays were performed, using as substrate a labeled hybrid 15-mer/21-mer DNA molecule (see *Materials and Methods*).

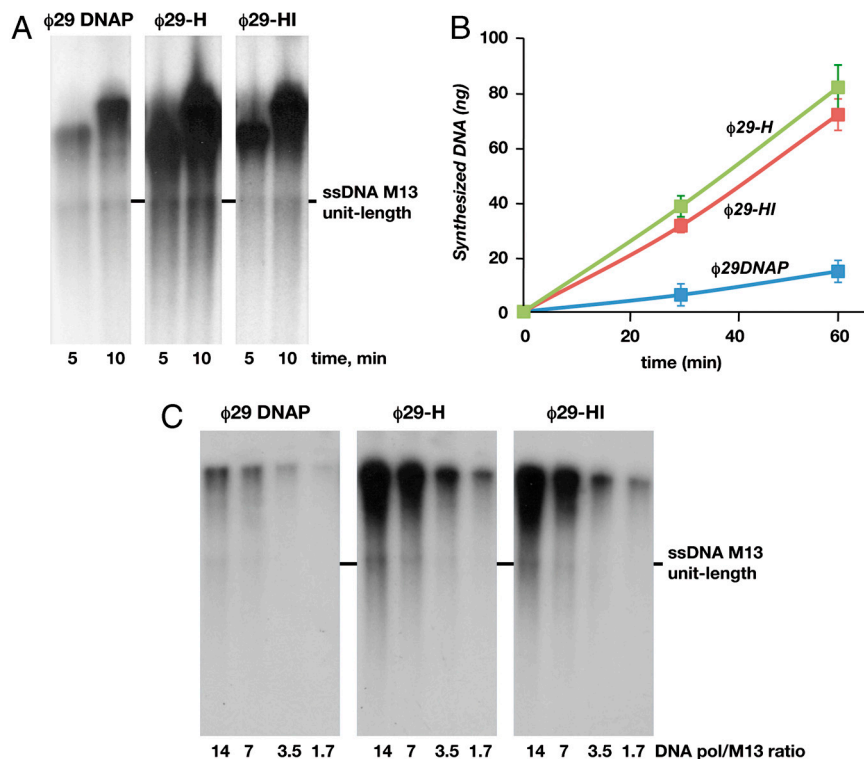
As shown in Fig. 2,  $\phi$ 29 DNA polymerase produces a single retardation band corresponding to a stable enzyme–DNA complex competent for polymerization (29) whose intensity depends on the concentration of enzyme added. Chimeras  $\phi$ 29-H and  $\phi$ 29-HI showed an improved DNA binding, requiring about twofold less enzyme concentration than the wild-type polymerase to give a similar shifted band. Thus, addition of (HhH)<sub>2</sub> domains H and HI from Topo V to the C-terminal end of the  $\phi$ 29 DNA polymerase endows the enzyme with an improved DNA binding.

**Rolling Circle Replication (RCR) by Chimerical DNA Polymerases.** As mentioned above, the use of  $\phi$ 29 DNA polymerase to perform successful DNA amplification relies on its faculty to couple processive polymerization to strand displacement by virtue of a simultaneous binding and translocation of the primer, template, and displaced strands through different regions of the polymerization domain (see Fig. 1). Thus, any alteration in this fine-tuned binding equilibrium could hinder the replication rate. To ascertain whether the improvement in DNA binding displayed by the chimerical polymerases affected the distinctive hallmarks of  $\phi$ 29 DNA polymerase, RCR assays were carried out using as substrate singly primed circular ssDNA of bacteriophage M13 (see *Materials and Methods*). In this assay the first replication round does not require any unwinding activity. However, to proceed through the next replication rounds the polymerase will have to deploy its active strand displacement (rolling circle type) (see *Materials and Methods*).

As it is shown in Fig. 3A, the length of the products synthesized by the three DNA polymerases was comparable. The fact that at the shortest reaction time products migrate at the resolving part of the gel indicates that the replication rate displayed by the chimeras was similar to that of  $\phi$ 29 DNA polymerase. This result indicates that, on the one hand, the (HhH)<sub>2</sub> domains interfere with neither the proper placement of the primer/template junction at the polymerization active site nor the strand displacement in chimerical DNA polymerases and, on the other hand, that the enhancement in DNA binding described above does not imply a tighter DNA polymerase/DNA interaction that could halt translocation of the enzyme along the DNA. Thus, considering the almost identical length of the products obtained, the increase in the total amount of DNA synthesized by chimeras  $\phi$ 29-H and  $\phi$ 29-HI



**Fig. 2.** Chimerical DNA polymerases have an improved DNA binding. The 5'-labeled hybrid molecule 15mer/21mer (dsDNA) was incubated with  $\phi$ 29 DNA polymerase or with the indicated chimerical DNA polymerase, under the conditions described in *Materials and Methods*. After gel electrophoresis, the mobility of free dsDNA and the polymerase–DNA complex was detected by autoradiography. The experiment shown is representative of several experiments.



**Fig. 3.** Chimerical DNA polymerases show an enhanced RCR efficiency. (A) Strand displacement-coupled M13 DNA replication by  $\phi$ 29 DNA polymerase and chimerical DNA polymerases. Replication of 250 ng of singly primed M13 DNA was carried out as described in *Materials and Methods* using 60 nM of  $\phi$ 29 wild-type or chimerical DNA polymerases. The position of unit-length M13 DNA is shown at the right. (B) Multiply primed RCR of M13 DNA by  $\phi$ 29 DNA polymerase and chimerical DNA polymerases. The assay was performed as described in *Materials and Methods*, in the presence of 250 ng of multiply primed M13 DNA as input and 60 nM of either  $\phi$ 29 DNA polymerase or the indicated chimerical DNA polymerase. Data are represented as Mean  $\pm$  SD. (C) Processive synthesis by  $\phi$ 29 DNA polymerase and chimerical DNA polymerases. The assay was performed as described in the text in the presence of 250 ng of singly primed M13 DNA and decreasing concentrations of the indicated DNA polymerase. After incubation at 30 °C for 20 min, samples were processed as described in A.

with respect to  $\phi$ 29 DNA polymerase (five- and fourfold, respectively) could be explained by a meliorated usage of the primer/template junctions, in agreement with their improved DNA binding.

The current  $\phi$ 29 DNA polymerase-based amplification protocols make use of random primers as short as hexamers. Thus, to ascertain whether the presence of (HhH)<sub>2</sub> domains confers a benefit on chimerical DNA polymerases also under these conditions, RCR assays were performed using as primers multiple hexamers (see *Materials and Methods*). As shown in Fig. 3B, chimerical DNA polymerases  $\phi$ 29-H and  $\phi$ 29-HI carried out RCR more efficiently than  $\phi$ 29 DNA polymerase, increasing the amount of DNA synthesized five- and sixfold, respectively.

Besides its intrinsic strand displacement capacity, the other specific feature that differentiates  $\phi$ 29 DNA polymerase is its processivity (13). To evaluate DNA polymerase processivity, the extension of each primer to one round of synthesis has to be assured so that the number of nucleotides added to each primer is equivalent to the processivity of the polymerase (30). For extremely processive DNA polymerases as  $\phi$ 29 DNA polymerase, this condition can be achieved by analyzing the distribution of products in a singly primed RCR assay under a range of ratios of DNA polymerase to substrate, a commonly used methodology (13, 30). A distributive (nonprocessive) pattern of elongation should give rise to a decrease in the DNA size concomitant with the polymerase dilution, because at low DNA polymerase concentrations the chance of dissociated DNA polymerase molecules to reassociate to an elongated primer is prevented. As shown in Fig. 3C, the reduction in the DNA polymerase/DNA molar ratio diminishes the total amount of DNA synthesized by  $\phi$ 29 DNA polymerase and by the two chimeras without encompassing shortening of the products, indicating that the same enzyme that starts replication proceeds with elongation of the growing chain without dissociation, i.e., processively.

Therefore, the fusion of the (HhH)<sub>2</sub> domains to the C-terminal end of  $\phi$ 29 DNA polymerase significantly improved its polymerization potential.

#### Multiply Primed RCA of Plasmid DNA by Chimerical DNA Polymerases.

The use of  $\phi$ 29 DNA polymerase in combination with 3'-protected random hexamers that allow synthesis of both strands has been the basis for the development of one of the most efficient procedures for isothermal dsDNA amplification of circular plasmids, named RCA (6, 7). In this methodology, the number of input DNA molecules used as substrate is reduced 10<sup>5</sup>-fold with respect to the RCR assays. Due to this significant difference between both protocols, an increase in the efficiency of chimerical DNA polymerases to perform RCA could not be anticipated. Therefore, RCA assays were set up in the presence of 1 ng (Fig. 4A) and 1 pg (Fig. 4B) of a dsDNA circular plasmid (see also *Materials and Methods*) and 3'-protected random hexamers. As shown in Fig. 4A,  $\phi$ 29 DNA polymerase gave rise to detectable amplification products using EtBrd staining at 2 h, and amplification continued for two more hours before reaching a plateau. Interestingly, such an amplification plateau was attained by both chimerical DNA polymerases in only 2 h. At this reaction time the amount of amplified DNA by the chimeras was fourfold higher than that rendered by  $\phi$ 29 DNA polymerase. Furthermore, chimera  $\phi$ 29-HI produced detectable amplification products at 1 h. In all cases the final amount of amplified material after 4 h was similar, most likely due to the exhaustion of the dNTPs provided. In the presence of 10<sup>3</sup>-fold lower input DNA (1 pg; Fig. 4B)  $\phi$ 29 DNA polymerase required 3 h to give an amplification product. Chimerical DNA polymerases rendered a perceptible amplification product from 1 h, reaching the amplification plateau at 3 h, the total amplification output at this reaction time being three- to fourfold higher than that obtained with  $\phi$ 29 DNA polymerase. With the three DNA polymerases, more than 80% of the amplified DNA was linearized after digestion with the endonuclease *EcoRI* rendering a 4.2 kbp dsDNA fragment, thus, most of the amplification products were tandem repeats of the original plasmid.

Additionally, 4,918 nonoverlapping nucleotides from the amplification reactions were sequenced as described (31) (see also *Materials and Methods*).  $\phi$ 29 DNA polymerase and the chimeras





**Multi-Primered RCA.** The incubation mixture contained, in 12.5  $\mu$ l, 37 mM Tris-HCl, pH 7.5, 0.025% Tween-20, 50 mM KCl, 5 mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM  $\text{MgCl}_2$ , 50  $\mu$ M of 3'-protected random hexamers, 500  $\mu$ M dNTPs and the indicated amount of plasmid DNA (4.2 kbp) as input. To denature DNA, samples were incubated for 3 min at 95 °C and afterward chilled on ice for 5 min. Reactions were started by adding 50 nM of  $\phi$ 29 DNA polymerase or the corresponding chimerical DNA polymerase. After incubation at 30 °C for the indicated times, reactions were stopped by incubating the samples for 10 min at 65 °C. One  $\mu$ l of each reaction was digested with *EcoRI* and further analyzed by electrophoresis in 0.7% agarose gels. After electrophoresis, the amplified DNA was detected by Etd bromide staining.

**Multi-Primered DNA Amplification of Genomic DNA.** The incubation mixture contained, in 12.5  $\mu$ l, 37 mM Tris-HCl, pH 7.5, 0.025% Tween-20, 50 mM KCl, 5 mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM  $\text{MgCl}_2$ , 50  $\mu$ M of 3'-protected random hexamers, 500  $\mu$ M dNTPs and 1 ng of *B. subtilis* genomic DNA. To denature DNA, the samples were incubated for 3 min at 95 °C and afterward chilled on ice for 5 min. Reactions were started by adding 50 nM of  $\phi$ 29 DNA polymerase or the corresponding chimerical DNA polymerase. After incubation at 30 °C for the indicated times, reactions were stopped by incubating samples for 10 min at 65 °C. One  $\mu$ l of each reaction was analyzed by electrophoresis in 0.7% agarose gels. After electrophoresis, the amplified DNA was detected by Etd bromide staining. In parallel, qPCR was carried out with a Roche Applied Science LightCycler following the manufacturer standard protocols, analyzing 1  $\mu$ l of a 250-fold dilution of each sample. The pair of specific primers amplifies a 700 bp region of the *B. subtilis* gene *ysHc*. The data obtained for the samples were interpolated to a standard curve obtained with

known amounts of *B. subtilis* genome in the same experiment. The results are represented as the amplification fold versus reaction time.

**Measurement of the Fidelity of the Chimerical Polymerases.** The protocol used is essentially as described in ref. 31. Thus, samples from the experiment corresponding to multiply primered RCA of plasmidic DNA by  $\phi$ 29 DNA polymerase and chimerical DNA polymerases, were taken (3  $\mu$ l) and mixed with 17  $\mu$ l of restriction mix (2  $\mu$ l New England Biolabs (NEB) 10X *EcoRI* Buffer, 0.5  $\mu$ l NEB *EcoRI* enzyme [10 units] and 14.5  $\mu$ l  $\text{H}_2\text{O}$ ) to get linear monomers of amplified plasmid. After incubation for 1 h at 37 °C, DNA was purified through Qiagen Gel-Extraction Kit Columns and eluted in 30  $\mu$ l of buffer 50 mM Tris-HCl pH 7.5, 1 mM EDTA. 10  $\mu$ l of each of the eluted samples were religated by mixing with 2  $\mu$ l NBE 10X Ligase Buffer, 8  $\mu$ l  $\text{H}_2\text{O}$ , and 0.5  $\mu$ l NEB Ligase (200 units). After overnight incubation at 16 °C, 2  $\mu$ l of each of the ligation reactions were transformed onto chemically competent *E. coli* cells XL-1 Blue. About 1,000 transformants were obtained using each of the amplified samples, whereas no transformant was obtained from control samples that contained the initial amount of plasmid pT7-4 and underwent the same procedure as the amplified samples. Two clones from each transformation were selected and the plasmid was prepared and sequenced according to standard procedures. In total, 4,918 nonoverlapping nucleotides were sequenced for each polymerase.

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