

ABSTRACT

We have modified bacterial, archaeal and viral polymerases used in sequencing and amplification. The enzymes were fused to one of three motifs: a single-stranded (ss) or double-stranded (ds) nucleic acid binding domain, or a new processivity-enhancing domain. Six modified DNA polymerases have been selected for further development. Most of these showed increased affinity for ssDNA and dsDNA in mobility shift and primer extension assays. Improvements were also seen in sequencing of recalcitrant templates that are either GC- or structure-rich. Higher affinity for either ssDNA or dsDNA templates also enabled Sanger sequencing directly from single bacterial colonies. Better affinity for dsDNA templates improved long PCR amplification. Interestingly, strand displacement activity correlated positively with affinity to dsDNA, but negatively with affinity to ssDNA. These newly developed reagents promise to advance a wide variety of next generation sequencing and amplification applications.

DNA polymerases (DNAP) are widely used for nucleic acid amplification, detection and sequencing. The most commonly used enzyme for Sanger sequencing is derived from *Thermus aquaticus*, whereas *Bacillus sterothrophilus* (Bst) DNAP is used in the 454/Roche pyrosequencing platform. Second and third generation instruments for massively parallel DNA sequencing can deliver megabases of data for a few dollars, with the promise of a human genome for a few thousand dollars in the near future. The development of a DNAP to match the technical capabilities of new instrument platforms has not kept pace. Achieving long and accurate reads using new solid phase template extension methods, terminator chemistries, and microfluidic flow technologies places new demands on the currently used enzymes. Polymerases with increased template affinity for DNA or RNA could provide important improvements in sequencing, amplification, and reverse transcription. We have engineered bacterial, archaeal, and viral polymerases to improve binding affinity. A new class of DNA polymerases and affinity modifications promises to improve a variety of next generation sequencing and amplification applications.

RESULTS

We have engineered high affinity binding for double stranded and/or single stranded DNA to improve the utility of several DNA polymerases. Double strand high affinity (DSHA) and single strand high affinity (SSHA) DNA polymerases were engineered from the following DNA polymerases: *Thermus aquaticus* (Taq), PyroPhage 3173, phage T4, and *E. coli* (Klenow fragment of Pol I).

Phage DNA polymerases, reverse transcriptases, and single strand and double strand binding motifs were obtained from thermal pools In YNP. Schoenfeld et al. 2008 Appl Environ Microbiol 74:4164-74.

dsDNA BINDING

DSHA Taq or WT Taq DNAP was incubated with a linear dsDNA template to assay for binding. Taq DNA polymerase did not demonstrate measurable mobility shift, indicating weak DNA binding. The DSHA derivative of Taq DNAP showed significant binding, as indicated by an upward shift in mobility (Figure 1). The DSHA derivative of T4 exo- DNAP showed a very strong binding, and the SSHA Klenow exo- enzyme showed weak binding (Figure 2).

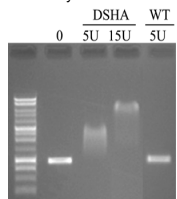


Figure 1. Binding of DSHA Taq DNAP to dsDNA. Enzymes were incubated with 20 ng of 1-kb PCR product in NaCl/MgCl₂ buffer for 20 minutes at 40°C. The mixture was subject to electrophoresis on a 1% agarose gel. Binding of DSHA Taq was not affected by varying the concentration of NaCl (50 to 200 mM) or MgCl₂ (0 to 2.0 mM).

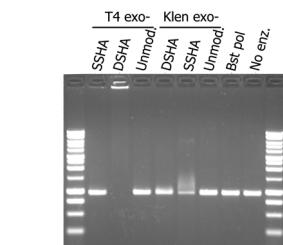


Figure 2. ds DNA binding of DSHA and SSHA Klenow exo- and T4 exo- DNAPs. A 1-kb dsDNA fragment was incubated with 5U of the indicated DNA DNAP in a standard buffer and run on a 1% agarose gel.

ssDNA BINDING

SSHA and DSHA modifications of T4 exo- and Klenow exo- resulted in a noticeable mobility shifts in the presence of ssDNA (Figure 3). In the presence of a primer, the ssDNA template is replicated to its dsDNA form (arrow). Bst pol showed no binding to ssDNA, and little extension of the replication primer.

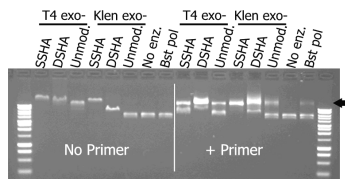


Figure 3. Single strand DNA binding and extension assay of DSHA and SSHA forms of Kenow exo- and T4 exo- DNAPs. M13 ssDNA was incubated with 5U of the indicated DNA DNAP in a standard buffer in the absence or presence of replication primer for 30 minutes at 37°C.

DNA SEQUENCING

DSHA Taq DNAP and SSHA PyroPhage 3173 DNAP are able to synthesize through a difficult hairpin that has confounded all other polymerases (Figure 4).

DSHA Taq DNAP was also able to sequence low amounts of template from crude lysates, a task for which Taq DNAP is inefficient (Figures 5 and 6).

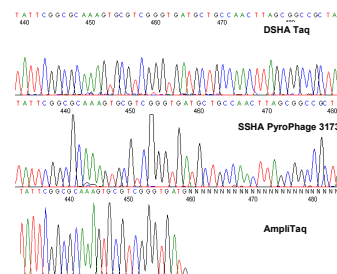


Figure 4. Improved affinity permits sequencing of hairpin DNA templates using DSHA Taq or SSHA PyroPhage 3173 DNAP. ABRF tough template #3 was sequenced with the reverse primer. ABRF study participants were unable to read through the 24bp hairpin, generating no acceptable data using existing DNAPs. (http://www.abrf.org/ResearchGroups/DNASequencing/EPosters/2008DSRG_Study_presentation.pdf) p19

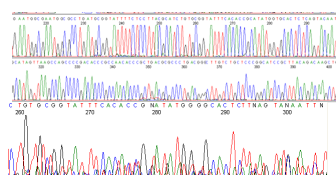


Figure 5. Single colony sequencing. A single bacterial colony containing pUC18 was sequenced directly using DSHA Taq (upper panel) or AmpliTaq DNAP (lower panel).

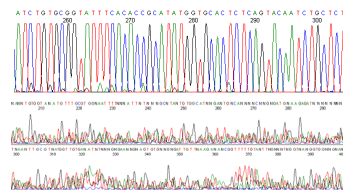


Figure 6. Direct sequencing from liquid cultures. *E. coli* containing pUC18 was grown overnight in LB. Five µl of culture was added directly to sequencing reactions containing DSHA Taq (upper panel) or AmpliTaq DNAP (lower panel).

LONG PCR AMPLIFICATION

The effect of increased affinity on long PCR was tested using DSHA and WT Taq DNAPs, as well as DSHA PyroPhage 3173 DNAP. Both DSHA enzymes generated a robust product (Figure 7).

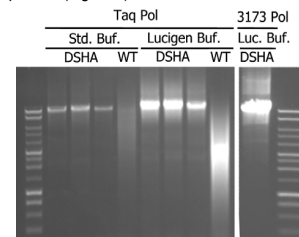


Figure 7. Long PCR using DSHA and WT Taq DNAP. Primers to lambda DNA were designed to produce a 10- kb amplicon. Amplification reactions were performed under standard conditions using two different buffers.

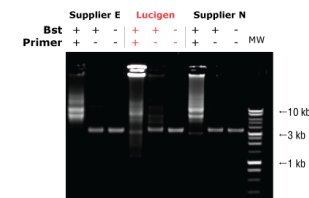


Figure 8. Lucigen Bst DNA Polymerase, exonuclease Minus, possesses superior strand displacement activity. M13 single stranded DNA was incubated with or without 8 units of Bst DNA Polymerase(±Bst) in the reaction buffer supplied by the manufacturer in the presence or absence of replication primer ± primer) for 30 minutes at 65°C. MW, 1 kb ladder.

CONCLUSIONS

Modifications of several DNAPs enhanced their ability to bind dsDNA or ssDNA.

DNAPs with enhanced binding showed significant improvement in sequencing templates that were difficult, impure, or present in low amounts.

Modification of DNAPs imparted the ability to amplify long templates by PCR.

These results suggest that DNAPs with enhanced binding to ssDNA or dsDNA should be applicable to next generation sequencing platforms.

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