Comparing the Speed and Product Yield of 7 high fidelity DNA Polymerases

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Introduction
Thermococcus kodakaraensis (strain KOD1), previously thought to be a Pyrococcus sp., is a hyperthermophilic archaea isolated from a solfataric hot spring on Kodakara Island, Japan (Atomi 2004). In preliminary studies to characterize the KOD1 DNA polymerase, researchers found that the enzyme had fidelity comparable to Pfu DNA polymerase, but with an extension rate (referred to as speed) 5 times higher and processivity 10 to 15 times higher than the Pfu enzyme (Takagi 1997). When amplifying DNA for cloning, high fidelity DNA polymerases, such as KOD polymerase, are recommended. If the enzyme is also fast and can generate high yields of full-length product, fewer amplification cycles are required and the probability of obtaining error-free clones is greatly increased.

Since the preliminary KOD DNA Polymerase studies, significant work has been done to optimize the PCR buffer and cycling parameters. Another improvement is KOD Hot Start DNA Polymerase, which is a premixed complex of KOD DNA Polymerase and two monoclonal antibodies. The antibodies inhibit the 3’→5’ exonuclease and DNA polymerase activities at ambient temperatures (Mizuguchi 1999), providing high template specificity by preventing primer degradation and mispriming events during reaction set-up. This report evaluates the speed and product yield of KOD Hot Start DNA Polymerase in an optimized reaction buffer and compares the enzyme to 6 other commercially available high fidelity thermostophilic DNA polymerases.

Materials and Methods
Thermocycler
DNA amplification was performed on MJ Research PTC-200 thermocyclers that had recently been calibrated by the manufacturer. Reactions were going to be done in tube strips, always using the same polymerase in the same location on each strip. To ensure no bias due to the location of the reaction in the strip or in the thermocycler, test reactions were performed using KOD Hot Start DNA Polymerase, and reaction yields measured. Yields were found to be comparable for all tube locations and wells tested (data not shown).

Table 1. Reaction components and cycling profile for each DNA polymerase based on manufacturers’ recommendations

<table>
<thead>
<tr>
<th>DNA Polymerase</th>
<th>Buffer</th>
<th>[Mg²⁺] in reaction (mM)</th>
<th>[dNTP] (mM each)</th>
<th>[Primer] (µM each)</th>
<th>Template/25-µl reaction (ng)</th>
<th>Polymerase’s Cycling profile†</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOD Hot Start</td>
<td>1X</td>
<td>1.5</td>
<td>0.2</td>
<td>0.3</td>
<td>5</td>
<td>0.5 U</td>
</tr>
<tr>
<td>KOD</td>
<td>1X</td>
<td>1.5</td>
<td>0.2</td>
<td>0.3</td>
<td>5</td>
<td>0.5 U</td>
</tr>
<tr>
<td>Platinum® Pfx</td>
<td>1X</td>
<td>1.0</td>
<td>0.3</td>
<td>0.3</td>
<td>5</td>
<td>0.5 U</td>
</tr>
<tr>
<td>PfxSO™</td>
<td>1X</td>
<td>in buffer</td>
<td>1.2</td>
<td>0.3</td>
<td>5</td>
<td>2.5 U</td>
</tr>
<tr>
<td>Phusion® Hot Start</td>
<td>1X</td>
<td>in buffer</td>
<td>1.5</td>
<td>0.2</td>
<td>5</td>
<td>0.5 U</td>
</tr>
<tr>
<td>PfuULtra™ Fusion Hot Start</td>
<td>1X</td>
<td>in buffer</td>
<td>2.0</td>
<td>0.25</td>
<td>0.2</td>
<td>5</td>
</tr>
<tr>
<td>PrimeSTAR® HS</td>
<td>1X</td>
<td>in buffer</td>
<td>1.0</td>
<td>0.2</td>
<td>0.3</td>
<td>5</td>
</tr>
</tbody>
</table>

*Manufacturer defined units, used as recommended by manufacturer. † See Table 2 for cycling parameters

Reaction volume
Reaction volumes of 50, 25, 20, and 10 µl were tested to determine an optimum volume for consistent PCR results (data not shown). Both 50- and 25-µl reactions gave consistent results, and the 25-µl volume was used for the remaining experiments.

Template
A 919-bp fragment of human GSK3α (glycogen synthase kinase 3α) catalytic domain ORF, 54% GC-content, in an Open Biosystems cDNA plasmid (MHS1010-7507851, GeneBank BC027984) was selected as the DNA template.

Primers/reagents
Primers used were HPLC purified: (35-mer sense) 5’-GACGACGACAAGATTTCCTAAGAAGTGGCTTACAC-3’ and (41-mer antisense) 5’-GAGGAGAAGCCGGTCTTACATCGCAGTTCATCAAAGAG-3’. Bases shown in bold are homologous to the ORF. Table 1 shows the reaction components used for each polymerase.

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Table 2. Cycling profiles used

<table>
<thead>
<tr>
<th>Cycle Profile 1</th>
<th>Cycle Profile 2</th>
<th>Cycle Profile 3</th>
<th>Cycle Profile 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>98°C 30 s</td>
<td>94°C 2 min</td>
<td>95°C 2 min</td>
</tr>
<tr>
<td>29 cycles</td>
<td>98°C 10 s</td>
<td>94°C 15 s</td>
<td>95°C 20 s</td>
</tr>
<tr>
<td></td>
<td>55°C 20 s</td>
<td>52°C 20 s</td>
<td>55°C 20 s</td>
</tr>
<tr>
<td></td>
<td>72°C 30 s</td>
<td>68°C 60 s</td>
<td>72°C 30 s</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C 5 min</td>
<td>68°C 5 min</td>
<td>72°C 3 min</td>
</tr>
</tbody>
</table>

A. 23 cycles 25 cycles
M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

B. 23 cycles 25 cycles
M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

C. 23 cycles 25 cycles
M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

D. 23 cycles 25 cycles
M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

M 1 2 3 4 M 5 6 7 M 1 2 3 4 M 5 6 7

Figure 1. PCR results from 7 high fidelity thermophilic DNA polymerases using 4 different cycling profiles

Cycling profiles

All 7 enzymes were tested in 4 different cycling protocols, which encompass the manufacturers’ recommended cycling conditions (Table 2).

Results and Discussion

Different cycling profiles

Multiple reaction strips were prepared using master mixes for each polymerase. After 19, 21, 23, 25, 27, and 29 cycles, reaction strips were removed and placed on ice. Samples (5 μl) from cycles 23, 25, 27, and 29 were assayed on 1.4% agarose/TAE gels containing ethidium bromide (Figure 1). Yield concentrations were determined on diluted samples from 19, 21, 23, 25, 27, and 29 cycles using a Quant-iT™ PicoGreen® ds DNA Assay Kit (Invitrogen) and a FLUOstar plate reader (BMG LABTECH).

New primers and enzyme kits were obtained and all experiments were repeated to verify initial results and trends. Results with the new reagents were comparable to the initial experiments (data not shown).
Yields generated by each enzyme at 19, 21, 23, 25, 27, and 29 cycles were plotted for each protocol. Not all enzymes gave their best yield using the manufacturer’s recommended cycling conditions, so the best yields for each enzyme, from any cycling protocol, were compared (Figure 2). For cloning purposes, fewer reaction cycles increase the potential for error-free clones. Cycles 19-25 have been shaded green on the graph to emphasize the reaction yields from these earlier cycles. KOD Hot Start DNA Polymerase consistently gave high yields in cycles 19-25 for all 4 profiles tested. (data not shown)

2-step PCR
Longer primers (generally ≥23 bases) can increase PCR specificity and, due to higher annealing temperatures, can be used in time-saving 2-step cycling profiles. KOD Hot Start DNA Polymerase and the other 6 high fidelity enzymes were tested in a 2-step protocol (initial denaturation at 95°C for 2 min, and 29 cycles of 95°C for 20 s, 68°C for 25 s). Figure 3 shows that not all enzymes functioned well with this 2-step protocol (lanes with little or not product). Other enzymes, including the 2 KOD enzymes, generated high yields comparable to the 3-step protocols shown in Figure 1.

KOD application - screening plaques
Testing the ability of KOD Hot Start DNA Polymerase to amplify a variety of DNA templates, the enzyme was used in a 2-step cycling profile to screen random clones from the T7Select® Human Brain cDNA Library (Cat. No. 70637). Plaques were eluted in 100 µl TE (10 mM Tris-HCl, 0.1 mM EDTA) and 5 µl eluate was used for PCR. Primers were: sense primer 5’-ACT TCC AAG CGG ACC AGA TTA TCG C-3’ and antisense primer 5’-AAC CCC TCA AGA CCC GTT TAG AGG-3’. Reactions were cycled with an initial denaturation at 95°C for 2 min, and 25 cycles of 95°C for 20 s, 68°C for 25 s. Of the 50 clones screened, KOD successfully amplified 49 inserts (Figure 4). Amplicons ranged in size from ~250-1800 bp.

Figure 2. Best yield for each high fidelity thermophilic enzyme from any cycling profile
Yields were determined by PicoGreen analysis after 19, 21, 23, 25, 27, and 29 cycles for all 4 cycling profiles (Table 2). The best yield data for each enzyme, from any cycling profile, was graphed. The cycling profile that gave the best yields is identified in parentheses. The green shaded area highlights yields in cycles 19–25, which would be preferable for cloning.

Figure 3. PCR results from 7 high fidelity enzymes using a 2-step cycling profile
PCR samples were removed after 23, 25, 27, and 29 cycles of a 2-step protocol and 5 µl were assayed on 1.4% agarose/TAE gels. Lanes indicate the enzyme used for the reaction.

Figure 4. Results from KOD Hot Start amplification of T7Select Human cDNA Library clones
PCR was performed as stated in the text; 5 µl of each 25-µl reaction were assayed on a 1.4% agarose/TAE gel. KOD Hot Start successfully amplified 49 of 50 clones with amplicons ranging from ~250-1800 bp.
KOD Fidelity

KOD DNA polymerase (pol) fidelity in PCR has been assayed by different methods. Initial studies by Takagi et al. (1997) measured the mutation frequency in amplicons after 30 PCR cycles using a plasmid template containing the lacZ gene. By comparing the number of white and light blue colonies (mutant) to the total number of colonies (including blue, intact lacZ colonies), they determined mutation rates of 2.8% for KOD DNA pol, 3.6% for Pfu DNA pol, and 48.0% for Taq DNA pol. Using the same blue/white assay method, but with 25 cycles, Nishioka et al. (2001) found mutation frequencies of 0.79% for KOD DNA pol and 28.1% for Taq DNA pol. Rual et al. (2004) directly sequenced ~70,000 bases and determined a misincorporation rate of 1 in 35,000 nucleotides for KOD DNA pol compared to 1 in 2,000 nucleotides for Platinum® Taq DNA Polymerase High Fidelity in amplicons generated after 20 cycles of PCR. Discrepancies in mutation rates can be due to the different assay methods, as well as to thermal degradation of DNA at high temperatures, which is not related to enzyme function (Tindall 1988, Pienaar 2006). What stands out in these independent assays is the consistent high fidelity of KOD DNA pol.

Conclusion

A number of independent studies have verified the extremely high fidelity of KOD DNA Polymerase. In addition to a low mutation frequency, the fast extension rate and high processivity of the KOD enzyme result in high yields of full-length product in fewer reaction cycles. Combined, these attributes have made KOD Hot Start DNA Polymerase the PCR enzyme of choice for many routine and high throughput cloning and structural proteomics studies.

REFERENCES