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# PCR with Degenerate Primers Containing Deoxyinosine Fails with Pfu DNA Polymerase

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Pfu DNA polymerase is a novel thermostable DNA polymerase that possesses a 3' → 5' exonuclease (proofreading) activity responsible for a low error rate per nucleotide. Here, we show that degenerate primers can also be used efficiently with Pfu DNA polymerase for PCR. However, in contrast with *Taq* DNA polymerase, Pfu DNA polymerase cannot amplify a target sequence using degenerate primers substituted at a single position with deoxyinosine.

PCR has become a powerful tool in molecular biology and is widely used for enzymatic amplification of specific sequences from small amounts of template DNA. Mixtures of primers representing all codon choices of a target sequence (degenerate primers) are often designed for PCR.<sup>(1)</sup> However, primer degeneracy can reduce PCR efficiency and specificity dramatically. Therefore, substitution of degenerate primer positions with deoxyinosine, which can base-pair with A, C, G, or T, has been used because it seems to increase PCR reliability and consistency.<sup>(2-4)</sup> Recently, a new thermostable DNA polymerase, termed Pfu DNA polymerase, has been isolated from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*.<sup>(5)</sup> In contrast with *Taq* DNA polymerase, Pfu DNA polymerase possesses a 3' → 5' exonuclease (proofreading) activity that is responsible for >10-fold improvement in error rate per nucleotide.<sup>(5)</sup> At present, it remains to be clarified whether PCR works with Pfu DNA polymerase using degenerate oligonucleotides or deoxyinosine-containing primers.

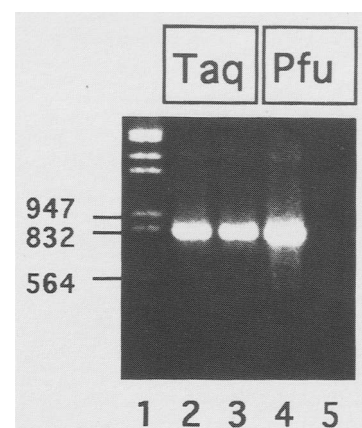
## MATERIALS AND METHODS

Degenerate sense (primer A) and antisense (primer B) oligonucleotide primers corresponding to conserved amino acid sequences within the DNA and hormone-binding regions of the rat glucocorticoid receptor (GR), respectively, had the following sequence: primer A, TG(C/T)AA(G/A)GT(A/C/G/T)TT(C/T)TT(C/T)AA; primer B, (T/C)TG(G/A)TA(G/A)AA(T/G/C/A)C(G/T)(T/C)TG CCA. Deoxyinosine was incorporated at position 10 in antisense primer B, creating primer BI with the sequence (T/C)TG(G/A)TA(G/A)AA IC(G/T)(T/C)TG CCA. PCR amplification was carried out in a 20- $\mu$ l reaction mixture containing 0.5 units of *Taq* DNA polymerase (Biofinex, Switzerland) or Pfu DNA polymerase (Stratagene), 1 pg

of plasmid containing a rat GR cDNA as target sequence, 200  $\mu$ M dNTPs, and 3  $\mu$ M of each primer. *Taq* DNA polymerase buffer was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl<sub>2</sub>, and 0.1 mg/ml gelatin. Pfu DNA polymerase buffer was composed of 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 0.1% Triton, and 0.1 mg/ml of BSA. Samples were amplified for 40 cycles in a DNA thermocycler using the following parameters: 94°C for 1 min; 45°C for 2 min; and 72°C for 2 min. In the first cycle incubation times were increased to 5 min, and following the final cycle PCR was completed with a 13-min incubation at 72°C. Four microliters of each reaction was fractionated on a 2% agarose gel containing 1  $\mu$ g/ml of ethidium bromide and visualized by UV fluorescence.

## RESULTS AND DISCUSSION

Using *Taq* or Pfu DNA polymerase and degenerate primers A and B, PCR yielded a single major DNA band of predicted size (828 bp) (Fig. 1). A single deoxyinosine substitution in primer B abolished amplification by Pfu DNA polymerase while *Taq* polymerase still worked with comparable efficiency (Fig. 1). This strik-



**FIGURE 1** Pfu DNA polymerase fails to amplify a target sequence using degenerate primers with a single deoxyinosine substitution. PCR reactions were performed using different DNA polymerases and degenerate primers with (primer BI) or without (primers A and B) deoxyinosine substitution (see Materials and Methods). Four microliters of each reaction was analyzed on a 2% agarose gel. (Lane 1) *Eco*RI/*Hind*III-digested  $\lambda$  DNA; (lanes 2,3) *Taq* DNA polymerase; (lanes 4,5) Pfu DNA polymerase. (Lanes 2,4) Primers A + B; (lanes 3,5) primers A + BI (deoxyinosine substituted).

ing difference between the two polymerases could not be overcome by varying PCR parameters (data not shown). Notably, we tested different primer sets and annealing temperatures (40°C, 45°C, 50°C), different concentrations of primers (0.3–6  $\mu$ M), template DNA (0.1–10 pg), dNTPs (100–200  $\mu$ M), magnesium ions (1.5–6 mM), and Pfu DNA polymerase (0.25–1 units). The difference also held up with the PCR “hot start” technique,<sup>(6)</sup> in the presence of 10% DMSO or when Pfu DNA polymerase was added after the annealing of the primers according to the manufacturer’s advice.

The fact that Pfu DNA polymerase cannot amplify a target sequence using deoxyinosine-containing primers may be the result of its 3' → 5' exonuclease activity. Pfu DNA polymerase might fail to incorporate a base complementary to deoxyinosine during consecutive rounds of PCR, preventing an exponential amplification of target DNA. It is quite likely that other thermostable DNA polymerases with proofreading activity would show the same limitations. However, it should be emphasized that Pfu DNA polymerase did work under all conditions tested using degenerate primers without deoxyinosine substitution. Therefore, its lower error rate per nucleotide, compared with that of *Taq* DNA polymerase, makes Pfu DNA polymerase a powerful tool for PCR employing degenerate primers, for example, in evolutionary PCR.

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