PCR with Deoxyinosine-containing Primers Using DNA Polymerases with Proofreading Activity

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As the application of PCR has expanded, more amplified products have been used for cloning and mutagenesis. Although *Taq* polymerase (from eubacterium *Thermus aquaticus*) is the most convenient and intensively studied enzyme to date, one of its drawbacks is a relatively high misincorporation (error) rate ($\sim 1 \times 10^{-5}$), which may be problematic in some applications. Recently several DNA polymerases with 3' $\rightarrow$ 5' exonuclease (proofreading) activity have become commercially available, namely Deep Vent (from archaeobacterium *Pyrococcus* sp.), *Pfu* (from archaeobacterium *Pyrococcus furiosus*), and *UITma* DNA polymerases (from eubacterium *Thermotoga maritima*). They could yield lower misincorporation rates ($\sim 2 \times 10^{-6}$) and greater stability at high temperature, according to the manufacturers' specification.

Degenerate primers with mixed bases have been used in many applications, such as identification of members of gene families,⁽¹⁾ and simultaneous detection of viruses with conserved genomic sequences.⁽²⁾ Substitution of a mixed-base position with deoxyinosine (dI) was reported to make the amplification more effective.⁽³⁾ Although we tried to identify paramyxovirus RNA in lupus erythematosus tissues with degenerate primers, we were able to amplify the sequence with *Taq* polymerase but not with *Pfu* or Deep Vent polymerases. In this report we present a successful amplification of a viral sequence with primers containing dI in various positions, using *UITma* DNA polymerase.

MATERIALS AND METHODS

Viral RNA was extracted from the supernatant of measles virus culture (Edmonston strain, American Type Culture Collection, Rockville, MD) with guanidium thiocyanate.⁽⁴⁾ The RNA was reverse transcribed with SuperScript II RNase H⁻ Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD) with random primers, and cDNA from ~ 1 ng of RNA was used as a template for amplification. The sense primer was positioned at codons 922–946 of measles NP gene (GenBank accession number K01711), and the antisense primer at codons 1105–1129. Five antisense primers were prepared to compare the effect of mixed bases or position of dI (Fig. 1). Forty cycles of hot-start PCR were performed in a TempCy-

sense primer (5' $\rightarrow$ 3')

Mea-922 ACTATTAAGTTTGGGATAGAACTA

antisense primer (5' $\rightarrow$ 3')

Mea-1105 CTCCTACTCCCATGGCATAGCTCCA

Mea-1105M CTCCTACTCCCATGGCATAACTCCA
GG A C A
G T

Mea-1110I CTCCTACTCCCATGGCATAICTCCA

Mea-1116I CTCCTACTCCCATIGCATAACTCCA

Mea-1125I CTCCTACTCCCATGGCATAGCTCCA

FIGURE 1 Primers used in this study.

cler II thermal cycler (Coy Laboratory Products Inc., Ann Arbor, MI) with a 50- μ l mixture of every combination of the following conditions: annealing for 1 min, from 40°C to 75°C in increments of 5°C; extension for 1 min at 75°C; denaturing for 30 sec at 95°C; MgCl₂ at 1, 2, or 3 mM; dNTPs at 50, 100, or 200 μ M each. Primers (1 μ M of each) and 1.5 units of *UITma* DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) were used. In the first cycle, both denaturation and annealing with lengthened to 5 min, and the last extension step was carried out for 10 min. A control amplification was performed with *Taq* Polymerase (Promega, Madison, WI). To compare exonuclease effect, recombinant *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and Deep Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA), both Exo⁺ and Exo⁻ forms, were used under comparable conditions. Immediately after the amplification, 5 μ l of each sample was run on a 2% agarose gel, electro-

FIGURE 2 Measles virus amplification with *Taq* or *UITma*. (Lane M) 123-bp DNA ladder; (lanes 1–5) *Taq*; (lanes 6–10) *UITma*; (lanes 1,6) Mea-1105 for antisense primer; (lanes 2,7) Mea-1105M; (lanes 3,8) Mea-1110I; (lanes 4,9) Mea-1120I; (lanes 5,10) Mea-1125I.

Technical Tips

FIGURE 3 With *Pfu* (Exo⁺ or Exo⁻) (Lane M) 123-bp DNA ladder; (lanes 1–5) Exo⁺ *Pfu*; (lanes 6–10) Exo⁻ *Pfu*; (lanes 1,6) Mea-1105 for antisense primer; (lanes 2,7) Mea-1105M; (lanes 3,8) Mea-1110I; (lanes 4,9) Mea-1116I; (lanes 5,10) Mea-1125I.

phoresed with ethidium bromide, and visualized on a UV illuminator.

RESULTS AND DISCUSSION

With both UITma and *Taq* polymerases, expected bands of 208 bp were obtained with every combination of primers, regardless of mixed bases or dI (Fig. 2). The most effective amplification was achieved at the annealing temperature of 55°C with both UITma and *Taq*. In contrast, with *Pfu* (Exo⁺ or Exo⁻) polymerase we obtained positive results with the primer with mixed bases, Mea-1105M, but not with the primers containing dI, Mea-1110I, 1116I, 1125I (Fig. 3). Deep Vent polymerase (Exo⁺) worked only with the nondegenerate primer, Mea-1105. Exo⁻ Deep Vent could amplify measles virus sequence with any of the primers (Fig. 4).

The failure of *Pfu* polymerase to amplify DNA with primers containing dI

was recently published, and the incompatibility of the proofreading enzyme and dI was suggested.⁽⁵⁾ Here, we demonstrated that UITma was the only Exo⁺ polymerase that was used successfully with degenerate primers. The position of dI in the primer did not affect the result, and when we incorporated up to seven dI in papilloma virus primers,⁽⁶⁾ UITma, but not *Pfu* or Deep Vent, amplified an 850 bp sequence as efficiently as *Taq* (H. Fujiwara, K. Fujiwara, and K. Hashimoto, unpubl.). According to the manufacturer, UITma was modified to reduce its 3' → 5' exonuclease activity relative to native DNA polymerase. Although the exonuclease activity is not the only factor that determines compatibility, as we found with *Pfu*, the reduction of the exonuclease activity could, at least partially, contribute to successful amplification, as shown with Exo⁻ Deep Vent. In the case of *Pfu* it is possible that the polymerase activity could not cross a dI on the template strand.

It is still necessary to determine optimal PCR conditions empirically.⁽⁷⁾ The choice of DNA polymerase should be made carefully, as each enzyme has its characteristic misincorporation,⁽⁸⁾ and if one uses a proofreading enzyme, careful optimization is required.⁽⁹⁾ Although the specifications of polymerases look similar on data sheets, one needs to choose a polymerase carefully for the specific needs of the application.

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FIGURE 4 With Deep Vent (Exo⁺ or Exo⁻). (Lane M) 123-bp DNA ladder; (lanes 1–5) Exo⁺ Deep Vent; (lanes 6–10) Exo⁻ Deep Vent; (lanes 1,6) Mea-1105 for antisense primer; (lanes 2,7) Mea-1105M; (lanes 3,8) Mea-1110I; (lanes 4,9) Mea-1116I; (lanes 5,10) Mea-1125I.

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