Benchmarks

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Cautionary Note on the Use of dUMP-Containing PCR Primers with *Pfu* and *Vent_R* DNA Polymerases

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High-fidelity amplification and cloning of polymerase chain reaction (PCR) fragments are often desirable in analyzing rare mutations in heterogeneous cell populations. PCR products can be cloned rapidly and efficiently using primers with dUMP-containing

12-base tails and the uracil DNA glycosylase (UDG)-cloning approach (4). Following amplification, PCR products and a compatible vector are annealed in the presence of UDG, which produces abasic sites at the dUMP positions and allows the complementary strands to anneal (4). Competent bacteria can be transformed with annealed products without prior ligation. To analyze mutations in DNA from cancer cells, we used the high-fidelity *Pfu* DNA polymerase from *Pyrococcus furiosus* (5) to minimize PCR-generated artifactual base changes. We also attempted to combine the use of *Pfu* DNA polymerase with the UDG cloning system, but to our dismay, we found that the dUMP-containing primers were incompatible with this enzyme as well as with at least one other high-fidelity thermostable DNA polymerase.

Primers were designed to amplify the helix-loop-helix (HLH) and leucine zipper (ZIP) domains of the transcriptional regulator MAD2 (also known as MXI1) (2,6). The primers contained the sequence (CUA)₄ at the 5' ends (Table 1). A similar set of primers, but devoid of the (CUA)₄ tails, was also compared by using *Pfu*, *Taq*, *Vent_R* and *Vent_R* (exo-) DNA polymerases and the eLONGase™ kit (Table 1), which contains a mixture of *Taq* and *Pyrococcus* GB-D DNA polymerases. Conditions for PCR are listed in Table 1. As shown in Figure 1A, *Pfu* DNA polymerase was unable to amplify a product of the correct size from human genomic MAD2 P1 clone DNA with the dUMP-containing primers (lanes 1 and 2). In contrast, *Taq* DNA polymerase, which does not possess 3' → 5' proofreading ability, amplifies a product from the same template regardless of whether the primers contain dUMP (lanes 5 and 6) or not (lanes 7 and 8). *Pfu* DNA polymerase was unable to utilize the dUMP-containing primers with a number of different annealing temperatures and MgSO₄ concentrations. However, *Pfu* DNA polymerase amplified the correct fragments using similar primers devoid of dUMP [i.e., lacking the (CUA)₄ tails] (Figure 1A, lanes 3 and 4). The eLONGase PCR amplification system contains a mixture of *Pyrococcus* GB-D DNA polymerase and *Taq* DNA polymerase, and dUMP-contain-

ing primers are not recommended by the manufacturer for use with this enzyme mixture. However, the eLONGase kit produced a robust DNA product using the dUMP-containing primers (Figure 1B, lanes 1 and 2 as compared with lanes 3 and 4).

Vent_R DNA polymerase from the archaeobacteria *Thermococcus litoralis* is a high-fidelity enzyme with a 3' → 5' proofreading exonuclease activity like *Pfu* DNA polymerase. *Vent_R* DNA polymerase was unable to utilize dUMP-containing primers for PCR amplification (Figure 1C, lanes 1 and 2 as compared with lanes 3 and 4). Based upon the results obtained with *Taq* DNA polymerase (Figure 1), we tested whether *Vent_R* (exo-) DNA polymerase, which also lacked a 3' → 5' proofreading exonuclease activity, could utilize the dUMP-containing primers.

Figure 1. Analysis of PCR on agarose gel. 20 µL of each reaction were analyzed on a 2.5% SeaKem® LE agarose gel (FMC BioProducts, Rockland, ME, USA) in 1× Tris-acetate-EDTA buffer (3). The gel was stained in 0.1 µg/mL ethidium bromide in distilled water and photographed. Thermostable DNA polymerases used for the reactions are: (A) *Pfu* (lanes 1–4) and *Taq* (lanes 5–8); (B) eLONGase kit; (C) *Vent_R*; and (D) *Vent_R* (exo-). Primers for each enzyme reaction are shown in the following order: HLH-U, ZIP-U, HLH and ZIP. M = 1-kb ladder (Life Technologies). Fragments generated with dUMP-containing primers migrate slightly slower than their non-dUMP-containing counterparts because of the additional two 12-base tails. Samples shown in each panel were run on different gels. HLH and ZIP PCR fragments were confirmed by DNA sequencing.

Benchmarks

Table 1. PCR Primers and Amplification Conditions

dUMP-Containing Primers^a:				
HLH-U forward:	(CUA) ₄	TAA	CCA	GAC
HLH-U reverse:	(CUA) ₄	CCA	GAA	CTG
ZIP-U forward:	(CUA) ₄	TTG	TTT	GTA
ZIP-U reverse:	(CUA) ₄	TGT	TTA	GTA
PCR amplification conditions^b:				
Primers	Enzyme; Units per Reaction	mM		Annealing Temperature
		MgCl ₂	MgSO ₄	
HLH-U	<i>Pfu</i>	0.5	2.0	58°C
	<i>Taq</i>	0.5	2.5	58°C
HLH	eLONGase	0.5	2.5	58°C
	Vent _R	0.4	2.0	48°C
	Vent _R (exo ⁻)	0.4	2.0	58°C
ZIP-U	<i>Pfu</i>	0.5	2.5	50°C
	<i>Taq</i>	0.5	2.5	58°C
ZIP	eLONGase	0.5	2.5	58°C
	Vent _R	0.4	2.0	48°C
	Vent _R (exo ⁻)	0.4	2.5	50°C

^aHLH and ZIP primers do not have the 12-base (CUA)₄ sequence.

^bReactions (20 µL) also contained 0.25–0.5 ng of human MAD2 P1 DNA, 0.2 mM dNTPs and 0.5 µM primers in 1× manufacturer's buffer. Cycling program: initial denaturation at 94°C for 30 s, followed by 35–45 cycles of 94°C for 30 s; the specified annealing temperature for 30 s; 72°C for 30 s; and a final incubation at 72°C for 10 min. Sources for thermostable enzymes and buffers are: *Pfu*, Stratagene (La Jolla, CA, USA); *Taq* and eLONGase kit, Life Technologies (Gaithersburg, MD, USA); Vent_R and Vent_R (exo⁻), New England Biolabs (Beverly, MA, USA).

However, Vent_R (exo⁻) DNA polymerase was also inhibited by the dUMP-containing primers (Figure 1D, lanes 1 and 2 as compared with lanes 3 and 4). It is also interesting to note that for any given primer set, significant differences in optimum annealing temperature (as much as 10°C) may sometimes exist among these thermostable DNA polymerases. Subtle differences in primer annealing temperatures may exist even for thermostable DNA polymerases that utilize the same buffer [Vent_R, Vent_R (exo⁻) and *Pfu*]. For example, *Pfu* and Vent_R (exo⁻) yielded products with HLH primers at 58°C, whereas results were more consistent with less lane background with Vent_R at 48°C annealing temperature (Table 1). *Taq* DNA polymerase amplified the template with ZIP primers annealed at

58°C (Table 1), whereas *Pfu* DNA polymerase poorly did so at the same temperature, unless the MgSO₄ concentration was raised to 6 mM (not shown). Consequently, PCR conditions should be carefully optimized for a given primer set when used with different thermostable DNA polymerases.

We conclude that the high-fidelity DNA polymerases *Pfu* and Vent_R, as well as Vent_R (exo⁻), are unable to utilize primers compatible with UDG cloning. The dUMP-containing primers tested had a contiguous region devoid of dUMP of 20–23 bases, perfectly complementary up to their 3' ends with the template. Despite this, they could not be extended successfully with either *Pfu*, Vent_R or Vent_R (exo⁻) DNA polymerases. The comparison of *Taq* vs. Vent_R (exo⁻) DNA polymerases suggest

that a lack of 3' → 5' exonuclease activity does not render the latter capable of utilizing the dUMP-containing primers. *Pfu*, Vent_R and Vent_R (exo⁻) DNA polymerases might become "frozen" on the template because of the 12-base dUMP-containing noncomplementary 5' tails of the primers. Consequently, for high-efficiency cloning with *Pfu* or Vent_R DNA polymerases, alternative systems should be considered (1).

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