Technical Overview

Technical notes provide unique applications, innovative methods, and clear protocols designed specifically for Agilent reagents and instruments.

High-Fidelity PCR Enzymes: Properties and Error Rate Determinations

The polymerase chain reaction (PCR) has revolutionized biological sciences, in particular genetics and proteomics. Since the introduction of Taq DNA polymerase in the late 1980s, significant progress has been made in developing PCR enzyme formulations with improved fidelity, PCR performance, and speed. This Technical Note surveys commercial PCR enzymes developed for high-fidelity PCR applications, such as cloning, mutation detection, and site-directed mutagenesis. We provide detailed information regarding the composition, PCR characteristics, and applications of proofreading DNA polymerases and DNA polymerase blends. We discuss methods for determining DNA polymerase error rates, and provide an in-depth description of the procedure and results obtained using the *lac*I-based phenotypic mutation assay.

Introduction

High-fidelity PCR enzymes are valuable for minimizing the introduction of amplification errors in products that will be cloned, sequenced, and expressed. Significant time and effort can be saved by employing high-fidelity amplification procedures that eliminate the need for downstream error-correction steps and minimize the number of clones that must be sequenced in order to obtain error free constructs or accurate consensus sequences. Moreover, the use of high-fidelity amplification conditions is essential when analyzing very small amounts of template DNA or rare molecules in heterogeneous populations[1]. Amplifications employing small amounts of template DNA are especially prone to high mutant frequencies due to PCR-generated errors in early cycles ("jackpot" artifacts) and high target doublings[1]. When analyzing rare sequences, such as allelic polymorphisms in individual mRNA transcripts[2], allelic stages of single cells[3], or rare mutations in human cells[4], it is essential that polymerase-generated errors ("PCR-induced noise") are minimized to prevent masking of rare DNA sequences.

PCR fidelity is largely determined by the intrinsic error rate of a DNA polymerase under the reaction conditions employed. Parameters contributing to DNA polymerase fidelity have been reviewed[5-8] and include the tendency of a polymerase to incorporate incorrect nucleotides, the rate at which the enzyme can extend from mispaired 3′ primer termini, and the presence of an integral 3′-5′ exonuclease (proofreading) activity, which can remove mispaired bases. The importance of proofreading is evident in comparisons of base substitution error rates between non-proofreading (10^{-2} to > 10^{-6}) and proofreading (10^{-6} to 10^{-7}) DNA polymerases[5,9]. DNA polymerase error rates are influenced by PCR reaction conditions, and can be minimized by optimizing pH, Mg^{2+} concentration, and nucleotide concentrations [9-12].

Taq DNA polymerase is suitable for a number of PCR applications, and is still considered by many to be the industry standard. However, the performance of *Taq* is limited in more challenging applications, such as those requiring high fidelity, synthesis of long (> 2 kb) amplicons, and amplification of GC-rich sequences. *Taq* DNA polymerase lacks proofreading activity, and as a result, exhibits relatively poor fidelity.

Background

Proofreading Archaeal DNA Polymerases

High-fidelity PCR enzymes include proofreading archaeal DNA polymerases (Table 1) and DNA polymerase blends (Table 2). Commercial proofreading DNA polymerases have been obtained from Thermococcus and Pyrococcus species of hyperthermophilic archaea and are classified as Family B-type DNA polymerases[13]. Unlike thermophilic eubacterial DNA polymerases (e.g., *Taq*), which may or may not possess 3′-5′ exonuclease activity, all archaeal B-type DNA polymerases possess proofreading activity and lack an associated 5′-3′ exonuclease activity.

The kinetic properties of several thermostable DNA polymerases have been reported[13-15]. Comparisons of steady-state kinetic parameters indicate that archaeal proofreading DNA polymerases exhibit lower K_m [DNA] values (0.01–0.7 nM) and similar K_m [dNTPs] values (16–57 µM) compared to those reported for *Taq* (1–4 nM, K_m [DNA]; 16–24 µM, K_m [dNTPs]). Most archaeal proofreading DNA polymerases (*Pfu*, Deep Vent) exhibit limited processivity (< 20 bases) in vitro (Table 1). The only known exceptions are KOD DNA polymerase, which is reported to be 10- to 15-fold more processive than *Pfu* and Deep Vent DNA polymerases[14], and archaeal DNA polymerases that have been engineered for increased processivity by fusion to DNA-binding proteins (see *Archaeal DNA Polymerase Fusions* section). Polymerization rates determined for thermostable DNA polymerases range from 9–25 nucleotides/second (*Pfu*) up to 47–61 nucleotides/second (*Taq*) and 106–138 nucleotides/second (KOD)[14, 15].

Unlike *Taq*, which possesses a structure-specific 5′–3′ endonuclease activity that cleaves 5′ flap structures[16], archaeal DNA polymerases exhibit temperature-dependent strand displacement activity (e.g., detectable at ≥ 70°C for *Pfu*[15,17]). *Taq* DNA polymerase also adds extra non-template directed nucleotide(s) to the 3′ ends of PCR fragments, and as a result, *Taq*-generated PCR products can be directly cloned into vectors containing 3′-T overhangs[18,19]. In contrast, archaeal DNA polymerases lack terminal extendase activity, and hence, produce blunt fragments that can be cloned directly into blunt-ended vectors[18, 20].

Uracil Poisoning of Archaeal DNA Polymerases

Unlike *Taq*, archaeal DNA polymerases possess a "read-ahead" function that detects uracil (dU) residues in the template strand and stalls synthesis[21]. Uracil detection is unique to archaeal DNA polymerases (e.g., *Pfu*), and is thought to represent the first step in a pathway to repair DNA cytosine deamination (dCMP → dUMP) in archaea[21]. Stalling of DNA synthesis opposite uracil has significant implications for high-fidelity amplification with archaeal DNA polymerases. Techniques requiring dUTP (e.g., dUTP/UDG decontamination methods[22]) or uracil-containing oligonucleotides cannot be performed with proofreading DNA polymerases[23,24]. Even more importantly, uracil stalling has been shown to compromise the performance of archaeal DNA polymerases under standard PCR conditions[25].

We found that during PCR amplification, a small amount of dCTP undergoes deamination to dUTP (%dUTP varies with cycling time), and is subsequently incorporated by archaeal DNA polymerases. Once incorporated, uracil-containing DNA inhibits archaeal DNA polymerases, limiting their efficiency. We found that adding a thermostable dUTPase (dUTP → dUMP + PPi) to amplification reactions carried out with *Pfu* and Deep Vent DNA polymerases significantly increases PCR product yields by preventing dUTP incorporation[25]. Moreover, the target-length capability of *Pfu* DNA polymerase is dramatically improved in the presence of dUTPase (e.g., increased from < 2 kb to 14 kb[25]). Long-range PCR is particularly susceptible to dUTP poisoning due to the use of prolonged extension times (1–2 minutes per kb at 72°C) that promote dUTP formation.

Archaeal DNA Polymerase Fusions

In an effort to increase processivity, various DNA-binding proteins have been fused to the termini of DNA polymerases to increase template binding affinity. For example, fusing the small basic chromatin-like *Sulfolobus solfataricus* 7d (Sso7d) protein to the C-terminus of *Pfu* was shown to increase processivity by 8.6-fold[26]. When tested in PCR, the resulting *Pfu*-Sso7d fusion amplified longer targets in less time compared to native (unfused) *Pfu*. Several archaeal DNA polymerase fusions have been commercialized that differ with respect to DNA polymerase and/or DNA-binding domain employed, and the inclusion of various PCR-enhancing supplements. For example, the *PfuUltra* II Fusion HS DNA Polymerase is formulated with a *Pfu*-based DNA polymerase fused to a proprietary double-stranded DNA binding protein (and supplemented with *P. furiosus* dUTPase and hotstart antibody; see paragraph below), while Phusion DNA Polymerase consists of a chimeric Deep Vent/*Pfu* (*Pyrococcus sp.* GB-D/*furiosus*) DNA polymerase fused to Sso7d[27]. Fusion DNA polymerases also differ with respect to target-length capability (Table 1); however, all fusions support the use of shorter extension times (15–30 seconds/kb), and thereby provide shorter time-to-results and increased throughput.

PCR Characteristics of Proofreading DNA Polymerases

The source, composition, and PCR characteristics of commercial proofreading enzymes are provided in Table 1. *PfuUltra* and *PfuUltra* II (fusion) DNA polymerases are formulated with a proprietary *Pfu* mutant that provides 3-fold higher fidelity than *Pfu*. In addition, the *PfuTurbo* and *PfuUltra* enzymes contain *P. furiosus* dUTPase (ArchaeMaxx Polymerase Enhancing Factor) to minimize uracil poisoning. As a result, both yield and target-length capability are vastly improved, and genomic targets up to 19 kb in length have been amplified[28,29]. With *PfuUltra* II fusion HS DNA polymerase, the use of shorter extension times (15 seconds/kb for < 10 kb targets) means that a 19 kb genomic fragment can be amplified in 5 hours (same-day analysis), instead of > 19 hours (next-day analysis) which is required for non-fusion archaeal DNA polymerases. Other archaeal DNA polymerase formulations that lack dUTPase exhibit comparatively shorter length-capability.

Several proofreading DNA polymerases are available as hotstart formulations. Heat-reversible inactivation is achieved by adding monoclonal antibodies that neutralize polymerase and 3´-5´ exonuclease activities (*PfuUltra* II fusion HS DNA polymerase, Platinum Superfi; no pre-activation required).

With proofreading DNA polymerases, high background and/or low product yield may result from extension of non-specifically annealed primers at ambient temperature (common with *Taq*;[30]) or from degradation of primers and DNA template during room-temperature reaction assembly (unique to proofreading enzymes). In our experience, hotstart formulations provide improved yield and/or specificity when amplifying low-copy-number targets in complex backgrounds[31] or longer targets with KOD DNA polymerase (B.Arezi and W. Xing, personal communication).

Each manufacturer recommends somewhat different PCR conditions for optimal performance (Table 1).
All manufacturers of proofreading enzymes recommend taking measures to minimize non-specific degradation of PCR primers or products, including using relatively high nucleotide concentrations (200–300 µM each), adding proofreading enzymes last to PCR reactions (after dNTPs), titrating the amount of enzyme, and using sufficient PCR primer concentrations. When testing different proofreading PCR enzymes, researchers are strongly encouraged to follow each manufacturer's recommendation for enzyme amount and extension time. With all proofreading enzymes, synthesizing longer targets or amplifying GC-rich (> 70 %) sequences typically requires additional optimization. In general, amplification of longer targets requires more enzyme units, higher nucleotide concentrations, and/or longer extension times. To enhance amplification of problematic or GC-rich templates, researchers can add DMSO to *Pfu* formulations (e.g., Herculase II fusion DNA polymerase plus 3–10 % DMSO; titrated in 1 % increments) or use the proprietary PCR additives that are provided with Phusion (GC buffer plus DMSO), Platinum Superfi (PCR_x Solution), and DNA polymerases (Table 1).

Table 1. Characteristics of High-Fidelity PCR Enzymes†

DNA Polymerase (Fusion Domain)	Exonuclease Activity		Processivity (bases)	Polymerization Rate (sec-1)	Uracil Stalling	Product Name (Manufacturer)	Notes and Recommendations for Use	Recommended Target Length	HotStart
	3'-5'	3'-5'							
P. furiosus	Yes	No	10^{15} < 20^{14} 6.4^{26}, 15^{47}	9.3^{15}, 25^{14}	Yes[25] (dU-DNA formuation minimized by ArchaeMaxx factor)	*PfuTurbo* DNA Polymerase	*Pfu* PCR buffer optimized for fidelity; Formulated with ArchaeMaxx factor; Genomic < 10 kb: use 2.5 U/50 µl, 200 µM dNTPs and either 1 min/kb (≤ 6 kb) or 2 min/kb (> 6 kb) at 72°C extensions; Genomic > 10 kb: use 5 U/50 µl rxn, 500 µM dNTPs and 2 min/kb at 68°C extensions	Up to 19 kb genomic[29]	Yes*
						PfuUltra DNA Polymerase	Formulated with ArchaeMaxx factor and *Pfu* mutant that improves fidelity; See *PfuTurbo* recommendations	Up to 17 kb genomic	Yes*
P. furiosus fusion (double-stranded DNA binding protein)	Yes	No	185^{47}	ND	Yes	*PfuUltra* II Fusion HS DNA Polymerase	Formulated with ArchaeMaxx factor, hotstart antibody, and *Pfu* mutant that improves fidelity; Unique 10X buffer required for optimal activity of fusion; Targets < 10 kb: use 1 µl/50 µl, 250 µM dNTPs, and 15 sec (< 1 kb) or 15 sec/kb (> 1 kb) at 72°C extensions; Targets > 10 kb: use 1 µl/50 µl rxn, 500 µM dNTPs and 30 sec/kb at 68°C extensions	Up to 19 kb genomic	Yes*
						Herculase II Fusion DNA Polymerase	Formulated with ArchaeMaxx factor; Includes unique 5X buffer and DMSO to enhance PCR of difficult targets; Targets < 12 kb: use 0.5 µl (< 1 kb) or 1 µl (> 1 kb)/50 µl, 250 µM dNTPs and 30 sec (< 1 kb) or 30 sec /kb (> 1 kb) at 72°C extensions; GC-rich targets: add DMSO (0-8 % in 1 % increments) and increase denaturation from 95°C to 98°C	Up to 12 kb genomic	No
P. sp. GB-D	Yes	No	< 20^{14}	23^{14}	Yes[25]	Deep Vent DNA Polymerase (New England BioLabs)	See manufacturer's recommendations	NR	No
P. sp. GB-D/ furiosus chimera fusion (Sso7d)	Yes	No	30-35[47] (relative processivity: 10X *Pfu*, 1.6X *Taq*)[48]	ND	Yes	Phusion DNA Polymerase (ThermoFisher, New England BioLabs); iProof DNA Polymerase (BioRad)	See manufacturer's recommendations	NR	No
T. kodakaraensis KOD1	Yes	No	> 300^{14}	106-138^{14}	Yes[25]	KOD HiFi (Millipore Sigma)	See manufacturer's recommendations	Up to 6 kb	Yes*
Thermus aquaticus	No	Yes	10^{15}, 42^{17}	46.7^{15}, 61^{14}	No	Numerous	See manufacturer's recommendations	Up to 5 kb	Yes

† Information from product manuals, unless otherwise specified; low-fidelity *Taq* included for comparative purposes
* Hotstart formulation contains polymerase- and exonuclease-neutralizing monoclonal antibodies

# Source identified by manufacturer as *Pyrococcus sp.* strain KOD, but reclassified as *T. kodakaraensis* KOD1[44]
NR = no recommendations provided by manufacturer;
ND = no data; *P. = Pyrococcus, T. = Thermococcus*

High-Fidelity DNA Polymerase Blends

In addition to proofreading DNA polymerases, several DNA polymerase blends have been introduced for high-fidelity PCR (Table 2). Commercial DNA polymerase blends consist predominantly of *Taq* plus a lesser amount of a proofreading DNA polymerase (e.g., *Pfu*, Deep Vent) to enhance PCR product yields, amplification of long targets, and fidelity[32]. The fidelity of *Taq*-based blends is typically improved by increasing the proportion of proofreading to non-proofreading DNA polymerase and by modifying the PCR reaction buffer to optimize yield. Since product yield and target-length capability decrease with increasing proofreading: non-proofreading polymerase ratios[32], higher fidelity *Taq*-based blends typically exhibit reduced performance compared to blends optimized for yield and length (i.e., blends with lower proofreading: non-proofreading polymerase ratios). In general, high-fidelity *Taq*-based blends provide superior performance compared to *Taq* alone with respect to fidelity, yield, and target-length capability (Table 2).

Table 2. Characteristics of High-Fidelity DNA Polymerase Blends†

DNA Polymerase (Manufacturer)	Blend Composition			HotStart*	Recommended Target Length
	Major Polymerase	Minor Polymerase	Additives		
TaqPlus Precision PCR System	Taq	Pfu	None	No	Up to 10 kb genomic and 15 kb vector
Expand High Fidelity PCR System (Millipore Sigma)	Taq	Tgo	None	No	Up to 5 kb genomic
Platinum *Taq* High Fidelity (Invitrogen/ThermoFisher)	Taq	Deep Vent	*Taq*- neutralizing mAb	Yes (only version available)	Up to 12 kb; up to 20 kb with optimization
Advantage HF 2 PCR Kit (Takara)	Titanium Taq	Proofreading DNA Polymerase	*Taq*- neutralizing mAb	Yes (only version available)	Up to 5 kb

† Information from manufacturers' catalog or product manual, unless otherwise specified; mAb, monoclonal antibody
* HotStart formulation contains *Pfu*- and *Taq*- neutralizing monoclonal antibodies

Protocol/Experimental Methods

Error Rate Measurements

DNA polymerase fidelity is expressed in terms of error rate, which corresponds to the number of misincorporated nucleotides per base synthesized. In PCR-based fidelity assays, error rate (E.R.) is calculated as:

$$\text{E.R.} = \frac{\text{number of mutations per bp}}{\text{number of amplicon doublings}}$$

where number of amplicon doublings (d) is quantified from the amount of input target DNA and amplicon yield, as:

$$2^d = \frac{\text{amplicon yield}}{\text{input target DNA}}$$

The error rates of *Pfu* and *Taq* DNA polymerases have been measured using several different methods, including DNA sequencing, denaturing gradient gel electrophoresis (DGGE), and phenotypic forward and reversion mutation assays[1]. Analyses employing direct sequencing or DGGE methods may provide more accurate estimates since all mutations, including silent and lethal mutations are taken into account. However, DNA sequencing is generally impractical for determining error rates of high-fidelity PCR enzymes due to the large number of clones that must be sequenced in order to obtain statistically significant results (e.g., > 23,000 clones must be sequenced to determine the error rate of the *PfuUltra* enzyme, assuming a mutation rate of 1 per 2.3 x 10^6 bases, 500 bases sequenced per clone, and 5X overage). Moreover, to minimize sequence bias, error rate measurements should employ multiple templates with varying sequence contexts (e.g., GC content, homopolymeric runs, etc.), which further increases cost and labor associated with direct methods. Indirect phenotypic methods are routinely employed by enzyme manufacturers for obvious reasons of simplicity and cost, and underestimates of mutation frequency can be avoided by choosing a well-characterized target gene, such as *lac*I.

The error rate of *Pfu* DNA polymerase has been estimated at 1.3 x 10^{-6} mutations per bp per doubling using a PCR-based phenotypic assay[9] (see *lac*I Phenotypic Mutation Assay below). This is consistent with estimates obtained from DGGE (0.7 x 10^{-6} for a 96 bp human mitochondrial sequence[4]; 1.8 x 10^{-6} for a 121 bp human APC cDNA sequence[35]) and from DNA sequencing (< 3 x 10^{-6})[36]. At this rate, the probability of a base being mutated in a single round of replication is ~1-3 per 1,500,000 nucleotides, and after 20 doublings (10^6-fold amplification), ~1-2.5 % of 1 kb amplification products will contain mutations. In comparison, published error rates for *Taq* range from 0.5-21 x 10^{-5} mutations per bp per doubling, and include: 7.2-21 x 10^{-5} using DGGE[12, 37] 0.8-1.0 x 10^{-5} (*lac*I) and 1.8 x 10^{-5} (p53) using PCR-based phenotypic assays[9,38,39], 2 x 10^{-5} using a gap-filling *lac*Z assay[10], and 0.5-2.7 x 10^{-5} by DNA sequencing of PCR products[36,40]. At these rates, anywhere from 10 % to 100 % of 1 kb products amplified with *Taq* will contain one or more mutations (doublings = 20; mutation-containing products = 10-420 %).

Variation in published error rates reflects differences in the reaction conditions (e.g., pH, [dNTPs], [Mg^{2+}], DNA template sequence) and types of fidelity assays employed[1,11,12]. Because different assays are likely to measure different parameters, error rates should only be compared among PCR enzymes tested in the same assay[13], and preferably, according to manufacturers' recommendations.

*lac*I Phenotypic Mutation Assay

Our laboratory routinely employs a PCR-based forward mutation assay that utilizes the well-characterized *lac*I target gene[9,38]. In this assay, a 1.9 kb sequence encoding *lac*IOZα is amplified and cloned, and the percentage of clones containing a mutation in *lac*I (% blue) is determined in a color-screening assay (Figure 1). To accurately determine mutation rates with a phenotypic assay, it is essential that the number of base changes producing a scorable mutant phenotype is known. Otherwise, mutation rates can be greatly underestimated by not taking into account silent mutations that alter DNA sequence without producing a change in protein sequence or function. The sensitivity of *lac*I to mutation is well known. More than 30,000 *lac*I mutants have been sequenced, and the results indicate that 349 single-base substitutions occurring at 179 amino acid positions in the 1080 bp *lac*I-coding region can be identified by color screening[41].

Therefore, in the *lac*I assay, error rates are calculated as mutation frequency per 349 bp per duplication:

$$\text{E.R.} = \frac{\textit{lac}\text{I- mutant frequency}}{(349 \text{ bases})\ (d)}$$

where (d) = the number of amplicon doublings

We have measured the error rates of several DNA polymerases using the *lac*I assay (Table 3). Error rates were measured in each enzyme's recommended PCR buffer, and whenever possible, identical PCR conditions were used,

including DNA template concentration, PCR cycling parameters, and number of PCR cycles performed. The only exceptions were that each manufacturer's recommendations were followed with respect to number of enzyme units, nucleotide concentration, primer concentration, extension temperature, and extension time (shorter times were employed with fusion enzymes) (Table 3). To allow assay-to-assay comparisons, *Pfu* DNA polymerase was run in every assay, and error rates were normalized relative to the mean value of 1.3 x 10^{-6} mutations per bp per doubling as determined for *Pfu* in study #1[9]. *Taq* DNA polymerase, serving as a second internal control, exhibited mean error rates of 8.0 x 10^{-6} (study #1; 11 PCRs) and 9.1 x 10^{-6} (mean of studies #2-5; 14 PCRs) mutations per bp per doubling.

Results

As expected, proofreading DNA polymerases exhibited significantly lower error rates (1-3 errors per 10^6 bases) compared to *Taq* DNA polymerase (8-9 errors per 10^6 bases). The *PfuUltra* mutant DNA polymerase (non-fusion and fusion) formulations exhibited error rates (4 x 10^{-7} mutations per bp per duplication) that were 3-fold lower than the error rates of *Pfu* and Phusion DNA polymerases. Relative differences in error rate observed with the *lac*I assay (Table 3) are consistent with those obtained using a p53-based forward mutation assay (e.g., *Pfu* < Taq[39]) and DGGE (e.g., *Pfu* < *Taq*[1]). In general, the error rates of high-fidelity DNA polymerase blends (3-6 errors per 10^6 bases) are intermediate between proofreading DNA polymerases and *Taq* (Table 3).

The use of high-fidelity DNA polymerases, especially those that support fast cycling, becomes increasingly important as amplicon size increases (Table 3). With *Taq*, the percentage of clones expected to contain mutations in a 10^6-fold amplification reaction increases from 4 % (for 250 bp amplicon) to 16 % (1 kb amplicon) to 80 % (5 kb amplicon), while the number of clones that should be sequenced to obtain an error-free clone (95 % confidence) increases from 1 to 2 to 14, respectively ($0.95=1-(1-f)^n$, where f = frequency of error-free clones and n = number of clones sequenced[42]). When amplifying a broader range of targets (0.25 to 10 kb) with high-fidelity blends (E.R.=2.8–5.8 x 10^{-6}), the percentage of clones likely to contain mutations increases from 1-3 % (250 bp amplicon) to 5–11 % (1 kb amplicon) to 28–58 % (5 kb amplicon) to 56–100 % (10 kb amplicon), and the number of clones that should be sequenced increases from 1–2 (up to 1 kb amplicon) to 3–5 (5 kb amplicon) to > 6 (10 kb amplicon). When amplifying similarly sized targets with the *PfuUltra* enzyme (E.R.=4 x 10^{-7}), the frequency of error-containing clones is: < 1 % (up to 1 kb amplicon), 4 % (5 kb amplicon), and 8 % (10 kb amplicon), and sequencing 1 (up to 6 kb amplicon) or 2 (6–10 kb amplicon) clones should be sufficient for identifying an error-free clone. In addition, with the faster *PfuUltra* II fusion HS DNA polymerase, long fragments can be amplified with the same degree of accuracy in a fraction of the time; for example, a 5 or 10 kb fragment can be amplified with PfuUltra II enzyme in 1 or 3 hours respectively, compared to 3 or 10 hours required for amplification by a non-fusion high-fidelity PCR enzyme.

Table 3. Error Rates of High-Fidelity PCR Enzymes.

DNA Polymerase	Number of Studies	Number of PCRs	Error Rate# (x 10^{-6} ± S.D.)	Accuracy (Error Rate-1 in Bases)	Percentage of Clones with Mutations (10^6-fold Amplification)		
					1 kb Amplicon	5 kb Amplicon	10 kb Amplicon
Proofreading DNA Polymerases							
PfuUltra II Fusion HS DNA Polymerase	6	8	0.4 ± 0.06	2,500,000	0.8	4	8
PfuUltra DNA Polymerase	5	12	0.4 ± 0.04	2,500,000	0.8	4	8
Pfu DNA Polymerase	1	10	1.3 ± 0.2[9,28]	770,000	2.6	13	26
Herculase II Fusion DNA Polymerase	6	6	1.3 ± 0.2	770,000	2.6	13	26
Phusion DNA Polymerase/ iProof DNA Polymerase	6	5	1.3 + 0.4	770,000	2.6	13	26
Deep Vent DNA Polymerase	1	4	2.7 ± 0.2[9]	370,000	5.4	NR	NR
High Fidelity Blends							
TaqPlus Precision PCR System	2–3	13	4.0 ± 1.3[33]	250,000	8	40	80
Platinum Taq High Fidelity	3	2	5.8 ± 0.3[33]	170,000	11.6	58	100
Advantage-HF	3	2	6.1 ± 0.0[33]	160,000	12.2	NR	NR
Taq DNA Polymerase	1	11	8.0 ± 3.9[9]	125,000	16	80	NR
	2–5	14	9.1 ± 2.4	110,000	18.2	91	NR

NR = not recommended for 5 to 10 kb target sizes

#Error rates were measured in each enzyme's recommended PCR buffer. Cycling conditions were described in[9], or were as follows: (*Taq*, *PfuUltra* DNA polymerases): 95°C 1 min. (1 cycle); 95°C 30 sec, 58°C 30 sec, 72°C 6 min. (30 cycles); 72°C 10 min. (1 cycle); (*PfuUltra* II, Herculase II, Phusion DNA polymerases): 95°C 1 min. (1 cycle); 95°C 30 sec, 58°C 30 sec, 72°C 45 sec (30 cycles); 72°C 5 min. (1 cycle); PCR reactions (50 µl) contained 0.2 µM each primer, 200 µM each nucleotide, 2.5 ng target DNA, and 2.5 U DNA polymerase, with the following manufacturer-recommended exceptions: Platinum Pfx- 300 µM each nucleotide, 1.25 U enzyme, and 68°C extension temperature; Deep Vent *1* U enzyme; *PfuUltra* II and Herculase II DNA polymerases- 1 µl; Phusion- 1 U

Table 4. Polymerase ordering guide. Volume can be customized to your needs.

PCR Enzyme	Fidelity	Speed	Yield	Target Length (genomic DNA)	Sensitivity
High-Fidelity & Difficult/GC Rich PCR					
PfuUltra II Fusion HotStart DNA Polymerase *Engineered to be the highest fidelity and fastest polymerase available*	1 error/2.5 million bp	15 sec/kb		0-19 kb	
Herculase II Fusion DNA Polymerase *High-fidelity polymerase for difficult targets. Provides superior yields over a broad range of targets. Economical enough for routine use*	1 error/770,000 bp	15 sec/kb		"0-12kb 12-23 kb (optimized)"	
PfuUltra High-Fidelity DNA Polymerase AD *Engineered for high-fidelity*	1 error/2.25 million bp	1 min/kb		19 kb (optimized)	
PfuTurbo DNA Polymerase AD *First high-fidelity polymerase to include the ArchaeMaxx Polymerase-Enhancing factor*	1 error/770,000 bp	1 min/kb		19 kb (optimized)	
Herculase Enhanced DNA Polymerase *Designed for difficult targets*	1 error/375,000 bp	1 min/kb		12 kb	
Cloned *Pfu* DNA Polymerase AD *Cloned to ensure ultrapure manufacturing of Pfu*	1 error/770,000 bp	2 min/kb		"1 kb 5 kb (optimized)"	
Pfu DNA Polymerase *Stratagene introduced the first thermophilic proofreading polymerase*	1 error/770,000 bp	2 min/kb		(up to 1 kb)	
Specialty Enzymes					
PfuTurbo Cx HotStart DNA Polymerase *The only high-fidelity polymerase that can read through dUTP in the template and extending strand*	1 error/770,000 bp	1 min/kb		0-10 kb	
PicoMaxx High-Fidelity PCR System *Most sensitive polymerase offered*	2x Taq	1 min/kb		0-10 kb	
Easy-A High-Fidelity PCR Cloning Enzyme *Proofreading DNA polymerase that adds 3'A overhangs to PCR amplicons*	1 error/770,000 bp	1 min/kb		0-6 kb	
Routine Enzymes					
Paq5000 DNA Polymerase *Fast and economical alternative to Taq*		30 sec/kb		0-6 kb	
*Taq*2000 DNA Polymerase *Ultrapure cloned Taq that eliminates unwanted background artifacts*		1 min/kb		"1 kb 4 kb (optimized)"	
Taq DNA Polymerase *First thermophilic PCR enzyme.*		1 min/kb		"1 kb 4 kb (optimized)"	

Table 5. Polymerase ordering guide. Reagents can be customized to your needs.

Blunt or 3'-A Ends	ArchaeMaxx Advantage	Enzyme Only		HotStart		Master Mix	PCR Enzyme
		100 U 1000 U	500 U 5000 U	100 U 1000 U	500 U 5000 U	100 rxn 400 rxn	
High-Fidelity & Difficult/GC Rich PCR							
Blunt	ArchaeMaxx Advantage			(40 rxn) 600670 (400 rxn) 600674	(200 rxn) 600672 –	600850 600852	*PfuUltra* II Fusion HotStart DNA Polymerase
Blunt	ArchaeMaxx Advantage	(40 rxn) 600675 (400 rxn) 600679	(200 rxn) 600677 –				Herculase II Fusion DNA Polymerase
Blunt	ArchaeMaxx Advantage	600385 600389	600387 –	600390 600394	600392 –	600630 –	*PfuUltra* High-Fidelity DNA Polymerase AD
Blunt	ArchaeMaxx Advantage	600255 600259	600257 –	600320 600324	600322 –		*PfuTurbo* DNA Polymerase AD
Mixed	ArchaeMaxx Advantage	600260 600264	600262 600266	600310 600314	600312 –		Herculase Enhanced DNA Polymerase
Blunt		600353 600357	600355 –				Cloned *Pfu* DNA Polymerase AD
Blunt		600135 600140	600136 –				Native *Pfu* DNA Polymerase
Specialty Enzymes							
Blunt	Alternative uracil resistance (Pfu mutation)			600410 600414	600412 –		*PfuTurbo* Cx HotStart DNA Polymerase
Mixed	ArchaeMaxx Advantage			600420 600424	600422 –	600650 –	PicoMaxx High-Fidelity PCR System
3'-A	ArchaeMaxx Advantage			600400 600404	600402 –	600640 600642	Easy-A High-Fidelity PCR Cloning Enzyme
Routine Enzymes							
Mixed	ArchaeMaxx Advantage	– 600682	600680 600684			600870 600872	Paq5000 DNA Polymerase
3'-A		600195 600197	600196 –	600280 600284	600282 –		*Taq*2000 DNA Polymerase
3'-A							*Taq* DNA Polymerase

Optimizing PCR Fidelity

PCR error rate can be minimized by employing the highest fidelity PCR enzyme available for the desired application. As discussed above, commercial high-fidelity DNA polymerases show considerable variation in error rates, ranging from 0.4–3.5 x 10^{-6} for proofreading DNA polymerases, up to 2.8–6.1 x 10^{-6} for DNA polymerase blends (Table 3). However, when selecting a PCR enzyme, parameters other than fidelity may have to be considered. Current high-fidelity PCR enzymes are incompatible with dUTP/UNG decontamination[22,23] and direct TA cloning methods[19]. However, post-amplification addition of 3´ A overhangs with *Taq* improves the TA cloning efficiency of bluntended fragments amplified with proofreading enzymes. (Post-amplification A-addition requires incubation for 8-10 minutes at 72°C; see the StrataClone PCR Cloning Kit instruction manual for details.) Alternatively, researchers can generate amplicons with 3´ A overhangs using the Easy-A DNA Polymerase, a proprietary PCR enzyme with *Pfu*-like fidelity. Thus, suitable high-fidelity enzyme formulations are available for nearly every PCR application.

In addition to enzyme choice, researchers should also consider optimizing reaction conditions to further reduce PCR mutation frequency. While error rate is an intrinsic property of DNA polymerases (under defined reaction conditions), observed mutation frequencies can vary from PCR to PCR, depending on the number of amplicon doublings. For example, assuming we amplify a 1 kb fragment using *Taq* (E.R., 8 x 10^{-6} mutations per bp per doubling), a PCR generating 5 µg of amplicon from 5 pg of target DNA has undergone 20 target doublings and produced 1.6 mutations per 10,000 bases (~3/20 clones with mutations). In comparison, a PCR generating 5 µg of amplicon from 75 ng target DNA has undergone 6 target doublings (67-fold amplification) and introduced 0.5 mutations per 10,000 bases (~1/20 clones with mutations). Therefore, researchers can minimize mutation frequency by limiting the number of target duplications, for example, by increasing the amount of input DNA template or reducing the number of PCR cycles.

Additional reductions in mutation frequency may be achieved by optimizing buffer composition, nucleotide concentration, or polymerase amount. As discussed above, the error rates shown in Table 3 were obtained using the PCR buffer and nucleotide concentration recommended by each manufacturer, which may or may not be optimal with respect to fidelity. High-fidelity PCR reaction conditions have been developed for *Taq*, Deep Vent, and *Pfu* DNA polymerases[9-12,43]. For example, the error rate of Pfu decreases from 2.6- to 1.1- x 10^{-6} as the nucleotide concentration is lowered from 1 mM to 100 µM each[9]. Even greater changes in *Pfu's* error rate were observed as the Mg^{2+} concentration was increased from 1 mM (4.9 x 10^{-6}) to 2 mM $MgSO_4$ (1.3 x 10^{-6}) (at 200 µM each dNTP, pH 8.8) and the pH was increased from pH 7.5 (8.2 x 10-6) to pH 8.8 (1.3 x 10-6) (at 200 µM each dNTP, 2 mM $MgSO_4$)[9]. For enzymes whose pH and Mg^{2+} optima are unknown, researchers can expect to achieve lower mutation frequencies by using the lowest balanced nucleotide concentration compatible with yield (e.g., 25–150 µM each). In addition, using lower enzyme concentrations is also likely to minimize polymerase extension from mispaired or misaligned primer termini[11].

Conclusion

Since the introduction of *Taq* DNA polymerase in the late 1980s, significant progress has been made in developing PCR enzyme formulations with improved fidelity, PCR performance, and speed. Proofreading DNA polymerases offer significantly higher fidelity compared to *Taq*, and initial problems associated with their use (low yield, unreliability, speed) have been largely overcome by reducing uracil poisoning (the *Pfu* formulations), preparing blends with *Taq* DNA polymerase, and developing faster, more processive proofreading DNA polymerase fusions. In fact, Agilent's new high-fidelity enzyme formulations provide significantly improved yield, throughput, and target-length capability compared to *Taq*.

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