Pfu DNA Polymerase

Instruction Manual

Catalog #600135, #600136, and #600140 (Native *Pfu* DNA Polymerase) and #600153, #600154, #600159, and #600160 (Cloned *Pfu* DNA Polymerase)

Revision G0

Laboratory Reagent.

600135-12

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Pfu DNA Polymerase

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MATERIALS PROVIDED

Native *Pfu* DNA Polymerase

Materials provided	Quantity		
	Catalog #600135	**Catalog #600136**	**Catalog #600140**
Native *Pfu* DNA polymerase (2.5 U/µl)	100 U[a]	500 U[b]	1000 U[c]
Native Plus 10× *Pfu* buffer	1 ml	2 × 1 ml	4 × 1 ml

[a] Sufficient native *Pfu* DNA polymerase is provided for up to 40 100-µl reactions.
[b] Sufficient native *Pfu* DNA polymerase is provided for up to 200 100-µl reactions.
[c] Sufficient native *Pfu* DNA polymerase is provided for up to 400 100-µl reactions.

Cloned *Pfu* DNA Polymerase

Materials provided	Quantity			
	Catalog #600153	**Catalog #600154**	**Catalog #600159**	**Catalog #600160**
Cloned *Pfu* DNA polymerase (2.5 U/µl)	100 U[a]	500 U[b]	1000 U[c]	5000 U[d]
10× Cloned *Pfu* buffer[e]	1 ml	2 × 1 ml	4 × 1 ml	20 × 1 ml

[a] Sufficient cloned *Pfu* DNA polymerase is provided for up to 40 100-µl reactions.
[b] Sufficient cloned *Pfu* DNA polymerase is provided for up to 200 100-µl reactions.
[c] Sufficient cloned *Pfu* DNA polymerase is provided for up to 400 100-µl reactions.
[d] Sufficient cloned *Pfu* DNA polymerase is provided for up to 2000 100-µl reactions.
[e] See *Preparation of Media and Reagents*.

STORAGE CONDITIONS

All components: –20°C

NOTICES TO PURCHASER

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Revision G0

INTRODUCTION

Pfu DNA polymerase, a proofreading DNA polymerase isolated from *Pyrococcus furiosus*, is an ideal choice for a variety of techniques requiring high-fidelity DNA synthesis by the polymerase chain reaction (PCR).[1–3] These applications include cloning, gene expression, and site-directed mutagenesis. Successful PCR using *Pfu* DNA polymerase is readily performed requiring only slight modifications from PCR protocols optimized with *Taq* DNA polymerase. Various PCR parameters that are important in increasing the yield and specificity of *Pfu* DNA polymerase-based PCR amplification reactions are described in this instruction manual.

CRITICAL OPTIMIZATION PARAMETERS FOR *Pfu* DNA POLYMERASE-BASED PCR

All PCR amplification reactions, whether performed using *Taq* or *Pfu* DNA polymerase, require optimization to achieve the highest product yield and specificity. Critical optimization parameters for successful PCR using *Pfu* DNA polymerase are outlined in the following sections and include the use of an extension time that is adequate for full-length DNA synthesis, sufficient enzyme concentration, optimization of the reaction buffer, adequate primer–template purity and concentration, and optimal primer design.

Extension Time

Extension time is the most critical parameter affecting the yield of PCR product obtained using *Pfu* DNA polymerase. For *Taq* DNA polymerase-based PCR amplifications, an extension time of 0.5-1.0 minute/kb of template amplified is usually sufficient for maximum synthesis of a PCR target. In contrast, *Pfu* DNA polymerase-based PCR amplifications require a minimum extension time of 1-2 minutes/kb of amplified template to achieve similar target synthesis.[4]

Enzyme Concentration

The concentration of *Pfu* DNA polymerase required for optimal PCR product yield and specificity depends on the individual target system to be amplified. Successful amplification can usually be achieved using 2.5–5.0 U of enzyme/100-µl reaction for PCR targets that are <2 kb. Further optimization will be required for targets greater than 2 kb.

TABLE I

Fidelity Comparison of Thermostable DNA Polymerases Using a *lac*IOZα-Based Fidelity Assay[a]

Thermostable DNA polymerase	Error rate[b]	Percentage (%) of mutated PCR products[c]
Pfu DNA polymerase	1.3×10^{-6}	2.6
Taq DNA polymerase	8.0×10^{-6}	16.0
$Vent_R$ DNA polymerase	2.8×10^{-6}	5.6
Deep $Vent_R$ DNA polymerase	2.7×10^{-6}	5.4

[a] Fidelity is measured using a PCR-based forward mutation assay based on the *lacI* target gene.[5]

[b] The error rate equals mutation frequency per base pair per duplication.

[c] The percentage of mutated PCR products after amplification of a 1-kb target sequence for 20 effective cycles.

Reaction Buffer

In *Pfu* DNA polymerase-based PCR, the reaction buffers for native and cloned *Pfu* DNA polymerases are formulated for optimal PCR yield and fidelity.[6] Use the 10× reaction buffer provided with each polymerase for all PCR applications.

If alterations in the *Pfu* reaction buffers are made, significant increases in the error rate of *Pfu* DNA polymerase can be avoided by maintaining the Mg^{2+} concentration above 1.5 mM, the total dNTP concentration at or below 1 mM, and the pH of Tris-based buffers above pH 8.0 when measured at 25°C. Deoxynucleoside triphosphate (dNTP) concentrations of 100–250 μM each dNTP generally result in the optimal balance of product yield (greatest at high dNTP concentrations) versus specificity and fidelity (highest at low dNTP concentration).[7]

Primer–Template Purity and Concentration

The most successful PCR results are achieved when the amplification reaction is performed using purified primers and templates that are essentially free of extraneous salts. Gel-purified primers, generally >18 nucleotides in length, are strongly recommended for use in *Pfu* DNA polymerase-based PCR.

Additionally, an adequate concentration of primers and template should be used to ensure a good yield of the desired PCR products. When DNA of known concentration is available, amounts of 50–200 ng of DNA template/100-µl reaction are typically used for amplifying single-copy chromosomal targets. Amplifying a single-copy target from complex genomic DNA is generally more difficult than amplifying a fragment from a plasmid or phage. Less DNA template can be used for amplifying lambda or plasmid PCR targets or for amplifying multicopy chromosomal genes (typically 10–100 ng).[7] The mutation frequency can be reduced by limiting the number of PCR cycles; however, a corresponding increase in DNA template concentration is required to achieve comparable yields of PCR product. We suggest using primers at a final concentration of 0.1–0.5 µM, which is equivalent to ~100–250 ng of an 18- to 25-mer oligonucleotide primer in a 100-µl reaction volume. Relatively high concentration of primers is typically required due to the fact that proofreading DNA polymerases such as *Pfu* DNA polymerase exhibit 3′- to 5′-exonuclease activity, which may contribute to a certain level of primer degradation. The use of primers with a phosphorothioate bond at the 3′-terminal internucleotide linkage reportedly minimizes primer degradation.[9]

Primer Design

Primer pairs that exhibit similar melting temperatures and are completely complementary to the template are recommended. Depending on the primer design and the desired specificity of the PCR amplification reaction, melting temperatures between 55° and 80°C generally yield the best results.[7] The following formula[10] is commonly used for estimating the melting temperature (T_m) of the primers:

$$T_m(^\circ C) \cong 2(N_A + N_T) + 4(N_G + N_C)$$

where N equals the number of primer adenine (A), thymidine (T), guanidine (G), or cytosine (C) bases. Several other articles present additional equations for estimating the melting temperature of the primers.[11,12] Finally, care must be taken when using degenerate primers. Degenerate primers should be designed with the least degeneracy at the 3′ end. Optimization of degenerate primer concentration is necessary.

Note *Because of the unique composistion of the Pfu buffer, the actual primer* T_m *may be 3°–5°C lower than that estimated by this forumla.*

ADDITIONAL OPTIMIZATION PARAMETERS FOR *Pfu* DNA POLYMERASE-BASED PCR

PCR Cycling Parameters

Standard PCR amplification reactions typically require 25–30 cycles to obtain a high yield of PCR product. Because high fidelity is a concern for certain PCR applications such as expression cloning, We suggest using a minimum number of cycles for *Pfu* DNA polymerase-based PCR to ensure the lowest number of errors. Thermal cycling parameters should be chosen carefully to ensure (1) the shortest denaturation times to avoid enzyme inactivation and/or template damage, (2) adequate extension times to achieve full-length target synthesis, and (3) the use of annealing temperatures near the primer melting temperature to improve specificity of the desired PCR product.

When performing PCR on a new target system, we suggest using an annealing temperature 5–10°C below the lowest primer melting temperature.

For best results, PCR primers should be designed with similar melting temperatures ranging from 55° to 80°C. The use of primers with melting temperatures within this range reduces false priming and ensures complete denaturation of unextended primers at 94–95°C (see also *Primer–Template Purity and Concentration* and *Primer Design*).

Order of Addition of Reaction Mixture Components

Because *Pfu* DNA polymerase exhibits 3′- to 5′-exonuclease activity that enables the polymerase to proofread nucleotide misincorporation errors, it is critical that *Pfu* DNA polymerase is the last component added to the PCR mixture (i.e., **after** the dNTPs). In the absence of dNTPs, the 3′- to 5′-exonuclease activity of proofreading DNA polymerases may degrade primers. When primers and nucleotides are present in the reaction mixture at recommended levels (i.e., primer concentrations of 0.1–0.5 μM and nucleotide concentrations of 100-250 μM), primer degradation is minimal.

Deoxynucleoside Triphosphates

For *Pfu* DNA polymerase-based PCR, we recommend using a dNTP concentration range of 100–250 μM each dNTP (0.4–1.0 mM total) in order to achieve the optimal balance between yield, specificity, and fidelity. Deoxynucleoside triphosphate concentrations of 100–250 μM each dNTP generally result in the optimal balance of product yield (greatest at high dNTP concentrations) versus specificity and fidelity (highest at low dNTP concentration).[7] The use of a balanced pool of dNTPs (equimolar amounts of each dNTP) ensures the lowest rate of misincorporation errors.

Salt Concentrations

Magnesium Concentration

Magnesium chloride concentration affects primer annealing and template denaturation, as well as enzyme activity and fidelity. Generally, excess Mg^{2+} concentration results in accumulation of nonspecific amplification products, whereas insufficient Mg^{2+} concentration results in reduced yield of the desired PCR product.[13] PCR amplification reactions should contain *free* Mg^{2+} in excess of the total dNTP concentration. For *Pfu* DNA polymerase-based PCR, yield is optimal when the *total* Mg^{2+} concentration is ~2 mM in a standard reaction mixture, and ~3 mM for amplification of cDNA. A 2 mM *total* Mg^{2+} concentration is present in the final 1× dilution of the 10× reaction buffer provided. For the amplification of cDNA, Mg^{2+} should be added to the PCR reaction to a final concentration of 3 mM.[7]

Adjuncts and Cosolvents

The adjuncts or cosolvents listed in the following table may be advantageous with respect to yield when used in the PCR buffer. Fidelity may or may not be affected by the presence of these adjuncts or cosolvents.

Adjunct or cosolvent	Optimal PCR final concentration
Bovine serum albumin (BSA)	10–100 µg/ml
Formamide	1.25–10%
Dimethylsulfoxide (DMSO)	1–10%
Glycerol	5–20%
Ammonium sulfate [$(NH_4)_2SO_4$]	15–30 mM
Perfect Match PCR enhancer	1 U/100-µl reaction (genomic DNA template) 0.01–1 U/100-µl reaction (plasmid DNA template)

Bovine Serum Albumin

Bovine serum albumin is a nonspecific enzyme stabilizer that also binds certain PCR inhibitors.[14]

Formamide

Formamide facilitates certain primer–template annealing reactions and also lowers the denaturing temperature of melt-resistant DNA.[15]

Dimethylsulfoxide and Glycerol

Cosolvents, such as DMSO and glycerol, improve the denaturation of GC-rich DNA and help overcome the difficulties of polymerase extension through secondary structures. Studies indicate that the presence of 1–10% DMSO in PCR may be essential for the amplification of the retinoblastoma gene[16] and may also enhance amplification of *Herpes simplex* virus (HSV) sequences. Glycerol is known to improve the yield of amplification products and also serves as an enzyme stabilizer.[17]

Ammonium Sulfate

Ammonium sulfate increases the ionic strength of the reaction mixture, which alters the denaturing and annealing temperatures of DNA, as well as enzyme activity.

Perfect Match PCR Enhancer

Perfect Match PCR enhancer improves the specificity of PCR products. This adjunct performs these functions by destabilizing mismatched primer–template complexes and helps to remove secondary structures that could impede normal extension.[18]

APPLICATION NOTES

Long PCR

Native *Pfu* DNA polymerase successfully synthesizes PCR targets up to 12-kb from either plasmid or genomic templates.[19] In long PCR amplification reactions, product yields are greatest when the reaction is performed using Agilent's Native *Pfu* DNA polymerase in combination with the Native Plus 10× *Pfu* buffer.

Thermostability

Pfu DNA polymerase is a highly thermostable enzyme, retaining 94-99% of its polymerase activity after 1 hour at 95°C. Unlike *Taq* DNA polymerase, denaturing temperatures up to 98°C can be used successfully with *Pfu* DNA polymerase to amplify GC-rich regions.[19,20]

Modified Nucleotide Incorporation

Pfu DNA polymerase successfully incorporates the following modified nucleotides: α-thionucleotides, 7-deaza-deoxyguanosine triphosphate (7-deaza-dGTP), and fluoresceinated and biotinylated nucleotides. Efficient incorporation of modified nucleotides may require optimization of the analog concentration. Exo^- *Pfu* DNA polymerase incorporates these modified nucleotides more efficiently than the Exo^+ version of the DNA polymerase due to the lack of an associated proofreading activity.

Terminal Transferase Activity

Studies demonstrate that thermostable DNA polymerases with the exception of *Pfu* DNA polymerase exhibit terminal deoxy-nucleotidyltransferase (TdT) activity, which is characterized by the addition of nontemplate-directed nucleotide(s) at the 3′ end of PCR-generated fragments.[21,22] *Pfu* DNA polymerase is devoid of TdT activity and generates blunt-ended PCR products exclusively. Therefore, this is the enzyme of choice for use with the PCR-Script Amp SK(+) cloning kit[23] and the PCR-Script Cam SK(+) cloning kit.[24] Alternatively, *Pfu* DNA polymerase can be used to remove 3′ overhangs (polishing) or to fill-in 5′ overhangs with greater efficiencies than either Klenow polymerase or T4 DNA polymerase.[25,26]

Reverse Transcriptase Activity

Pfu DNA polymerase lacks detectable reverse transcriptase activity.

PCR PROTOCOL USING *Pfu* DNA POLYMERASE

1. Prepare a reaction mixture for the appropriate number of samples to be amplified. Add the components *in order* while mixing gently. Table II provides an example of a reaction mixture for the amplification of a typical single-copy chromosomal target. The recipes listed in Table II are for one reaction and must be adjusted for multiple samples. The final volume of each sample reaction is 100 µl.

   Note *The volumes of each component in the reaction mixture may also be decreased proportionally to a 50-µl final volume.*

2. Immediately before thermal cycling, aliquot 100 µl of the reaction mixture into the appropriate number of reaction tubes.

3. Perform PCR using optimized cycling conditions. Suggested cycling parameters are indicated in Table III.

4. Analyze the PCR amplification products on a 0.7–1.0% (w/v) agarose gel.

TABLE II

Reaction Mixture for a Typical Single-Copy Chromosomal Locus PCR Amplification

Component	Amount per reaction
Distilled water (dH_2O)	81.2 µl
10× buffer[a]	10.0 µl
dNTPs (25 mM each NTP)	0.8 µl
DNA template (100 ng/µl)	1.0 µl[b]
Primer #1 (100 ng/µl)	2.5 µl[c]
Primer #2 (100 ng/µl)	2.5 µl[c]
Native or cloned *Pfu* DNA polymerase (2.5 U/µl)	2.0 µl (5.0 U)[d]
Total reaction volume	100 µl

[a] The 10× buffer provides a final 1× Mg^{2+} concentration of 2 mM. To amplify cDNA, Mg^{2+} may need to be added to a final 1× concentration of 3 mM.

[b] The amount of DNA template required varies depending on the type of DNA being amplified. Generally 50–200 ng of genomic DNA template is recommended; however, less DNA template (typically 1–100 ng) can be used for amplification of lambda or plasmid PCR targets or for amplification of multicopy chromosomal genes.

[c] Primer concentrations between 0.1 and 0.5 µM are recommended (generally 100–250 ng for typical 18- to 25-mer oligonucleotide primers in a 100-µl reaction volume).

[d] The amount of *Pfu* DNA polymerase varies depending on the length of the template to be amplified. A typical starting point is 5.0 U. Successful amplification can usually be achieved using 2.5–5.0 U of enzyme/100-µl reaction for PCR targets that are <2 kb. Further optimization will be required for targets greater than 2 kb.

TABLE III

Suggested Cycling Parameters for PCR Using *Pfu* DNA Polymerase

Segment	Number of cycles	Temperature	Duration
1	1	94–98°C[a]	45 seconds
2	25–30	94–98°C Primer T_m – 5°C[b] 72°C	45 seconds 45 seconds 1–2 minutes/kb of PCR target
3	1	72°C	10 minutes

[a] Denaturing temperatures above 95°C are recommended only for GC-rich templates.

[b] The annealing temperature may be lowered further if necessary to obtain optimal results. Typically annealing temperatures will range between 55° and 72°C.[9]

TROUBLESHOOTING

Observation	Suggestion(s)
No product or low yield	Allow an extension time of at least 1-2 minutes/kb of PCR target
	Optimize annealing temperature by lowering it in 5°C increments
	Ensure that the appropriate buffer is used (i.e., use Native Plus 10× *Pfu* buffer with native *Pfu* DNA polymerase and use 10× cloned *Pfu* buffer with cloned *Pfu* DNA polymerase)
	Add *Pfu* DNA polymerase last to the reaction mixture to minimize any potential primer degradation
	To minimize the effects of high-GC content or secondary structure use higher denaturing temperatures (94–98°C)
	To minimize the effects of high-GC content or secondary structure, use cosolvents such as DMSO in a 1–10% (v/v) final concentration or glycerol in a 5–20% (v/v) final concentration (see *Dimethylsulfoxide and Glycerol*)
	Ensure that primer concentration is not too low. Use the recommended primer concentrations between 0.1 and 0.5 μM (generally 100–250 ng for typical 18- to 25-mer oligonucleotide primers in a 100-μl reaction volume)
	Ensure that primers used are of high quality
	Optimize primers with respect to melting temperature, purity, GC content, and length
	Consider using adjuncts to optimize PCR [e.g., use 1–2 U of Perfect Match PCR enhancer or a low concentration (1–5%) of formamide]
	Ensure the ionic strength of the reaction mix is correct. Remove extraneous salts from the PCR primers and DNA preparations
	Optimize denaturation time. Denaturation times of 30–60 seconds at 94–95°C are usually sufficient while longer denaturation times may damage the DNA template; use the shortest denaturation time compatible with successful PCR on the thermal cycler
	Increase the amount of *Pfu* DNA polymerase
	Ensure that template used is intact and of high purity. Use template at an adequate concentration (see *Primer–Template Purity and Concentration* and *Primer Design*)
	Titrate the amount of DNA template to ensure template concentration is not too high
	See the *Adjuncts and Cosolvents* section for information about optimal adjuncts and solvents
Multiple bands	Optimize primer annealing temperature by increasing the annealing temperature in 5°C increments and/or use a hot start[27,28]
	Check for nonspecific primer-template annealing. Use Perfect Match PCR enhancer to improve PCR product specificity
Artifactual smears	Decrease the amount of *Pfu* DNA polymerase
	Ensure that extension time is not too long.

PREPARATION OF MEDIA AND REAGENTS

10× Cloned *Pfu* Buffer

200 mM Tris-HCl (pH 8.8)
20 mM $MgSO_4$
100 mM KCl
100 mM $(NH_4)_2SO_4$
1% Triton X-100
1 mg/ml nuclease-free BSA

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Material Safety Data Sheets (MSDSs) are provided online at *www.agilent.com*. MSDS documents are not included with product shipments.