# *PfuUltra* Hotstart DNA Polymerase

## Instruction Manual

**Catalog #600390, #600392, and #600394**

Revision E.0

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600390-12

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# *PfuUltra* Hotstart DNA Polymerase

## CONTENTS

# *PfuUltra* Hotstart DNA Polymerase

## MATERIALS PROVIDED

| Materials provided | Quantity | | |
|---|---|---|---|
| | Catalog #600390 | Catalog #600392 | Catalog #600394 |
| *PfuUltra* hotstart DNA polymerase (2.5 U/µl) | 100 U | 500 U | 1000 U |
| 10× *PfuUltra* HF reaction buffer | 1 ml | 2 × 1 ml | 4 × 1 ml |

## STORAGE CONDITIONS

**All components:** –20°C

## ADDITIONAL MATERIALS REQUIRED

Temperature cycler
PCR tubes
PCR primers
Deoxynucleoside triphosphates (dNTPs)

Revision E.0

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# INTRODUCTION

*PfuUltra* hotstart DNA polymerase* features a genetically engineered mutant of *Pfu* DNA polymerase** and the ArchaeMaxx polymerase-enhancing factor and is formulated for hot start activity. *PfuUltra* hotstart DNA polymerase exhibits an average error rate three-fold lower than *PfuTurbo* DNA polymerase and 18-fold lower than *Taq* DNA polymerase, making it the highest fidelity enzyme available (see Table I). Amplification of a 500-bp fragment using *PfuUltra* hotstart DNA polymerase results in errors in less than 0.5% of full-length PCR products, making this the ideal enzyme for PCR cloning.

In addition to high accuracy, *PfuUltra* hotstart DNA polymerase provides robust amplification of long, complex genomic targets. A key component of *PfuUltra* hotstart DNA polymerase is the ArchaeMaxx polymerase-enhancing factor. The ArchaeMaxx factor eliminates a PCR inhibitor and promotes shorter extension times, higher yield, and greater target length capabilities. The ArchaeMaxx factor improves the yield of products by overcoming dUTP poisoning, which is caused by dUTP accumulation during PCR through dCTP deamination.[1] Once incorporated, dU-containing DNA inhibits *Pfu* and most archaeal proofreading DNA polymerases, such as Vent$_R$® and Deep Vent$_R$® DNA polymerases, limiting their efficiency.[1] The ArchaeMaxx factor functions as a dUTPase, converting poisonous dUTP to harmless dUMP and inorganic pyrophosphate, resulting in improved overall PCR performance.

*PfuUltra* hotstart DNA polymerase is formulated with heat labile monoclonal antibodies that, at room temperature, effectively neutralize DNA polymerase and 3´-5´ exonuclease (proofreading) activities, facilitating room temperature assembly. Full enzyme activity is regained upon denaturation of the antibody during the initial denaturation step. (See Table II for a comparison of Agilent hot start PCR enzymes.) *PfuUltra* hotstart DNA polymerase provides reduced background and improved detection sensitivity by preventing priming until stringent primer annealing temperatures are reached. Amplification systems that are most likely to benefit from the hot start capability of *PfuUltra* hotstart DNA polymerase are those (1) designed to detect low-copy-number targets in complex DNA backgrounds, (2) prone to primer-dimer formation, and (3) assembled robotically during high throughput PCR procedures.

* U.S. Patent Nos 6,734,293, 6,444,428, 6,183,997, and 5,948,663.

** U.S. Patent No. 5,948,663.

**TABLE I**

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

| Thermostable DNA polymerase | Error rate[b] | Percentage (%) of mutated 1-kb PCR products[c] |
|---|---|---|
| *PfuUltra* hotstart DNA polymerase | $4.3 \times 10^{-7}$ | 0.9 |
| *PfuTurbo* DNA polymerase | $1.3 \times 10^{-6}$ | 2.6 |
| *Pfu* DNA polymerase | $1.3 \times 10^{-6}$ | 2.6 |
| Tgo DNA polymerase | $2.1 \times 10^{-6}$ | 4.3 |
| Deep Vent$_R$® DNA polymerase | $2.7 \times 10^{-6}$ | 5.4 |
| Vent$_R$® DNA polymerase | $2.8 \times 10^{-6}$ | 5.6 |
| PLATINUM® *Pfx* | $3.5 \times 10^{-6}$ | 7.0 |
| KOD DNA polymerase | $3.5 \times 10^{-6}$ | 7.0 |
| *Taq* DNA polymerase | $8.0 \times 10^{-6}$ | 16.0 |

[a] Fidelity is measured using a published PCR forward mutation assay that is based on the *lac*I target gene.[2]

[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 ($2^{20}$- or $10^6$-fold amplification).

**TABLE II**

**Properties of Agilent Hot Start PCR Enzymes**

| Hot Start PCR enzyme | Hot Start Method | Activities Neutralized | Activation Procedure[a] | Enzyme Applications |
|---|---|---|---|---|
| *PfuUltra* hotstart DNA polymerase | Antibody | DNA polymerase, 3′-5′ exonuclease | PCR Activation<br>30 cycles | • highest fidelity |
| *PfuTurbo* hotstart DNA polymerase | Antibody | DNA polymerase, 3′-5′ exonuclease | PCR Activation<br>30 cycles | • genomic DNA templates up to 19 kb<br>• high fidelity |
| Herculase hotstart DNA polymerase | Antibody | DNA polymerase, 3′-5′ exonuclease | PCR Activation<br>30 cycles | • challenging cloning targets<br>• long and/or GC-rich targets<br>• higher fidelity than *Taq* DNA polymerase |
| SureStart *Taq* DNA polymerase | Chemical | DNA polymerase, 5′-3′ exonuclease | Pre-PCR Activation<br>(9–12 minutes @ 95°C)<br>30 cycles<br>or<br>PCR Activation<br>40 cycles | • routine PCR up to 3 kb |

[a] PCR activation means that full enzyme activity is recovered during temperature cycling, either during the initial denaturation step (antibody-based formulations) or within the first 5–15 cycles (chemical hot start). For SureStart *Taq*, slow enzyme activation during temperature cycling typically necessitates the use of additional PCR cycles to achieve desired product yield (35–45 cycles). In the Pre-PCR activation method, the enzyme is activated prior to temperature cycling, and no additional cycles are necessary.

# CRITICAL OPTIMIZATION PARAMETERS FOR *PFUULTRA* HOTSTART DNA POLYMERASE-BASED PCR

All PCR amplification reactions require optimization to achieve the highest product yield and specificity. Critical optimization parameters for successful PCR using *PfuUltra* hotstart DNA polymerase are outlined in Table III and are discussed in the following section. These parameters include the use of an extension time that is adequate for full-length DNA synthesis, sufficient enzyme concentration, adequate primer–template purity and concentration, optimal primer design, and appropriate nucleotide concentration.

**TABLE III**

**Optimization Parameters and Suggested Reaction Conditions (50 µl reaction volume)**

| Parameter | Targets: <10 kb (vector DNA) or <6 kb (genomic DNA) | Targets: >10 kb (vector DNA) or >6 kb (genomic DNA) |
|---|---|---|
| Extension time | 1 min per kb | 2 min per kb |
| *PfuUltra* hotstart DNA polymerase | 2.5 U | 5.0 U |
| Input template | 50–100 ng genomic DNA[a] | 200–250 ng genomic DNA[a] |
| Primers (each) | 100–200 ng (0.2–0.5 µM) | 200 ng (0.5 µM) |
| dNTP concentration | 200–250 µM each dNTP (0.8–1.0 mM total) | 500 µM each dNTP (2 mM total) |
| Final reaction buffer concentration | 1.0× | 1.5× (genomic DNA targets)<br>1.0× (vector DNA targets) |
| Denaturing temperature | 95°C | 92°C |
| Extension temperature | 72°C | 68°C |

[a] See *Primer-Template Purity and Concentration* for recommended amounts of other forms of template DNA.

## Extension Time

Extension time is one of the most critical parameters affecting the yield of PCR product obtained using *PfuUltra* hotstart DNA polymerase. For optimal yield, use an extension time of 1.0 minute per kb for vector targets up to 10 kb and genomic targets up to 6 kb. When amplifying vector targets greater than 10 kb or genomic targets greater than 6 kb in length, use an extension time of 2.0 minutes per kb.

## Enzyme Concentration

The concentration of *PfuUltra* hotstart DNA polymerase required for optimal PCR product yield and specificity depends on the individual target system to be amplified. Most amplifications are successful with 2.5 U of enzyme per 50 µl reaction for vector targets up to 17 kb and for genomic targets up to 6 kb.

## 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 *PfuUltra* hotstart 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 50–100 ng of DNA template per 50-µl reaction are typically used for amplifications of single-copy chromosomal targets. When amplifying genomic targets greater than 6 kb, increase the template amount to 200–250 ng. The amplification of a single-copy target from complex genomic DNA is generally more difficult than amplification of a fragment from a plasmid or phage. Less DNA template can be used for amplification of lambda (1–30 ng) or plasmid (100 pg–10 ng) PCR targets or for amplification of multicopy chromosomal genes (10–100 ng).[3]

Use primers at a final concentration of 0.2–0.5 µM, which is equivalent to ~100–200 ng of an 18- to 25-mer oligonucleotide primer in a 50-µl reaction volume.

## 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.[3] The following formula[4] 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 primers.[5, 6] 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.

## Deoxynucleoside Triphosphates

For *PfuUltra* hotstart DNA polymerase-based PCR, Use a dNTP concentration range of 200–250 µM each dNTP (0.8–1.0 mM total) for optimal product yield. Supplying dNTPs at 100–175 µM each dNTP generally results in good product yield; however, at these dNTP concentrations, yields achieved in amplifications performed using *PfuUltra* hotstart DNA polymerase may be lower compared to identical amplifications performed using *PfuTurbo* DNA polymerase. The yield of genomic targets >6 kb in length can be improved by increasing nucleotide concentration to 500 µM (each dNTP) and the reaction buffer to 1.5× final concentration.[7] The use of a balanced pool of dNTPs (equimolar amounts of each dNTP) ensures the lowest rate of misincorporation errors.

## Reaction Buffer

The reaction buffer provided with this enzyme has been formulated for optimal PCR yield and fidelity when performing PCR amplification using *PfuUltra* hotstart DNA polymerase. If alterations in the buffer are made, significant increases in the error rate of *PfuUltra* hotstart DNA polymerase can be avoided by maintaining the $Mg^{2+}$ concentration above 1.5 mM, the total dNTP concentration at 0.4–1.0 mM, and the pH of Tris-based buffers above pH 8.0 when measured at 25°C.[2]

To improve yields of genomic targets >6 kb, increase the final concentration of reaction buffer from 1× to 1.5×.[7]

## PCR Cycling Parameters

Standard PCR amplification reactions typically require 25–30 cycles to obtain a high yield of PCR product. Thermal cycling parameters should be chosen carefully to ensure (1) the shortest denaturation times to avoid 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 yield of the desired PCR product.

When performing PCR on a new target system, we recommend using an annealing temperature 5°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*).

See Table V for suggested PCR cycling parameters, depending upon template size and complexity. Optimized cycling parameters are not necessarily transferable between thermal cyclers designed by different manufacturers. Therefore, each manufacturer's recommendations for optimal cycling parameters should be consulted.

### Amplification of Genomic Targets >6 kb

To improve yields of genomic targets >6 kb, increase the amount of *PfuUltra* hotstart DNA polymerase from 2.5 U to 5.0 U, and increase the final concentration of reaction buffer from 1× to 1.5×.[7] Use 200–250 ng of genomic template DNA, 200 ng of each primer, and 500 µM each dNTP. Use a denaturing temperature of 92°C, an extension temperature of 68°C, and an extension time of 2.0 minutes per kilobase. Finally, overlay each reaction with ~50 µl of RNase/DNase-free mineral oil prior to thermal cycling.

## ADDITIONAL OPTIMIZATION PARAMETERS

### Magnesium Ion Concentration

Magnesium ion concentration affects primer annealing and template denaturation, as well as enzyme activity and fidelity. Generally, excess $Mg^{2+}$ results in accumulation of nonspecific amplification products, whereas insufficient $Mg^{2+}$ results in reduced yield of the desired PCR product.[8] PCR amplification reactions should contain free $Mg^{2+}$ in excess of the total dNTP concentration. For *PfuUltra* hotstart 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× *PfuUltra* HF reaction buffer. For the amplification of cDNA, $Mg^{2+}$ should be added to the PCR reaction to a final concentration of 3 mM.[8]

### 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 |
|---|---|
| Formamide | 1.25–10% |
| Dimethylsulfoxide (DMSO) | 1–10% |
| Glycerol | 5–20% |
| Perfect Match PCR enhancer | 1 U per 50-µl reaction (genomic DNA template)<br>0.01–1 U per 50-µl reaction (plasmid DNA template) |

#### Formamide

Formamide facilitates certain primer–template annealing reactions and also lowers the denaturing temperature of melt-resistant DNA.[9]

### 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[10] and may also enhance amplification of *Herpes simplex* virus (HSV) sequences.[11] Glycerol is known to improve the yield of amplification products and also serves as an enzyme stabilizer.[11]

### Perfect Match PCR Enhancer

Perfect Match PCR enhancer improves the specificity of PCR products. This adjunct performs this function by destabilizing mismatched primer-template complexes and by helping to remove secondary structures that could impede normal extension.[12]

## APPLICATION NOTES

### 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 *PfuUltra* hotstart DNA polymerase to amplify GC-rich regions.[13, 14]

### Terminal Transferase Activity

Studies demonstrate that thermostable DNA polymerases, with the exception of *Pfu* DNA polymerase, exhibit terminal deoxynucleotidyl-transferase (TdT) activity, which is characterized by the addition of nontemplate-directed nucleotide(s) at the 3´ end of PCR-generated fragments.[15, 16] *PfuUltra* hotstart DNA polymerase, like *Pfu* and *PfuTurbo* DNA polymerases, is devoid of TdT activity and generates blunt-ended PCR products exclusively.

In addition, *PfuUltra, PfuTurbo* or *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.[17, 18]

### Reverse Transcriptase Activity

*PfuUltra* hotstart DNA polymerase lacks detectable reverse transcriptase activity.

1. Prepare a reaction mixture for the appropriate number of samples to be amplified. Add the components in order while mixing gently. Table IV provides an example of a reaction mixture for the amplification of a typical single-copy chromosomal target. The recipe listed in Table IV is for one reaction and must be adjusted for multiple samples. The final volume of each sample reaction is 50 µl.

**TABLE IV**

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

| Component | Amount per reaction |
|---|---|
| Distilled water ($dH_2O$) | 40.6 µl |
| 10× *PfuUltra* HF reaction buffer[a] | 5.0 µl |
| dNTPs (25 mM each dNTP) | 0.4 µl |
| DNA template (100 ng/µl) | 1.0 µl[b] |
| Primer #1 (100 ng/µl) | 1.0 µl[c] |
| Primer #2 (100 ng/µl) | 1.0 µl[c] |
| *PfuUltra* hotstart DNA polymerase (2.5 U/µl) | 1.0 µl (2.5 U)[d] |
| Total reaction volume | 50 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 50–100 ng of genomic DNA template is recommended. Less DNA template (typically 0.1–30 ng) can be used for amplification of lambda or plasmid PCR targets or 10-100 ng for amplification of multicopy chromosomal genes.

[c] Primer concentrations between 0.2 and 0.5 µM are recommended (this corresponds to 100–250 ng for typical 18- to 25-mer oligonucleotide primers in a 50-µl reaction volume).

[d] The amount of *PfuUltra* hotstart DNA polymerase varies depending on the length of the template to be amplified. The standard amount for vector targets up to 17 kb and genomic targets up to 6 kb in length is 1 µl (2.5 U).

2. Before thermal cycling, aliquot 50 µl of the reaction mixture into the appropriate number of sterile thin-wall PCR tubes or standard 0.5-ml microcentrifuge tubes.

3. If the extension times are >15 minutes, overlay each reaction with DNase-, RNase-, and protease-free mineral oil (Sigma, St. Louis, Missouri) even if the temperature-cycler is equipped with a heated cover.

4. Perform PCR using optimized cycling conditions (see also *PCR Cycling Parameters*). Suggested cycling parameters are indicated in Table V.

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

**TABLE V**

## PCR Cycling Parameters for *PfuUltra* Hotstart DNA Polymerase with Single-Block Temperature Cyclers[a,b]

### A. Targets <10 kb (vector DNA) or <6 kb (genomic DNA)

| Segment | Number of cycles | Temperature | Duration |
|---|---|---|---|
| 1 | 1 | 95°C[c] | 2 minutes |
| 2 | 30 | 95°C | 30 seconds |
| | | Primer $T_m$ – 5°C[d] | 30 seconds |
| | | 72°C | 1 minute for targets ≤1 kb<br>1 minute per kb for targets >1 kb[e] |
| 3 | 1 | 72°C | 10 minutes |

### B. Targets >10 kb (vector DNA) or >6 kb (genomic DNA)

| Segment | Number of cycles | Temperature | Duration |
|---|---|---|---|
| 1 | 1 | 92°C | 2 minutes |
| 2 | 10 | 92°C | 10 seconds |
| | | Primer $T_m$ – 5°C[e] | 30 seconds |
| | | 68°C | 2 minutes per kb |
| 3 | 20 | 92°C | 10 seconds |
| | | Primer $T_m$ – 5°C[e] | 30 seconds |
| | | 68°C | 2 minutes per kb plus<br>10 seconds/cycle |

[a] Thin-wall PCR tubes are highly recommended. These PCR tubes are optimized to ensure more efficient heat transfer and to maximize thermal-cycling performance.

[b] Optimized cycling parameters are not necessarily transferable between thermal cyclers designed by different manufacturers; therefore, each manufacturer's recommendations for optimal cycling parameters should be consulted.

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

[d] The annealing temperature may require optimization. Typically annealing temperatures will range between 55° and 72°C.[3]

[e] The annealing temperature may require optimization. Typical annealing temperatures will range between 60 and 65°C.

# TROUBLESHOOTING

| Observation | Solution(s) |
|---|---|
| No product or low yield | Increase extension time to 2 minutes per kb of PCR target. |
| | Use the recommended amount of DNA template amounts. Use of excess template can reduce PCR product yield. |
| | Lower the annealing temperature in 5°C increments. |
| | Use a high-quality dNTP mix to supply a final concentration of ≥200 μM each dNTP. |
| | Ensure that 10× *PfuUltra* HF reaction buffer is used. |
| | Remove extraneous salts from the PCR primers and DNA preparations. |
| | Use higher denaturing temperatures (94–98°C). |
| | 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*). |
| | Use the recommended primer concentrations between 0.2 and 0.5 μM (corresponding to 100–200 ng for typical 18- to 25-mer oligonucleotide primers in a 50-μl reaction volume). |
| | Use high-quality primers. |
| | Check the melting temperature, purity, GC content, and length of the primers. |
| | Consider using adjuncts or cosolvents such as Perfect Match PCR Enhancer or formamide (see *Adjuncts and Cosolvents* ). |
| | Perform further buffer optimization if necessary. |
| | 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 *PfuUltra* hotstart DNA polymerase. |
| | Use intact and highly purified templates at an adequate concentration (see *Primer-Template Purity and Concentration*). |
| Multiple bands | Increase the annealing temperature in 5°C increments. |
| | Use Perfect Match PCR enhancer to improve PCR product specificity. |
| Artifactual smears | Decrease the amount of *PfuUltra* hotstart DNA polymerase. |
| | Reduce the extension time utilized. |

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## ENDNOTES

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