PfuTurbo Hotstart DNA Polymerase

INSTRUCTION MANUAL

Catalog #600320, #600322, and #600324
Revision C

For In Vitro Use Only
600320-12

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PfuTurbo Hotstart DNA Polymerase

Contents

PfuTurbo Hotstart DNA Polymerase

MATERIALS PROVIDED

Materials provided	Quantity		
	Catalog #600320	**Catalog #600322**	**Catalog #600324**
PfuTurbo hotstart DNA polymerase (2.5 U/µl)	100 U	500 U	1000 U
10× Cloned *Pfu* DNA polymerase reaction buffer[a]	1 ml	2 × 1 ml	4 × 1 ml

[a] See *Preparation of Media and Reagents*.

STORAGE CONDITIONS

All components: –20°C

ADDITIONAL MATERIALS REQUIRED

Temperature cycler
PCR tubes[||]
PCR primers
Deoxynucleoside triphosphates (dNTP's)

[||] Thin-walled PCR tubes are highly recommended for use with Stratagene RoboCycler thermal cyclers. These PCR tubes are optimized to ensure ideal contact with the multiblock design to permit more efficient heat transfer and to maximize thermal-cycling performance.

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Revision C

INTRODUCTION

PfuTurbo hotstart DNA polymerase is a special formulation of Stratagene high fidelity *PfuTurbo** DNA polymerase with hot start activity. (See Table I for a comparison of Stratagene hot start PCR enzymes.) *PfuTurbo* hotstart DNA polymerase is formulated with heat labile monoclonal antibodies that, at room temperature, effectively neutralize DNA polymerase and 3´-5´ exonuclease (proofreading) activities. Full enzyme activity is regained upon denaturation of the antibody during the initial denaturation step. *PfuTurbo* hotstart DNA polymerase retains the high fidelity, sensitivity and yield of *PfuTurbo* DNA polymerase, while facilitating room temperature PCR assembly. *PfuTurbo* 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 *PfuTurbo* 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.

PfuTurbo DNA polymerase has an error rate six-fold lower than *Taq* DNA polymerase, and significantly lower than the error rates of most other proofreading enzymes or DNA polymerase mixtures.[1–3] (See Table II for a comparison of the fidelity characteristics of many of the commercially available DNA polymerases.) Finally, the enhanced performance of *PfuTurbo* DNA polymerase allows the use of shorter extension times, fewer PCR cycles, and lower DNA template concentrations than are required for *Pfu* DNA polymerase, making it ideally suited for high-performance PCR applications.

PfuTurbo hotstart DNA polymerase provides robust amplification of long, complex genomic targets. A key component of *PfuTurbo* hotstart DNA polymerase is the exclusive thermostable 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.[4] Once incorporated, dU-containing DNA inhibits *Pfu* and most archaeal proofreading DNA polymerases, such as Vent® and Deep Vent® DNA polymerases, limiting their efficiency.[4] The ArchaeMaxx factor functions as a dUTPase, converting poisonous dUTP to harmless dUMP and inorganic pyrophosphate, resulting in improved overall PCR performance.

* U.S. Patent Nos. 7,045,328, 6,734,293, 6,489,150, 6,444,428, 6,183,997, 5,948,663, 5,866,395, 5,556,772, 5,545,552 and patents pending.

TABLE I

Properties of Stratagene Hot Start PCR Enzymes

Hot Start PCR enzyme	Hot Start Method	Activities Neutralized	Activation Procedure[a]	Enzyme Applications
PfuTurbo hotstart DNA polymerase	Antibody	DNA polymerase, 3′-5′ exonuclease	PCR Activation 30 cycles	• highest fidelity • genomic DNA templates up to 19 kb
Herculase hotstart DNA polymerase	Antibody	DNA polymerase, 3′-5′ exonuclease	PCR Activation 30 cycles	• challenging cloning targets • long and/or GC-rich targets • higher fidelity than *Taq* DNA polymerase
SureStart *Taq* DNA polymerase	Chemical	DNA polymerase, 5′-3′ exonuclease	Pre-PCR Activation (9–12 minutes @ 95°C) 30 cycles or PCR Activation 40 cycles	• routine PCR up to 3 kb
YieldAce hotstart DNA polymerase	Antibody	DNA polymerase	PCR Activation 30 cycles	• maximum target yields • superior yield compared to *Taq* DNA polymerase • amplification of clones for microarrays

[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.

TABLE II

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 high-fidelity DNA polymerase	4.3×10^{-7}	0.9
PfuTurbo DNA polymerase	1.3×10^{-6}	2.6
Pfu DNA polymerase	1.3×10^{-6}	2.6
Tgo DNA polymerase	2.1×10^{-6}	4.3
Deep Vent$_R$® DNA polymerase	2.7×10^{-6}	5.4
Vent$_R$® DNA polymerase	2.8×10^{-6}	5.6
PLATINUM® *Pfx*	3.5×10^{-6}	5.6
KOD DNA polymerase	3.5×10^{-6}	5.6
Taq DNA polymerase	8.0×10^{-6}	16.0

[a] Fidelity is measured using a published PCR forward mutation assay that is based on the *lacI* 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).

CRITICAL OPTIMIZATION PARAMETERS FOR *PFUTURBO* 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 *PfuTurbo* 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
PfuTurbo 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	100–250 μM each dNTP (0.4–1.0 mM total)	500 μM each dNTP (2 mM total)
Final reaction buffer concentration	1.0×	1.5× (genomic DNA targets) 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 *PfuTurbo* 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 *PfuTurbo* 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 19 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 *PfuTurbo* 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).[5]

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.[5] The following formula[6] is commonly used for estimating the melting temperature (T_m) of the primers:

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

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.[7, 8] 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 *PfuTurbo* hotstart DNA polymerase-based PCR, use a dNTP concentration range of 100–250 μM each dNTP (0.4–1.0 mM total) for optimal product yield. Supplying dNTPs in this concentration range generally results in the optimal balance of product yield (greatest at high dNTP concentrations) versus specificity and fidelity (highest at low dNTP concentration).[2, 5] The yield produced from 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.[1] 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 *PfuTurbo* hotstart DNA polymerase. Use this reaction buffer in standard PCR amplification reactions. If alterations are made to the buffer, significant increases in the error rate of *Pfu* 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×.[1]

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.[9] PCR amplification reactions should contain *free* Mg^{2+} in excess of the total dNTP concentration. For *PfuTurbo* 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× cloned *Pfu* DNA polymerase reaction buffer. For the amplification of cDNA, Mg^{2+} should be added to the PCR reaction to a final concentration of 3 mM.

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 suggests 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 Tables IV and V for suggested PCR cycling parameters, depending upon template size and thermal cycler. 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 *PfuTurbo* hotstart DNA polymerase from 2.5 U to 5.0 U, and increasing the final concentration of reaction buffer from 1× to 1.5×.[1] 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.

Application Notes

Thermostability

PfuTurbo hotstart DNA polymerase is a highly thermostable enzyme, retaining 94–99% of its polymerase activity after 1 hour at 95°C. The half life of *Pfu* DNA polymerase is approximately 19 hours at 95°C. Unlike *Taq* DNA polymerase, denaturing temperatures up to 98°C can be used successfully with *PfuTurbo* hotstart DNA polymerase to amplify GC-rich regions.[10, 11]

Terminal Transferase Activity

Studies demonstrate that thermostable DNA polymerases, with the exception of *Pfu* DNA polymerase, exhibit terminal deoxynucleotidyltransferase (TdT) activity, which is characterized by the addition of nontemplate-directed nucleotide(s) at the 3´ end of PCR-generated fragments.[12, 13] *Pfu* DNA polymerase is devoid of TdT activity and *PfuTurbo* hotstart DNA polymerase generates blunt-ended PCR products exclusively. Therefore, *PfuTurbo* hotstart DNA polymerase is optimal for use with the PCR-Script Amp SK(+) cloning kit[14], and the PCR-Script Cam SK(+) cloning kit.[15], Alternatively, *PfuTurbo* hotstart 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.[16, 17]

Reverse Transcriptase Activity

PfuTurbo hotstart DNA polymerase lacks detectable reverse transcriptase activity.

PCR PROTOCOL USING *PFUTURBO* HOTSTART DNA POLYMERASE

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

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

Component	Amount per reaction
Distilled water (dH_2O)	40.6 μl
10× cloned *Pfu* 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]
PfuTurbo 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 *PfuTurbo* hotstart DNA polymerase varies depending on the length of the template to be amplified. The standard amount for vector targets up to 19 kb and genomic targets up to 6 kb in length is 1 μl (2.5 U).

2. 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 temperature cycler is not equipped with a heated cover, overlay each reaction with ~50 μl of DNase-, RNase-, and protease-free mineral oil (Sigma, St. Louis, Missouri). If the extension times are >15 minutes, overlay each reaction with mineral oil 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 for *PfuTurbo* hotstart DNA polymerase-based PCR using single-block temperature cyclers and Stratagene RoboCycler temperature cyclers are indicated in Table IV and Table V, respectively.

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

TABLE IV

PCR Cycling Parameters for *PfuTurbo* 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 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 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.[5]

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

TABLE V

PCR Cycling Parameters for *PfuTurbo* DNA Polymerase with Stratagene RoboCycler Temperature Cycler[a]

Segment	Number of cycles	Temperature	Duration
1	1	95°C[b]	1 minute
2	25–30	95°C	1 minute
		Primer T_m – 5°C[c]	1 minute
		72°C	1 minute for targets ≤1 kb 1 minute/kb for targets > 1 kb[d]
3	1	72°C	10 minutes

[a] Thin-wall PCR tubes are highly recommended for use with Stratagene RoboCycler thermal cyclers.
These PCR tubes are optimized to ensure ideal contact with the multiblock design to permit more efficient heat transfer and to maximize thermal-cycling performance.

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

[c] The annealing temperature may require optimization. Typical annealing temperatures will range between 55° and 72°C.[5]

[d] For genomic targets >6 kb and vector targets >10 kb, use an extension time of 2 minutes per kb.

TROUBLESHOOTING

Observation	Solution(s)
No product or low yield	Increase extension time to 2 minutes/kb of PCR target
	Lower the annealing temperature in 5°C increments
	Ensure that 10× cloned *Pfu* DNA polymerase reaction buffer is used
	Use higher denaturing temperatures (94–98°C) if the template DNA contains a high GC content or secondary structures (see also Reference 12)
	Use the recommended primer concentrations between 0.2 and 0.5 μM (generally 100–200 ng for typical 18- to 25-mer oligonucleotide primers in a 50-μl reaction volume)
	Use only high quality, gel purified primers (see *Primer–Template Purity and Concentration* and *Primer Design*)
	Check the melting temperature, purity, GC content, and length of the primers
	Remove extraneous salts from the PCR primers and DNA preparations. High ionic strength can inhibit reactions
	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 *PfuTurbo* hotstart DNA polymerase
	Use intact and highly purified templates at an adequate concentration (see *Primer–Template Purity and Concentration* and *Primer Design*)
	Excessive template DNA can be inhibitory. Follow the recommendations given in the manual for template amount
	Insufficient heat exchange between the reaction tubes and the thermal cycler can cause inadequate temperature ramping. Use thin-wall PCR tubes. These PCR tubes are optimized to ensure ideal contact with the multiblock design to permit more efficient heat transfer and to maximize thermal-cycling performance
Multiple bands	Increase the annealing temperature in 5°C increments to ensure sufficient primer annealing
	Use Perfect Match PCR enhancer to improve PCR product specificity
Artifactual smears	Decrease the amount of *PfuTurbo* hotstart DNA polymerase
	Reduce the extension time utilized

PREPARATION OF MEDIA AND REAGENTS

10× Cloned *Pfu* DNA Polymerase Reaction 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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ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.