PfuTurbo C_x Hotstart DNA Polymerase

Instruction Manual

Catalog #600410, #600412, and #600414

Revision C.0

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600410-12

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PfuTurbo C_x Hotstart DNA Polymerase

CONTENTS

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MATERIALS PROVIDED

Materials provided	Quantity		
	Catalog #600410	Catalog #600412	Catalog #600414
PfuTurbo C_x hotstart DNA polymerase (2.5 U/µl)	100 U	500 U	1000 U
10× *PfuTurbo* C_x reaction buffer	1 ml	2 × 1 ml	4 × 1 ml
Dimethyl sulfoxide (DMSO)	1 ml	1 ml	1 ml

STORAGE CONDITIONS

All components: –20°C

ADDITIONAL MATERIALS REQUIRED

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

Revision C.0

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INTRODUCTION

Deamination of cytosine to uracil has been shown to occur during PCR and to impact the performance of proofreading archaeal DNA polymerases by a phenomenon known as PCR poisoning.[1] Cytosine deamination at elevated temperatures can occur either in the nucleotide pool (dCTP→dUTP) or in the template strand (dCMP→dUMP).[2] The dUTP that arises during PCR is efficiently incorporated by archaeal DNA polymerases (e.g., *Pfu* DNA polymerase,* $Vent_R$® DNA polymerase, Tgo DNA polymerase).[1, 3] However, this group of polymerases also exhibits the unique tendency of stalling replication opposite uracil in the template strand, thereby limiting PCR product yield.[4] Unlike dUTP incorporation opposite dAMP, which is a non-mutagenic event (A/T→A/U), deamination of dCMP to dUMP in DNA is a major promutagenic event, generating G/C→A/T mutations if not repaired before DNA replication. Although uracil stalling limits PCR product yield, this unique property is expected to contribute to the high fidelity of *Pfu* DNA polymerase.[4, 5]

Agilent *PfuTurbo* C_x hotstart DNA polymerase** is formulated with a mutant of *Pfu* DNA polymerase that overcomes uracil stalling completely, allowing the polymerase to read through uracil located in the template strand or incorporated into the extending strand. Since uracil read-through can introduce mutations that adversely impact fidelity, we have further engineered the mutant polymerase to increase the level of proofreading activity, thereby reducing the error rate of *PfuTurbo* C_x hotstart DNA polymerase to that of the original *PfuTurbo* DNA polymerase.*** (See Table I for a comparison of the fidelity characteristics of many commercially available DNA polymerases.) Thus *PfuTurbo* C_x hotstart DNA polymerase improves the overall reliability of high-fidelity PCR and exhibits more robust performance, affording higher product yields from templates of 5–10 kb in length as well as from more difficult systems including targets with high GC-content, without sacrificing accuracy. Although most systems amplify successfully without the need for additives, DMSO is included with *PfuTurbo* C_x hotstart DNA polymerase to further enhance yields of long, high-complexity, or GC-rich target sequences.[6, 7]

Unlike many proofreading enzymes, the enhanced performance of *PfuTurbo* C_x hotstart DNA polymerase requires minimal optimization, and allows the use of shorter extension times, fewer PCR cycles, and a wide range of DNA template concentrations, making it ideally suited for high-performance PCR applications.

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

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

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

PfuTurbo C_x 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 setup. Full enzyme activity is regained upon denaturation of the antibody during the initial denaturation step. Preventing priming until stringent primer annealing temperatures are reached provides reduced background and improved detection sensitivity.[8]

Because *PfuTurbo* C_x hotstart DNA polymerase is able to accommodate uracil-containing DNA, it can be used in conjunction with dUTP and uracil-N-glycosylase (UNG) to prevent carry-over contamination.[9,10] With dUTP/UNG procedures, carry-over PCR product contamination is eliminated while template DNA is left intact, preventing false-positives due to cross-contamination.

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 high-fidelity DNA polymerase	4.3×10^{-7}	0.9
Easy-A high-fidelity PCR cloning enzyme	1.3×10^{-6}	2.6
PfuTurbo C_x hotstart DNA polymerase	1.3×10^{-6}	2.6
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* 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.[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 (2^{20}- or 10^6-fold amplification).

OPTIMIZATION PARAMETERS FOR *PFUTURBO* C_x HOTSTART DNA POLYMERASE-BASED PCR

All PCR amplification reactions require optimization to achieve the highest product yield and specificity. Optimization parameters for successful PCR using *PfuTurbo* C_x hotstart DNA polymerase are outlined in Table II and are discussed in the following sections. These parameters include:

TABLE II

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

Parameter	Reaction conditions for targets <10kb
PfuTurbo C_x hotstart DNA polymerase	2.5 U
Input template	50–100 ng genomic DNA[a]
Primers (each)	100–200 ng (0.2–0.5 µM)
dNTP concentration	200–250 µM each dNTP (0.8–1.0 mM total)
Final reaction buffer concentration	1.0×
Denaturing temperature	95°C[b]
Extension time	1 min for targets ≤ 1 kb 1 min per kb for targets >1 kb 2 min per kb for 6–10 kb genomic templates
Extension temperature	72°C

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

[b] See *Amplification of Difficult or GC-Rich Targets* for alternative denaturing temperatures when amplifying from difficult or GC-rich templates.

Enzyme Concentration

The concentration of *PfuTurbo* C_x 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.

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* C_x 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).[11]

We suggest using 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.[11] The following formula[12] 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.[13, 14] 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 reactions using *PfuTurbo* C_x hotstart DNA polymerase, use a dNTP concentration range of 200–250 µM each dNTP (0.8–1.0 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).[5,11] 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* C_x hotstart DNA polymerase. If alterations in this buffer are made, significant increases in the error rate of *PfuTurbo* C_x hotstart DNA polymerase can be avoided by maintaining the Mg^{2+} concentration above 1.5 mM, the total dNTP concentration at 0.8–1.0 mM, and the pH of Tris-based buffers above pH 8.0 when measured at 25°C.[5]

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.[15] PCR amplification reactions should contain *free* Mg^{2+} in excess of the total dNTP concentration (i.e., an optimal *free* Mg^{2+} concentration between 0.5 and 2.5 mM).[11] For *PfuTurbo* C_x hotstart DNA polymerase-based PCR, fidelity is optimal when the *total* Mg^{2+} concentration is ~2 mM in a standard reaction mixture. This *total* Mg^{2+} concentration is present in the final 1× dilution of the *PfuTurbo* C_x 10× reaction buffer. The fidelity of *PfuTurbo* C_x hotstart DNA polymerase drops significantly at a *total* Mg^{2+} concentration of ≤1 mM in the presence of 200 µM each dNTP.

Note that the presence of ethylenediaminetetraacetic acid (EDTA) or other metal chelating agents lowers the effective concentration of Mg^{2+} as do excessive levels of dNTPs. The concentration of metal chelators and total dNTPs should be taken into account when determining the final Mg^{2+} concentration required for PCR.

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, use 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 III 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.

Extension Time

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

Amplification of Difficult or GC-Rich Targets

Increase the initial denaturation temperature and duration to 96–98°C for 5 minutes if the template DNA contains a high GC content or secondary structures.

DMSO is provided as a means of obtaining higher yields of PCR product with extra-long or GC-rich targets. The DMSO concentration must be titrated for each application in the range of 1–10%, since the degree to which DMSO enhances product yield and specificity varies according to target length, complexity, and GC content. For GC-rich targets, DMSO at 4–8% is generally recommended.

Note *The addition of 5% DMSO has no effect on the fidelity of* PfuTurbo C_x *hotstart DNA polymerase. However, the addition of >5% DMSO may increase the error rate of the* PfuTurbo C_x *hotstart DNA polymerase slightly. The use of >5% DMSO is discouraged when the highest fidelity is essential.*

Preventing Template Cross-Contamination

Because *PfuTurbo* C_x hotstart DNA polymerase is able to accommodate uracil-containing DNA, it can be used to prevent carry-over contamination in conjunction with uracil-N-glycosylase (UNG). This method involves the use of dUTP in place of dTTP in the nucleotide pool. When dUTP replaces dTTP in PCR amplification, UNG can be used to hydrolyze uracil-glycosidic bonds in single- and double-stranded dU-containing DNA so that carry-over PCR products will be subsequently degraded and not act as template in future testing assays. The heat-labile UNG is then inactivated during the initial denaturation step of the PCR cycling reaction. *Pfu Turbo* C_x DNA polymerase can be used as a replacement for *Taq*-based enzymes in dUTP/UNG decontamination protocols to provide higher fidelity and amplification of longer targets than can be amplified with *Taq* DNA polymerase.

After degrading dU-containing DNA and inactivating UNG following the UNG manufacturer's protocol, amplify with 200 µM of each nucleotide (dUTP, dATP, dGTP, and dCTP) in the PCR. For PCR targets up to 4 kb, use an extension time of 1 minute per kb. For PCR targets of 4–6 kb, an extension time of 2 minutes per kb is recommended.[9, 16, 17]

Notes PfuTurbo C_x *hotstart DNA polymerase successfully incorporates dUTP in targets up to 6 kb in length. However, for targets >1 kb, PCR product yields with dUTP are less than with dTTP.*

If low product yields are obtained when incorporating dUTP, relative to dTTP-incorporation, try increasing the PfuTurbo C_x *hotstart DNA polymerase to 5.0 U and/or increasing the extension time to 2 minutes per kb.*

Amplification of Genomic Targets >6 kb

To improve yields of genomic targets >6 kb, increase the amount of *PfuTurbo* C_x hotstart DNA polymerase from 2.5 U to 5.0 U, and increasing the final concentration of reaction buffer from 1× to 1.5×. 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 kb.

APPLICATION NOTES

Thermostability

PfuTurbo C_x 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* C_x hotstart DNA polymerase to amplify GC-rich regions.[6, 18]

Reverse Transcriptase Activity

PfuTurbo C_x 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. The table shown below provides an example 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× *PfuTurbo* C_x reaction buffer	5.0 µl
dNTPs (25 mM each dNTP)	0.4 µl
DNA template (100 ng/µl)	1.0 µl[a]
Primer #1 (100 ng/µl)	1.0 µl[b]
Primer #2 (100 ng/µl)	1.0 µl[b]
PfuTurbo C_x hotstart DNA polymerase (2.5 U/µl)	1.0 µl (2.5 U)
Total reaction volume	50 µl

[a] 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.

[b] Primer concentrations between 0.2 and 0.5 µM are recommended (this corresponds to 100–200 ng for typical 18- to 25-mer oligonucleotide primers in a 50-µl reaction volume).

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 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 III.

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

TABLE III

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

Segment	Number of cycles	Temperature	Duration
1	1	95°C[c]	2 minutes
2	30	95°C	30 seconds
		Primer T_m – 5°C	30 seconds
		72°C	1 minute for targets ≤1 kb 1 minute per kb for targets >1 kb 2 minute per kb for 6–10 kb genomic templates
3	1	72°C	10 minutes

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× *PfuTurbo* C_x reaction buffer is used.
	Use DMSO in the PCR mixture; titrate the DMSO concentration in 1% increments.
	Increase the initial denaturation temperature and duration to 96–98°C for 5–10 minutes if the template DNA contains a high GC content or secondary structures (see also Reference 19).
	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* C_x hotstart DNA polymerase to 5.0 U per 50 μl reaction.
	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.
Multiple bands	Increase the annealing temperature in 5°C increments to ensure sufficient primer annealing.
Artifactual smears	Decrease the amount of *PfuTurbo* C_x hotstart DNA polymerase.
	Reduce the extension time utilized.

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ENDNOTES

MSDS INFORMATION

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

PfuTurbo C_x Hotstart DNA Polymerase

Catalog #600410, 600412, 600414

QUICK-REFERENCE PROTOCOL

- Prepare reaction mixtures according to the table below, mix gently, and place in thin-walled PCR tubes.

Component	Amount per reaction
Distilled water (dH_2O)	40.6 µl
10× *PfuTurbo* C_x reaction buffer	5.0 µl
dNTPs (25 mM each dNTP)	0.4 µl
DNA template (100 ng/µl)	1.0 µl
Primer #1 (100 ng/µl)	1.0 µl
Primer #2 (100 ng/µl)	1.0 µl
PfuTurbo C_x hotstart DNA polymerase (2.5 U/µl)	1.0 µl (2.5 U)
Total reaction volume	50 µl

- Perform PCR using the cycling conditions according to the following table:

PCR Cycling Parameters Using Single-Block Temperature Cyclers

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	30	95°C	30 seconds
		Primer T_m – 5°C	30 seconds
		72°C	1 minute for targets ≤1 kb 1 minute per kb for targets >1 kb
3	1	72°C	10 minutes

- Analyze the PCR amplification products by gel electrophoresis.