



Herculase Hotstart DNA Polymerase

Instruction Manual

Catalog ##600310 (100 U), #600312 (500 U), and #600314 (1000 U)

Revision C.0

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600310-12



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CONTENTS

Materials Provided	1
Notices to Purchaser	1
Introduction	2
Properties of Agilent Hot Start PCR Enzymes	3
Critical Optimization Parameters	4
DNA Template Quality and Concentration	4
Enzyme Concentration	5
DMSO	5
Primer Design and Concentration	5
Deoxynucleotide Concentrations.....	5
Cycling Parameters.....	6
GC-Rich Targets.....	6
Protocol	7
Troubleshooting	9
References	10
Endnotes	10
MSDS Information	10
Quick-Reference Protocol	11

Herculase Hotstart DNA Polymerase

MATERIALS PROVIDED

Material provided	Quantity		
	Catalog #600310 (100 U)	Catalog #600312 (500 U)	Catalog #600314 (1000 U)
Herculase hotstart DNA polymerase (5 U/ μ l)	20 μ l	100 μ l	200 μ l
10 \times Herculase reaction buffer	1 ml	2 \times 1 ml	4 \times 1 ml
Dimethyl Sulfoxide (DMSO)	1 ml	1 ml	1 ml

STORAGE CONDITIONS

All Components: -20°C

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INTRODUCTION

Herculase hotstart DNA polymerase* provides superior performance in applications requiring high fidelity amplification over a broad range of target lengths and complexities (0.1–48 kb).^{1, 2} Herculase hotstart DNA polymerase features a novel DNA polymerase composition that consists predominantly of *Pfu* DNA polymerase, combined the exclusive thermostable ArchaeMaxx polymerase-enhancing factor and *Taq2000* DNA polymerase. This unique *Pfu*-based formulation is provided with a buffer optimized to promote high yield, specificity, and amplification of a wide range of targets. As a result, Herculase hotstart DNA polymerase can be used to successfully amplify small targets as well as genomic targets up to 37 kb and vector targets up to 48 kb, while maintaining an accuracy greater than *Taq* DNA polymerase and other DNA polymerase mixtures.

A key component of Herculase 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.³ Once incorporated, dU-containing DNA inhibits *Pfu* and most archaeal proofreading DNA polymerases, such as Vent® and Deep Vent® DNA polymerases, limiting their efficiency.³ The ArchaeMaxx factor functions as a dUTPase, converting poisonous dUTP to harmless dUMP and inorganic pyrophosphate, resulting in improved overall PCR performance.

Herculase hotstart DNA polymerase provides high specificity and detection sensitivity, while facilitating high-throughput robotic PCR applications. Herculase hotstart DNA polymerase is formulated with a combination of antibodies that effectively neutralize DNA polymerase and 3'-5' exonuclease (proofreading) activities. Full enzyme activity is regained upon denaturation of the antibodies during the initial denaturation step. See the table in *Properties of Agilent Hot Start PCR Enzymes*, below, for a comparison of the Hot-Start PCR enzymes offered by Agilent.

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

PROPERTIES OF AGILENT HOT START PCR ENZYMES

Hot Start PCR enzyme	Hot Start Method	Activities Neutralized	Activation Procedure ^o	Applications
Herculase hotstart DNA polymerase	Antibody	DNA polymerase, 3'-5' exonuclease	PCR Activation 30 cycles	<ul style="list-style-type: none"> challenging cloning targets long and/or GC-rich targets higher fidelity than <i>Taq</i> DNA polymerase
<i>PfuTurbo</i> hotstart DNA polymerase	Antibody	DNA polymerase, 3'-5' exonuclease	PCR Activation 30 cycles	<ul style="list-style-type: none"> Highest fidelity Genomic DNA templates up to 19 kb
SureStart <i>Taq</i> DNA polymerase	Chemical	DNA polymerase, 5'-3' exonuclease	Pre-PCR Activation (9–12 minutes @ 95°C) 30 cycles or PCR Activation 40 cycles	<ul style="list-style-type: none"> Routine PCR up to 3 kb

^o 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* DNA polymerase, 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

All PCR amplification reactions require optimization to achieve the highest product yield and specificity. Critical optimization parameters for successful PCR using Herculase hotstart DNA polymerase are outlined in Table I and discussed in the following section. The provided Herculase hotstart buffer contains the magnesium ion concentration that is optimal for the enzyme. Adjusting the magnesium concentration is not recommended.

TABLE I

Optimization Parameters and Suggested Reaction Conditions (50 μ l reaction volume)

Parameter	Typical Targets ≤ 10 kb	G-C Rich Targets < 10 kb	Targets > 10 kb
Input template	100–200 ng genomic DNA 1–15 ng vector DNA	100–200 ng genomic DNA 1–15 ng vector DNA	250–1000 ng genomic DNA 15–60 ng vector DNA
Herculase Hotstart DNA polymerase	2.5 U	2.5 U	5.0 U
DMSO concentration ^a	0%	4–8%	0–3% for genomic targets < 23 kb 3–6% for genomic targets > 23 kb 5–7% for lambda targets > 30 kb 4–8% for G-C rich targets
Primers (each)	~ 100 ng (0.25 μ M)	~ 100 ng (0.25 μ M)	~ 200 ng (0.5 μ M)
dNTP concentration	200 μ M each dNTP (0.8 mM total)	200 μ M each dNTP (0.8 mM total)	500 μ M each dNTP (2 mM total)
Extension time	1 min per kb	1 min per kb	≥ 1 min per kb, not to exceed 1 hour
Extension temperature	72°C	72°C	68°C
Denaturing temperature	92–95°C	92–98°C	92°C

^a Titrate the DMSO in 1% increments over the indicated range for each set of templates/primer pairs.

DNA Template Quality and Concentration

Successful amplification is dependent upon the purity, integrity, concentration, and molecular weight of the DNA template. Isolation of intact, high molecular weight genomic DNA may be achieved by using the Agilent DNA Extraction Kit or the RecoverEase DNA isolation kit. Potential shearing of the genomic DNA template is minimized by the use of wide-bore tips for pipetting or mixing of the template. Additionally, freezing of high molecular weight templates should be avoided; storage at 4°C is recommended. The length of an intact genomic DNA template should be > 50 kb.

For amplifying genomic DNA templates, use 100–250 ng of template for targets of ≤ 10 kb. Optimal concentrations of template for longer complex targets, up to 37 kb, may range between 250 ng and 1 μ g using reaction volumes of 50 μ l. To amplify low-complexity targets (for example, lambda DNA or cloned DNA), use 1–15 ng for targets ≤ 10 kb and 15–60 ng for targets > 10 kb. Excess template DNA can inhibit the PCR reaction.

Enzyme Concentration

Robust product yield requires an adequate DNA polymerase concentration. The use of 2.5 U/50- μ l reaction consistently generates high yield of templates \leq 10 kb. Longer templates require 5 U of Herculase hotstart DNA polymerase per 50- μ l reaction for optimal results.

DMSO

DMSO is provided as a means of obtaining higher yields of PCR product with extra-long targets or GC-rich targets. The DMSO concentration must be titrated for each application, since the degree to which DMSO enhances product yield and specificity varies according to target length, complexity, and GC content.

For genomic DNA, begin with the optimization guidelines of 0–3% DMSO for 10–23 kb targets, and 3–6% DMSO for targets $>$ 23 kb. For lambda DNA targets $>$ 30 kb, begin optimization using 5–7% DMSO. For GC-rich targets, DMSO at 4–8% is generally recommended. The DMSO concentration should be titrated in the specified range in 1% increments.

Note *The addition of DMSO may increase the error rate of the Herculase polymerase slightly (<50% increase with 3% DMSO). The use of DMSO is discouraged when the highest fidelity is essential.*

Primer Design and Concentration

Primers should be \geq 23 bp in length with a balanced $T_m \geq 60^\circ\text{C}$. The resulting high annealing temperature promotes specificity and discourages secondary structure formation. Further, primer sequences should be analyzed for potential duplex and hairpin formation as well as false priming sites in order to obtain the highest yield of specific PCR products.

Use \sim 0.25 μM final concentration of each primer for targets $<$ 10 kb, and \sim 0.5 μM final concentration of each primer for targets $>$ 10 kb. When using 25-mer oligonucleotide primers in a 50- μ l reaction volume, this is equivalent to \sim 100 ng or \sim 200 ng, respectively, of each primer.

Deoxynucleotide Concentrations

Amplification efficiencies are influenced by deoxynucleotide (dNTP) concentrations. Insufficient concentrations of dNTPs may result in lower yields. For targets \leq 10 kb, 200 μM each dNTP is recommended; for targets \geq 10 kb, the use of 500 μM each dNTP is optimal.

Cycling Parameters

As with all PCR reactions, cycling parameters are critical for successful amplification and may require further optimization.

Extension Time

Maintain an extension time of 1.0 minute/kb of template for general applications; longer extension times may produce higher yields, however, an extension time exceeding one hour provides no further benefit.

Extension Temperature

Extension temperatures also have a critical effect on amplicon yield. An extension temperature of 72°C should be used with templates less than 10 kb, while templates greater than 10 kb in length require an extension temperature of 68°C.

Denaturation Temperature

High denaturation temperatures damage DNA templates, so the denaturation temperature should be as low as possible. A denaturation temperature of 92°C works well for most targets, and is sufficient to denature the hotstart antibodies. For GC-rich targets, which are difficult to melt, a denaturation temperature of 98°C is recommended.

GC-Rich Targets

For amplification of GC-rich targets, we recommend using DMSO in the reaction at a concentration of 4–8% and using a PCR program with a denaturation temperature of 98°C and an extension temperature of 72°C.

PROTOCOL

1. Prepare a reaction mixture for the appropriate number of samples to be amplified. The following table provides an example of a reaction mixture for the amplification of targets ≤ 10 kb, targets > 10 kb, and GC-rich targets (which are typically ≤ 10 kb). The recipe listed in the table is for one reaction and can be adjusted for multiple samples. Add the components *in order* and mix gently.

Component	Quantity per reaction		
	≤ 10 -kb targets	> 10 -kb targets	GC-rich targets ≤ 10 kb
Distilled water	X μ l to final volume of 50.0 μ l	X μ l to final volume of 50.0 μ l	X μ l to final volume of 50.0 μ l
10 \times Herculase reaction buffer	5.0 μ l	5.0 μ l	5.0 μ l
dNTP mix (25 mM of each dNTP)	0.4 μ l	1.0 μ l	0.4 μ l
DNA template: Genomic DNA Low-complexity templates (λ DNA or cloned DNA)	100–250 ng 1–15 ng	250–1000 ng 15–60 ng	100–250 ng 1–15 ng
Primer #1	100 ng	200 ng	100 ng
Primer #2	100 ng	200 ng	100 ng
Herculase hotstart polymerase (5 U/ μ l)	0.5 μ l	1.0 μ l	0.5 μ l
DMSO ^a	—	Either 0–3%, 3–6%, 5–7% or 4–8%	4–8%
Total reaction volume	50.0 μ l	50.0 μ l	50.0 μ l

^a The optimal DMSO concentration must be determined by titration in 1% increments for each primer-template set. See *Critical Optimization Parameters* for DMSO concentration range recommendations for specific targets.

2. Before thermal cycling, aliquot 50 μ l of the master mixture into sterile thin-walled PCR 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. Suggested cycling parameters are given in the following tables for (A) targets with average base composition; and (B) GC-rich targets.

(A)**Average Base Composition Targets (>10 kb or ≤10 kb)**

Segment	Number of cycles	Temperature		Duration	
		Targets >10 kb	Targets ≤10 kb	Targets >10 kb	Targets ≤10 kb
1	1	92°C	95°C	2 minutes	2 minutes
2	10	92°C	95°C	10 seconds	30 seconds
		Primer $T_m - 5^\circ\text{C}^\circ$	Primer $T_m - 5^\circ\text{C}^\circ$	30 seconds	30 seconds
		68°C	72°C	60 seconds/kb of PCR target	60 seconds/kb of PCR target
3	20	92°C	95°C	10 seconds	30 seconds
		Primer $T_m - 5^\circ\text{C}$	Primer $T_m - 5^\circ\text{C}$	30 seconds	30 seconds
		68°C	72°C	60 seconds/kb of PCR target plus 10 seconds/cycle	60 seconds/kb of PCR target plus 10 seconds/cycle

^o The annealing temperature may be lowered or raised further if necessary to obtain optimal results. Typical annealing temperatures will range between 60 and 65°C.

(B)**GC-Rich Targets (≤10 kb)**

Segment	Number of cycles	Temperature	Duration
1	1	98°C	3 minutes
2	10	98°C	40 seconds
		Primer $T_m - 5^\circ\text{C}$	30 seconds
		72°C	60 seconds/kb of PCR target ^o
3	20–25	98°C	40 seconds
		Primer $T_m - 5^\circ\text{C}$	30 seconds
		72°C	60 seconds/kb of PCR target plus 10 seconds/cycle ^o
4	1	72°C	10 minutes

^o 1 minute minimum extension.

- Analyze the PCR amplification products by electrophoresis using an appropriate percentage acrylamide or agarose gel. Long PCR products greater than 17 kb in length may be separated on a 0.6% agarose gel, however a 0.8% agarose gel may be used if higher resolution with less separation is desired. For maximum separation and resolution, pulse field gel electrophoresis with a 1.0% gel is recommended.

TROUBLESHOOTING

Observations	Suggestions
No PCR product or lower yield than expected	Increase the amount of Herculanase hotstart polymerase (up to 10 U can be used for targets >23 kb)
	Increase the number of cycles up to a maximum of 40 cycles
	Use intact and highly purified DNA templates
	Store the template at 4°C; do not freeze the template
	Lower the annealing temperature in 5°C increments
	Allow at least 60 seconds of extension time for each kilobase to be amplified (90 seconds of extension time per kilobase may also be helpful for difficult templates). For GC-rich targets, use a minimum extension time of 1 minute
	For targets > 10 kb, denaturation times of 10 seconds at 92°C are usually sufficient, while longer denaturation times or higher denaturation temperatures may damage the DNA template; use the shortest denaturation time compatible with successful PCR on the thermal cycler
	For targets > 10 kb, denaturation times of 30 seconds at 95°C are usually sufficient. For GC-rich targets, denaturation times of 40 seconds at 98°C are usually sufficient
	Primer pairs exhibiting matched primer melting temperatures (T_m) and complete complementarity to the template are recommended
	Analyze the primers to ensure that duplexes or hairpins do not form
	Gel-purified or HPLC-purified primers ≥ 23 nucleotides in length are recommended
	Purify the primers by PAGE or HPLC
	Artifactual PCR smears
Decrease the amount of Herculanase hotstart polymerase	
Reduce the extension time	
Multiple bands	Optimize the cycling parameters specifically for the primer–template set and the thermal cycler used
	Increase the annealing temperature in 5°C increments
	Use Perfect Match PCR enhancer to improve PCR product specificity
	Use DMSO in the PCR mixture; titrate the DMSO concentration
	Verify that the primers hybridize only to the desired sequences on the template

REFERENCES

1. Borns, M. and Hogrefe, H. H. (2000) *Strategies* 13(1):1-3.
2. Borns, M. and Hogrefe, H. H. (2001) *Strategies* 14(2):41-42.
3. Hogrefe, H. H., Hansen, C. J., Scott, B. R. and Nielson, K. B. (2002) *Proc Natl Acad Sci U S A* 99(2):596-601.

ENDNOTES

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MSDS INFORMATION

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

Herculase Hotstart DNA Polymerase

Catalog #600310, 600312, 600314

QUICK-REFERENCE PROTOCOL (TEMPLATES ≤ 10 KB)*

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

Component	Quantity per reaction	
	Average composition targets	GC-rich targets
Distilled water	X μ l to final volume of 50.0 μ l	X μ l to final volume of 50.0 μ l
10 \times Herculase reaction buffer	5.0 μ l	5.0 μ l
dNTP mix (25 mM of each dNTP)	0.4 μ l	0.4 μ l
DNA template: Genomic DNA	100–250 ng	100–250 ng
Low-complexity templates	1–15 ng	1–15 ng
Primer #1	100 ng	100 ng
Primer #2	100 ng	100 ng
Herculase hotstart polymerase (5 U/ μ l)	0.5 μ l	0.5 μ l
DMSO	—	4–8%
Total reaction volume	50.0 μ l	50.0 μ l

- ◆ If extension times are > 15 minutes, overlay each reaction mixture with 50 μ l mineral oil.
- ◆ Perform PCR using the cycling conditions appropriate for your temperature cycler and target base composition, according to the following tables:
 - (A) Single block temperature cyclers (average base composition targets)
 - (B) Single block temperature cyclers (GC-rich targets)

* For targets > 10 kb, see *Protocol* section of manual.

(A) Single-Block Temperature Cyclers, Average Base Composition Targets ≤ 10 kb

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	10	95°C	30 seconds
		Primer $T_m - 5^\circ\text{C}$	30 seconds
		72°C	60 seconds/kb target
3	20	95°C	30 seconds
		Primer $T_m - 5^\circ\text{C}$	30 seconds
		72°C	60 seconds/kb target plus 10 seconds per cycle

(B) Single-Block Temperature Cyclers, GC-Rich Targets ≤ 10 kb

Segment	Number of cycles	Temperature	Duration
1	1	98°C	3 minutes
2	10	98°C	40 seconds
		Primer $T_m - 5^\circ\text{C}$	30 seconds
		72°C	60 seconds/kb target
3	20–25	98°C	40 seconds
		Primer $T_m - 5^\circ\text{C}$	30 seconds
		72°C	60 seconds/kb target plus 10 seconds per cycle
4	1	72°C	10 minutes

- ◆ Analyze the PCR amplification products by gel electrophoresis