



Herculase Enhanced DNA Polymerase

Instruction Manual

Catalog #600260 (100 U), #600262 (500 U), #600264 (1000 U), and #600266 (5000 U)

Revision C.0

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Herculase Enhanced DNA Polymerase

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Herculase Enhanced DNA Polymerase

MATERIALS PROVIDED

| Materials provided | Quantity | | | |
|--|-------------------------|-------------------------|--------------------------|--------------------------|
| | Catalog #600260 (100 U) | Catalog #600262 (500 U) | Catalog #600264 (1000 U) | Catalog #600266 (5000 U) |
| Herculase enhanced DNA polymerase (5 U/ μ l) | 20 μ l | 100 μ l | 200 μ l | 5 \times 200 μ l |
| 10 \times Herculase reaction buffer | 1 ml | 2 \times 1 ml | 4 \times 1 ml | 20 \times 1 ml |
| Dimethyl Sulfoxide (DMSO) | 1 ml | 1 ml | 1 ml | 5 \times 1 ml |

STORAGE CONDITIONS

All Components: -20°C

NOTICES TO PURCHASER

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INTRODUCTION

Herculase enhanced 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 Herculase polymerase features a novel DNA polymerase composition that consists predominantly of *Pfu* DNA polymerase, combined with the exclusive thermostable ArchaeMaxx polymerase-enhancing factor** and a small amount of *Taq2000* DNA polymerase. Our unique *Pfu*-based formulation is provided with a buffer optimized to promote high yield, specificity, and amplification of extra long targets. As a result, the Herculase enhanced 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 a lower error rate than other DNA polymerase mixtures.

A key component of Herculase enhanced 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.² Once incorporated, dU-containing DNA inhibits *Pfu* and most archaeal proofreading DNA polymerases, such as Vent[®] and Deep Vent[®] DNA polymerases, limiting their efficiency.² 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. 6,734,293, 6,444,428, 6,379,553, 6,333,165, 6,183,997, and 5,948,663.

** U.S. Patent Nos. 6,444,428, 6,379,553, 6,333,165, and 6,183,997.

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 DNA polymerase are outlined in Table I and are discussed in the following section. The provided Herculase 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 \leq 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 Enhanced 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 | \geq 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 We suggest titration of 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, we recommend using 100–250 ng of template for targets of \leq 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), we recommend using 1–15 ng for targets \leq 10 kb and 15–60 ng for targets >10 kb in a 50- μ l reaction volume. 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 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.

We suggest using \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

Use 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. 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 including 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 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.

- Perform PCR using optimized cycling conditions. Suggested cycling parameters are given below 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 Herculase polymerase (up to 10 U can be used for targets >23 kb) |
| | Increase the amount of full-length intact DNA template and/or 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 at 92°C for 10 seconds is usually sufficient. 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 at 95°C for 30 seconds is usually sufficient. |
| | For GC-rich targets, denaturation at 98°C for 40 seconds is 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 |
| | Adjust the ratio of primer versus template to optimize yield of the desired product |
| Use DMSO in the PCR mixture; titrate the DMSO concentration in 1% increments | |
| Artifactual PCR smears | Decrease the amount of Herculase polymerase |
| | Reduce the extension time |
| | Optimize the cycling parameters specifically for the primer–template set and the thermal cycler used |
| Multiple bands | 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. Borna, M. and Hogrefe, H. H. (2000) *Strategies* 13(1):1-3.
2. 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 Enhanced DNA Polymerase

Catalog #600260, #600262, #600264, and #600266

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 |

Perform PCR using the cycling conditions appropriate for your 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) 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°C | 30 seconds |
| | | 72°C | 60 seconds/kb target |
| 3 | 20 | 95°C | 30 seconds |
| | | Primer T_m – 5°C | 30 seconds |
| | | 72°C | 60 seconds/kb target plus 10 seconds per cycle |

(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°C | 30 seconds |
| | | 72°C | 60 seconds/kb target |
| 3 | 20–25 | 98°C | 40 seconds |
| | | Primer T_m – 5°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