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Product Information

Restorase® DNA Polymerase with 10× Reaction Buffer

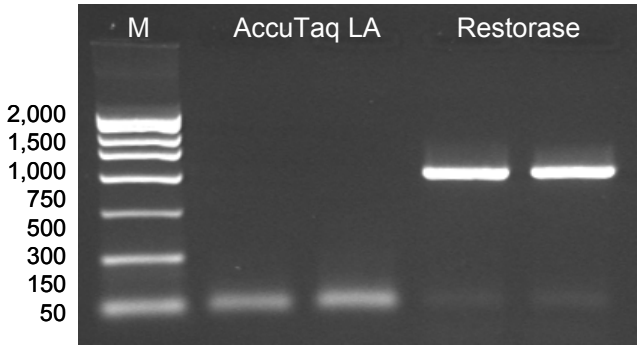
Catalog Number **R1028**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Restorase DNA Polymerase with 10× Reaction Buffer combines Sigma's Long and Accurate enzyme technology with a DNA repair enzyme resulting in a blend that facilitates repair and amplification of damaged DNA. Even though Restorase has been shown to increase yield and specificity of presumably "undamaged" DNA templates, it is not generally recommended to be used in place of standard amplification enzymes on truly undamaged templates.

DNA templates are compromised when damaged by exposure to acid, alkylating agents, heat or light. These damages block the progression of DNA polymerases, thereby affecting PCR efficiency. Restorase functions by modifying the damaged sites allowing subsequent template copying. Restorase can successfully produce PCR products from 0.2 to 20 kb in length.



AccuTaq™ LA vs. Restorase Amplification of a 500 bp fragment from formic acid damaged lambda DNA.

The level of template damage will dictate optimal Restorase treatment of the DNA. Lightly damaged DNA will require a shorter pretreatment step than templates containing heavier damage. DNA repair begins the moment enzyme is added and ends when the reaction is heated to >50 °C. While Restorase can restore the ability to amplify many damaged DNAs, some templates are irretrievably damaged.

Reagents Provided

- Restorase DNA Polymerase, Catalog Number L4165. 2.5 units/µL in 10 mM Tris-HCl, pH 8.0, 2.5 mM potassium phosphate, 150 mM KCl, 0.075 mM EDTA, 1 mM DTT, 100 µg/µl BSA, 0.25% TWEEN® 20, 0.25% IGEPAL® CA-630, 50% glycerol, AccuTaq™ LA DNA polymerase, a repair enzyme, and mesophilic polymerase.
- 10× Restorase Buffer, Catalog Number R8402. 0.5 ml/vial; 500 mM Tris-HCl, 150 mM ammonium sulfate (pH 9.3, adjusted with NH_4OH), 25 mM $MgCl_2$, 1% TWEEN 20. Provided as 1 vial/250 units

Materials and reagents required, but not provided

- Deoxynucleotide Mix, Catalog Number D7295 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM TTP
- Water, PCR Reagent, Catalog Number W1754
- Primers (Recommended source - Sigma-Genosys)
- DNA to be amplified
- Dedicated pipettes
- PCR pipette tips
- 200 µl or 500 µL thin-walled PCR microcentrifuge tubes, PCR plates or strips
- Thermal cycler

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Warning statements are included on the label or in the components section of this bulletin where applicable. If using radioactively labeled nucleic acids, standard procedures for safely handling radioactive materials should be followed.

Note: Please read the **Preparation Instructions** and **Procedure** sections before beginning experiments.

Preparation Instructions

Primer design

It is highly recommended that thiophosphate primers, available from Sigma-Genosys, http://www.sigma-genosys.com, be used in Restorase protocols. Thiophosphate linkages are recommended at the antepenultimate and final phosphodiester linkages:

5'- NpNpNpN…pNsNpNsN -3'

N= nucleotide, p= phosphodiester, s=thiophosphate

These modifications help ensure primer stability during the pre-incubation/repair step. Primer dimer formation during preincubation due to the mesophilic polymerase may be avoided by judicious primer design or Manual Primer Hot Start. **Primer Hot Start or increased primer concentrations should be used for unmodified primers** (see Procedure Step 3 for additional information).

In general, the primers are 21 to 34 bases in length and nominally have a GC content of 50%. Melting temperatures (T_M) of primer pairs should be within 3 °C of each other and between 60 and 72 °C.[1] Primers should also be free of stable secondary structures (e.g. hairpins) or inter/intrahybridization domains that may lead to primer dimer formation. Priming efficiency can be increased by including GC clamps (i.e. 3-prime CC, GG, CG, or GC).[2] Using a primer design software is highly recommended.

Template

Restorase is often used to attempt PCR with rare and/or valuable templates that have failed amplifications using standard PCR enzymes. It is prudent to verify thermal cycling conditions using Restorase with easily replenished templates before attempting restorative PCR with irreplaceable templates.

Nicked and/or base damaged DNA can be repaired in most cases. DNA sheared between primer hybridization sites is unrecoverable. Some DNA templates may be damaged beyond repair.

Due to the sources of damaged templates, template quantity is often difficult to measure. Even if the amount of template is known, the extent of damage is likely unknown. For these reasons it is not possible to recommend absolute quantities of template to add to a restorative PCR. In model studies, very lightly depurinated human genomic DNA was successfully amplified from as little as 100 pg while more severe damage required up to 100 ng of template. Thus, for most samples, greater success is realized by using larger quantities (up to 100 ng) of template.

Pre-incubation

Reliable amplification of damaged DNA sequences requires efficient repair and is dependent on the proper pre-incubation time of the reaction. The extent of DNA damage will determine optimal pre-incubation conditions.

Typical pre-incubation with Restorase is 10 minutes at 37 °C. Lightly damaged DNA will be rescued best by using short incubation times and/or lower temperatures. Heavily damaged DNA may require longer incubation times for optimal rescue.

If an initial amplification fails, the time of pre-incubation should be investigated to discover an optimal rescue protocol for a particular sample. See Troubleshooting Guide for details on insufficient pre-incubation.

Cycle Number

It is typical that thermal cycling is carried for 30 cycles. In some instances, it may be beneficial to perform additional cycles of PCR. Cycle number optimization may be attempted by performing additional cycles in increments of 5-10 cycles. For damaged DNA samples, it is prudent to withdraw aliquots from the reactions for analysis returning the rest of the reaction for additional cycles. While primer dimer and other primer design artifacts may be magnified by this approach, it may be possible to prepare amplicons that are otherwise missed at lower cycling numbers.

Denaturation

Acid catalyzed depurination is minimized by short denaturation times using Tris buffers pH 9.0 (25 °C) and above. It is recommended that Restorase DNA Polymerase only be used with the supplied magnesium concentration optimized buffer. Follow the protocol below if magnesium optimization is required.

Magnesium concentration

Optimization may be necessary for some template/primer combinations. Magnesium concentration may be optimized by preparing a buffer without magnesium chloride. Magnesium optimization will generally be reached by performing parallel PCR between 1 and 5 mM at 0.5 mM increments (i.e. 1, 1.5, 2 …5 mM).

Buffer preparation
The 10× Restorase Buffer is at a relatively high pH, which may allow precipitation of magnesium hydroxide [$Mg(OH)_2$]. Thaw the buffer at room temperature before use and vigorously vortex to dissolve any precipitated $Mg(OH)_2$. Alternatively, warm the buffer at 37 °C for 15 minutes and then vortex.
Note: It is important that the buffer be homogeneous before reaction assembly.

If titrating pre-incubation times and other PCR optimization experiments fail, it is likely the template is damaged beyond repair and rescue with Restorase.

Storage/Stability
Store Restorase DNA Polymerase and Restorase 10× Buffer at –20 °C.

Procedure
Note: Repair is initiated the moment template and Restorase are mixed. The time between initiation and the initial denaturation step should be tightly controlled.

1. Add the following reagents to a PCR microcentrifuge tube, strip or plate at ambient temperature:

Volume	Reagent	Final Concentration
5 µL	10× Restorase reaction buffer	1×
1 µL	dNTP Mix (10 mM each)	200 µM (each)
1 µL	10 µM forward thiophosphate primer*	0.2 µM
1 µL	10 µM reverse thiophosphate primer*	0.2 µM
36 µL	Water (W1754)	----
1 µL	Restorase	0.05 units/µL
5 µL	Template DNA** (≥10 ng/µl)	1-2 ng/µL:
50 µL	Total Volume***	

* Use of thiophosphate primers, Manual Primer Hot Start, or elevated primer concentration is necessary to minimize the effects of primer degradation by the DNA repair enzyme during pre-incubation. Elevated primer concentrations are dependent upon pre-incubation times. 2 µM is recommended for unprotected primers for 10-minute pre-incubations. Longer pre-incubations require higher primer concentrations, up to 10 µM.
Note: If thiophosphate primers are not used, do not add primers until after the initial denaturation step.

** Up to 200 ng of template DNA may be necessary for amplification of complex genomes (e.g., human).

***Assumed - Other reaction volumes may be used by scaling this protocol. If a master mix is desired, add template/primers or template to a mix containing buffer, dNTPs and enzyme.

2. If pre-incubation is less than 3 minutes mix the reaction by re-pipetting after addition of the last reagent (either enzyme or template) at the pre-incubation temperature. Longer pre-incubations may be vortex mixed and briefly centrifuged.
   Note: Pre-incubation begins the moment enzyme and template are mixed.

3. Cycling conditions - While amplification parameters may require optimization for specific primers, templates, and thermal cyclers, the protocol below is a good starting point and will suffice in many instances.
   Note: Pre-incubation time and temperature may be altered depending on the extent of DNA damage (see Pre-incubation under Preparation Instructions).

Pre-incubation	37 °C 72 °C	10 min 5 min
Initial denaturation	94 °C	30 sec
Primer Hot Start (Not thiophosphate primers)****	Between 4 and 75 °C	Until restart
For cycles 1-30		
Denaturation	94 °C	30 sec
Annealing	T_M-5 °C	30 sec
Extension	72 °C	1 min/kb
Final extension	72 °C	1 min/kb

****Eliminate the Primer Hot Start step above when using thiophosphate primers

Primer Hot Start

Manual Primer Hot Start

While paused at the Primer Hot Start step, add 1 µL each of 10 µM forward and reverse primers. The temperature at which primers are added is dependent upon primer design. Primers that yield single product amplicons can be added at low temperatures. Primers that form primer dimer and/or alternate amplicons must be added at elevated temperatures. Resume cycling.

4. Evaluate the amplified DNA by standard techniques such as agarose gel electrophoresis.[3]

References

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4. Don, R.H., et al. "Touchdown" PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res*., **19,** 4008 (1991).
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Label License Statement

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Troubleshooting Guide

Problem	Possible Causes	Solution
No PCR product observed.	PCR component missing or degraded.	Always run a positive control to insure components are functioning. A checklist is also recommended when assembling reactions.
	Too few thermal cycles.	Increase the number of cycles (5-10 additional cycles at a time). See Cycle Number section under Preparation Instructions.
	Annealing temperature too high.	Decrease annealing temperature in 2-4 °C increments. Use a temperature gradient thermal cycler.
	Primer design.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primer to 27-33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primer with a GC content of 45-60%.
	Too little template.	If increasing the number of cycles does not lead to a higher PCR product yield, repeat the PCR with a five fold, or higher, concentration of template.
	Insufficient Pre-Incubation	Pre-incubations can be investigated by titrating times up to 6 hours. Optimal conditions can be screened by splitting a 50 µL reaction into 10 µL aliquots and preincubating in 2 to 3 fold increments, such as 15, 35, 75, 150 and 300 minutes.
	Template too damaged.	Attempt Restorase optimization methods. See Preparation Instructions.
	Inappropriate denaturation temperature.	Optimize the denaturation temperature by increasing or decreasing the temperature in 1 °C increments or on a temperature gradient thermal cycler.
	Denaturation time too long or too short.	Optimize the denaturation time by increasing in 10-second increments.
	Extension time too short.	Increase the extension time. In general, increasing the extension time 1 minute per 1 kb is sufficient; however, longer extensions times may be beneficial.
	Mg^{2+} concentration too low.	This is unlikely if the 10× reaction buffer (provided) is used and the deoxynucleotides do not exceed a concentration of 0.6 mM each. However, if the concentration of EDTA in the sample is greater than 5 mM, this can reduce the effective concentration of magnesium.
	Deoxynucleotide concentrations too low.	This is unlikely if the final concentration of each deoxynucleotide is 0.2 mM. This concentration of dNTPs is suitable for a wide range of applications. If the dNTPs are being prepared in the laboratory, be sure that the final concentration of each deoxynucleotide is 0.2 mM. If the concentration of dNTPs is increased, the Mg^{2+} concentration will need to be increased proportionately.

Troubleshooting Guide (continued)

Problem	Possible Causes	Solution
Multiple products	Too many thermal cycles.	By reducing the cycle number, the nonspecific bands may be eliminated.
	Annealing temperature too low.	Increase the annealing/extension temperature in increments of 2-3 °C.
	Sub-optimal primer design.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primers to 27-33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primers with a GC content of 45-60%.
Products are smeared	Too many thermal cycles.	Reduce the cycle number in 5-10 cycle increments.
	Denaturation temperature too low.	Increase the denaturation temperature in 1 °C increments.
	Extension time too long.	Decrease the extension time in 1-2 minute increments
	Multiple PCR products generated	Perform Touchdown PCR (see ref. 4)
	Too much enzyme.	1 µL (2.5 units) is sufficient for most applications. However, this concentration may be too high for some applications. It is recommended the cycling parameters be optimized first, as described above, then, reduce the enzyme concentration to 0.5-0.2×.
	Mg^{2+} concentration too high.	In general magnesium should be 0.7 mM above the dNTP concentration. Some reactions may benefit from higher concentrations. Titrate Mg^{2+} in 0.2 mM increments.
	Incubation times too long	Reduce incubation times.
	Template concentration too high.	Reduce template concentration in a series of 10 fold dilutions.
Product is wrong size	Sub-optimal primer design.	See recommendations under “Multiple Products” for procedure.
	Extension time too short.	Increase the extension times or use touchdown PCR (see ref. 4).
Faint Product	Template concentration too low.	Add additional template in 50 ng increments for genomic or 1-2 ng for viral or plasmid DNA
	Too few thermal cycles.	Increase the cycle number in 5-10 cycle increments
	Extension time too short.	Increase the extension times in 2 minute increments
	Sub-optimal reaction conditions.	Add PCR enhancers, e.g., 1-4% dimethyl sulfoxide (DMSO) or 0.8-1.3 M betaine, final concentration. Add enhancers after the pre-incubation and first denaturation.

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