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The Perkin-Elmer Cetus *GeneAmplimer*™ HIV-1 Reagents are designed for use in detecting the presence of HIV-1 *gag* gene sequences in experimental samples using the *GeneAmp*™ Polymerase Chain Reaction (PCR) process. These reagents include both positive and negative HIV-1 control DNA templates. The positive control is plasmid DNA that contains the entire genome of the HIVZ6 isolate. The HIVZ6 genome has been rearranged to interrupt the *pol* gene region and thus block infectivity. The plasmid has been shown to be non-infectious in cell culture.[1] Human placental DNA that is free of HIV-1 sequences is provided as a negative control. Included are primers SK38 and SK39 which can be used to amplify a 115 bp sequence in the *gag* region of the HIV-1 genome.[2] Detection of the 115 bp PCR product can be achieved using the SK19 probe after suitable labeling. The ***GeneAmplimer* HIV-1 Reagents are optimized for use with the *GeneAmp*® PCR Core Reagents (Part No. N808-0009) or the *GeneAmp* PCR Reagent Kits with Native *Taq* DNA Polymerase (Part No. N801-0043) or *AmpliTaq*® DNA Polymerase (Part No. N801-0055).** Sufficient primer and probe reagents are provided for 100 amplification reactions of 100 µL each. Depending upon the dilutions used, sufficient control materials are provided for 10–100 amplification reactions.

The *GeneAmp* PCR process is a simple and powerful method[3], invented by K. Mullis and patented by Cetus Corporation, which allows *in vitro* amplification of DNA segments[1,2] (including cDNA)[3] through a succession of incubation steps at different temperatures. Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at a low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle. For the *GeneAmplimer* HIV-1 Reagents it is possible to simultaneously anneal and extend at the intermediate temperature thereby reducing the number of steps by one, leading to a decrease in cycling time while still maintaining specificity and the level of amplification.

LIST OF KIT COMPONENTS

Store the *GeneAmplimer* HIV-1 Reagents at −20°C in a constant temperature freezer. If stored under the proper conditions, the product will maintain performance until the control date printed on the label.

Reagent	*Amount*	*Volume*	*Concentration*	*Comments*
HIV-1 Positive Control DNA	10^5 copies	100 µL	10^3 copies/µL	In 10 µg/mL human placental DNA, 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8.0.
HIV-1 Negative Control DNA from human placenta	1 µg	100 µL	10 µg/mL	In 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8.0.
HIV-1 Primer 1 SK38	5 nmoles	200 µL	25 µM	In 10 mM Tris-HCl, pH 8.3.
HIV-1 Primer 2 SK39	5 nmoles	200 µL	25 µM	In 10 mM Tris-HCl, pH 8.3.
HIV-1 Probe SK19	100 pmoles	50 µL	2 µM	In 10 mM Tris-HCl, pH 8.3.

REAGENTS NEEDED BUT SOLD SEPARATELY

Reagent	Part No.
AmpliTaq® DNA Polymerase*	N801-0060
GeneAmp dNTPs*	N808-0007
GeneAmp 10X PCR Buffer II & $MgCl_2$ Solution*	N808-0010
*These items can be purchased together by ordering the *GeneAmp* PCR Core Reagents	N808-0009

Notice to Purchaser: Disclaimer of License

This product is designed for use in the *GeneAmp*™ Polymerase Chain Reaction (PCR) process covered by patents issued to Cetus Corporation, but no license under these patents to use the *GeneAmp* PCR process is conveyed by the purchase of this product. A license to use the *GeneAmp* PCR process for certain research and development activities accompanies the purchase and use of Perkin-Elmer Cetus' *GeneAmp*® PCR Core Reagents, *GeneAmp*® PCR Reagent Kits, and certain other products, or is available from Cetus Corporation. For information contact Cetus Director of Business Development, PCR Division, at 1400 Fifty-Third Street, Emeryville, CA 94608. Tel. (415) 420-3300.

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PROTOCOL FOR DNA AMPLIFICATION

I. General Advice

Due to the enormous amplification possible with the *GeneAmp*® PCR process, small levels of DNA contamination, especially from previous PCR amplification reactions and positive control templates, can result in product even in the absence of purposefully added template DNA.* If possible, all reactions should be set up in an area separate from PCR product analysis or sample preparation. Dedicated pipettes, vessels and solutions for DNA preparation, reaction mixing, and sample analysis will minimize cross contamination.

A master mix of reagents (water, buffer, dNTPs, primers and enzyme) for all samples can be prepared first, then aliquoted to individual tubes. Magnesium chloride and the template DNA are then added. Using such mixes will minimize reagent pipetting losses, increase accuracy, and reduce the number of reagent transfers.

Amplifications are performed in 100 µL of reaction mix in capped 0.5 mL polypropylene microcentrifuge tubes. Perkin-Elmer Cetus 0.5 mL *GeneAmp*® Reaction Tubes **(Part No. N801-0180)** provide the best heat transfer when using the Perkin-Elmer Cetus DNA Thermal Cycler, because of their uniform fit in the wells. Since DNA may stick to plastic and since nucleases are often found on surfaces, it may be preferable to use sterile, siliconized tubes and pipette tips.

II. Preparation of Stock Solutions

The positive control reagent contains 10^4 copies/10 µL of the HIV-1 control plasmid DNA and can be used directly. Further dilutions of the positive control can be prepared by diluting into either TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) or the negative control DNA.

If these control reagents are used with the *GeneAmp* PCR Reagent Kits with either native *Taq* DNA polymerase **(Part No. N801-0043)** or *AmpliTaq*® DNA polymerase **(Part No. N801-0055)**, a sterile 10 mM $MgCl_2$ solution is needed, to supplement the $MgCl_2$ already present in the [10x] Reaction buffer. Dissolve 203 mg $MgCl_2$-$6H_2O$ in 100 mL distilled water, filter through a 0.2 µm filter and autoclave at 121°C at 15 PSI for 20 minutes.

III. Reaction Mix using the *GeneAmp* PCR Core Reagents (Part No. N808-0009)

	Component	*Addition Order*	*Volume*	*Final Concentration*
Master Mix	Distilled Sterile Water	1	57.5 µL*	
	10X PCR Buffer II	2	10 µL	1X
	dATP	3	2 µL	200 µM
	dCTP		2 µL	200 µM
	dGTP		2 µL	200 µM
	dTTP		2 µL	200 µM
	HIV-1 Primer 1 (SK38)	4	2 µL	0.5 µM
	HIV-1 Primer 2 (SK39)	5	2 µL	0.5 µM
	AmpliTaq DNA Polymerase	6	0.5 µL	2.5 Units/100 µL
	25 mM $MgCl_2$ Solution	7	10 µL	2.5 mM
	Positive Control DNA		10 µL	10^4 copies/100 µL
	-or-		-or-	
	Negative Control DNA	8	10 µL	0.1 µg human placental DNA/100 µL
	-or-		-or-	
	Experimental sample		10 µL*	
	Total Mix		100 µL	

*Any combination of sterile distilled water and experimental sample volumes can be used as long as the total combined volume equals 67.5 µL.

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IV. **Reaction Mix using the *GeneAmp*® PCR Reagent Kits (Part Nos. N801-0043 & N801-0055)**

	Component	Addition Order	Volume	Final Concentration
Master Mix	Distilled Sterile Water	1	57.5 µL*	
	[10x] Reaction Buffer	2	10 µL	1X
	dATP	3	2 µL	200 µM
	dCTP		2 µL	200 µM
	dGTP		2 µL	200 µM
	dTTP		2 µL	200 µM
	HIV-1 Primer 1 (SK38)	4	2 µL	0.5 µM
	HIV-1 Primer 2 (SK39)	5	2 µL	0.5 µM
	AmpliTaq® or Native *Taq* DNA Polymerase	6	0.5 µL	2.5 Units/100 µL
	10 mM $MgCl_2$ Solution	7	10 µL	2.5 mM (including $MgCl_2$ in [10x] reaction buffer)
	Positive Control		10 µL	10^4 copies/100 µL
	-or-		-or-	
	Negative Control	8	10 µL	0.1 µg human placental DNA/100 µL
	-or-		-or-	
	Experimental sample		10 µL*	
	Total Mix		100 µL	

*Any combination of sterile distilled water and experimental sample volumes can be used as long as the total combined volume equals 67.5 µL.

V. **Notes on Reaction Mix for Controls**

Vortex briefly (2 seconds; avoid generating bubbles) the *AmpliTaq* or native *Taq* DNA Polymerase solutions or other recently thawed reagents, then spin down in a microcentrifuge before pipetting. Pipette enzyme carefully and slowly. The viscosity of the 50% glycerol in the buffer can lead to errors. If possible use a positive displacement pipette. Using master mixes (see above) will increase accuracy, reduce reagent loss on tips, and reduce tube-to-tube variability.

To reduce evaporation or refluxing, overlay the mix with with 50 to 100 µL of mineral oil **(Part No. 0186-2302)**. The oil should not interfere when withdrawing samples. If the entire volume is to be recovered, 100 µL of high purity chloroform should be added after amplification. The aqueous phase containing the DNA will then float on the chloroform-oil mixture, allowing easy collection.

VI. **Temperature Cycling for the Control Reagents**

Optimum performance of the *GeneAmp*™ PCR Process is achieved using the Perkin-Elmer Cetus DNA Thermal Cycler. For amplification using the *GeneAmplimer*™ HIV-1 Reagents, program the Perkin-Elmer Cetus DNA Thermal Cycler for 4 linked files.

File # 11 STEP-CYCLE FILE PARAMETERS
Temp = 95C
Seg #1 1min 0sec
Temp = 0C
Seg #2 0min 0sec
Auto Segment Extension: OFF
Cycle Count = 1
Linked to file #12

File # 12 STEP-CYCLE FILE PARAMETERS
Temp = 95C
Seg #1 1min 0sec
Temp = 60C
Seg #2 1min 0sec
Temp = 0C
Seg #3 0min 0sec
Auto Segment Extension: OFF
Cycle Count = 30
Linked to file #13

File # 13 STEP-CYCLE FILE PARAMETERS
Temp = 60C
Seg #1 10min 0sec
Temp = 0C
Seg #2 0min 0sec
Auto Segment Extension: OFF
Cycle Count = 1
Linked to file #14

File # 14 SOAK FILE PARAMETERS
Temp = 4C

Refer to the Thermal Cycler User Manual for additional programming instructions.

The samples can be stored at 2° to 8°C until subsequent analysis. The selection of 60°C for the anneal-extend temperature is optimal for amplification of the positive control template. Due to genetic variation in the HIV-1 genome[*,11] a lower anneal-extend temperature in the range of 52° to 60°C may be necessary to achieve amplification of viral sequences in experimental samples[*].

VII. Performance Claims

Using the amplification conditions described above and the Perkin-Elmer Cetus DNA Thermal Cycler and starting with 50 copies of the positive control template, sufficient amounts of the 115 bp PCR product will be generated to allow specific detection by the oligomer-hybridization procedure.[14] Detailed procedures for labeling the SK19 probe with γ-[^{32}P]-ATP can be found in references 14 and 15.

VIII. Sequences

The sequences of the HIV-1 primers and probes and their location in the HIV-1 genome of isolate ARV-2[11], GenBank accession number KO2007, are shown below.

Primer or Probe	Sequence (5'-3')	Location
SK38 Primer	ATAATCCACCTATCCCAGTAGGAGAAAT	*gag* 1551-1578
SK39 Primer	TTTGGTCCTTGTCTTATGTCCAGAATGC	*gag* 1638-1665
SK19 Probe	ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC	*gag* 1595-1635

The North American consensus sequence of the 115 bp target[13] segment of the HIV-1 *gag* gene and the sequence of the 115 bp PCR product obtained using the SK38 and SK39 primers with the positive control plasmid DNA are shown below. Information on the extent of sequence conservation in this region among different HIV-1 isolates can be found in References 9 and 13.

Sequence

North American Consensus

(5') ·TAATCCACC TA·CCCAGTA GGAGAAAT·T ATAAAAG·TG
GATAAT·CTG GG·TTAAATA AAATAGTAAG ·ATGTATAG·
CC···CAGCA TTCT·GACAT AAGACAAGGA CC·AA (3')

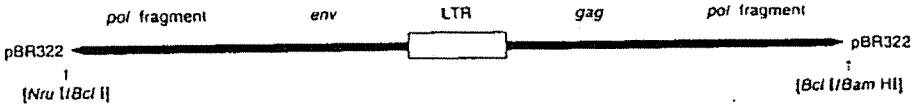
· nonconsensus nucleotide

115 bp PCR Product

| < ——————Region for SK38—————— > |
(5') ATAATCCACC TATCCCAGTA GGAGAAATCT ATAAAAGATG
GATAATCCTG GGATTAAATA AAATAGTAAG AATGTATAGC
CCTGTCAGCA TTCTGGACAT AAGACAAGGA CCAAA (3')
| < ——————Region for SK39—————— > |

IX. HIV-1 Positive Control Plasmid

The following schematic diagram (not drawn to scale) shows the general rearrangement of the HIV-1 genome in the control plasmid. The *Bam* HI - *Nru* I region of plasmid pBR322 has been substituted with the disrupted (in *pol* gene) and rearranged HIVZ6 sequence.



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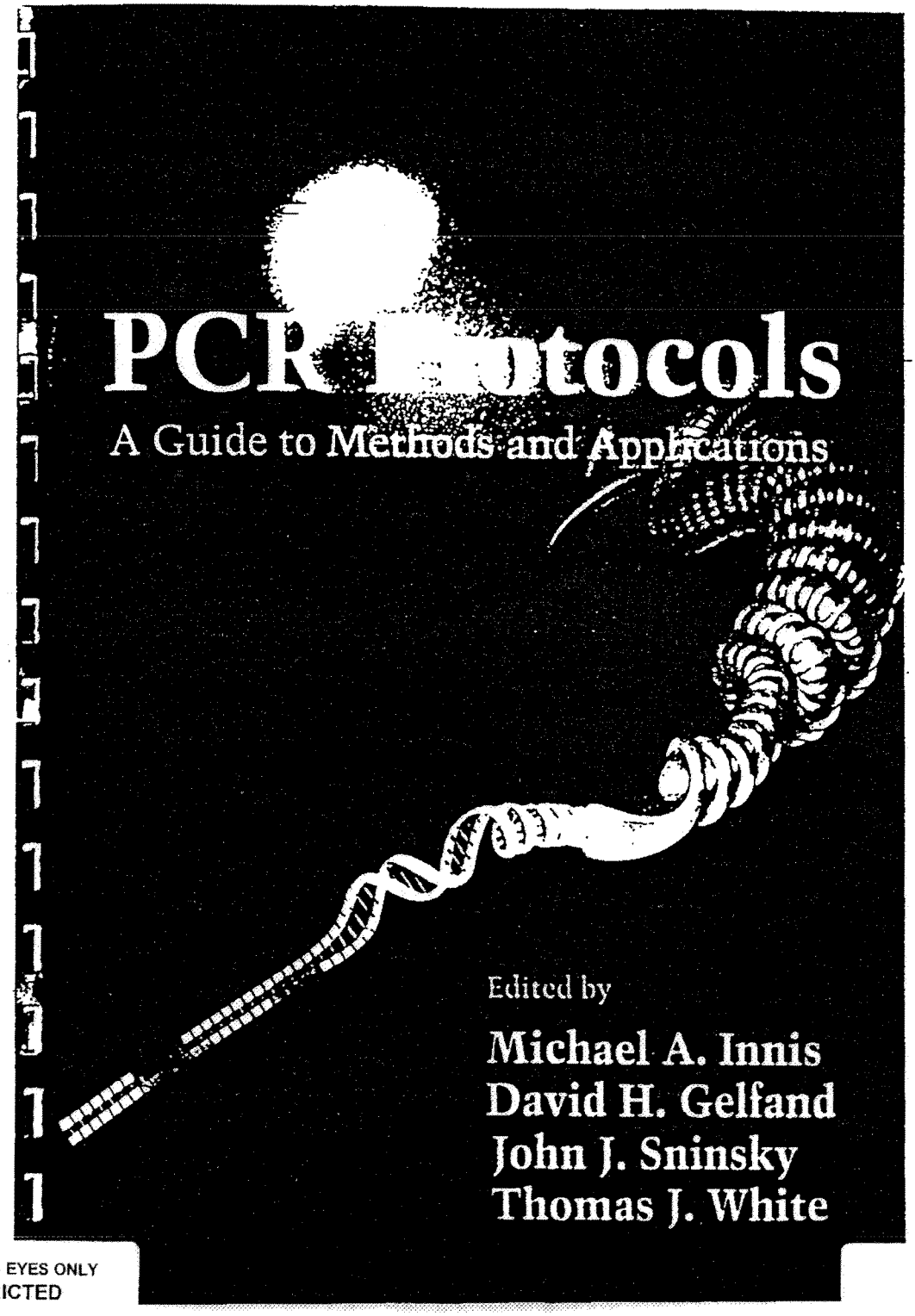

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The capability for single molecule amplification provided by the GeneAmp™ Polymerase Chain Reaction (PCR) process[1-3] has required the adoption of special laboratory practices to avoid false positive amplifications.[4] A primary source of exogenous templates leading to false positive amplifications has been identified as products from previous PCR amplifications.[5] The Perkin-Elmer Cetus GeneAmp® Carry-over Prevention Kit has been developed to provide a means for ensuring that products of previous PCR amplifications cannot be reamplified to produce false positive results. The GeneAmp Carry-over Prevention Kit uses enzymatic and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to degrade specifically PCR products from previous PCR amplifications, but not degrade native nucleic acid templates. The method used to make PCR products susceptible to degradation involves substituting dUTP for dTTP in the PCR reaction mix and treating subsequent PCR amplifications with the enzyme Uracil N-glycosylase (UNG, EC 3.2.2.-) prior to amplification.

The UNG provided in this kit is a pure, nuclease free, 26 kDa enzyme encoded by the *Escherichia coli* Uracil N-glycosylase gene[6] which has been inserted into an *E. coli* host to direct the expression of the native form of the enzyme.[7] When dUTP replaces dTTP as a dNTP substrate in PCR and the method described below is used, UNG treatment can prevent the subsequent reamplification of dU-containing PCR products.[7] This insert contains important details about the use of dUTP and UNG and the properties of dU-containing oligonucleotides. ***The entire product insert should be read prior to using the reagents provided.***

INTRODUCTION

Bacterial cells are particularly efficient at (i) degrading incoming phage DNA if the viral genomes are not methylated[8-10] and (ii) removing by enzymatic excision modified bases that result from inappropriate insertion by the replication apparatus or from damage by mutagens.[11] Warner and colleagues, for example, demonstrated that T4[12] and T5[13] phage containing about 30% of the thymine replaced by uracil were restricted by wild type *E. coli* but were capable of nearly equivalent infection as phage lacking uracil in *E. coli* cells deficient in UNG. In addition, Kunkel[14] exploited the *in vivo* degradation of uracil-containing DNA to enhance the recovery of recombinant M13 phage from *in vitro* mutagenesis reactions. The GeneAmp PCR Carry-over Prevention Kit adapts the UNG and dU-containing DNA cellular restriction-excision system to provide a means of degrading PCR products from previous reactions.

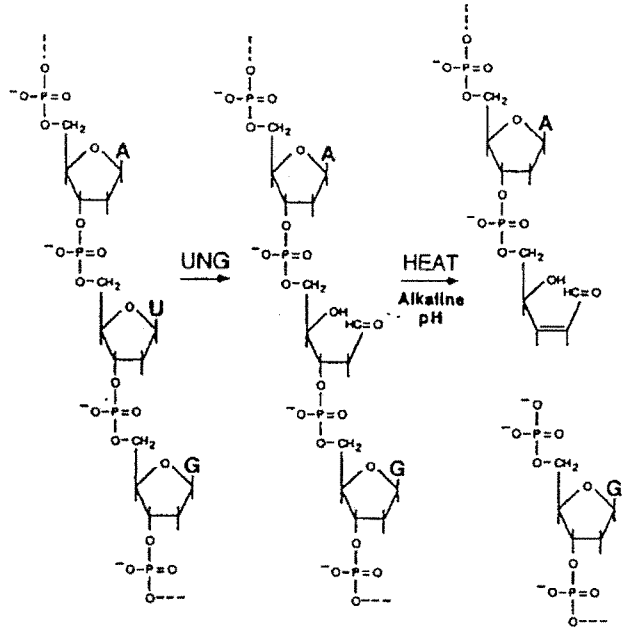

Figure 1. Cleavage of dU-containing PCR products by UNG in concert with alkaline pH and heat.

The distinguishing characteristic of PCR product relative to native DNA template is that PCR product is newly synthesized and thus modified nucleotides other than the typical four nucleotides (dA, dG, dT and dC) can be incorporated. Incorporation of a novel or modified nucleotide in the PCR product thus serves as the basis for distinguishing the newly synthesized PCR product from the starting native DNA template. Further, since enzymes of the excision-repair system can either remove the altered base and/or cleave the polynucleotide backbone, specific degradation of the PCR product instead of the starting native DNA template can be achieved. Abasic polynucleotides are susceptible to hydrolysis in alkaline solutions especially at elevated temperatures.[1] [2] The alkaline pH of the PCR buffer and the elevated temperature of the initial denaturation step (> 94°C) are more than sufficient to result in abasic polynucleotide strand scission. The reagents in this kit can be used to modify PCR amplifications such that the PCR products from previous amplifications are susceptible to enzymatic excision and chemical cleavage without degrading native DNA templates.

To render selectively PCR products susceptible to cleavage, the modification of PCR amplifications begins with the substitution of dUTP for dTTP in the PCR reaction mix. UNG is added to the PCR reaction mix. A short room temperature incubation step is performed prior to the start of the PCR. This allows time for UNG to excise the uracil in carried-over dU-containing PCR products should they be present in the reaction. The excised dU-containing PCR products are refractory to further PCR amplification because of the frequent stalling of DNA polymerases at the abasic sites[1,2,3] and/or cleavage of the abasic polynucleotide strands due to the inherent alkaline and thermal lability of the resulting abasic linkage. Since UNG is active on single- and double-stranded DNA[4], the procedure should work on dU-containing PCR products from standard or asymmetric PCR amplifications. Ribouracil residues in RNA and dUTP are not substrates for UNG.[4] Figure 1 shows the reactions involved in the UNG catalyzed degradation of dU-containing DNA.

LIST OF COMPONENTS

Store UNG and dUTP solutions at −20°C in a constant temperature freezer. If stored under the proper conditions, the product will maintain performance until the control date printed on the label.

Reagent	*Volume*	*Concentration*	*Comments*
Uracil N-glycosylase	100 µL	1 Unit/µL	See **Uracil N-glycosylase Characteristics**
dUTP	320 µL	20 mM	Deoxynucleotide dissolved in glass distilled water; titrated with NaOH to pH 7.0

NOTICE TO PURCHASER: DISCLAIMER OF LICENSE

This product is optimized for use in the *GeneAmp*™ Polymerase Chain Reaction (PCR) process covered by patents issued to Cetus Corporation, but no license under these patents to use the *GeneAmp* PCR process is conveyed by the purchase of this product. A license to use the *GeneAmp* PCR process for certain research and development activities accompanies the purchase and use of Perkin-Elmer Cetus' *GeneAmp* PCR Core Reagents, *GeneAmp* PCR Reagent Kits, and certain other products, or is available from Cetus Corporation. For information contact Cetus Director of Business Development, PCR Division, at 1400 Fifty-Third Street, Emeryville, CA 94608. Tel. (415) 420-3300.

URACIL N-GLYCOSYLASE CHARACTERISTICS

Concentration:	1 Unit/µL
Unit Definition:	One unit of enzyme is defined as the amount that releases 1 nmole of uracil from a dU-containing DNA template into acid soluble material per 60 minutes at 37°C under the analysis conditions below.
UNG Activity Analysis Conditions:	10 mM Tris-HCl, pH 8.3 (at room temperature); 50 mM KCl; and 100 pmoles dUMP substrate (dU-containing Lambda PCR product, a mix of unlabeled and [^{3}H-Uracil]-labeled), in a final volume of 50 µL and incubated at 37°C for 10 min. The dU-containing 500 bp Lambda PCR product is prepared by following the procedure for amplifying the Control Template provided with the *GeneAmp*® PCR Reagent Kit (**Part No. N801-0055**) except that a mixture of dUTP and [^{3}H-Uracil]-dUTP are substituted for dTTP.
Storage Buffer:	5% Glycerol (w/v) 150 mM NaCl 30 mM Tris-HCl, pH 7.5 (at room temperature) 1.0 mM EDTA (Ethylenediamine-tetraacetic acid) 1 mM DTT (Dithiothreitol) 0.05% Tween 20®
Storage Temperature:	Store UNG at −20°C, in a constant temperature freezer. If stored under proper conditions, the enzyme will remain active until the control date printed on the label.
Associated Activities:	No single-stranded or double-stranded endonuclease activities were detected when 5 Units of UNG were incubated at 37°C for 1 hour with 1.2 µg of circular M13 DNA or 600 ng supercoiled pBR322 (dcm$^-$, dam$^-$), respectively, in a final volume of 50 µL. No double-stranded exonuclease activity was detected when 5 Units of UNG were incubated at 37°C for 30 minutes with 1 pmole of [^{3}H]-dT-labeled 500 bp Lambda PCR product in a final volume of 50 µL. No 5'-single-stranded exonuclease activity was detected when 5 Units of UNG were incubated at 37°C for 30 minutes with 0.3 pmoles of a 5'-[^{32}P]-labeled oligonucleotide (41-mer) in a final volume of 50 µL. No 3'-single-stranded exonuclease activity was detected when 5 Units of UNG were incubated at 37°C for 30 minutes with an oligonucleotide (40-mer) labeled at the 3' end with α-[^{32}P]-Cordycepin in a final volume of 50 µL.

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PROTOCOL FOR DNA AMPLIFICATION

I. Materials Necessary but Not Provided

The following materials are needed in order to amplify target DNA segments with dUTP

- *GeneAmp*® PCR Core Reagents (Part No. N808-0009)

-or-

- *GeneAmp* PCR Reagent Kit with *AmpliTaq*® DNA Polymerase (Part No. N801-0055)

-or-

- *GeneAmp* PCR Reagent Kit with Native *Taq* DNA Polymerase (Part No. N801-0043)

-and-

- Primers and Experimental template.

II. General Advice

Because of the enormous amplification possible with the *GeneAmp*™ PCR process, small levels of DNA carry over from samples with high DNA levels, positive control templates, or from previous PCR amplifications can result in product even in the absence of purposefully added template DNA. Although the protocol and reagents described here are capable of degrading or eliminating large numbers of carried over PCR products, we encourage users to continue using the specific devices and suggestions described in the package inserts provided with the products listed in **Section I** and in References 4 and 5 to minimize cross-contamination from non-dU-containing PCR products or other samples. Briefly, the use of dedicated or disposable vessels, solutions, and pipettes (preferably positive displacement pipettes) for DNA preparation, reaction mixing, and sample analysis is still recommended. In addition, all reactions should be set up in an area separate from PCR product analysis.

A master mix of reagents (water, buffer, dNTPs [dATP, dCTP, dGTP, and dUTP], primers, *AmpliTaq*® DNA Polymerase, and UNG) for all samples can be prepared first, then aliquoted to individual tubes. Magnesium chloride and template DNA are then added. Using such mixes will minimize pipetting losses, increase accuracy, and reduce the number of reagent transfers.

Amplifications are performed in 100 µL of reaction mix in capped 0.5 mL polypropylene microcentrifuge tubes. Perkin-Elmer Cetus 0.5 mL *GeneAmp*™ Reaction Tubes (Part No. N801-0180) provide the best heat transfer when using the Perkin-Elmer Cetus DNA Thermal Cycler, because of their uniform fit in the wells. Since DNA may stick to plastic and since nucleases are often found on surfaces, it may be preferable to use sterile, siliconized tubes and pipette tips.

III. General Protocol using the GeneAmp PCR Carry-over Prevention Kit and GeneAmp PCR Core Reagents

Reaction Mix

	Component	*Addition Order*	*Volume*	*Final Concentration*
Master Mix	Sterile Distilled Water	1	*	
	10X PCR Buffer II	2	10 µL	1X
	dATP, 10 mM		2 µL	200 µM
	dCTP, 10 mM	3	2 µL	200 µM
	dGTP, 10 mM		2 µL	200 µM
	dUTP, 20 mM		1-5 µL	200-1000 µM**
	Primer 1	4	1-5 µL	0.2-1.0 µM
	Primer 2	5	1-5 µL	0.2-1.0 µM
	AmpliTaq® DNA Polymerase	6	0.5 µL	2.5 Units/100 µL
	UNG	7	1 µL	1 Unit/100 µL***
	25 mM $MgCl_2$	8	4-16 µL	1.0-4.0 mM****
	Experimental template	9	*	<1 µg/100 µL
	Total Mix		100 µL	

* Any combination of sterile distilled water and experimental template volumes can be used as long as the total volume of the reaction (including buffer, dNTPs, primers, enzymes and $MgCl_2$ solutions) equals 100 µL.

** Although many PCR systems are amplified efficiently when an equimolar concentration of dUTP (200 µM) is substituted for dTTP (200 µM) in the standard reaction mix, some PCR systems require increasing the dUTP concentration 2 to 5 fold (e. g., 400-1000 µM) for optimal amplification efficiencies. Increase $MgCl_2$ on an equimolar basis, when an increased dUTP concentration is required. In some systems, the level of amplification obtained even with 1000 µM dUTP may be less than the level obtained with 200 µM dTTP. Sufficient 20 mM dUTP solution is provided for 100 PCR amplifications (final volume of 100 µL) at 600 µM dUTP.

*** Depending on the level of potential dU-containing PCR product cross-contamination, the required amount of UNG may vary. With a short incubation step (0-10 minutes at room temperature) 1 Unit/100 µL reaction is generally more than sufficient to preclude subsequent reamplification of high levels (1 x 10^8 copies) of dU-containing PCR product. If further optimization of the concentration of UNG is desired, concentrations in the range of 1.0 to 2.0 Units/100 µL have been shown to be effective (see **Section IV.3**).

**** The optimal magnesium chloride concentration may vary, depending on the total dNTP concentration and on the primer and templates used and must be determined empirically. In most cases a final concentration of $MgCl_2$ in the range of 1.0 to 5.0 mM in the reaction mix will work well. The addition of UNG will not affect the magnesium chloride optimum of your specific PCR amplification. Magnesium ion is not required for UNG activity.

UNG Reaction Conditions

After the addition of $MgCl_2$ and Experimental template DNA begin with a 1 to 10 minute incubation at room temperature (18° to 22°C) to allow UNG to excise uracil from any contaminating dU-containing PCR product from previous amplifications. The time of this incubation depends on the level of UNG used and the potential for primer-dimer formation in your the PCR system (see **Section IV.3**). Follow the room temperature incubation with at least a 10 minute incubation at 95°C using a soak file in the DNA Thermal Cycler process. Incubation at 95°C for at least 10 minutes is necessary to cleave the abasic dU-containing PCR product generated in the room temperature incubation, to inactivate UNG and to denature the native DNA in the experimental sample. If a

shorter incubation at 95°C is used, UNG may not be completely inactivated, leading to degradation of newly synthesized dU-containing PCR product during the PCR process. The 10 minute incubation at 95°C does not affect significantly the activity of *AmpliTaq*® DNA Polymerase, which has a half-life of >45 minutes at 95°C.

Since UNG has activity below 55°C, the annealing temperature used for the PCR amplification should be at or above this temperature, in order to avoid degradation of newly synthesized dU-containing PCR products by residual UNG activity.

VERY IMPORTANT

After the final PCR cycle, use a soak file to hold PCR reaction mixes in which dUTP and UNG are present at 72°C until they are removed from the Perkin-Elmer Cetus DNA Thermal Cycler. Store reaction mixes immediately at −20°C or add an equal volume of chloroform to prevent any degradation of dU-containing PCR products by residual or reactivated UNG.

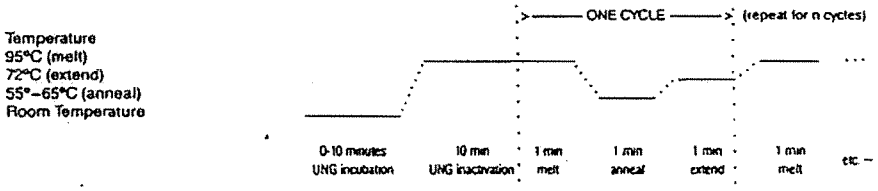
IV. Notes on Preventing PCR Product Carry-over

1. One should determine if the sequence being amplified contains dA and dT nucleotides. Only DNA sequences that have dA and dT will lead to dU-containing PCR products that can be degraded.
2. The primers that are used should contain dA nucleotides near the 3' termini so that any primer-dimer generated is efficiently degraded by UNG at least as well as any dU-containing PCR products. The further a dA nucleotide is from the 3' terminus the more likely that partially degraded primer-dimer molecules may serve as templates for a subsequent PCR amplification. Production of primer-dimer could compromise the amplification of the desired target region. If primers can not be selected with dA nucleotides near the termini, the use of primers with 3' terminal dU nucleotides should be considered. Terminal dU nucleotides are not substrates for UNG[11] and thus the primers will not be degraded. Biotin-dUMP derivatives are not substrates for UNG.
3. The incubation time of the UNG reaction may affect the subsequent PCR amplification efficiency. When 1 Unit/100 µL of UNG is used, a 10 minute incubation at room temperature will be sufficient to degrade 10^8 copies of cross-contaminating dU-containing PCR products. ***The concentration of UNG and the time of the incubation step necessary to prevent amplification of contaminating dU-containing PCR products depends on the PCR conditions necessary to amplify your particular DNA sequence and the level of contamination to be expected.*** In most cases, the general recommendation of using UNG at 1 Unit/100 µL reaction and incubation at room temperature for 10 minutes will suffice. If the PCR system is prone to primer-dimer formation, a 10 minute incubation at room temperature may result in the preferential amplification of primer-dimer sequences instead of the desired target sequence. If primer-dimer amplification dominates the PCR, reduce the incubation time at room temperature with or without increasing the UNG concentration to 2 Units/100 µL reaction.

V. Temperature Cycling and Cycle Optimization

The performance of the *GeneAmp*® PCR Carry-over Prevention Kit is optimized and quality control tested for performance on the Perkin-Elmer Cetus DNA Thermal Cycler. Optimal performance of the *GeneAmp*™ PCR process is influenced by choice of temperature, time at temperature, and length of time between temperatures for each step in the cycle.

A typical cycling profile for utilizing UNG to degrade contaminating dU-containing PCR products from previous amplifications prior to PCR amplification of freshly added native DNA Experimental Templates is shown below:



VERY IMPORTANT

After the final PCR cycle, use a soak file to hold PCR reaction mixes in which dUTP and UNG are present at 72°C until they are removed from the Perkin-Elmer Cetus DNA Thermal Cycler. Store reaction mixes immediately at −20°C or add an equal volume of chloroform to prevent any degradation of dU-containing PCR product by residual or reactivated UNG.

VI. Properties of dU-containing DNA

The substitution of dUTP for dTTP in PCR amplifications leads to the generation of PCR products containing dU instead of dT. Although this renders the dU-containing PCR products susceptible to degradation by UNG, most other properties of dU-containing PCR products are largely not affected.

- dU-containing PCR products will serve in an equivalent manner as dT-containing PCR products as hybridization targets.[7,11]
- dU-containing PCR products should also serve as templates for dideoxy-terminated sequencing reactions.
- dU-containing DNA can be cloned directly, if transformed into an *ung*⁻ bacterial host.[12,13]
- The recognition of dU-containing DNA by restriction endonucleases has been studied.[14–16] Depending on the specific endonuclease there may be no effect of the substitution of dU for dT on enzyme activity (*e.g.*, *Eco*RI and *Bam*HI) or the dU-containing DNA is cleaved more slowly than dT-containing DNA (*e.g.*, *Hpa*I, *Hind*II, and *Hind*III). For other endonucleases the effect of substituting dU for dT on enzyme activity will need to be examined empirically on an individual enzyme basis.

The use of PCR products containing exclusively uracil is not recommended for protein binding or site-specific recognition studies (*e.g.*, operators[17] and promoters[18], restriction endonucleases[14–16], or methylases). The absence of the C5 methyl group of thymine in the major groove, the face of the helix most frequently involved in protein-nucleic acid interactions[19, 20], and/or distortion of the glycosidic bonds of the dA:dU (and adjacent base pairs)[21] may impair recognition by proteins.

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