

RDMC INTERIM MEETING
12/7/87 --2

(3) Reference Lab

Although establishment of an EK/Cetus lab is still under study, there is a decided preference to contract or establish a JV with an existing lab. This would be quicker and less expensive up front. Candidates are: Pathology Institutes, Berkeley or Dianon, Connecticut. Other proposals are being considered. The areas of infectious diseases (HIV/HPV, cancer, genetics and forensics) may lead to more than one relationship with laboratories. Some options are expected to be selected before the next RDMC meeting (February 1 and 2, at Cetus).

(4) Renegotiation of the EK/Cetus Agreement

The major concern is less than adequate R&D support to fully exploit the opportunities provided by PCR technology. Cetus encouraged Kodak to increase PCR license or sell the technology in order to realize the commercial opportunities before competing technology is developed. PCR strategy statement will be prepared in draft form for discussion at the February RDMC and in final form two weeks later.

(5) Blood Bank

FDA approval of HTLV-I test is not expected before February 1988. Continued delays in revenue are disappointing and will lead to a review of options at the end of the first quarter 1988. Under any circumstances Kodak recommends not withdrawing from this market before SureCell products are available, in order to minimize adverse image in the marketplace. Bill Gerber, Paul Law and John Knowles will explore options to become OEM supplier to Olympus and/or Smith-Kline Beckman.

ATTORNEY'S EYES ONLY
RESTRICTED

001154
CETUS

RMS 71126

RDMC INTERIM MEETING
12/7/87 --3

(6) Reporting Format

A format for financial and project milestone reporting has been established and agreed upon. The first "live" report will be issued in late January and will review 1987 financials and current time lines. Fred Marcellus (EK) will issue the report with input from Cetus.

Scientific reports at Kodak will be altered in order to better communicate with Cetus.

(7) Public Relations and Marketing Review Procedure

The attached procedure (Attachment IV) was accepted for Kodak releases relating to the venture. This should provide adequate means for comments, revisions and notification before release.

Marketing promotional material is not subject to this procedure but will be offered for review of technical content when appropriate.

(8) LRPD

A loss of \$140K is forecast. This is less than the earlier estimate of \$200-300K loss. All responsibilities will be in the hands of LRPD in 1988. Current transfer price for new kits has been increased to reflect Cetus' actual costs. The inventory of reagents will be bought by LRPD in January for approximately \$70K.

(9) Licensing PCR

A list of requests is attached (Attachment V) along with the guidelines for granting licenses. Bill Gerber is managing this process.

PAL:llf

cc: RDMC Members

ATTORNEY'S EYES ONLY
RESTRICTED

001155
CETUS

RMS 71127

Attachment I

<u>Target</u>	<u>Formula Decision</u>	<u>Internal Trials</u>	<u>Clinical Trials</u>	<u>FDA Subm.</u>	<u>Prodn.</u>	<u>FDA Approval</u>	<u>Sales</u>
Strep A	2/88	5/88	5/88	6/88	8/88	9/88	9/88
Chlamydia	12/15/87	1/88	1/88	2/88	4/88	5/88	5/88
hCG				11/87	12/87	1/88	1/88
HSV (510K) (IND)	12/25/87	1/88	1/88	3/88	4/88	4/88 7/88-10/88	4-10/88 (FDA)
GC (from Cetus 1/88)	3/88	1/88	3/88	4/88	6-7/88	7-8/88	7-8/88
HIV-Rapid	1/15	1/88	2/88	5/15	4/88	8/88-2/89	8/88-2/89 (FDA)
HIV-PCR	4/88						

• Possible FY88 Cetus Revenue. No Revenue forecasted from FDA to Cetus

ATTORNEY'S EYES ONLY -
RESTRICTED

301156
CETUS

RMS 71128

Attachment 1

Joint Development Program (K\$)
(12/10/87)

	<u>87 AOP</u>	<u>87 E Y/E</u>	<u>88 FCST.</u>	
	7100	7500	7950	(incl. forensics 530, HRI pmts 350)
	5100	6400	6500	(delayed milestones)
		250	700	
		200	50	
		50	-150	
	<u>12200</u>	<u>14500</u>	<u>15650</u>	

e Oct. RDMC

CETUS
Kodak
CPI - rapid test milestones
CPI - COMBI & Western Blots
Bagshaw Patent

Dec. 7 Agreement

CETUS
Kodak
CPI - rapid test milestones
CPI - COMBI & Western Blots
Contract R&D
Bagshaw Patent

	<u>87 AOP</u>	<u>87 E Y/E</u>	<u>88 FCST.</u>	
	7600	7600	7470	(HRI pmts to Special Items of Cost)
	5900	5900	6250	
	250	250	700	(slower spending rate)
	100	100	150	(HRI)
	0	0	380	(to Special Items of Cost)
	0	0	0	
	<u>13750</u>	<u>13750</u>	<u>14950</u>	

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71129

001157
CETUS

Attachment VI

Critical Success

ATTORNEY'S EYES ONLY
RESTRICTED

Cetus/EK Joint Dev. (K\$)

Oxus
Kodak
Payments to CPI
Total

Oxus Share @ 35%
Kodak Share @ 65%

1987 AOP	7100	5100	12200	4270	7930
----------	------	------	-------	------	------

	87 E-Y/E (12/5/87)		88 est (12/5/87)		89 est (12/5/87)	
	Cetus/EK	Blood Bank	87 Total Est. Y/E	Cetus/EK	Blood Bank	1988 Total
	7600	0	7600	7600	0	7600
	5700	100	5800	6500	0	6500
	250	100	350	700	150	850
	13550	200	13750	14800	150	14950
	4743	70	4813	5180	53	5233
	9808	130	9938	9620	98	9718
	5285	35	5320	5285	35	5320
	9815	65	9880	9815	65	9880
	15100	100	15200	15100	100	15200

Cetus/EK Joint Profit (K\$)

Sales (Gross @ 30% Margin)

Cost of Goods
Ment Dev & Pre-Production
HRI & Bagshaw Payments
SADA (50% of Sales)
Royalty (9% of Sales)
Gross Profit

Loss carry forward
EK ROI (20% of Capital exp.)
EK ROI (20% of net book value)
Cetus ROI (20% of \$60F)
Net Profit/Partnership

Cetus Share @ 35%
Kodak Share @ 65%

800	300	0	0	-100	100
-----	-----	---	---	------	-----

	1987 E-Y/E		1988 est		1989 est	
	Cetus/EK	Blood Bank	1987 Total	Cetus/EK	Blood Bank	1988 Total
	0	100	100	18900	5870	24770
	0	50	50	3692	2233	5925
	800	0	800	745	0	745
	50	0	50	800	0	800
	0	50	50	9450	2935	12385
	0	8	8	0	470	470
	-850	-8	-858	-4213	232	-4445
	0	0	0	1187	8	1195
	325	0	325	100	0	100
	0	0	0	723	0	723
	12	0	12	12	0	12
	-1187	-8	-1195	2191	224	2415
	-415	-3	-418	767	79	846
	-772	-5	-777	1424	146	1570
	0			3614		3614
	1627			500		1627
	1600			4960		4960
				0		0

(E) Capital - Net Book Value)
(E) Capital - Capital Expend)

Admin 1 SA / File (70 70)

Answer there?

001158
CETUS

RMS 71130

Attachment IV

APPROVAL PROCESS FOR BOX PUBLIC ANNOUNCEMENTS

The approval process for public information relating to products, technology or plans of Biological Diagnostics will be a 3-part process as follows (normally will take two weeks from date of first draft):

(1) Preliminary Interactive Editing (Parallel Process)

- The draft announcement and/or text along with the intended media targets will be sent by Paul Law or Linda Ferry to the following people for comment; a target date of release will be prominently noted, normally two weeks after first draft.

Cetus	Katharine Russell (via FAX)
	Bill Gerber (via FAX)
CPI	Jeff Meshulam (via FAX)
	Richard Montagna (via FAX)
EK	John Zeman (via PROFS)
	Paul Law (via PROFS)
	Paul Schnipelsky (via PROFS)
	David Frank (via PROFS)
	Art Rosenstein (via PROFS)
	Dan Snilt (via PROFS)
	Dennis Delahunty (via PROFS)
	John Knowles (via PROFS)
	Gerry Battist (via PROFS)

- A response via FAX, PROFS, or KMX will be expected to Paul Law (258-0351) or Linda Ferry (258-0282) within five working days of transmission. The due date for comments will be presented.

(2) Final Approval

- At least two full days prior to release, the announcement and/or text will be delivered by Jim Blamphin (or designate) to the following people:

Cetus	Bill Gerber
CPI	Jeff Meshulam
EK	Kay Whitmore
	Frank Strong
	John Zeman
	Paul Law

- Stopping the release will be an exception and done by notifying Paul Law (KMX 258-0351).

(3) Release

- Jim Blamphin will release announcements on the designated date.

001159
CETUS

12/15/87

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71131

EXHIBIT 1

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71132

Exhibit I to the Sninsky Affidavit
is a publication entitled "PCR Protocols:
A Guide to Methods and Applications"
Edited by Sninsky, et al.

A copy of the exhibit is contained
within an envelope attached to the
original affidavit filed with the
Register in Chancery, a copy of the
Affidavit delivered to Vice Chancellor
Berger and one of the copies of the
Affidavit served upon Plaintiff's
counsel and upon Intervenor's counsel

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71133

PCR Protocols

A Guide to Methods and Applications

Michael A. Innis
David H. Gelland
John J. Smitsky
Thomas J. White

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71134

PCR PROTOCOLS

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71135

PCR PROTOCOLS

A GUIDE TO METHODS AND APPLICATIONS

Edited by

Michael A. Innis,
David H. Gelfand, John J. Sninsky
Cetus Corporation, Emeryville, California

Thomas J. White
Hofmann-La Roche, Inc., Emeryville, California



ACADEMIC PRESS, INC.

Harcourt Brace Jovanovich, Publishers

San Diego New York Berkeley Boston London Sydney Tokyo Toronto

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71136

This book is printed on acid-free paper. ∞

Copyright © 1990 by Academic Press, Inc.
All Rights Reserved.
No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without permission in writing from the publisher.

Academic Press, Inc., San Diego, California 92101
United Kingdom Edition published by
Academic Press Limited, 24-28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

PCR protocols.
Includes index.
1. Polymerase chain reaction. 2. Gene amplification.
I. Innis, Michael A. [DNLM: 1. DNA Polymerases.
2. Gene Amplification--methods. 3. Genetic Engineering
--methods. 4. RNA Polymerases. QH 442 P3479]
QP606.D46P36 1989 574.87'328 89-6938
ISBN 0-12-372180-6 (alk. paper)
ISBN 0-12-372181-4 (pbk. : alk. paper)

Printed in the United States of America
89 90 91 92 9 8 7 6 5 4 3 2 1

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 711:

CONTENTS

Contributors xi
Preface xvii

Part One

BASIC METHODOLOGY

1. Optimization of PCRs 3
Michael A. Innis and David H. Gelfand
2. Amplification of Genomic DNA 13
Randall K. Saiki
3. Amplification of RNA 21
Ernest S. Kawasaki
4. RACE: Rapid Amplification of cDNA Ends 28
Michael A. Frohman
5. Degenerate Primers for DNA Amplification 39
Teresa Compton
6. cDNA Cloning Using Degenerate Primers 46
Cheng Chi Lee and C. Thomas Caskey
7. PCR with 7-Deaza-2'-Deoxyguanosine
Triphosphate 54
Michael A. Innis
8. Competitive PCR for Quantitation of mRNA 60
Gary Gilliland, Steven Perrin, and H. Franklin Bunn

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71138

vi Contents

- 9. Quantitative PCR 70
Alice M. Wang and David F. Mark
- 10. Production of Single-Stranded DNA by Asymmetric PCR 76
Peter C. McCabe
- 11. Cloning with PCR 84
Stephen J. Scharf
- 12. Oligonucleotide Ligation Assay 92
Ulf Landegren, Robert Kaiser, and Leroy Hood
- 13. Nonisotopically Labeled Probes and Primers 99
Corey Levenson and Chu-an Chang
- 14. Incorporation of Biotinylated dUTP 113
Y.-M. Dennis Lo, Wajahat Z. Mehal, and Kenneth A. Fleming
- 15. Nonisotopic Detection of PCR Products 119
Rhea Helmuth
- 16. Thermostable DNA Polymerases 129
David H. Gelfand and Thomas J. White
- 17. Procedures to Minimize PCR-Product Carry-Over 142
Shirley Kwok
- 18. Sample Preparation from Blood, Cells, and Other Fluids 146
Ernest S. Kawasaki
- 19. Sample Preparation from Paraffin-Embedded Tissues 153
Deann K. Wright and M. Michele Manos
- 20. Amplifying Ancient DNA 159
Svante Pääbo

RF
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 7113

Part Two
RESEARCH APPLICATIONS

- 21. *In Vitro* Transcription of PCR Templates 169
Michael I. Holland and Michael A. Innis
- 22. Recombinant PCR 177
Russell Higuchi
- 23. DNase I Footprinting 184
Barbara Krummel
- 24. Sequencing with Taq DNA Polymerase 189
Mary Ann D. Brow
- 25. Direct Sequencing with the Aid of Phage Promoters 197
Steve S. Sommer, Gobinda Sarkar, Dwight D. Koeberl,
Cynthia D. K. Bottema, Jean-Marie Buerstedde,
David B. Schowalter, and Joslyn D. Cassidy
- 26. Identifying DNA Polymorphisms by Denaturing Gradient Gel Electrophoresis 206
Val C. Sheffield, David R. Cox, and Richard M. Myers
- 27. Amplification of Flanking Sequences by Inverse PCR 219
Howard Ochman, Meetha M. Medhora, Dan Garza, and Daniel L. Hartl
- 28. Detection of Homologous Recombinants 228
Michael A. Frohman and Gail R. Martin
- 29. RNA Processing: Apo-B 237
Lyn M. Powell
- 30. A Transcription-Based Amplification System 245
T. R. Gingeras, G. R. Davis, K. M. Whitfield, H. L. Chappelle,
L. I. DiMichele, and D. Y. Kwok

ATTORNEY'S EYES ONLY
RESTRICTED

R

viii Contents

31. Screening of λ gt11 Libraries 253
Kenneth D. Friedman, Nancy L. Rosen, Peter J. Newman, and Robert R. Montgomery

Part Three _____
GENETICS AND EVOLUTION

32. HLA DNA Typing 261
Henry A. Erlich and Teodorica L. Bugawan
33. Multiplex PCR for the Diagnosis of Duchenne Muscular Dystrophy 272
Jeffrey S. Chamberlain, Richard A. Gibbs, Joel E. Ranier, and C. Thomas Caskey
34. Isolation of DNA from Fungal Mycelia and Single Spores 282
Steven B. Lee and John W. Taylor
35. Genetic Prediction of Hemophilia A 288
Scott C. Kogan and Jane Gitschier
36. Haplotype Analysis from Single Sperm or Diploid Cells 300
Ulf Gyllenstein
37. Amplification of Ribosomal RNA Genes for Molecular Evolution Studies 307
Mitchell L. Sogin
38. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics 315
T. J. White, T. Bruns, S. Lee, and J. Taylor

Part Four _____
DIAGNOSTICS AND FORENSICS

39. Detection of Human T-Cell Lymphoma/Leukemia Viruses 325
Garth D. Ehrlich, Steven Greenberg, and Mark A. Abbott

- 40. Detection of Human Immunodeficiency Virus 337
David E. Kellogg and Shirley Kwok
- 41. Detection of Hepatitis B Virus 348
I. Baginski, A. Ferrie, R. Watson, and D. Mack
- 42. Detection and Typing of Genital Human Papillomaviruses 356
Yi Ting and M. Michele Manos
- 43. Detection of Human Cytomegalovirus 368
Darryl Shibata
- 44. PCR Amplification of Enteroviruses 372
Harley A. Rotbart
- 45. Novel Viruses 378
David Mack, Oh-Sik Kwon, and Fred Faloona
- 46. Analysis of *ras* Gene Point Mutations by PCR and Oligonucleotide Hybridization 386
John Lyons
- 47. B-Cell Lymphoma: t(14;18) Chromosome Rearrangement 392
Marco Crescenzi
- 48. Detecting Bacterial Pathogens in Environmental Water Samples by Using PCR and Gene Probes 399
Ronald M. Atlas and Asim K. Bei
- 49. PCR in the Diagnosis of Retinoblastoma 407
Sang-Ho Park
- 50. Determination of Familial Relationships 416
Cristián Orrego and Mary Claire King

x Contents

Part Five
INSTRUMENTATION AND SUPPLIES

- 51. PCR in a Teacup: A Simple and Inexpensive Method for Thermocycling PCRs 429
Robert Watson
 - 52. A Low-Cost Air-Driven Cycling Oven 435
Peter Denton and H. Reisner
 - 53. Modification of a Histokinette for Use as an Automated PCR Machine 442
N. C. P. Cross, N. S. Foulkes, D. Chappel, J. McDonnell, and L. Luzzatto
 - 54. Organizing a Laboratory for PCR Work 447
Cristián Orrego
 - 55. Basic Equipment and Supplies 455
Roberta Madej and Stephen Scharf
- Index 461



ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71144

EXHIBIT J

PERKIN ELMER CETUS
761 Main Ave., Norwalk, CT 06859

GeneAmp[®] PCR Reagent Kit with

AmpliTa^q DNA Polymerase (250 Units)

(Deoxynucleoside triphosphate: DNA Deoxynucleotidyltransferase, EC 2.7.7.7)

NOT FOR DIAGNOSTIC OR MEDICAL USE
RESEARCH USE ONLY
See notice to purchaser

Part No. N801-0055

The Perkin-Elmer Cetus GeneAmp PCR Reagent Kit is designed to perform the GeneAmp[™] Polymerase Chain Reaction (PCR) process on all DNA templates. It provides sufficient reagents for 100 amplifications of 100 μ L each. It includes full length lambda DNA and control primers for 10 control amplifications.

The GeneAmp PCR process is a simple and powerful method,* invented by K. Mullis and patented by Cetus Corporation, which allows *in vitro* amplification of DNA segments (including cDNA)[†] 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 low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle. The GeneAmp PCR process is based on the repetition of this cycle and can amplify DNA segments by at least 10⁴ fold, and potentially as high as 10⁸ fold,* under the conditions described below.

LIST OF KIT COMPONENTS

Store the GeneAmp PCR Reagent Kit and AmpliTaq DNA Polymerase at -20°C in a constant temperature freezer. If stored under proper conditions, the product will maintain performance through the control date printed on the label.

Reagent	Volume	Concentration	Comments
AmpliTa ^q DNA Polymerase	50 μ L	5 U/ μ L	Extends primer during PCR amplification
dATP	320 μ L	10 mM	Deoxynucleosidetriphosphates dissolved in water. pH 7.0.
dCTP	320 μ L	10 mM	
dGTP	320 μ L	10 mM	
dTTP	320 μ L	10 mM	
[10X] Reaction Buffer	1.4 mL		Provides preferred pH and ionic strength for amplification.
Control Template	100 μ L	1 μ g/mL	Whole bacteriophage lambda DNA as a template for PCR.
Control Primer #1	50 μ L	20 μ M	Primer pair defines a 500 base segment on the lambda target DNA: nucleotides 7131 through 7630 (see on page 4).
Control Primer #2	50 μ L	20 μ M	

NOTE: [10X] Reaction Buffer composition,
(solution has been autoclaved)

100 mM Tris-HCl, pH 8.3 (at 25°C)
500 mM KCl
15 mM MgCl ₂
0.01% (w/v) gelatin (Sigma, Cat. No. G2500, St. Louis, MO)

AmpliTa^q DNA Polymerase

The key component in the GeneAmp PCR Reagent Kit is AmpliTaq DNA Polymerase (Part No. N801-0050). Perkin-Elmer Cetus' AmpliTaq DNA Polymerase is a recombinant, thermostable, 94kDa DNA polymerase encoded by a modified form of the *Thermus aquaticus* DNA polymerase gene which has been inserted into an *Escherichia coli* host.[†] AmpliTaq DNA Polymerase and native Taq DNA Polymerase are covered by U.S. Patent 4,889,818 issued to Cetus Corporation, and licensed to Perkin-Elmer Cetus Instruments. Additional patent applications on genes encoding thermostable DNA polymerases, and methods and formulations for stable preparations of thermostable DNA polymerase are pending. Its features are described on page 4.

NOTICE TO PURCHASER ABOUT LIMITED LICENSE

This product is designed for use in the GeneAmp Polymerase Chain Reaction (PCR) process covered by patents issued to Cetus Corporation. The seller has a limited license under such patent rights, and purchase of this product includes a fully paid-up, limited, non-exclusive sublicense under such patent rights to use this product to perform the GeneAmp PCR process only for research and development activities and for industrial quality assurance testing. For this purpose the license for "research and development" includes the development of commercial products, such as new therapeutic products. No license is granted for any other uses, for example (1) the diagnosis or detection of disease or other health-related condition in humans, animals or plants, or (2) genetic analysis for identification of individuals for forensic or other purposes. No other license is granted expressly, impliedly, or by estoppel. For information concerning the availability of additional licenses to practice the PCR process, contact Cetus Corporation, Director of Business Development, PCR Division, at 1400 Fifty-Third Street, Emeryville, CA 94608, Tel. (415) 420-3300.

ATTORNEY'S EYES ONLY
RESTRICTED

RMS 71145

PERKIN ELMER CETUS**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 reactions and Positive Control templates, can result in product formation even in the absence of purposely added template DNA. If possible, set up reaction mixes in areas separate from other DNA handling. The use of dedicated or disposable vessels, solutions, and pipettes (preferably positive displacement pipettes) for DNA preparation, reaction mixing, and sample analysis will minimize cross contamination. Use of the *GeneAmp* PCR Carry-over Prevention Kit (Part No. N808-0068) will prevent reamplification of PCR products from previous amplifications.

Perform reactions with 100 μ L 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 Perkin-Elmer Cetus DNA Thermal Cyclers, 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.

A Master Mix of reagents for all samples (water, buffer, dNTP's and enzyme) can be prepared first, then aliquoted to the individual tubes. Then add the primers and template DNA. Using such mixes will minimize reagent losses on pipette tips and increase pipetting accuracy.

II. Dilution of Control Template

Prepare a 10-fold dilution of the Control Template in 10mM Tris-HCl pH 8.0 (room temperature), 1 mM EDTA, 10 mM NaCl. Additional dilutions may be used as controls for dilute DNA.

III. Reaction Mix

Component	Addition Order	Volume	Final Concentration
Autoclaved ultra-filtered water	1	61.5 μ L	
[10x] Reaction Buffer	2	10 μ L	1X
Master Mix { dATP dCTP dGTP dTTP	3	2 μ L	200 μ M
		2 μ L	200 μ M
		2 μ L	200 μ M
		2 μ L	200 μ M
<i>AmpliTag</i> [®] DNA Polymerase	4	0.5 μ L	2.5 Units/100 μ L
Control Primer #1	5	5 μ L	1.0 μ M
Control Primer #2		5 μ L	1.0 μ M
Control Template, 1:10 dilution	6	10 μ L	1 ng/100 μ L
Total Mix		100 μ L	

IV. Notes on Reaction Mix for Controls

- Mix gently (avoid generating bubbles) the *AmpliTag* DNA Polymerase or other recently thawed reagents, then spin down in a microcentrifuge before pipetting. Pipette the enzyme carefully and slowly; the viscosity of the 50% glycerol in the buffer can lead to pipetting errors. If possible, use a positive displacement pipette. Using Master Mixes (see above) will increase accuracy and reduce enzyme loss on tips, and reduce tube-to-tube variability.
- To reduce evaporation or refluxing, overlay the mix with 50 to 100 μ L of mineral oil (Sigma Chemical Co., St. Louis, MO). The oil should not interfere when withdrawing samples. If the entire volume is to be recovered, 100 μ L of high purity chloroform can be added after amplification. The aqueous phase containing the DNA will then float on the chloroform-oil mixture, allowing easy collection.

V. Reaction Mix for Amplifying DNA Templates

- The conditions described above are also a useful starting place for amplification of different DNA targets using primers designed by the user. Optimization of reactions for each primer-template pair may be necessary and can be achieved by varying magnesium chloride concentration, primer concentration, dNTP concentration and anneal-extend (Section VII) temperature. The effect of these variations can be monitored by examining the intensity and distribution of product samples electrophoresed on 3% NuSieve[®]/1% Seakem[®] agarose (FMC Corp., Rockland, ME) gels and visualized with ethidium bromide staining.
- The DNA segment to be amplified from the template can be up to 10 kb long, although 100 to 1000 bases are more typical and easier to amplify. Start with enough copies of the template to be sure of obtaining a signal after 25 or 30 cycles; preferably > 10⁴ copies but less than 1 μ g total sample DNA per 100 μ L. Low concentrations of target DNA may require up to 35 or more cycles to produce sufficient product for analysis.
- If proteases are present in the sample DNA (such as impure genomic DNA), inactivate the proteases by heating samples to 95°C for 5 minutes before adding the *AmpliTag* DNA Polymerase. This step can be carried out automatically with Perkin-Elmer Cetus DNA Thermal Cyclers using a time delay file linked to the PCR cycle file.
- The single-strand DNA primers should be 15 to 30 bases in length. The %G+C of primers should be near 50%, to maximize specificity. To avoid potential problems, primers should be purified by gel electrophoresis or HPLC ion-exchange chromatography. The optimal primer concentrations need to be determined empirically, by testing concentrations in the range of 0.1 to 1.0 μ M. Primer concentrations that are too low will result in little or no PCR product, while concentrations that are too high may result in amplification of non-target sequences. Primer concentrations in the range of 0.2 to 0.5 μ M will work for most PCR amplifications. Primer sequences should not complement within themselves or to each other, particularly at the 3' ends. The primers in the *GeneAmp*[®] PCR Reagent Kit have been designed with a GG/CC overlap at the 3' end, to show one consequence of

PERKIN ELMER CETUS

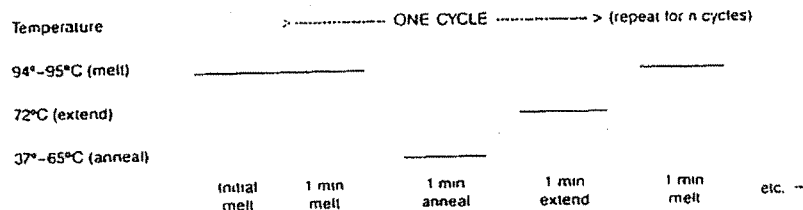
complementary sequences. Their use results in production of 46 to 50 base pair products called primer dimer. This product may occur to some extent even without such an overlap. Reducing the primer concentration (up to 5 fold, to 0.2 μ M) will greatly reduce such products.

5. The optimal magnesium concentration needs to be determined empirically, by testing concentrations up to 4 mM MgCl₂ for each primer set. Too little or too much MgCl₂ could reduce amplification efficiency or result in non-specific products. If the samples contain EDTA or other chelators, raise the MgCl₂ concentration in the reaction mix proportionately. Magnesium Chloride concentrations should also be adjusted in parallel with significant changes in the concentrations (higher or lower) of sample DNA and dNTPs.
6. Keep concentrations of dNTPs in the reaction mix balanced; if the concentration of any one is significantly different from the rest, the *AmpliTaq*[®] DNA Polymerase will tend to misincorporate, slow down and terminate prematurely.¹
7. Reactions can be performed in volumes from 20 to over 200 μ L per tube. If different volumes of primer or template DNA are used in the Master Mix, adjust the volume of water in the Master Mix by an equivalent amount to keep the concentrations of other reactants constant.

VI. Temperature Cycling for the Control Reagents

The *GeneAmp*[®] PCR Reagent Kit is optimized and quality control tested for performance on Perkin-Elmer Cetus *GeneAmp*[®] PCR Instrument Systems. 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.

Begin with a template melting step (at least 1 minute at 94° to 95°C), then start cycling. A typical starting cycle profile for the DNA Thermal Cycler and DNA Thermal Cycler 480 will be:



After the last cycle, extend the polymerization step by several more minutes, to complete all strands. The samples can be stored at -20° until subsequent analysis.

If using the *GeneAmp* PCR System 9600, please refer to Perkin-Elmer Cetus Technical Data Sheet L-1405 entitled "The *GeneAmp* PCR System 9600: Transportability of Protocols" for guidelines concerning how to adjust protocols for optimal PCR performance.

VII. Cycle Optimization for Amplification of other DNA Templates

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. These parameters may be efficiently optimized using Perkin-Elmer Cetus *GeneAmp* PCR Instrument Systems, which come with operating instructions.

The following paragraphs discuss the effect of changing various conditions.

High G+C content DNA needs very high annealing (> 60°C) and melting temperatures or the use of 7-deaza-2'-deoxy-GTP mixed with dGTP, to overcome secondary structure.¹¹ The half life of *AmpliTaq* DNA Polymerase (< 35 minutes at > 95°C)¹² suggests 95°C as the maximum practical melting temperature. It is very important in the early cycles to be sure to completely melt the template DNA. When genomic DNA is used as the starting template, melting at 97°C for the first few cycles will ensure single stranded template for the PCR reaction. The melting temperature should be reduced for the later cycles because the smaller PCR product usually melts completely at a lower temperature (unless the PCR product is excessively G+C rich) than the starting genomic DNA.

Higher annealing temperatures (45° to 65°C) generally result in much more specific product.¹³ The optimal anneal temperature can be determined empirically by testing at 5°C or smaller increments until the maximum in specificity is reached. At these temperatures *AmpliTaq* DNA Polymerase has significant activity and extension of primed templates is occurring. For some primer-template pairs extension is completed during the 1 min annealing step and a separate extension step is unnecessary. Two temperature PCR cycles consist of 1 min at 94° to 95°C followed by 1 min at 45° to 65°C; no hold time at 72°C is necessary.

The length of the target sequence will affect the required extension time. Typically, *AmpliTaq* DNA Polymerase has an extension rate of 2,000 to 4,000 bases per minute at 70° to 80°C. Polymerization rates are significant even below 55°C and with some templates, up to 85°C.¹⁴ As the amount of DNA increases in later cycles, the number of *AmpliTaq* DNA Polymerase molecules may become limiting for the extension time allotted. Increasing the extend times in later cycles may be needed to maintain efficiency of amplification. This can be achieved by using the auto-segment extension feature of Perkin-Elmer Cetus DNA Thermal Cyclers.

PERKIN ELMER CETUS

PERFORMANCE CLAIM

Under the conditions described above, with 2 minutes for the 72°C extension step, the *GeneAmp* PCR Reagent Kit will yield a 10⁵ fold amplification efficiency, in 25 cycles, starting with 1 ng of control template, and using 100 pmoles of each control primer and 2.5 units of *AmpliQaq* DNA polymerase.

***AmpliQaq* ENZYME CHARACTERISTICS**

- Concentration:** 5 Units/μL
- Unit Definition:** One unit of enzyme is defined as the amount that will incorporate 10 nmoles of dNTPs into acid insoluble material per 30 minutes at 74°C under the analysis conditions below.
- Analysis conditions:*** 25 mM TAPS (*tris*-(hydroxymethyl)-methyl-amino-propanesulfonic acid, sodium salt) pH 9.3 (at 25°C), 50 mM KCl, 2 mM MgCl₂; 1 mM β-mercaptoethanol; 200 μM each dATP, dGTP, dTTP; 100 μM [³²P]-dCTP (0.05 to 0.1 Ci/mole); activated salmon sperm DNA, in a final volume of 50 μL and incubated at 74°C for 10 minutes. The salmon sperm DNA is activated by a modification of the methods in reference 11
- Storage buffer:** 50% glycerol (v/v)
100 mM KCl
20 mM Tris-HCl pH 8.0
0.1 mM EDTA (ethylenediaminetetraacetic acid)
1 mM DTT (Dithiothreitol)
0.5% Tween 20^{*}
0.5% Nonidet P40^{*}
- Storage temperature:** Store *AmpliQaq* DNA Polymerase at -20°C, in a constant temperature freezer. If stored under proper conditions, the enzyme will remain active through the control date printed on the label.
- Associated activities:** Non-specific endonuclease and exonuclease activities were not detectable after one hour incubation of 600 ng of supercoiled pBR322 (dam⁻, dcm⁻) or 600 ng of *MspI*-digested pBR322 DNA, respectively, at 74°C, in the presence of 8 units of *AmpliQaq* DNA Polymerase. The enzyme has a 5' to 3' nick translation or a polymerization enhanced 5' to 3' nuclease activity** but lacks a 3' to 5' exonuclease activity.**

SEQUENCES

Sequence** (+ strand) of 500 nucleotide target segment of bacteriophage lambda target segment amplified using primers in the *GeneAmp* PCR Reagent Kit.

```

(5') <-----Region for Control Primer 1----->
7131      GATGAGTTTCG TGTCGGTACA ACTGGCGTAA TCATGGCCCT
7171 TCGGGGCCAT TGTTTCTCTG TGGAGGAGTC CATGACGAAA GATGAACTGA TTGCCCGTCT
7231 CCGCTCGCTG GGTGAACAAC TGAACCGTGA TGTCAGCCTG ACGGGGACGA AAGAAGA ACT
7291 GGCCTCCCGT GTGGCAGAGC TGAAGAGGGA GCTTGATGAC ACGGATGAAA CTGCCGGTCA
7351 GGACACCCCT CTCAGCCGGG AAAATGTGCT GACCCGACAT GAAAATGAGG TGGGATCAGC
7411 GCAGCCGGAT ACCGTGATTC TGGATACGTC TGAACCTGGTC ACGGTCGTGG CACTGGTGAA
7471 GCTGCATACT GATGCACTTC ACGCCACGCG GGATGAACCT GTGGCATTGT TGCTGCCGGG
7531 AACGGCGTTC CGTGTCTCTG CCGGTGTGGC AGCCGAAATG ACAGAGCGCG GCCTGGCCAG
7591 AATGCAATAA CGGGAGGCGC TGTGGCTGAT TTCGATAACC (nucleotide 7630)
<-----Region for Control Primer 2-----> (3')
    
```

ACTUAL PRIMER SEQUENCES

Primer	Nucleotides	Sequence	Strand
Control Primer 1	7131 - 7155	(5')GATGAGTTTCGTGTCGGTACA ACTGG(3')	(Complement of - Sequence)
Control Primer 2	7606 - 7630	(5')GGTTATCGAAATCAGCCACAGCGCC(3')	(Complement of + Sequence)

Note: 1) The 3' ends have a GG/CC overlap enhancing production of 46 to 50 bp products: Primer Dimer.
2) Lambda genome is ~48.5 Kb. Target Segment (including primers) is 500 bp (~1% of total)