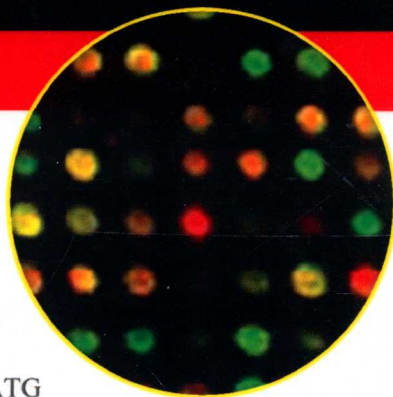
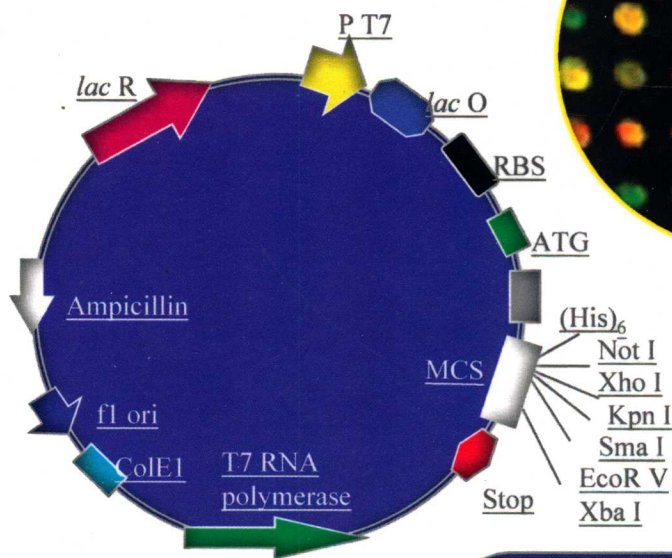
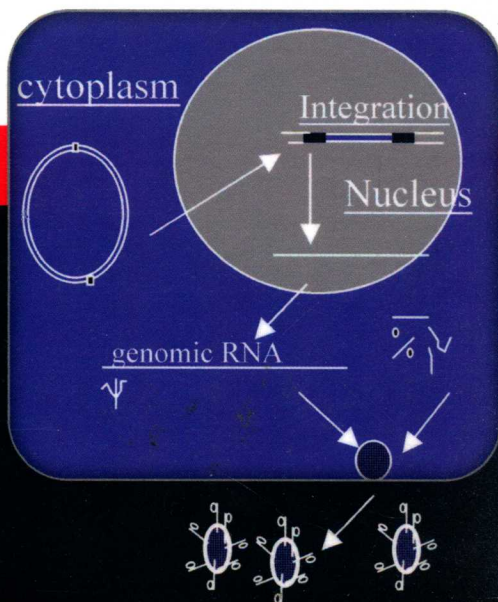


# Gene Biotechnology

Third Edition



**William Wu**  
**Helen H. Zhang**  
**Michael J. Welsh**  
**Peter B. Kaufman**



CRC Press  
Taylor & Francis Group

# Gene Biotechnology

---

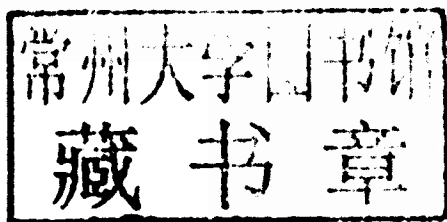
Third Edition

William Wu

Helen H. Zhang

Michael J. Welsh

Peter B. Kaufman



**CRC Press**

Taylor & Francis Group

Boca Raton London New York

---

CRC Press is an imprint of the  
Taylor & Francis Group, an **informa** business

CRC Press  
Taylor & Francis Group  
6000 Broken Sound Parkway NW, Suite 300  
Boca Raton, FL 33487-2742

© 2011 by Taylor and Francis Group, LLC  
CRC Press is an imprint of Taylor & Francis Group, an Informa business

No claim to original U.S. Government works

Printed in the United States of America on acid-free paper  
10 9 8 7 6 5 4 3 2 1

International Standard Book Number: 978-1-4398-4830-2 (Hardback)

This book contains information obtained from authentic and highly regarded sources. Reasonable efforts have been made to publish reliable data and information, but the author and publisher cannot assume responsibility for the validity of all materials or the consequences of their use. The authors and publishers have attempted to trace the copyright holders of all material reproduced in this publication and apologize to copyright holders if permission to publish in this form has not been obtained. If any copyright material has not been acknowledged please write and let us know so we may rectify in any future reprint.

Except as permitted under U.S. Copyright Law, no part of this book may be reprinted, reproduced, transmitted, or utilized in any form by any electronic, mechanical, or other means, now known or hereafter invented, including photocopying, micro-filming, and recording, or in any information storage or retrieval system, without written permission from the publishers.

For permission to photocopy or use material electronically from this work, please access [www.copyright.com](http://www.copyright.com) (<http://www.copyright.com/>) or contact the Copyright Clearance Center, Inc. (CCC), 222 Rosewood Drive, Danvers, MA 01923, 978-750-8400. CCC is a not-for-profit organization that provides licenses and registration for a variety of users. For organizations that have been granted a photocopy license by the CCC, a separate system of payment has been arranged.

**Trademark Notice:** Product or corporate names may be trademarks or registered trademarks, and are used only for identification and explanation without intent to infringe.

---

#### Library of Congress Cataloging-in-Publication Data

---

Wu, William, author.

Gene biotechnology / William Wu, Helen H. Zhang, Michael J. Welsh, and Peter B. Kaufman. -- Third Edition.

p. ; cm.

"A CRC title."

Includes bibliographical references and index.

Summary: "The third edition of this popular reference covers a variety of techniques related to gene manipulation, including DNA isolation, preparation, screening, and analysis. Topics range from very basic methods to current and sophisticated technologies, including methodologies created and tested by the authors. Other topics include approaches to grant funding and SiRNA technology. The authors offer detailed, step-by-step explanations of protocols and helpful troubleshooting guides. This edition features new techniques for every chapter, as well as several new chapters"--Provided by publisher.

ISBN 978-1-4398-4830-2 (hardcover : alk. paper)

1. Genetic engineering--Laboratory manuals. 2. Molecular biology--Laboratory manuals. I. Zhang, Helen H., author. II. Welsh, Michael J., author. III. Kaufman, Peter B., author. IV. Gene biotechnology. revision of (work) V. Title.

[DNLM: 1. Genetic Engineering--methods--Laboratory Manuals. 2.

Biotechnology--methods--Laboratory Manuals. QU 25]

QH442.M475 2011

660.6'5078--dc22

2010051443

---

Visit the Taylor & Francis Web site at  
<http://www.taylorandfrancis.com>

and the CRC Press Web site at  
<http://www.crcpress.com>

---

# Preface

We are living in an era of biotechnology revolution. To many molecular and cellular biologists, the flood of information on human genome and new methodologies is particularly overwhelming because biotechnology is forging ahead and bringing rapid changes every day. Because every single organism depends on molecular actions for survival, molecular biology research has become more and more dominant in multiple disciplines. In fact, there is a general trend for the U.S. National Institutes of Health (NIH) and other funding agencies to award grants with high priority to those research proposals using molecular biology approaches. How can one catch up with new biotechnologies, and use the most recent, proven techniques for novel research? One of the aims of this book is to provide investigators with the tools needed for modern molecular and cellular biology research.

Another aim of this book is to guide graduate students in their thesis research. In our experience, good graduate training mandates independent performance with minimal advice from one's mentor. How do you select a novel research project for your thesis? What are your hypotheses, objectives, and experimental designs? How can you grasp technical problems and master current techniques? Where do you begin and what are the predicted results? How do you write your thesis and research manuscripts for publications? It is apparent that a graduate student needs some help with these questions. This book will provide the clues.

This book covers a wide range of current biotechnologies and methods that have been developed and are widely used in molecular biology, biochemistry, cell biology, immunology, recombinant protein biochemistry, biopharmaceutics, etc. The methods and protocols described in the appropriate chapters include the following: approaches to novel research, rapid isolation of specific cDNAs or genes by PCR; construction and screening of cDNA and genomic DNA libraries; preparation of DNA constructs; nonisotopic and isotopic DNA or RNA sequencing; information superhighway and computer databases of nucleic acids and proteins; characterization of DNA, RNA, or proteins by Southern, northern, or western blot hybridization; fluorescent techniques; gene overexpression; gene underexpression; and gene knockout in mammalian systems; analysis of cellular DNA or abundance of mRNA by radioactivity *in situ* hybridization; localization of DNA or abundance mRNA by fluorescence *in situ* hybridization; *in situ* PCR hybridization of low copy genes and *in situ* RT-PCR detection of low abundance mRNAs; strategies for gene double knockout, large-scale expression, and purification of recombinant proteins in cultured cells; high-throughput analysis of gene expression by real-time RT-PCR; gene expression profiling via DNA microarray, phage display, and siRNA technology; protein-protein binding and proteomic interactions; conditional gene knockout; and determination of gene copy numbers. Each chapter covers the principle(s) underlying methods and techniques, detailed step-by-step descriptions of each protocol, notes, tips, and a troubleshooting guide. We have observed that many of the currently available books in molecular biology contain only protocol recipes. Unfortunately, many

fail to explain the principles and concepts behind the methods outlined or to inform the reader about the possible pitfalls in the methods described. We intend to fill these gaps.

More importantly, this technology book provides unique features: (1) how to write a research paper for publication in English journals, (2) how to protect research discoveries and inventions via patents, and (3) discussion on practical biocalculation. The authors believe that research paper publication, intellectual property (IP) protection via patent(s), and biocalculations are very important topics to graduate students, researchers, and university faculty members.

For the information of the reader, Chapter 1 was written by William Wu, Peter B. Kaufman, and Michael J. Welsh. Chapters 2, 3, 6, 7, 9, 10, 13–17, 19–22, 23, 25, and 26 were written by William Wu. Chapter 8 was written by William Wu and Michael J. Welsh. Chapter 5 was written by William Wu and Peter B. Kaufman. Chapters 4, 11, 12, 18, 24, and 27 were written by William Wu and Helen Zhang. Nonetheless, all four authors have worked as a team.

---

# Authors

**William Wu** has extensive experience in both academic and industrial settings. Throughout the years, Dr. Wu has held various positions in several organizations, including investigator and research assistant professor at the University of Michigan Medical School, Ann Arbor, Michigan; professor at the Hunan Normal University, Hunan, China; and senior scientist, team leader, and chief technology officer in several biomedical and pharmaceutical companies. He received his master's in biology from the Hunan Normal University, Hunan, China, in 1984. In 1992, he received his PhD in molecular and cellular biology at The Ohio University, Athens, Ohio, USA. This was followed by 3 years of postdoctoral training in molecular biology at the University of Michigan.

Dr. Wu is an internationally recognized expert in molecular biology, cell biology, protein biochemistry, immunology, and biotechnology. He has presented and published more than 35 research papers and abstracts at scientific meetings and in national and international journals. As a senior author, Dr. Wu has contributed 39 chapters on molecular and cellular biology methodologies in four books. He has an extensive knowledge and profound understanding of molecular biology, biotechnology, protein biochemistry, cellular biology, and molecular genetics. He is highly experienced and has extensive hands-on expertise in a variety of current molecular biology technologies in both academic and bioindustrial settings. As inventor and coinventor, Dr. Wu has five biomedical patents granted or in pending status.

**Helen H. Zhang** is a senior research associate at the University of Michigan Medical School, Ann Arbor, Michigan. In 1996, she received her MS in molecular and cellular biology at the Eastern Michigan University, Ypsilanti, Michigan.

Zhang has a strong background in molecular biology, microbiology, biochemistry, and immunology. She is highly experienced in DNA recombination, polymerase chain reaction (PCR) applications, cell transfection, and gene expression in bacterial and animal systems. Additionally, she has extensive experience in protein-peptide purification, enzymatic assays, and analysis of drug-protein binding as well as cell culture techniques. As a coauthor, she has published many research papers in internationally recognized journals.

**Michael J. Welsh** is a professor of cell biology in the Department of Cell and Developmental Biology at the University of Michigan Medical School, Ann Arbor, Michigan. He is also a professor of toxicology in the School of Public Health at the University of Michigan. Dr. Welsh received his PhD in 1977 from the University of Western Ontario, London, Ontario, Canada.

Dr. Welsh is an internationally recognized top scientist in molecular and cellular biology. He has published more than 120 research papers and has contributed chapters in several reference books. Dr. Welsh is a leading authority on mammalian heat shock protein (HSP27) and is a recipient of several major grants from the U.S.

National Institutes of Health; some of these projects utilized his expertise to examine the function and mechanisms of the HSP27 gene. He is also a fellow of the American Association for the Advancement of Science.

**Peter B. Kaufman** is a professor of biology in the Plant Cellular and Molecular Biology Program in the Department of Biology and a member of the faculty of the Bioengineering Program at the University of Michigan, Ann Arbor, Michigan. Dr. Kaufman received his PhD in 1954 in plant biology at the University of California, Davis, California.

Dr. Kaufman is an internationally recognized scholar in the field of molecular biology and physiology. He is a fellow of the American Association for the Advancement of Science and Secretary-Treasurer of the American Society for Gravitational and Space Biology. He has served on the editorial board of *Plant Physiology* for 10 years, has published more than 190 research papers and seven professional books. Dr. Kaufman teaches a popular course in plant biotechnology yearly at the University of Michigan. He has been awarded research grants from the National Science Foundation, the National Aeronautics and Space Administration, the U.S. Department of Agriculture, and Parke-Davis Pharmaceutical Research Laboratories in Ann Arbor, Michigan.

---

# Contents

Preface.....	xxvii
Authors.....	xxix

<b>Chapter 1</b>	Strategies for Novel Research Projects and/or Research Grant Funding.....	1
1.1	Introduction .....	1
	References .....	14
<b>Chapter 2</b>	Rapid Isolation of Specific cDNAs or Genes by PCR.....	17
2.1	Introduction .....	17
2.2	Isolation of Specific Full-Length cDNAs by RT-PCR	
	Method.....	17
2.2.1	Isolation of RNAs.....	18
2.2.2	Design and Synthesis of Specific Forward and Reverse Primers.....	18
2.2.3	Amplification of cDNA of Interest by RT-PCR .....	20
2.2.4	Purification of PCR Products by High-Speed Centrifugation of Agarose Gel Slices .....	22
2.3	Amplification and Isolation of cDNA Ends by 5'-RACE .....	23
2.4	Amplification and Isolation of cDNA Ends by 3'-RACE.....	24
2.5	Isolation of Gene of Interest by PCR.....	24
2.5.1	Isolation of Genomic DNA.....	24
2.5.2	Partial Digestion of Genomic DNA Using <i>Sau3AI</i> .....	26
	2.5.2.1 Optimization of Partial Digestion of Genomic DNA with <i>Sau3AI</i> .....	26
	2.5.2.2 Large-Scale Preparation of Partially Digested Genomic DNA .....	27
2.5.3	Design and Synthesis of Specific Forward and Reverse Primers.....	28
	2.5.3.1 Amplification and Isolation of Exon and Intron Sequences.....	28
	2.5.3.2 Amplification and Isolation of Promoter Sequence .....	29
2.5.4	Amplification of Specific DNA Fragments by PCR .....	29
2.5.5	Purification of PCR Products by Agarose Gels .....	30
2.6	Subcloning of cDNA or Gene of Interest .....	30
2.7	Characterization of PCR Products .....	30
	References .....	30



<b>Chapter 3</b>	<b>Construction and Screening of Subtracted and Complete Expression cDNA Libraries .....</b>	<b>33</b>
3.1	Introduction .....	33
3.2	Construction and Screening of a Subtracted cDNA Library .....	36
3.2.1	Isolation of Total RNAs from Cell- or Tissue-Type A and B of Interest .....	36
3.2.1.1	Protocol A. Rapid Isolation of Total RNA by Acid Guanidinium Thiocyanate–Phenol–Chloroform Method.....	38
3.2.1.2	Protocol B. Rapid Isolation of Total RNA Using Trizol Reagent™.....	39
3.2.1.3	Protocol C. Measurement of RNAs.....	40
3.2.2	Purification of mRNAs from Total RNAs .....	42
3.2.2.1	Protocol A. Purification of Poly(A) <sup>+</sup> RNAs Using Oligo(dT)-Cellulose Column .....	42
3.2.2.2	Protocol B. Minipurification of mRNAs Using Oligo(dT) Cellulose Resin..	44
3.2.3	Synthesis of First Strand cDNAs from Cell/Tissue Type A or B.....	45
3.2.3.1	Protocol A. Synthesis of First Strand cDNAs from mRNAs .....	45
3.2.3.2	Protocol B. TCA Assay and Calculation of cDNA Yield.....	47
3.2.3.3	Protocol C. Analysis of cDNAs by Alkaline Agarose Gel Electrophoresis .....	47
3.2.4	Hybridization of cDNAs from Cell/Tissue Type A or B with mRNAs from Cell/Tissue Type B or A, or Vice Versa .....	49
3.2.5	Separation of cDNA/mRNA Hybrids from Single-Stranded cDNAs by HAP Chromatography ...	50
3.2.6	Synthesis of Double-Stranded cDNAs from Subtracted cDNAs.....	50
3.2.7	Ligation of cDNAs to Lambda gt10 or Appropriate Vectors .....	52
3.2.7.1	Ligation of <i>Eco</i> RI Linkers/Adapters to Double-Stranded, Blunt-End cDNAs.....	52
3.2.7.2	Restriction Enzyme Digestion of Vectors .....	56
3.2.7.3	Ligation of cDNAs to Vectors .....	56
3.2.8	Generation of a Subtracted cDNA Library .....	57
3.2.8.1	<i>In Vitro</i> Packaging .....	57
3.2.8.2	Titration of Packaged Phage .....	58

- 3.2.8.3 Amplification of cDNA Library (Optional)..... 60
    - 3.2.9 Isolation of Specific cDNA from the Subtracted cDNA Library ..... 60
    - 3.2.10 Characterization of cDNA..... 60
  - 3.3 Construction and Screening of a Complete Expression cDNA Library..... 61
    - 3.3.1 General Principles and Considerations of an Expression cDNA Library ..... 61
      - 3.3.2 Isolation of Total RNAs and Purification of mRNAs from Cells or Tissues of Interest ..... 61
      - 3.3.3 Synthesis of cDNAs from mRNAs ..... 61
      - 3.3.4 Ligation of cDNAs to  $\lambda$ gt11 Expression Vectors..... 61
      - 3.3.5 Generation of an Expression cDNA Library ..... 61
      - 3.3.6 Screening of the Expression Library and Isolation of the cDNA of Interest ..... 63
        - 3.3.6.1 Method A. Immunoscreening of Expression cDNA Library Using Specific Antibodies..... 63
        - 3.3.6.2 Method B. Screening a cDNA Library Using  $^{32}\text{P}$ -Labeled DNA as a Probe ..... 67
        - 3.3.6.3 Method C. Screening a cDNA Library Using a Nonradioactive Probe ..... 67
        - 3.3.6.4 Method D. Isolation of  $\lambda$  Phage DNAs by the Liquid Method ..... 67
    - 3.3.7 Characterization of cDNA..... 69
  - References ..... 71

- Chapter 4 Subcloning of Genes or DNA Fragments..... 73**
  - 4.1 Introduction ..... 73
  - 4.2 Restriction Enzyme Digestion of Vector or DNA Insert for Subcloning ..... 73
    - 4.2.1 Selection of Restriction Enzymes ..... 73
    - 4.2.2 Selection of Cloning Vectors..... 75
    - 4.2.3 Protocols for Restriction Enzyme Digestion..... 76
  - 4.3 Purification of DNA Fragments from Agarose Gels ..... 78
    - 4.3.1 Elution of DNA Bands by High-Speed Centrifugation of Agarose Gel Slices ..... 78
    - 4.3.2 Elution of DNA Fragment by Melting and Thawing of Agarose Gel Slices..... 80
    - 4.3.3 Elution of DNA Fragment Using NA45 DEAE Membrane..... 81
    - 4.3.4 Elution of DNA Fragments in Agarose Gel Well..... 81
  - 4.4 Ligation of DNA Fragments ..... 81
  - 4.5 Single-Step Cloning by PCR ..... 83

4.6	Transformation of Ligated DNA into Bacteria.....	85
4.6.1	Protocol 1. Preparation of Competent Cells for Transformation .....	85
4.6.1.1	Competent Cells for $\text{CaCl}_2$ Transformation before Ligation.....	85
4.6.1.2	Preparation of Competent Cells for Electroporation .....	86
4.6.2	Protocol 2. Transformation of Cells by $\text{CaCl}_2$ Method .....	86
4.6.3	Protocol 3. Transformation by Electroporation.....	87
4.7	Isolation and Purification of Plasmid DNA by Alkaline Method.....	88
4.7.1	Protocol 1. Minipreparation of Plasmid DNA .....	88
4.7.2	Protocol 2. Large-Scale Preparation of Plasmid DNA .....	91
4.7.3	Protocol 3. Purification of Plasmid DNA by $\text{CsCl}$ Gradient.....	92
4.8	Verification of DNA Insertion by Restriction Enzyme Digestion and Agarose Gel Electrophoresis.....	94
4.9	Verification of Insertion Site by DNA Sequencing .....	95
	References .....	96

## **Chapter 5** Nonisotopic and Isotopic DNA or RNA Sequencing..... 97

5.1	Introduction .....	97
5.2	Nonisotopic DNA Sequencing Method.....	99
5.2.1	Protocol 1. Preparation of DNA Templates for Sequencing .....	99
5.2.1.1	Purification of Double-Stranded Plasmid DNA Using the Alkaline Method.....	99
5.2.1.2	Purification of ssDNA.....	102
5.2.2	Protocol 2. Sequencing Reactions.....	103
5.2.2.1	Method A. Sequencing Reactions for Double-Stranded Plasmid DNA .....	103
5.2.2.2	Method B. Sequencing Reactions for ssDNA.....	107
5.2.3	Protocol 3. Preparation of Sequencing Gels.....	108
5.2.3.1	Method A. Pouring the Gel Mixture Horizontally into the Sandwich.....	109
5.2.3.2	Method B. Pouring the Gel at an Angle ...	110
5.2.4	Protocol 4. Electrophoresis .....	112
5.2.5	Protocol 5. Transferring of DNA from Gel onto a Nylon Membrane.....	114
5.2.6	Protocol 6. Detection.....	115

- 5.2.6.1 Method A. Chemiluminescent Detection..... 115
  - 5.2.6.2 Method B. Colorimetric Detection..... 116
- 5.3 Isotopic DNA Sequencing Method..... 117
- 5.4 Use of Formamide Gels..... 119
- 5.5 Extending Sequencing Far from the Primers ..... 120
- 5.6 DNA Sequencing by Primer Walking ..... 120
- 5.7 DNA Sequencing by Unidirectional Deletions ..... 120
  - 5.7.1 Protocol 1. Performing a Series of Deletions of the Linearized DNA with Exonuclease III and Recircularization of DNA with T4 DNA Ligase..... 122
- 5.8 Direct DNA Sequencing by PCR ..... 124
- 5.9 RNA Sequencing ..... 127
  - 5.9.1 Protocol 1. Annealing of Primer and RNA Template ..... 127
  - 5.9.2 Protocol 2. Labeling Reactions ..... 127
  - 5.9.3 Protocol 3. Termination Reaction..... 128
- References ..... 132

**Chapter 6** Bioinformation Superhighway and Computer Databases of Nucleic Acids and Proteins ..... 133

- 6.1 Introduction ..... 133
- 6.2 Communication with GenBank via the Internet ..... 133
  - 6.2.1 Submission of a Sequence to GenBank ..... 133
  - 6.2.2 Sequence Similarity Searching Using BLAST Programs ..... 135
    - 6.2.2.1 BLASTN..... 135
    - 6.2.2.2 BLASTX..... 138
    - 6.2.2.3 BLASTP ..... 139
- 6.3 Computer Analysis of DNA Sequences by the GCG Program ..... 140
  - 6.3.1 Entry and Editing of a Sequence Using the GCG Program..... 140
    - 6.3.1.1 Sequence Entry ..... 140
    - 6.3.1.2 Sequence Editing or Modification ..... 142
    - 6.3.1.3 Review of Sequence Output..... 142
  - 6.3.2 Combination or Assembly of Multiple Fragments into a Single Sequence ..... 142
    - 6.3.2.1 Generating a New Project File Using the GelStart Program..... 142
    - 6.3.2.2 Enter Sequences to Be Assembled into the Project File Generated in the Previous Section (e.g., FRAGMENT) Using the GelEnter Program ..... 143

6.3.2.3	Compare and Identify Overlap Points of the Entered Fragments Using the GelMerge or GelOverlap Program .....	144
6.3.2.4	Assembly and Review of the Combined Sequence Using the GelAssemble Program .....	145
6.3.3	Identification of Restriction Enzyme Digestion Sites, Fragment Sizes, and Potential Protein Translations of a DNA Sequence .....	145
6.3.3.1	Exhibition of Restriction Enzymes above Both Strands of a DNA Sequence and Possible Protein Translation below the Sequence Using the Map Program .....	146
6.3.3.2	Identification of Specific Restriction Enzyme Cutting Sites and Sizes of Fragments Using MapSort Program .....	147
6.3.4	Comparison of Similarity between Two Sequences .....	148
6.3.5	Translation of Nucleic Acid Sequences into Amino Acid Sequences or from an Amino Acid Sequence into a Nucleic Acid Sequence .....	150
6.3.5.1	Translate .....	150
6.3.5.2	BackTranslate .....	150
6.3.6	Identification of Enzyme Digestion Sites within a Peptide or Protein .....	151
6.3.7	Obtaining Nucleotide and Amino Acid Sequences from GenBank .....	152
	References .....	153

<b>Chapter 7</b>	<b>Characterization of DNA or Genes by Southern Blot Hybridization .....</b>	<b>155</b>
7.1	Introduction .....	155
7.2	Principles and General Considerations .....	155
7.3	Isolation of DNA for Analysis .....	157
7.4	Restriction Enzyme Digestion of DNA .....	157
7.5	Agarose Gel Electrophoresis of DNAs .....	157
7.6	Blotting of DNAs onto Nylon Membranes .....	159
7.6.1	Method A. Upward Capillary Transfer (6 to 12 h) ...	159
7.6.2	Method B. Downward Capillary Transfer (1 to 1.5 h Using Alkaline Buffer or 3 h Using Neutral Buffer) .....	161
7.7	Preparation of Probes .....	163
7.7.1	Preparation of Nonisotopic DNA Probes .....	163

- 7.7.1.1 Protocol 1. Direct Labeling of ssDNA  
Using the ECL Kit ..... 163
    - 7.7.1.2 Protocol 2. Random Primer  
Digoxigenin Labeling of dsDNA..... 165
    - 7.7.1.3 Protocol 3. Nick Translation Labeling  
of dsDNA with Biotin-11-dUTP or  
Digoxigenin-11-dUTP ..... 167
  - 7.7.2 Preparation of Isotopic DNA Probes..... 168
    - 7.7.2.1 Protocol 1. Nick Translation Labeling  
of dsDNA ..... 168
    - 7.7.2.2 Protocol 2. DE-81 Filter-Binding Assay... 168
    - 7.7.2.3 Protocol 3. Trichloroacetic Acid  
Precipitation..... 168
    - 7.7.2.4 Protocol 4. Random Primer Labeling  
of dsDNA ..... 170
    - 7.7.2.5 Protocol 5. 3'-End Labeling of ssDNA  
(Oligonucleotides) with a Terminal  
Transferase..... 170
- 7.8 Prehybridization and Hybridization ..... 171
- 7.9 Washing and/or Incubation of Antibodies..... 172
  - 7.9.1 Protocol A. Washing of Filters Hybridized with  
ECL-Labeled Probes ..... 172
  - 7.9.2 Protocol B. Washing and Antibody Incubation  
of Filters Hybridized with Biotin-dUTP- or  
DIG-UTP-Labeled Probes..... 173
  - 7.9.3 Protocol C. Washing of Filters Hybridized with  
Isotopic Probes ..... 173
- 7.10 Detection of Hybridized Signal(s) ..... 173
  - 7.10.1 Method A. Chemiluminescence Detection ..... 173
  - 7.10.2 Method B. Colorimetric Detection of Filters  
Hybridized with Antibody-Conjugated Probes..... 174
  - 7.10.3 Method C. Detection of Signals by  
Autoradiography..... 174
- References ..... 179

- Chapter 8** Gene Overexpression by Sense RNA in Mammalian Systems..... 181
  - 8.1 Introduction ..... 181
  - 8.2 Design and Selection of Plasmid-Based Expression  
Vectors..... 183
    - 8.2.1 Constitutive Promoter Vectors ..... 183
      - 8.2.1.1 Constitutive Promoters ..... 183
      - 8.2.1.2 Selectable Marker Genes ..... 184
      - 8.2.1.3 Reporter Genes ..... 184
      - 8.2.1.4 Splicing Regions ..... 185

	8.2.1.5	Kozak Sequence and Enhancer Element .....	185
	8.2.2	Inducible Promoter Vectors .....	185
	8.2.3	Retrovirus Vectors .....	186
8.3		Preparation of Plasmid Sense cDNA Constructs .....	190
8.4		Transient Transfection of Mammalian Cells with Sense Constructs .....	190
	8.4.1	Method A. Transfection by Calcium Phosphate Precipitation .....	190
	8.4.2	Method B. Transfection by Retrovirus Vectors .....	193
	8.4.2.1	Protocol 1. Preparation of Viral Supernatant by Transient Transfection of a Packaging Cell Line with Retrovirus Vector Constructs .....	193
	8.4.2.2	Protocol 2. Production of Stably Transfected Producer Cell Lines .....	194
	8.4.2.3	Protocol 3. Determination of Viral Titer .....	195
	8.4.2.4	Protocol 4. Amplification of Virus Stock by Serial Reinfection of Fresh Target Cells .....	196
	8.4.2.5	Protocol 5. Transfection of Cells of Interest with the High Titer Stock of Replication-Incompetent Retroviruses .....	197
8.5		Stable Transfection of Mammalian Cells with Sense DNA Constructs .....	198
	8.5.1	Method A. Transfection by Liposomes .....	198
	8.5.2	Method B. Transfection by Electroporation .....	198
	8.5.3	Method C. Transfection by Retrovirus Vectors .....	199
8.6		Selection of Stably Transfected Cell Lines with Appropriate Drugs .....	199
8.7		Characterization of Stably Transfected Cell Clones .....	200
	8.7.1	Analysis of Gene Overexpression at the Protein Level by Western Blotting .....	201
	8.7.2	Examination of the Expression of Sense RNA by Northern Blotting .....	202
	8.7.3	Determination of Integration Copy Number by Southern Blot Analysis .....	202
	8.7.3.1	Isolation of Genomic DNA from Cultured Cells .....	202
	8.7.3.2	Analysis of Southern Blot Data .....	203
	8.7.4	Expression Assay of a Reporter Gene .....	203
	8.7.4.1	Activity Assay of CAT .....	203
	8.7.4.2	Luciferase Assay .....	204
	8.7.4.3	$\beta$ -Galactosidase Assay .....	205
	8.7.4.4	$\beta$ -Galactosidase Staining of Cells .....	206

8.8	Generation of Transgenic Mice from Sense ES Clones .....	207
8.9	Characterization of Transgenic Mice .....	207
	References .....	208

<b>Chapter 9</b>	<b>Gene Underexpression in Cultured Cells and Animals by Antisense DNA and RNA Strategies .....</b>	<b>209</b>
9.1	Introduction .....	209
9.2	Antisense Oligonucleotide Approaches.....	209
9.2.1	Design and Synthesis of Antisense Oligonucleotides.....	209
9.2.2	Treatment of Cultured Cells with Antisense Oligomers and Determination of the Optimum Dose of Oligomers by Western Blot Analysis.....	211
9.2.3	Treatment of Cultured Cells Using an Optimum Dose of Oligomers.....	213
9.2.4	Analysis of Inhibition of Gene Expression by Western Blotting.....	213
9.3	Design and Selection of Plasmid-Based Expression Vectors.....	214
9.4	Preparation of Plasmid Antisense cDNA Constructs.....	214
9.5	Transient Transfection of Cultured Cells with Antisense Constructs.....	215
9.6	Stable Transfection of Cultured Cells with Antisense DNA Constructs .....	215
9.6.1	Method A. Transfection by Liposomes .....	215
9.6.2	Method B. Transfection by Microinjection .....	217
9.6.3	Method C. Transfection by Electroporation .....	218
9.6.4	Method D. Transfection by Retrovirus Vectors .....	218
9.7	Selection of Stably Transfected Cell Lines with Appropriate Drugs.....	218
9.8	Characterization of Stably Transfected Cell Clones .....	218
9.8.1	Analysis of Gene Underexpression at the Protein Level by Western Blotting.....	218
9.8.2	Examination of the Expression of Antisense RNA by Northern Blotting.....	219
9.8.3	Determination of Integration Copy Number by Southern Blot Analysis.....	219
9.8.4	Expression Assay of Reporter Genes .....	220
9.9	Generation of Transgenic Mice .....	220
9.9.1	Method A. Production of Transgenic Mice from Stably Transfected ES Cells .....	220
9.9.1.1	Selection of C57BL/6J Estrous Females .....	220
9.9.1.2	Preparation of a Bank of Sterile Males by Vasectomy.....	221



9.9.1.3	Pairing of Estrous Females and Sterile Males.....	222
9.9.1.4	Preparation of Blastocyst-Stage Embryos from Pseudopregnant Mice .....	222
9.9.1.5	Preparation of Micromanipulation Apparatus.....	223
9.9.1.6	Injection of ES Cells into Blastocysts.....	224
9.9.1.7	Reimplantation of the Injected Blastocysts into the Uterus of Recipient Females .....	225
9.9.2	Method B. Production of Transgenic Mice from Oocytes.....	225
9.9.2.1	Preparation of Oocytes .....	225
9.9.2.2	Microinjection of DNA Constructs into Oocytes .....	227
9.9.2.3	Reimplantation of Injected Eggs into Recipient Female Mice and Generation of Founder Mice .....	227
9.10	Characterization of Transgenic Mice .....	228
	References .....	229

<b>Chapter 10</b>	Analysis of Gene Expression at Functional Genomic Level Using Northern Blotting or PCR.....	231
10.1	Introduction .....	231
10.2	Principles and General Considerations .....	232
10.3	Isolation of Total RNAs and/or Purification of mRNAs .....	234
10.4	Electrophoresis of RNAs Using Formaldehyde Agarose Gels.....	234
10.5	Blotting of RNAs onto Nylon Membranes by the Capillary Method .....	237
10.6	Preparation of Isotopic or Nonisotopic DNA/RNA Probes .....	237
10.6.1	Protocol A. Preparation of DNA Probes .....	237
10.6.2	Protocol B. Preparation of RNA Probes by Transcription <i>in Vitro</i> .....	238
10.7	Hybridization and Detection of Signals .....	241
10.8	Analysis of mRNA Expression by a Semiquantitative PCR Approach.....	241
	References .....	246

<b>Chapter 11</b>	Analysis of Gene Expression at Proteomic Level via Western Blotting.....	249
11.1	Introduction .....	249
11.2	Principles .....	251