Introduction to Molecular Cloning Techniques



Gérard Lucotte François Baneyx





Introduction to Molecular Cloning Techniques

by

Gérard Lucotte and François Banaya

江苏工业学院图书馆

藏书章



Gérard Lucotte Laboratoire d'Anthologie Moléculaire CHU de Cochin-Port Royale 24, rue du Fb. St. Jacques 75004 Paris France

François Baneyx Department of Chemical Engineering University of Washington Seattle, WA 98195

This book is printed on acid-free paper. 🗪



Library of Congress Cataloging-in-Publication Data

Lucotte, Gérard

[Techniques de clonage moléculaire. English]

Introduction to molecular cloning techniques / by Gérard Lucotte and François Baneyx.

Includes bibliographical references and index.

ISBN 1-56081-613-9. ISBN 3-527-89613-9 (Weinheim)

1. Molecular cloning—Laboratory manuals. I. Baneyx, François. II. Title

OH442.2L8313 1993 574.87'328'0724-dc20

93-22418

CIP

Originally published as "Techniques de clonage moléculaire" by Technique et Documentation Lavoisier, Paris, 11, rue Lavoisier, 75008 Paris, France and Éditions, Médicales Internationales, Allée de la Croix Bossée, 94234 Cachan Cedex, France

© 1993 VCH Publishers, Inc.

This work is subject to copyright.

All rights are reserved, whether the whole or part of the material is concerned, specifically those of translation, reprinting, re-use of illustrations, broadcasting, reproduction by photocopying machine or similar means, and storage in data banks.

Registered names, trademark, etc. used in this book, even when not specifically marked as such, are not to be considered unprotected by law.

Printed in the United States of America.

ISBN 1-56081-613-9 VCH Publishers ISBN 3-527-89613-9 VCH Verlagsgesellschaft

Printing History 10 9 8 7 6 5 4 3 2 1

Published jointly by:

VCH Publishers, Inc. 220 East 23rd Street New York, New York 10010-4606

VCH Verlagsgesellschaft mbH P.O. Box 10 11 61 D-69451 Weinheim Federal Republic of Germany

VCH Publishers (UK) Ltd. 8 Wellington Court Cambridge CB1 1HZ United Kingdom

Introduction to Molecular Cloning Techniques

Preface

This book offers a simplified summary of the basic principles and techiques of genetic engineering. It is focused entirely on the most widely used host, the gram-negative bacterium *Escherichia coli*. In addition to extensive descriptions of cloning vectors and essential recombinant DNA methodologies, the steps involved in the construction of genomic, cDNA and cosmid libraries are discussed. The different chapters tackle important aspects of molecular cloning by providing the necessary biochemistry and microbiology background in an effort to clearly introduce the pertinent genetic engineering concepts. Examples of routinely used experimental protocols and solved problems are also provided at the end of each chapter in order to extend their theoretical content and familiarize the reader with laboratory techniques. The text relies heavily on more than ten years of teaching experience by Dr. Lucotte in the biology, pharmaceutical and medical fields and contains a number of original experimental protocols.

G. Lucotte F. Baneyx

April, 1993

Contents

PART I The Host 1

CHAPTER 1. Escherichia coli: a Host for Genetic Engineering 3

6
1.1. Structure, Growth Conditions, Genetic Markers, and Nomenclature 3
1.2. Resistance to Antibiotics 5
1.3. Bacterial Growth 5
1.4. Host Restriction and Modification 7
1.5. Host Recombination 7
Experiments 7
1.1 Growth Media 7
1.2 Spreading 9
1.3 Isolation of a Single Colony 9
1.4 Use of Antibiotics 10
1.5 Storage of Bacterial Strains 11
Problems 11
1.1 Strain Phenotype 11
1.2 Characteristics of the Exponential Phase 12

PART II Restriction Enzymes, Methylation, Electrophoresis Techniques 13

CHAPTER 2. Restriction Enzymes	15	
2.1. Host Restriction and Modification	15	
2.2. Isolation of Type II Restriction Endo	nucleases	17
2.3. Properties of the Recognition Site	18	

2.3.	Propertie	s of the
Exp	eriments	19
2.	1 Purity	19
2.	2 Storage	19
2	3 Unit De	finitio

2.3 Unit Definition 19

2.4 Measuring Restriction Enzyme Activity2.5 Reaction Conditions 20

Problems 21

2.1 E	nzymes	and	Extension	is 21
-------	--------	-----	-----------	-------

2.2 Enzymes with Multiple Recognition Sites 21

2.3 Rotational Symmetry of Restriction Enzymes 21

2.4 Isoschizomery 22

2.5 Theoretical Frequency of Restriction Cuts 22

2.6 Theoretical Symmetrical Sites 22

2.7 Theoretical vs. Actual Recognition Sequences 22

2.8 Enzymatic Units Necessary to Digest a Given Amount of DNA 23

CHAPTER 3. Methylation 25

3.1. Prokaryotic Methylases 25

3.2. Methylation by Eukaryotes

Experiments 26

3.1 Example: *Eco*RI Methylase 26

3.2 Unit Definition 27

3.3 Reaction Conditions 27

Problems 27

3.1 Methylation Sites 27

3.2 Selecting Restriction Enzymes to Study Adenine Methylation

27

3.3 Effect of the dam Methylase on Restriction Enzymes 27

3.4 Protecting Restriction Sites by Methylation 27

3.5 Methylation and Cleavage of Eukaryotic DNA 27

CHAPTER 4. Electrophoresis and Blotting Techniques 29

4.1. DNA Electrophoresis 29

4.2. Protein Electrophoresis 32

4.3. Blotting Techniques 33

CONTENTS ix

Experiments 36 4.1 Electrophoresis Cells 36 4.2 Agarose 38 4.3 Electrophoresis Buffers 38 4.4 Casting DNA Gels and Loading the Samples 40 4.5 Visualization of DNA Bands 41 4.6 Migration Anomalies 41 Problems 42 4.1 DNA Molecular Weight Markers for Agarose Gels 42 4.2 DNA Molecular Weight Markers for Polyacrylamide Gels 4.3 Criteria for Complete Digestion of Eukaryotic DNA 43	42
PART III Cloning Vectors 45	
CHAPTER 5. Bacteriophage λ 47	
 5.1. Genes Involved in the Alternative Life Cycles of λ 47 5.2. Gene Map 49 5.3. Plaque Formation 50 Experiments 51 5.1 Infection and Plaque Formation 51 5.2 Plaque Excision 51 5.3 Preparation of Lysate Stock Solutions 51 Problems 52 5.1 Stages in the Lytic Cycle and Encapsidation 52 5.2 Phage Adsorption and Entry 52 5.3 Increasing Plaque Size 52 	
CHAPTER 6. Bacteriophage Cloning Vectors 53	
 6.1. Removal of Restriction Sites 53 6.2. Phage Vectors Derived from λ 54 6.3. Categories of λ Vectors 54 Experiments 61 6.1 Transfection and Bacterial Lysis 61 6.2 Bacteriophage Purification 63 	
6.3 Prophage Induction 64	
6.4 Isolation of Phage λ DNA6.5 Preparation of Phage Arms65	
Problems 67	
 6.1 Cloning Capacity at Different Sites 67 6.2 Blue/Clear Plaque Selection 67 6.3 Advantages of <i>Bam</i>HI Insertion Vectors 67 	

CHA	PTE	7	Plasm	nids	69

_	2	~ 1	-	
1	1	(teneral	Properties	69
/ .		Ochiciai	1 TODGI IICS	02

- 7.2. Naturally Occurring Plasmids 71
- 7.3. Characteristics of the Ideal Plasmid 72
- 7.4. Plasmid pBR322 73
- 7.5. Other Useful Plasmids 75

Experiments 79

7.1 Linearization of pBR322 with *Pst*I 79

Problems 80

- 7.1 Characteristics of Plasmids as Cloning Vectors 80
- 7.2 Advantages of Small Plasmids 80
- 7.3 Directed Cloning 80
- 7.4 The Multiple Cloning Site of pUC18 81

CHAPTER 8. Purification of Plasmid DNA 83

- 8.1 Bacterial Growth and Plasmid Amplification 83
- 8.2 Cell Lysis 84
- 8.3 Plasmid DNA Purification 84

Experiments 86

- 8.1 Isolation of Plasmid DNA from Medium-Scale Cultures (>200 ml) by Ultracentrifugation on Cesium Chloride Gradients in the Presence of Ethidium Bromide 86
- 8.2 Use of A50 Columns 86
- 8.3 Isolation of Plasmid DNA from Small-Volume Cultures by Alkaline Lysis 87

Problems 88

- 8.1 Relevance of Plasmid and Chromosomal DNA Separation 88
- 8.2 Plasmic Conformation 88
- 8.3 Differential Sedimentation of Chromosomal and Plasmid DNA on a CsCl Gradient in the Presence of Ethidium Bromide 88
- 8.4 Relative Position of DNA Bands Following Ultracentrifugation on CsCl Gradient in a Fixed-Angle or Swinging-Bucket Rotor 88
- 8.5 Plasmid DNA Purity 88

CHAPTER 9. Transforming E. coli with Plasmid DNA 89

Experiments 91

- 9.1 Typical Transformation Protocol 91
- 9.2 Storage of Competent Cells 91

Problems 92

- 9.1 General Transformation Scheme in Gram-Positive Organisms 92
- 9.2 Frequency of Transformation 92

CONTENTS xi

9.3 Calculating the Frequency of Transformation9.4 Calculating the Fraction of Competent Cells92
CHAPTER 10. In Vitro DNA Encapsidation 93
Experiments 96 Problems 96 10.1 Verifying the Genotype of Encapsidation Strains 96 10.2 Efficiency of DNA Encapsidation and Amount of Encapsidated DNA 97 10.3 Encapsidation Selectivity vs. DNA Size 97
PART IV DNA and RNA Preparation 99
CHAPTER 11. Extraction and Characterization of DNA from Tissues 101
11.1 Enzyme Preparation 102 11.2 DNA Extraction 103 11.3 Spectrophotometric Assay of DNA 103 11.4 Chemical Assay of DNA Content 103 11.5 Concentrating DNA Samples 104 11.6 Ethanol Precipitation of DNA 105 11.7 Storage of DNA Samples 105 Problems 105 11.1 Absorbance Spectrum of a DNA Sample 105 11.2 RNA Contamination 105 11.3 Total Yield of DNA Extracted from Blood 105
CHAPTER 12. Extraction of DNA from Gels 107
12.1. DNA Extraction from Polyacrylamide Gels 107 12.2. DNA Extraction from Agarose Gels 108 Experiments 109 12.1 Elution of DNA from Acrylamide Gels 109 12.2 Electroelution from Agarose Gels Using DEAE Paper 109 12.3 Electroelution Using Dialysis Tubing 109 12.4 Elution from Low-Melting-Point Agarose 110

CHAPTER 13. Isolation and Characterization of Messenger RNA 111

- 13.1. Isolation of mRNA by Affinity Chromatography 112
- 13.2. Analysis of RNA Samples by Sedimentation on Sucrose Gradients
- 13.3. Assay of mRNA Content by Hybridization to Poly(U) 113

Experiments 115

- 13.1 Isolation of Total Cellular RNA 115
- 13.2 Spectrophotometric Assay of RNA 115
- 13.3 Affinity Chromatography on Oligo(dT) Columns 115
- 13.4 Sedimentation of RNA Solutions on Sucrose Gradients 116
- 13.5 Assay of the Poly(A) Content 116

Problems 116

- 13.1 RNA Integrity 116
- 13.2 Calculating the Concentration of Total Extracted RNA 117
- 13.3 Poly(A) Yield 117
- 13.4 Poly(A) Contamination by Ribosomal RNA 117

CHAPTER 14. RNA Electrophoresis 119

Experiments 120

- 14.1 Sample Preparation 120
- 14.2 Methylmercuric Hydroxide Gels 120
- 14.3 Formaldehyde Gels 120
- 14.4 Use of Glyoxal 120
- 14.5 Preparative Electrophoresis Using Low-Melting-Point Agarose in the Presence of Methylmercuric Hydroxide 121
- 14.6 Formamide Polyacrylamide Gels 121
- 14.7 Urea Polyacrylamide Gels 121

CHAPTER 15. In Vitro Protein Synthesis 123

- 15.1. Different Types of In Vitro Translation Systems 123
- 15.2. Optimization of the Reaction Conditions 124
- 15.3. Analysis of the Translation Products 125

Experiments 127

Problems 129

- 15.1 Effect of the Potassium Concentration on the Efficiency of Translation 129
- 15.2 Advantages of [35S]-Methionine 129
- 15.3 Assaying Different Incorporations 130
- 15.4 Assaying the Amount of Immunoprecipitated Protein 132
- 15.5 Characterization by Electrophoresis 132

CONTENTS xiii

CHAPTER 16.	Enriching for Specific mRNA Molecules
	133

16.1. Tissue Selection 133 16.2. Preparation of Polysomes and Extraction of Polysomal RNA 133 16.3. Affinity Chromatography of Oligo(dT) Columns 135 Experiments 135 16.1 Polysome Isolation 135 16.2 Extraction of Polysomal RNA 137
Problems 137
16.1 Polysome Integrity 137
16.2 Artifacts in the Sucrose Gradient 137
16.3 Purification of Albumin mRNA by Second Affinity
Chromatography of the Poly(A) ⁺ Fraction 137
CHAPTER 17. Complementary DNA Synthesis 139
 17.1. Synthesis of cDNA from Poly(A)⁺ mRNA 139 17.2. Synthesis of Double-Stranded DNA 141 17.3. S1 Nuclease Digestion 143 17.4. Selection of cDNA According to Size 144 17.5. Isolation of Full-Length cDNA 144 Experiments 145
17.1 Estimating the Required Amount of Reverse Transcriptase 145
17.2 Synthesis of the First cDNA Strand 146
17.3 Synthesis of the Second cDNA Strand 147
17.4 S1 Nuclease Digestion 148
Problems 149
17.1 Yield of Single-Stranded cDNA 149
17.2 Yield of Double-Stranded cDNA 150
17.3 Verifying the cDNA Digestion by S1 Nuclease 150
17.4 Heterogeneous Nature of Double-Stranded cDNA Prepared from
Total Liver Poly(A) ⁺ mRNA 150
17.5 Incomplete Double-Stranded cDNA 150
PART V Cloning Techniques 151

P

CHAPTER 18. Ligation 153

- 18.1. Ligases 153
- 18.2. DNA Molecules Involved in Ligations 154
- 18.3. In Vivo vs. In Vitro Ligation 154
- 18.4. Ligation Parameters 156
- 18.5. Increasing the Cloning Efficiency by the Simultaneous Use of Two **Restriction Enzymes** 158

18.6. Treatment of Linearized DNA with Alkaline Phosphatase 159
18.7. Blunt-End Ligations 159
Experiments 160
18.1 Typical Ligation Protocol 160
18.2 Blunt-End Ligation 160
18.3 Vector Dephosphorylation 160
Problems 160
18.1 Schematic Representation of Ligation Reactions 160
18.2 Efficiency of Ligation and Nature of Overhangs 160
18.3 Restriction Enzymes and Ligation 161
18.4 Determination of End Concentrations 161
18.5 Calculating j_{λ} 161
18.6 Influence of the DNA Concentration on the j/i Ratio 161
CHAPTER 19. Cloning by Addition of Complementary
Homopolymeric Sequences 163
Experiments 166
19.1 Synthesis of Poly(dC) Homopolymeric Tracts at the 3' End of the
Double-Stranded cDNA Molecules 166
19.2 Preparing pBR322 for the Cloning Step 167
19.3 Hybridization and Formation of the Recombinant Plasmid
167
Problems 167
19.1 Number of dNTP Residues Synthesized per 3' End 167
19.2 Cloning at the <i>Hin</i> dIII Site of pBR322 Using dA/dT Tracts 168
19.3 Number of 3' Ends Available for Generating Homopolymeric
Tracts 168
CHAPTER 20. Cloning by Addition of Synthetic Sequences
169
20.1. Generating Blunt Ends from Staggered Ends 169
20.2. Synthetic Linkers 170
20.3. Adaptors 173
Experiments 173
20.1 Phosphorylation of Synthetic Linkers 173
20.2 Ligation of a Phosphorylated Linker to a Blunt-Ended cDNA
Molecule 173
Problems 174
20.1 Regenerating a Restriction Site with Polymerase 174
20.2 Linker Purity 176
20.3 Linker Sites 176
20.4 Hexameric Linkers 176
20.5 Linker Manipulations 176

CONTENTS xv

20.6 Insertion of a <i>Hpa</i> II Fragment in a Vector Linearized with <i>Eco</i> RI
176
20.7 Adaptor Sites 176
20.8 Use of a Single-Stranded Adaptor 176
20.9 Simultaneous Use of Two Adaptors 177 20.10 Adaptor-Mediated cDNA Cloning 177
20.10 Adaptor-Mediated CDNA Cloning 177
PART VI Libraries 179
CHAPTED 91 Companie Libraria 191
CHAPTER 21. Genomic Libraries 181
21.1. Example: Construction of a Human Genomic Library 182
21.2. Steps Involved in the Construction of a Genomic Library 182 21.3. EMBL Vectors 184
Experiments 186
21.1 DNA Fractionation on a Sodium Chloride Gradient 186
21.2 Preparation of the Phage Arms and Removal of the Central
Fragment 186
21.3 Ligation of Passenger DNA to the Phage Arms 187
Problems 187 21.1 Ideal Genomic Library 187
21.1 Ideal Genomic Library 167 21.2 Number of <i>EcoRI</i> Recombinants to Obtain a Given Sequence
187
21.3 Number of 20-kbp Fragments to Obtain a Complete Library
187
21.4 Probability of Obtaining a Complete Genomic Library 188
21.5 Complete Genomic Libraries in Different Organisms 18821.6 Lower Theoretical Limit for the Construction of a Genomic
Library 188
21.7 Advantages of EMBL Vectors 188
CHAPTER 22. cDNA Libraries 189
22.1. Requirements for a Complete cDNA Library 189
22.2. Eliminating the S1 Nuclease Step 191
22.3. Cloning cDNA into λgt10, λgt11, and λZAPII 194
Experiments 196
22.1 Synthesis of a Full-Length Complementary Strand22.2 Using λgt10 and λgt11197
Problems 198
22.1 Importance of cDNA Libraries 198
22.2 Obtaining Full-Length cDNA Molecules 198
22.3 pUC Cloning Vectors 198
22.4 Length of the Complementary cDNA Strand 198
22.5 λgt10 vs. λgt11 198

CH

CHAPTER 23. Cosmid Libraries 199
Experiments 203 23.1 Digestion of Genomic DNA with Sau3A 203 23.2 Isolation of Restriction Fragments about 45 kDA in size 204 23.3 Cosmid Preparation and Ligation 204 Problems 204 23.1 Theoretical Cloning Capacity of Cosmids 204 23.2 Features of Sau3A 204
23.3 Complete Cosmid Genomic Library 205
APPENDICES 207
 Commonly Used Bacterial Strains 207 Some of the Known Restriction Enzymes 209 Examples of Isoschizomers 219 Methylation Chart 223 Detailed Genetic and Physical Map of λDNA 227 Simplified Restriction Map of λDNA 229 Map of the First 21 Vectors of the Charon Series 231 Complete Nucleotide Sequence of pBR322 235 Detailed Restriction Map of pBR322 239 Structure of the pUC Plasmid Series 241 Proteinase K 243 Extraction of Lymphocyte DNA 245 RNase A 247 DNase I 249 Preparation of Rabbit Reticulocyte Extract 251 S1 Nuclease 253 Reverse Transcriptase 255 T4 DNA Ligase 257 E coli Alkaline Phosphatase 259 Deoxynucleotidyl Terminal Transferase 261 The Polymerase Chain Reaction 263 Example of Adaptor Molecules of Different Sizes 265 Ribonuclease H 267 Characteristics of a Few Commonly Used Cosmids 269
REFERENCES 287

INDEX 293

Part I The Host