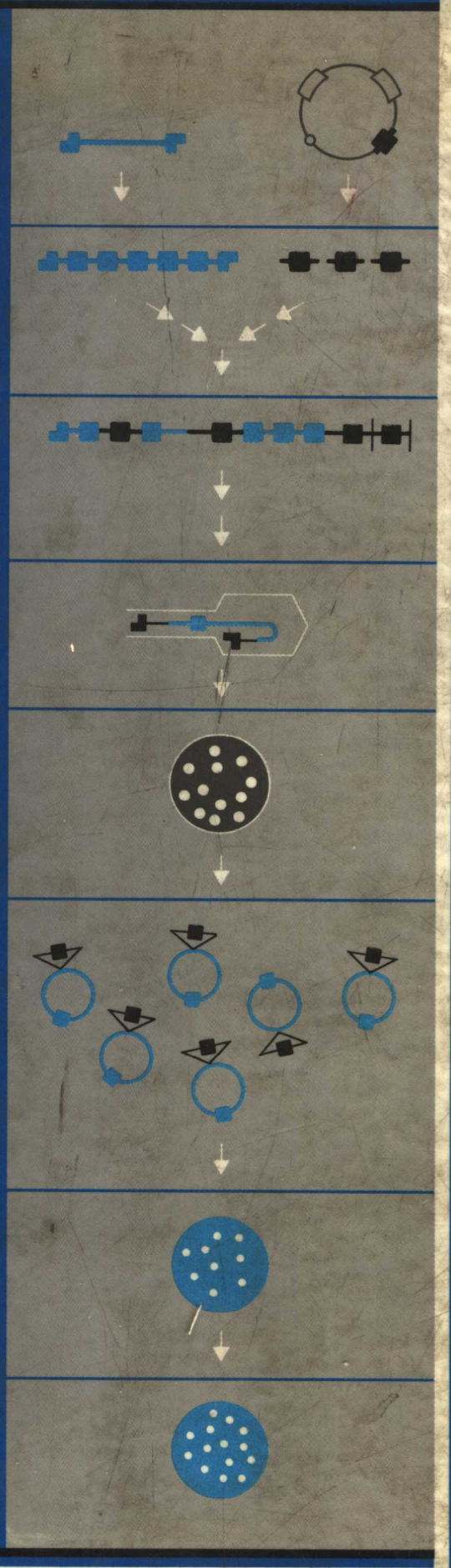
SECOND EDITION

A PRACTICAL GUIDE TO MOLECULAR CLONING



Bernard Perbal

A PRACTICAL GUIDE TO MOLECULAR CLONING

SECOND EDITION

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The drawing represents the construction of a shuttle phasmid vector to introduce foreign DNA into mycobacteria. This novel strategy opens the possibility of developing recombinant multivaccine vehicles (Jacobs *et al.* 1987) Proc. Natl. Acad. Sci. USA 327,532

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PREFACE

Molecular cloning has become a fundamental technique in all biological fields and probably represents the most powerful tool ever developed not only for understanding the basis of life but also for modifying or creating living species. Cloning of genes has already provided a tremendous body of information for important medical problems. For instance, cancer research has been progressing notably with the isolation of activated oncogenes and the identification of many chromosomal breakage points associated with the tumoral state. The DNA sequences responsible for other severe diseases such as Duchenne distrophy or retinoblastoma have now been identified, and the recent cloning of DNA sequences encoding for factor VIII will allow treatment of hemophiles without injecting human blood compounds. This is especially important to consider now that we know of the potential existence of several infectious new human viruses such as HIV.

Another very promising application of cloning is exemplified by the introduction and expression of a functional cloned gene in defective cells so that the expression of the wild type gene compensates the cellular defect and therefore restores the normal activity. It has already been shown that treatment of anemia, such as thalassemia, is possible by this method. The low-cost mass-production of biological molecules such as interferon, insulin, and antibiotics for use in human therapy has also been made possible by the use of cloning procedures. Engineering of genes has also been applied successfully to the production of vaccines for humans. For example, the production of hepatitis B envelope protein by yeasts has led to the production of a vaccine commercialized this year in the United States. Also very promising is the use of vaccinia virus recombinants that express cloned genes of unrelated infectious agents to develop live vaccines. This approach, which is being used with recombinants expressing proteins from human immunodeficiency virus (HIV) and Hepatitis B virus, might become more powerful with the use of vaccinia recombinants also carrying genes encoding for lymphokines. Even more spectacular is the recent description of a strategy allowing construction of a multivaccine vehicle which might permit a multiple-long-term immunization following a single injection. No doubt that molecular cloning will permit a considerable improvement of our life in the near future.

Aside from medical applications, molecular cloning is also applied at a considerably expanding rate to biotechnology of animals and plants. Since the description of transgenic giant mice in 1982, human growth hormone has been expressed in transgenic rabbits, sheep, and pigs, while transgenic cows will be used for the production of foreign proteins directly excreted in milk. Transgenic plants include cereals, beetroot, tobacco plants, carrots, salads, and petunias. For example, a tobacco plant containing Tobacco Mosaic Virus (TMV) genes has been completely reconstructed in vitro and has given rise to a plant which is more resistant to TMV infection.

Other unexpected achievements permitted by gene cloning include the use of individual-specific

"fingerprints" of human DNA in police investigation and expression of 2500-year-old mummy genes. The combined use of computerized programs and genetic engineering will certainly permit in the near future the creation of synthetic proteins with new activities adapted to particular needs of our society, such as elimination of hydrocarbons or heavy metals and the production of new medications.

This second edition of ***A Practical Guide to Molecular Cloning*** is intended to provide both the basic information needed for inexperienced people to perform cloning of any DNA fragment and eventually study its expression products in transformed (or transfected) cells, and the most sophisticated updated techniques for researchers who are already familiar with molecular biology. Therefore, this book should be very useful to students, technical staff members, and senior scientists. We have deliberately chosen to describe only a selection of techniques which have proved to be, in our hands, the most reliable and easiest to use. In a few cases, selected protocols were obtained from specialists in their fields. Because of the considerable amount of information that was added to the previous edition, we have changed its format and tried to make it even more pleasant to use. We have kept a larger printing size for the protocol sections so that the different steps in the procedures can be checked at a glance when necessary during the experimentation. A wider margin has also been set at the side of the protocols to provide space to write notes or comments. Finally, we have used a spiral binding for the regular edition to fill the need of many colleagues who prefer to open the book flat on a bench.

Aside from these changes, the content of the book has been largely reviewed and updated. A new chapter describes basic methods used in molecular biology. The aim of this chapter is to provide very basic principles of some current methods (e.g., the importance of working at an accurate pH, choosing correct buffers, determining the best conditions for electrophoresis and centrifugation, etc.). This is particularly important to beginners in the field, since many cloning experiments may fail due to a lack of knowledge in this basic matter.

Modification and specific digestion of DNA are two basic steps in molecular cloning. An updated review of commercial modifying enzymes is presented in Chapter 4 (commercial availability in both the United States and Europe being indicated). The combined use of bacterial strains expressing cloned genes and sophisticated purification methods has allowed marketing of high quality products at very reasonable prices. More and more suppliers also offer kits containing all components needed in addition to the enzyme itself. Before using such kits, make sure you can obtain individual components without having to buy the whole package again!

Because restriction endonucleases remain an essential tool in molecular cloning, we have devoted a complete chapter to describe their main properties. The number of commercial restriction endonucleases has increased considerably since the first edition was completed. We have now provided the reader with a concise table indicating commercial availability (adjusted to meet a 1988 publication date) and specific comments for all enzymes from many different sources. Several other tables provide information on thermosensitivity, salt effects, the number of cutting sites in main vectors, and an alphabetical listing of all known restriction endonucleases and isoschizomers. Special attention has been given to the search for compatibility of different restriction sites and the nature of sites generated after combining two compatible ends. This information is particularly useful when one wants to ligate DNA fragments harboring different but compatible ends without using modifying enzymes. A computerized program has been developed especially for this purpose in collaboration with C. Mugnier (CITI 2, PARIS) to provide all compatibilities between commercial enzymes listed in this manual and the resulting restriction sites generated after ligation.

Because so many different new vectors appear every month in literature, we did not feel that a complete review should be included in this book. Rather, we have presented a listing of the main commercial vectors representing the different kinds of systems being used today. Their practical advantages and limitations are also discussed.

Many of the new methods, which have been described in the past few years to obtain rapidly better yields of high quality vector and passenger DNA, have been included in this manual, with new procedures for separating DNA fragments by electrophoresis. These new techniques, which offer the ability to identify genes on intact chromosomes, open very promising roads to the understanding of genome organization and expression. We have also described new methods for propagation of recombinant DNA molecules and in situ hybridization techniques.

Labeling of DNA fragments is another essential step in molecular cloning. Because it is used on a daily basis by most researchers in various laboratories, several methods have been developed for labeling DNA with nonradioactive precursors. Their advantages and conditions for use are described in this

manual. These procedures may be of great interest to laboratories involved in diagnosis. Also of considerable interest is the in vitro synthesis of labeled probes from cloned genes by SP6, T7, and T3 RNA polymerases. The methods described in this manual allow the synthesis of riboprobes with very high specific radioactivity, therefore providing increased sensitivity without high backgrounds. These procedure are suitable for in situ hybridization, as well as synthesis of probes to search genomic sequences directly on chromosomes.

When the use of purified DNA fragments is not possible, cloning the genes of interest is made possible by preparing genomic libraries in which any gene is theoretically represented or by cloning cDNA species. For the first time in literature, we give the results of a comparative test which has been performed in our laboratory with the different "lambda in vitro packaging extracts" commercially available. We hope that our experience will help our colleagues in choosing the source of extract being the most appropriate to their own needs. Performing cDNA cloning is now greatly eased by the use of specially tailored vectors which are also commercially available and proved to be of satisfactory quality for reliable, safe, and fast cloning. We describe in this manual two protocols based on the use of commercial and "house-made" vectors.

Nucleotide sequencing of DNA and RNA has been improved by the introduction of new versatile vectors, the use of reverse transcriptases and techniques allowing direct sequencing of double stranded DNA. Use of ^{35}S-labeled nucleotides and reverse transcriptases, direct sequencing of double stranded DNA, sequencing in both orientations, sequencing in microtiter plates, and other new rapid ways of sequencing are among the techniques included in this manual. Because the large amount of information obtained in sequencing often leads to use of computerized systems, we present here an updated brief review of what can be gained by using computerized programs. This chapter does not intend to review all programs, but should allow beginners to become familiar with these techniques.

Cloning of genes may be directed towards modification of living cells (see above) to improve or create new species. Mutagenesis of cloned DNA fragments is possible by several techniques which are described in this book. New techniques, such as "TAB linker mutagenesis" and "random introduction of single base mutations in defined fragments" are described in detail.

We also felt that protocols for studying DNA-protein interactions should be presented in this manual because regulatory proteins involved in differentiation processes or growth cycle regulation are often described as DNA-binding proteins. Techniques for purification of protein-DNA complexes and characterization of the DNA sequences involved in the complex are currently used by leading laboratories, and no current book describes them in detail.

Finally, in many instances, the ultimate goal of cloning remains the production of large quantities of a pure product (for research or industrial purposes). The last chapter has been completely reorganized to provide the reader with an updated review of different ways to express cloned genes in various procaryotic and eucaryotic systems. Protocols for in vitro transcription and translation of cloned sequences are described in detail. Among them are the recent techniques for the addition of cap structures to eucaryotic mRNAs and direct in vitro synthesis of capped mRNA species. We have also described in this chapter two procedures for the in vitro translation of messenger RNAs in both reticulocyte and wheat germ extracts.

Different kinds of procaryotic and eucaryotic expression vectors have become commercially available. We present a brief review of their advantages and main properties. For example, expression vectors have been designed recently to allow an easy recovery of proteins. The use of these vectors in combination with the techniques that have been described to recover intact gene products from fusion proteins should prove to be very helpful in isolating gene products corresponding to cloned sequences of various origins.

In addition to these protocols, we also describe several different techniques for the introduction of foreign DNA in animal or plant cells by means of transfection and for the characterization of polypeptides expressed in transfected or transformed cells by immunoprecipitation and immunoblotting.

The protocols described in this book have been used successfully in the past few years to characterize the cellular localization, the nature, and the function of many gene products in both procaryotes and eucaryotes.

I hope this manual will be helpful not only to all those who are about to start using molecular cloning, but also to the researchers already involved in this field. I am indebted to all the colleagues who helped me select the methods described in this manual, discussed the relative advantages and disadvantages of different techniques, and provided unpublished protocols or experimental data. I wish to thank Johann Soret for reading the manuscript, André Sentenac and collaborators who provided pro-

tocols for the study of DNA-proteins interactions, Claude Mugnier and P. Le Beux for allowing us to use the CITI2 facility programs, Anne-Lise Haenni who provided the protocol for wheat-germ extract preparation, and all the members of my laboratory for their criticism, support, and suggestions. I also wish to thank S. Kudzin for his support, stimulating discussions, and help in designing this book, and A. Besnard, C. Leray, and N. Frey for skillful typing assistance. The members of the production department at Wiley have done a great job with my original manuscript. I thank all of them sincerely.

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Again, this is my opportunity to thank most particularly my children, Sabine, Sébastien, Séverine, and Sonia, and my wife Annick for their constant support, patience, and help during the preparation of this book.

Orsay, France
January, 1988

BERNARD PERBAL

CONTENTS

1 LABORATORY EQUIPMENT NEEDED FOR MOLECULAR CLONING 1

2 A FEW WORDS ABOUT SAFETY 4

Laboratory Hoods, 4
Biological Safety Cabinets, 4
Gloves, 5
Ultraviolet Light, 5
- Germicidal Lights, 6
- Detection of DNA by UV Illumination, 6

Phenol, 6
- First Aid, 6
- Redistillation of Phenol, 6

Liquid Scintillation Cocktails, 7
Chloroform, 7
Isoamyl Alcohol, 7
Ethyl Ether, 8
Diethyl Pyrocarbonate, 8
Methylmercury, 8
Plasticware and Chemicals, 8
Working with ^{32}P-Labeled Compounds, 8
Working with Known or Potential Carcinogens, 8

3 BASIC METHODS IN MOLECULAR BIOLOGY 11

Gel Electrophoresis, 11
- Introduction to Electrophoretic Theory, 11
- SDS Gel Electrophoresis: Separation of Proteins on the Basis of Molecular Weight, 14
- Continuous and Discontinuous Buffer Systems, 19
- Two-Dimensional Gel Electrophoresis: To Separate Complex Mixtures of Proteins into Many Components, 23
- Electrophoretic Transfer, 32
- Separation of Low Molecular Weight Proteins by Polyacrylamide Gel Electrophoresis, 34

Separation Methods in Preparative Ultracentrifuges, 38
- Differential Centrifugation, 38
- Density Gradient Centrifugation, 38
- Selection of Rotors, 40
- Pelleting, 40
- Isopycnic Separations (Usually Using Cesium Chloride Gradients), 40
- Rate Zonal Separations (Usually Using Sucrose-Gradients), 40

Gel Filtration with Sephadex, 43
Chemical and Physical Properties of Sephadex, 43
Ion Exchange Chromatography, 44
The Theory of Ion Exchange, 45
Sepharose Ion Exchangers, 45
Chemical and Physical Properties, 45
NACS Ion Exchangers, 46
Quantification of Proteins, 46
Lowry Procedure, 46
Bradford Procedure, 47
Choice of Buffers, 49
Autoradiography, 51
Choosing the Correct Exposure Conditions, 51
Estimating Exposure Times, 52
Fluorography Techniques, 54
Quantification of Results, 55
Intensifying X-ray Films, 55
Trouble-Shooting Guide, 57
Physical Properties of Some Radionuclides Used in Molecular Cloning, 59
Physical Constants of Nucleosides and Nucleotides, 59
Phenol Extraction of Nucleic Acids, 73
Preparation of Phenol for Nucleic Acid Extractions, 73
Extraction of Nucleic Acids, 74
Ethanol Precipitation of Small Amounts of Nucleic Acids, 76
Some Useful Hints and Units, 79

4 USEFUL MODIFYING ENZYMES FOR MOLECULAR CLONING 80

T4 DNA Ligase, 80
E. coli DNA Ligase, 82
T4 DNA Polymerase, 82
E. coli DNA Polymerases, 84
Modified Bacteriophage T7 DNA Polymerase, 85
Alkaline Phosphatase, 86
T4 Polynucleotide Kinase, 86
Terminal Deoxynucleotidyl Transferase, 87
Exonuclease III, 89
Nuclease Bal 31, 89
Exonuclease VII, 90
Tobacco Acid Pyrophosphatase, 90
Ribonuclease H, 91
Ribonuclease Phy I, 91
RNAse CL3, 91
B. cereus Ribonuclease, 91
Ribonuclease PHY M, 91
RNAse U2, 91
RNAse T1, 92
RNAse T2, 92
DNAse I, 92
S1 Nuclease, 92
Mung Bean Nuclease, 93
Topoisomerase I, 93
Topoisomerase II (DNA gyrase), 93
Guanylyltransferase from Vaccinia Virus, 94
Polyadenylate Polymerase of *E. coli*, 94
Reverse Transcriptase, 94
SP6 Polymerase, 96
T7 RNA Polymerase, 97
Methylases, 97
Listing of Some Modifying Enzymes Commercially Available, 102

5 RESTRICTION ENDONUCLEASES 107

6 VECTORS FOR MOLECULAR CLONING 201

Plasmid Vectors, 201
Bacteriophage-Derived Vectors, 234
Bacteriophage λ, 234
Cosmid Vectors, 265
The Use of Cosmids as Cloning Vehicles, 265
Cosmid Vectors, 269
Host Strains and Cosmid Stability, 269
In Vivo Packaging of Cosmids, 270
Recombination Screening, 270
Other Cloning Vectors, 273
Procaryotic Vectors, 273
Yeast Cloning Vectors, 274

Plant Vectors, 275
Viral Vectors, 277
Cloning Vectors from Commercial Sources, 278

7 PURIFICATION AND CHARACTERIZATION OF VECTOR AND PASSENGER DNA 297

Isolation of Plasmid DNA Using Cesium Chloride Gradient Centrifugation, 297
Rapid Procedure for Isolation of Plasmid DNA, 304
Removal of RNA Species from DNA Preparations, 307
CsTFA for Isolation and Separation of Nucleic Acids by Isopycnic Centrifugation, 309
Product Description, 310
Specifications for CsTFA, 310
Instructions for Use, 310
Purification of DNA Restriction Fragments, Plasmid DNA, and Chromosomal DNA by Sephacryl Gel Chromatography, 313
Preparation of λ Phage Stocks and DNA, 313
Titration of Initial Seed Stock and Preparation of Large Stocks, 313
Purification of High Molecular Weight DNA from Eucaryotic Cells, 324

8 DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES 327

Enzymatic Units, 327
Conditions for Optimal Enzymatic Activity, 327
Multiple Digestion of DNA with Restriction Endonucleases, 331
Stability of Restriction Endonuclease Activity During Extended Digestions, 335
Partial Digestion of DNA with Restriction Endonucleases, 336
Incubation in the Presence of Ethidium Bromide, 338

9 SEPARATION OF DNA FRAGMENTS BY ELECTROPHORESIS 340

Agarose Gels, 340
Gel Concentration, 340
Preparation of the Agarose Solution, 340
Gel Beds, for Horizontal Electrophoresis, 340
Combs, 344
Vertical Agarose Gels, 344
Separation of DNA Fragments by Pulse and Reversed Field Electrophoresis, 344
Staining of DNA Fragments, 347
Photography of DNA Fragments, 349
Polyacrylamide Gels, 349
Gel Concentration, 349
Preparation of Gels, 349
Anomalous Mobilities of DNA Restriction Fragments on Polyacrylamide Gels, 350
Staining of DNA Fragments, 355
Size Markers for DNA Fragments Run in Agarose and Polyacrylamide Gels, 356
Agarose Gels, 356
Polyacrylamide Gels, 360
Quantification of DNA Fragments on Agarose Gels, 361

10 ORDERING THE RESTRICTION DNA FRAGMENTS IN PHYSICAL MAPS 363

Double Digestion of DNA Fragments, 363
Mapping by Further Digestion of Purified DNA Fragments, 363
Mapping by Partial Digestion of End-Labeled DNA, 365
Labeling of the DNA Ends, 365
Asymmetric Digestion of Labeled DNA, 365
Partial Digestion of Purified Labeled Fragments, 365

Mapping Restriction Sites with Bal 31, 365
Mapping by Two-Dimensional Analysis of End-Labeled DNA, 366

11 PURIFICATION OF DNA FRAGMENTS 368

Purification of DNA Fragments from Regular Agarose Gels, 368
Purification of DNA Fragments from Low-Melting Agarose Gels, 370
Purification of DNA from Agarose or Acrylamide Gels Using DEAE Paper, 372
Eution of DNA Species from Polyacrylamide Gels, 374

12 MODIFICATION OF DNA FRAGMENTS WITH COHESIVE TERMINI 375

Modification of 3′ and 5′ DNA Termini with DNA Polymerase, 375
Modification of Cohesive Ends with 3′ Extension, 375
Modification of Cohesive Ends with 5′ Extension, 377
Digestion of DNA with Bal 31 Nuclease, 377
Addition of Molecular Linkers to DNA Fragments, 379
Use of Adapters for Cloning, 385

13 LIGATION 394

Theory, 394
Practice, 397

14 PROPAGATION OF RECOMBINANT DNA MOLECULES 402

Reduction of Transformation Background Due to Vector Recircularization—Use of Alkaline Phosphatase, 402
Cloning in Tailed Vector, 405
Tailing Conditions and Calculations, 408
Transformation of *E. coli* Strains with Plasmid DNA, 411
Transformation of Competent Cells Following Direct Ligation in Low Melting Point Agarose, 416

15 CHARACTERIZATION OF RECOMBINANT CLONES 418

Rapid Purification of Recombinant Plasmid DNA, 418
Direct Analysis of Recombinant Plasmids by Vertical Electrophoresis on Agarose Gels, 420
Screening of Recombinant Bacteria on Nitrocellulose Filters, 421
Capillary Transfer of DNA Fragments to Nitrocellulose Membranes, 423
Electrophoretic DNA Transfer to Nitrocellulose Membranes, 430
Preparation and Activation of Chemically Modified Papers for Nucleic Acids Transfer, 431
DNA Transfer on Modified Nylon Membranes, 436
Dot Blotting, 438
Slot Blotting, 439
Analysis of Recombinant DNA by Hybridization with Specific Probes, 439
Rehybridization of Filters, 445
Direct Hybridization of Labeled DNA to DNA in Agarose Gels, 446
Hybridization Histochemistry, 448
Preparation of Tissue, 448
Preparation of Sections, 448
cDNA Probe Labeling, 450
Synthesis of DNA Probes, 450
Washing Conditions, 451
Autoradiography, 451
Staining, 451
Preparation of Whole Mouse Sections, 451

16 LABELING OF NUCLEIC ACIDS 453

Preparation of Radioactive Probes, 453
Nick-Translation, 453
Labeling DNA by the Replacement Synthesis Method, 458

End Labeling of DNA Fragments, 461
Procedures for Nonradioactive Labeling of Probes, 469
In Vitro Synthesis of Labeled RNA Probes, 471
Radiolabeled RNA Probe Synthesis with SP6, T3, and T7 RNA Polymerases, 473
In Vitro Synthesis of Nonradioactive Labeled RNA Probes with SP6, T3, and T7 RNA Polymerases, 479

17 PREPARATION OF GENOMIC LIBRARIES 480

Theoretical Study of the Fraction of a Long-Chain DNA That Can Be Incorporated in a Recombinant DNA Partial-Digest Library, 480
Theory, 480
Comparison of the Estimates, 489
Representation of DNA Sequences in Recombinant DNA Libraries Prepared by Restriction-Enzyme Partial Digestion, 491
Appendix, 495
Preparation of Libraries with λ Charon Phages, 497
Isolation of the DNA to Be Cloned, 497
Preparation of λ Charon Arms, 501
Ligation of Digested DNA to λ Charon Arms, 503
Lambda In Vitro Packaging System, 503
Amplification of Lambda Libraries, 506
Efficiency of Commercial Packaging Extracts, 508
Parameters Affecting Efficiency of the Packaging Reaction, 508
Screening of Lambda Libraries by Hybridization with Specific Probes, 510

18 PURIFICATION AND CHARACTERIZATION OF RNA SPECIES 516

Purification of Intact RNA Species by Ultracentrifugation, 516
Purification of RNA Species for Quick Blot, 518
Preparation of Cytoplasmic RNA from Eucaryotic Cells, 518
Isolation of Cytoplasmic Polyadenylated RNA Species, 522
Isolation of Specific RNA Species, 524
Formaldehyde Gels for RNA, 526
Methyl Mercury Gels for RNA, 527
Glyoxal Gels for RNA, 530
Size Markers for RNA Species Run in Denaturing Agarose Gels, 532
Transfer of RNA to Nitrocellulose, 533
Methyl Mercury Gels, 533
Glyoxal or Formaldehyde Gels, 534
Transfer of RNA to DBM or DPT Paper, 535
Methyl Mercury Gels, 535
Glyoxal or Formaldehyde Gels, 536
Transfer of RNA to Nylon Membranes, 537
Hybridization to mRNA Immobilized on Hybond Map, 538
Mapping RNAs by Digestion of DNA–RNA Hybrids, 542
Nuclease S1 Mapping of DNA Sequences Homologous to RNA Species, 543

19 CLONING OF cDNA SPECIES 550

Synthesis of cDNA Species with Reverse Transcriptase, 550
Cloning of cDNA Sequences in Plasmid Vectors, 553
Preparation of a cDNA Library in Lambda gt11 Vector, 569
Characterization of the Recombinant Clones, 574
Freezing Bacterial Colonies Directly on Nitrocellulose Filters, 576

20 SEQUENCING OF DNA 578

Sequencing of DNA by Base-Specific Cleavage, 578
Large-Scale Production of Unique DNA Fragments, 578

End Labeling of the DNA Fragments, 578
Separation of Labeled Ends, 579
Chemical Cleavage Reactions, 580
Detailed Procedures, 580
Modified Maxam–Gilbert Sequencing Procedure for Use with Oligodeoxynucleotides, 584
Gel Electrophoresis and Autoradiography, 584
DNA Cloning in M13 Phage for Sequencing, 585
Preparation of Vector DNA, 585
Preparation of DNA Fragments to Be Inserted, 593
Ligation of DNA Fragments to Vector DNA, 594
Preparation of Competent Cells, Transformation, 595
Rapid Deletion Subcloning System for Sequencing, 599
Characterization of M13 DNA Recombinants, 605
Sequencing of Recombinant DNA Obtained with M13 Cloning, 610
Procedure for Performing Dideoxy Sequencing Reactions in Microtiter Plates, 618
Polyacrylamide Gel Electrophoresis, 619
Autoradiography, 624
Interpretation of the Autoradiograms, 628
Some Common Problems Encountered When Performing Sequencing. Trouble Shooting Guide, 630
Sequencing with Reverse Transcriptase, 631
Direct Sequencing of DNA Fragments Cloned in Plasmids, 634
Sequencing of Single Stranded DNA in Both Directions, 636

21 RNA SEQUENCING 641

Chemical Specific Cleavage of RNA for Sequencing, 641
Labeling of RNA 3′ Terminus, 641
Base-Specific Cleavage of Labeled RNA, 642
Enzymatic Sequencing of RNA, 647
Procedures for Enzymatic Sequencing, 649
Use of Reverse Transcriptase for RNA Sequencing, 650

22 USE OF COMPUTERIZED PROGRAMS IN THE TREATMENT OF DNA SEQUENCE INFORMATION 654

Sequencing Programs for Shotgun Sequencing, 655
Manual Shotgun Sequencing, 655
Automatic Shotgun Sequencing, 655
Search for Occurrences of Nucleotides and Base Sequences in DNA, 656
Base Frequencies, 656
Restriction Endonuclease Maps, 656
Consensus Sequences, 658
Determination of Secondary Structures, 659
Identification of Putative Gene Products, 659
Primary Structure, Molecular Weight, 660
Proteolytic Digestion, 660
Localization of Potential Antigenic Epitops, 661
Localization of Secondary Structures, 661
Comparison of Sequences, 666
Comparison of Sequences with the Whole Content of a Data Bank, 675
Data Sources, 675

23 LOCALIZED MUTAGENESIS 685

Oligonucleotide-Directed Mutagenesis, 685
Linkers Mutagenesis, 693
Linker-Scanning Mutagenesis, 693
TAB Linker Mutagenesis, 695

Random Introduction of Single Base Mutations in a Defined DNA Fragment, 707
Specific Points to Consider for the Synthesis of the Mutagenized Oligonucleotide, 708

24 METHODS FOR STUDYING DNA–PROTEIN INTERACTIONS 719

Analysis of Protein–DNA Complexes by Polyacrylamide Gel Electrophoresis (Gel Retardation), 719
DNAse I Footprinting, 723
Localization of Contact Points by Dimethylsulfate Resistance, 725
Isolation of DNA–Protein Complexes for the Analysis of Footprints Following DNAse I Digestion or Protection from DMS Modification, 728

25 EXPRESSION OF CLONED DNA SEQUENCES IN VITRO OR IN PROCARYOTIC AND EUCARYOTIC CELLS 731

In Vitro Transcription of Cloned Genes, 734
In Vitro Translation of RNA Expressed from Cloned Genes, 740
Expression Vectors for the Characterization of Cloned Gene Products, 751
Expression in Procaryotic Systems, 751
Vectors for Mammalian Cells, 758
Recovery of Intact Gene Products from Fusion Proteins, 776
Transfection of Eucaryotic Cells with Purified DNA, 780
Transfection of Cells Following DEAE Dextran Treatment, 780
DNA–Calcium Phosphate Coprecipitation Technique with Cell Monolayers, 781
Transfection of Cells in Suspension with DNA–Calcium Phosphate Precipitates, 782
Liposome-Mediated DNA Transfection, 783
Direct Transfer of Plasmid DNA from Bacterial to Mammalian Cells, 784
Selection of Cells Expressing Newly Introduced DNA, 785
Construction of Cosmid Libraries Used to Transform Eucaryotic Cells, 786
Detection of Polypeptide Synthesis by Immune Precipitation, 788
Detection of Polypeptide Synthesis by Immunoblotting, 792

EPILOGUE: IF YOU WANT AVA I TO MEET BAM HI . . . 795

APPENDIX: ADDRESSES OF MAIN OFFICES FOR SUPPLIERS CITED IN THIS MANUAL 798

INDEX 801

1

LABORATORY EQUIPMENT FOR MOLECULAR CLONING

The following laboratory equipment is used in molecular cloning:

Autoclave(s).

Centrifuges for ultracentrifugation (e.g., Beckman, Sorvall, MSE).

Rotors such as Beckman 60 or 70 Ti, SW41, SW28, SW50, or equivalent. Vertical rotors are also very useful.

Centrifuges for high-speed centrifugation, such as RC5B (Sorvall) or J21 (Beckman), with rotors for large volumes (Sorvall GS3 = 6 × 500 ml, GSA = 6 × 250 ml, Beckman JS4.2 = 6 × 1000 ml or 6 × 500 ml or 6 × 250 ml with adapters) and rotors for small volumes (Sorvall SS34 or Beckman JA20), allowing runs up to 50,000 × *g*.

The new TL100 ultracentrifuge (Beckman) is particularly adapted to manipulation of small samples, and allows a considerable reduction of centrifugation times.

Centrifuge for microfuge tubes (1.5 ml and 1 ml). Rotors allowing horizontal centrifugation will permit pellet precipitates at the bottom of the tubes.

Centrifuge for low-speed centrifugation (used for pelleting bacteria, pelleting cells, phenol extraction, etc.).

Computer (e.g., IBM or Apple).

Sequence analysis software (e.g., IBI/Pustell).

Laminar flow hood and chemical hood.

Orbital shaker for bacterial cultures (e.g., New Brunswick), with thermostat for use anywhere; without thermostat if used in warm room.

CO_2 incubator for cell cultures.

Refrigerators and freezers (4°C, −25°C, −80°C).

Power supplies for electrophoresis. Consider buying one delivering high amperage (up to 200 mA) and high voltage (2500–3000 V) for sequencing (e.g., Bio-Rad, LKB, Pharmacia).

Power supply delivering at least 250 mA for Western blotting.

Pulse controller (such as Hoeffer PC 750) for reverse field electrophoresis (requires a 750-V DC power supply).

Apparatus for electrophoretic transfer of proteins and nucleic acids (e.g., BioRad, and Hoefer). The transphor system from Hoefer comes with a convenient power lid designed to provide the high current required for efficient and even transfer.

Camera such as Polaroid MP4 with instant type 57 (positive) and/or type 55 (positive/negative) films.

Benchtop orbital shaker for staining, destaining, and washing.

Transilluminator (long-wave UV will avoid damaging DNA).

Vacuum oven (80°C).

Microwave oven.

Gel beds for regular agarose and acrylamide gels and for sequencing gels.

Miniature electrophoresis apparatus for rapid screening of proteins (such as "mighty small" SE250 from Hoefer).

Apparatus for slot blotting (Schleicher & Schuell, BRL).

Microscope (with inverted light for examination of cell cultures).

Refractometer.

Desiccator.

Speed Vac concentrator/evaporator (Savant).

Equipment for redistillation of chemicals.

Equipment for deionization or distillation of water.

Water bath at 37°C.

Refrigerated water bath for temperatures of 10–18°C.

Heating block to keep tubes warm and dry (e.g., for phage plating).

Water bath at 80°C.

Water bath at 45°C.

Water bath with agitation.

Automatic pipettemen (e.g., Gilson or Eppendorf) for volumes of 0–20 µl, 0–100 µl, 0–200 µl, 0–1000 µl. Yellow and blue tips for pipetteman.

Cassettes for autoradiography, with intensifying screens for ^{32}P (DuPont) and films (Kodak or Cronex). Keep cassettes for ^{35}S sequencing separately.

Vacuum pump.

Liquid nitrogen tank.

pH meter and pH indicator paper.

Spectrophotometer (UV and visible).

Multicanal peristaltic pump.

Radioactivity counter.

Radioactivity monitor.

Plastic shield for protection against radiation.

Pipette acid (e.g., Drummond).

Vortexer.

Magnetic stirrer.

Balances.

Sealing-bag system and plastic bags (e.g., Sears or Krups).

Timer.

Slab gel dryers (Hoefer). Consider buying one for sequencing gels and one for protein gels.

Apparatus for electroelution, and concentration of samples to small volumes (e.g., Biotrap, Schleicher & Schuell).

Gel reader for sequencing gels. Some of them may be related directly to computers for safe analysis (IBI, Beckman).

Filtration units (e.g., Millipore).

Polypropylene bottles for centrifugation (1 liter, 500 ml, 250 ml).

Polypropylene tubes (5 ml, 15 ml, 50 ml).

Polypropylene flasks, cylinders, and beakers.

Ultraclear tubes (Beckman) for high-speed centrifugation.

Quick seal unit and tubes (Beckman).

Corex tubes (30 ml and 15 ml) with adapters.

Whatman 3MM paper.

Nitrocellulose filters.

Millipore flat forceps to manipulate filters.

BenchKote (Whatman).

Plastic disposable pipettes (10 ml, 5 ml, 1 ml).

Glass disposable pipettes (20 ml, 1 ml).

Glass plates for electrophoresis.

Combs for electrophoresis, sharkstooth combs for sequencing.

Spacers for electrophoresis, wedge spacers for sequencing.

Glass slides for minigels.

Polypropylene microfuge tubes (1.5 ml, 0.75 ml) such as Eppendorf or Beckman.

Dialyzing tubing (2.5-cm and 1-cm diameter).

Portable UV lamp.

Syringes (50 ml, 10 ml, 5 ml, 1 ml).

Hamilton syringes (5 µl, 10 µl).

Needles (18-, 21-, and 25-gauge).

Giemsa rapid stain for staining cells.

Xylene cyanol and Bromophenol blue (dyes for electrophoresis).

Gradient former and collector.

Microcaps (1–5 µl, 10 µl, 50µl, 100µl).

Adhesive tape (regular and indicating radioactivity or biohazard).

Sterilization tape (for 120°C and 180°C).

Scalpels and blades, forceps and scissors.

Plastic autoclaving bags (to eliminate biohazardous material).

Loops to seed bacterial cultures and pick colonies.

Petri dishes for eucaryotic cell cultures (special plastic) and for bacteria plating.

Isothermic containers.

All glassware to be used for RNA work should be baked at 180°C for at least 2 hours. Glassware for DNA work should be siliconized by immersion in 5% dichloromethylsilane in chloroform, followed by extensive rinsing with deionized water and drying in the oven.