An Introduction to Recombinant DNA Techniques

Basic Experiments in Gene Manipulation

HACKETT · FUCHS · MESSING

An Introduction to Recombinant DNA Techniques

Basic Experiments in Gene Manipulation

Perry B. Hackett

James A. Fuchs

Joachim W. Messing

University of Minnesota

The Benjamin/Cummings Publishing Company, Inc.
Menlo Park, California • Reading, Massachusetts
London • Amsterdam • Don Mills, Ontario • Sydney

Sponsoring Editor: Jane R. Gillen
Production Coordinator: Greg Hubit
Copy Editor: Debra Myson-Etherington
Book Designer: Susan Rogin
Cover Design: Henry Breuer
Artists: Scientific Illustrators and Kris Kohn

Library of Congress Cataloging in Publication Data

Hackett, Perry B.
An introduction to recombinant DNA techniques.
(Benjamin/Cummings series in the life sciences)
Includes bibliographical references.
1. Recombinant DNA—Experiments. 2. Recombinant DNA—Laboratory manuals. 3. Genetic engineering—Experiments. 4. Genetic engineering—Laboratory manuals. I. Fuchs, James A. II. Messing, Joachim W. III. Title. IV. Series.
QH442.H33 1984 574.87'3283 83-21421
ISBN 0-8053-3672-9

abcdefghij-AL-8987654

The Benjamin/Cummings Publishing Company, Inc.
2727 Sand Hill Road
Menlo Park, California 94025

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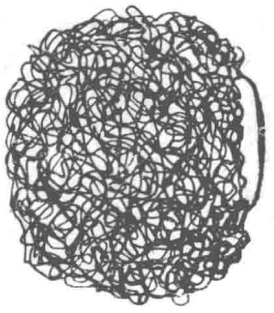

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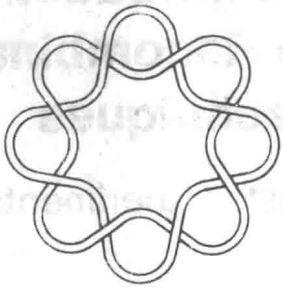

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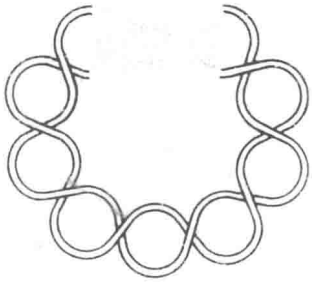

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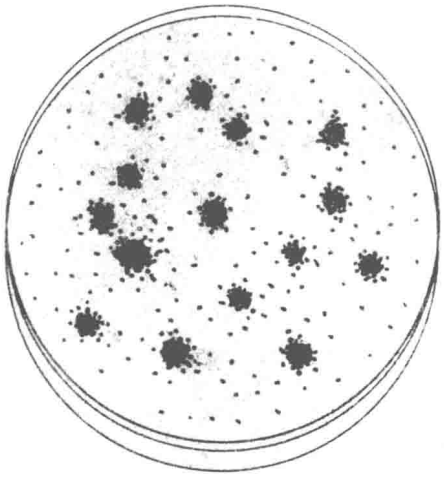

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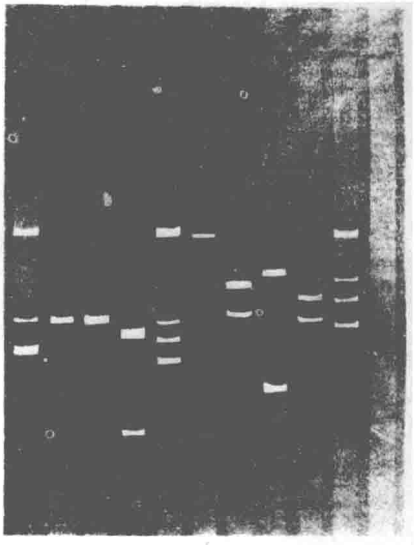

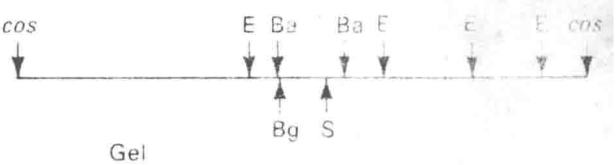

Gel
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Preface

In the past decade, the use of genetic engineering has spread from university research laboratories to industrial laboratories and, just recently, back into the curricula of college biology departments. For the past several years, we have taught at the University of Minnesota a laboratory course in recombinant DNA techniques, a ten-week course with two formal laboratory sessions per week. Because the theoretical foundations for the recombinant DNA methodologies are in principle quite simple, the course prerequisites do not go beyond a good college background in biology, chemistry, and genetics. Enrolled in our course have been a diverse group of graduate and advanced undergraduate students, many of whom have come to the course with minimal laboratory experience in molecular biology. We found that the available methods manuals and more descriptive treatises on gene manipulation, while excellent for reference purposes, were not appropriate lab manuals for our course. Accordingly, we developed our own sequence of experiments and a number of simplified protocols. After many revisions and refinements, the result is this book. We hope it will prove useful to other instructors and their students, as well as to scientists who want an efficient way to learn basic recombinant DNA techniques independently.

SCOPE AND SEQUENCE

Part One of this book consists of five chapters that introduce the basic principles of gene cloning, give essential background on working with *E. coli*, and describe the three cloning systems to be used. By reading these chapters, students acquire an understanding of the basic principles of the course without distraction by procedural details.

In Part Two, a unique sequence of carefully designed experiments enables the user of this book to become familiar with a variety of tech-

niques in a relatively short time. In only eighteen experiments (twenty periods), students are taken from a simple entry point—determining the number of viable bacteria in a given volume—through cloning of an *E. coli* gene in the three major types of cloning vectors: λ phage, plasmids, and the single-stranded DNA phage M13. By the time the last experiment (on site-specific mutagenesis) is completed, students have achieved a sophisticated level of laboratory expertise in gene manipulation and a solid understanding of the principles of genetic engineering.

The individual protocols are designed to be effective, reliable, fast, and as inexpensive as possible. Protocols requiring ultracentrifugation have been avoided. By using *E. coli* genes—the genes encoding ribonucleotide reductase, *nrdA* and *nrdB*—the experiments are exempt from federal guidelines concerning recombinant DNA, and there are no problems with biohazards. In addition, the *nrd* genes can be identified by their biological properties, thus obviating the need for radioisotopes. Part Three contains appendices providing detailed information about reagents and their sources, necessary equipment, and some of the key procedures.

MATERIALS FOR THE LABORATORY

Our goal is to make this manual usable in any college teaching laboratory. To help the instructor, we have arranged for Bethesda Research Laboratories (BRL) to assemble reagent packages, which will provide all the specialized materials needed for a class of 20 students. Three separate packages are available: (1) a biological package containing all required bacterial phage strains, enzymes, and nucleic acids; (2) a chemicals package containing antibiotics and ultrapure chemical reagents; and (3) a media package. For further information about the packages, please see Appendix A and contact BRL directly at the address given on the next page.

While we believe there are obvious benefits to using the entire sequence of experiments, we realize that this is not always possible or desirable. Therefore, the BRL biological package will include nucleic acid intermediates that will allow performance of experiments out of sequence.

ACKNOWLEDGMENTS

We acknowledge with gratitude the assistance of numerous people in the development of this book. We are grateful to the members of our laboratories who helped improve the techniques used in this book; in particular, we want to thank Betsy Kren for constructing a number of *E. coli* strains and testing some techniques. We owe a major debt to the students and teaching assistants who participated in the previous offerings of our course at the University of Minnesota; as a result of their suggestions, the final version of this manual is significantly better than the original version. Extremely valuable suggestions were also made by the reviewers of the manuscript: David Freifelder of the University of California, San Diego; Joyce Maxwell of California State University at Northridge; John Reeve of Ohio State University; LeLeng To of Gaucher College; and William Wood of the

University of Colorado. We are especially grateful to David Freifelder, who went over an early draft of the manuscript with a fine-tooth comb.

The artwork in this manual was designed and executed by Kris Kohn and Scientific Illustrators, Inc. We are also indebted to Jane Gillen of The Benjamin/Cummings Publishing Company for her efforts and encouragement in directing this project through the long process leading to publication.

Perry B. Hackett
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Joachim W. Messing

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Part One

Basic Principles

Chapter 1
Introduction

Recombinant DNA technology has revolutionized molecular biology and genetics. Today, virtually any segment of DNA, the genetic material of all cells and of most viruses, can be isolated and replicated to provide sufficient quantities of genes to study their structure and expression. Furthermore, cellular systems can be designed to produce large quantities of particular biological substances. Recently, recombinant DNA techniques have been used in new industrial and medical ventures to produce economically important substances of high purity.

The purpose of this laboratory course is to introduce you to several of the many techniques for gene cloning using three different types of carrier DNA molecules. These carrier molecules, which are called ***cloning vectors***, are used to introduce DNA fragments into cells for replication and amplification. The process of insertion followed by establishment of the hybrid vector in a cell is called ***molecular cloning*** or, simply, ***cloning***. In this manual, we will focus on the three cloning vectors most frequently used—double-stranded DNA genomes of viruses, single-stranded DNA genomes of viruses and plasmids. An understanding of the properties of these vectors is essential for anyone interested in using molecular cloning to obtain various biological products. In the exercises described in this course, two genes of the bacterium *Escherichia coli* (*E. coli*)—*nrdA* and *nrdB*, which encode the B1 and B2 subunits of ribonucleotide reductase and which have already been cloned in bacteriophage λ—will be sequentially transferred (subcloned) from the double-stranded DNA of phage λ to the plasmid pBR325, and then from the plasmid DNA into the single-stranded DNA of phage M13.

We will present methods for growing and isolating these cloning vectors, for cleaving DNA into fragments that can be moved from one cloning vector to the next, and for selecting specific recombinant DNA molecules.

As you move the genes from one cloning vector to the next, you will analyze recombinant DNA molecules by several techniques—biological tests for gene complementation and drug resistance, physical tests for size determinations by electrophoresis, and mapping of restriction-endonuclease cleavage sites.

A summary of the course is presented as a flowchart in Figure 1-1. This complex figure, with symbols for various recombinant DNA molecules and vectors, should be used throughout the course as a map to locate where you are at any particular time and to see where you are going. You will appreciate the figure more fully when you have completed Part One of the manual.

You may wish to consult textbooks of molecular biology, microbiology, and biochemistry for help in understanding the fundamental concepts underlying the experiments and procedures used in this manual. In addition, we recommend that you obtain a text that covers the principles of cloning. Several useful books and review articles are listed in the references at the end of this manual. Furthermore, you can obtain—free of charge—informative catalogues from companies selling reagents and materials used in gene cloning. Some companies are Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, and New England Biolabs (see Appendix A). These catalogues contain a wealth of practical information not presented in this manual.

This book is divided into three major sections. The first section consists of five chapters that contain a brief discussion of the microbiological techniques required in the course and descriptions of the three cloning systems to be used. Double-stranded DNA vectors—in particular, plasmids and phage λ—have been used for gene cloning for the past several years, and an extensive literature on their use is available. The employment of single-stranded phage DNA as a cloning vector is quite recent so there are fewer publications to which you can refer. Thus, the discussion of M13 will be more extensive than that of plasmid and phage λ DNA.

Figure 1-1 Flowchart of experiments described in this manual. The chart shows the progressive subcloning of the *E. coli nrdA* and *nrdB* genes, which are abbreviated *nrd*. Initially, a recombinant λ phage lysogen that contains the *nrd* genes, designated λd*nrd*$^+$, is induced, the phage is harvested, and the *nrd* genes are recombined with plasmid pBR325 to form the recombinant plasmid pBR*nrd*. The *nrd* genes are then further subcloned into the single-stranded phage M13 to form the recombinant phage M13*nrd*. In addition, an experiment illustrating the technique of site-specific mutagenesis is shown in the lower right corner of the figure. DNA molecules cleaved by specific restriction endonucleases are designated by (DNA) × (endonuclease); thus, λ phage cleaved by the restriction enzyme *Eco*RI is designated λ × *Eco*RI. The numbers in parentheses indicate the laboratory period during which the particular step is accomplished. The letters in parentheses indicate the procedures used: (a) biological selection or analysis, (b) induction of phage, (c) isolation of DNA, (d) restriction endonuclease cleavage of DNA, (e) ligation of DNA fragments, (f) transformation of *E. coli* cells with recombinant DNAs, (g) electrophoretic analysis of DNAs.