# RECOMBINANT DNA LABORATORY MANUAL

# RECOMBINANT DNA LABORATORY MANUAL

*Revised Edition*

**Judith W. Zyskind**
**Sanford I. Bernstein**
*San Diego State University*
*San Diego, California*

**Academic Press, Inc.**
*Harcourt Brace Jovanovich, Publishers*
San Diego New York Boston London Sidney Tokyo Toronto

This book is printed on acid-free paper. ♾



Academic Press, Inc.
1250 Sixth Avenue
San Diego, California 92101

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

Zyskind, Judith W.
Recombinant DNA laboratory manual / Judith W. Zyskind and Sanford I. Bernstein. -- Rev. ed.
p. cm.
Includes index.
ISBN 0-12-784401-5
1. Recombinant DNA—Laboratory manuals. I. Bernstein, Sanford I. II. Title.
QH442.Z97 1992
574.87'3282'078--dc20 91-40989
CIP

Printed in the United States of America
92 93 94 95 9 8 7 6 5 4 3 2 1

# PREFACE

This book represents the culmination of more than seven years of effort directed at initiating and refining a laboratory course in recombinant DNA techniques. We have approached this course from two different backgrounds: J.W.Z. utilizes bacterial genetics and molecular biology in her research program; S.I.B. is a specialist in eukaryotic gene manipulation. As such, we have attempted to incorporate procedures that are useful to individuals studying either prokaryotes or eukaryotes, keeping in mind that the ability to manipulate *E. coli* and its phages is requisite for any DNA molecular biologist. The experiments have been tested for several semesters in the classroom and are designed to teach the fundamentals of recombinant DNA technology. These techniques include basic microbiological manipulations; extractions of chromosomal, plasmid, M13, and lambda phage DNA; gel electrophoresis; *in vivo* mutagenesis; restriction mapping; isolation and cloning of restriction fragments; DNA sequencing; probe labeling (with both biotin and radioactive precursors); DNA gel blotting; phage plaque blotting; and molecular hybridization. This revised edition of the text includes a new laboratory experiment using the polymerase chain reaction (PCR), a procedure that has revolutionized gene analysis. We are indebted to Dr. Phillip Singer, who taught the laboratory course for the past two years, for developing the PCR experiment. Dr. Douglas Smith has written a chapter on computer analysis of DNA/protein sequences. As computer literacy is an integral part of the modern scientific approach, we include several hands-on computer sessions in our course in which students enter and manipulate sequence information. These computer exercises are specific for our particular program and computer configuration and thus are not included in this manual.

We have designed this manual for those who have a background in molecular biology but have limited experience in manipulating DNA molecules. As such, this book should also prove useful to the advanced undergraduate or beginning graduate student. The techniques described in the laboratory exercises and those in the appendix will allow this manual to be useful beyond the classroom situation. It should be particularly helpful to established investigators who are changing their research focus. In the revised edition we have included additional appendix procedures on electroporation, rapid plasmid minipreps, double-stranded DNA sequencing, and DNA labeling by the random priming method.

The experiments are designed to be included in a semester-long course that meets twice weekly for three hours per session. As a guide to the instructor, we have included the schedule we utilize for teaching our course. This can, of course, be modified to accommodate the quarter system or laboratory periods of a different length than ours.

The laboratory course (along with a lecture course we teach) serves as the major element of a Recombinant DNA Certificate program at San Diego State University. We began the program in 1984, and many of our students have gone on to advanced degree programs or careers in the biotechnology industry. In designing such a program, we regard it as essential that both technological and basic research advances be presented to the students. A lecture course is necessary for providing such a background, and we urge prospective instructors to incorporate oral and written student presentations and analysis of journal articles as a major component of such a course.

We are indebted to members of the Biology Department at SDSU and Deans Donald Short and James Neel for helping us to initiate the Recombinant DNA Certificate program. The contributions of several graduate assistants (Teresa Larsen, Elisabeth Roche, Martine Uittenbogaard, Rafael Soto-Gil, Sandy Putirka, Patrick O'Donnell, David Becker, Annabeth Fieck, and Paul Thomas) who aided us in designing and troubleshooting the laboratory experiments have been essential to the success of our course. We also thank Robert Phelps, whose organizational and preparative skills have made the laboratory class flow much more easily. We are pleased that International Biotechnology Incorporated (a Kodak company) of New Haven, Connecticut has sponsored our laboratory course. We are indebted to Matthew Biery, Michael McCaffery, William Kronert, Phillip Singer, and Kouakou Golly for their help in preparing some of the figures and to Anne Chiaramello, Paul Thomas, and Norbert Hess for help in preparing the protocols in the appendix. In revising the first edition, we included numerous updates, changes, and corrections to the procedures. We appreciate Dr. Phillip Singer's invaluable suggestions in this regard. Finally, we thank Charles Arthur, who as editor for this book always had encouragement when we most needed it.

Judith W. Zyskind
Sanford I. Bernstein

# SCHEDULE OF LABORATORY EXERCISES

| Period | Lab I | Lab II | Lab III | Lab IV | Lab V | Lab VI | Appendix |
|---|---|---|---|---|---|---|---|
| 1 | (Introduction) | | | | | | |
| 2 | Day 1 | | | | | | |
| 3 | Day 2 | Day 1 | | | | | |
| 4 | | Day 2 | Day 1 | | | | |
| 5 | | Day 3 | Day 2 | Day 1 | | | |
| 6 | | Day 4 | | Day 2 | | | |
| 7 | | Day 5 | | | Day 1 | | |
| 8 | | | | | Day 2 | Day 1. Part A | |
| 9 | | | | | Day 3 | Day 1. Part B | |
| 10 | | | | | Day 4 | Day 2. Part A | |
| 11 | | | | | Day 5 | Day 2. Part B<br>Day 3 | |
| 12 | | | | | | Day 4 | |
| 13 | | | | | | Day 5 | |
| | | | | | | Day 6 | |

*Schedule of Laboratory Exercises*

| Period | Lab VI | Lab VII | Lab VIII | Lab IX | Lab X | Appendix |
|---|---|---|---|---|---|---|
| 15 | Day 7 | | | | | Large Scale Plasmid Prep Day 2 |
| 16 | (Checkout) | Day 1 | | | | Day 3 |
| 17 | | Day 2 | | | | Day 4 |
| 18 | | Day 3 | | | | Day 5 |
| 19 | | Day 4 | | | | Day 6 |
| 20 | | Day 5 | | A. Day −1 | | Day 7 |
| 21 | | | | A. Day 0 | | |
| 22 | | | Day 0 | A. Day 1<br>B. Day 1 | Day 1 | |
| 23 | | | Day 1 | A. Day 2<br>B. Day 2 | Day 2 | |
| 24 | | | Day 2 | B. Day 3 | | |
| 25 | | | Day 3 | B. Day 4 | | |
| 26 | | | Day 4 | B. Day 5 | | |
| 27 | | | Day 5 | | | |
| 28 | (Checkout) | | | | | |

# CONTENTS

# BACTERIAL GROWTH PARAMETERS

It is often important to be able to determine three properties of a bacterial cell culture: its phase of growth, the growth rate, and the cell concentration. Generally, because experiments performed on exponentially growing cells are most readily reproducible, many procedures start with cells in this phase of growth. Also, the success of many DNA isolation procedures depends upon using a given number of cells. This experiment will provide you with the tools for easily determining these parameters for a particular strain of bacteria grown in a specific medium at a certain temperature. The approach is to correlate the number of cells in a growing culture of *Escherichia coli* with the optical density (OD) at 450 nm of that culture and, by following the changes in $OD_{450}$ and cell number during growth, to determine the growth rate. In future experiments, if the strain of *E. coli*, the growth medium, the temperature at which the cells are grown, and the wavelength you use to determine the culture turbidity all remain unchanged, then the growth phase, growth rate, and cell density can be determined by simply following the $OD_{450}$ of the cell culture. If you always start with a culture grown overnight (overnight culture) under the same conditions and inoculate the new culture from the overnight culture using the same dilution factor, then you can predict at approximately what time the culture will be ready for the experiment.

In this experiment, the OD of the bacterial culture is measured in a spectrophotometer. The OD of a culture is primarily the result of light scattering by the bacterial cells, because most bacteria do not absorb visible light. When the OD is plotted on the log scale and time is plotted on the linear scale of semilog paper, it is easy to estimate the time it takes for the OD to double, and because OD is directly proportional to cell number in the exponential growth phase, this is the generation time ($g$) of the culture.

**DAY 1**

# Measuring Bacterial Cell Growth

## Materials

- ❒ Exponential phase culture of *E. coli* strain LE392 *lacY galK2 galT22 metB1 trpR55 supE44 supF58 hsdR514* ($r_k^-$, $m_k^+$)
- ❒ 50 ml of sterile LB medium in a 250-ml culture flask
- ❒ LB agar plates
- ❒ Dilution tubes (sterile)
- ❒ Sterile M9 salts for making dilutions (6 g/l $Na_2HPO_4$, 3 g/l $KH_2PO_4$, 0.5 g/l NaCl, 1 g/l $NH_4Cl$)
- ❒ Glass petri plates containing 95% ethanol
- ❒ Bent glass rods for spreading bacteria on plates
- ❒ 37°C shaker incubator
- ❒ Spectrophotometer
- ❒ 37°C air incubator
- ❒ 5 ml pipets

**Note**: An overnight culture is made by inoculating a small volume of culture medium (5–10 ml) with cells from a single colony. After the culture has grown overnight, it is then diluted (usually 1:100) into fresh medium and grown to exponential phase.

## Protocol

1. Add 1 ml of the exponential phase culture of *E. coli* strain LE392 to 50 ml sterile LB broth. Start timing the age of the culture at this point, and shake the culture at 37°C, except when samples are removed for optical density (OD) measurements and viable cell counts.

**Note**: The starting culture is in exponential phase in order to minimize the length of the lag phase so that the experiment can be accomplished in 3–4 hours. Normally, you would be diluting from an overnight culture if you were in an experimental laboratory.

2. Measure the OD at 450 nm of a 5.0-ml aliquot of the *E. coli* strain LE392 culture at 0 time and at 30, 60, 90, and 120 minutes to determine turbidity.

3. Plate dilutions of the culture as described in Table 1-1 to determine cell number. In plating dilutions, 0.1 ml of the dilution is spread on the agar plate. Thus, for a final dilution of $10^{-5}$, you would plate 0.1 ml of the $10^{-2}$ dilution. Spread the bacteria around the agar plate using a bent glass rod that you have sterilized by dipping in ethanol and flaming. To prevent a fire, never return the glass rod to the ethanol solution until the flame is extinguished.

4. Incubate the plates inverted at 37°C overnight or at room temperature for two days.

**Note:** The OD of LB varies from batch to batch. To get an accurate 0 time measurement, use LB as a blank from the same batch as the growth media.

**Caution:** In plating dilutions, sterility must be maintained. For example, if you placed the 5 ml for the OD reading in an unsterile spectrophotometer tube and then removed cells for dilution from this tube, your dilutions would be contaminated.

**Table 1-1. Plating Procedure for Determining Cells/ml**

| Time | Dilutions to make | Final dilution after spreading 0.1 ml of last dilution on plates |
|---|---|---|
| 0, 30, 60 minutes | $10^{-3}$ (1:100, 1:10) | $10^{-4}$ |
| | $10^{-4}$ (1:100, 1:10, 1:10) | $10^{-5}$ |
| | $10^{-5}$ (1:100, 1:10, 1:10, 1:10) | $10^{-6}$ |
| 90, 120 minutes | $10^{-4}$ (1:100, 1:100) | $10^{-5}$ |
| | $10^{-5}$ (1:100, 1:100, 1:10) | $10^{-6}$ |
| | $10^{-6}$ (1:100, 1:100, 1:10, 1:10) | $10^{-7}$ |

**DAY 2**

# Plotting Cell Growth Data

## Protocol

1. Count the colonies on the plates. Plot the following:
   a. $OD_{450}$ ($y$) vs. time ($x$) on semilog graph paper;
   b. cells/ml ($y$) vs. time ($x$) on semilog graph paper;
   c. $\log_{10}$ cells/ml ($y$) vs. time ($x$) on linear graph paper;
   d. $OD_{450}$ ($y$) vs. cells/ml ($x$) on linear graph paper. Draw a line through the data points after you calculate the slope ($m$) using the least-squares equation.

$$m = \frac{\Sigma(x)\Sigma(y) - n\Sigma(xy)}{[\Sigma(x)]^2 - n\Sigma(x^2)} \qquad (1\text{-}1)$$

   The intercept, $b$, is expected to be 0 for the definition of this line, $y = mx + b$; $n$ is the number of data points. In the future, you can use the slope of this line, $m$, to calculate the number of cells present in your culture from $OD_{450}$ measurements.

2. Calculate the time it takes for the cell number to double (doubling or generation time, $g$) from the following equation using either the cell number at 60 minutes for $N_0$ and the cell number at 90 minutes for $N$, or, if these values do not fall on the linear part of the growth curve in the exponential growth phase, use values for $N_0$ and $N$ that do. See Ingraham *et al.* (1983) for a detailed explanation of the exponential growth phase.

$$\ln N - \ln N_0 = kt \qquad g = 0.693/k \qquad (1\text{-}2)$$

3. Confirm that $g$ is correct from the graphs drawn in steps 1a and 1b. Indicate different growth phases present in graphs a, b, and c as shown in Fig. 1-1.

**Note**: There are two possible reasons why your data may not fit a straight line in the plot of $OD_{450}$ vs. cells/ml: (1) the size of the cell changes throughout the cell cycle; cells in the lag and stationary growth phases are smaller than those in the exponential phase. (2) At higher cell densities, a ray of light has an increased probability of being doubly scattered so that the chance of reaching the photodetection system is increased. You may, therefore, want to connect the data points and use this line in the future to determine the cell density from OD measurements rather than using the slope obtained from the least-squares equation.

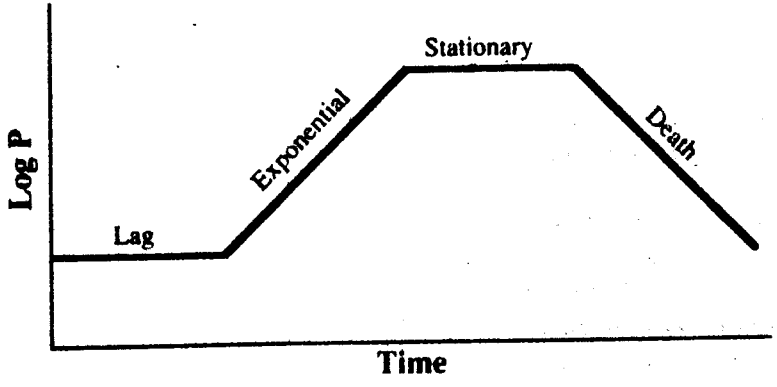


**Figure 1-1**. Growth curve of a bacterial culture showing lag, exponential, stationary, and death phases. P refers to any parameter of biomass being measured including viable cell number, dry weight, $OD_{450}$, or some cell component such as DNA, RNA, or protein. The transition from one growth phase to another varies, depending upon the parameter being measured. This is especially true for cell mass and cell number. During the lag phase, the increase in cell mass occurs much earlier than cell division, and in the transition to the stationary phase, the increase in cell mass falls behind the rate of cell division. However, during exponential growth, cell mass and cell number increase at the same rate. These differences should be reflected in the growth curves derived from $OD_{450}$ (a) or cell number (b and c) measurements if you start with an inoculum from an overnight culture and follow the growth of the culture into the stationary phase.

4. Determine how you would use the data from this experiment in the future when a procedure calls for *E. coli* cells in a certain phase of growth at a certain cell density.

## ADDENDUM

### A. Choosing an *E. coli* strain for cloning

The most important phenotype required for successful cloning of heterologous DNA into *E. coli* cells involves the *Eco*K restriction–modification system encoded on the *E. coli* chromosome. There are three genes in the *Eco*K restriction–modification system: *hsdR*, *hsdM*, and *hsdS*. Mutations in the *hsdR* gene are deficient in the *Eco*K endonuclease activity but still retain the *Eco*K methylase activity giving the phenotype r⁻ m⁺. Mutations in either *hsdM* or *hsdS* lead to loss in both the endonuclease and methylase and have the phenotype r⁻ m⁻. The optimum phenotype is r⁻ m⁺, because DNA isolated from this strain will be methylated at *Eco*K sites and can be introduced into r⁺ m⁺ *E. coli* strains without concern for degradation by the *Eco*K endonuclease. Although heterologous DNA can be successfully cloned in *hsdM* or *hsdS* mutants, DNA isolated from these mutants is not methylated at *Eco*K sites and will be degraded just as foreign DNA will be when introduced into r⁺ m⁺ *E. coli* strains. The optimum mutation, therefore, is in the *hsdR* gene, which is in the strain used in this experiment, LE392. See Raleigh (1987) for a discussion of the interference with cloning caused by the r⁺ phenotype in *E. coli*.

Other *E. coli* mutations of interest and concern when cloning include:

1. *recA* mutants. Mutations in the *recA* gene lead to loss of 98% of homologous recombination activity. This is an especially important mutation to have in the recipient strain when cloning homologous DNA, for example, when cloning *E. coli* genes, when maintaining two plasmids with regions of homology, or to prevent loss of direct repeats.
2. *polA* mutants. The *polA* gene codes for DNA polymerase I, an enzyme required for the ColE1-type plasmid of replication. If you are us-