Molecular Cloning

A LABORATORY MANUAL

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Front cover: The electron micrograph of bacteriophage λ particles stained with uranyl acetate was digitized and assigned false color by computer. *Thomas R. Broker, Louise T. Chow, and James I. Garrels*

Back cover: E. coli DH1 with fimbriae was negatively stained with phosphotungstic acid and the electron micrograph was digitized and assigned false color by computer. *Jeffrey A. Engler, Thomas R. Broker, and James I. Garrels*

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Preface

This manual began as a collection of laboratory protocols that were used during the 1980 Cold Spring Harbor course on the Molecular Cloning of Eukaryotic Genes. These procedures had been in use in our laboratories at that time but were scattered throughout the notebooks of many different people. In 1981 we decided to produce a more complete and up-to-date manual not only for use in the next Cold Spring Harbor course, but also for eventual publication. Out of the many permutations of the methods being used, we assembled a set of "consensus protocols," which were photocopied and widely distributed to many laboratories even as the 1981 course was underway. Then in the winter of 1981-1982, the manual was substantially rewritten, and new or revised protocols and figures, as well as entirely new chapters, were added.

Even since this last rewriting, however, the field has progressed: New methods are constantly being invented and existing techniques are altered in response to changing needs. Although we have included in this manual only those protocols that have been thoroughly tested and used successfully in our laboratories, we make no claim that they are inviolable or perfect. We would welcome suggestions for improvements, and we would be grateful to be told about any new procedures that are devised.

The evolution of protocols poses the difficult problem of attribution. We have tried to give credit at appropriate places in the text to the people who originally developed the procedures presented here, but in many cases tracing a particular method to its undisputed roots has proved to be impossible. We therefore wish to apologize—and to express gratitude—to those we have been unable to acknowledge for an idea, procedure, or recipe. Our major function has been to compile, to verify, and, we hope, to clarify; less frequently we have introduced modifications, and only in rare instances have we devised new protocols. In large part, then, the manual is based on procedures developed by others, and it is to them that any credit belongs.

Because the manual was originally written to serve as a guide to those who had little experience in molecular cloning, it contains much basic material. However, the current version also deals in detail with almost every laboratory task currently used in molecular cloning. We therefore hope that newcomers to cloning and veterans alike will find material of value in this book.

Although molecular cloning seems straightforward on paper, it is more difficult to put into practice. Most protocols involve a large number of individual steps and a problem with any one of them can lead the experimenter into difficulty. It is a good idea to verify the products of each step and to include controls to check the efficiency of each reaction. To deal with these problems, a well-founded understanding of the principles underlying each

procedure is essential. We have therefore provided background information and references that may be useful should trouble occur.

This manual could not have been written without the help and advice of members of our laboratories and contributions from many others. We therefore wish to thank Joan Brooks, John Fiddes, Mary-Jane Gething, Tom Gingeras, David Goldberg, Steve Hughes, David Ish-Horowicz, Mike Mathews, Patty Reichel, Joe Sorge, Jim Stringer, Richard Treisman, and Nigel Whittle. We wish particularly to thank Arg Efstratiadis for his helpful discussions and criticisms of Chapter 7; Brian Seed for permission to include a description of his unpublished procedure for screening libraries by recombination (Chapter 10) and many other useful suggestions; Doug Hanahan for advice on transformation (Chapter 8); Bryan Roberts for suggestions on methods of hybrid-selection and cDNA cloning; Doug Melton for providing a protocol for injection of *Xenopus* oocytes; Ronni Greene for suggesting improvements to many protocols; Nina Irwin for providing a critical anthology of methods available for expressing eukaryotic proteins in bacteria (Chapter 12); Rich Roberts for supplying the computer analysis of the sequence of pBR322; Barbara Bachmann and Ahmad Bukhari for reviewing and correcting the list of *E. coli* strains; and Tom Broker, Louise Chow, Jeff Engler, and Jim Garrels for producing the elegant photographs used for the front and back covers.

We also thank all those who participated in the Cold Spring Harbor Molecular Cloning courses of 1980 and 1981. They were an excellent group of students, who struggled through the first two drafts of the manual and made many useful suggestions. We also thank Nancy Hopkins, who helped us to teach the course the first year and convinced us that producing a manual would be a worthwhile task. In 1981 Doug Engel helped teach the course and suggested many improvements to the manual. Contributing to the success of both courses were the efforts of the teaching assistants, who were Catherine O'Connell and Helen Doris Keller in the summer of 1980 and Susan Vande-Woude, Paul Bates, and Michael Weiss in 1981.

We wish to thank Patti Barkley and Marilyn Goodwin for their cheerfulness and forbearance during the typing of successive revisions of the manuscript. Our artists, Fran Cefalu and Mike Ockler, worked with great dedication and perseverance to produce the drawings for the manual. Joan Ebert kept track of the many references added to and deleted from the text and assembled the reference list. We are also grateful to Nancy Ford, Director of Publications, Cold Spring Harbor Laboratory, for her encouragement and support. Finally, without the patience, skill, and diplomacy of Doug Owen, who prepared the manuscript for the printer and helped us in many other ways, this book would not exist.

Tom Maniatis
Ed Fritsch
Joe Sambrook

Contents

1 Vector-Host Systems

Four types of vector can be used to clone fragments of foreign DNA and propagate them in *Escherichia coli:*

plasmids
bacteriophage λ
cosmids
bacteriophage M13

Although very different in size and structure, these four types of vector share the following properties:

1. They can replicate autonomously in *E. coli* (i.e., they are replicons in their own right), even when joined covalently to a foreign DNA fragment.

2. They can be easily separated from bacterial nucleic acids and purified.

3. They contain regions of DNA that are not essential for propagation in bacteria. Foreign DNA inserted into these regions is replicated and propagated as if it were a normal component of the vector.

Each type of vector has particular biological features that make it useful for different purposes. In this chapter we describe these cloning vectors and discuss the principles of their application to problems of molecular cloning.

PLASMIDS

Plasmids are extrachromosomal genetic elements found in a variety of bacterial species. They are double-stranded, closed circular DNA molecules that range in size from 1 kb to greater than 200 kb. Often, plasmids contain genes coding for enzymes that, under certain circumstances, are advantageous to the bacterial host. Among the phenotypes conferred by different plasmids are:

resistance to antibiotics
production of antibiotics
degradation of complex organic compounds
production of colicins
production of enterotoxins
production of restriction and modification enzymes

Under natural conditions, many plasmids are transmitted to new hosts by a process similar to bacterial conjugation. In the laboratory, however, plasmids can be transferred to bacteria by an artificial process, known as transformation, in which they are introduced into bacteria that have been treated in ways that make some of the cells temporarily permeable to small DNA molecules. The new phenotype conferred upon the recipients by the plasmid (e.g., resistance to an antibiotic) allows simple selection of bacteria that have been successfully transformed.

For the most part, replication of plasmid DNA is carried out by the same set of enzymes used to duplicate the bacterial chromosome. Some plasmids are under "stringent control," which means that their replication is coupled to that of the host so that only one or at most a few copies of the plasmid will be present in each bacterial cell (for review, see Novick et al. 1976). Plasmids under "relaxed control," on the other hand, have copy numbers of 10–200. More importantly, the copy number of "relaxed" plasmids can be increased to several thousand per cell if host protein synthesis is stopped (e.g., by treatment with chloramphenicol) (Clewell 1972). In the absence of protein synthesis, replication of relaxed plasmids continues, whereas replication of chromosomal DNA and of stringent plasmids ceases.

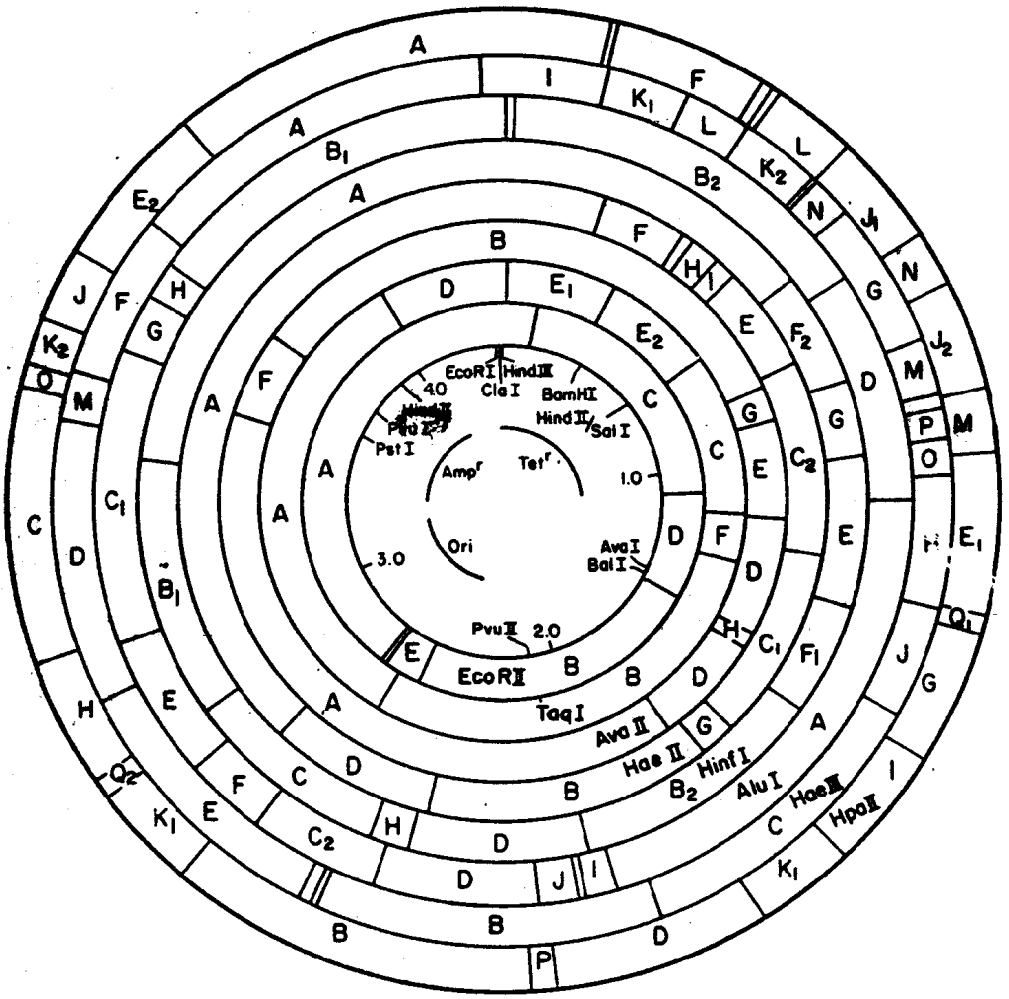
To be useful as a cloning vector, a plasmid should possess several properties. It should be relatively small and should replicate in a relaxed fashion. It should carry one or more selectable markers to allow identification of transformants and to maintain the plasmid in the bacterial population. Finally, it should contain a single recognition site for one or more restriction enzymes in regions of the plasmid that are not essential for replication. Preferably, these restriction sites, into which foreign DNA can be inserted, should be located within the genes coding for selectable markers so that insertion of a foreign DNA fragment inactivates the gene.

Below we describe a number of versatile cloning vectors that embody many of these properties (for reviews, see Bolivar and Backman 1979 and Bernard and Helinski 1980). The most widely used vector is pBR322, a plasmid under relaxed control that contains both ampicillin- and tetracycline-resistance genes and a number of convenient restriction sites (Bolivar et al. 1977) (see page 5). The complete nucleotide sequence of pBR322 is known (Sutcliffe 1978) and is given in Appendix B.

Recently, two derivatives of pBR322 have become available that have the advantage of replicating to even higher copy numbers. One derivative, pAT153, was constructed (Twigg and Sherratt 1980) by deleting the *Hae*IIB and G (A. Cowie and E. Ruley, pers. comm.) fragments, which span a region of the plasmid genome involved in control of copy number (see page 6). About 1.5 to 3.0 times as many copies of pAT153 are present per cell than pBR322. The second derivative, pXf3 (D. Hanahan, unpubl.), is even smaller than pAT153 (see page 6).

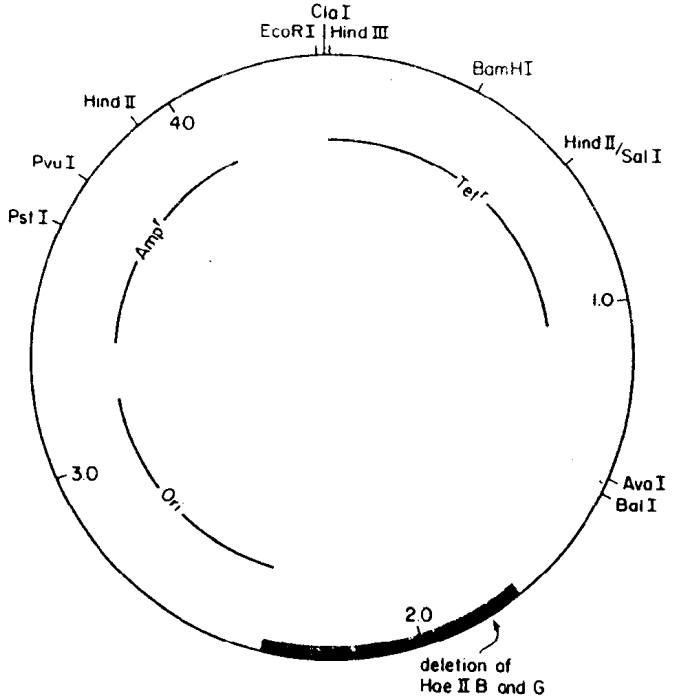
The advantages of small size are manifold: The plasmid DNA is easier to handle in that it is less susceptible to physical damage, and it has a simpler restriction map. Furthermore, the fact that smaller plasmids generally have higher copy numbers increases the sensitivity with which bacteria carrying foreign DNA sequences can be identified using radiolabeled hybridization probes. However, reduction in size may lead to the elimination of useful cloning sites. pXf3, for example, lacks the *Ball* and *Aval* sites that are present in pBR322 and pAT153. To extend the range of useful cloning sites, polylinkers have been inserted into several small plasmids. Polylinkers are segments of DNA that contain closely spaced sites for several restriction enzymes. An example of a plasmid, plink322 (B. Seed, unpubl.), carrying a polylinker is shown on page 7.

Also shown are diagrams of plasmids that, although not as widely used as pBR322 and its derivatives, are extremely useful for particular cloning purposes. pMK16 (see page 7), for example, contains single *Sma*I and *Xho*I restriction sites in a gene coding for kanamycin resistance. pKC7 and pACYC184 (see page 8) carry useful cloning sites within a gene coding for chloramphenicol resistance. The large plasmids pCRl (11.4 kb) and pSC101 (9.9 kb; see page 9) are not routinely used in cloning experiments but have the unique property of not hybridizing to one another. Thus, it is possible to clone DNA fragments from one source in pSC101 and from another source in pCRl and then carry out cross-hybridization experiments between the inserted DNAs without interference from plasmid DNA sequences. Perhaps the most specialized of all plasmids is πVX, which is used to select from a population of recombinant λ bacteriophages those phages that contain DNA sequences homologous to a foreign DNA segment inserted into the plasmid (B. Seed, unpubl.) (see page 10).



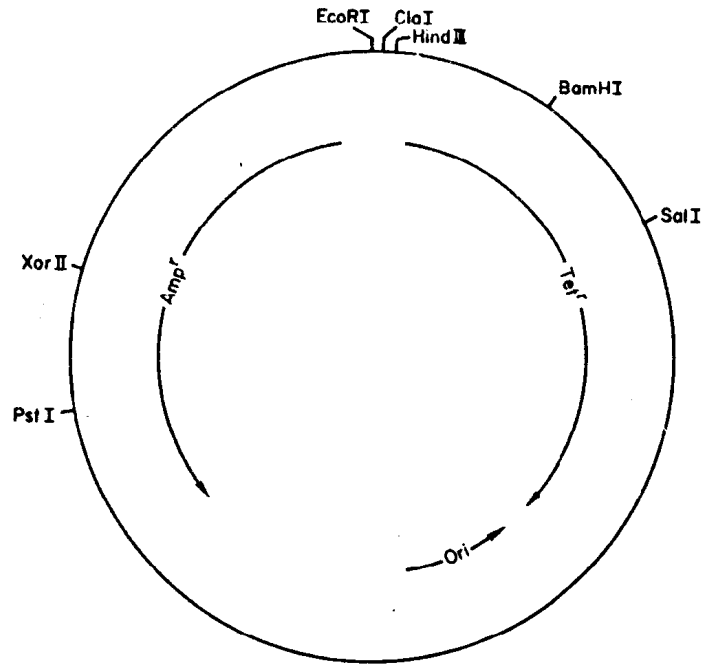
pBR322

Size: 4.3 kb
Replicon: ColE1, relaxed
Selective markers: Amp^r, Tet^r
Single sites: Ava I, Pst I, BamHI, Pvu II, Cla I, Sal I, EcoRI, Hind III
Insertional inactivation: Amp^r - Pst I
Tet^r - BamHI, Hind III (variable), Sal I
References: Bolivar et al. (1977); Sutcliffe (1978, 1979).
Comments: pBR322 is the most versatile of the plasmid cloning vectors. Its complete nucleotide sequence is known (Sutcliffe 1979).



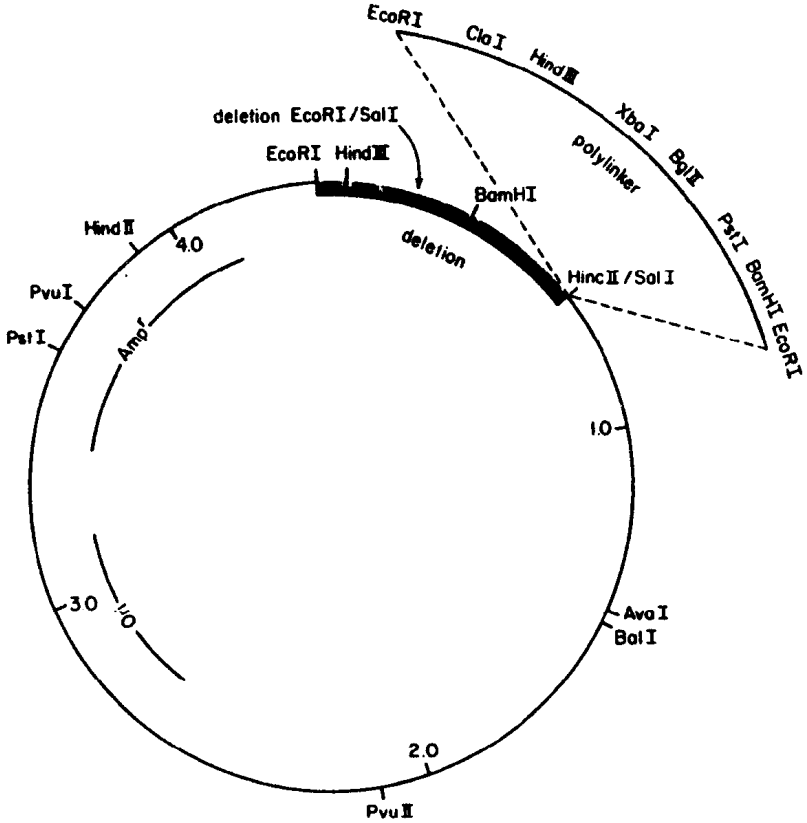
pAT153

Size: 3.6 kb
Replicon: ColE1, relaxed
Selective markers: Amp^r, Tet^r
Single sites: AvaI, PstI, BamHI, ClaI, SalI, EcoRI, HindIII
Insertional inactivation: Amp^r - PstI
Tet^r - BamHI, HindIII (variable), SalI
Reference: Twigg and Sherratt (1980)
Comment: A high-copy variant of pBR322



pXf3

Size: 3.16 kb
Replicon: ColE1, relaxed
Selective markers: Amp^r, Tet^r
Single sites: EcoRI, ClaI, HindIII
Insertional inactivation: Tet^r - BamHI, SalI
Amp^r - PstI, XorII
Reference: Sutcliffe (1978), D. Hanahan (pers. comm.)
Comment: pXf3 is a derivative of pBR322 in which the ThaI-A fragment containing the replicon was fused to an AvaI to Sau3A fragment that encodes resistance to ampicillin and tetracycline, generating a plasmid of ~3160 bp.



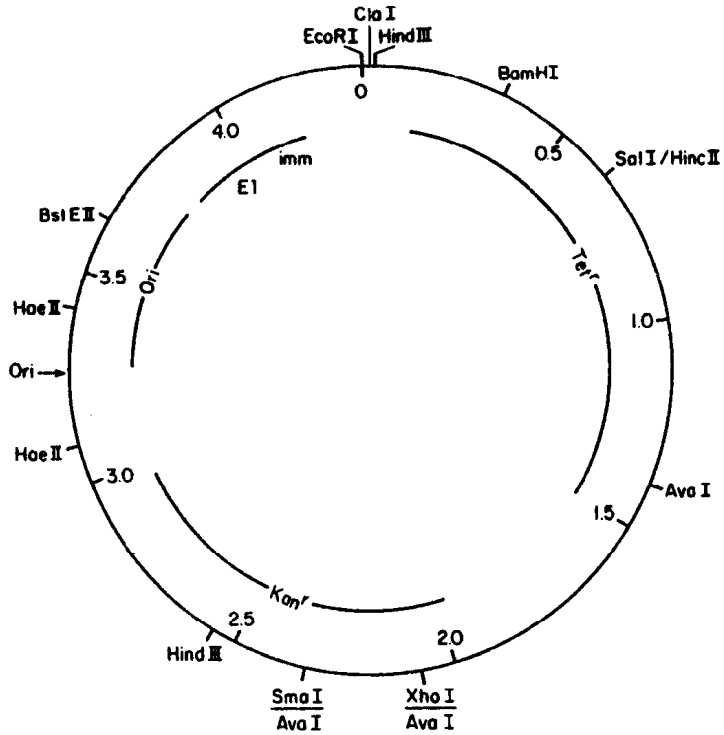
plink 322

Size: 3.8 kb
Replicon: ColE1, relaxed
Selection: Amp^r
Single sites: ClaI, HindIII, XbaI, BglII, BamI
plink322, a variant of pBR322, contains a polylinker which increases the number of useful cloning sites.
Reference: B. Seed (unpubl.)
Polylinker sequence:

GAATTCTCATGTTTGACAGCTTATCATCGATAAGCTTCTAGAGATCT
EcoRI ClaI HindIII XbaI BglII

TCCATACCTACCAGTTCTCCGCCTGCAGCAATGGCAACAACGTTGCC
PstI

CGGATCCGGTCGCGCGAATTC
BamHI EcoRI



pMK16

Size: 4.6 kb
Replicon: ColE1, relaxed
Selective markers: ColE1 imm, Kan^r, Tet^r
Single sites: BamHI, SmaI, EcoRI, XhoI, HincII, SalI, BstEII,
Insertion into the BstEII site interferes with replication.
Insertional inactivation: Tet^r - BamHI, HincII, SalI
Kan^r - SmaI, XhoI
Insertion of foreign DNA into the XhoI site inactivates the Kan^r gene.
Reference: Kahn et al. (1979).
Comment: The best available vector for cloning fragments of DNA with SmaI or XhoI termini.