



# 噬菌体表达

Phage Display: A Laboratory Manual

## 实验室手册

Carlos F.Barbas III

Dennis R.Burton

Jamie K.Scott

Gregg J.Silverman

世界图书出版公司



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*Simon Fraser University*

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*University of California, San Diego*

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### Associate Authors

Carlos F. Barbas III  
*The Scripps Research Institute*

Jamie K. Scott  
*Simon Fraser University*

Dennis R. Burton  
*The Scripps Research Institute*

Gregg J. Silverman  
*University of California, San Diego*

Developmental Editor Tracy Kuhlman

Project Coordinator Mary Cozza

Production Editor Patricia Barker

Desktop Editor Danny deBruin

Interior Designer Denise Weiss

Cover Designer Tony Uργο

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# Preface

**M**OLECULAR RECOGNITION IS CENTRAL TO BIOLOGY, and the discovery and characterization of interacting partners are major endeavors of biological scientists. Phage display, largely developed in the 1990s, has begun to make critical contributions to these endeavors. The approach is based on two pivotal concepts. The first is that phage, viruses that infect bacteria, can be used to link protein recognition and DNA replication. The protein (or peptide) is displayed on the surface of the phage particle and the genes encoding it are contained within the particle. The second concept is that large libraries of the DNA sequences encoding these molecules can be cloned into phage. Individual phage can then be rescued from libraries by virtue of interaction of the displayed protein with the cognate ligand, and the phage can be amplified by infection of bacteria.

The broad strategy is one that was adopted long ago by nature in the immune system. There, vast immune repertoires or libraries of molecules (antibodies, T-cell receptors) permit recognition of virtually any foreign entity. Protein recognition and replication are then linked; for example, when specific antibody-producing cells are stimulated to divide by interaction of antigen and antibody cell-surface receptors for antigen. The result is a system for efficiently generating molecular species capable of specifically recognizing almost any molecular shape.

In 1985, George Smith first showed that the linkage between phenotype and genotype could be established in filamentous bacteriophage and gave birth to the new technology of phage display. Smith showed that foreign DNA fragments could be inserted into filamentous phage *gene III*, which codes for the phage coat protein pIII, to create a fusion protein with the foreign sequence in the amino-terminal domain. The fusion protein was incorporated into the virion, which retained infectivity and displayed the foreign peptide in a form accessible to specific antibody to the peptide. This "fusion phage" could be greatly enriched relative to ordinary phage by affinity selection on immobilized antibody (a process usually termed "panning"). Subsequently, in 1990, Scott and Smith, Dower and colleagues, and Devlin and colleagues independently cloned libraries of peptides and showed that peptides of specific activity could be retrieved from these libraries by panning. Concurrent with these developments, in 1989, Richard Lerner and colleagues reported that libraries of randomly recombined

antibody heavy and light chains could provide an alternative route to monoclonal antibodies of defined specificity. These studies were performed by cloning into phage lambda and involved plaque screening rather than phage display. The expression of proteins such as antibodies as phage-displayed libraries followed shortly thereafter. These two types of libraries created an explosion of activity in the area.

Despite rapid growth in the field, there has been a relative dearth of publications dealing with practical aspects of phage display. The technology has the reputation of requiring some considerable technical expertise. The aim of this manual is to provide comprehensive instruction in theoretical and applied aspects of phage-display technologies, so that any scientist with even modest molecular biology experience can effectively employ them.

This manual is the direct descendant of materials prepared for the Cold Spring Harbor Laboratory course on "Phage Display of Combinatorial Antibody Libraries." Following a conversation between Jim Watson of Cold Spring Harbor Laboratory and Richard Lerner of The Scripps Research Institute, the first course was presented by two of us (C.F.B. and D.R.B.) in the fall of 1992. Thanks to the outstanding support of the CSHL staff, the course was a success and has been modified every year since then to take account of the experience and comments of the students, and to reflect developments in the field. Much of this manual is thus the result of nearly a decade of experience with students of greatly varying technical expertise and experience from all over the world. All of these students made the writing of this manual possible. In addition to antibody libraries, the content of this manual has been expanded to include other types of libraries displayed on phage. We have included our most up-to-date laboratory protocols, and the accompanying didactic material provides all of the essential information and references needed by both the novice and the experienced practitioner of expression-cloning techniques to design experiments of their own.

This manual is divided into five sections. The first gives an overview of some of the key aspects of phage display. This manual was not intended to reproduce all the information contained in the many excellent reviews that are currently available. Rather, we felt it appropriate to present the more important concepts in phage display in one text as a background for understanding the practical approaches described. Thus, this section reviews phage structure, genetics, and physiology and, within this context, presents the phage vectors. The crucial features of antibody, peptide, protein fragment, and cDNA libraries are also summarized, and the section ends with an overview of emerging technologies.

The second section deals with the construction, screening, and analysis of antibody libraries on the surface of phage. These libraries are expressed as fusions with the pIII coat protein of filamentous phage. This section includes chapters on the production and purification of recombinant antibody fragments, as well as antibody engineering and the construction of specialty libraries.

The third section deals with the construction, screening, and analysis of phage-displayed peptide libraries. The focus is principally on the use of peptide expressed as fusions with the pVIII phage coat protein. A chapter is also included that considers peptide libraries constructed by fusion with the pIII phage coat protein.

The fourth section covers the construction, screening, and analysis of gene-fragment and cDNA-expression libraries. It also deals with affinity selection of libraries with more complex targets (namely, cells) as well as *in vivo* selection techniques. The final section includes a number of appendices that summarize commonly used experimental procedures, data, recipes, suppliers, and important precautions.

We gratefully acknowledge the excellent contributions of our friends and collaborators who took the time to share their practical experience. They have made important contributions to the CSHL Antibody Library course and have provided outstanding chapters to this manual.

We acknowledge Dr. George Smith, from whose work all phage-display methods derive. He demonstrated the concept of phage display using expressed cDNA fragments on phage, developed affinity-selection methods for phage, and invented the concept of peptide libraries on filamentous phage. With outstanding collegiate spirit, he has freely shared all of his inventions, vectors, and libraries with the scientific community.

We are indebted to Dr. Richard Lerner for his contributions to and support of this work, and especially for his longstanding vision of antibody libraries. He fostered the group at The Scripps Research Institute that designed and constructed the first Fab libraries from immune antibody responses. That work, along with the pComb3 phage-display vector that emerged from it, has made possible the CSHL Antibody Display course and this manual. We acknowledge the help and support of John Inglis, Mary Cozza, Tracy Kuhlman, Danny deBruin, and Pat Barker, who have nurtured this project to completion.

This work is dedicated to our teachers, colleagues, collaborators, and students for their many contributions over the years; and to our families for their patience, encouragement, and support.

**Carlos F. Barbas III**  
**Dennis R. Burton**  
**Jamie K. Scott**  
**Gregg J. Silverman**

# Abbreviations

(+)	viral strand of DNA
(-)	complementary strand of DNA
ABTS	2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)]
ANP	atrial nautreic peptide
APS	ammonium persulfate
AU <sub>260</sub>	absorbance unit at 260 nm
B	biotinylated
bp	base pair
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
C	complement
carb	carbenicillin
ccc	covalently closed circular
CD	circular dichroism
cDNA	complementary DNA
CDR	complementarity determining region
CFA	complete Freund's adjuvant
cfu	colony-forming unit
C <sub>H</sub>	constant region, heavy chain
C <sub>κ</sub>	constant region, kappa light chain
C <sub>L</sub>	constant region, light chain
CNTF	ciliary neurotrophic factor
CT	carboxy-terminal (domain of pIII)
CWS	cell wall skeleton
D	diversity (gene segment)
D1, D2, D3	domain 1, 2, 3
DIRE	direct interaction rescue
DMEM	Dulbecco's modified Eagle's medium
DMP	dimethyl pimelimidate
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DO	dissolved oxygen



dsDNA	double-stranded DNA
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
ERD	ERF repressor domain
Fab	fragment antigen binding
Fc	fragment crystalline
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FR	framework region
gIII	gene III
gVIII	gene VIII
GAP	glyceraldehyde-3-phosphate dehydrogenase promoter
GH	growth hormone
GST	glutathione S-transferase
H	heavy (chain)
HA	hemagglutinin
HABA	4'-hydroxyazobenzene-2-carboxylic acid
HCDR3	heavy chain CDR3
hGH	human growth hormone
HNE	human neutrophil elastase
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
IgG	immunoglobulin G
IL	interleukin
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
J	joining (gene segment)
J <sub>H</sub>	heavy-chain joining
J <sub>L</sub>	light-chain joining
Kb	kilobase
Kbp	kilobase pair
K <sub>d</sub>	dissociation constant
KRAB	Krüppel-associated box
L	light (chain)
LB	Luria broth
LCDR3	light chain CDR3
LES	lipid emulsion system
LL	long linker
mA	milliampere
mAb	monoclonal antibody
MBP	maltose-binding protein
moi	multiplicity of infection
MPL	monophosphoryl lipid A



mRNA	messenger RNA
MW	molecular weight
MWM	molecular weight marker
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
NPR	naturetic peptide receptor
NTA	nitrilotriacetic acid
oc	open-circular
OD	optical density
OD <sub>260</sub>	optical density at 260 nm
OMP	outer membrane protein
OPD	<i>o</i> -phenylenediamine
ORF	open reading frame
pIII	protein III
PA	plasminogen activator
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque-forming unit
PI	protease inhibitors
PMSF	phenylmethylsulfonyl fluoride
PPI	peptidylprolyl isomerase
PrP	prion protein
PS	packaging signal
PSM	prostate-specific membrane
PSTI	pancreatic secretory trypsin inhibitor
RBC	red blood cell
RF	replicative form
Rh	Rhesus
RNase	ribonuclease
Sa	streptavidin
SAP	selective amplification of phages
SAS	saturated ammonium sulfate
SB	super broth
scFv	single-chain Fv
SD	Shine-Dalgarno
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SID	mSIN3 interaction domain
SIP	selectively infective phages
SL	short linker
SLE	systemic lupus erythematosus
SM	screening molecule
SpA	staphylococcal protein A

SRP	signal recognition particle
ss	single-stranded
STM	<i>Salmonella typhimurium</i> mitogen
$t_{1/2}$	half-life
TDM	trehalose dicorynomycolate
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethylenediamine
tet	tetracycline
TPO	thrombopoietin
TU	transducing unit
V	variable gene segment
V <sub>H</sub>	variable domain, heavy chain
V <sub>L</sub>	variable domain, light chain
(v/v)	volume/volume
(w/v)	weight/volume
X	variable or randomized residue/amino acid

## Phage Display: A Laboratory Manual

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# 1 Filamentous Phage Biology

ROBERT WEBSTER

Duke University Medical Center, Department of Biochemistry, Durham,  
North Carolina 27710

THE FILAMENTOUS BACTERIOPHAGES (GENUS *INOVIRUS*) are a group of viruses that contain a circular single-stranded DNA genome encased in a long protein capsid cylinder. Many use some type of bacterial pilus to facilitate the infection process. The Ff class of the filamentous phages (f1, fd, and M13) have been the most extensively studied. As the name implies, these bacteriophage use the tip of the F conjugative pilus as a receptor and thus are specific for *Escherichia coli* containing the F plasmid. The DNA sequence of these three phages shows them to be 98% homologous; consequently, the protein sequences of the gene products are practically the same.

The Ff phages do not kill their host during productive infection. The single-stranded viral DNA is replicated via a double-stranded intermediate by a mixture of bacterial and phage-encoded components. The result of this replicative process is a newly synthesized viral single-stranded DNA in a complex with many copies of a phage-encoded single-stranded DNA-binding protein. The capsid proteins are all synthesized as integral membrane proteins that remain in the membrane until they are assembled around the DNA. Assembly occurs at specific sites in the bacterial envelope where the cytoplasmic and outer membranes are in close contact. During the assembly process, the viral DNA is extruded through the membrane-associated assembly site, where the phage DNA-binding proteins are removed and the capsid proteins are packaged around the DNA. This process continues until the end of the DNA is reached, so there is little if any constraint on the size of the DNA packaged. The bacteria tolerate this process quite well and continue to grow and divide with a generation time approximately 50% longer than that of uninfected bacteria. There is a burst of about 1000 phage particles produced in the first generation after infection, and then the bacteria produce about 100–200 particles per generation. This continues for many generations, resulting in titers of  $10^{11}$  to  $10^{12}$  particles per ml. The plaques are turbid and of varying size and contain about  $10^8$  infective phage particles.

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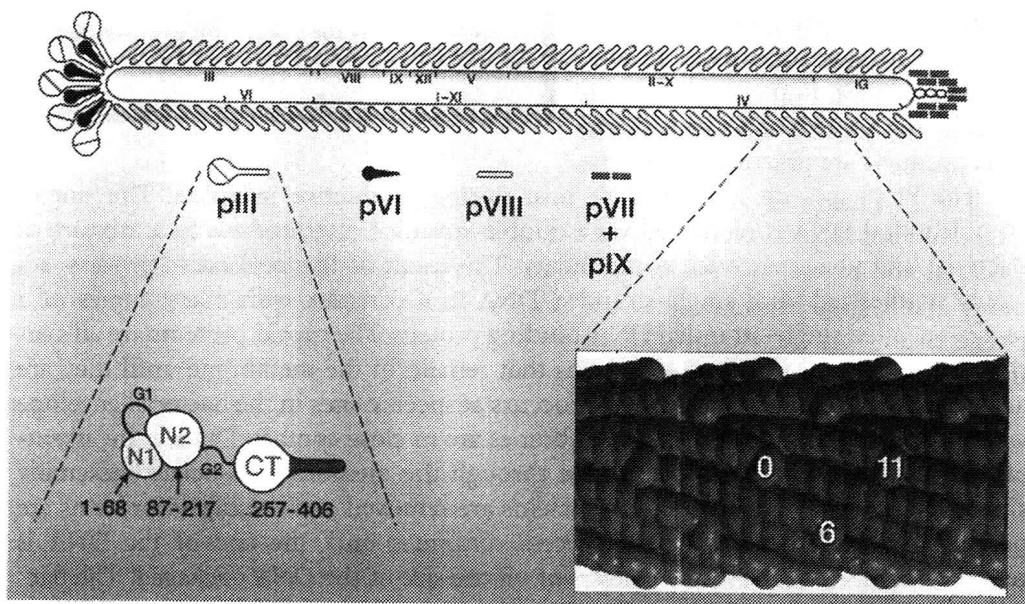
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The phage structure and its mode of replication have made it a valuable tool for biological research. Phage can be used as cloning vehicles, because insertion of DNA into a nonessential region of the phage genome results in a longer phage that contains a single-stranded copy of the inserted DNA. The ability to isolate the single-stranded viral DNA and its double-stranded replication intermediate makes it possible to easily create substrates for studying recombination and repair of mismatches in DNA. The membrane-associated assembly process has made it possible to display foreign peptides or proteins on the surface of the phage particle, as described in this manual. To aid in understanding the techniques involved in "phage display," this chapter describes aspects of the biology of the phage and bacteria. In the first section, the phage particle and its life cycle are described. The next section relates the phage life cycle to some of the basic principles involved in displaying proteins on the phage surface. Because the replication of phage is governed to a great extent by the physiology of bacteria, the last section briefly discusses some aspects of bacterial biology that can have a direct relation to the phage-display technique.

This chapter is intended to give the reader only an overview of the biology of the Ff bacteriophage. Therefore, it is brief and does not fully discuss all aspects of the subject or the many papers that have contributed to the study of this organism. In some



**Figure 1.1.** The Ff bacteriophage particle. Schematic representation of the phage particle showing the location of the capsid proteins and the orientation of the DNA. The lower left is a schematic of the structure of pIII. N1, N2, and CT refer to domains, and G1 and G2 refer to glycine-rich regions. The lower right is a representation of the orientation of the pVIII molecules along the cylinder part of the phage. (Adapted, with permission, from Marvin 1998 © Elsevier Science.) The three nearest neighbors indexed as 0, 6, and 11 are indicated. Because the amino-terminal regions face to the right, this depiction of the phage would have the cone end at the pVII–pIX end of the particle.

cases, conclusions may be stated that probably are correct but are not absolutely proven by the present experimental data. For readers wanting to explore a particular area more deeply, each section mentions a number of recent reviews or papers with good introductions related to the various topics discussed.

## THE Ff BACTERIOPHAGE

### Structure of the Bacteriophage

The Ff phage particle is approximately 6.5 nm in diameter and 930 nm in length (Fig. 1.1). The mass of the particle is approximately 16.3 MD, of which 87% is contributed by protein. The genome is a single-stranded, covalently closed DNA molecule of about 6400 nucleotides that is encased in a somewhat flexible protein cylinder. The length of the cylinder consists of approximately 2700 molecules of the 50-amino-acid major coat protein, also called *gene VIII* protein (pVIII). At one end of the particle, there are about 5 molecules each of the 33-residue *gene VII* protein (pVII) and the 32-residue *gene IX* protein (pIX). The other end contains approximately 5 molecules each of the 406-residue *gene III* and 112-residue *gene VI* proteins (pIII and pVI). The DNA is oriented within the virion such that a 78-nucleotide hairpin region called the packaging signal (PS) is always located at the end of the particle containing the pVII and pIX proteins.

There now exists a fairly complete description of the pVIII cylinder portion of the virion (Marvin et al. 1994; Overman and Thomas 1995; Williams et al. 1995; Marvin 1998). The pVIII monomers are present in the particle as an uninterrupted  $\alpha$ -helix except for the amino-terminal 5 residues. The proteins are arranged in an overlapping shingle-type array with a symmetry defined by a fivefold rotational axis with a twofold screw axis of pitch 3.2 nm (Fig. 1.1, lower right). The axis of the helical pVIII monomer is tilted approximately  $20^\circ$  to the long axis of the particle, gently wrapping around the long axis of the virus in a right-handed way. The pVIII molecules are packed quite tightly, as only the outside 3 residues are accessible to digestion by proteases (Terry et al. 1997). The carboxy-terminal 10–13 residues of pVIII form the inside wall of the cylinder. This region contains 4 positively charged lysine residues that reside on one face of an amphiphilic helix. These positive charges interact with the sugar phosphate backbone of the DNA that is present in the particle with the bases pointed inward (Greenwood et al. 1991a; Marvin et al. 1994). The amino-terminal portion of pVIII is present on the outside of the particle. The residues connecting the amino and carboxyl regions of pVIII interact with the same region of other pVIII molecules to form the stable inner core of the protein cylinder. Most of this middle portion of pVIII spans the cytoplasmic membrane before being assembled into phage particles.

One end of the particle has approximately 5 molecules each of the small hydrophobic pVII and pIX proteins. This end contains the PS and is the first part of the phage to be assembled. It is not known how these two proteins are arranged at the end of the phage or how they interact with the pVIII cylinder. Attempts to model the ends of the particle suggest that one of these proteins must be buried close to the DNA, whereas the other is exposed at the surface (Makowski 1992). The observation that antibodies to pIX but not pVII are able to interact with one end of the phage particle