Preface

Exciting new developments in recombinant DNA research allow the isolation and amplification of specific genes or DNA segments from almost any living organism. These new developments have revolutionized our approaches to solving complex biological problems and have opened up new possibilities for producing new and better products in the areas of health, agriculture, and industry.

Volumes 100 and 101 supplement Volumes 65 and 68 of *Methods in Enzymology*. During the last three years, many new or improved methods on recombinant DNA or nucleic acids have appeared, and they are included in these two volumes. Volume 100 covers the use of enzymes in recombinant DNA research, enzymes affecting the gross morphology of DNA, proteins with specialized functions acting at specific loci, new methods for DNA isolation, hybridization, and cloning, analytical methods for gene products, and mutagenesis: *in vitro* and *in vivo*. Volume 101 includes sections on new vectors for cloning genes, cloning of genes into yeast cells, and systems for monitoring cloned gene expression.

RAY WU
LAWRENCE GROSSMAN
KIVIE MOLDAVE

GERHARD SCHMIDT
1901–1981

Gerhard Schmidt (1901–1981)

This hundredth volume of *Methods in Enzymology* is dedicated to the memory of a dear friend and colleague whose pioneering work on the nucleic acids was important to the development of the techniques described in this and related volumes. Gerhard Schmidt was among the first to recognize the power of a combined chemical and enzymatic approach to the analysis of the structure of the nucleic acids. The importance of his work was belatedly recognized by his election to the National Academy of Sciences in 1976. In his classic work in 1928, while in Frankfurt in Embden's laboratory, he demonstrated the deamination of "muscle adenylic acid" by a highly specific enzyme which fails to deaminate "yeast adenylic acid." He speculated (correctly) that the two adenylic acids differed in the position of the phosphate group. He is probably best known for his development in 1945, while at the Boston Dispensary, of the method for determining the RNA, DNA, and phosphoproteins in tissues by phosphorus analysis (the Schmidt–Thannhauser method). He made many other contributions in the nucleic acid field, beginning with his studies with P. A. Levene at the Rockefeller Institute in 1938–1939 on the enzymatic depolymerization of RNA and DNA, and extending into the 1970s when he published some of the first definitive work on the nature of DNA–histone complexes.

Schmidt's research was by no means limited to the nucleic acids. He was almost equally involved in studies on the structure and measurement of the complex lipids. He also made important observations on the accumulation of inorganic polyphosphates in living cells. During the period between his forced flight from Germany in 1933 when the Nazis came to power and his employment by Thannhauser at the Boston Dispensary in 1940, he had a variety of research fellowships in Italy, Sweden, Canada, and the United States, including one in 1939–1940 in the laboratory of Carl and Gerty Cori in St. Louis, where he worked on the enzymatic breakdown of glycogen by the muscle and liver phosphorylases.

It was during this St. Louis period that one of us (SPC), then a graduate student in the Cori laboratory, came to know Gerhard intimately. In the mid-1940s, the other one of us (NOK), then a postdoctoral fellow with Fritz Lipmann at the Massachusetts General Hospital, also developed close scientific and personal ties with Gerhard. In the early 1950s, when we had joined the McCollum–Pratt Institute, Gerhard was invited to participate in the Symposia on Phosphorus Metabolism where he presented a

monumental review on the polyphosphates and metaphosphates, and was also a central figure in the discussions on the nucleic acids. In the late 1950s and the 1960s, when NOK returned to Boston to be on the Brandeis faculty, the close ties with Gerhard were renewed. In the early 1960s, shortly after SPC joined the Vanderbilt faculty, Gerhard was invited there as a visiting professor and gave a series of memorable lectures on the nucleic acids which also formed the basis for his typically thorough chapter on that subject which appeared in *Annual Reviews of Biochemistry* for 1964.

During all the years from 1940 on, Gerhard did his research at the Boston Dispensary where Thannhauser had established a clinical chemistry laboratory. Throughout that time, Gerhard also held a joint appointment in biochemistry at the Tufts University School of Medicine where he participated in the teaching of medical students and the training of graduate students. He enjoyed a good relationship with the successive Chairmen of that department, three of whom, Alton Meister, Morris Friedkin, and Henry Mautner, were especially helpful. Dr. Mautner was instrumental in establishing the Gerhard Schmidt Memorial Lectureship which was initiated in December, 1981.

Gerhard was one of the most universally beloved figures in biochemistry. Perhaps this was because he lacked the "operator" gene. He would never have been comfortable as Chairman of a department or as President of a genetic engineering company. He liked to laugh, especially at himself. He identified with Laurel and Hardy, and once injured his jaw while rocking with laughter at one of their movies. He had a delightful collection of anecdotes, which, like his lectures, were carefully constructed and overly lengthy, but always well received by the Schmidt-story afficionados. He was enthusiastic about many things in addition to science, but he attacked with special gusto the playing of good chamber music or the eating of a good Liederkranz.

We present this dedication to his wife, Edith, and his sons, Michael and Milton, all of whom he loved very much, perhaps even more than his science, his music, and his Liederkranz.

SIDNEY P. COLOWICK
NATHAN O. KAPLAN

Contributors to Volume 100

Article numbers are in parentheses following the names of contributors.
Affiliations listed are current.

A. BECKER (12), *Department of Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada*

MICHAEL D. BEEN (8), *Department of Microbiology and Immunology, School of Medicine, University of Washington, Seattle, Washington 98195*

GERALD A. BELTZ (19), *Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138*

H. C. BIRNBOIM (17), *Radiation Biology Branch, Atomic Energy of Canada Limited, Chalk River, Ontario KOJ 1JO, Canada*

ROBERT BLAKESLEY (1, 26), *Bethesda Research Laboratories, Inc., Gaithersburg, Maryland 20877*

DAVID BOTSTEIN (31), *Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

CATHERINE A. BRENNAN (2), *Department of Biochemistry, School of Basic Medical Sciences and School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801*

BONITA J. BREWER (8), *Department of Genetics, University of Washington, Seattle, Washington 98195*

DAVID R. BROWN (16), *Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461*

HANS BÜNEMANN (27), *Institut für Genetik, Universität Düsseldorf, D-4000 Düsseldorf, Federal Republic of Germany*

MALCOLM J. CASADABAN (21), *Department of Biophysics and Theoretical Biology, University of Chicago, Chicago, Illinois 60637*

JAMES J. CHAMPOUX (8), *Department of Microbiology and Immunology, School of Medicine, University of Washington, Seattle, Washington 98195*

PETER T. CHERBAS (19), *Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138*

JOANY CHOU (21), *Department of Biophysics and Theoretical Biology, University of Chicago, Chicago, Illinois 60637*

R. JOHN COLLIER (25), *Department of Microbiology and The Molecular Biology Institute, University of California, Los Angeles, California 90024*

NICHOLAS R. COZZARELLI (11), *Department of Molecular Biology, University of California, Berkeley, California 94720*

ALBERT E. DAHLBERG (23), *Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912*

GUO-REN DENG (5), *Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853*

ALAN DIAMOND (30), *Sidney Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115*

JOHN E. DONELSON (6), *Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242*

K. DORAN (26), *Bethesda Research Laboratories, Inc., Gaithersburg, Maryland 20877*

BERNARD DUDOCK (30), *Department of Biochemistry, State University of New York, Stony Brook, New York 11794*

THOMAS H. EICKBUSH (19), *Department of Biology, University of Rochester, Rochester, New York 14627*

STUART G. FISCHER (29), *Department of Biological Sciences, Center for Biological Macromolecules, State University of New York, Albany, New York 12222*

ERICH FREI (22), *Department of Cell Biology, Biocenter of the University, CH-4056 Basel, Switzerland*

ROY FUCHS (1), *Corporate Research and Development, Monsanto Company, St. Louis, Missouri 63166*

JAMES I. GARRELS (28), *Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724*

M. GOLD (12), *Department of Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada*

PETER GOWLAND (22), *Department of Cell Biology, Biocenter of the University, CH-4056 Basel, Switzerland*

LAWRENCE GREENFIELD (25), *Cetus Corporation, Berkeley, California 94710*

MANUEL GREZ (20), *Department of Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033*

RICHARD I. GUMPORT (2), *Department of Biochemistry, School of Basic Medical Sciences and School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801*

LI-HE GUO (4), *Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853*

DOUGLAS HANAHAN (24), *Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724*

JAMES L. HARTLEY (6), *Bethesda Research Laboratories Inc., Gaithersburg, Maryland 20877*

HANSJÖRG HAUSER (20), *Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig, Federal Republic of Germany*

C. J. HOUGH (26), *Bethesda Research Laboratories, Inc., Gaithersburg, Maryland 20877*

TAO-SHIH HSIEH (10), *Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710*

JERARD HURWITZ (16), *Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461*

KENNETH A. JACOBS (19), *Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138*

CORNELIS VICTOR JONGENEEL (9), *Department of Biochemistry/Biophysics, University of California, San Francisco, San Francisco, California 94143*

FOTIS C. KAFATOS (19), *Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138*

DONALD A. KAPLAN (25), *Cetus Corporation, Berkeley, California 94710*

KENNETH N. KREUZER (9), *Department of Biochemistry/Biophysics, University of California, San Francisco, San Francisco, California 94143*

JUDY H. KRUEGER (33), *Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

HARTMUT LAND (20), *Center of Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

ABRAHAM LEVY (22), *Friedrich-Meischer-Institut, Ciba-Geigy, CH-4058 Basel, Switzerland*

WERNER LINDENMAIER (20), *Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig, Federal Republic of Germany*

LEROY F. LIU (7), *Department of Physiological Chemistry, Johns Hopkins University Medical School, Baltimore, Maryland 21205*

ALICE E. MANTHEY (2), *Department of Biochemistry, School of Basic Medical Sciences and School of Chemical Sciences,*

University of Illinois, Urbana, Illinois 61801

SUSAN R. MARTIN (8), *Genetic Systems Corp., 3005 First Avenue, Seattle, Washington 98121*

ALFONSO MARTINEZ-ARIAS (21), *Department of Biophysics and Theoretical Biology, University of Chicago, Chicago, Illinois 60637*

BETTY L. MCCONAUGHY (8), *Department of Genetics, University of Washington, Seattle, Washington, 98195*

WILLIAM K. MCCOUBREY, JR. (8), *Department of Microbiology and Immunology, School of Medicine, University of Washington, Seattle, Washington 98195*

MATTHEW MESELSON (24), *Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138*

HOWARD A. NASH (15), *Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20205*

MARKUS NOLL (22), *Department of Cell Biology, Biocenter of the University, CH-4056 Basel, Switzerland*

LYNN OSBER (14), *Departments of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510*

RICHARD OTTER (11), *Department of Molecular Biology, University of California, Berkeley, California 94720*

W. PARRIS (12), *Department of Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada*

CHARLES M. RADDING (14), *Departments of Human Genetics and of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06510*

RANDALL R. REED (13), *Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115*

DANNY REINBERG (16), *Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461*

PAUL J. ROMANIUK (3), *Department of Biochemistry, University of Illinois, Urbana, Illinois 61801*

THOMAS SCHMIDT-GLENEWINKEL (16), *Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461*

GÜNTHER SCHÜTZ (20), *Institut für Zell- und Tumorbiologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany*

STUART K. SHAPIRA (21), *Committee on Genetics, University of Chicago, Chicago, Illinois 60637*

TAKEHIKO SHIBATA (14), *Department of Microbiology, The Institute of Physical and Chemical Research, Saitama 351, Japan*

DAVID SHORTLE (31), *Department of Microbiology, State University of New York, Stony Brook, New York 11794*

MICHAEL SMITH (32), *Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada*

EDMUND J. STELLWAG (23), *Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455*

PATRICIA S. THOMAS (18), *Genetic Systems Corporation, 3005 First Avenue, Seattle, Washington 98121*

J. A. THOMPSON (26), *Bethesda Research Laboratories, Inc., Gaithersburg, Maryland 20877*

OLKE C. UHLENBECK (3), *Department of Biochemistry, University of Illinois, Urbana, Illinois 61801*

GRAHAM C. WALKER (33), *Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

ROBERT D. WELLS (26), *Department of Biochemistry, Schools of Medicine and Dentistry, University of Alabama, Birming-*

ham, University Station, Birmingham, Alabama 35294

PETER WESTHOFF (27), *Botanik IV, Universität Düsseldorf, D-4000 Düsseldorf, Federal Republic of Germany*

RAY WU (4, 5), *Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853*

LISA S. YOUNG (8), *Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403*

STEPHEN L. ZIPURSKY (16), *Division of Biology, California Institute of Technology, Pasadena, California 90025*

MARK J. ZOLLER (32), *Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada*

[1] Guide to the Use of Type II Restriction Endonucleases

By ROY FUCHS and ROBERT BLAKESLEY

Type II restriction endonucleases are DNases that recognize specific oligonucleotide sequences, make double-strand cleavages, and generate unique, equal molar fragments of a DNA molecule. By the nature of their controllable, predictable, infrequent, and site-specific cleavage of DNA, restriction endonucleases proved to be extremely useful as tools in dissecting, analyzing, and reconfiguring genetic information at the molecular level. Over 350 different restriction endonucleases have been isolated from a wide variety of prokaryotic sources, representing at least 85 different recognition sequences.[1,2] A number of excellent reviews detail the variety of restriction enzymes and their sources,[2,3] their purification and determination of their sequence specificity,[4,5] and their physical properties, kinetics, and reaction mechanism.[6] Here we provide a summary, based on the literature and our experience in this laboratory, emphasizing the practical aspects for using restriction endonucleases as tools. This review focuses on the reaction, its components and the conditions that affect enzymic activity and sequence fidelity, methods for terminating the reaction, some reaction variations, and a troubleshooting guide to help identify and solve restriction endonuclease-related problems.

The Reaction

Despite the diversity of the source and specificity for the over 350 type II restriction endonucleases identified to date,[1,2] their reaction conditions are remarkably similar. Compared to other classes of enzymes these conditions are also very simple. The restriction endonuclease reaction (Table I) is typically composed of the substrate DNA incubated at 37° in a solution buffered near pH 7.5, containing Mg^{2+}, frequently Na^{+}, and the selected restriction enzyme. Specific reaction details as found in the liter-

[1] R. Blakesley, *in* "Gene Amplification and Analysis," Vol. 1: "Restriction Endonucleases" (J. G. Chirikjian, ed.), p. 1. Elsevier/North-Holland, Amsterdam, 1981.
[2] R. J. Roberts, *Nucleic Acids Res.* **10,** r117 (1982).
[3] J. G. Chirikjian, "Gene Amplification and Analysis," Vol. 1: "Restriction Endonucleases." Elsevier/North-Holland, Amsterdam, 1981.
[4] R. J. Roberts, *CRC Crit. Rev. Biochem.* **4,** 123 (1976).
[5] This series, Vol. 65, several articles.
[6] R. D. Wells, R. D. Klein, and C. K. Singleton, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 14, Part A, p. 157. Academic Press, New York, 1981.

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TABLE I
GENERALIZED REACTION CONDITIONS FOR RESTRICTION ENDONUCLEASES

Conditions	Reaction type	
	Analytical	Preparative
Volume	20–100 μl	0.5–5 ml
DNA	0.1–10 μg	10–500 μg
Enzyme	1–5 units/μg DNA	1–5 units/μg DNA
Tris-HCl (pH 7.5)	20–50 m*M*	50 m*M*
$MgCl_2$	5–10 m*M*	10 m*M*
2-Mercaptoethanol	5–10 m*M*	5–10 m*M*
Bovine serum albumin	50–500 μg/ml	200–500 μg/ml
Glycerol	<5% (v/v)	<5% (v/v)
NaCl	As required	As required
Time	1 hr	1–5 hr
Temperature	37°	37°

ature for the more frequently used enzymes are listed in Table II. Note that in most cases these data do not represent optimal reaction conditions.

By convention, a unit of restriction endonuclease activity is usually defined as that amount of enzyme required to digest completely 1 μg of DNA (usually of bacteriophage lambda) in 1 hr.[4] This definition was chosen for convenience, since the useful, readily measurable end result of a restriction endonuclease reaction is completely cleaved DNA. However, a unit defined in this manner measures enzyme activity by an end point rather than by the classical initial rate term. Thus, traditional kinetic arguments based upon substrate saturating (initial rate) conditions cannot be applied to restriction endonucleases defined in this (enzyme saturating) manner.

One reason why there are few proper kinetic data on restriction endonucleases lies in the difficulty in measuring restriction enzyme activities during the linear portion of the reaction when using the standard enzyme assay.[7] The strong emphasis placed on their use as research tools in molecular biology rather than on investigation of their biochemical properties also contributed to the deficiency. Hence we lack good experimental data on conditions for optimal activity. For most newly isolated restriction endonucleases, assay buffers were selected for convenience during enzyme isolation rather than for optimal reactivity. These conditions have persisted as dogma. Thus, the implied precision and unique-

[7] P. A. Sharp, B. Sugden, and J. Sambrook, *Biochemistry* **12**, 3055 (1973).

ness of these values, e.g., pH 7.2 vs pH 7.4, is frequently without experimental basis. In fact, where investigated, restriction endonucleases usually show relatively broad activity profiles for the various reaction parameters.[8-10]

The fact that restriction endonucleases are active under a variety of conditions indicates that, similar to other nucleases, they are rather hardy enzymes. From an enzymologist's viewpoint, these enzymes can be mishandled and still demonstrate activity. But to achieve reproducible, efficient, and specific DNA cleavages, certain factors concerning restriction enzyme reactions should be considered. From our experience the most important factors for proper restriction endonuclease use are (*a*) the purity and physical characteristics of the substrate DNA; (*b*) the reagents used in the reaction; (*c*) the assay volume and associated errors; and (*d*) the time and temperature of incubation.

In the following sections each of these reaction parameters is discussed in detail. General conclusions are drawn in order to provide the researcher a framework in which properly to use restriction endonucleases. However, one must always be cognizant of the fact that each restriction endonuclease represents a unique enzymic protein. Any kinetic or biochemical generalization applied to the over 350 restriction enzymes will find exceptions.

DNA

The single most critical component of a restriction endonuclease reaction is the DNA substrate. DNA products generated in the reaction are directly affected by the degree of purity of the DNA substrate. Improperly prepared DNA samples will be cleaved poorly, if at all, producing partially digested DNA. In addition to DNA purity, other DNA-associated parameters that affect the products of the restriction endonuclease reaction include: DNA concentration, the specific sequence at and adjacent to the recognition site (including nucleotide modifications), and the secondary/tertiary DNA structure. Physical data pertaining to the DNA to be cleaved, if known, can guide one in choosing appropriate reaction conditions or prereaction treatments. Conversely, the response of a DNA of unknown physical properties to a standard restriction endonuclease digest can suggest certain characteristics of the DNA, e.g., the extent of methylation (see below).

[8] R. W. Blakesley, J. B. Dodgson, I. F. Nes, and R. D. Wells, *J. Biol. Chem.* **252,** 7300 (1977).

[9] P. J. Greene, M. S. Poonian, A. L. Nussbaum, L. Tobias, D. E. Garfin, H. W. Boyer, and H. M. Goodman, *J. Mol. Biol.* **99,** 237 (1975).

[10] B. Hinsch and M.-R. Kula, *Nucleic Acids Res.* **8,** 623 (1980).

TABLE II

REACTION CONDITIONS FOR CERTAIN RESTRICTION ENDONUCLEASES SELECTED FROM THE LITERATURE

Enzyme	Temperature (°C)	pH	Tris-HCl (m*M*)	$MgCl_2$ (m*M*)	NaCl (m*M*)	2-Mercapto-ethanol (m*M*)	Notes	Reference*
*Alu*I	37	7.9	6	6	—	6	—	1
*Asu*I	37	7.5	20	10	100	—	—	2
*Ava*I	37	7.5	20	10	100	20	—	3
*Ava*II	37	7.5	20	10	100	20	—	3
*Bal*I	37	7.9	6	6	—	6	—	4
*BamH*I	37	7.3	10	13[a]	50–100[a]	—	—	5
*BamH*I·1[b]	37	8.5	20	10	—	2	*c*	6
*Bcl*I	50[a]	7.4	12	12	—	0.5 m*M* DTT[b]	—	7
*Bgl*I	30[a]	9.5[a]	20 m*M* GOH[b]	20[a]	150[a]	7	—	8
*Bgl*II	30[a]	9.5[a]	20 m*M* GOH[b]	10[a]	—	—	*d, e*	8
*Bsp*I	37	8[a]	25	20[a]	50[a]	—	*e, f*	9
*Bst*I	37–50[a]	7–9.5[a]	100	0.5–2[a]	—	—	*g*	10
*Bst*I*[b]	37	9	100	>10	—	—	*h*	10
*Bst*15031	65[a]	7.8[a]	10[a]	0.2[a]	—	6.6	—	11
*Bsu*I	37	7.4[a]	10	10[a]	150[a]	1 m*M* DTT[b]	—	12
*Bsu*I*[b]	37	8.5	25	10	—	5	*i*	12
*Cla*I	37	7.4	6	6	50	6	—	13
*Dde*I	37	7.5	100	5	100	—	*j*	14
*Dpn*I	37	7.5	50	5	50	—	*k, l*	15
*Dpn*II	37	7.5	50	5	50	—	*k, l*	15
*Eca*I	37	8	10	10	—	—	*l*	16
*Eco*RI	37	7.1–7.5[a]	100	5[a]	50[a]	—	—	17
*Eco*RI*[b]	37	8.5	25	2	—	—	*e*	18
*Eco*RII	37	7.4	25	5	—	—	—	19
*Fnu*DII	37	7.9	6	6	50–150[a]	6	—	20
*Hae*II	37	7.9	6	6	—	6	—	21

*Hae*III	70[a]	7.5[a]	50[a]	5[a]	—	0.5 m*M* DTT	—	22
*Hga*I	37	7.6	10	5[a]	—	7	*m*	23
*Hgi*AI	30–45[a]	7.5–8.5[a]	10	10	100–150[a]	10	—	24
*Hha*I	37	7.9	6	6	—	6	—	25
*Hin*cII	37	7.9	10	6.6	60	6	—	26
*Hin*dIII	37	8.5[a]	10	10[a]	60[a]	7	*e, n*	27
*Hin*fI	37	7.5	6.6	10	50	6.6	*j*	28
*Hpa*I	45[a]	7.7–8.1[a]	10	5[a]	—	10	*k, o*	29
*Hpa*II	37	7.5	10	10	—	10	*k, p*	29
*Hph*I	37	7.4	10	10	6 m*M* KCl	10	—	30
*Kpn*I	37	7.9	6	6	—	6	—	31
*Mbo*I	37	7.9	6	6	—	6	—	32
*Mbo*II	37	7.9	6	6	—	6	—	32
*Mla*I	40	7.4	6.7	6.7	60 m*M* KCl	6.7	—	33
*Msp*I	37	8.0	20	10	5	10	*q*	34
*Nci*I	37	7.5	6	6	6	6	*k*	35
*Ngo*II	55[a]	8.5[a]	100	1[a]	20	—	*k*	36
*Pst*I	37	7.4	6.6	6.6	50	—	—	37
*Pvu*I	37	7.9	6	6	—	6	—	38
*Pvu*II	37	7.9	6	6	—	6	—	38
*Rsa*I	34[a]	7.9[a]	10	6	—	0.5 m*M* DTT[b]	*o*	39
*Rsh*I	30–37[a]	7.9	10	6	—	0.5 m*M* DTT[b]	*l*	40
*Sal*I	37	7.9	6	6	—	6	—	41
*Sau*3AI	30	7.5	6	15	60	6	—	42
*Sau*96I	30	7.4	6	15	60	6	—	43
*Sma*I	37	7.5	10	10	50	—	—	44
*Sph*I	37	7.3–7.8[a]	6	6[a]	50[a]	6	*l*	45
*Sst*I	37	7.5	10	10	100	10	—	46
*Stu*I	37	7.9	10	10	100	—	—	47
*Taq*I	37	7.4	10	10	—	10	*r*	48
*Tha*I	60[a]	7.4	10	10	—	—	—	49
*Tth*I	60[a]	7.5–8.5[a]	20	5	50	10	*e, s*	50
*Tth*111, I	65[a]	7.4	8	8[a]	50[a]	8	*t*	51

(*continued*)

TABLE II (*continued*)

Enzyme	Temperature (°C)	pH	Tris-HCl (m*M*)	$MgCl_2$ (m*M*)	NaCl (m*M*)	2-Mercapto-ethanol (m*M*)	Notes	Reference*
*Tth*111, II	65[a]	7.4	6	6	120–150[a]	6	*r*	52
*Xba*I	37	7.9	6	6	—	6	—	53
*Xho*I	37	7.9	6	6	—	6	—	54
*Xma*I	37	7.9	6	6	—	6	—	44
*Xor*II	37	7.4	6	12–24[a]	—	6	*e*	55

[a] Optimal condition.
[b] Abbreviations: DTT, dithiothreitol; GOH, glycine-NaOH; *Bam*HI.1, *Bst*I*, *Bsu*I*, *Eco*RI*, the secondary, "star" activities of *Bam*HI, *Bst*I, *Bsu*I, and *Eco*RI, respectively.
[c] In addition, 36% (v/v) glycerol and >20 × excess of enzyme are needed.
[d] Activity is stimulated twofold with 200 m*M* NaCl.
[e] Mn^{2+} can substitute for Mg^{2+}.
[f] Zn^{2+} inhibits activity.
[g] Activity is inhibited 50% by 50 m*M* NaCl.
[h] In addition, >5% glycerol is needed.
[i] In addition, 25% glycerol and 20–40 × excess of enzyme are needed.
[j] In addition, 500 μg of bovine serum albumin are needed per milliliter.
[k] In addition, 100 μg of bovine serum albumin are needed per milliliter.
[l] Activity is inhibited by ≥100 m*M* NaCl.
[m] Active to 500 m*M* KCl.
[n] Activity is inhibited by >250 m*M* NaCl or pH <7.
[o] Active to 200 m*M* NaCl.
[p] Activity is inhibited by >60 m*M* NaCl.
[q] In addition, 50 μg of bovine serum albumin are needed per milliliter.
[r] Active at 70°.
[s] Active to 300 m*M* NaCl.
[t] Activity is inhibited by >200 m*M* NaCl.
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Depending upon the subsequent use of the cleaved DNA, the demands on the purity of the DNA may vary. Generally, RNA and/or DNA contamination does not significantly interfere with the apparent restriction reaction rate as measured by digest completion. This is in spite of the fact that nonspecific binding to nucleic acids reduces the effective concentration of a restriction endonuclease. Contaminating nucleic acids more often interfere by obscuring the detection or selection of reaction products. For example, positive clones screened by rapid lysis methods[11] may be difficult to identify if the insert DNA excised by restriction endonuclease cleavage migrates in the same region as the intense broad tRNA band upon agarose gel electrophoresis. In such cases, treatment with DNase-free RNase or purification with a quick minicolumn using RPC-5 ANALOG[12] is recommended. On the other hand, sequencing protocols, e.g., the M13mp7 dideoxy method,[13] require highly purified DNA as restriction cleavage products. Protein contaminations are tolerated in a restriction reaction as long as the products eventually are protein-free. It should be noted, however, that the presence of other nucleases will reduce the integrity of the product, whereas proteins tightly bound to the DNA may lessen or block the cleavage reaction. DNAs are customarily deproteinized by phenol extraction prior to restriction endonuclease treatment.

Compounds involved in DNA isolation should be rigorously removed by dialysis or by ethanol precipitation and drying prior to addition of the DNA sample to the restriction endonuclease reaction. For example, Hg^{2+}, phenol, chloroform, ethanol, ethylene(diaminetetraacetic) acid (EDTA), sodium dodecyl sulfate (SDS), and NaCl at high levels interfere with restriction reactions, and some can alter the recognition specificity of restriction endonucleases. Drugs frequently used in DNA studies, e.g., actinomycin and distamycin A,[14] also influence restriction endonuclease activity.

In a typical reaction, the restriction endonuclease is in considerable molar excess of the substrate DNA. Therefore, consideration of DNA concentration usually is not required. In fact, it was necessary to dilute *Hae*III[8] or *Bam*HI[15] approximately 1000-fold from typical unit assay conditions in order to observe a substrate cleavage rate proportional to the

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[12] J. A. Thompson, R. W. Blakesley, K. Doran, C. J. Hough, and R. D. Wells, this volume [26].

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amount of enzyme added to the reaction. Further, caution must be exercised when attempting to extrapolate the amount of enzyme required for a complete digest based upon the number of recognition sites in a particular DNA. Preliminary observations using the enzyme-saturated, end point-dependent unit assay indicates that apparently no general correlation exists between recognition site density and restriction enzyme units required.[16]

By exception, the concentration of the substrate DNA did influence the apparent reaction rate for *Hin*dIII under enzyme-saturating conditions. A typical reaction for unit determination contains 1 μg of lambda DNA in a 50-μl reaction volume (20 μg/ml). One unit, but not 0.5 unit, of *Hin*dIII completely cleaves 1 μg of lambda DNA. One unit of *Hin*dIII also completely cleaves 4 μg (80 μg/ml) of lambda DNA under these conditions.[16] This peculiar response in *Hin*dIII activity cannot be attributed to enzyme : DNA concentration ratios, but is assumed to reflect the absolute DNA concentration dependence of *Hin*dIII. In contrast to the increased *Hin*dIII activity in the presence of increased DNA, 10 units of *Hpa*I, *Kpn*I, or *Sau*3AI proved to be insufficient to cleave completely 4 μg (80 μg/ml) of lambda DNA in a 15-hr reaction.[16] This phenomenon may be attributed to the viscosity produced by high concentrations of high molecular weight DNA (e.g., lambda DNA), which can inhibit enzyme diffusion and, therefore, inhibit some enzyme activities. These apparently anomalous results point out that one cannot directly compare units determined by titrating enzyme with those obtained by titrating (changing the concentration of) DNA. Further, DNA concentrations near or below the K_m of a restriction enzyme (1–10 nM[6]) could also inhibit apparent enzyme cleavage. However, for lambda DNA the K_m is approximately 1000-fold less than the concentration used in the standard reaction for unit determination. From these observations it is recommended that the DNA concentration be at or near that used in the unit assay reaction for the particular restriction endonuclease.

Restriction endonucleases probably show their greatest sensitivity to the DNA sequence. Obviously, the sequence of the recognition site is essentially invariant, as this distinguishes type II restriction endonucleases from other nucleases. The stringent sequence requirement frequently can be relaxed by alterations of the reaction environment, generating the "star" activity (see below) observed for a number of enzymes, *Eco*RI being the most notable. Sequences adjacent to the recognition site also influence the rate of cleavage. A nearly 10-fold difference in reaction rate was observed between two of the *Eco*RI sites in lambda DNA.[17] A

[16] This laboratory, unpublished results, 1981.

[17] M. Thomas and R. W. Davis, *J. Mol. Biol.* **91**, 315 (1975).

TABLE III
EFFECT OF BASE ANALOG SUBSTITUTIONS IN DNA ON RESTRICTION ENDONUCLEASE ACTIVITY

Enzyme	Recognition sequence	Relative activity of base analogs[a-c]				
		HMC	GHMC	U	HMU	BrdU
*Bam*HI[d]	GGATCC		−	++	+	+
*Eco*RI[d-f]	GAATTC	++	−	++	+	+
*Hae*II[d]	PuGCGCPy		−		+	+
*Hha*I[d]	GCGC		−		++	
*Hin*dII[d,e]	GTPyPuAC	−	−	+	+	
*Hin*dIII[d-f]	AAGCTT	−	−	+	+	+
*Hpa*I[d,g]	GTTAAC		−	+	+	+
*Hpa*II[d]	CCGG		−		++	+
*Mbo*I[g]	GATC					Enhanced 5-fold

[a] *Activity symbols:* ++, full activity; +, diminished activity; −, no activity; blank, not tested.
[b] In these studies, HMC or GHMC were in place of cytosine, while U, HMU, or BrdU replaced thymidine in the tested DNAs.
[c] Abbreviations used: HMC, 5-hydroxymethylcytosine; GHMC, glucosylated 5-hydroxymethylcytosine; U, uridine; HMU, 5-hydroxymethyluridine; BrdU, 5-bromodeoxyuridine; Py, pyrimidine; Pu, purine.
[d] K. L. Berkner and W. R. Folk, *J. Biol. Chem.* **254,** 2551 (1979).
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similar effect was reported for *Pst*I.[18] In addition, thymine substituted by 5-bromodeoxyuridine prevented cleavage of some *Sma*I sites in the DNA tested, even though the 5-bromodeoxyuridine was not part of the canonical recognition sequence (CCCGGG).[19]

Nucleotide changes within the recognition sequence more directly affect the restriction endonuclease reaction (Tables III and IV). For *Eco*RI, cleavage was unaffected by 5-hydroxymethylcytosine substitution for cytosine[20] or by the absence or the presence of the 2-amino group of guanine.[21] Glycosylation of 5-hydroxymethylcytosine, however, made the DNA resistant to cleavage by *Eco*RI as well as by *Hpa*I, *Hin*dII, *Hin*dIII, *Bam*HI, *Hae*II, *Hpa*II and *Hha*I.[22] Substitution of thymine with

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5-hydroxymethyluridine diminished activities of enzymes with AT-containing sites, whereas a differential effect was observed for uridine and 5-bromodeoxyuridine substitutions.[22] Methylation of nucleotides within restriction endonuclease recognition sequences, occurring almost exclusively as 5-methylcytosine or N^6-methyladenine, prevented most

TABLE IV
METHYLATED DNAs AS SUBSTRATES FOR RESTRICTION ENDONUCLEASES[a]

	Sequences containing 5-methylcytosine or N^6-methyladenine[b]		
Enzyme	Cleaved	Not cleaved	References
*Aos*II	—	GPu^{m}CGPyC	*d, e*
*Ava*I	—	CPy^{m}CGPuG	*f*
*Ava*II	—	GG(A)C^{m}C (T)	*g*
*Bst*NI	C^{m}C(A)GG (T)	—	*h*
*Eco*RII	—	C^{m}C(A)GG (T)	*h, i*
*Hae*II	—	PuG^{m}CGCPy	*e, f*
*Hae*III	GGC^{m}C	GG^{m}CC	*j, k*
*Hap*II	—	C^{m}CGG	*e, l*
*Hha*I	—	G^{m}CGC	*f, j*
*Hpa*II	^{m}CCGG	C^{m}CGG	*j, l*
*Msp*I	C^{m}CGG	^{m}CCGG	*l, m*
*Pst*I	—	^{m}CTGCAG	*n*
*Pvu*II	—	^{m}CAGCTG	*n*
*Sal*I	—	GT^{m}CGAC	*d, e*
*Sma*I	—	CC^{m}CGGG	*e, u*
*Taq*I	T^{m}CGA	—	*o*
*Xho*I	—	CT^{m}CGAG	*d, e*
*Xma*I	CC^{m}CGGG	—	*u*
*Bam*HI	GG^{m}ATCC	—	*g, p*
*Bgl*II	AG^{m}ATCT	—	*p*
*Dpn*I	G^{m}ATC[c]	—	*o, q*
*Dpn*II	—	G^{m}ATC	*q*
*Eco*RI	—	GA^{m}ATTC	*r*
*Fnu*EI	G^{m}ATC	—	*s*
*Hin*dII	—	GTPyPu^{m}AC	*k*
*Hin*dIII	—	^{m}AAGCTT	*k*
*Hpa*I	—	GTTA^{m}AC	*t*
*Mbo*I	—	G^{m}ATC	*p, s*
*Mbo*II	—	GAAG^{m}A	*g*
*Sau*3AI	G^{m}ATC	—	*o, p*
*Taq*I	—	TCG^{m}A	*d, o*