LIFE TECHNOLOGIES™

BULK RATE
US POSTAGE
PAID
Rockville, MD
Permit #3539

FOCUS®

PUBLISHED FOR THE LIFE SCIENTIST BY LIFE TECHNOLOGIES, INC.

VOLUME 20 • NUMBER 3 • 1998

Featured Papers:

Enabling Technologies of Molecular Biology – *58*

CONCERT™ Plasmid Purification Systems – *68*

Archiving DNA at Room Temperature – *72*

Electroporation of Large Plasmids – *77*

RNA Isolation from Pancreas Tissue – *82*

Quantitation of Oligonucleotides – *84*

γ-Irradiated FBS – *86*

1978 · 20 YEARS OF FOCUS · *1998*

ABOUT THE COVER:
From DNA to sequencing the human genome.
See article on page 58.

FOCUS®

FOCUS contains manuscripts describing novel techniques, improvements of common techniques, simplified protocols, and troubleshooting. "Instructions to Authors" are available on the Internet or from the editor:

***FOCUS* Editor:**
Dr. Doreen Cupo
Life Technologies, Inc.
9800 Medical Center Drive
Rockville, MD 20849-6482
(800) 828-6686
(301) 610-8000 outside the U.S.
E-mail: dcupo@lifetech.com

Assistant to the Editor:
Karen Carstensen

Editorial Review Board:
Holly Anderson, Paul Battista, Stephen Gorfien, James Hartley, Curtis Henrich, Larry Mertz, Frank Swartzwelder

Contributing Editorial Reviewers:
Val Ciccarone, Donna Fox, J.J. Lin, and David Schuster

FOCUS® is published triannually by Life Technologies, Inc.

POSTMASTER: Send address changes to
FOCUS, Life Technologies, Inc.
P.O. Box 6482, Rockville, MD
20849-6482

Requests for subscriptions and address changes should be directed to the nearest Life Technologies office:

U.S.A. ORDERS
To Order/TECH-LINE:℠
(800) 828-6686
Fax: (800) 331-2286

U.S. INDUSTRIAL ORDERS
To Order/TECH-LINE:
(800) 874-4226
Fax: (800) 352-1468

Internet
info@lifetech.com
http://www.lifetech.com

INTERNATIONAL ORDERS
For **Latin America** and other locations not listed below.
Rockville, MD U.S.A.
Atten: International Customer Services
To Order/TECH-LINE: (301) 610-8709
Fax: (301) 610-8724

AUSTRALIA
Melbourne
To Order/TECH-LINE: 1800 331 627
Tel: (03) 9562 8245
Fax: (03) 9562 7773

CANADA
Burlington, Ontario
To Order: (800) 263-6236
TECH-LINE: (800) 757-8257
Fax: (800) 387-1007

EUROPE
Paisley, Scotland
To Order: 0800 269210
TECH-LINE: 0800 838380
Fax: 0800 243485

HONG KONG
Tsuen Wan
Tel: (852) 2407-8450
Fax: (852) 2408-2280/2409-6043

INDIA
New Delhi
To Order: 91-11-373-7749
Fax: 91-11-373-7748

JAPAN
Tokyo
Tel: (81-3) 3580-5429
Fax: (81-3) 3580-5428

NEW ZEALAND
Auckland
To Order/TECH-LINE: 0800 600 200
Tel: (09) 579 3024
Fax: (09) 579 3119

PEOPLE'S REPUBLIC OF CHINA
Beijing
To Order: (86-10) 6256-3836
Fax: (86-10) 6256-4852

PEOPLE'S REPUBLIC OF CHINA
Shanghai
To Order: (86-21) 6471-1313
Fax: (86-21) 6471-1313

TAIWAN
Taipei
Tel: (886-2) 2698-8111
Fax: (886-2) 2698-3700

20 YEARS OF MOLECULAR BIOLOGY

The Genomic Revolution: Enabling Technologies of the Human Genome Project **58**
Jeffrey W. Touchman and Eric D. Green

The Two-Hybrid System: Finding Likely Partners for Lonely Proteins **62**
Peter H. Uetz, Robert E. Hughes, and Stanley Fields

Reverse Transcriptase: A Historical Perspective **65**
Gary F. Gerard

DNA PURIFICATION AND STORAGE

High Purity Plasmid DNA from Anion Exchange Chromatography **68**
Mindy D. Goldsborough, Lisha Xu, Krista Evans, and Alice Young

Rapid Purification of Plasmid DNA Using Spin Cartridges **70**
Mindy D. Goldsborough, Lisha Xu, and Alice Young

Simple Archiving of Bacterial and Plasmid DNAs for Future Use **72**
Pamela Hansen and Robert Blakesley

TRANSFORMATION

ELECTROMAX™ DH5α-E™ Cells: A New Addition to the DH5α Family **75**
Robert A. Donahue, Jr., and Fredric R. Bloom

Transformation Efficiency of E. coli *Electroporated with Large Plasmid DNA* **77**
Robert A. Donahue, Jr., and Fredric R. Bloom

TRANSFECTION

LIPOFECTAMINE™ Reagent for Stable Co-transfection in an SV40-Transformed Human Cell Line **79**
Vaseem Palejwala, Satnam S. Banga, Lisa M. Bloch, Tanya Dasgupta, Cesar Guerra, Bohdan Harvey, Alexandra Soggiu, Krishna K. Jha, and Harvey L. Ozer

RNA PURIFICATION

Modified TRIZOL® Reagent Protocol for Large mRNA **80**
Daisuke Nakajima, Manabu Nakayama, and Osamu Ohara

Isolation and Long-Term Storage of RNA from Ribonuclease-Rich Pancreas Tissue **82**
Alma M. Bracete, Donna K. Fox, and Domenica Simms

PCR

Effect of Base Composition on the Quantitation of Oligonucleotides **84**
Donna Fox

CELL CULTURE

Virus Inactivation by Gamma Irradiation of Fetal Bovine Serum **86**
John P. Daley, Douglas J. Danner, David J. Weppner, and Mark Z. Plavsic

Printed on recycled paper

LIFE TECHNOLOGIES™

Jeffrey W. Touchman and **Eric D. Green**
National Human Genome Research Institute and
National Institutes of Health Intramural Sequencing Center
National Institutes of Health
Bethesda, Maryland 20892

The Genomic Revolution:
Enabling Technologies of the Human Genome Project

from the editor

Twenty years ago, *FOCUS* magazine began as a newsletter to provide valuable information on key techniques and applications for Life Technologies' customers. In this milestone issue of *FOCUS* magazine, several prominent scientists describe some key enabling technologies, what they have meant to science, and how they will lead to discoveries in the future—from DNA sequence information to understanding gene function.

The following article by Touchman and Green shows how the development of a group of molecular biology techniques and advances in computer technology have turned the idea of mapping and sequencing the human genome into a reality. The compilation of volumes of sequence information can accelerate discovery of the function of specific genes.

In the article by Gerard (page 65), he recounts the history of reverse transcriptases (RTs). RTs have enabled work with cDNA sequences to identify the expressed regions of the genome.

On page 62, Uetz, Hughes, and Fields describe a burgeoning area of research—unraveling protein functions by identifying protein-protein interactions using two-hybrid technology.

As these and other exciting advances continue to occur, *FOCUS* magazine will continue to communicate them to you.

Doreen Cupo
Editor

Shortly after the advent of recombinant DNA cloning, the revolution of "molecular biology" swept through myriad disciplines of biomedical research. As molecular cloning, DNA sequencing, and various other DNA manipulation methods became standard operating procedures for numerous laboratories, a spectacular era of science began that established a foundation for untangling the complexities of gene structure and function. However, the molecular biology revolution of the 1970s and 1980s was in many ways a preview of more exciting times to come, with the launch of the Human Genome Project (HGP) in the early 1990s and the arrival of its associated "genomic revolution."

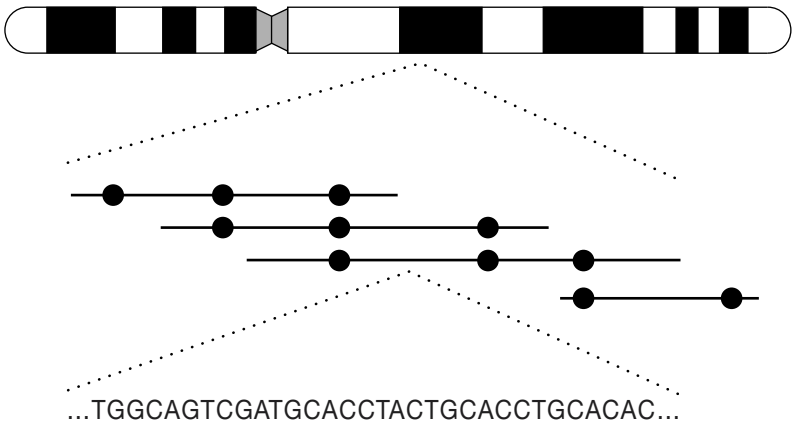
In retrospect, the notion of embarking on the ***Human Genome Project without PCR is a terrifying one.***

With the fundamental goal to elucidate the genetic blueprint of humans and several well-studied model organisms, the HGP is roughly at its halfway point and is achieving virtually all of its ambitious goals (1). Central to its remarkable achievements in the mapping and sequencing of complex genomes have been numerous key technologies that have paved the way for major advances in genome analysis. Here, we briefly highlight several of these technologies; in particular, those that are permeating beyond genomics laboratories to become enabling technologies in broader areas of scientific research.

PCR. Few (if any) experimental methods have had as dramatic an impact on biomedical research as the polymerase chain reaction (PCR). However, as astonishing as it may seem, PCR (2,3) was not yet invented when the initial proposals and earliest plans for the HGP were first being discussed (4–6). In retrospect, the notion of embarking on the HGP without PCR is a terrifying one.

The HGP has been a particularly fortunate beneficiary of PCR and its related technologies. Because of its sensitivity, specificity, and potential for automation, PCR rapidly became the front-line analytical method for detecting the landmarks on which genetic and physical maps of mammalian chromosomes were based [*e.g.,* microsatellite-based genetic markers (7) and sequence-tagged sites (STSs) (8), respectively]. Specifically, PCR has been routinely applied in a large-scale fashion to analyze genomic DNA samples for constructing genetic maps (9), large-insert clones for assembling clone-based physical maps (10), and panels of radiation hybrid cell lines for deriving radiation hybrid maps (11).

As with most critical technologies, continued incremental improvements in the basic methods have escalated the power and usage of PCR (12). For example, the development of improved thermostable polymerases and associated PCR-enhancing reagents has resulted in the ability to generate larger, more authentic, and purer PCR products. In addition, more sophisticated instruments for thermal cycling have become available, providing better functionality, shorter cycling times, and increased capacity. Finally, new approaches for oligonucleotide synthesis have been designed (13), and they have directly resulted in a dramatic decrease in the costs for synthesizing PCR primers. Together, these advances have catapulted PCR to become one of the most widely used experimental methods in research today.



CLONING SYSTEMS. Several major advances in the technologies available for constructing recombinant DNA libraries have been critical for the mapping and sequencing components of the HGP. Among these was the establishment of new large-insert cloning systems. For example, the ability to construct complete physical maps of complex genomes was greatly facilitated by the development of yeast artificial chromosomes (YACs) (14,15). With YAC cloning, segments of DNA that are 100 kb to over 1,000 kb can be harnessed with the appropriate structural elements and propagated as artificial chromosomes in yeast. YACs played a central role in constructing the first-generation physical maps of the human and mouse genomes and are now being used for mapping the genomes of numerous other organisms (15). The next-generation physical maps of the human and mouse (as well as other) genomes are, for the most part, being assembled with the intention of using the resulting mapped clones for systematic genomic sequencing. Here, the recently developed bacterial artificial chromosome (BAC) (16) and the closely related P1-derived artificial chromosome (PAC) (17) cloning systems provide the ability to isolate relatively large DNA segments (averaging 100 to 150 kb) in a stable form and in a reliable fashion. BACs and PACs are already proving well suited for use in constructing high-resolution physical maps and as starting clones for genomic sequencing.

Another set of advances in cloning systems relates to the construction of cDNA libraries. Here, significantly refined preparations of enzymes for DNA manipulation such as reverse transcriptases (see page 65 in this issue) have allowed the generation of longer and more authentic primary cDNA, which in turn has profoundly improved the quality of the resulting cDNA libraries. Similarly, the establishment of sophisticated protocols for generating "normalized" cDNA libraries (where individual transcripts are more equally represented) (18) has been critical for establishing complex gene inventories via the large-scale generation of expressed-sequence tags (ESTs) (19,20). The latter efforts have collectively produced enormous databases of gene sequences (21,22) (*e.g.,* dbEST; see http://www.ncbi.nlm.nih.gov/dbEST) and facilitated the construction of a preliminary "transcript map" of the human genome (23) (see http://www.ncbi.nlm.nih.gov/genemap98), which in turn are providing valuable insight about the repertoire of genes expressed in various organisms, tissues, and disease states.

DNA SEQUENCING. A central goal of the HGP is to elucidate the complete genomic sequences of humans and several other organisms. Enroute toward such an achievement, significant advances have been made in the technologies used in large-scale DNA sequencing. Despite efforts to establish revolutionary new DNA sequencing methods, the classic technique of dideoxynucleotide chain termination sequencing, developed by Sanger and coworkers in 1977 (24), remains the dominant approach for DNA sequencing. However, successive and incremental improvements in the protocols used for large-scale DNA sequencing have been made in recent years. Among these advances have been the development of semi-automated, laser-based instruments for performing fluorescent-based DNA sequencing (25,26), the generation of fluorescent-based dye primer and dye terminator molecules with increasingly superior spectral characteristics, and continued improvements in sequencing enzymes with respect to the resulting read lengths and accuracy (27). Additional evolutionary advances in DNA sequencing abound on the horizon, with newer capillary-based instruments and even better sequencing enzymes and associated reagents becoming available.

Together, the numerous advances in sequencing technology coupled with the productiveness of a handful of large sequencing groups have resulted in the recent generation of an impressive amount of DNA sequence data. Notable accomplishments include completing the sequence of the yeast *S. cerevisiae* genome (http://genome-www.stanford.edu/Saccharomyces), numerous bacterial genomes (including *E. coli*; see http://www.tigr.org/tdb), and the nematode

The Genomic Revolution continued

C. elegans genome (http://www.sanger.ac.uk; http://genome.wustl.edu/gsc/gschmpg.html), as well as generating literally hundreds of thousands of ESTs from various organisms and tissues (http://www.ncbi.nlm.nih.gov/dbEST). However, the current efforts of the HGP are now firmly focused on sequencing the human genome, with completion targeted for shortly after the turn of the century.

***The prototypic biomedical researcher in the 21st century** will likely be seen in the laboratory with one hand on a pipette and the other on a computer mouse.*

DNA CHIPS. Among the more exciting new genomic technologies are "DNA chips," consisting of miniaturized microarrays that can be used for DNA analysis (28,29). With DNA chips, relatively standard techniques (*e.g.,* DNA-DNA hybridization and fluorescence-based image acquisition) are implemented on a microscale, allowing analyses to be performed in a massively parallel fashion. Two major types of DNA chips have been developed. The first contains a high-density array of short oligonucleotides, for example, created by a process known as photolithography (30). Such arrays are made with 100,000 to 400,000 oligonucleotides immobilized within a 1.28-cm^2 area. PCR-amplified, fluorescently tagged target DNA is then hybridized to the oligonucleotide array, with the resulting hybridization pattern microscopically captured and used to deduce the target DNA sequence. Thus, oligonucleotide-based DNA chips can be used for resequencing DNA. The power of such an efficient, high-throughput approach for DNA analysis is quickly being realized for numerous applications, such as detecting important sequences within pathogens (31,32), resequencing targeted regions of human DNA (33,34), simultaneously studying the expression of large numbers of genes (35,36), and performing large-scale analyses of single nucleotide polymorphisms (SNPs) (37).

The second major type of DNA chip contains a high-density array of short double-stranded DNA fragments, most often derived from cDNA (38,39). These chips are typically made by robotically spotting PCR-amplified DNA fragments onto glass or nylon surfaces. For gene expression studies, hybridization is typically carried out with fluorescently labeled, mRNA-derived probes, with a two-color fluorescence detection scheme often employed to examine simultaneously parallel biological samples. cDNA microarrays can be used for various applications, such as studying gene expression patterns under different metabolic conditions (39,40) or in human cancer (41). The true power of cDNA chips resides in their ability to analyze large numbers of genes in parallel, thereby gaining insight about the complex cellular networks regulating gene expression. The further development and refinement of DNA chip technologies will undoubtedly represent important and active areas of genome research in the coming years.

COMPUTATIONAL GENOMICS. Among the most critical, rapidly evolving genomic technologies are those centered on computational-based analyses, storage, and retrieval of the massive amounts of mapping and sequencing data being generated by the HGP. It is quite fitting that the HGP and a major growth spurt of the "information superhighway" (the Internet) are occurring in parallel; in fact, the former would be greatly weakened by the absence of the latter. The rapidly growing field of computational genomics (or bioinformatics), which encompasses everything from software tools for analyzing maps and sequences to the construction and dissemination of sophisticated databases of genomic information, now represents an essential and well-respected discipline of biomedical research (42). Already, practicing molecular geneticists and, increasingly, researchers from all scientific disciplines spend sizable amounts of time in front of computer screens retrieving, analyzing, and manipulating mapping and sequencing data. The fundamental information being produced by the HGP and its related activities is becoming the currency from which scientific inquiry is initiated. Undoubtedly, this trend will intensify in the near future, as the amount of sequence data available for numerous organisms increases in an unprecedented fashion. The prototypic biomedical researcher in the 21st century will likely be seen in the laboratory with one hand on a pipette and the other on a computer mouse.

SUMMARY

The technologies of the HGP and its associated "genomic revolution" are becoming standard tools in the typical biologist's armamentarium. In this regard, the fruits of the HGP include both the mapping and sequencing information it produces and the enabling technologies that empower researchers with the ability to probe the genetic bases of important biomedical problems. Further technical developments and refinements of these current methodologies should help to usher in a new era of scientific inquiry.

The views expressed in this article are those of the authors and do not represent the views of the National Human Genome Research Institute, the National Institutes of Health, or the Department of Health and Human Services. FOCUS

REFERENCES

1. Green, E.D., Cox, D.R., and Myers, R.M. (1998) in *The Genetic Basis of Human Cancer* (Vogelstein, B. and Kinzler, K.W., eds.), McGraw-Hill, New York. p. 33.
2. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985) *Science 230*, 1350.
3. Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1986) *Cold Spring Harbor Symp. Quant. Biol. LI*, 263.
4. Cook-Deegan, R.M. (1989) *Genomics 5*, 661.
5. Sinsheimer, R.L. (1989) *Genomics 5*, 954.
6. Dulbecco, R. (1986) *Science 231*, 1055.
7. Weber, J.L. and May, P.E. (1989) *Am. J. Hum. Genet 44*, 388.
8. Olson, M., Hood, L., Cantor, C., and Botstein, D. (1989) *Science 245*, 1434.
9. Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morissette, J., and Weissenbach, J. (1996) *Nature 380*, 152.
10. Green, E.D. and Olson, M.V. (1990) *Science 250*, 94.
11. Cox, D.R., Burmeister, M., Price, E.R., Kim, S., and Myers, R.M. (1990) *Science 250*, 245.
12. Fanning, S. and Gibbs, R.A. (1997) in *Genome Analysis: A Laboratory Manual. Vol. 1. Analyzing DNA* (Birren, B., Green, E.D., Klapholz, S., Myers, R.M., and Roskams, J., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. p. 249.
13. Lashkari, D.A., Hunicke-Smith, S.P., Norgren, R.M., Davis, R.W., and Brennan, T. (1995) *Proc. Natl. Acad. Sci. USA 92*, 7912.
14. Burke, D.T., Carle, G.F., and Olson, M.V. (1987) *Science 236*, 806.
15. Green, E.D., Hieter, P., and Spencer, F.A. (1998) in *Genome Analysis: A Laboratory Manual. Vol. 3. Cloning Systems* (Birren, B., Green, E.D., Klapholz, S., Myers, R.M., Riethman, H., and Roskams, J., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. p. 297.
16. Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiiri, Y., and Simon, M. (1992) *Proc. Natl. Acad. Sci. USA 89*, 8794.
17. Ioannou, P.A., Amemiya, C.T., Garnes, J., Kroisel, P.M., Shizuya, H., Chen, C., Batzer, M.A., and de Jong, P.J. (1994) *Nat. Genet. 6*, 84.
18. Soares, M.B. and Bonaldo, M.F. (1998) in *Genome Analysis: A Laboratory Manual. Vol. 2. Detecting Genes* (Birren, B., Green, E.D., Klapholz, S., Myers, R.M., and Roskams, J., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. p. 49.
19. Hillier, L., Lennon, G., Becker, M., Bonaldo, M., Chiapelli, B., Chissoe, S., Dietrich, N., DuBuque, T., Favello, A., Gish, W., Hawkins, M., Hultman, M., Kucaba, T., Lacy, M., Le, M., Le, N., Mardis, E., Moore, B., Morris, M., Parsons, J., Prange, C., Rifkin, L., Rohlfing, T., Schellenberg, K., Soares, M.B., Tan, F., Thierry-Meg, J., Trevaskis, E., Underwood, K., Wohldman, P., Waterston, R., Wilson, R., and Marra, M. (1996) *Genome Res. 6*, 807.
20. Adams, M.D., Kerlavage, A.R., Fleischmann, R.D., Fuldner, R.A., Bult, C.J., Lee, N.H., Kirkness, E.F., Weinstock, K.J., Gocayne, J.D., White, O., Sutton, G., Blake, J.A., Brandon, R.C., Chiu, M.W., Clayton, R.A., Cline, R.T., Cotton, M.D., Earle-Hughes, J., Fine, L.D., FitzGerald, L.M., FitzHugh, W.M., Fritchman, J.L., Geoghagen, N.S.M., Glodek, A., Gnehm, C.L., Hanna, M.C., Hedblom, E., Hinkle, P.S., Jr., Kelley, J.M., Klimek, K.M., Kelley, J.C., Liu, L.I., Marmaros, S.M., Merrick, J.M., Moreno-Palanques, R.F., McDonald, L.A., Nguyen, D.T., Pellegrino, S.M., Phillips, C.A., Ryder, S.E., Scott, J.L., Saudek, D.M., Shirley, R., Small, K.V., Spriggs, T.A., Utterback, T.R., Weldman, J.F., Li, Y., Barthlow, R., Bednarik, D.P., Cao, L., Cepeda, M.A., Coleman, T.A., Collins, E.J., Dimke, D., Feng, P., Ferrie, A., Fischer, C., Hastings, G.A., He, W.W., Hu, J.S., Huddleston, K.A., Greene, J.M., Gruber, J., Hudson, P., Kim, A., Kozak, D.L., Kunsch, C., Ji, H., Li, H., Meissner, P.S., Olsen, H., Raymond, L., Wei, Y.F., Wing, J., Xu, C., Yu, G.L., Ruben, S.M., Dillon, P.J., Fannon, M.R., Rosen, C.A., Haseltine, W.A., Fields, C., Fraser, C.M., and Venter, J.C. (1995) *Nature 377*, 3.
21. Boguski, M.S. and Schuler, G.D. (1995) *Nat. Genet. 10*, 369.
22. Boguski, M.S. (1995) *Trends Biochem. Sci. 20*, 295.
23. Deloukas, P., Schuler, G.D., Gyapay, G., Beasley, E.M., Soderlund, C., Rodriguez-Tome, P., Hui, L., Matise, T.C., McKusick, K.B., Beckmann, J.S., Bentolila, S., Bihoreau, M.T., Birren, B.B., Browne, J., Butler, A., Castle, A.B., Chiannikulchai, N., Clee, C., Day, P.J.R., Dehejia, A., Dibling, T., Drouot, N., Duprat, S., Fizames, C., Fox, S., Gelling, S., Green, L., Harison, P., Hocking, R., Holloway, E., Hunt, S., Keil, S., Lijnzaad, P., Louis-Dit-Sully, C., Ma, J., Mendis, A., Miller, J., Morissette, J., Muselet, D., Nusbaum, H.C., Peck, A., Rozen, S., Simon, D., Slonim, D.K., Staples, R., Stein, L., Stewart, E.A., Suchard, M.A., Thangarajah, T., Vega-Czarny, N., Webber, C., Wu, X., Auffray, C., Nomura, N., Sikela, J.M., Polymeropoulos, M.H., James, M.R., Lander, E.S., Hudson, T.J., Myers, R.M., Cox, D.R., Weissenbach, J., Boguski, M.S., and Bentley, D.R. (1998) *Science,* in press.
24. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA 74*, 5463.
25. Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B.H., and Hood, L.E. (1986) *Nature 321*, 674.
26. Hunkapiller, T., Kaiser, R.J., Koop, B.F., and Hood, L. (1991) *Science 254*, 59.
27. Wilson, R.K. and Mardis, E.R. (1997) in *Genome Analysis: A Laboratory Manual. Vol. 1. Analyzing DNA* (Birren, B., Green, E.D., Klapholz, S., Myers, R.M., and Roskams, J., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. p. 301.
28. Johnston, M. (1998) *Curr. Biol. 8*, R171.
29. Castellino, A.M. (1997) *Genome Res. 7*, 943.
30. Fodor, S.P.A., Read, J.L., Pirrung, M.C., Stryer, L., Lu, A.T., and Solas, D. (1991) *Science 251*, 767.
31. Kozal, M.J., Shah, N., Shen, N., Yang, R., Fucini, R., Merigan, T.C., Richman, D.D., Morris, D., Hubbell, E., Chee, M., and Gingeras, T.R. (1996) *Nat. Med. 2*, 753.
32. Gingeras, T.R., Ghandour, G., Wang, E., Berno, A., Small, P.M., Drobniewski, F., Alland, D., Desmond, E., Holodniy, M., and Drenkow, J. (1998) *Genome Res. 8*, 435.
33. Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhart, D.J., Morris, M.S., and Fodor, S.P.A. (1996) *Science 274*, 610.
34. Hacia, J.G., Brody, L.C., Chee, M.S., Fodor, S.P.A., and Collins, F.S. (1996) *Nat. Genet. 14*, 441.
35. Lockhart, D.J., Dong, H., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E.L. (1996) *Nat. Biotechnol. 14*, 1675.
36. Wodicka, L., Dong, H., Mittmann, M., Ho, M.H., and Lockhart, D.J. (1997) *Nat. Biotechnol. 15*, 1359.
37. Wang, D.G., Fan, J.-B., Siao, C.-J., Berno, A., Young, P., Sapolsky, R., Ghandour, G., Perkins, N., Winchester, E., Spencer, J., Kruglyak, L., Stein, L., Hsie, L., Topaloglou, T., Hubbell, E., Robinson, E., Mittmann, M., Morris, M.S., Shen, N., Kilburn, D., Rioux, J., Nusbaum, C., Rozen, S., Hudson, T.J., Lipshutz, R., Chee, M., and Lander, E.S. (1998) *Science 280*, 1077.
38. Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995) *Science 270*, 467.
39. Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P.O., and Davis, R.W. (1996) *Proc. Natl. Acad. Sci. USA 93*, 10614.
40. DeRisi, J.L., Iyer, V.R., and Brown, P.O. (1997) *Science 278*, 680.
41. DeRisi, J., Penland, L., Brown, P.O., Bittner, M.L., Meltzer, P.S., Ray, M., Chen, Y., Su, Y.A., and Trent, J.M. (1996) *Nat. Genet. 14*, 457.
42. *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins* (1998) (Baxevanis, A.D. and Ouellette, B.F.F., eds.), John Wiley and Sons, New York.

The Two-Hybrid System:
Finding Likely Partners for Lonely Proteins

Peter H. Uetz and **Robert E. Hughes**
Departments of Genetics and Medicine
University of Washington

Stanley Fields
Departments of Genetics and Medicine
University of Washington and
Howard Hughes Medical Institute
Seattle, Washington 98195
E-mail: fields@genetics.washington.edu

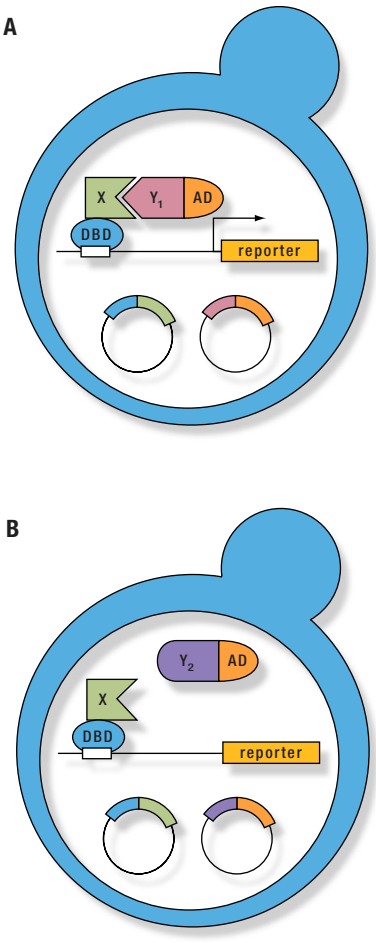


FIGURE 1. How the two-hybrid system works. *Panel A.* Protein X (the bait protein) is fused to a DNA-binding domain (DBD) and protein Y_1 is fused to a transcription activation domain (AD). Both hybrid proteins are expressed in a yeast cell from plasmids. Interaction of X and Y_1 leads to activation of a reporter gene that allows the yeast to grow on a defined medium. *Panel B.* Protein X and protein Y_2 do not interact; consequently, the reporter gene is not expressed.

A common response of the biologist first scoring a successful two-hybrid search is outright disbelief that an assay so simple can yield results so revealing of the innermost circuitry of a cell. That the method, described nearly a decade ago (1), often works precisely as depicted in its standard cartoon version *(figure 1)* is a testament to the tolerance of the eukaryotic transcriptional complex for proteins that have no business residing in the vicinity of a promoter.

Three features are key to why searching for partners with this assay means that your protein won't be home alone on a Saturday night. The first is that DNA-binding domains like those of the yeast Gal4 and *E. coli* LexA proteins can drag all sorts of unwitting protein X's to the regulatory region of a yeast reporter gene.

The second is that transcription activation domains derived from Gal4 or Herpes virus VP16, for example, can be recruited to this same reporter gene when fused to some protein Y, provided that Y can cozy up to X.

Finally, when the two hybrid proteins show up together at the reporter, the transcription machinery joins the party and the gene is expressed. While the readout of the assay is yeast that can grow on a defined medium or turn blue with the right substrate, the outcome of the experiment is a set of cDNA clones in hand encoding proteins that bind to your favorite protein X.

SOME HISTORY

As with any new technology, the two-hybrid system had its origins in a host of previous experiments and methodologies. One was the glorious history of genetic selections in microbial organisms: for bacteria and phage, these selections were essential to decipher the fundamental language underlying the flow of genetic information but are now used principally for the mundane manipulations of recombinant DNA. In yeast, complementation of cell cycle defects with human cDNA libraries (*e.g.*, reference 2) was a major contribution to an understanding of how animal cells control their division.

Two, the use of fusion proteins to analyze transcription, translation, protein localization, and other processes has a long and productive track record. In particular, β-galactosidase—the workhorse of fusion technology—played a central role both in the dissection of Gal4 protein function and as a simple reporter for Gal4-dependent transcription.

Three, by the late 1980s we were more than a decade into the revolution brought about by DNA cloning. Recombinant DNA-based methods were becoming ever more prevalent in the identification of genes through the screening of cDNA, genomic, or λ expression libraries.

Four, and of particular relevance, were the ground-breaking studies on the mechanism of transcriptional activation. That a transcription factor like Gal4 has small, discrete domains responsible for contacting DNA and for recruiting the general transcription machinery was itself a striking finding, but the demonstration that an artificial factor could be created, by fusing a DNA-binding domain to a transcription activation domain, was a remarkable result (3). While this "swap" experiment provided the concept of a covalent hybrid activator, other experiments (4) revealed that proteins with activation domains could work by associating noncovalently with DNA-bound proteins.

Finally, the pressure to generate fundable ideas in order to maintain a laboratory (as recounted in reference 5) provided a major stimulus to the development of this assay.

SOME VIRTUES

The two-hybrid system possesses several virtues that have led to its popularity. First, it came along at a timely moment, as a burgeoning supply of proteins to analyze was becoming available, initially from more traditional biochemical and genetic approaches and more recently from genome sequencing. Not only did the supply of proteins grow, but the demand for interacting partners accelerated as awareness increased of the role of protein complexes in growth control, signal transduction, and other cellular processes.

***The two-hybrid system** came along at a timely moment, as a burgeoning supply of proteins to analyze was becoming available.*

Second, the yeast assay is easy to perform, allowing geneticists, cell biologists, and molecular biologists to succeed in a task formerly the sole province of competent biochemists. As a method that could be readily packaged into a set of strains, plasmids, libraries, and an accompanying guidebook, the yeast assay has unintentionally contributed to the current kit-based mentality of molecular biology. In this regard, hybrid proteins have many friends, including glutathione S-transferase, hexahistidine, and an assortment of epitope tags.

Third, the two-hybrid system is enormously adaptable, proving to be the basic platform upon which were built a series of related approaches (reviewed in 6) to analyze DNA-protein, RNA-protein, and small molecule-protein interactions. Other developments include reverse assays designed to identify mutations, peptides, or drugs that disrupt interactions, and three plasmid assays to express bridging proteins or modifying activities. In addition to transcription factor activity, the functions of other proteins like ubiquitin, β-galactosidase, and SOS have proven capable of reconstitution via a protein-protein interaction.

Finally, at a time when genomics is being superseded by proteomics, the method can be carried out on a grand scale. In the works are two-hybrid searches to identify interactions for the complete protein complements of model organisms such as yeast up to humans.

SOME OUTCOMES

What is the ideal outcome for a two-hybrid search with a "bait" protein dear to your heart? Twenty positives are found, defining six different proteins, each of these as a set of overlapping clones that defines a minimal domain of interaction. These six fall into three classes as follows. The first comprises three known proteins, one previously shown to bind to your bait by co-immunoprecipitation, and two others that make sense from the genetics of your system. These validate your search and provide confirmatory data for a long-held hypothesis. The second class consists of a totally surprising, but in retrospect plausible, well-known protein that links your bait to what was thought to be an unconnected cellular process. Further work confirms this unexpected cross-connection and suggests innovative possibilities for the treatment of human disease. The third class defines two novel proteins with no significant homology to any others in the database. These provide grist for John Miller, a graduate student in your lab, who turns them into a stellar degree.

While we know of no such single search, each of the individual outcomes has been described. For example, one early success was the identification of the Raf protein kinase as a partner for the oncoprotein Ras (7). Raf had been shown to be active downstream of Ras in *Drosophila, Xenopus, C. elegans,* and mammalian cells. Thus, the finding that an 81-residue region of Raf bound in the two-hybrid assay to wild-type, but not an effector domain mutant of Ras, was a satisfying result. Another early search, using the HIV gag protein as bait, unexpectedly turned up cyclophilins A and B (8), suggesting that these host proteins may act during the retroviral life cycle.

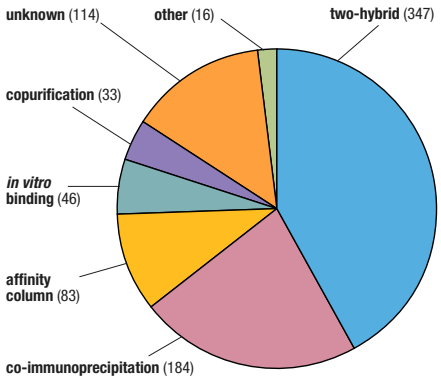


FIGURE 2. Frequency of methods used to detect protein-protein interactions of yeast proteins. Some of these interactions have been detected by multiple methods. Note that some methods such as affinity columns and *in vitro* binding are based on similar principles. The popularity of these methods to detect interactions may differ for proteins from other organisms.

Recently, a two-hybrid search with GATA4, a protein required for the upregulation of genes implicated in cardiac hypertrophy, found NF-AT3, a member of the family of nuclear factors of activated T cells (9). Just as T cell signaling requires dephosphorylation of NF-AT factors by the calcineurin protein phosphatase, the heart pathology involves calcineurin signaling through NF-AT3 and GATA4. Furthermore, the immunosuppressant cyclosporin A, which inhibits calcineurin in T cells, suppresses cardiac hypertrophy in an animal model of the disease.

The Two-Hybrid System continued

The two-hybrid system has become popular along with other simple methodologies that employ expression plasmids to attach convenient handles to proteins. In the yeast world, where use of the protocols required has no energy barrier, two-hybrid has been used to detect about half of the interactions, for which a method is provided, that are listed in the Yeast Proteome Handbook (10)*(figure 2)*. In the biological community as a whole, a clear sign of the system's commonality is the decreasing frequency with which it is cited in the literature. After a few years in the early '90s of exponential citation growth, references to the original description of the assay are now plateauing and should soon decrease, replaced by reviews, literature from supply companies, or common knowledge. Similarly, methods sections of papers no longer provide detailed descriptions of the search procedures, but merely indicate which version of the assay was used.

SOME FINAL THOUGHTS

As users well know, two-hybrid searches are not without pitfalls (*e.g.,* reference 11), and more than one promising hypothesis has evaporated following the performance of additional tests on a hopeful candidate from a library screen. But if you're persistent—if not with one bait then with another—it's likely that something worthwhile and perhaps even invaluable will turn up. Indeed, once you've mastered the technique, you can apply it rapidly to all manner of proteins in which you have a passing fancy. That the technique is nearly immune to the unique characteristics of proteins that make them so interesting is perhaps its greatest strength, as long as the two-hybrid practitioner realizes that more than the life or death of a yeast cell will be required for proteins to give up more of their secrets. FOCUS

REFERENCES

1. Fields, S. and Song, O. (1989) *Nature 340*, 245.
2. Lee, M.G. and Nurse, P. (1987) *Nature 327*, 31.
3. Brent, R. and Ptashne, M. (1985) *Cell 43*, 729.
4. Triezenberg, S.J., Kingsbury, R.C., and McKnight, S.L. (1988) *Genes Dev. 2*, 718.
5. Fields, S. and Bartel, P.L. (1997) In *The Yeast Two-Hybrid System*. Oxford: Oxford University Press, p. 3.
6. Frederickson, R.M. (1998) *Curr. Opin. Biotechnol. 9*, 90.
7. Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A. (1993) *Cell 74*, 205.
8. Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V., and Goff, S.P. (1993) *Cell 73*, 1067.
9. Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., and Olson, E.N. (1998) *Cell 93*, 215.
10. *Yeast Proteome Handbook* (1997) Beverly, Massachusetts: Proteome, Inc.
11. Allen, J.B., Walberg, M.W., Edwards, M.C., and Elledge S.J. (1995) *Trends Biochem. Sci. 20*, 511.

"That's Wyatt, he does all of our Western blots."

Gary F. Gerard
Protein Engineering and Analysis
Life Technologies, Inc.
Rockville, Maryland 20849

Reverse Transcriptase: *A Historical Perspective*

The discovery, study, and use of reverse transcriptase (RT) has had a significant impact on a number of areas of molecular biology (reviewed in 1), *see page 58 in this issue*. The purpose of this brief perspective is to highlight some of the most important events in the development of RT as a tool for conversion of RNA to DNA (see reference 2 for recent protocols) and to delineate what remains to be done to create the ideal RT for use as a tool. Some of the most significant historical milestones are summarized in the timeline on the following pages.

THE BEGINNING YEARS

1964 TO 1971. Howard Temin predicted the existence in the mid-1960s of an enzymatic process to convert RNA to DNA as part of his DNA provirus hypothesis, which explained the ability of "positive-strand" RNA retroviruses to transform infected cells permanently (3,4). This work culminated in his discovery of RT in 1970 (5). Following paths suggested by work on "negative-strand" RNA viruses, David Baltimore discovered RT in retroviruses at the same time (6). These discoveries were followed quickly by a demonstration that, in addition to RNA-directed and DNA-directed DNA polymerase activity, RT also possesses a ribonuclease activity specific to RNA in RNA-DNA, RNase H (7). The significance of this discovery to the effective use of RT would become apparent. Foundational to subsequent study and application of RT was a program set up by Dr. J.W. Beard in 1971 to supply avian myeloblastosis virus (AMV) and later purified AMV RT to the research community. For a number of years this was the major source of RT in the United States. AMV is not easily obtained in individual laboratories, since it is generated by infecting a special strain of day-old male chicks with AMV and subsequently harvesting virus from chick blood.

A TOOL FOR cDNA LIBRARY PREPARATION

1972 TO 1987. Demonstrations quickly followed in 1972 that purified AMV RT could be used to synthesize cDNA from mRNA (9–11). Thus began a period of ~15 years during which techniques and reagents were perfected to prepare cDNA libraries (reviewed in 22). A highlight among many significant advances was the development by Gubler and Hoffman (20) of a simple method for the conversion of first-strand to double-stranded cDNA by RNA-primed nick translation (19) without significant loss of sequence information (21).

NEW USES FOR RT

1987 TO PRESENT. The marriage of reverse transcription and PCR in 1987 (26) opened new vistas for the sensitive detection and analysis of RNA molecules. RT-PCR adapted to a plethora of novel approaches has greatly enhanced our ability to dissect patterns of gene expression in cells (33).

IMPROVING RT

1985 TO PRESENT. RT was first cloned and overexpressed in *E. coli* in 1985 (23,24). This opened the door to several approaches for improving RT. First, extremely pure preparations of large quantities of RT could be generated. This reduced problems sometimes experienced with native AMV RT preparations during cDNA synthesis caused by contaminating ribonucleases. Crystallization and three-dimensional structural analysis of RT were then possible (28–30), which provided a road map to structure-function studies of RT. Second, genetic engineering to improve RT became possible. A critical improvement came with the development of RT having active polymerase but lacking RNase H activity (27). Berger and colleagues (18) demonstrated that the RNase H activity of RT reduces the efficiency of reverse transcription. Moreover, removal of the RT RNase H activity improved the efficiency of cDNA synthesis substantially (34,35). Unexpectedly, inactivating the RNase H domain of RT by deletion or point mutation increased the thermal activity of RT (34,35).

THE IDEAL RT

Despite these advances, more remains to be done to engineer a better RT for copying mRNA. There are at least 3 characteristics of RT that, if improved, will contribute to a better enzyme: a relatively high error rate, low thermal stability, and a strong tendency to produce truncated cDNA products because of pausing. The three-dimensional structure of HIV RT (28,29) has been and will be important in guiding engineering efforts for HIV RT. However, a more complete three-dimensional structure of M-MLV RT (30), as well as the structure of AMV RT, which have different subunit and domain structures than HIV RT, will be needed to engineer improved forms of these enzymes.

The overall error rates of M-MLV and AMV RT on a DNA template are 1/30,000 and 1/17,000, respectively (36,37). On RNA, the error rate of M-MLV RT is 1/37,000 (37). Efforts have just begun to understand which amino acids in RT influence fidelity (38,39). These kinds of studies have yielded some success with HIV RT and might also be successful with other RTs. For HIV RT, there is a correlation between the location of RT pause sites in template sequence and the location of frameshift mutation hot spots (40,41). It is possible, therefore, that changes engineered in RT amino acids that decrease pausing will also decrease the frequency of this type of mutation.

Reverse Transcriptase continued

The ability to carry out reverse transcription reactions at elevated temperatures at which RNA is still stable (below 65°C) is important for several reasons: primer binding specificity is increased, reducing the chances of oligo(dT) priming internally at short stretches of As in mRNA and reducing background during RT-PCR with gene-specific primers; and RNA secondary structure is reduced, which in turn reduces one type of RT pausing (42). Typically, M-MLV RT has been used in the 37°C to 42°C range, and AMV RT in the 37°C to 45°C range. Reactions can be carried out with RNase H-minus forms of M-MLV RT at 50°C (34,35). New RNase H-minus forms of avian RT can be used at 55°C to 60°C, and higher (31,32). It might be possible by a directed evolution approach (43) to develop a retroviral RT stable for extended periods at 60°C. A second approach useful in reducing RT pausing at RNA secondary structural regions is to unwind the RNA by addition of retroviral nucleocapsid protein (44–46).

The final characteristic for improvement is the tendency of RT to terminate cDNA synthesis at homopolymer runs in template RNA (47). Nucleocapsid protein does not appear to help in reducing this type of RT pausing (48). Since at least some pauses are unique to a particular RT (49), *e.g.,* M-MLV versus AMV RT, using two different RNase H-minus RTs together in the same reaction may reduce pausing. In light of our lack of understanding of the mechanism of pausing, another approach might be to use directed evolution of random mutations to generate the desired enzyme. However, it is difficult to envision a biological selection or screen that will yield an RT without this pausing tendency. Solutions to this problem await detailed information about the RT amino acids involved in the multiple steps of DNA polymerization. **FOCUS**

REFERENCES

1. Skalka, A.M. and Goff, S.P. (1993) in *Reverse Transcriptase*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. Gerard, G.F., Fox, D.K., Nathan, M., and D'Alessio, J.M. (1997) *Molecular Biotechnology 8*, 61.
3. Temin, H. (1964) *Virology 23*, 486.
4. Green, M. (1970) *Ann. Rev. Biochem. 39*, 701.
5. Temin, H.M. and Mitzutani, S. (1970) *Nature 226*, 1211.
6. Baltimore, D. (1970) *Nature 226*, 1209.
7. Moelling, K., Bolognesi, D.P., Bauer, H., Busen, W., Plassmann, H.W., and Hausen, P. (1971) *Nature New Biology 234*, 240.
8. Kacian, D.L., Watson, K.F., Burney, A., and Spiegelman, S. (1971) *Biochim. Biophys. Acta 246*, 365.
9. Ross, J., Aviv, H., Scolnick, E., and Leder, P. (1972) *Proc. Natl. Acad. Sci. USA 69*, 264.
10. Verma, I.M., Temple, G.F., Fan, H., and Baltimore, D. (1972) *Nature New Biology 235*, 163.
11. Kacian, D.L., Spiegelman, S., Bank, A., Terada, M., Metafora, S., Dow, L., and Marks, P.A. (1972) *Nature New Biology 235*, 167.
12. Faust, C.H., Diggelmann, H., and Mach, B. (1973) *Biochemistry 12*, 925.
13. Efstratiadis, A., Maniatis, T., Kafatos, F.C., Jeffrey, A., and Vournakis, J.N. (1975) *Cell 4*, 367.
14. Kacian, D.L. and Myers, J.C. (1976) *Proc. Natl. Acad. Sci. USA 73*, 2191.
15. Myers, J.C., Spiegelman, S., and Kacian, D.L. (1977) *Proc. Natl. Acad. Sci. USA 74*, 2840.

Reverse Transcriptase Historical Highlights

1964
Existence of reverse transcriptase (RT) predicted (3; reviewed in 4)

1970
RT discovered independently in murine and avian retroviruses (5,6)

1971
RT shown to have ribonuclease H (RNase H) activity (7)

1971
Program started by Dr. J. W. Beard (Duke University and Life Sciences) to supply AMV RT for study and use; program met required demand during the next 10 years before RT commercialized

1971
Highly purified preparations of AMV RT became available for use (8)

1972
Purified RT first used to synthesize cDNA from purified mRNA *in vitro* (9–11)

1973-1980
Reaction conditions established for AMV RT optimal for synthesis of cDNA (12–16)

1976
Double-stranded cDNA synthesized and cloned in *E. coli* (17)

1983
RT-associated RNase H demonstrated to be detrimental to cDNA synthesis from mRNA (18)

16. Retzel, E.F., Collett, M.S., and Faras, A.J. (1980) *Biochemistry 19*, 513.
17. Maniatis, T., Kee, S.G., Efstratiadis, A., and Kafatos, F.C. (1976) *Cell 8*, 163.
18. Berger, S.L., Wallace, D.M., Puskas, R.S., and Eschenfeldt, W.H. (1983) *Biochemistry 22*, 2365.
19. Okayoma, H. and Berg, P. (1982) *Mol. Cell. Biol. 2*, 161.
20. Gubler, U. and Hoffman, B.J. (1983) *Gene 25*, 263.
21. D'Alessio, J.M. and Gerard, G.F. (1983) *Nucleic Acids Res. 16*, 1999.
22. Kimmel, A.R. and Berger, S.L. (1987) *Methods Enz. 152*, 307.
23. Kotewicz, M.L., D'Alessio, J.M., Driftmier, K.M., Blodgett, K.P., and Gerard, G.F. (1985) *Gene 35*, 249.
24. Roth, M.J., Tanese, N., and Goff, S.P. (1985) *J. Biol. Chem. 260*, 9326.
25. Tanese, N., Sodroski, J., Haseltine, W.A., and Goff, S.P. (1986) *J. Virol. 59*, 743.
26. Veres, Q., Gibbs, R.A., Scherer, S.E., and Caskey, C.T. (1987) *Science 237*, 415.
27. Kotewicz, M.L., Sampson, C.M., D'Alessio, J.M., and Gerard, G.F. (1988) *Nucleic Acids Res. 16*, 265.
28. Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A., and Steitz, T.A. (1992) *Science 256*, 1783.
29. Arnold, E., Jacobo-Molina, A., Nanni, R.G., Williams, R.L., Lu, X., Ding, J., Clark, A.D., Jr., Zhang, A., Ferris, A.L., Clark, P., Hizi, A., and Hughes, S.H. (1992) *Nature 357*, 85.
30. Georgiadis, M.M., Jessen, S.M., Ogata, C.M., Telesnitsky, A., Goff, S.P., and Hendrickson, W.A. (1995) *Structure 3*, 879.
31. Gerard, G., Potter, R., Smith, M., Rosenthal, K., and Chatterjee, D. (in preparation).
32. Schwabe, W., Lee, J.E., Xu, R.H., Sitaraman, K., Smith, M., Potter, R.J., Rosenthal, K., Rashtchian, A., and Gerard, G.F. (1998) *FOCUS 20*, 30.
33. Montarras, D., Pinset, C., Chelly, J., and Kahn, A. (1994) in *The Polymerase Chain Reaction* (Mullis, K.B., Ferre, F., and Gibbs, R.A., eds.) Birkhauser, Boston, Massachusetts, p. 277.
34. Gerard, G.F., D'Alessio, J.M., and Kotewicz, M.L. (1989) *FOCUS 11*, 66.
35. Gerard, G.F., Schmidt, B.J., Kotewicz, M.L., and Campbell, J.H. (1992) *FOCUS 14*, 91.
36. Roberts, J.D., Bebenek, K., and Kunkel, T.A. (1988) *Science 242*, 1171.
37. Ji, J. and Loeb, L.A. (1992) *Biochemistry 31*, 954.
38. Dresopoulos, W.C. and Prasad, V.R. (1998) *J. Virol. 72*, 4224.
39. Kim, B., Hathaway, J.R., and Loeb, L.A. (1998) *Biochemistry 37*, 5831.
40. Bebenek, K., Abbotts, J., Roberts, J.D., Wilson, S.H., and Kunkle, T.A. (1989) *J. Biol. Chem. 264*, 16948.
41. Bebenek, K., Abbotts, J., Wilson, S.H., and Kunkel, T.A. (1993) *J. Biol. Chem. 268*, 10324.
42. Myers, T.W. and Gelfand, D.H. (1991) *Biochemistry 30*, 7661.
43. Arnold, F.H. (1998) *Acc. Chem. Res. 31*, 125.
44. Tanchou, V., Gabus, C., Rogemond, V., and Darlix, J.-L. (1995) *J. Mol. Biol. 252*, 563.
45. Wu, W., Henderson, L.E., Copeland, T.D., Gorelick, R.J., Bosche, W.J., Rein, A., and Levin, J.G. (1996) *J. Virol. 70*, 7132.
46. Ji, X., Klarmann, G.J., and Preston, B.D. (1996) *Biochemistry 35*, 132.
47. Klarman, G.J., Schauber, C.A., and Preston, B.D. (1993) *J. Biol. Chem. 268*, 9793.
48. DeStefano, J.J. (1995) *Arch. Virol. 140*, 1775.
49. DeStefano, J.J., Buiser, R.G., Mallaber, L.M., Myers, T.W., Bambara, R.A., and Fay, P.J. (1991) *J. Biol. Chem. 266*, 7423.

1983

Efficient methods developed to synthesize double-stranded DNA from first-strand cDNA involving minimal loss of sequence information (19–21)

1983-1987

Multiple methods developed to generate plasmid and phage cDNA libraries (22)

1985

Fully active murine RT cloned and overexpressed in *E. coli* (23,24)

1986

Active HIV RT cloned in *E. coli* (25)

1987

Reverse transcription and PCR combined to amplify mRNA sequences (26)

1987

Cloned murine RT engineered to maintain polymerase and eliminate RNase H activity (27)

1992

The three-dimensional structure of HIV RT elucidated (28,29)

1995

The three-dimensional structure of a catalytically active fragment of murine RT elucidated (30)

1998

Cloned avian RT with fully active polymerase and minimized RNase H activity engineered (31,32)

High Purity Plasmid DNA from Anion Exchange Chromatography

Mindy D. Goldsborough, Lisha Xu, Krista Evans, and **Alice Young**
Molecular and Cell Biology
Research and Development
Life Technologies, Inc.
Rockville, Maryland 20849

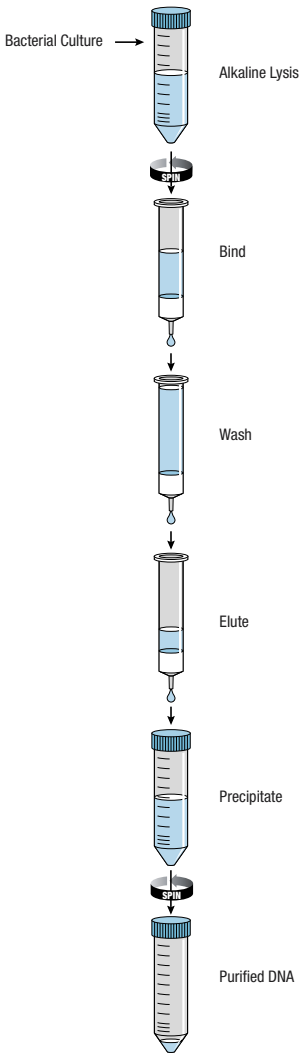


FIGURE 1. Schematic of plasmid DNA purification protocol.

Preparation of high purity supercoiled plasmid DNA can be a laborious process, involving multiple high-speed CsCl centrifugations, dialysis, and concentration. Alternatively, the same quality DNA can be obtained using anion exchange chromatography in <2 h. The new CONCERT™ High Purity Plasmid Purification Systems provide qualified reagents for preparing a cleared lysate and a unique anion exchange resin for producing DNA of the highest quality. DNA purified by the CONCERT High Purity Plasmid Purification Systems is suitable for a wide variety of applications, including the demanding application of eukaryotic cell transfection.

METHODS

All media, sera, enzymes, agarose, buffers, and reagents were GIBCO BRL brand unless otherwise noted. All plasmids were grown in *E. coli* DH10B™ cells from single colonies or glycerol stocks to stationary phase at 37°C using LB broth supplemented with appropriate antibiotics. Plasmids included pCMVβ (1), pCMVSPORTPP2A, pSUsrmB2, and pMAB32.

Plasmid purification was performed using the CONCERT High Purity Plasmid Purification Systems. There are 3 sizes: Mini (up to 20 µg DNA, Cat. No. 11449), Midi (up to 100 µg DNA, Cat. No. 11451), and Maxi (up to 500 µg DNA, Cat. No. 11452). The purification process is outlined in *figure 1*. Cleared lysate from 3 ml, 25 ml, or 100 ml of culture was applied to mini-, midi-, and maxiprep columns, respectively. CsCl-purified DNA was purified by 2 passes through a CsCl gradient. DNA was quantitated using Hoechst dye 33258. Restriction endonuclease digestions were at 37°C for 1 h using 100 ng of DNA and 1 unit of enzyme. DNA was electrophoresed in 1% (w/v) agarose TAE gels, stained with ethidium bromide, and photographed under UV transillumination.

For transfection, MDCK canine kidney cells were cultured in D-MEM, high glucose, with 0.1 mM NEAA, and 10% FBS (2). Cells were plated one day prior to transfection at 6×10^4 cells/well in 24-well plates and 1.5×10^5 cells/well in 12-well plates to be 70% confluent on the day of transfection.

For 24-well plate transfections, 0.4 µg pCMVβ DNA, 4.5 µl LIPOFECTAMINE™ Reagent, and 7.5 µl PLUS Reagent were used in serum-free D-MEM with NEAA (total volume 300 µl) (2). At 28 h post-transfection, cells were assayed for β-galactosidase (β-gal) activity (3). Cell extracts were assayed for soluble protein content using the Bradford assay (4).

For 12-well plate transfections, 0.8 µg pCMVβ DNA, 5 µl LIPOFECTAMINE Reagent, and 10 µl PLUS Reagent were used with serum-free D-MEM with NEAA (total volume 500 µl) (2). At 28 h post-transfection, cells were fixed and stained *in situ* with X-gal (5).

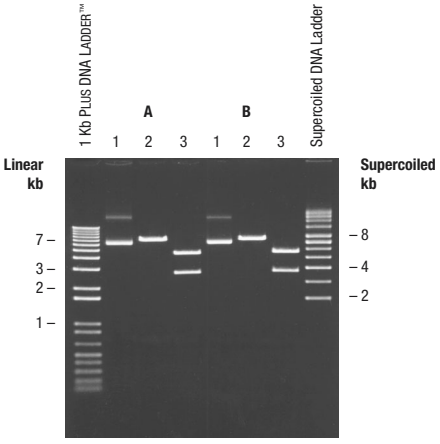


FIGURE 2. Restriction endonuclease digestion of purified plasmid DNA. Each sample contains 100 ng of a 7.2-kb plasmid, pCMVβ. *Panel A.* Miniprep-purified DNA. *Panel B.* Maxiprep-purified DNA. *Lane 1,* undigested purified plasmid; *lane 2,* purified plasmid digested with *Eco*R I; *lane 3,* purified plasmid digested with *Pst* I.

RESULTS AND DISCUSSION

High-quality DNA was obtained from all 3 sizes of CONCERT High Purity columns. For example, *figure 2* shows plasmid purity from mini- and maxiprep columns. The plasmid DNA was predominantly supercoiled with no RNA contamination. *Eco*R I digests pCMVβ once to produce a linear DNA of 7.2 kb. *Pst* I digestion produces 2 fragments, 4.5 and 2.7 kb. Plasmid DNA was digested to completion for both purifications and both enzymes.

Difficult-to-transfect MDCK cells were transfected in triplicate wells with supercoiled plasmid DNA purified by the CONCERT High Purity System to measure plasmid purity in a demanding system. The transfection efficiency measured by the percent of blue cells for maxiprep DNA was 49 ± 11% (mean ± S.D.) (*figure 3*). Transfection efficiency was similar for mini- and midiprep purified DNA, 40 ± 8% and 42 ± 1%, respectively, and compared favorably to DNA purified by CsCl with 40 ± 4%.

Quantitative measurement of the transfected β-gal reporter gene activity gave results equivalent to CsCl-purified DNA (*figure 4*). The average percent cell protein was compared to untransfected control cells (set at 100%). CsCl-purified DNA was 99 ± 2%, maxiprep-purified DNA was 122 ± 6%, midiprep DNA was 108 ± 2%, and miniprep DNA was 91 ± 2% of the control. Thus, DNA purified with the CONCERT High Purity Plasmid Purification Systems transfected as well as CsCl-purified DNA and had no effect on cell viability.

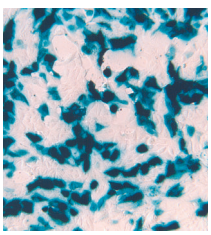


FIGURE 3. Transgene expression. MDCK cells were transfected with maxiprep column-purified pCMVβ using LIPOFECTAMINE PLUS Reagent in 12-well plates. The photograph taken was the best field-of-view using a 10X objective on a Nikon microscope with Hoffman optics.

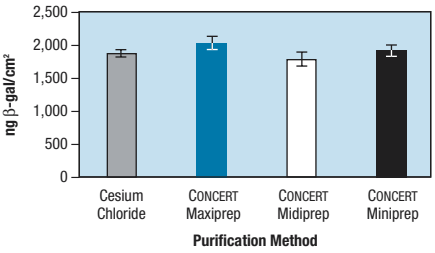


FIGURE 4. Effect of DNA preparation on transfection. MDCK cells were transfected with purified pCMVβ plasmid DNA using LIPOFECTAMINE PLUS Reagent in 24-well plates. Cells were harvested and assayed for β-gal activity 28 h after transfection. Results are mean ± SD for N = 3.

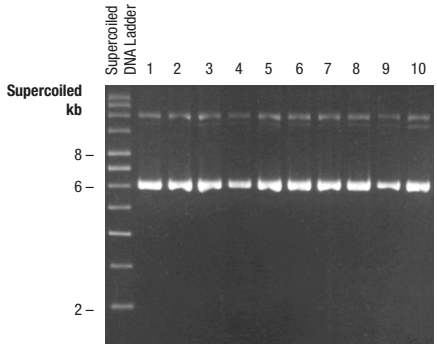


FIGURE 5. Reproducibility of plasmid minipreps. Equal volumes of samples (1 µl of 25) from 10 replicate miniprep column plasmid purifications of a 6.2-kb plasmid, pCMVSPORTPP2A.

Reproducibility of the CONCERT Miniprep System was investigated. Ten individual 3-ml aliquots from a single bacterial growth were purified on 10 columns (*figure 5*). The average yield of DNA from the replicates was 3 ± 0.5 µg. In addition, CONCERT High Purity Plasmid Purification Systems had consistent column-to-column flow rates (data not shown).

The yield of DNA from any bacterial culture is affected by several factors, including the copy number of the plasmid, host cell genotype, growth conditions, and plasmid size. The effect of copy number was investigated (*table 1*). The total DNA yield for a miniprep column was 1.5 µg for the low-copy-number plasmid and 28 µg for the high-copy-number plasmid. While the yields were different, the standard deviation values showed the purifications were consistent for the plasmids.

CONCERT High Purity Plasmid Purification Systems offer a quick, reliable, and scalable alternative to CsCl for plasmid DNA purification requiring high-quality DNA. DNAs purified by miniprep, midi-prep, and maxiprep columns were shown to be of very high purity and suitable for digestion with restriction endonucleases or for transfection into cultured cells. DNA yield was highly consistent between columns. However, specific yield is dependent on the characteristics of the particular plasmid being purified.

ACKNOWLEDGEMENTS

The authors thank Mike Brasch, Mary Longo, and Debbie Polayes for bacterial plasmids and Robert Blakesley and Pam Hawley-Nelson for helpful discussions. FOCUS

REFERENCES

1. MacGregor, G.R. and Caskey, C.T. (1989) *Nucleic Acids Res. 17*, 2365.
2. Shih, P.J., Evans, K., Schifferli, K., Ciccarone, V., Lichaa, F., Masoud, M., Lan, J., and Hawley-Nelson, P. (1997) *FOCUS 19*, 52.
3. Jain, V.K. and Magrath, I.T. (1991) *Anal. Biochem. 199,* 119.
4. Bradford, M.A. (1976) *Anal. Biochem. 72*, 248.
5. Sanes, J.R., Rubenstein, L.R., and Nicolas, J.F. (1986) *EMBO J. 5*, 3133.

Plasmid	Size (kb)	Plasmid Copy Number (at 37°C)	DNA Yield Total DNA (µg)	DNA Yield µg/ml of Culture
pSUsrmB2	5.31	6–10	1.5 ± 0.1	0.5 ± 0.02
pMAB32	11.2	>100	28.9 ± 0.5	9.6 ± 0.2

TABLE 1. Average DNA yield for different plasmids. Cultures containing the plasmids were grown to comparable cell densities and 3-ml aliquots were purified on CONCERT miniprep columns. pSUsrmB2 has a pACYC origin of replication and pMAB32 has a pUC origin of replication. Results are mean ± SD for N = 3.

Rapid Purification of Plasmid DNA Using Spin Cartridges

Mindy D. Goldsborough, Lisha Xu,
and **Alice Young**
Molecular and Cell Biology
Research and Development
Life Technologies, Inc.
Rockville, Maryland 20849

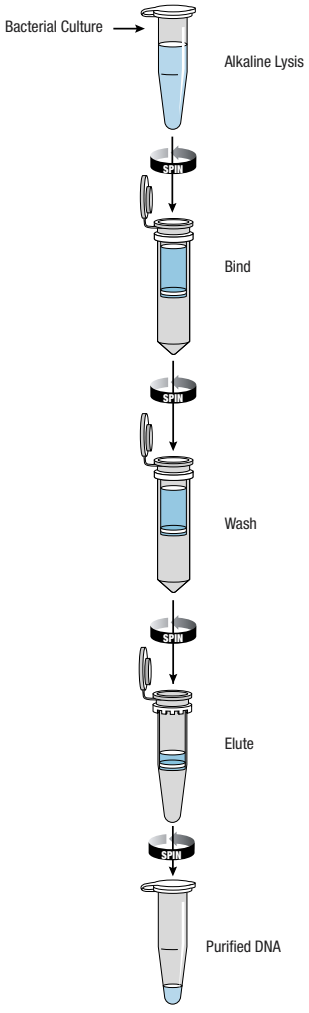


FIGURE 1. Schematic of plasmid DNA purification protocol.

Applications that require DNA of the highest purity necessitate a time investment for purification from hours to days. However, for other applications the CONCERT™ Rapid Plasmid Purification Systems purify plasmid DNA in as little as 30 min. Qualified reagents are provided to produce a cleared lysate, and plasmid DNA is then purified by a silica-based membrane (1) spin cartridge. The DNA is eluted, ready-to-use, at high concentration in low ionic strength buffer. DNA purified by the CONCERT Rapid Plasmid Purification Systems is suitable for a wide variety of applications, including restriction endonuclease digestion, PCR, and sequencing.

METHODS

All media, enzymes, agarose, buffers, and reagents were GIBCO BRL brand unless otherwise noted. All plasmids were grown in *E. coli* DH10B™ cells from single colonies or glycerol stocks to stationary phase at 37°C using LB broth supplemented with appropriate antibiotics. Plasmids included pCMVβ (2), pCMVSPORTPP2A, pSUsrmB2, and pMAB32.

Plasmid purification was performed using the CONCERT Rapid Plasmid Purification Systems. There are 3 sizes: Mini (up to 30 µg DNA, Cat. No. 11453), Midi (up to 100 µg DNA, Cat. No. 11454), and Maxi (up to 500 µg DNA, Cat. No. 11455). The purification procedure is outlined in *figure 1*. DNA was quantitated using Hoechst dye 33258. Restriction endonuclease digestions were at 37°C for 1 h using 100 ng of DNA and 1 unit of enzyme. DNA was electrophoresed in 1% (w/v) agarose TAE gels, stained with ethidium bromide, and photographed under UV transillumination.

Automated fluorescent sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, ~400 ng template DNA from miniprep cartridges, and 3.2 pmol M13/pUC Forward 23-Base Sequencing Primer.

RESULTS AND DISCUSSION

Plasmid DNA purified from all 3 sizes of CONCERT Rapid cartridges was free of enzymatic inhibitors as measured by restriction endonuclease digestion. As an example, *figure 2* shows purified plasmid DNA was completely digested. The plasmid DNA was predominantly supercoiled with no RNA contamination. *Eco*R I digests pCMVβ once to produce a linear DNA of 7.2 kb.

DNA obtained from the CONCERT Rapid Plasmid Purification Systems is of high quality and suitable for automated fluorescent sequencing. As a challenging example, a low copy number plasmid, pSUsrmB2 (6–10 copies per cells), was selected for sequencing *(figure 3)*. The average percent accuracy over a 600-nucleotide read-length was 98.6 ± 0.5% (mean ± SD, N = 3). The data show plasmid DNA prepared with the CONCERT Rapid Plasmid

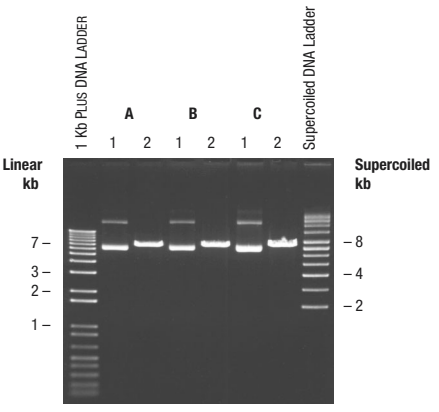


FIGURE 2. Restriction endonuclease digestion of purified plasmid DNA. Each sample contains 100 ng of a 7.2-kb plasmid, pCMVβ. *Panel A*. Miniprep-purified DNA. *Panel B*. Midiprep-purified DNA. *Panel C*. Maxiprep-purified DNA. *Lane 1,* undigested purified plasmid; *lane 2,* purified plasmid digested with *Eco*R I.

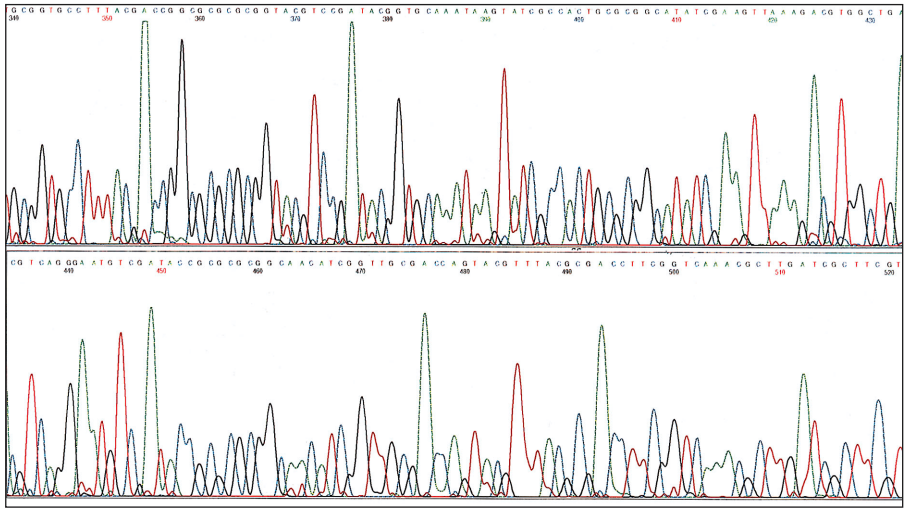


FIGURE 3. DNA sequence. A section of the electropherogram (nucleotide 340-530) obtained using purified pSUsrmB2 DNA.

Purification Systems did not inhibit this *Taq* DNA polymerase-based enzymatic reaction.

Reproducibility of the CONCERT Miniprep System was investigated. Ten individual 3-ml aliquots from a single bacterial growth were purified in 10 cartridges *(figure 4)*. The average DNA yield from the replicates was 1.7 ± 0.3 µg, verifying the reproducibility of this method.

The yield of DNA from a bacterial culture is affected by several factors, including the copy number of the plasmid, host cell genotype, growth conditions, and plasmid size. The effect of copy number was investigated *(table 1)*. The total DNA yield from a miniprep cartridge was 0.8 µg for the low copy number plasmid and 26 µg for the high copy number plasmid. As expected, yields were highly influenced by copy number.

CONCERT Rapid Plasmid Purification Systems offer a quick, reliable, and scalable method to purify plasmid DNA of high quality that is compatible with enzyme-based post-purification applications. DNA yield was highly consistent between cartridges. However, specific yield is dependent on the characteristics of the particular plasmid being purified.

ACKNOWLEDGEMENTS

The authors thank Mike Brasch, Mary Longo, and Debbie Polayes for bacterial plasmids and Robert Blakesley for helpful discussions. FOCUS

REFERENCES

1. Vogelstein, B. and Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA 76*, 615.
2. MacGregor, G.R. and Caskey, C.T. (1989) *Nucleic Acids Res. 17*, 2365.

Plasmid	Size (kb)	Plasmid Copy Number (at 37°C)	DNA Yield Total DNA (µg)	DNA Yield µg/ml of Culture
pSUsrmB2	5.31	6–10	0.8 ± 0.3	0.3 ± 0.1
pMAB32	11.2	>100	26 ± 6	9 ± 2

TABLE 1. Average DNA yield for different plasmids. Cultures containing the plasmids were grown to comparable cell densities and 3-ml aliquots were purified on CONCERT miniprep cartridges. pSUsrmB2 has a pACYC origin of replication and pMAB32 has a pUC origin of replication. Results are mean ± SD for N = 3.

The Help Box from Your Technical Support & Training Team

Growing Bacteria for Plasmid Purification

Q. Can I grow my *E. coli* cultures in rich medium (*e.g.*, terrific broth) to increase cell yield prior to DNA purification?

A. Although one can grow plasmids, especially high-copy-number plasmids, to higher cell densities using rich medium, DNA quality suffers when column capacity is outstripped. Following recommended conditions (growth in LB medium to an A_{600} of 1 to 1.5) and using a column of the correct capacity ensures optimal purification.

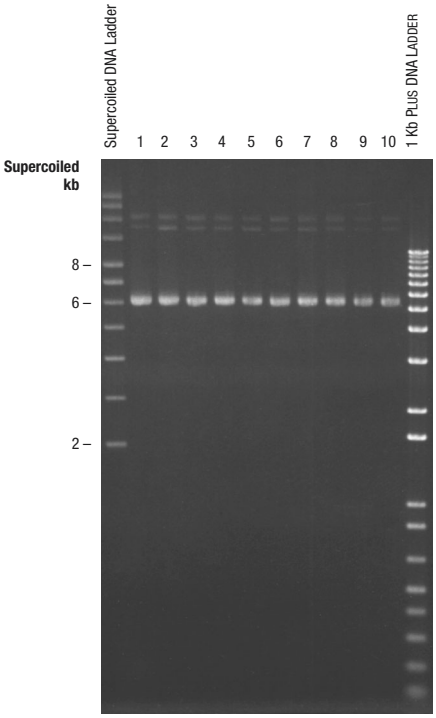
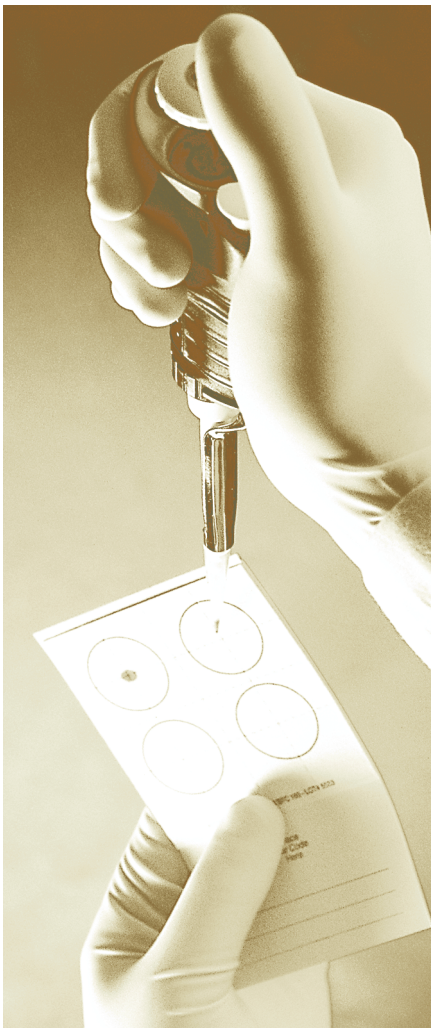


FIGURE 4. Reproducibility of plasmid minipreps. Equal volumes of samples (4 µl of 75) from 10 replicate miniprep cartridge purifications of a 6.2-kb plasmid, pCMVSPORTPP2A.

Pamela Hansen and **Robert Blakesley**
Molecular and Cell Biology
Research and Development
Life Technologies, Inc.
Rockville, Maryland 20849

Simple Archiving of Bacterial and Plasmid DNAs for Future Use



For many projects, generation of numerous DNA samples from biological specimens is routine. Handling and archiving a large collection can become a logistical problem for the laboratory. One solution, used in forensic labs, is the blood-storage medium FTA™ Cards. An FTA Card safely stores genomic DNA in the form of dried spots of human whole blood, the cells of which were lysed on the paper. Stored at room temperature, genomic DNA on FTA paper is reported to be stable at least 7.5 years (1). Before analysis of the captured DNA, a few simple washing steps remove the stabilizing chemicals and cellular inhibitors of enzymatic reactions. Since the DNA remains with the paper, the manipulations to purify the DNA are simplified and amenable to automation. DNA samples on FTA Cards offer a very compact archival system compared to glass vials or plastic tubes located in precious freezer space.

Bacterial DNAs spotted on FTA Cards may be an efficient system for storage and retrieval as well. Recently, Rogers and Burgoyne (2) characterized by PCR-ribotyping culture samples of several bacterial strains of *Staphylococcus* and *E. coli* stored on FTA Cards. Further, purified plasmid DNA was efficiently recovered after spotting on treated paper; however, encasement of the paper in polystyrene was used for storage (3). In this paper, we extend these observations to colonies from plates, rather than cultures, and to a few other bacteria of interest, namely, *Agrobacter tumefaciens* and *Streptomyces* species. We also examined whether unpurified plasmid DNAs harbored by bacteria could be simply stored and easily processed using a standard FTA Card.

	Amplicon		
	Target	Size (bp)	Primer Sequence
Agrobacter tumefaciens	16S rRNA	284	GGGAA AGATT TATCG GGGAT G GGCTG CTGGC ACGAA GTTA
E. coli DH10B cells	rrsE	372	CTGAG ACACG GTCCA GACTC CTACG TCACC GCTAC ACCTG GGATT CTACC
Streptomyces	16S rRNA	1,500	AGAGT TTGAT GATCC TGGCT CAG AAGGA GGTGA TCCAG CCGCA
Plasmid	pSUsrmB2	1,503	CCCAG TCACG ACGTT GTAAA ACG AGCGG ATAAC AATTT CACAC AGG
Plasmid	pMAB32	1,800	GAATA AGTGC GACAT CATCA TC GTAAA TTTCT GGCAA GGTAG AC
Plasmid	pJH11104	1,200	ACTTC TTCGC CCCCG TTTTC GCTGA CTTGA CGGGA CGGCG

TABLE 1. Primer sequences.

METHODS

Reagents were from Life Technologies unless otherwise noted.

PREPARATION OF SAMPLES ON AN FTA CARD. From bacterial cultures grown overnight, 5 µl were spotted in separate locations on an FTA Card (Cat. No. 10786) and allowed to dry overnight at room temperature. Bacterial colonies from petri dishes were spotted onto an FTA Card by suspending a colony in 5 µl of PBS, applying the entire volume as a single spot, then allowing the paper to dry overnight at room temperature. Using a HARRIS MICRO-PUNCH™ Apparatus with mat, a 2-mm punch was taken from a dried bacterial spot (~6 mm diameter), then washed with FTA Purification Reagent (Cat. No. 10876) and TE according to manufacturer's recommendations. The washed punch was air dried for 1 h at room temperature or 30 min at 60°C. Processed FTA Card punches were either assayed immediately or stored at 4°C.

AMPLIFICATION. DNA was amplified directly from a washed FTA Card punch placed in 50 µl of 1X PCR buffer, 1.5 mM Mg^{++}; 0.2 µM dNTPs, 1.25 units PLATINUM® *Taq* DNA Polymerase, and 0.2 µM primers *(table 1)*. For *Agrobacter tumefaciens* genomic sequence, amplification was one cycle of 94°C for 1 min; 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 3 min; and one cycle of 72°C

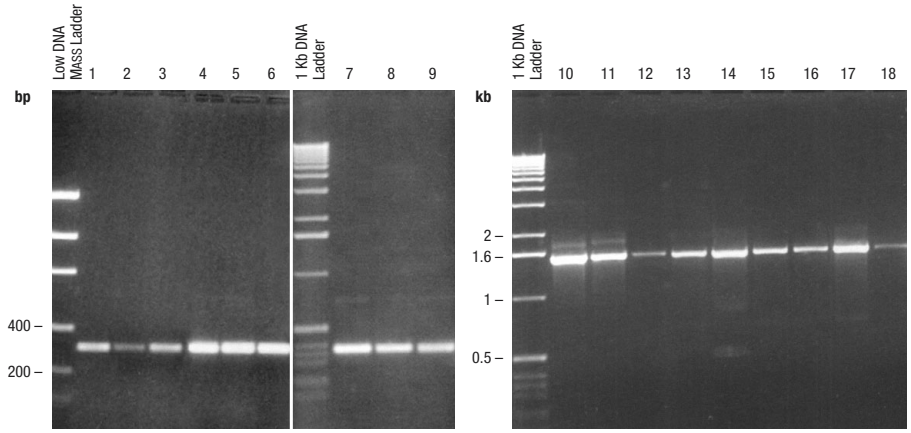


FIGURE 1. PCR analysis of bacterial genomic DNA on an FTA Card. Agarose gel analysis of the amplification products from *Agrobacter tumefaciens*, strain LBA4404 *(lanes 1–3)* and strain EHA101 *(lanes 4–6)*; *E. coli* DH10B strain *(lanes 7–9)*; and *Streptomyces coelicolor (lanes 10–12)*, *S. lividans (lanes 13–15)*, and *S. paralus (lanes 16–18)*.

for 10 min. For *E. coli* DH10B™ genomic DNA and *Streptomyces* samples, amplification was one cycle of 94°C for 1 min; 36 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and one cycle of 72°C for 10 min.

Plasmids pSUsrmB2 and pJH11104 were amplified in the same reaction mixture described above. Amplification of pMAB32 was in a reaction mixture using ELONGASE® Amplification System at 1.5 mM Mg^{++}. For all three plasmids, the incubation profile was one cycle of 94°C for 1 min, one cycle of 94°C for 15 s, and 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 3 min.

Aliquots of each reaction product were analyzed by electrophoresis in a 1.5% (w/v) agarose/TAE gel.

TRANSFORMATION. Cells were transformed with plasmid DNA from washed FTA Card punches, either by adding a punch directly to the reaction or by adding 5 µl of a TE buffer extract, which had soaked the punch for 20 min at room temperature. Both MAX EFFICIENCY® DH5α™ and MAX EFFICIENCY DH10B cells were transformed according to manufacturer's recommendations. Various dilutions of the final 1-ml transformation reaction volume (100 µl of a 1/100 dilution, 100 µl of a 1/10 dilution, 100 µl of undiluted cells, and 10 µl of undiluted cells) were plated on the appropriate medium and incubated overnight at 37°C.

RESULTS AND DISCUSSION

Using samples from overnight liquid cultures spotted on FTA Cards, Rogers and Burgoyne (2) generated the expected diagnostic PCR patterns for the six bacterial strains tested. Encouraged by their success with the DNA equivalent of only ~3 µl of culture and by the fact that bacterial DNA is readily amplified when colonies are introduced directly into PCR, we tested bacterial colonies placed on an FTA Card. Unlike Rogers and Burgoyne, who tested two complex methods of processing, all bacteria-spotted FTA Cards in this study were processed with simple washes.

***Plasmid DNA** was retained on the FTA Card, as the expected amplicons were observed.*

Bacterial genomic DNA targets amplified directly from washed punches gave the expected bands (*figure 1*). FTA Cards were useful for screening and identifying colonies of an *Agrobacter* bacterium from plants, a gram-negative *E. coli* bacterium, and a difficult-to-lyse, gram-positive *Streptomyces* bacterium. Our success was due in part to the robust nature of the amplification reaction, not requiring quantitation of the DNA beforehand. It is reasonable to assume that liquid cultures of the same bacteria spotted on the FTA Card would work as well as the colonies. Also, it is anticipated that transfer of bacteria by colony lift from the solid growth medium surface with an appropriate-sized FTA Card would be successful. Consistent with the known properties of FTA Cards, we were unable to rescue bacteria after washed, bacteria-spotted punches were placed on solid growth medium and incubated for >3 days.

Retention of plasmid DNA on FTA Cards was tested. Burgoyne reported (3) that purified plasmid DNA spotted on treated paper was efficiently removed with TE buffer as measured by PCR and trans-

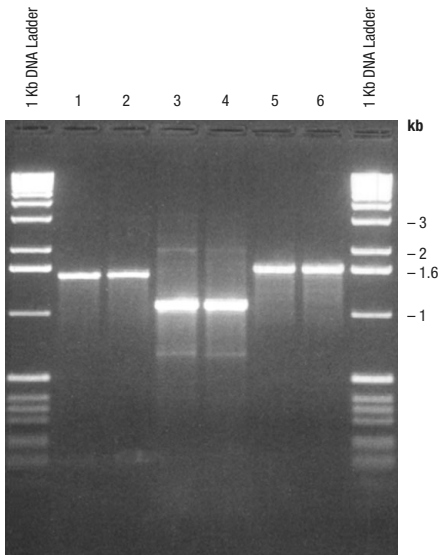


FIGURE 2. PCR analysis of plasmid DNA on an FTA Card. Agarose gel analysis of the amplification products pSUsrmB2 *(lanes 1 and 2)*, pJH11104 *(lanes 3 and 4)*, and pMAB32 *(lanes 5 and 6)*.

Simple Archiving continued

Plasmid	Size (kb)	Copy Number	Transformants/Punch
pSUsrmB2	5.3	6–10	7,000
pJH11104	2.7	~100	28,000
pMAB32	11.2	>100	5,700

TABLE 2. Transformation of DH5α cells with plasmids on an FTA Card punch. Results are the average from 4 dilutions.

formation. However, using purified DNA did not employ the advantage of directly spotting plasmid-containing bacteria on an FTA Card. Three cultures of *E. coli* were spotted onto an FTA Card, and processed punches were amplified. Plasmid DNA was retained on the FTA Card, as the expected amplicons were observed *(figure 2)*. However, the amount of plasmid DNA remaining with the paper is probably low. Standard fluorescent cycle sequencing of plasmid DNA directly from a washed punch did not detect any signal (data not shown) even though plasmid DNA in a single colony has been shown to be sufficient for cycle sequencing (4).

Although some plasmid DNA remained with the FTA Card through an extensive wash procedure, it is probable the DNA is loosely associated and some of it releases during each wash. This was confirmed by generation of the correct amplicon when a 5-µl TE buffer extraction of a washed punch was amplified by PCR (data not shown). This result suggested that the released plasmid DNA from washed punches might also be recovered by biological amplification, *i.e.,* bacterial transformation. DH5α and DH10B cells were successfully transformed with 5 µl from a TE-buffer extraction of a washed punch previously spotted with bacteria harboring one of three plasmids (data not shown). Successful rescue of the three plasmids led to testing a washed punch introduced directly into the cell transformation reaction *(table 2)*. Transformation reactions tested (two cell types and two DNA introduction methods) gave comparable results (data not shown). In all cases, bacterial clones were easily recovered, making FTA Cards potentially very convenient and cost effective for the long-term storage of bacterial clones. Similarly, it is possible other bacterial clones harboring DNAs such as cosmids, BACs, or PACs could be recovered from samples archived on FTA paper.

The FTA Card is known for its utility in archiving, its ease of sample preparation, and its elimination of potential biohazards in DNA from blood samples. We have shown that the FTA Card also is useful for archiving DNA from bacterial sources, both genomic and plasmid. The FTA Card represents a method for convenient and efficient ambient temperature storage, processing, and recovery of plasmid clones. This method could be very useful in storage of the vast numbers of subclones generated, for example, in large-scale genome sequencing projects.

NOTE ADDED IN PROOF: Recent experiments demonstrated equivalent results in PCR and transformation when the processing of punches of bacterial samples was reduced to simply two washes with TE. However, samples which contain blood must follow the full processing protocol with FTA Purification Reagent to obtain optimal results.

ACKNOWLEDGEMENTS

The authors thank S. Cohen and E. Nester for providing bacterial strains; M. Longo, A. Hu, and L. Mohelski for providing plasmids used in this study; and D. Carlson for helpful discussions. FOCUS

REFERENCES

1. Burgoyne, L.A., Carroll, D.J., Rogers, C., and Turner, J. (1997) Conventional DNA Collection and Processing: Disposable Toothbrushes and FTA™ Paper as a Non-threating Buccal-Cell Collection Kit Compatible with Automatable DNA Processing. 8th International Symposium on Human Identification, September 17–20, 1997.
2. Rogers, C. and Burgoyne, L. (1997) *Anal. Biochem. 247*, 223.
3. Burgoyne, L.A. Solid Medium and Method for DNA Storage. U.S. Patent 5,496,562.
4. Young, A. and Blakesley, R. (1991) *FOCUS 13*, 137.

The Help Box from Your Technical Support & Training Team

For More Information…

…On using the FTA Card for blood samples, look in the TECH-ONLINE section of www.lifetech.com.

Also, the following references may be useful:

1. Belgrader, P., Del Rio, S.A., Turner, K.A., Marino, M.A., Weaver, K.R., and Williams, P.E. (1995) *BioTechniques 19,* 426. "Automated DNA Purification and Amplification for Blood-Stained Cards Using a Robotic Workstation."
2. Del Rio, S.A., Marino, M.A., and Belgrader, P. (1996) *BioTechniques 20,* 970. "Reusing the Same Blood-Stained Punch for Sequential DNA Amplifications and Typing."

Robert A. Donahue, Jr. and **Fredric R. Bloom**
Competent Cells
Research and Development
Life Technologies, Inc.
Rockville, Maryland 20849

ELECTROMAX™ DH5α-E™ Cells:
A New Addition to the DH5α Family

The high transformation efficiency of electrocompetent cells (1) and their ease of use make them an ideal choice for construction of libraries. Electrocompetent cells make it feasible to construct cDNA libraries with 1 electroporation reaction.

The *E. coli* DH5α™ strain has been used to generate cDNA libraries due to the availability of high efficiency chemically competent cells (3) and several key genetic properties. The strain carries the *hsd*R mutation to prevent restriction of foreign DNA and the *end*A1 mutation that increases the yield of plasmid DNA prepared using miniprep protocols (4). DH5α cells contain the *deo*R mutation, for more efficient uptake of large plasmid DNAs (4), and the *lac*ZΔM15 deletion, which allows for blue/white screening on X-gal plates by α-complementation (5). A derivative of the DH5α (DH5α-E) strain was constructed for electroporation. This isolate is highly electrocompetent (>1 × 10^{10} transformants/µg pUC19), making it ideal for production of cDNA libraries. This paper demonstrates the attributes of ELECTROMAX DH5α-E cells (Cat. No. 11319).

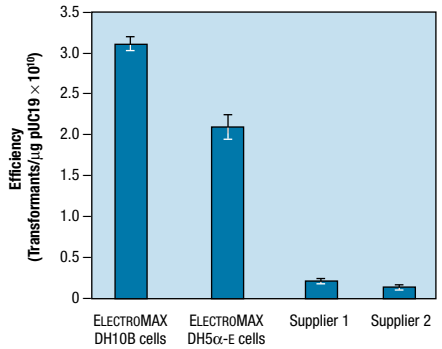


FIGURE 1. Transformation efficiencies under limiting DNA amounts. Electrocompetent cells were electroporated with 10 pg pUC19/20 µl. Results are mean of at least 6 electroporations and the error bars represent ± 1 SE.

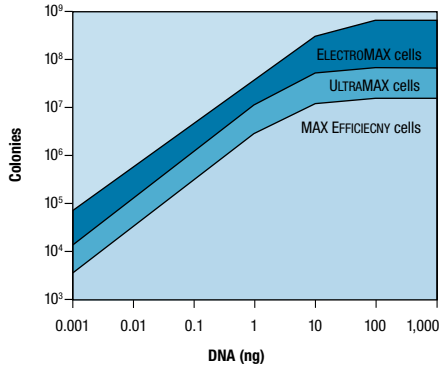


FIGURE 2. Colony output comparison. A saturation curve was constructed for several DH5α cells. The total number of colonies obtained from 1 transformation of ELECTROMAX DH5α-E cells compared to chemically competent ULTRAMAX™ DH5α-FT™ cells and MAX EFFICIENCY® DH5α cells. Results are the average for N = 6.

***ELECTROMAX DH5α-E cells** are ideal competent cells for difficult cloning applications.*

METHODS

TRANSFORMATIONS. Cells were electroporated according to the manufacturer's instructions with either a limiting amount of pUC19 DNA (10 pg/20 µl) or varying amounts of pUC19 DNA (0.001 ng to 1,000 ng). 10 µl of the electroporated cell mixture was expressed in 1 ml GIBCO BRL S.O.C. Medium at 37°C, 250 rpm for 1 h. The cells were diluted for plating based on the reported transformation efficiency, plated in duplicate on LB plates with 100 µg/ml ampicillin, and incubated for 16 h to 20 h at 37°C.

TRANSFORMATION WITH cDNA LIGATIONS. The cDNA was generated from purified HeLa poly(A⁺) RNA (a generous gift from S. Simms, Life Technologies) using the SUPERSCRIPT™ Plasmid cDNA Synthesis and Cloning System and inserted into pSPORT1 (6). The ligation mixtures were pooled, ethanol precipitated, and concentrated (500 µl of ligation was dissolved in 100 µl of TE). Competent cells were transformed with ~15 ng/reaction (1 µl) of the cDNA ligation mixture. The cells were plated as described above.

RESULTS AND DISCUSSION

To evaluate the transformation efficiencies of competent cells, the first benchmark is to use limiting amounts (subsaturating conditions) of DNA. ELECTROMAX DH5α-E cells resulted in an efficiency similar to ELECTROMAX DH10B™ cells, which have been used previously for demanding transformation applications *(figure 1)*.

To further evaluate the usefulness of competent cells, a saturation curve is constructed *(figure 2)*. For ELECTROMAX DH5α-E cells, 10- to 50-times greater colonies were obtained in a single transformation than using the chemically competent DH5α cells.

When enough cDNA is available, it is optimal to transform competent cells with saturating amounts of DNA to get maximal colony output per reaction. We typically use 15 ng of cDNA for library construction. Under these conditions, both the ELECTROMAX DH5α-E and ELECTROMAX DH10B cells generated >5 ×10^6 colonies/reaction *(figure 3)*. This

ELECTROMAX DH5α-E Cells continued

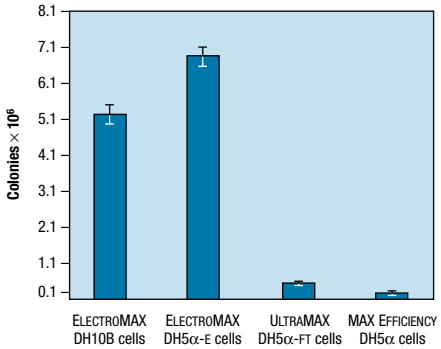


FIGURE 3. cDNA library construction. ~15 ng of the cDNA ligation mixture was transformed into various cells. Results are mean of 6 transformations and the error bars represent ± 1 SE.

allows production of a representative library in 1 reaction, unlike chemically competent cells. In fact, as little as 1 ng of a cDNA ligation reaction can generate a library of $>5 \times 10^5$ colonies in a single electroporation (1).

In summary, ELECTROMAX DH5α-E cells combine the power of electroporation for high efficiency with the genetic markers of the DH5α strain. This makes them ideal competent cells for difficult cloning applications, including cDNA library construction. FOCUS

REFERENCES

1. Smith, M., Jessee, J., Landers, T., and Jordan, J. (1990) *FOCUS 12*, 38.
2. Hanahan, D., Jessee, J., and Bloom, F.B. (1991) *Meth. Enzymol. 204*, 63.
3. Donahue, R.A., Jr., and Bebee, R.L. (1997) *FOCUS 19*, 22.
4. Grant, S.G.N., Jessee, J., Bloom, F.R., and Hanahan, D. (1990) *Proc. Natl. Acad. Sci. USA 87*,4645.
5. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene 33*,103.
6. D'Alessio, J.M., Gruber, C.E., Cain, C., and Noon, M.C. (1990) *FOCUS 12*, 47.

Choosing a Host Strain for Library Construction

Criteria used to select a host strain for cDNA library construction include the ability to generate large numbers of transformed colonies from a single reaction to minimize the number of transformations. Also, the ability to maintain large inserts improves the chances of finding the full-length sequence, as well as the ability to minimize degradation of the plasmid DNA to facilitate plasmid purification. The ELECTROMAX DH5α-E strain has the ability to generate a large number of transformants ($>5 \times 10^6$ transformants/reaction with 15 ng cDNA), and has a genetic background engineered to maintain large plasmids (*deo*R) and to reduce the degradation and increase the yield of the transformed plasmid DNAs (*end*A1).

The same features are found in 2 other electrocompetent strains, DH10B cells and DH12S™ cells *(table 1)*. In addition, DH10B cells contain the *mcr*A, *mcr*BC, and *mrr* mutations that allow the cloning of methylated DNA, making them the preferred host for cloning genomic DNA sequences and constructing genomic libraries. ELECTROMAX DH10B cells have been used to construct BAC and P1-based libraries. The DH12S strain is a derivative of the DH10B strain containing an F′ episome to produce single-stranded DNA for sequencing and phage display when transformed with a phagemid vector.

Application	Strain DH5α-E Cells	DH10B Cells	DH12S Cells
cDNA Libraries	X	X	X
Genomic Libraries		X	X
Producing ss DNA			X
Cloning Large DNA* (>30 kb)		X	

*See article on page 77 in this issue.

TABLE 1. Choosing electrocompetent cells.

The Help Box from Your Technical Support & Training Team

Handling Electrocompetent Cells

Q. How do you recommend handling ELECTROMAX cells ?

A. Thaw cells on wet ice and electroporate immediately upon thawing. Do not vortex cells. Precool the electroporation chambers to 4°C before adding cells and DNA.

Q. How do you recommend storing ELECTROMAX cells?

A. Store cells at –70°C. They are stable for a year. Do not store cells in liquid nitrogen or at –20°C. Do not refreeze ELECTROMAX cells. Freeze/thaw reduces efficiency up to 10 times.

Robert A. Donahue, Jr., and **Fredric R. Bloom**
Competent Cells
Research and Development
Life Technologies, Inc.
Rockville, Maryland 20849

Transformation Efficiency of E. coli *Electroporated with Large Plasmid DNA*

The ability to efficiently electroporate large plasmid DNA molecules into *E. coli* cells is important for the construction of cDNA and genomic libraries. Emphasis on the uptake of large plasmid molecules into *E. coli* cells has led to several articles studying the effect of plasmid size on transformation efficiency. These studies have shown that the transformation efficiency (transformants/µg DNA) decreased as the size of the plasmid DNA increased (1–3). In these studies, a single strain was analyzed using a series of plasmids based on the same backbone sequence in the size range (4 to 14 kb and 52 to 132 kb). In this paper, we characterize the transformation efficiency using 3 electrotransformable *E. coli* strains and 3 sets of plasmids to cover a range of 8 kb to 210 kb.

METHODS

ELECTROMAX™ DH5α-E™ (4) cells, DH10B™ (2) cells, and DH12S™ (5) cells were electroporated using the manufacturer's recommended conditions.

Plasmid DNA was prepared from DH5α or DH10B cells grown overnight at 30°C in LB medium. The DNA was isolated using a modified alkaline lysis method developed for the purification of large plasmid DNA (6). Plasmid DNA concentration was determined spectrophotometrically, and plasmid size was confirmed by sizing digested and undigested DNA using agarose gel electrophoresis. The pDELTA1-based plasmids (7) were provided by Alice Young (Life Technologies), the cosmid clones are from a chromosome 21 library constructed in the Lawrist 4 vector (8) and provided by Nicoletta Sacchi (Department of Biology and Genetics, School of Medicine, University of Milan, Italy), and the P1 plasmids (9) were provided by Pieter deJong (Human Genetics Department, Roswell Park Cancer Institute, Buffalo, New York). The plasmids were diluted in 0.5X TE to 2 ng/µl for the pDELTA1 plasmids, 5 ng/µl for the cosmids, and 10 ng/µl for the P1 plasmids.

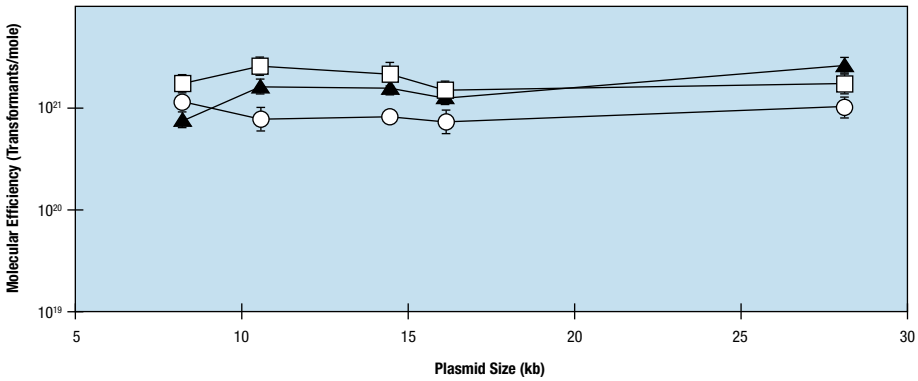


FIGURE 1. Electroporation of pDELTA1 plasmids. Strains used were DH5α-E (○), DH10B (▲), and DH12S (□) cells. Results are the mean ± SE for N = 4.

The frozen electrotransformable cells were thawed on ice for 20 min. The cells were gently mixed, and 40 µl of cells were added to 2 µl of plasmid DNA. The DNA was gently mixed with the cells, and 20 µl transferred to an electroporation chamber. After pulsing the cells, 10 µl of cells were added to a round-bottom 15-ml polypropylene tube containing 1 ml of S.O.C. Medium (Cat. No. 15544). The cells were incubated for 1 h at 37°C at 250 rpm. The cells were diluted in S.O.C. and plated on a selective medium containing either 100 µg/ml ampicillin or 50 µg/ml kanamycin. The plates were incubated at 37°C for 16 to 24 h before counting (some of the cosmid clones required 24 h to 48 h of growth after transformation into DH10B or DH12S cells).

RESULTS AND DISCUSSION

To evaluate the efficiency over a broad

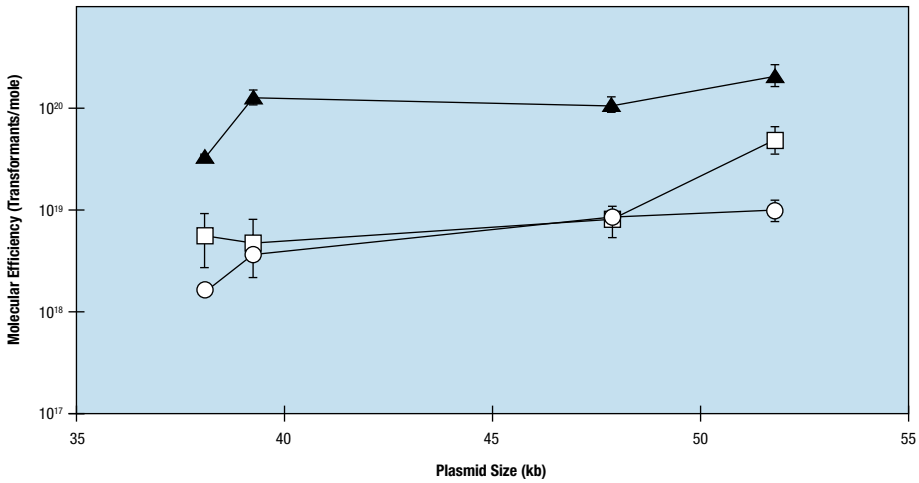


FIGURE 2. Electroporation of cosmid clones. Strains used were DH5α-E (○), DH10B (▲), and DH12S (□) cells. Results are mean ± 1 SE for N = 6.

Transformation Efficiency of Large Plasmids continued

size range, it is preferable to compare the molecular transformation efficiency (transformants/mole DNA) to minimize the variation of the number of molecules/µg DNA. The molecular transformation efficiency of plasmids with sizes from 8 kb to 28 kb was the same for the 3 strains *(figure 1)*. Since cDNA libraries usually do not contain clones over 30 kb, these *E. coli* strains can be used for cDNA libraries.

As the size of the plasmids increased over 30 kb (cosmid clones), some differences became apparent *(figure 2)*. While the molecular efficiency did not appear to decrease as the size of the cosmid DNA increased, the efficiency was highest with DH10B cells. Also, some of the transformations required 48 h to generate colonies, while others produced colonies of highly varying sizes.

With plasmids over 50 kb (P1 clones), the molecular transformation efficiency decreased as the plasmid size increased from 50 to 200 kb *(figure 3)*. The extent of the efficiency decrease appeared to be strain specific. DH10B cells retained the highest molecular efficiency. Previous studies have shown that plasmid DNA molecules of 100 to 750 kb have been maintained in *E. coli* DH10B cells using electroporation (1,9,10).

In summary, the molecular transformation efficiency was independent of size for plasmids <30 kb in the DH10B, DH5α-E, and DH12S cells. For plasmids >30 kb, DH10B cells maintained the highest transformation efficiency.

EDITOR'S NOTE: For more information on choosing strains for library construction, see page 76 in this issue. FOCUS

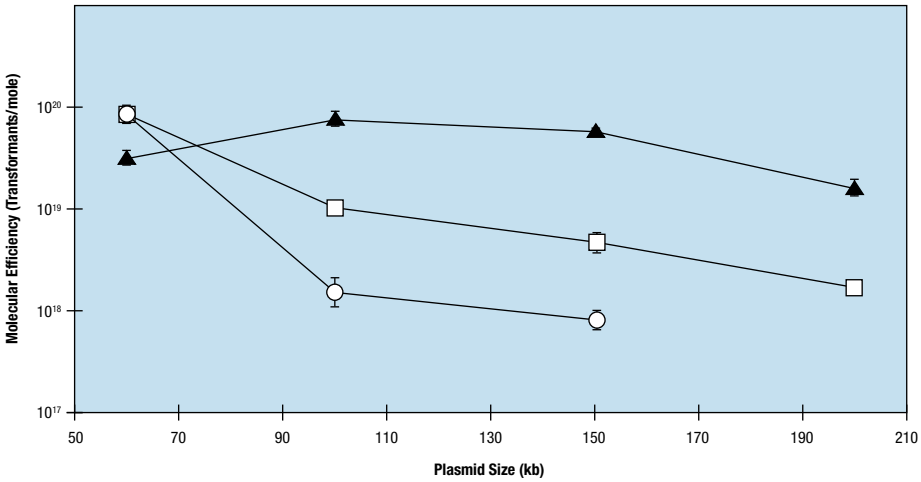


FIGURE 3. Electroporation of P1 plasmids. Strains used were DH5α-E (○), DH10B (▲), and DH12S (□) cells. Results are mean ± 1 SE for N = 6.

REFERENCES

1. Leonardo, E.D. and Sedivy, J.M. (1990) *BioTechnology 8*, 841.
2. Smith, M.J., Jessee, J., Landers, T., and Jordan, J. (1990) *FOCUS 12*, 38.
3. Siguret,V., Ribba, A.S., Chérel, G., Meyer, D., and Piétu, G. (1994) *BioTechniques 16*, 422.
4. Donahue, R.A., Jr., and Bloom, F.R. (1998) *FOCUS 20*, 75.
5. Lin, J.J., Jessee, J., and Bloom, F. (1992) *FOCUS 14*, 101.
6. Sternberg, N. (1994) in *Current Protocols in Human Genetics*. Supplement 1, pp 5.3.1–5.3.24.
7. Wang, G., Berg, C.M., Chen, J., Young, A.C., Blakesley, R.W., Lee, Y.L., and Berg, D.E. (1993) *FOCUS 15*, 47.
8. Lehrach, H., Demanac, R., Hoheisal, J., Larin, Z., Lennon, G., Monaco, A.P., Nizetic, D., Zehetner, G., and Poustka, A. (1990) in *Genome Analysis: Vol. 1. Genetic and Physical Mapping* (Davies, K.E. and Tilgham, S.M., eds.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 39–81.
9. Ioannou, P.A., Amemiya, C.T., Garnes, J., Kroisel, P.M., Shizuya, H., Chen, C., Batzer, M.A., and de Jong, P.J. (1994) *Nature Genetics 6*, 84.
10. Zimmer, R. and Verrinder Gibbins, A.M. (1997) *Genomics 42*, 217.

Vaseem Palejwala, Satnam S. Banga, Lisa M. Bloch, Tanya Dasgupta, Cesar Guerra, Bohdan Harvey, Alexandra Soggiu, Krishna K. Jha, and **Harvey L. Ozer**
Department of Microbiology and Molecular Genetics
UMDNJ-New Jersey Medical School
185 South Orange Avenue
Newark, New Jersey 07103
E-mail: jha@umdnj.edu

LIPOFECTAMINE™ Reagent

For Stable Co-transfection in an SV40-Transformed Human Cell Line

Co-tranfection for introduction of two unlinked pieces of DNA into mammalian cells, with one fragment or plasmid coding for a selectable marker, was developed by Wigler and co-workers (1). Their technique involved a modified calcium phosphate co-precipitation method of van der Eb and Graham (2). We report here that LIPOFECTAMINE Reagent is a convenient and efficient reagent for stable co-transfection.

METHODS

An SV40-transformed immortal cell line, C1.39T, was used (3). A clone designated as C1.39T/tTA3 was obtained by infection of C1.39T cells with a dicistronic retrovirus (LNCT). This clone is resistant to GENETICIN® Selective Antibiotic and expresses tetracycline-controlled transcription activator protein (tTA) from the internal CMV promoter. The CMV promoter and the tTA coding DNA were derived from the plasmid pUMD15-1 (4). Cells were cultured in Ham's F10/Dulbecco's MEM (high glucose) with 10% FBS at 37°C in a 7.5% CO_2 humidified incubator.

The selectable phenotype was resistance to hygromycin B (tgCMV/HyTK, ~5 kb, reference 5; or pUCHygro, ~5.2 kb, reference 6). Co-transfected plasmids were pUHD13-3 (~5 kb) that expresses luciferase activity when the tTA protein activates transcription in the absence of tetracycline (4), pUHD10-3M/AF6 (~8.2 kb) that similarly expresses AF6 mRNA (7) in the absence of tetracycline, and GIBCO BRL pGREENLANTERN®-1 (~5 kb) that constitutively expresses green fluorescent protein (GFP).

Co-transfections used a total of 2 µg DNA (1 µg of each plasmid) complexed with 12 µl of LIPOFECTAMINE Reagent in 600 µl D-MEM/F10 without FBS. Plasmid DNA was purified by anion exchange chromatography. Approximately 1.5×10^6 cells/60-mm dish, plated the day before, were washed with serum-free medium and re-fed with 2.4 ml serum-free medium. LIPOFECTAMINE Reagent-DNA complexes were added to the culture for 3 h, transfection medium was removed, and 5 ml of complete medium supplemented with 1 µg/ml tetracycline was added. On the next day, cells were trypsinized and distributed into five 100-mm dishes with tetracycline-supplemented complete medium. After 48 h, 75 µg/ml hygromycin was added. Cultures were re-fed every 3 to 4 days, and hygromycin-resistant clones were picked, grown, and tested for expression of the unselected gene in cells grown in the presence or absence of tetracycline. Expression of GFP in living cells was examined with an Olympus Epifluorescence Microscope, Model BX40, equipped with recommended filters.

Luciferase assays were performed with cell lysates. Relative luminescence units were obtained for each lysate using a Lumat luminometer.

AF6 cDNA was identified using PCR primers for the 5′ and 3′ ends of genomic and cDNA sequences.

Plasmids	Hygromycin-Resistant Clones	Clones Positive for Unselected DNA
tgCMV/HyTK (s) + pUHD13-3 (s)	35	35
pUC Hygro (l) + pUHD 10-3M/AF6 (l)	20	20
pUC Hygro (l) + pUHD 10-3M/AF6 (s)	21	21
pUC Hygro (l) + pGREENLANTERN 1 (s)	108	79

TABLE. Frequency of co-transfection. Plasmids were transfected as supercoiled (s) or linear (l) DNA.

RESULTS

Results of the co-transfections are summarized in *table 1*. 75% to 100% of the hygromycin-resistant clones were positive for the co-transfected plasmid. Results were similar with linear and supercoiled DNA. The frequency of hygromycin-resistant clones was ~1/10,000 transfected cells. Induction of luciferase activity was 5– to 280–times uninduced activity.

In summary, LIPOFECTAMINE Reagent-mediated co-transfection was efficient for C1.39 cells. The experimental conditions used here were the initial conditions, and no further optimization was necessary for our application. Specific conditions of co-transfections should be established for each cell line of interest.

ACKNOWLEDGEMENTS

We thank A.D. Miller, M. Gossen, H. Bujard, S.D. Lupton, C.M. Croce, and E. Canaani for the plasmids. This work was supported by grants from the National Institute on Aging, National Institutes of Health AG 04821 and AG 00398 to HLO. FOCUS

REFERENCES

1. Wigler, M., Sweet, R., Sim, G.K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S., and Axel, R. (1979) *Cell 16*, 777.
2. Graham, F.L. and van der Eb, A.J. (1973) *Virology 52*, 156.
3. Neufeld, D.S., Ripley, S., Henderson, A., and Ozer, H.L. (1987) *Mol. Cell. Biol. 7*, 2794.
4. Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA 89*, 5547.
5. Lupton, S.D., Brunton, L.L., Kalberg, V.A., and Overell, R.W. (1991) *Mol. Cell. Biol. 11*, 3374.
6. Chowdary, D.R., Dermody J.J., Jha, K.K., and Ozer H.L. (1994) *Mol. Cell. Biol. 14*, 1997.
7. Prasad, R., Alder, H., Nakamura, T., Canaani, O., Saito, H., Huebner, K., Gale, R.P., Nowell, P.C., Kuriyama, K., Miyazaki, Y., Croce, C.M., and Canaani, E. (1993) *Cancer Res. 53*, 5624.

Daisuke Nakajima, Manabu Nakayama,
and **Osamu Ohara**
Laboratory of DNA Technology
Kazusa DNA Research Institute
1532-3 Yana, Kisarazu
Chiba 292-0812 Japan
E-mail: ohara@kazusa.or.jp

Modified TRIZOL® Reagent Protocol for Large mRNA

One of the popular methods for preparation of high-quality RNA uses guanidinium thiocyanate (GTC) as a protein denaturant in combination with acidic phenol extraction (1). TRIZOL Reagent is a modification of the original Chomczynski and Sacchi method designed for optimal single-step isolation of RNA and has gained popularity as the reagent of choice for high-quality RNA preparation (2). However, Gruffat *et al.* (3) reported that the method of Chomczynski and Sacchi and its variations yielded large mRNAs than the method of Chirgwin *et al.* (4). Since we sequence cDNA derived from large transcripts (>4 kb) (5), it was critical to establish a reliable RNA isolation protocol to prepare high-quality large mRNAs. By trial and error, we found that some simple modifications in the original TRIZOL Reagent protocol could improve the quality of large mRNAs without user-to-user variations.

METHODS

ISOLATION OF TOTAL RNA FROM RAT BRAIN. Strict attention was given to follow the general guidelines for preventing external ribonuclease (RNase) contamination described in the instruction sheet with GIBCO BRL TRIZOL Reagent. Rat brain (~1 g) was homogenized in 20 ml of TRIZOL Reagent as described in the instructions through the RNA precipitation step with isopropanol. After recovering RNA by the first isopropanol precipitation and completely removing residual liquids on the wall of the centrifugation tube, the RNA was dissolved completely in 3 ml of GTC solution [4 M GTC, 25 mM sodium citrate (pH 7.0), 0.5% N-lauroylsarcosine, and 0.1 M 2-mercaptoethanol]. The solution was transferred to a new tube and precipitated with 3 ml of isopropanol. The isopropanol precipitation from the GTC solution was repeated. The recovered RNA was washed with 75% ethanol, briefly air-dried, dissolved in 400 µl of GIBCO BRL Formamide, and stored in aliquots at –80°C.

After heat denaturation in the presence of formaldehyde, the RNA was subjected to electrophoresis on a formaldehyde denaturing gel. Before being used for enzymatic reactions such as cDNA synthesis, the RNA was recovered from the formamide by ethanol precipitation (addition of 4 volumes of ethanol) (6).

***Formamide used for storage of RNA** must not contain impurities that cause RNA degradation.*

ANALYSIS OF RNA. RNA samples were electrophoresed on a formaldehyde denaturing agarose gel, transferred onto a nylon membrane, and cross-linked by UV irradiation (7). RNA staining with methylene blue on the membrane was performed prior to hybridization for examination of the integrity of RNA species present in large amounts (18S and 28S ribosomal RNAs) (7). cDNA fragments of rat dynein (GenBank accession number, d13896.gb_ro; nucleotides, 14344-15422) and rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH; GenBank accession number, M11561; nucleotides, 125-329) were used as probes for hybridization analysis after being ^{32}P-labeled with the GIBCO BRL RadPrime DNA Labeling System. After hybridization, the membrane was extensively washed with 0.1X SSC containing 0.1% SDS at 65°C.

RESULTS AND DISCUSSION

It is well known that isolation of intact mRNA is more difficult as the size increases. A report describing the variability of yield and integrity of large mRNA depending on preparation methods (3) did not include TRIZOL Reagent, so we examined the results with TRIZOL Reagent. A literature report showed that TRIZOL Reagent isolated large intact mRNA (12 kb) as detected by RT-PCR (8). Our results indicated that the original TRIZOL protocol worked well for large mRNA (>10 kb) in most preparations but did not obtain high-quality large mRNA in some preparations, even though the integrity of smaller RNA (<4 kb) was always good. A likely cause of run-to-run and/or user-to-user degradation of large mRNA in the original protocol is carry-over of a trace amount of RNase. To minimize RNase carry-over, 2 additional isopropanol precipitations of RNA from a GTC solution were added. Also, to avoid RNA degradation after the preparation (6), the RNA was stored in formamide. Formamide used for storage of RNA must not contain impurities that cause RNA degradation (formamide from Life Technologies satisfies this requirement).

Comparison of rat brain RNA samples prepared by the modified and the original protocols demonstrated similar RNA staining patterns with strong 18S and 28S rRNA bands *(figure 1A)*. Hybridization with the 1.4-kb rat G3PDH cDNA probe detected similar amounts of G3PDH mRNA in both protocols *(figure 1B)*, indicating that the protocols were equally reliable for preparation of relatively small mRNAs. However, a difference was seen between these RNA preparations when the same membrane was subjected to hybridization with a cDNA probe derived from a 16-kb mRNA *(figure 1C)*. The 16-kb dynein mRNA band was detected in the sample prepared by the modified protocol, but no discrete band was seen at the corresponding position in the sample prepared by the original protocol. It should be noted

that this sample was the worst one obtained by the original protocol. Since using the modified protocol, the 16-kb dynein mRNA band has been visible in every experiment. The modified protocol has been used successfully for RNA preparation from rat lung, kidney, skeletal muscle, heart, ovary, testis, spleen, and pancreas.

To our knowledge, procedures to isolate intact large mRNA (>10 kb) have not been adequately addressed in the literature. In fact, most RNA preparation kits certify performance by showing intactness of rRNAs or relatively small housekeeping gene transcripts (*e.g.,* β–actin and G3PDH). Over 20% of expressed genes are expected to generate large mRNA (>5 kb), at least in brain (Ohara, O., unpublished result), and frequently encode biologically important proteins (5). Accordingly, there will be a greater need for analysis of large mRNA as the gene discovery efforts continue to accelerate. The modified protocol presented here helped to minimize user-to-user and preparation-to-preparation variations in the quality of large mRNA without sacrificing the rapidity and simplicity of the TRIZOL Reagent method.

ACKNOWLEDGEMENT

We thank Dr. P.E. Cizdziel for his careful review of the manuscript. FOCUS

REFERENCES

1. Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem. 162*, 156.
2. Simms, D., Cizdziel, P.E., and Chomczynski, P. (1993) *FOCUS 15*, 99.
3. Gruffat, D., Piot, C., Durand, D., and Bauchart, D. (1996) *Anal. Biochem. 242*, 77.
4. Chirgwin, J.M., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) *Biochemistry 18*, 5294.
5. Ohara, O., Nagase, T., Ishikawa, K.I., Nakajima, D., Ohira, M., Seki, N., and Nomura, N. (1997) *DNA Res. 4*, 53.
6. Chomczynski, P. (1992) *Nucleic Acids Res. 20*, 3791.
7. Herrin, D.L. and Schmidt, G.W. (1988) *BioTechniques 6*, 196.
8. Nathan, M., Mertz, L., and Fox, D. (1995) *FOCUS 17*, 78.

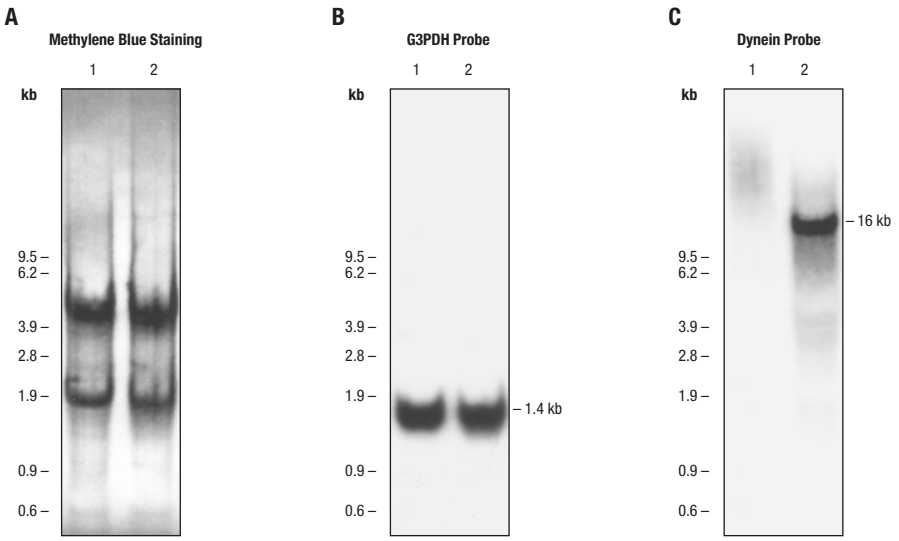
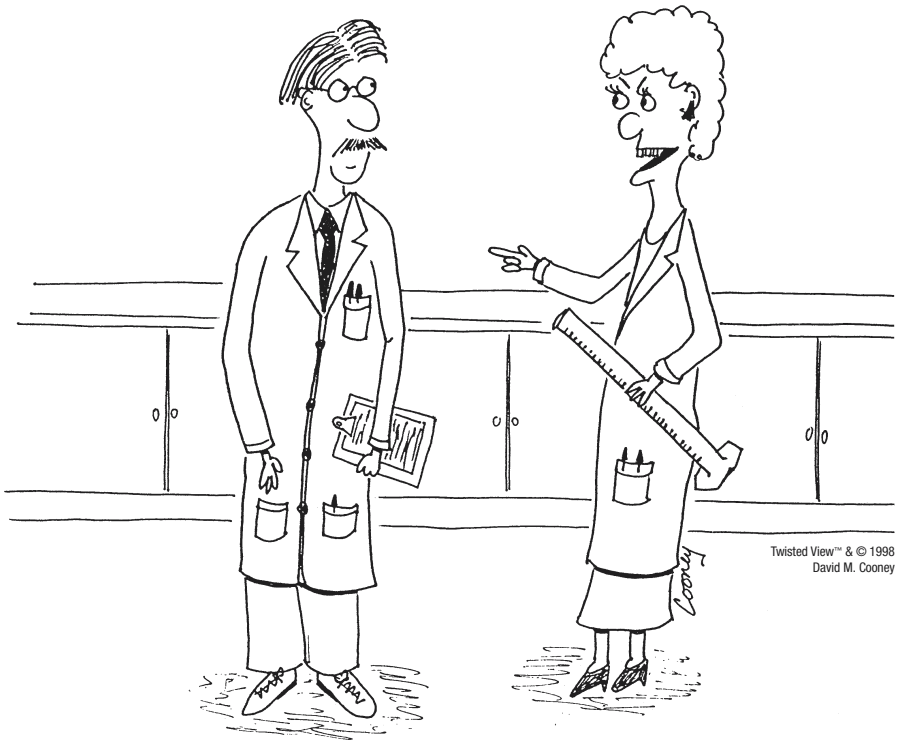


FIGURE 1. Analysis of total RNA. RNA was prepared by the original *(lane 1)* or the modified protocols *(lane 2)* for TRIZOL Reagent. After formaldehyde denaturing gel electrophoresis, total RNA (10 µg) was blotted on a nylon membrane. *Panel A.* RNA stained on the membrane with methylene blue. *Panel B.* Hybridization with a 1.4-kb G3PDH cDNA probe. *Panel C.* Hybridization with a 16-kb dynein cDNA probe.



"My recipe for Elution Buffer is off limits!
It's been a family secret for three generations and I'm not about to give it up."

Isolation and Long-Term Storage of RNA from Ribonuclease-Rich Pancreas Tissue

Alma M. Bracete and **Donna K. Fox**
Applications Laboratory, part of the
Technical Support and Training Team
Life Technologies, Inc.
Rockville, Maryland 20849

Domenica Simms
Research and Development
Life Technologies, Inc.
Rockville, Maryland 20849

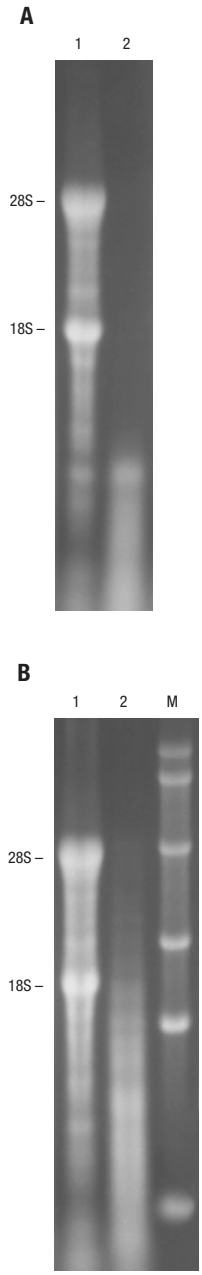


FIGURE 1. Gel electrophoresis of rat pancreas RNA. Total RNA (2 µg) was electrophoresed per lane on a 1.5% agarose-formaldehyde gel. *Lane 1.* TRIZOL Reagent-purified RNA. *Lane 2.* Spin cartridge-isolated RNA. RNA was dissolved in water *(panel A)* or formamide *(panel B)*. *Lane M.* 0.25–9.5 Kb RNA Ladder.

Maintaining the integrity of the RNA during purification and storage is more challenging in RNase-rich tissues such as rat pancreas. While several methods of RNA purification work for most tissues, not all methods may be stringent enough for tissues with high RNases. TRIZOL® Reagent is a simple method for the isolation of high-quality total RNA in high yield (1). In this study, we compared TRIZOL Reagent with a spin cartridge isolation system to isolate RNA from rat pancreas. Also, the storage of TRIZOL-purified rat pancreas RNA was investigated in RNase-free water or formamide.

METHODS

RNA ISOLATION. Rat pancreas was purchased from Pel-Freeze. Total RNA was isolated from 0.5 g of rat pancreas by TRIZOL Reagent (1) or by a spin cartridge RNA isolation system that binds RNA to a silica membrane in the presence of a chaotropic agent. All steps with TRIZOL Reagent were performed at 4°C. After purification, RNA samples were divided into 2 aliquots and stored at –70°C in either GIBCO BRL RNase-Free Distilled Water or 100% GIBCO BRL Formamide. RNA concentrations were determined by A_{260}.

RNA was isolated from 1 g of rat liver with TRIZOL Reagent, dissolved in water, and stored at –70°C.

AGAROSE GEL ELECTROPHORESIS. RNA samples were analyzed on a 1.5% (w/v) agarose/1.1% (v/v) formaldehyde gel in MOPS buffer [20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA] and stained by ethidium bromide.

RT-PCR. First-strand cDNA synthesis was performed in a 20-µl volume at 45°C with SUPERSCRIPT™ II RT, oligo$(dT)_{12\text{-}18}$, and 5 µg of total RNA (2). The RT was inactivated at 70°C for 15 min. The samples were treated with 3 units of RNase H for 30 min at 37°C. Parallel reactions without SUPERSCRIPT II RT were performed to check for trace genomic DNA contamination. (No contamination was detected.)

Between 1 and 5 µl of the cDNA reaction was amplified in 50 µl containing 200 µM each dNTPs, 200 nM of rat clathrin primers, and 1 unit of the ELONGASE® Enzyme Mix in buffer B. After an initial denaturation step at 94°C for 2 min, PCR was 35 cycles of 94°C for 20 s, 60°C for 30 s, and 68°C for 7 min.

RESULTS AND DISCUSSION

The integrity of the RNA was determined by formaldehyde gel electrophoresis. Total RNA isolated from rat pancreas was higher quality than RNA isolated with the silica spin cartridge *(figure 1)*. RNA recoveries by TRIZOL Reagent were typically 450 to 500 µg, compared with 100 to 200 µg using the spin cartridge system.

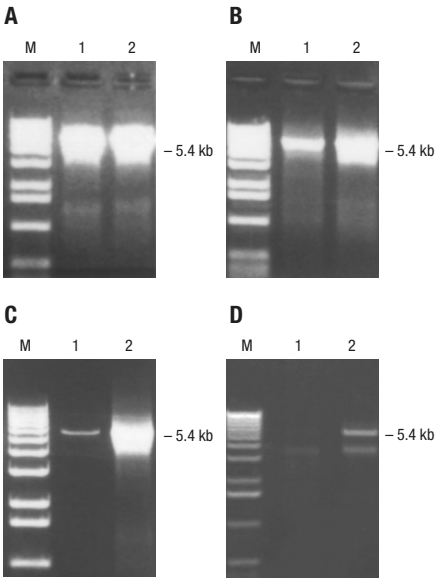


FIGURE 2. Clathrin RT-PCR products from rat pancreas RNA. Total RNA was isolated with TRIZOL Reagent. Amplification products (15 µl) were analyzed on a 1% agarose-TAE gel. RNA stored in water *(lane 1)* or 100% formamide *(lane 2)*. Fresh RNA *(A)*, 1 week *(B)*, 1 month *(C)*, and 3 years *(D)* after isolation. *Lane M.* 1 Kb DNA Ladder.

The integrity of the RNA was analyzed by long RT-PCR *(figure 2A)*. Clathrin cDNA prepared from freshly isolated total RNA using TRIZOL Reagent was efficiently amplified. Negligible or no amplification products were detected when cDNA prepared from RNA isolated with the spin cartridge was used in PCR even for a short 550-bp GAPDH target (data not shown). These results demonstrate that TRIZOL Reagent isolated a high yield of full-length RNA from rat pancreas.

With tissues like rat liver, which have relatively low amounts of RNases, isolated RNA is commonly stored in RNase-free water even for long-term storage. For example, rat liver RNA isolated with TRIZOL Reagent and stored in water for 3 years produced the clathrin RT-PCR product *(figure 3)*.

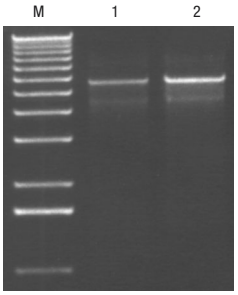


FIGURE 3. Clathrin RT-PCR products from rat liver RNA. Total RNA was isolated with TRIZOL Reagent and dissolved in water. *Lane 1.* RNA isolated 3 years ago. *Lane 2.* Freshly isolated RNA.

Since pancreas is high in RNases, we investigated storage solutions for RNA. The advantages of long-term storage of RNA in formamide have been reported previously for RNA from MCF-7 cells (3). After 1 week, the RNA from rat pancreas stored in water showed substantial degradation that resulted in a decrease in the cDNA amplification product when compared to RNA stored in formamide *(figure 2B)*. Agarose gel analysis of the RNA (data not shown) also showed degradation of the RNA in water. By 1 month, the RNA stored in water showed almost complete degradation *(figure 2C)*. Even after 3 years of storage in formamide, the rat pancreas RNA was efficiently amplified *(figure 2D)*.

In summary, TRIZOL Reagent is a fast, reliable method for isolating intact RNA from tissues with a high content of ribonucleases like rat pancreas. The integrity of pancreatic RNA can be maintained for at least 3 years when the RNA is stored in formamide. FOCUS

REFERENCES

1. Simms, D., Cizdziel, P., and Chomczynski, P. (1993) *FOCUS 15*, 99.
2. Fox, D.K. and Nathan, M. (1997) *FOCUS 19*, 49.
3. Chomczynski, P. (1992) *Nucleic Acids Res. 20*, 3791.

Advanced Technical Support for Customers

Life Technologies' unsurpassed customer technical support is much more than a phone line. Our TECH-LINE℠ Technical Services, Customer Training Center, and Product Applications Laboratory staff are integrated into out Technical Support and Customer Training Team. Team members actively participate in many facets of technical support to serve your needs. Each staff member has expertise in multiple areas to serve your technical needs in the following functions:

- Worldwide customer training in our workshop programs.
- Direct laboratory investigation of GIBCO BRL product applications to provide you with more technical tips and protocols.
- TECH-LINE and TECH-ONLINE℠ technical support.
- Creating applications guides, *FOCUS*® articles, technical tips, and bulletins to communicate new information and protocols to you. (Many of these tips are found in the Help Boxes in *SPECTRUM* and *FOCUS*.)

Donna Fox
Applications Laboratory, part of the
Technical Support and Training Team
Life Technologies, Inc.
Rockville, Maryland 20849

Effect of Base Composition on the Quantitation of Oligonucleotides

The absorbance of a DNA sample at 260 nm is used to calculate the concentration when the extinction coefficient is known. This calculation is based on the Beer-Lambert Law:

$A = Ecl$
where A = absorbance,
E = extinction coefficient,
c = concentration, and
l = pathlength of the cuvette (usually 1 cm).

When the concentration is given in moles/liter and the pathlength is 1 cm, E is a molar extinction coefficient and has the units of $M^{-1}cm^{-1}$. More commonly for oligonucleotides, concentration is reported as mmol/liter (mM), and E is a millimolar extinction coefficient.

EXTINCTION COEFFICIENTS FOR OLIGONUCLEOTIDES

For large double-stranded DNA molecules, an average extinction coefficient of 50 $(\mu g/ml)^{-1}$ cm^{-1} is used. This represents an average of the unique extinction coefficients for each nucleotide, and since the DNA molecules are large the composition results in minimal variation from that average value. In contrast, the extinction coefficient for small molecules such as oligonucleotides will be different for each unique sequence since A_{260} is different for each nucleotide. Therefore, for accurate determination of oligonucleotide concentration, calculate the extinction coefficient for each oligonucleotide using an equation that incorporates the contribution of each base, such as:

Millimolar Extinction Coefficient of oligonucleotide =
A(15.2) + C(7.05) + G(12.01) + T(8.4) at pH 8.0
where A, C, G, and T are the number of dAs, dCs, dGs, and dTs (1,2).

The numbers in parentheses are the millimolar extinction coefficients for each deoxynucleotide. If other bases such as uracil or inosine are present, include them in the equation. The value of the extinction coefficient at 260 nm will change with pH. When these values are given, often the conditions at which they were determined are not given. A reference for these values is *Data for Biochemical Research* (3).

To estimate the extinction coefficient, an average mmolar extinction coefficient of 10 for each nucleotide has been assumed, so:

Millimolar Extinction Coefficient = N(10)
where N is the total number of bases.

This is a reasonable approximation for oligonucleotides having an equal number of each base. However, as shown in *table 1*, large errors in E can occur if one base predominates. This leads to errors in calculated DNA concentration *(see below)*. We recommend using the extinction coefficient that is individually calculated for each oligonucleotide.

Oligonucleotide	Calculated Extinction Coefficient (mM)	Average Extinction Coefficient (mM)	Measured 260/280	dA (%)	dC (%)	dG (%)	dT (%)	Number of Bases
1	304	200	3.27	100	0	0	0	20
2	212.4	180	1.94	50	0	0	50	18
3	270.5	240	1.96	38	21	17	25	24
4	253.6	220	1.91	32	14	32	23	22
5	265.3	250	1.81	28	20	16	36	25
6	213.3	200	1.79	25	25	25	25	20
7	213.3	200	1.80	25	25	25	25	20
8	266.6	250	1.74	25	25	25	25	25
9	160	150	1.73	25	25	25	25	15
10	215.2	190	1.81	21	16	47	16	19
11	208.8	200	1.74	15	20	20	20	20
12	216.5	220	1.63	14	23	23	41	22
13	296	320	1.59	13	0	0	88	32
14	201	200	1.78	10	35	40	15	20
15	181.6	200	1.54	10	40	15	35	20
16	240.2	200	1.86	0	0	100	0	20
17	183.7	180	1.59	0	0	50	50	18
18	283.2	320	1.54	0	0	13	88	32
19	168	200	1.48	0	0	0	100	20
20	141	200	1.15	0	100	0	0	20

TABLE 1. Comparison of measurements on different oglionucleotides.

CALCULATION OF OLIGONUCLEOTIDE CONCENTRATION

Dissolve the oligonucleotide in 0.5 ml to 1 ml of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. For a 50-nmole-scale synthesis, 0.5–1 ml gives a final concentration of 50 to

400 µm. Prepare a dilution of the oligonucleotide so that the absorbance at 260 nm is between 0.1 and 0.5. Generally a 1:100 (10 µl + 990 µl of TE) or a 1:50 (20 µl + 980 µl of TE) dilution is sufficient. Read the absorbance at 260 nm.

$$c = \frac{(A_{260})(\text{dilution factor})}{El}$$

Example: A 1:100 dilution of oligonucleotide 3 (final volume 1 ml) had an A_{260} of 0.3. The calculated E was 270.5.

$$c = \frac{(0.3)(100)}{(270.5\ mM^{-1}cm^{-1})(1\ cm)}$$

or c = 0.111 mM or 111 µM

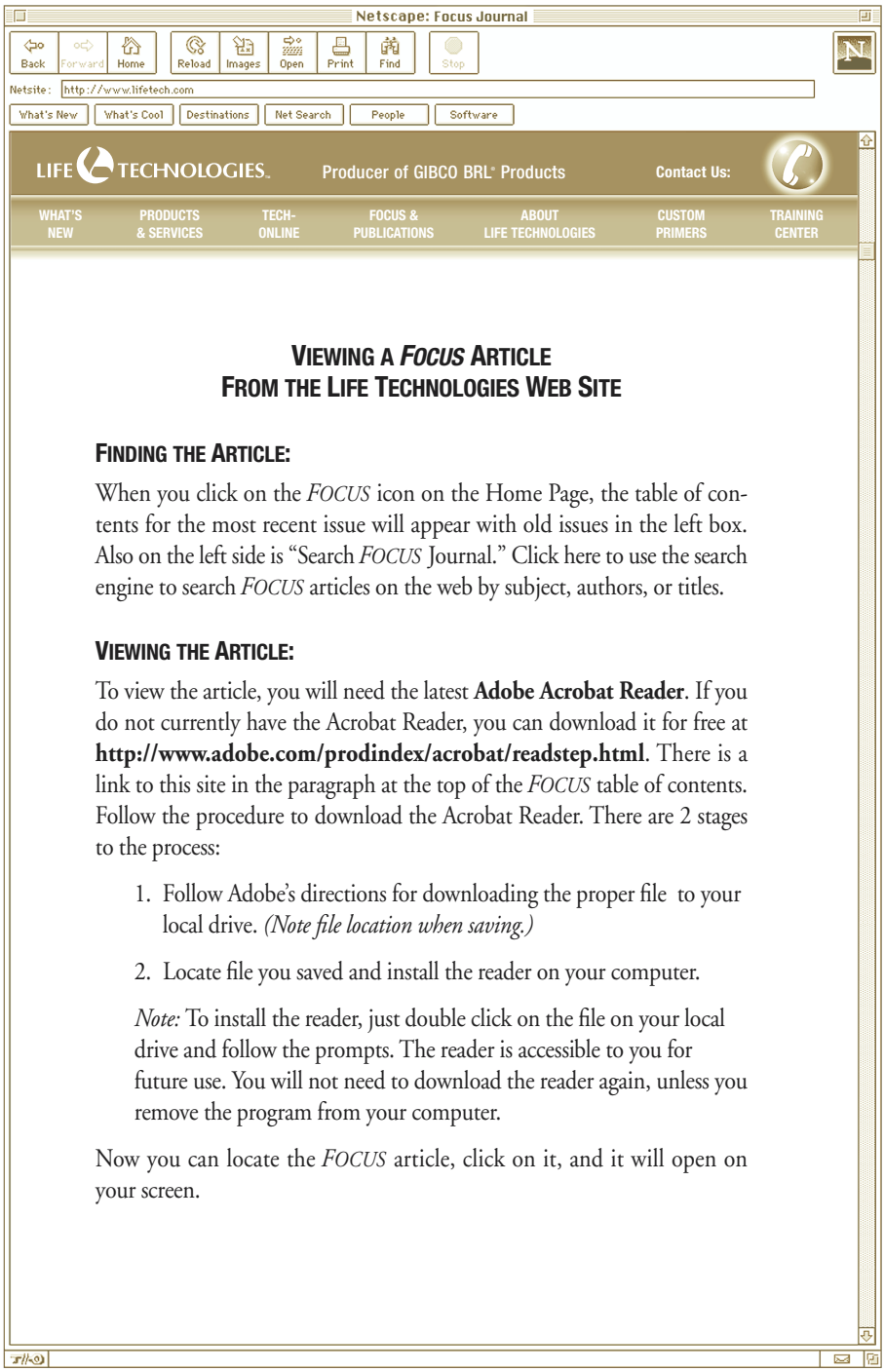
Note: Using the average E of 240, the concentration of oligonucleotide 3 was 0.125 mM. This represents an error of ~11%.

EFFECT OF SEQUENCE ON THE A_{260}/A_{280} RATIO OF OLIGONUCLEOTIDES.

The $A_{260/280}$ ratio can be used for large DNA molecules as an indicator of purity. For oligonucleotides, the $A_{260/280}$ ratio is not an indicator of purity because the ratio of a given oligonucleotide is highly sequence dependent. The reason for this is the large differences in extinction coefficients of individual nucleotides. The $A_{260/280}$ ratio increases with increasing amount of dAs and dGs and decreases with increasing amount of dCs *(table 1)*. The extreme differences are shown by the values between homopolymers. The ratio for poly(dA)$_{20}$ is ~3–times higher than that for poly(dC)$_{20}$. FOCUS

REFERENCES

1. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1994) *Current Protocols in Molecular Biology,* Wiley & Sons, p. A.3D.L.
2. Berger, S.L. and Kimmel, A.R. (1987) *Meth. Enzymol. 152,* 438.
3. Dawson, R.M.C., Elliott, D.C., Elliott, W.H., and Jones, K.M., eds. (1996) *Data for Biochemical Research,* 2nd Edition, Oxford University Press, London.



Virus Inactivation by Gamma Irradiation of Fetal Bovine Serum

John P. Daley and **Douglas J. Danner**
Cell Culture Research and Development
Life Technologies, Inc.

David J. Weppner
Quality Engineering Department
Life Technologies, Inc.

Mark Z. Plavsic
Cell Culture Research and Development
Life Technologies, Inc.
Grand Island, New York 14072

Cell culture is routinely used in a variety of applications, including production of animal vaccines and production of recombinant molecules. Cell culture systems commonly use medium supplemented with animal sera, the most common being fetal bovine serum (FBS). Animal sera can harbor adventitious viruses that cannot be totally removed even by the most rigorous aseptic manufacturing procedures. The presence of viruses not only interferes with the cellular studies but also interferes with the production of biologically active products, thus raising regulatory concerns in the manufacture of human or animal therapeutic products (1,2).

Several approaches to inactivate viruses from biologicals have been developed. These include physical separation or partition techniques (3), chemical disinfectants (4,5), and heat, γ-radiation, or UV light (6,7). Because FBS is a complex and undefined mixture of many biologically active compounds such as nutrients, vitamins, growth factors, and attachment factors, many of the treatments used to eliminate adventitious viruses negatively impact the growth-promoting properties of sera (8).

Gamma irradiation has been found to be an effective way to inactivate both animal and human viruses (4,9,10). We have developed an irradiation protocol for FBS that demonstrates optimal viral killing for 5 model viruses. This protocol did not significantly reduce the ability of the serum to promote cell growth of a wide range of cell lines in different culture applications.

METHODS

SPIKING AND γ-IRRADIATION OF FBS. The model viruses reflect the spectrum of physiochemical characteristics by which viruses are identified and classified and represent the viral families likely to be found in FBS *(table 1)*. The spiking of FBS and all subsequent viral titer studies were done by an outside testing laboratory using validated standard operating procedures in accordance with 9CFR section 113, parts 46 and 47. Determination of viral titers used standard quantitative $TCID_{50}$ or plaque assays. Viruses were grown to high titers prior to spiking. Each virus was spiked, 1:10 (v/v) into five 500-ml bottles of FBS (titers of 2.8×10^7 to 1.3×10^8). The Reduction Factor (RF) was calculated as the log of the ratio of the virus load in the starting material and the virus load in the final material (11).

Virus	Type	Size (nm)
Infectious Bovine Rhinotracheitis	*Herpetoviridae*, ds-DNA, enveloped	100
Bovine Viral Diarrhea Virus	*Flaviviridae*, ss-RNA, enveloped	30–60
Bovine Reovirus-3	*Reoviridae*, ds-RNA, non-enveloped	75–80
Canine Adenovirus	*Adenoviridae*, ds-DNA, non-enveloped	75
Porcine Parvovirus	*Parvoviridae*, ss-DNA, non-enveloped	18–24

TABLE 1. Physiochemical characteristics of virus panel.

$$RF = \text{Log}([V_1 \times T_1] \div [V_2 \times T_2])$$

V_1, volume of starting material;
T_1, starting viral titer;
V_2, volume of the final material;
T_2, viral titer in the final material.

γ-irradiation of spiked and nonspiked FBS was performed at a commercial irradiation facility using a Cobalt-60 source. GIBCO BRL Qualified FBS was irradiated as final product in 500-ml PET bottles using the method developed by Life Technologies.

BIOCHEMICAL ANALYSIS. An extensive biochemical analysis of the irradiated and control sera was performed by an outside testing laboratory and included quantitative analysis of amino acids, enzyme levels, and hormone levels.

BIOLOGICAL PERFORMANCE TESTING. The ability of γ-irradiated FBS to support cell growth was tested on the following cell lines: Baby Hamster Kidney cells (BHK-21; ATCC, CCL10), African Green Monkey cells (VERO; CCL 81), Chinese Hamster Ovary cells (CHO-K1; CCL 61), murine myeloma SP2/0-Ag14 (CRL 1581), human lung carcinoma A549 (CCL 185), and human diploid fibroblast cell lines WI-38 (CCL 75) and MCR-5 (CCL 171). The biological tests were cloning assays, plating assays, and serial passage long-term growth assays used to quality test GIBCO BRL FBS. All procedures were carried out according to the standard operating procedures for FBS testing.

CLONING ASSAY. Cells were plated in 96-well, flat-bottomed microtiter plates at 1 or 5 cells/well in medium with 10% or 4% FBS, respectively, and grown for 12 days. The higher serum concentration of 10% is representative of many routine procedures in hybridoma selection, while the 4% FBS is a more stringent test designed to detect subtle variations in serum. All plates were incubated at 37°C, 5% CO_2 in humidified air.

LOW-DENSITY CELL GROWTH. Cells were seeded into 6-well plates at 100 or 200 cells/well in medium with control or γ-irradiated FBS at 10% or 4%, respectively. Plates were incubated at 37°C, 5% CO_2 in humidified air for 14 days.

SERIAL SUBCULTURING OF CELLS. Cells were inoculated into duplicate T25 flasks at densities of 1×10^5 to 1.5×10^5 cells/flask (depending upon cell line) containing the appropriate medium and 5% FBS. The flasks were incubated, loosely capped, at 37°C in a humidified incubator with 5% CO_2 and air. The cells were harvested from duplicate flasks every 4 or 7 days (depending upon cell line), counted via Coulter counter, and subcultured for 3 passages.

RESULTS AND DISCUSSION

DOSE-DEPENDENT DECLINE IN VIRAL TITER. While γ-irradiation has been an effective method for viral inactivation (6,9,10), the challenge was to develop a method that would inactivate a panel of representative viruses from FBS and maintain the growth-promoting properties of FBS in a wide spectrum of cell culture assays. Viral inactivation by γ-irradiation was investigated for 5 viruses. A dose-dependent decline in survival was seen for enveloped and non-enveloped viruses *(figure 1)*. The viruses showed a significant decline in titer at 25 kGy and were below the level of detection (0.5 $TCID_{50}$/ml) at 35 kGy. Reduction factors were 6.7- to 7-fold.

BIOCHEMICAL PROFILE OF IRRADIATED FBS. Fetal bovine serum is a complex mixture of many biologically active molecules of both a defined and undefined nature. All of these components together are responsible for the growth-promoting properties of the sera. Of the 34 components tested, only 4 component levels declined: alkaline phosphatase (12–39%), alanine amino transferase (20–37%), aspartate amino transferase (8–49%), and lactate dehydrogenase (20–36%). The reduction was dose dependent. Hemoglobin levels decreased slightly as a function of dose in 2 of the 3 lots of FBS. No changes were observed in physiochemical parameters such as pH or osmolality. In addition, γ-irradiation did not alter endotoxin levels or the appearance of the FBS in any way.

γ-irradiation Dose (kGy)	Relative Cloning Efficiency SP2/0 Cells		Relative Plating Efficiency A549 Cells	
	10% FBS 1 cell/well	4% FBS 5 cells/well	10% FBS 100 cells/well	4% FBS 200 cells/well
0	1.12 ± 0.01	1.06 ± 0.01	0.89 ± 0.01	0.92 ± 0.01
25	1.07 ± 0.03	1.04 ± 0.02	0.97 ± 0.04	0.96 ± 0.03
35	1.05 ± 0.04	1.07 ± 0.02	0.92 ± 0.02	0.93 ± 0.02
45	1.11 ± 0.05	1.08 ± 0.01	0.94 ± 0.06	0.93 ± 0.03

TABLE 2. Cell growth at single-cell (cloning efficiency) or low-cell (plating efficiency) seeding densities. Results are mean ± SE of 3 different lots of FBS.

CELL GROWTH AT SINGLE-CELL AND LOW-CELL DENSITY. The growth-promoting activity of FBS is most rigorously tested when cells are plated at clonal densities (1 or 5 cells/well). γ-irradiation did not decrease the ability of the FBS to support cell growth in these most rigorous conditions *(table 2)*. Also, γ-irradiated serum supported the growth of adherent cells seeded at low density (100 or 200 cells/well) at levels comparable to the non-irradiated sera *(table 2)*.

CELL GROWTH IN MULTIPLE PASSAGES. A panel of continuous cell lines routinely used in the production of animal vaccines was tested to determine if γ-irradiated serum supported sequential subculturing. Growth was determined from the average of the second and third sequential subcultures of irradiated FBS compared to non-irradiated FBS to minimize any effect(s) due to serum carry-over from earlier maintenance of the cells.

Cell morphology in the γ-irradiated FBS was consistent with that of the control cultures. VERO, WI-38, and MRC-5 cells

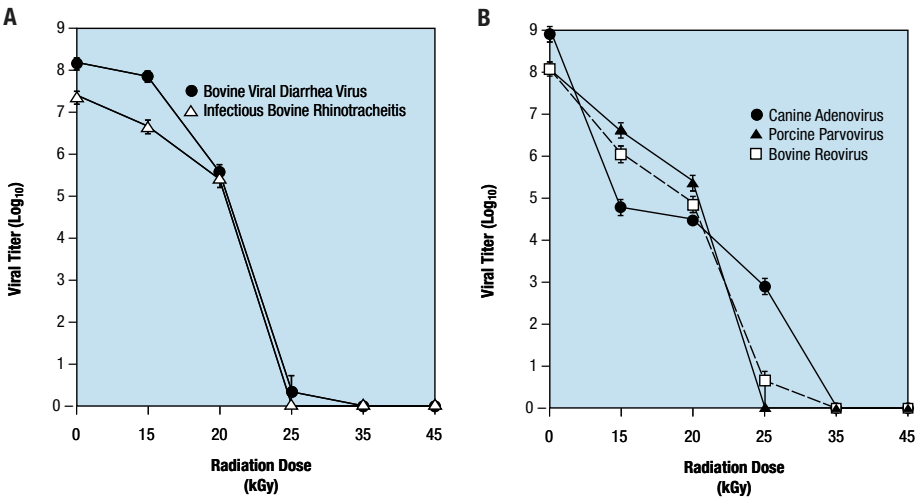


FIGURE 1. Dose-dependent virus inactivation by γ-irradiation. Enveloped (panel A) or non-enveloped (panel B) viruses were spiked into FBS and γ-irradiated. Titers were determined by the quantitative plaque assay in panel A and the $TCID_{50}$ assay in panel B. Results are mean ± SE for 3 lots of FBS.

Virus Inactivation continued

Cell Line	Radiation Dose (kGy)	Growth (Percent of Control)	Cell Line	Radiation Dose kGy)	Growth (Percent of Control)
	15	97 ± 4		15	95 ± 5*
	20	102 ± 6		20	95 ± 7*
VERO	25	90 ± 10	**BHK**	25	84 ± 9*
	35	91 ± 8	(Low-Passage Number)	35	76 ± 12*
	45	86 ± 8*		45	62 ± 6*
	15	107 ± 6		15	97 ± 5
	20	108 ± 3		20	103 ± 9
WI-38	25	104 ± 6	**BHK**	25	102 ± 10
	35	104 ± 6	(High-Passage Number)	35	107 ± 11
	45	103 ± 11		45	101 ± 10
	15	99 ± 4		15	93 ± 6
	20	104 ± 2		20	86 ± 7
MRC-5	25	99 ± 9	**CHO**	25	80 ± 5*
	35	96 ± 9		35	72 ± 7*
	45	98 ± 14		45	64 ± 16*

*$p \leq 0.05$ relative to control non-irradiated FBS.

TABLE 3. Long-term cell growth. Results are mean ± SE for passages 2 and 3 for 3 lots of FBS.

grew equally well in irradiated or control FBS *(table 3)*. Of interest is that WI-38 and MRC-5 did not display any decline in growth as a function of γ-irradiated FBS, since these fibroblasts are used because of their sensitivities to toxic factors. The ability of BHK cells to grow in γ-irradiated FBS depended upon the length of time the cells had been in culture. Low-passage BHK cells (≤3 passages from thawing) displayed a dose-dependent decline in growth. However, high-passage BHK cells (passaged twice weekly for ~2 months) showed no difference in growth. CHO-K1 cells were sensitive to γ-irradiated FBS and displayed a dose-dependent decrease in growth. However, in these assays cells were cultured with 5% FBS and the diminished growth may be minimized by supplementation with 10% FBS.

In summary, the method developed by Life Technologies for γ-irradiation of FBS achieved a 6- to 7-fold reduction in viral titer without a significant loss in the growth promotion for most of the cell lines studied. Even in clonal density culture, no decline in serum performance was seen for γ-irradiated FBS.

EDITOR'S NOTE: γ-irradiated FBS is available as a custom service. Researchers can test the FBS to determine a lot that meets their criteria and then we will γ-irradiate that lot. Life Technologies' method for γ-irradiation of FBS as final product is a validated process based on published ICH (11) and CPMP (12) guidelines. FOCUS

REFERENCES

1. Revised CPMP Guideline on Medicinal Products Derived from Human Blood and Plasma. CPMP/269 (1996).
2. Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. ICH/Q5H (1997).
3. Aranha-Creado, H., and Fennington, G.J. (1997) *J. Pharm. Sci. Tech. 51*, 208.
4. Spire, B., Barre-Sinoussi, F., Montagnier, L., and Chermann, J.C. (1984) *Lancet 2 (8408)*, 899.
5. Dichtelmuller, H., Rudnick, D., Breuer, B., and Ganshirt, K.H. (1993) *Biologicals 21*, 259.
6. House, C., House, J.A., and Yedloutschnig, R.J. (1990) *Can. J. Microbiol. 36*, 737.
7. Spire, B., Dormont, D., Barre-Sinoussi, F., Montagnier. L., and Chermann, J.C. (1985) *Lancet 1 (8422)*,188.
8. Giard, D.J. (1987) *In Vitro Cell Devel. Biol. 23(10)*, 691.
9. Sullivan, R., Fassolitis, A.C., Larkin, E.P., Read, R.B., and Peeler, J.T. (1971) *Applied Micro. 22(1)*, 61.
10. Thomas, F.C., Davies, A.G., Dulac, G.C., Willis, N.G., Papp-Vid, G., and Girard, A. (1981) *Can. J. Comp. Med. 45*, 397.
11. Note for Guidance on Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. CPMP/ICH/295/95 (1997).
12. Note for Guidance on Virus Validation Studies: The Design, Contribution, and Interpretation of Studies Validating the Inactivation and Removal of Viruses. CPMP/BWP/268/95.