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GENE EXPRESSION TOOLS FROM mRNA →cDNA→PROTEIN

LONG PCR

AFLP

95

VOLUME 17 NUMBER 2

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Techniques for identifying genetic markers in plants are being influenced by PCR. See article on page 66. Photograph by Uniphoto.

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Gene Expression Tools

Gene expression encompasses a breadth of techniques from making and screening cDNA to producing recombinant proteins. This issue of *Focus* describes some advances in these areas to facilitate studying gene expression.

For cDNA libraries, high-quality mRNA is an essential precursor. The new MessageMaker mRNA Isolation System, described by Simms, results in greater poly(A)$^+$ enrichment than other methods. An alternative to making your own libraries is to acquire a premade library. Several human cDNA libraries (see page 43) are available that are made with SuperScript™ II RT for longer cDNA and cloning procedures to yield higher levels of primary clones.

Screening cDNA libraries is difficult and time consuming. Li *et al.* show that when using the new GeneTrapper™ System, it is possible to enrich your target population for a cDNA sequence many thousand-fold, enabling you to isolate a full-length clone in as little as 3 days. Also, some tips on immunoscreening of libraries are presented by Nathan and Robinson.

After isolating a clone of interest, a common next step involves producing protein. The use of insect cells with baculovirus expression vectors is a rapidly growing system for recombinant protein production. Creating recombinant baculovirus DNA by homologous recombination can be considered an art. Anderson *et al.* use site-specific recombination in specially engineered *E. coli* to revolutionize the generation of recombinant baculovirus DNA. Screening for the recombinant is done in bacteria, and efficient transfection into insect cells eliminates the need for viral amplification. Another factor important to producing large amounts of recombinant protein is to use optimized cell culture medium for the insect cells. A new medium, Express Five™ SFM, described by Godwin *et al.* for BTI-TN-5B1-4 insect cells is used to express high levels of recombinant protein from baculovirus clones.

Technologies including transfection, DNA purification, and PCR support gene expression, as well as other applications. Hawley-Nelson and Shih examine how transfection efficiency can decrease as cell cultures age. Ally and Chomczynski describe a new DNA purification reagent that simplifies DNA isolation from cells or tissues. RT-PCR of fragments up to 8.9 kb using eLONGase™ Reagents is demonstrated by Westfall *et al.* These new and improved techniques provide a powerful set of tools for the identification, isolation, and expression of new protein and DNA sequences.

Doreen Cupo

mRNA Isolation for High-Quality cDNA

Domenica Simms
Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

Poly(A)$^+$ RNA comprises ~1% to 4% of total RNA (1). To prepare cDNA libraries or detect less abundant mRNA transcripts by Northern blot analysis, poly(A)$^+$ RNA is routinely isolated from total RNA using affinity chromatography reagents. Although highly enriched for poly(A)$^+$ RNA, these samples contain residual ribosomal RNA (rRNA). cDNA synthesized in the presence of rRNA generates ribosomal cDNA. The greater the ribosomal cDNA contamination, the greater the effort required to isolate rare transcripts from cDNA libraries.

MessageMaker™ mRNA Isolation System prepares high-quality mRNA, that is, intact mRNA containing very low levels of rRNA. This method begins with intact total RNA, isolated with TRIzol® Reagent (2) or an equivalent method. The poly(A)$^+$ RNA is selected using oligo(dT) cellulose. A syringe filter unit is used to simplify the washes and elution of poly(A)$^+$-selected RNA. A second poly(A)$^+$ selection effectively removes >90% of the rRNA.

Methods

Isolation of total RNA. Total RNA from cells and tissue samples was isolated using the Gibco BRL TRIzol Reagent (2).

Isolation of mRNA. mRNA was prepared from total RNA with the Gibco BRL MessageMaker mRNA Isolation System (Cat. No. 10298, table 1), as described in the product profile. Briefly, RNA was adjusted to 0.5 mg/ml with distilled water, heated for 5 min at 65°C, chilled on ice, and the salt concentration adjusted to 0.5 M. The settled oligo(dT) cellulose was resuspended by mixing, the appropriate volume was added to the RNA, and the tube was incubated at 37°C for 10 min. The oligo(dT) cellulose/RNA mixture was transferred to a syringe filter, and the liquid was expelled. The oligo(dT) cellulose/RNA hybrid was washed with wash buffer 1 and wash buffer 2. mRNA was eluted with 65°C DEPC-treated water.

If a second purification was done, the oligo(dT) cellulose in the syringe filter was resuspended in wash buffer 1. The RNA isolated from the first purification was heated for 5 min at 65°C, chilled on ice, and its salt concentration adjusted to 0.5 M. The RNA was transferred to the syringe filter. After 10 min, the liquid was expelled, the oligo(dT) cellulose was washed, and the RNA was eluted with distilled water.

Before quantitating the amount of mRNA obtained, the oligo(dT) cellulose fines were removed by centrifuging the eluted RNA for 2 to 3 min at 2,300 × *g*. The A_{260} was measured on the supernate. The RNA was precipitated using 0.1 volume of 7.5 M ammonium acetate and 2 volumes of –20°C ethanol, washed with 75% ethanol, and dissolved in distilled water.

Other forms of oligo(dT) tested included oligo(dT) magnetic beads, oligo(dT) latex beads, and biotinylated-oligo(dT) with streptavidin magnetic beads. The protocols used were as recommended by the manufacturer except for the oligo(dT) magnetic beads, where quantity of magnetic beads per starting RNA was increased to 2.5 times that recommended.

The MessageMaker System was also compared to direct mRNA isolation systems that isolate total RNA with guanidine isothiocyanate or proteinase K-SDS and then poly(A)$^+$ select with oligo(dT) cellulose.

Northern blot. Total RNA and mRNA (2 µg each) were electrophoresed on agarose gels in MOPS buffer and transferred onto nylon membranes. The membranes were hybridized in

TABLE 1. MessageMaker mRNA Isolation System.

Components	Amount
syringe filter	10
oligo(dT) cellulose (1 g/10 ml) contains 0.05% sodium azide	10 ml
5 M NaCl	3 ml
disposable beakers	100
wash buffer 1 [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl]	90 ml
wash buffer 2 [20 mM Tris-HCl (pH 7.5), 0.1 M NaCl]	60 ml
distilled water	60 ml
7.5 M ammonium acetate	1 ml

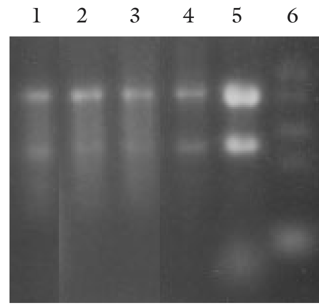


FIGURE 1. Gel analysis of poly(A)+ HeLa RNA using various affinity matrices. Two micrograms of RNA were loaded per lane on a 1.5% agarose gel. Poly(A)+ RNA isolated with oligo(dT) cellulose (lane 1), biotinylated-oligo(dT) primer/streptavidin magnetic beads (lane 2), oligo(dT) magnetic beads (lane 3), and oligo(dT) latex beads (lane 4). Lane 5. Total HeLa RNA. Lane 6. 0.24–9.5-Kb RNA Ladder.

the presence of 2×10^6 cpm/ml denatured ^{32}P-labeled GAPDH probe (specific activity 4×10^9 cpm/µg), prepared using the GIBCO BRL PCR Radioactive Labeling System.

First strand cDNA synthesis. The 20-µl first strand reactions contained RNA (1 to 5 µg); 0.5 µg of oligo$(dT)_{12–18}$; 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM $MgCl_2$; 10 mM DTT; 500 µM each dATP, dCTP, dGTP, and dTTP; 200 U of SUPERSCRIPT™ II RT per µg of RNA; and 1 µCi of [α-^{33}P]dATP (or 1 µCi of [α-^{32}P]dCTP) and were incubated at 37°C for 1 h. An aliquot of each reaction was used to determine input radioactivity and acid-precipitable radioactivity. Samples were electrophoresed on alkaline 1.4% agarose gels, which were either dried for direct

FIGURE 2. Comparison of mRNA quality with different systems. Panel A. Gel analysis. Two micrograms of HeLa RNA was loaded in each lane. Lane 1. Single-selected with the MESSAGEMAKER System after TRIZOL isolation. Lane 2. Single-selected with the guanidine isothiocyanate method. Lane 3. Single-selected with the proteinase K-SDS method. Lanes 4 and 5. Double-selected with the MESSAGEMAKER System. Lane 6. 2 µg total HeLa RNA. Lane 7. 2 µg 0.24–9.5-Kb RNA Ladder. Panel B. Northern blot of gel A probed with ^{32}P-labeled GAPDH DNA. Panel C. Blot B was stripped and reprobed with a ^{32}P-labeled 1.4-kb fragment of 28S human ribosomal DNA. Panel D. GAPDH/rRNA ratio. The ratio was determined using the 28S rRNA band.

detection of product or blotted onto nylon for Southern analysis.

Southern blot. The ^{33}P-labeled cDNA present on the membrane was detected by autoradiography. Presence of ^{32}P-labeled 1-Kb DNA Ladder helped determine conditions under which the ^{33}P-signal was completely quenched. Hybridization using a ^{32}P-labeled 1.4-kb *Bam*H I restriction fragment of the 28S human rDNA as probe allowed the detection of ribosomal cDNA. The same blot was stripped and reprobed with a ^{32}P-labeled 377-b PCR fragment generated using primers for human DNA polymerase ε (4).

Results and Discussion

Several affinity chromatography materials were tested for poly(A)+ RNA selection from total HeLa RNA. The yields of mRNA using oligo(dT) magnetic beads, biotinylated-oligo(dT) primer/streptavidin magnetic beads, and oligo(dT) cellulose [batch protocol (3)] clustered at 3%. Gel analysis showed that residual rRNA was present and visible in all of these poly(A)+ RNA samples (figure 1). Therefore, a single poly(A)+ RNA selection, regardless of affinity chromatography matrix used, removed only a portion of the rRNA.

The enrichment of mRNA using several mRNA isolation systems was compared. Gel analysis showed that the single-selected mRNA samples contained some rRNA (figure 2). Northern blot analysis of this gel showed varying amounts of the specific GAPDH mRNA. Poly(A)+-selected RNA using the MessageMaker System gave a GAPDH signal that was 5.6 times greater than for total RNA and 13.4 and 4.9 times greater than the guanidine isothiocyanate or proteinase K-SDS prepared mRNA, respectively. This blot was reprobed with 28S human rDNA (figure 2C). The ratio of GAPDH signal to rRNA signal was used as a measure of enrichment (figure 2D). Single selection with the MessageMaker System gave mRNA with a GAPDH/rRNA ratio 32.0 and 5.3 times greater than for the guanidine isothiocyanate system and the proteinase K-SDS system, respectively. In addition, the GAPDH/rRNA ratio was increased from 10.7 to 85.4 times greater than that of total RNA by selecting the poly(A)+ RNA a second time with the MessageMaker System.

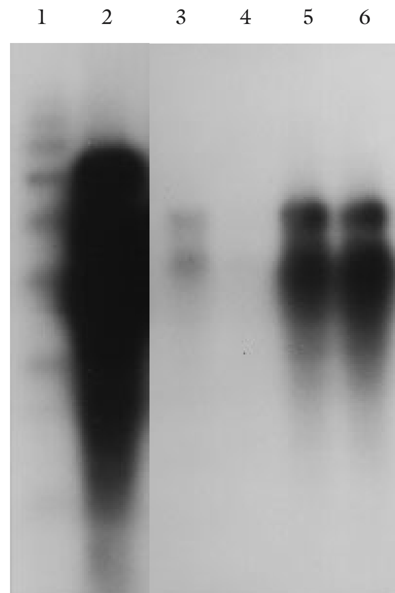


FIGURE 3. Level of ribosomal cDNA synthesized. Trace levels of [α-^{33}P]dATP were used to monitor the first strand synthesis reactions. After removal of unincorporated nucleotides by ethanol precipitation, the samples were loaded on an alkaline agarose gel and electrophoresed. The cDNA was transferred to a nylon membrane and probed with a ^{32}P-labeled 1.4-kb fragment of the 28S human rDNA. ^{33}P has much lower energy than ^{32}P, so that either short exposures or a barrier of plastic completely quenches its signal. Lane 1. ^{32}P-labeled 1-Kb DNA Ladder. Ribosomal cDNA synthesized from 5 µg total HeLa RNA (lane 2), 2 µg double-selected HeLa mRNA using the MessageMaker System (lanes 3 and 4), and 2 µg single-selected HeLa (lanes 5 and 6).

A single purification enriched with poly(A)+ RNA is suitable for RT-PCR and Northern analysis. Synthesis of high-quality cDNA libraries requires full-length mRNA containing as little rRNA as possible. A second selection for poly(A)+ RNA with oligo(dT) cellulose minimized rRNA contamination, which is more critical for cDNA libraries. The amount of ribosomal cDNA synthesized was compared for single- and double-selected poly(A)+ (figure 3) for the MessageMaker System. The single-selected samples had reduced ribosomal cDNA compared to total RNA. Double-selected mRNA had either a trace or no ribosomal cDNA. The same blot stripped and reprobed with a ^{32}P-labeled RT-PCR product from the human DNA polymerse ε mRNA, a long transcript of low abundance, shows a band at 8 kb only in lanes containing cDNA from poly(A)+ selected samples (data not shown). Table 2 demonstrates the yields obtained with the MessageMaker System for single- and double-selected RNA from total RNA from various sources.

TABLE 2. mRNA yields.

Total RNA (mg)	Number of Selections	mRNA (µg)
HeLa		
1.0	1	17
	1	17
2.0	1	32
	2	19
	1	36
	2	24
Rat Liver		
2.0	1	38
	2	22
	1	39
	2	22
	1	48
	2	17
Rat Brain		
2.0	1	44
	2	26

First strand cDNA was prepared from double-selected RNA and examined by electrophoresis on an alkaline agarose gel (figure 4). Percent cDNA synthesized was 42.5% and 42.0% for the double-selected mRNA and 11.0% for total HeLa RNA.

In summary, the MESSAGEMAKER mRNA Isolation System is fast, simple, and does not require repeated centrifugations or excessive sample manipulations. The rRNA is reduced by >90% when double selection is performed. cDNA prepared from double-selected HeLa mRNA shows either a trace or no ribosomal cDNA present.

ACKNOWLEDGEMENTS

I thank Dr. Christian Gruber for providing the culture seed for 28S human ribosomal DNA, and Ms. Margret Nathan for the human DNA polymerase ε data. I thank Dr. Christian Gruber, Dr. Robert Blakesley, and Dr. Reynaldo Pless for their helpful suggestions concerning this work.

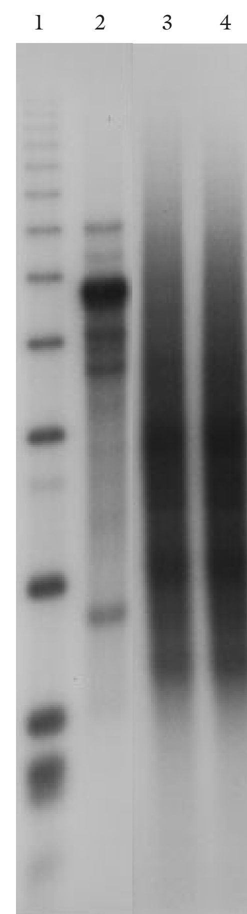


FIGURE 4. cDNA synthesis. Lane 1. ^{32}P-labeled 1-Kb DNA Ladder. Lane 2. cDNA from 5 µg total HeLa RNA. Lanes 3 and 4. cDNA from 2 µg double-selected HeLa mRNA using the MESSAGEMAKER System.

REFERENCES

1. Farrell, R.E., Jr. (1993) *RNA Methodologies*, Academic Press, New York.
2. Simms, D., Cizdziel, P.E., and Chomczynski, P. (1993) *FOCUS 15*, 99.
3. Celano, P., Vertino, P.M., and Casero, R.A. (1993) *BioTechniques 15*, 27.
4. Kesti, T., Frantti, H., and Syvioja, J. E. (1993) *J. Biol.Chem.268*, 10238.

CONSTRUCTION OF SUPERSCRIPT™ HUMAN cDNA LIBRARIES

Christian E. Gruber
Deborah Polayes
Marian C. Noon
Paul Nisson
Wu-Bo Li
Joel Jessee
Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20877

Lang Best
Patrick J. Dillon
Human Genome Sciences, Inc.
Rockville, Maryland 20850

Quality human cDNA libraries have many applications. The commonly used human tissues for cDNA libraries (table 1) represent >50% of the estimated 100,000 human transcripts (1). To enhance the number of full-length cDNAs represented in the library, we have constructed GIBCO BRL Human cDNA Libraries using SUPERSCRIPT II RT.

Library construction was carried out by isolating poly(A)$^+$ RNA from human tissues using the TRIZOL® Reagent in combination with double selection on GIBCO BRL Oligo(dT) Cellulose columns. cDNA was synthesized with the SUPERSCRIPT Plasmid System for cDNA Synthesis and Plasmid Cloning (2) to construct directional (*Not* I–*Sal* I) human cDNA libraries in the eukaryotic expression vector pCMV•SPORT. To produce libraries containing the largest cDNAs, only the first cDNA fractions (6 to 8 = 410 µl void volume) from the size fractionation columns were pooled together and ligated into the plasmid vector.

The pCMV•SPORT vector (figure 1) contains the strong cytomegalovirus (CMV) promoter for the transient high-level expression of recombinant cDNAs in COS cells to facilitate identification of specific proteins (3,4). This plasmid contains an f1 origin for the production of single-stranded DNA to isolate desired cDNA clones with the GENETRAPPER™ cDNA Positive Selection System (see page 45, this issue) or for the production of subtractive cDNA libraries (5,6). The vector also has T7 and SP6 RNA polymerase promoters flanking the multiple cloning site for the *in vitro* transcription of RNA.

After determining the optimal pCMV•SPORT vector:cDNA mass ratio, multiple ligation reactions were performed and the DNA was electroporated into DH12S cells to generate $>1 \times 10^7$ independent clones. Following electroporation, at least 1×10^7 independent clones were amplified once by a semi-solid procedure (7). This method, comparable to library amplification on plates, minimizes cDNA clone loss that might occur with liquid amplifications. After amplification, cDNA libraries are routinely $>5 \times 10^9$ colony-forming units (cfu) per ml (this represents 5,000 copies of a cDNA expressed at 1 copy per 1×10^6 clones per ml of library cells).

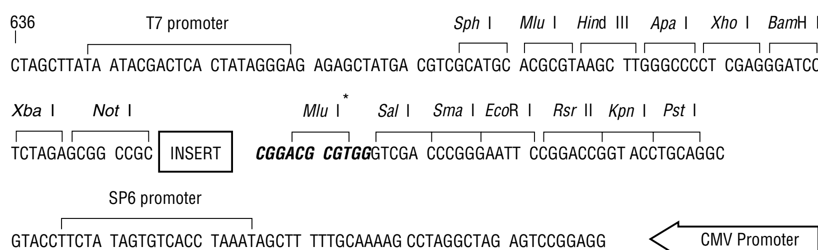
The presence of certain cDNAs was confirmed to validate the tissue specificity of the cDNA libraries using PCR (table 1). PCR was also used to determine the percent background and the cDNA insert size (table 2). The other five human cDNA libraries also displayed a low percent background of <5% and contained average cDNA insert size of >1,200 bp (data not shown).

The presence of the mRNA cap-binding protein (eIF-4E) (8), the replication protein A (RPA) (9), and the liver phosphatase 2A

TABLE 1. SUPERSCRIPT Human cDNA Libraries and the tissue-specific genes identified.

Human cDNA Library	Tissue-Specific Gene
Liver	Apolipoprotein C, serum albumin
Leukocyte	Platelet receptor
Brain	Myelin basic protein
Kidney	Uromodulin
Lung	Surfactant D
Spleen	Kappa immunoglobulin
Testes	RNA-binding protein (YRRM1)
Heart	Cardiac troponin C, cardiac troponin T

The presence of the tissue-specific genes within the human cDNA libraries was identified with gene-specific primers to verify the origin of each library.



*This *Mlu* I restriction site contained within the *Sal* I adapter is introduced into the pCMV•SPORT vector upon ligation of the cDNA insert. Due to flanking sites, *Mlu* I, by itself, or combined *Not* I–*Sal* I digestion can be used to completely excise the cDNA insert.

FIGURE 1. Multiple cloning site sequence of pCMV•SPORT.

TABLE 2. Characteristics of SUPERSCRIPT Human cDNA Libraries.

	Liver	Leukocyte	Brain
Number of clones with inserts	46/46	43/46	44/46
Average insert size	1,720 bp	1,660 bp	1,340 bp
Largest insert out of 46 clones	4,300 bp	3,400 bp	3,200 bp
Number of inserts >1,500 bp	28	20	19
Number of inserts >2,000 bp	15	11	7

The cDNA inserts were sized by using the SP6 (AGC TAT TTA GGT GAC ACT ATA G) and T7 (TAA TAC GAC TCA CTA TAG GGA GA) promoter primers and 40 cycles of PCR on 46 randomly chosen colonies from each library.

(PP2A) (10) cDNAs in the liver and leukocyte cDNA libraries was verified by PCR analysis (figure 2). Also, these three cDNAs (~2 kb each) were isolated from the liver and leukocyte

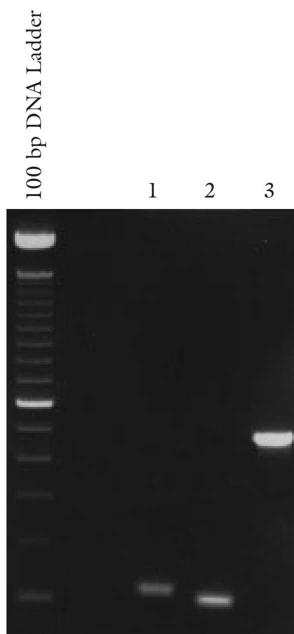


FIGURE 2. Presence of several different cDNAs in the SUPERSCRIPT Human Liver cDNA Library. Plasmid DNA from the Human Liver cDNA Library was used as a template for PCR screening with the specific gene primer sets. After 35 cycles of amplification, samples were electrophoresed on a 1.5% agarose-1X TAE gel. Lane 1. eIF-4E. Lane 2. RPA. Lane 3. PP2A.

Is there any possibility of finding a yeast sequence in the SUPERSCRIPT Human cDNA Libraries?

No, since yeast tRNA was not used during the construction of these libraries.

cDNA libraries with the GENETRAPPER System (see page 45, this issue). Using 5′ oligonucleotides, the rare eIF-4E (0.002%), RPA (0.001%), and PP2A (0.001%) cDNAs were enriched 25,000-fold and 90 to 100% of these clones were shown to contain 5′ untranslated and coding sequences by colony hybridization.

In this paper, we show that rare cDNAs are present and can be isolated from high-quality GIBCO BRL SUPERSCRIPT Human cDNA Libraries. Currently, we are assessing these libraries for the presence of large (5 to 12 kb) and small (0.5 kb) cDNAs.

NOTE:

The cDNA inserts can be sequenced from ds DNA using cycle sequencing and the SP6 or T7 dye primers (Applied Biosystems). For the SP6 dye primer use and anealing temperature of 50°C not 55°C.

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REFERENCES

1. Fields, C., Adams, M.D., White, O., and Venter, J.C. (1994) *Nature Genetics 7*, 345.
2. D'Alessio, J.M., Gruber, C.E., Cain, C., and Noon, M.C. (1990) *FOCUS 12*, 47.
3. Xie, G.-X., Miyajima, A., and Goldstein, A. (1992) *Proc. Natl. Acad. Sci. USA 89*, 4124.
4. Tanaka, K., Satokata, I., Ogita, Z., Uchida, T., and Okada, Y. (1989) *Proc. Natl. Acad. Sci. USA 86*, 5512.
5. Gruber, C.E., Li, W.-B., Lin, J.-J., and D'Alessio, J.M. (1993) *FOCUS 15*, 59.
6. Li, W.-B., Gruber, C.E., Lin, J.-J., Lim, R., D'Alessio, J.M., and Jessee, J.A. (1994) *BioTechniques 16*, 722.
7. Kriegler, M. (1990) *Gene Transfer and Expression: A Laboratory Manual.* Stockton Press, New York, New York.
8. Rychlik, W., Domier, L.L., Gardner, P.R., Hellmann, G.M., and Rhoads, R.E. (1987) *Proc. Natl. Acad. Sci. USA 84*, 945.
9. Erdile, L.F., Wold, M.S., and Kelly, T.J. (1990) *J. Biol. Chem. 265*, 3177.
10. Arino, J., Woon, C.W., Brautigan, D.L., Miller, T.B., and Johnson, G.L. (1988) *Proc. Natl. Acad. Sci. USA 85*, 4252.

The Rapid Isolation of Specific Genes from cDNA Libraries with the GeneTrapper™ cDNA Positive Selection System

Wu-Bo Li
Christian E. Gruber
Marian C. Noon
Deborah Polayes
Brian Schmidt
Joel Jessee
Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

The most popular screening method to identify cDNA clones is *in situ* hybridization. In this technique, nucleic acid or antibody probes are hybridized to dense populations of bacteria or phage that have been grown on plates and transferred to membranes (1). The isolation of the pure target clones usually requires secondary and tertiary screenings. Generally, this process can accommodate up to 2×10^6 clones and may take weeks or, if the cDNA is rare or large, months of screening to yield pure full-length target clones. Recently, several methods that enrich the target population have been reported (2–4). Here, we report a more rapid and efficient method using the GeneTrapper cDNA Positive Selection System.

In our novel approach (figure 1), a complex population of single-stranded (ss) phagemid DNA containing directional cDNA inserts is enriched for the target sequence by hybridization in solution to a biotinylated oligonucleotide probe complementary to the target sequence. The hybrids are captured on streptavidin-coated paramagnetic beads. A magnet retrieves the paramagnetic beads from the solution, leaving nonhybridized ssDNA behind. Subsequently, the captured ssDNA target is released from the biotinylated oligonucleotide. After release, the cDNA clone is further enriched by using a nonbiotinylated target oligonucleotide to specifically prime conversion of the recovered ssDNA target to double-stranded (ds) DNA. Following transformation and plating, typically, 20% to 100% of the colonies represent the cDNA clone of interest.

Methods

Preparation of ds plasmid-cDNA libraries. The Gibco BRL GeneTrapper System (Cat. No. 10356, patent pending) contains detailed instructions for all the steps described briefly here. Luria Broth or Terrific Broth (100 ml) was inoculated with cells containing the plasmid-cDNA library, and grown to saturation at 30°C. The ds plasmid DNA was prepared as described in the GeneTrapper System and dissolved in 400 µl of TE buffer. The concentration of the dsDNA was ≥1 µg/µl.

Design and purification of oligonucleotides. To avoid hairpin and dimer formation, an oligonucleotide design program was used. Gibco BRL Custom Primers were used and the sequences are listed in table 1. All

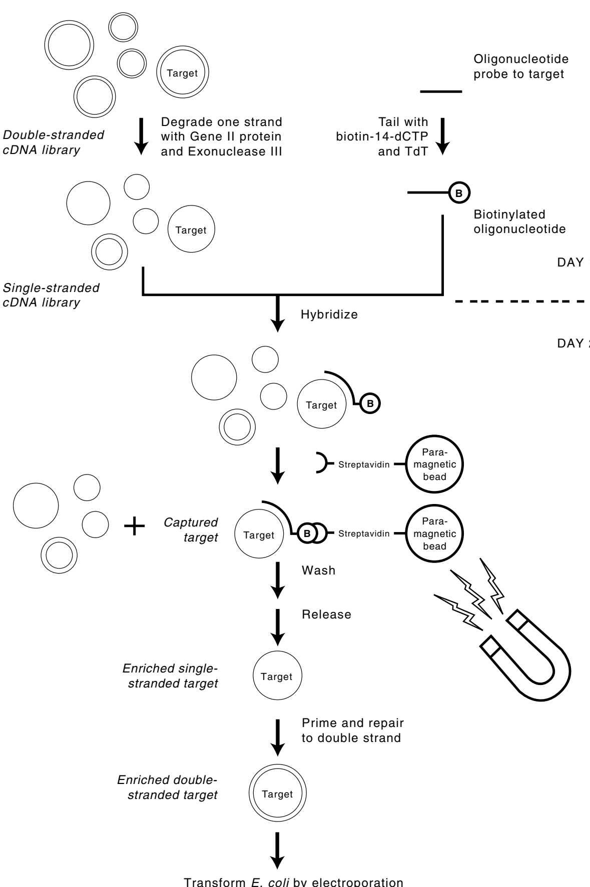


FIGURE 1. Schematic of specific cDNA isolation with GeneTrapper System. The dsDNA and ssDNA are represented by the double circle and single circle, respectively. The abbreviations represent: B, biotin attached to the oligonucleotide; Target, the target cDNA to be isolated.

TABLE 1. Oligonucleotide sequences.

For the GeneTrapper Procedure

Oligonucleotide	Nucleotide Sequence
942	GAC CGT TCA GCT GGA TAT TAC GGC C
1051-D8	GTN TGT/CGA T/CG GNT TT/C CAT/CGT CGG
1051-D10	GTN TGT /CGA T/CG GNT TT/C CAT /CGT NGG
O-eIF4E-3	CAG CTA CTA AGA GCG GCT CCA C
eIF4E-2	CAG ATC GAT CTA AGA TGG CG
O-RPA-3	ATG TCT GTA TCC TCA ATC AAG C
RPA-2	CAG CAC ATT GTG CCC TGT AC
O-eIF4E-d1	TAT /CCA A/GT /AG/C NCA T/CG CNG AT/C AC

For Colony PCR

Oligonucleotide	Nucleotide Sequence	Gene Position
O-eIF4E-1	CAG TCC CAC GCA GAC ACA GCT AC	eIF-4E #593-615
eIF4E-1	TCT CGA TTG CTT GAC GCA GTC TCC	eIF-4E #705-682
O-RPA-4	AAC TTT CAG GAT CTC AAG AAC CAG	RP-A #753-776
RPA-1	GTA GAA TAG ATG TGC CCC TCA TT	RP-A #850-828

oligonucleotides used with the GeneTrapper System were purified on a 12% acrylamide 8 M urea gel. Oligonucleotides (5 to 10 A_{260} units) in 25 µl of TE were denatured with an equal volume of formamide before electrophoresis. The full-length oligonucleotide was eluted from the gel slice by crushing and soaking in 1 ml of TE overnight in a shaking incubator at 37°C. After elution, the oligonucleotide solution was passed through a PD-10 column. The purified oligonucleotide was dried and dissolved in 100 µl TE, followed by one phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. The oligonucleotide was dissolved in 60 µl TE and stored at –20°C.

Preparation of biotinylated oligonucleotides. The oligonucleotide probes were biotin-labeled with biotin-14-dCTP and terminal deoxynucleotidyl transferase (TdT) (5). Usually, 0.3 to 0.5 nmol (~3 µg) of each oligonucleotide (16 to 25mer) was labeled. Typically, greater than 80% of the oligonucleotide was biotinylated and from 1 to 6 biotin residues were added. The concentration of the labeled probes was determined by comparison to the known starting material.

Generation of ssDNA with gene II and exonuclease III. The ssDNA was generated by the combined action of gene II nicking, followed by exonuclease III (Exo III) digestion from the 3´ end. Routinely, 5 µg of ds phagemid DNA was digested with 1 µl of gene II protein followed by treatment with Exo III.

GeneTrapper hybridization. The ssDNA was hybridized to the biotinylated oligonucleotide probe and captured with streptavidin paramagnetic beads. The captured ssDNA pellet was dissolved in 10 µl of TE buffer and stored at 4°C.

Repair of captured ss cDNA. Five microliters of the captured ss cDNA were converted to dsDNA with a DNA polymerase. The repaired DNA was dissolved in 10 µl of TE.

Electroporation of the captured and repaired DNA. A 2-µl aliquot of repaired DNA sample was mixed with 23 µl of Gibco BRL ElectroMAX DH10B™ cells. The cell mixture was electroporated using a Gibco BRL Cell-Porator® *E. coli* Electroporation System as described in the apparatus operating instructions. The cells were plated onto LB plates containing 100 µg/ml ampicillin. If the pSPORT1-CAT DNA control was captured, an additional 100 µl and 200 µl were plated onto LB plates containing 7.5 mg/ml of chloramphenicol and 100 µg/ml of ampicillin. All the plates were incubated overnight at 37°C. Because the colonies on chloramphenicol plates grow slowly, a longer growing period may be required.

Identification of desired cDNA clones. To identify the target gene, the colonies on the plates were screened by colony hybridization and colony PCR.

For colony hybridization, the colonies were screened as described by Sambrook *et al.* (1). The membranes were prehybridized in 1X Gibco BRL Prehybridization Buffer and 50% formamide for 1 h at 42°C and hybridized overnight in the same buffer containing a ^{32}P-labeled DNA fragment probe at 42°C. After hybridization, the membranes were washed once in a solution of 2X SSC and 0.1% SDS at room temperature for 30 min and once in a fresh solution at 55°C for 30 min. The membranes were finally washed in 0.1X SSC and 0.1% SDS at 68°C for 30 min.

For oligonucleotide colony hybridization and colony PCR, the colonies were screened as described in the GeneTrapper System.

Results and Discussion

In order to evaluate the GeneTrapper System, model cDNA libraries were made with different amounts of ds pSPORT1-CAT cDNA

and a ds pSPORT1-HeLa phagemid cDNA library. The CAT cDNA served as a pseudo target cDNA in each CAT:HeLa library. After electroporation of the captured and repaired DNA, the transformed cells were differentially plated onto ampicillin and chloramphenicol plates. Because plasmid pSPORT1 confers ampicillin resistance, all the transformants will grow on ampicillin plates (Ap^R). However, only those cells that have been transformed with the pSPORT1-CAT cDNA will grow on the chloramphenicol plates (Cm^R). From this study, the CAT clone was shown to be enriched 4,800-fold for the 1:5,000, 40,000-fold for the 1:50,000, and 10^5-fold for the 1:500,000 library (table 2). Furthermore, 600 CAT clones were recovered from the library with 0.0002% CAT.

TABLE 2. The enrichment of the CAT cDNA from CAT/HeLa cDNA libraries.

Library (CAT/HeLa)	Ap^R colonies/reaction	Cm^R colonies/reaction	CAT colonies (%)
0.02% (1:5,000)	1.84×10^4	1.75×10^4	95
0.002% (1:50,000)	5.5×10^3	4.3×10^3	78
0.0002% (1:500,000)	2×10^3	600	30

The CAT/HeLa cDNA libraries were prepared by mixing the pSPORT1-CAT plasmid with the HeLa cDNA plasmid. The CAT oligonucleotide #942 was used. The abbreviations represent: Ap^R, ampicillin-resistant colonies; Cm^R, chloramphenicol-resistant colonies. The total Ap^R and Cm^R was calculated for each hybridization. The percentage of positive CAT clone after selection was measured by dividing the total chloramphenicol-resistant colonies by the total ampicillin-resistant colonies in each reaction.

Several specific cDNAs have been isolated from different cDNA libraries using the GeneTrapper System, including the 2.0-kb mRNA cap-binding protein eIF-4E (6) and the 1.6-kb replication protein A (RP-A) gene (7). For each gene, 2 oligonucleotides were designed complementary to the sequence near the 3´ and 5´ coding regions, respectively. These oligonucleotides captured eIF-4E and RPA cDNAs from a human liver cDNA library (see page 43, this issue). Colony hybridization showed the abundance level of the eIF-4E cDNA in the original library was ~1/50,000 (figure 2). The colony hybridization demonstrated that 55.8% of the clones isolated by the eIF-4E 3´ probe were positive (figure 2). The GeneTrapper System results are summarized in table 3. For eIF-4E, 50% of the positive clones were full length, containing the complete coding sequence, 50 to 200 bp of the 5´ untranslated sequence, and the 3´ untranslated sequence. Additionally, 47.1% of the clones isolated by the eIF-4E 5´ probe were positive, and 100% of these positive clones were full length. Similar results were observed for the RP-A gene. It is not surprising that oligonucleotides designed to the 3´ coding sequences captured partial and full-length clones, while the 5´ probes selected only those clones containing the 5´-most sequences. This is a useful approach to isolate full-length sequences of cDNA from a complex population, particularly when the full-length transcripts are rare or large. Furthermore, using a 5´ oligonucleotide, we have successfully isolated the full-length clone of the 1.8-kb human protein phosphatase 2Aα subunit gene (8), a rare gene (1/100,000) from the human liver cDNA library. Interestingly, the percentage of positive clones detected by colony PCR was nearly identical to the percentage detected by colony hybridization. Therefore, colony PCR may be a convenient tool to detect desired clones after capture and repair, if enough sequence information is available. In addition to

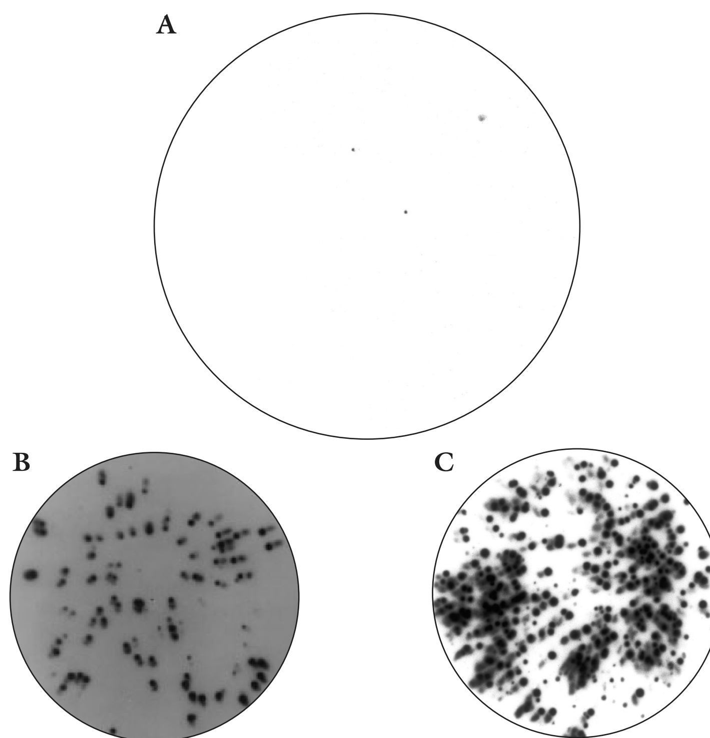


FIGURE 2. Colony hybridization of the captured clones with a ^{32}P-labeled oligonucleotide probe. The eIF-4E cDNA was isolated with biotinylated oligonucleotide O-eIF4E-3. After electroporation, the colonies were transferred to membranes and hybridized with a ^{32}P-labeled oligonucleotide O-eIF4E-3. Membrane A. 50,000 colonies of the original human liver cDNA library. Membranes B and C. 140 and ~900 colonies after cDNA selection.

TABLE 3. The enrichment of the genes from human liver and mouse cDNA libraries.

cDNA	Oligonucleotide	Positive by Colony Hybridization (%)	Positive by PCR (%)	Enrichment Factor	Total Positive Clones	Full-Length (%)
eIF-4E	O-eIF4E-3 (3′ end)	55.8	25.2	28,000	8,000	50.0
eIF-4E	eIF4E-2 (5′ end)	47.1	40.9	47,000	6,700	100
RP-A	O-RPA-3 (3′ end)	61.1	58.3	31,000	6,550	50.0
RP-A	RPA-2 (5′ end)	28.7	33.3	28,700	1,850	90.0
PP2Aα	PP2Aα-1 (5′ end)	20.5	N/A	21,000	1,400	87.5
HLH	HLH-2 (3′ end)	62.5	N/A	5,200	27,000	83.3
Nur	Nur-2 (3′ end)	77.0	N/A	30,000	9,500	41.7

The eIF-4E, RP-A, and PP2Aα cDNA were isolated from a human liver cDNA library. The abundance levels of these cDNA clones are approximately: eIF-4E, 0.002%; RP-A, 0.001%; PP2Aα, 0.001%. The HLH and Nur cDNA were isolated from the mouse brain tumor derived muscle-like cell line, BC_3H1 cDNA library. The abundance levels of both cDNA clones are approximately 0.012% and 0.0025%. The percentage of full-length cDNA clones was determined by restriction digestion and sequencing the 5′ regions of the clones.

human genes, two mouse genes, the 950-bp helix-loop-helix protein (HLH) and the 2.5-kb nuclear receptor (Nur) genes (table 3), were isolated from a BC_3H1 cDNA library (9).

Degenerate oligonucleotides were used to capture the CAT cDNA from a CAT:HeLa (1:50,000) cDNA library. To optimize the amount of degenerate biotinylated probe, the range of 0.1 ng to 200 ng of degenerate biotinylated oligonucleotide was used. By using only 2 ng of degenerate oligonucleotide in the hybridization, the background colonies were tremendously reduced versus 20 ng (as recommended for specific oligonucleotides). However, decreasing the amount of oligonucleotide tended to reduce the recovery of the target gene. Since the amount of the degenerate biotinylated oligonucleotide is critical for the target gene enrichment, multiple simultaneous hybridizations should be performed using from 1 to 5 ng of the biotinylated probe. Using the CAT degenerate oligonucleotides, 1051-D8 (degeneracy = 256) and 1051-D10 (degeneracy = 1024), the CAT cDNA was enriched from a starting representation of 0.002% to 9.1% and 2.3%, respectively. Additionally, the eIF-4E cDNA from the human liver cDNA library was enriched using 2 ng of the degenerate oligonucleotide, eIF4E-d1 (degeneracy = 1024). Colony hybridization and PCR detection revealed that 10% of the population represented the eIF-4E clone.

Different phagemid vector-based cDNA libraries were used as a source of ss cDNA. The cDNA libraries pSPORT1-HeLa, pCMVSPORT-leukocyte, pBluescript® SK(-)-T cell (from λZAP™ mass excision), and λZipLox®-HeLa were converted to ssDNA and hybridized to the biotinylated oligonucleotide O-eIF4E-3. As determined by colony hybridization, the percentage of positive colonies and the total positive clones obtained from pSPORT1-HeLa, pCMVSPORT-leukocyte, and pBluescript SK(-)-T cell libraries were very similar (data not shown). The total clones and the percentage of positive clones were lower in the λZipLox library compared to the other libraries. This observation may be caused by the formation of phagemid multimers in the λZipLox library (10).

Oligonucleotide size could affect the success of isolating cDNA from libraries. The minimum size was investigated with CAT cDNA from a CAT:HeLa (1:50,000) cDNA library. The percentage of positive clones and the total recovery were nearly identical using oligonucleotide lengths ≥16 (table 4). The recovery of the CAT cDNA was 10- to 20-fold lower using a 14mer.

This technology has advantages over conventional cDNA library screening. Since the hybridization is performed in solution, up to 10^{12} phagemid molecules (5 to 10 µg of the ds

TABLE 4. The effect of oligonucleotide length on enrichment.

Oligonucleotide	Total Clones	CAT	Positive Clones (%)
12mer	80	0	0
14mer	170	40	24
16mer	540	370	69
18mer	710	530	75
20mer	780	550	71

The oligonucleotides were designed from the same region of the CAT gene. The sequences of these oligonucleotides were: 12mer, GGC TTC ATG TC; 14mer, ATG GCT TCC ATG TC; 16mer, TGA TGG CTT CCA TGT TC; 18mer, TGT GAT GGC TTC CAT GTC; 20mer, TCT GTG ATG GCT TCC ATG TC. The library is HeLa cDNA mixed with CAT cDNA (CAT:HeLa = 0.002%).

phagemid cDNA) can be used in each enrichment reaction, resulting in hundreds to thousands of positive clones. The recovery of large numbers of positive clones enhances the isolation of full-length cDNAs. The conversion of the enriched ssDNA to dsDNA before transformation reduces the background and is particularly useful for rare cDNAs or when the background of nonspecifically captured species is high. In summary, the GENETRAPPER System can enrich target cDNAs many thousand-fold from a library, making isolation of full-length cDNAs easier and faster than conventional screening methods.

References

1. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
2. Rigas, B., Welcher, A.A., Ward, D.C., and Weissman, S.M. (1986) *Proc. Natl. Acad. Sci. USA 83*, 9591.
3. Pruitt, S.C. (1988) *Gene 66*, 121.
4. Ito, T., Smith, C.L., and Cantor, C.R. (1992) *Proc. Natl. Acad. Sci. USA 89*, 495.
5. Flickinger, J.L., Gebeyehu, G., Buchman, G., Haces, A., and Rashtchian, A. (1992) *Nucleic Acids Res. 20*, 2382.
6. Rychlik, W., Domier, L.L., Gardner, P.R., Hellmann, G.M., and Rhoads, R.E. (1987) *Proc. Natl. Acad. Sci. USA 84*, 945.
7. Erdile, L.F., Wold, M.S., and Kelly, T.J. (1990) *J. Biol. Chem. 265*, 3177.
8. Arino, J., Woon, C.W., Brautigan, D.L., Miller, T.B., and Johnson, T.L. (1988) *Proc. Natl. Acad. Sci. USA 85*, 4252.
9. Li, W.-B., Gruber, C.E., Lin, J.-J., Lim, R., D'Alessio, J.M., and Jessee, J.A. (1994) *BioTechniques 16*, 722.
10. D'Alessio, J.M., Bebee, R., Hartley, J.L., Noon, M.C., and Polayes, D. (1992) *FOCUS 14*, 76.

Optimized Immunoscreening of cDNA Libraries

Margret Nathan
Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

Robin Robinson
Training Center
Life Technologies, Inc.
Germantown, Maryland 20876

Considerations affecting immunoscreening of libraries include the nature of the cDNA library, level and regulation of recombinant gene expression, protein stability, antibody specificity/avidity, and antigen/antibody detection methods (1–6). Therefore, optimization of individual steps in immunoscreening is important to obtain true positive clones.

The first step in immunoscreening is the expression and binding of recombinant proteins to membranes. Following protein-membrane binding, a cascade amplification of protein-protein binding reactions begins with the binding of nonspecific blocking proteins to unoccupied sites on the membranes. Subsequently, primary antibodies bind specifically to recombinant proteins immobilized on the membranes. The resulting antigen/antibody (Ag/Ab) complexes are detected with radiolabeled Protein A or a secondary antibody raised against the primary antibody. The secondary antibody may be biotinylated and detected with streptavidin conjugated to a reporter enzyme such as alkaline phosphatase. Alternatively, the secondary antibody may be conjugated directly to the reporter enzyme. The reporter enzyme catalyzes the conversion of a chromogenic substrate to a colored insoluble precipitate or a chemiluminescent substrate to a light-emitting species for detection of positive clones.

In this study, several immunoscreening steps were analyzed, including library plating densities, membrane binding properties, blocking agents, primary and secondary antibody selection, and chromogenic/chemiluminescent detection. Plating density, preadsorption of antibodies, and detection method were key for successful immunoscreening.

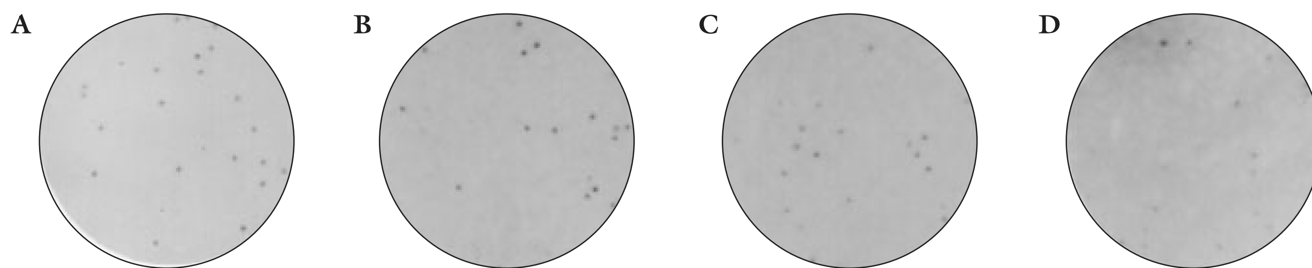


FIGURE 1. The effect of density on immunoscreening of plaques. 35 pfu of a λZipLox vimentin clone were plated on a background of (A) 1.7×10^3, (B) 3.3×10^3, (C) 6.6×10^3, or (D) 1.25×10^4 pfu of a λZipLox HeLa cDNA library. Plaques were transferred to PVDF membranes soaked in 10 mM IPTG. Membranes were blocked with Blocking Solution for Immunoblotting. Positive plaques were detected with preadsorbed monoclonal antibody to vimentin, alkaline phosphatase conjugated to goat anti-mouse IgG, and LumiPhos 530.

Methods

cDNA expression libraries. cDNA was generated from HeLa cell poly(A)$^+$ RNA and inserted into λZipLox and pSPORT1 vectors. *E. coli* Y1090 were infected with 10,000 pfu (or as indicated) from the λZipLox cDNA library and 10 to 50 pfu of a vimentin λ phage clone, plated on LB agar with 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ using 0.7% LB top agar, and permitted to form plaques initially at 42°C for 3 to 4 h, followed by incubation at 37°C for 5 to 8 h prior to IPTG induction (37°C for 2 h). The plasmid pSPORT1 library (5,000 cfu or as indicated) in *E. coli* DH5αF′™ and 10 to 20 cfu of a vimentin pSPORT plasmid clone were plated on membranes saturated with IPTG and incubated for 10 to 12 h. Colonies were permeabilized by chloroform vapor treatment for 20 min at room temperature and lysed *in situ* by treatment with DNase I (1 µg/ml) and lysozyme (40 µg/ml) in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20®] for 2 to 12 h at room temperature. The cDNA libraries were grown on membranes directly (colonies) or replica plated onto membranes (plaques). Membranes included Gibco BRL Nitrocellulose-1, Gibco BRL Supported Nitrocellulose-1, and PVDF.

Membrane blocking. Membranes were blocked in 25 ml/membrane of blocking solution prepared in TBST for 90 min at room temperature on a rocking platform. The blocking agents included Gibco BRL Blocking Solution for Immunoblotting (Cat. No. 19952), 5% nonfat dry milk, 2% Gibco BRL Bovine Serum Albumin, Fraction V (Cat. No. 11018), and 20% Gibco BRL Fetal Bovine Serum (FBS).

Primary antibody binding. The primary antibody to vimentin at 10 ng/ml (murine MAb to bovine lens vimentin, clone Vim 3B4) was incubated in 10 ml/membrane of TBST with 0.2X blocking solution for 2 to 12 h at 4°C on a rocking platform shaker. Antisera was preadsorbed with sonicated *E. coli* lysates for 30 min at 4°C at 1 mg/ml of bacterial protein lysate per 100 ml of diluted antisera. Antisera was clarified by passage through a Sepharose® 4B-20 column equilibrated with TBST and evaluated for antibody binding by immunoblotting using purified vimentin.

Secondary antibody binding. Following three washes in TBST with 0.2X blocking solution for 5 min each at room temperature, the Ag/Ab complexes were incubated with 0.2 µg/ml secondary antibody in 10 ml/membrane TBST with 0.2X blocking solution for 60 min on a rocker platform shaker. Secondary antibodies included Gibco BRL Goat Anti-Mouse IgG (H+L) - Alkaline Phosphatase Conjugate (Cat. No. 13864) and Biotinylated Goat Anti-Mouse IgG (Cat. No. 19590). For the biotinylated antibody, an incubation with 0.1 µg/ml streptavidin-alkaline phosphatase (SA-AP) in TBST with 0.2X blocking solution (10 ml/membrane) for 30 to 60 min at room temperature on a rocking platform was performed after antibody incubation. Then, the membranes were washed three times with TBST and once with TBS for 5 min. Prior to detection the membranes were equilibrated in alkaline phosphatase substrate buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$] for 10 min.

Chromogenic and chemiluminescent detection. Membranes were incubated for 5 to 30 min at room temperature in 25 ml/mem-

brane of 75 µg/ml NBT and 50 µg/ml BCIP (Cat. No. 18280). The reaction was stopped by immersion in 10 mM EDTA for 5 to 10 min. Alternatively, PVDF membranes were blotted, immersed briefly in 10 ml of LumiPhos® 530 to coat the membrane, blotted to remove excess reagent, and placed in a development folder. Membranes were incubated at 37°C for 30 min and exposed 0.5 to 2 h at room temperature to Kodak X-OMAT AR film.

Results and Discussion

The bovine vimentin gene, cloned previously in λZipLox and pSPORT1 vectors, was expressed as a fusion protein with ß-galactosidase under the control of the *lacZ* promoter. The availability of antisera, recombinant phage, and plasmid clones afforded a model system to evaluate the immunoscreening technique.

Plating density. Optimal plating density for immunoscreening was evaluated. For chromogenic detection with nitrocellulose membranes, detection of positive colonies was better at lower plating densities (3,300 pfu/100-mm plate). Where plaque confluency occurred (≥6,600 pfu/100-mm plate), the chromogenic signal of individual plaques was greatly diminished and the appearance of plaques was irregular (data not shown).

Chemiluminescent detection was more sensitive than chromogenic detection and permitted detection of positive plaques with normal morphology at 12,500 pfu/100-mm plate (figure 1). The greater sensitivity of the chemiluminescent method may be attributable to both the higher binding capacity of PVDF membrane and the greater sensitivity of the LumiPhos 530 substrate. In contrast, the maximal number of colonies per 100-mm plate for immunoscreening was 5,000 for chemiluminescent detection (data not shown). Higher plating densities caused colony smearing during processing and low-level expression of recombinant proteins from individual colonies.

Membranes. Immunoscreening may be affected by the quality and binding properties of membranes. cDNA libraries were transferred onto nitrocellulose or supported nitrocellulose membranes. The signal intensity for vimentin expressed in pSPORT1 colonies was enhanced on nitrocellulose membranes as compared to supported nitrocellulose (figure 2). However, nitrocellulose membranes were less durable during processing than supported nitrocellulose. No differences in the expected positive clone number, signal intensity, or background were noted between these two membrane types with λZipLox plaques (data not shown).

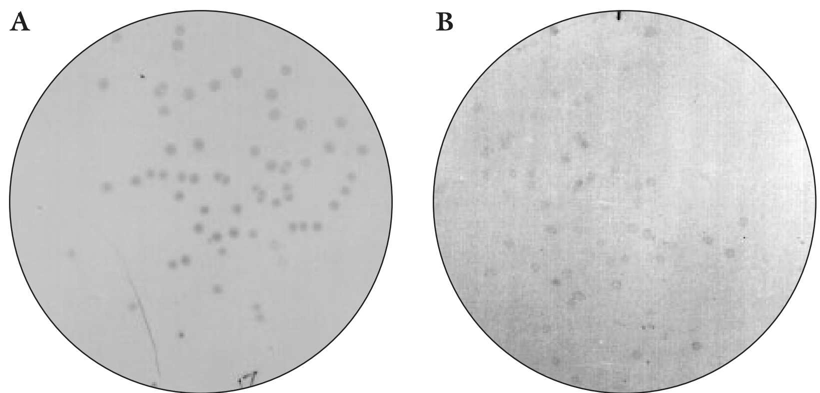


FIGURE 2. The effect of membranes on immunoscreening of colonies. A pSPORT1 HeLa cDNA library was plated on nitrocellulose (A) or supported nitrocellulose (B) membranes and induced with IPTG. Membranes were blocked with Blocking Solution for Immunoblotting and colonies expressing vimentin were detected with preadsorbed monoclonal antibody to vimentin, alkaline phosphatase conjugated to goat anti-mouse IgG, and NBT/BCIP.

Blocking agents. Positive colonies or plaques can be obscured by high background over the entire membrane or apparent substrate conversion for every colony/plaque. This background can be caused by nonspecific protein binding to the membranes or bacterial proteins during the antibody incubations. To minimize background, BSA, FBS, nonfat dry milk, and Blocking Solution for Immunoblotting were tested as blocking agents with each membrane type during the immunoscreening of both colonies and plaques. FBS and purified BSA effectively prevented high membrane backgrounds, but failed to block nonspecific substrate conversion on colonies and plaques, resulting in false positives. Blocking Solution for Immunoblotting and nonfat dry milk were more effective blocking almost all substrate conversion on nonpositive plaques, and they yielded a lower background on positive colonies (data not shown).

Antisera adsorption. One common source of false positives for immunoscreening cDNA libraries is antisera contaminated with antibodies to *E. coli* proteins. Adsorption of polyclonal and ascites sera with sonicated bacterial lysates effectively removed this background (data not

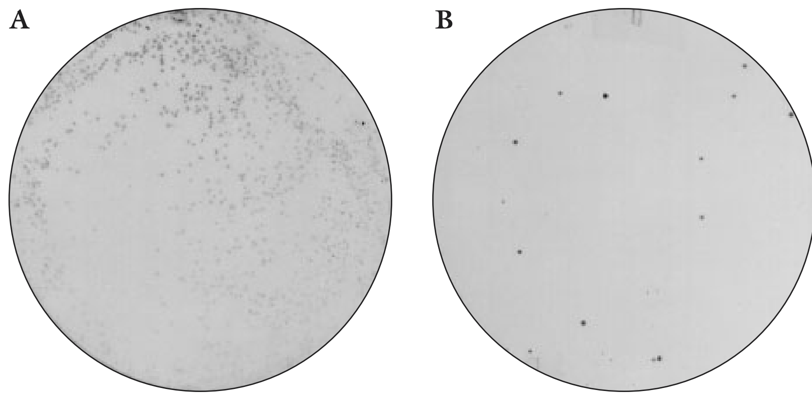


FIGURE 3. Immunoscreening colonies using nonadsorbed and preadsorbed primary antisera. A pSPORT1 HeLa cDNA library was plated on supported nitrocellulose membranes and detected with (A) nonadsorbed monoclonal antibody to vimentin or (B) the same antibody preadsorbed against an *E. coli* lysate.

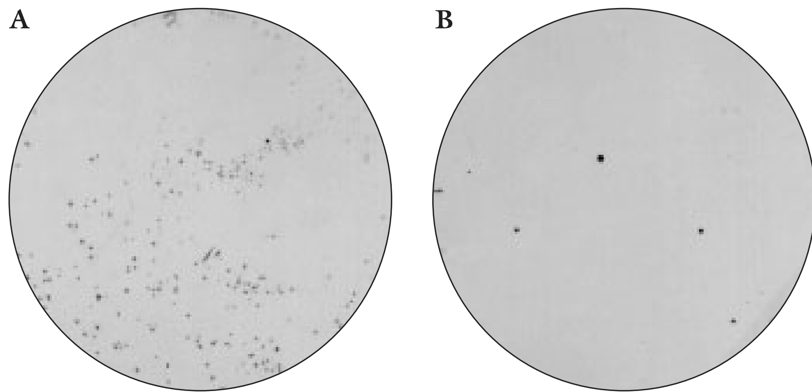


FIGURE 4. Immunoscreening colonies using different secondary antibody detection systems. Recombinant proteins on supported nitrocellulose membranes were bound to primary antisera and detected using BCIP/NBT by incubating with (A) biotinylated goat anti-mouse IgG, then SA-AP or (B) goat anti-mouse IgG conjugated directly to alkaline phosphatase. The plating density was 5,000 colonies/100-mm plate.

shown). Also, preadsorption was required to remove background reactivity from a monoclonal antibody (figure 3). Several of these colonies were used for minipreps and restriction analysis to confirm that the positives from the adsorbed sera did contain vimentin. 4 of the 5 colonies screened contained the expected bands (data not shown).

Secondary antibody. Different secondary antibody detection schemes were evaluated for their sensitivity and specificity (figure 4). Using a biotinylated goat secondary antibody and streptavidin-alkaline phosphatase, many positive signals were seen. Alternatively, with goat secondary antibody conjugated to alkaline phosphatase, the expected 0.1% to 1% of the colonies were positive. Similar results were seen with plaques (data not shown). This disparity was probably due to the high prevalence of biotin-binding proteins in *E. coli* that remain on the membranes causing false positive signals.

In summary, optimization of plating density, antisera adsorption, and detection methods are important for successful immunoscreening of cDNA libraries. Determination of the primary and secondary antibody dilutions with cell extracts or purified protein prior to immunoscreening ensures high specificity and low background.

References

1. Sikela, J.M. and Hahn, W.E. (1987) *Proc. Natl. Acad. Sci. USA 84*, 3038.
2. McGregor, P.F., Abate, C., and Curran, T. (1990) *Oncogene 5*, 451.
3. Blackwood, E.M. and Eisenmann, R.N. (1991) *Science 251*, 1211.
4. Skolnik, E. (1991) *Cell 65*, 83.
5. Defoe-Jones, D., Huang, P.S., Jones, R.E., Haskell, K.M., Vuocolo, G.A., Hanobik, M.G., Huber, H.E., and Oliff, A. (1991) *Nature 352*, 251.
6. Bowen, H.J. and Hearl, W.G. (1993) *Focus 15*, 87.

Rapid Generation of Recombinant Baculovirus and Expression of Foreign Genes Using the Bac-to-Bac™ Baculovirus Expression System

Dina Anderson
Molecular Biology Research and Development
Life Technologies, Inc.

Ray Harris
Research and Development
Life Technologies, Ltd.
Paisley, Scotland

Deborah Polayes
Valentina Ciccarone
Robert Donahue
Gary Gerard
Joel Jessee
Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

Verne Luckow
Molecular and Cellular Biology
Monsanto/Searle
Chesterfield, Missouri 63198

Abstract

The Bac-to-Bac Baculovirus Expression System is a novel gene expression system that allows the rapid and efficient generation of recombinant baculovirus DNAs by site-specific transposition in *E. coli*, rather than homologous recombination in insect cells. This article highlights the experimental procedures utilized in this system and demonstrates generation of recombinant baculoviruses and expression of β-glucuronidase and reverse transcriptase in three insect cell lines.

Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. Heterologous genes placed under the transcriptional control of the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) are often abundantly expressed during the late stages of infection. In most cases, the recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts (1–7).

Recombinant baculoviruses are traditionally constructed in two steps. The gene to be expressed is first cloned into a plasmid transfer vector downstream from a baculovirus promoter that is flanked by baculovirus DNA derived from a nonessential locus, usually the polyhedrin gene. This plasmid is then introduced into insect cells along with circular wild-type genomic viral DNA for homologous recombination to occur. Typically, 0.1% to 1% of the resulting progeny are recombinant. The fraction of recombinant progeny virus can be improved to nearly 30% by using a parent virus that is linearized at one or more unique sites (8,9). A higher proportion of recombinant viruses (80% or higher) can be achieved using linearized viral DNA that is missing an essential portion of the baculovirus genome downstream from the polyhedrin gene (10). While the frequency is improved, the actual number of recombinant plaques remains low and sequential plaque assays are required to purify the recombinant virus away from the nonrecombinant parental virus. Viral amplification is also necessary to obtain a sufficient amount of virus for protein expression. Plaque purifying the desired recombinant virus and confirming its DNA structure or using immunological methods to identify recombinant viruses expressing the desired protein can easily take more than a month to complete (5–7).

Recently, a rapid and efficient method to generate recombinant baculoviruses was developed (11) (figure 1). It is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid, bMON14272) propagated in *E. coli*. The bacmid contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and *lac*Zα from pUC. Inserted into the N-terminus of the *lac*Zα gene is the attachment site for the bacterial transposon Tn7 (mini-*att*Tn*7*) that does not disrupt the reading frame of the *lac*Zα peptide. The bacmid propagates in *E. coli* DH10Bac™ cells as a large plasmid that confers resistance to kanamycin and can complement a *lac*Z deletion present on the chromosome to form colonies that are blue (*Lac*$^{+}$) in the presence of Bluo-gal and the inducer IPTG. Recombinant bacmids are constructed by transposing a mini-Tn7 from a donor plasmid (pFastBac™1) to the mini-*att*Tn7 attachment site on the bacmid with Tn7 transposition functions provided in *trans* by a helper plasmid (pMON7124). The mini-Tn7 in pFastBac1 (figure 2) contains an expression cassette

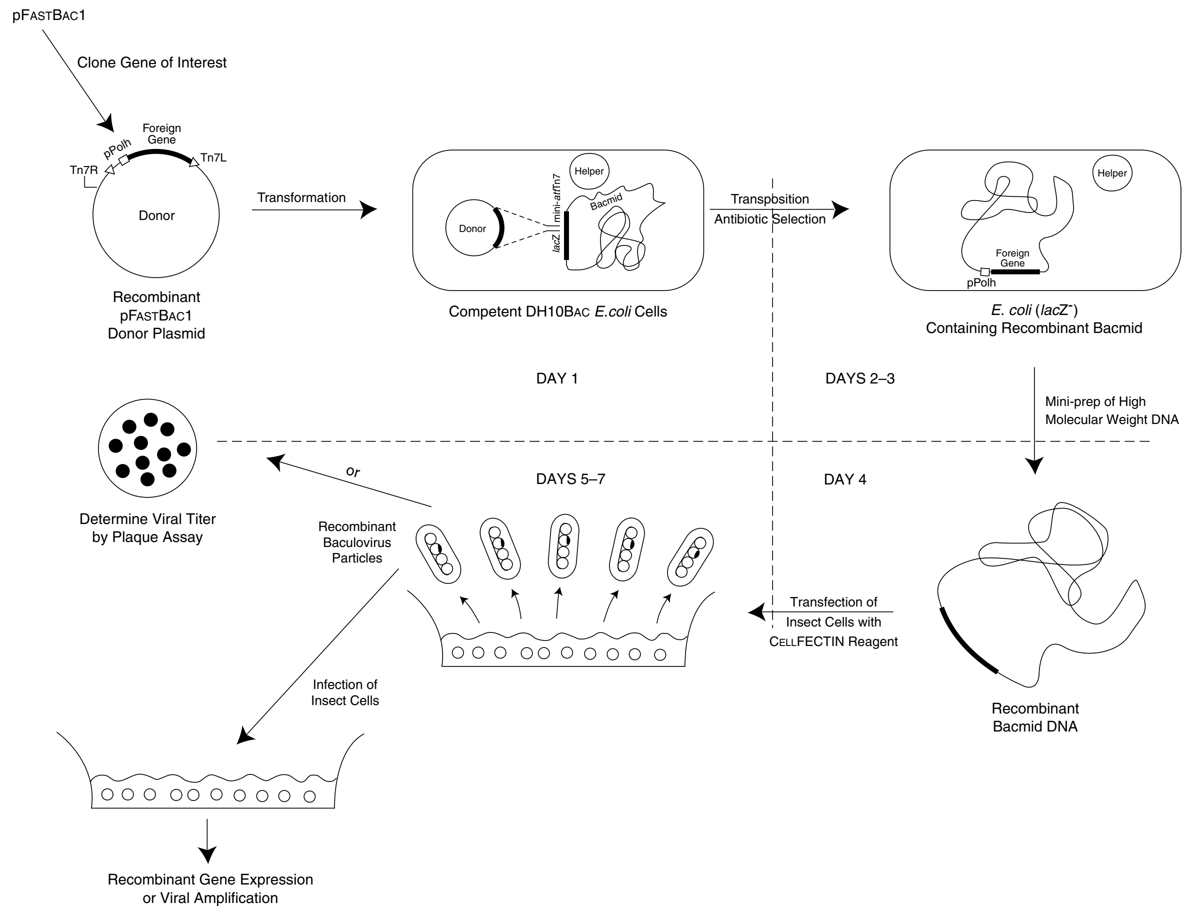


FIGURE 1. Generation of recombinant baculoviruses and gene expression with the BAC-TO-BAC system.

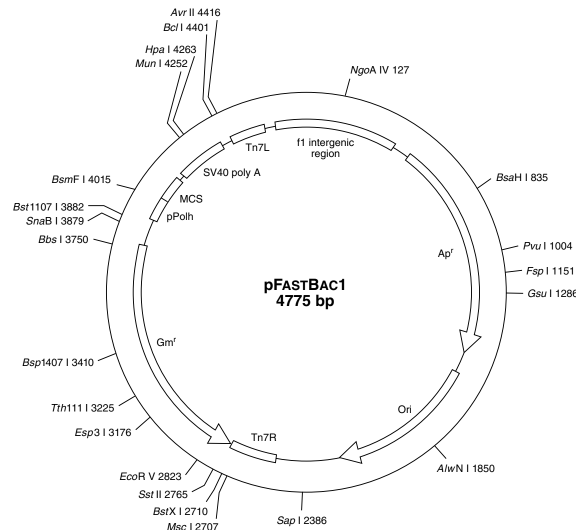
consisting of a gentamicin resistance gene (Gm^r), the polyhedrin promoter from AcNPV, a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7. Genes to be expressed are inserted into the multiple cloning site of pFASTBAC1 downstream from the polyhedrin promoter. Insertion of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupts expression of the *lac*Zα peptide, so that colonies containing the recombinant bacmid are white. Recombinant bacmid DNA can be rapidly isolated from small-scale cultures and used to transfect insect cells. Viral stocks (>10^7 pfu/ml) harvested from the transfected cells can be used to infect fresh insect cells for subsequent protein expression, purification, and analysis.

METHODS

The GIBCO BRL BAC-TO-BAC Baculovirus Expression System (Cat. No. 10359) consists of the cloning vector, pFASTBAC1; MAX EFFICIENCY DH10BAC™ Competent Cells harboring the bMON14272 (11) bacmid DNA and the pMON7124 (11) helper plasmid; the control vector, pFASTBAC-*gus*, containing the *uid*A (*gus*A) gene (encoding β-glucuronidase) inserted into the *Bam*H I–*Eco*R I site of pFASTBAC1; and CELLFECTIN™ Reagent. The AMV Reverse Transcriptase (AMV RT) gene was cloned into the *Bam*H I–*Xho* I site of pFASTBAC1.

Insect cell culture. Spodoptera frugiperda (Sf9) cells (ATCC, CRL 1711) and Sf21 cells were cultured at 27°C in Sf-900 II SFM. BTI-TN-5B1-4 (High Five™) cells were cultured at 27°C in EXPRESS FIVE™ SFM. All media were supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin. All cell culture media and reagents were GIBCO BRL brand.

Isolation of recombinant bacmid DNA. Recombinant pFASTBAC1 plasmids were transformed into MAX EFFICIENCY DH10BAC Competent Cells and plated on Miller's Luria



pFastBac1 MCS and promoter region:

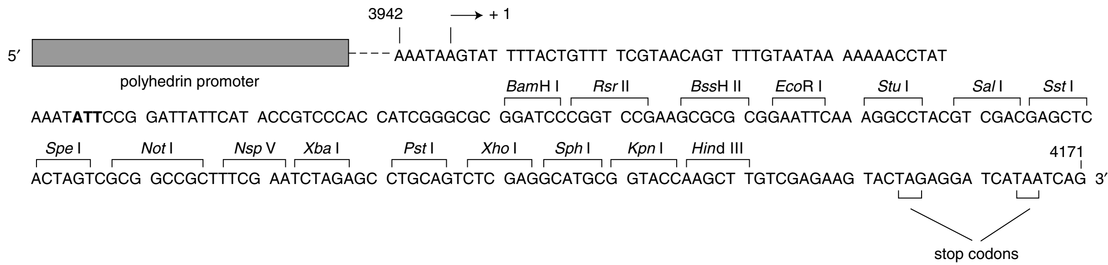


FIGURE 2. Map and MCS of pFastBac1. Transcriptional start for the polyhedrin promoter corresponds to +1. ATT marks the site of the original translational start of the polyhedrin gene. The ATG was mutated to an ATT. Single restriction endonuclease recognition sites are designated on the plasmid map.

Agar (LA) plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100–300 µg/ml Bluo-gal, and 40 µg/ml IPTG. After a 24 to 48-h incubation at 37°C, large, white (*lacZ*$^{-}$) colonies were selected for overnight culture. High-molecular-weight DNA was isolated from the overnight cultures as described in the manual.

Transfections. The minipreparations of recombinant bacmid DNA were transfected into insect cells using CellFECTIN Reagent. For each transfection, 9×10^5 cells were seeded in 35-mm wells of a 6-well plate and allowed to attach for ~1 h. Lipid reagent and DNA were diluted separately into 100 µl of Sf-900 II SFM (for Sf9 and Sf21 cells) or Express Five SFM (for BTI-TN-5B1-4 cells), then combined to form lipid-DNA complexes. The lipid-DNA complexes were diluted to 1 ml with SFM and added to cells. Cells were incubated 5 h at 27°C. The transfection medium was removed and replaced with fresh medium. Cells were analyzed for protein expression at 24 to 72 h post-transfection. Viral supernatant was collected at 72 h post-transfection.

In situ β-glucuronidase (β-gluc) staining. To demonstrate β-gluc expression *in situ*, a modified procedure of Sanes *et al.* was used (12). Cells were rinsed with Dulbecco's Phosphate Buffered Saline (PBS); fixed for 5 min in 2% formaldehyde, 0.2% glutaraldehyde in PBS; rinsed twice with PBS; and stained 2 h to overnight with 0.1% X-gluc, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS.

MUG assay for β-gluc. β-gluc was quantitated by harvesting cells directly in 1% Nonidet® P40 (v/v), 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 5 µg/ml aprotinin. Cell extracts were cleared by centrifugation, and aliquots were assayed (13) using the substrate MUG (methylumbelliferyl-

FIGURE 3. DH10Bac colonies following transposition with recombinant pFastBac-*gus*. DH10Bac Competent Cells (100 µl) were transformed with 1 ng pFastBac-*gus*. After a 4-h outgrowth, 100 µl of a 1:100 dilution was plated on an LA agar plate containing antibiotics and Bluo-gal. The plate was incubated at 37°C for 24 h.

β-D-galactopyranoside).

Assay for reverse transcriptase. Crude cell extracts were diluted 1:10 in 0.2 M KPO_4 (pH 7.0), 0.1 mM EDTA, 0.01% Nonidet P40, 1 mM DTT, 20 mM Tris-HCl (pH 7.4), and 10% glycerol. Aliquots (2 µl) of diluted extract were assayed in reaction mixtures [50 µl containing 50 mM Tris-HCl (pH 8.4), 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 100 µM [^{3}H]dGTP (100 cpm/pmole), 60 µM poly(C), and 24 µM oligo$(dG)_{12-18}$]. Incubation was for 10 min at 37°C. Amount of TCA-insoluble product was determined by precipitation of reaction mixtures on GF/C filters. With this template-primer (poly(C)-oligo$(dG)_{12-18}$) under these reaction conditions, only reverse transcriptase catalyzes significant amounts of [^{3}H]poly(dG) product.

SDS-PAGE protein analysis. Control or infected cells were washed with PBS. To extract total soluble protein, cells were lysed directly in 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and boiled for 10 min. Cell extracts were cleared by centrifugation, and samples were analyzed on 8–16% acrylamide gels (14).

Table 1. Virus yield from insect cells following transfection with bacmid DNA. Viral titers were determined by a plaque assay.

Cell Line	Viral Titer (pfu/ml) Bacmid DNA Transfected 1 µl	5 µl	10 µl
Sf9	2.7×10^7	3.1×10^7	2.2×10^7
Sf21	2.6×10^7	2.5×10^7	2.7×10^7
BTI-TN-5B1-4	8.0×10^5	1.3×10^6	6.5×10^5

Results and Discussion

Following transformation and plating of cells on agar plates, transposed colonies were visible as large, white (*lacZ*$^-$) colonies among a background of blue colonies (figure 3). Blue colonies represent either nontransposed DH10bac colonies or colonies in which transposition occurred into the *E. coli* chromosome rather than the bacmid DNA.

Recombinant bacmid DNA was isolated from overnight cultures and transfected into insect cells. Sf9 and Sf21 cells generated viral titers of 2×10^7 to 3×10^7 pfu/ml (table 1). BTI-TN-5B1-4 cells produced titers that were 10-fold lower, suggesting that Sf9 or Sf21 cells are better candidates for generation of recombinant virus. The yields of recombinant virus were similar when cells were transfected with 1 to 10 µl of bacmid DNA, suggesting that there was flexibility in the amount of bacmid DNA transfected. The bacmid DNA-transfected cells from which viral supernatant was collected were also analyzed for protein expression by *in situ* staining for β-gluc activity (data not shown). Significant protein expression was detected in these cells. Therefore, recombinant protein expression can be evaluated at 2 to 3 days after transfection of bacmid DNA, when the viral supernatant is collected. This allows rapid qualitative analysis of the recombinant protein without viral amplification or titering.

To express recombinant protein by infection, insect cells were infected with β-gluc- and AMV RT-recombinant baculoviruses. Maximal expression levels of RT were obtained at 72 h post-infection, whereas 48 h was optimal for β-gluc expression. For the peak expression levels, RT expression was 2-fold higher in Sf21 and BTI-TN-5B1-4 cells than in Sf9 cells (figure 4). In contrast, β-gluc expression was optimal in BTI-TN-5B1-4 cells. These differences in expression among different cell lines were consistent at all time points analyzed (data not shown). The level of β-gluc in infected cells was analyzed by SDS-PAGE (figure 5). Consistent with the enzyme activity, the highest

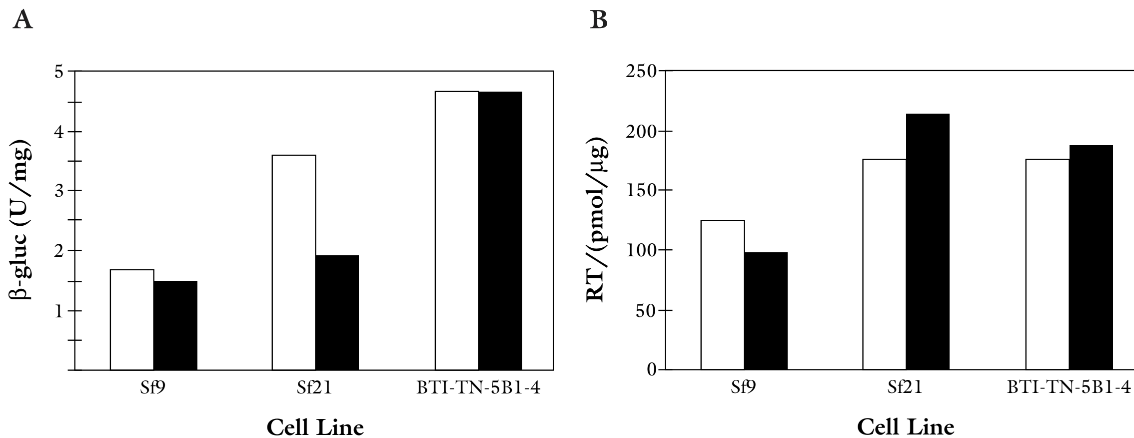


FIGURE 4. Expression of β-gluc and RT. Sf9, Sf21, and BTI-TN-5B1-4 cells were infected in 50-ml shaker cultures at MOIs of 5 (□) and 10 (■) with recombinant virus coding for β-gluc and RT. Samples were harvested at 24, 48, and 72 h post-infection and assayed for functional activity. Panel A. β-gluc activity at 48 h post-infection. Panel B. RT activity at 72 h post-infection.

level of recombinant protein was observed in BTI-TN-5B1-4 cells. These results indicate that recombinant protein expression must be optimized to determine the optimal cell line and time (post-infection) to recover protein. In these studies, 48 to 72 h post-infection resulted in the highest protein expression levels, and Sf21 and BTI-TN-5B1-4 cells produced higher protein levels than Sf9 cells.

To verify that recombinant baculovirus particles produced with the BAC-TO-BAC system yielded the same level of recombinant protein expression as virus generated by traditional recombination methodologies, reverse transcriptase- and β-gluc-recombinant baculoviruses were generated by homologous recombination. Once the recombinant transfer vectors were constructed, the time required to generate high-titer virus was 27 days, as 3 rounds of viral amplification were required. No significant differences were seen in the amount of recombinant protein expressed when Sf9 cells were infected with virus generated by the two methods (data not shown).

CONCLUSION

We have demonstrated the expression of two recombinant proteins using the BAC-TO-BAC System. This novel expression system utilizes site-specific transposition in *E. coli* and allows rapid and efficient generation of recombinant baculoviruses. With this system, recombinant virus DNA isolated from selected colonies is not mixed with parental, nonrecombinant virus, eliminating the need for multiple rounds of plaque purification. Also, high-titer virus is produced from the initial transfection and transfected cells can be analyzed for recombinant protein. These features reduce the time to identify and purify a recombinant virus from 4 to 6 weeks (typical for conventional methods) to 7 to 10 days. This method permits the rapid and simultaneous isolation of multiple recombinant viruses and is particularly suited for

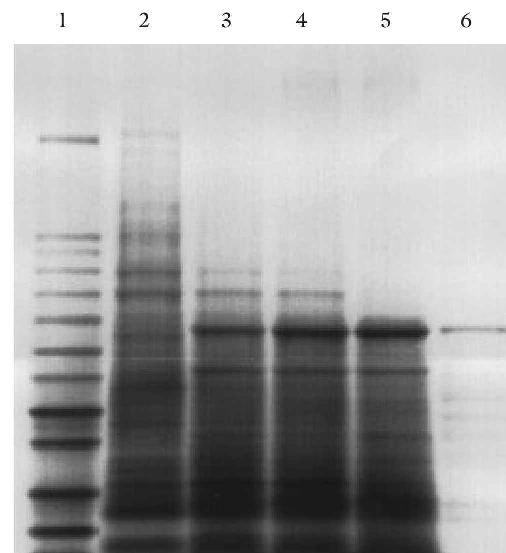


FIGURE 5. SDS-PAGE analysis of β-gluc expression. Sf9, Sf21, and BTI-TN-5B1-4 cells were infected at an MOI of 10 with β-gluc-recombinant virus. Samples were analyzed by SDS-PAGE. 25 μg protein were loaded into each lane (48 h post-infection). Lane 1. 10 kDa Protein Ladder. Lane 2. Uninfected Sf9. Lane 3. Sf9 infected cells. Lane 4. Sf21 infected cells. Lane 5. BTI-TN-5B1-4 infected cells. Lane 6. 2 μg purified β-gluc.

the expression of protein variants for structure/function studies.

Acknowledgements

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The Bac-to-Bac Baculovirus Expression System is sold under patent license from Monsanto Co. for research purposes only. U.S. Patent No. 5,348,886.

References

1. Luckow, V.A. (1991) in *Recombinant DNA Technology and Applications* (Prokop, A., Bajpai, R.K., and Ho, C., eds.), McGraw-Hill, New York.
2. O'Reilly, D.R., Miller, L.K., and Luckow, V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W.H. Freeman and Company, New York.
3. King, L.A. and Possee, R.D. (1992) *The Baculovirus Expression System: A Laboratory Guide*, Chapman & Hall, London.
4. Luckow, V.A. (1993) *Curr. Opin. Biotechnol. 4*, 564.
5. Davies, A.H. (1994) *Bio/Technology 12*, 47.
6. Luckow, V.A. (1995) in *Baculovirus Expression Systems and Biopesticides* (Shuler, M.L., Wood, H.A., Granados, R.R., and Hammer, D.A., eds.), Wiley-Liss, Inc., New York.
7. Luckow, V.A. (1995) in *Principles and Practice of Protein Engineering* (Cleland, J.L. and Craik, C.S., eds.), John Wiley and Sons, New York.
8. Kitts, P.A., Ayres, M.D., and Possee, R.D. (1990) *Nucleic Acids Res. 18*, 5667.
9. Hartig, P.C. and Cardon, M.C. (1992) *J. Virol. Methods 38*, 61.
10. Kitts, P.A. and Possee, R.D. (1993) *BioTechniques 14*, 810.
11. Luckow, V.A., Lee, S.C., Barry, G.F., and Olins, P.O. (1993) *J. Virol. 67*, 4566.
12. Sanes, J.R., Rubenstein, J.L.R., and Nicolas, J.F. (1986) *EMBO J. 5*, 3133.
13. Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987) *EMBO J. 6*, 3901.
14. Laemmli, U.K. (1970) *Nature 227*, 680.

Express Five™ SFM: A New Serum-Free Medium for Growth of BTI-TN-5B1-4 Cells and Expression of Recombinant Proteins

Glenn Godwin
Doug Danner
Stephen Gorfien
Cell Culture Research and Development
Life Technologies, Inc.
Grand Island, New York 14072

The traditional cell lines for the Baculovirus Expression Vector System (BEVS) have been Sf9 and Sf21, derived from the fall armyworm *Spodoptera frugiperda*. A number of new invertebrate cell lines have been described and evaluated for use in BEVS (1–3). Recently, recombinant protein expression levels 1- to 10-fold higher have been reported in the *Trichoplusia ni* (cabbage looper) cell line, BTI-TN-5B1-4 (4,5). Although current serum-free media formulations support serum-free suspension growth of BTI-TN-5B1-4 cells, these cells have different nutritional requirements from the classic cell lines. Express Five SFM has been optimized to achieve rapid doubling times and high peak cell densities of BTI-TN-5B1-4 cells in suspension culture allowing infection at higher cell densities, thus resulting in higher recombinant protein yields.

Methods

Culturing cells. Monolayer cultures of BTI-TN-5B1-4 cells (High Five™ cells) were adapted to suspension growth in Gibco BRL Sf-900 II SFM. Cells were seeded at a density of 3×10^5 cells/ml in 125-ml shake flasks (35-ml volume). The cultures were incubated at 27°C in ambient CO_2 on an orbital shaker platform rotating at 130 to 150 rpm. On day 3 or 4 postplanting, when the cells had reached densities of 2×10^6 to 3×10^6/ml, the cells were

subcultured. Following adaptation to suspension culture in Sf-900 II SFM, triplicate 75-ml shake flask cultures of BTI-TN-5B1-4 cells were seeded at 3×10^5 viable cells/ml in GIBCO BRL EXPRESS FIVE SFM, Sf-900 II SFM, or supplier A SFM. EXPRESS FIVE SFM is formulated without L-glutamine and supplemented with 2.8 g/L L-glutamine at the time of use to prevent problems associated with L-glutamine breakdown (6,7). The cells were subcultured every 3 days for 3 passages. During the third passage, total and viable cell counts were monitored daily.

Minimizing cell clumping. BTI-TN-5B1-4 cells tend to form very large cell clumps (>50 cells) during the adaptation to suspension culture and recovery from cryopreservation. The clumps eventually form aggregates that can interfere with culturing and infection steps. Clumping was decreased by modifying the subculture procedure slightly during the first few subcultures. The cells were allowed to settle for 1 to 2 min. The clumps that settled to the bottom were broken up using a 10-ml pipet by first drawing up the cells into the pipet and then pressing the pipet tip against the bottom of the flask and discharging the cells back into the medium. This can be repeated one or two more times with minimal adverse effects on the cells. The cells may be reincubated or counted and subcultured. Smaller clumps were removed by allowing the culture to settle for 30 to 60 s and taking the sample for counting and subculturing from the upper layer of the culture.

Recombinant protein production. Replicate cultures of BTI-TN-5B1-4 cells and Sf9 cells were infected with rAcNPV (Clone VL-941, expressing β-galactosidase) at a multiplicity of infection (MOI) of 5.0 when the cultures were in mid-exponential growth and at densities of 1.5×10^6 viable cells/ml. Cultures were sampled every 24 h through day 3 post-infection. β-galactosidase levels were quantitated from 72 h post-infection samples.

For BTI-TN-5B1-4 cells, viral titers are 10- to 1,000-fold lower than with Sf9 and Sf21 cells. To compensate for the lower titers, lower MOIs were used or baculovirus stocks were produced in Sf9 or Sf21 cell lines. Because rAcNPV is infectious to *Spodoptera frugiperda* and *Trichoplusia ni* cell lines, the virus stocks produced in Sf9 or Sf21 cells can be used

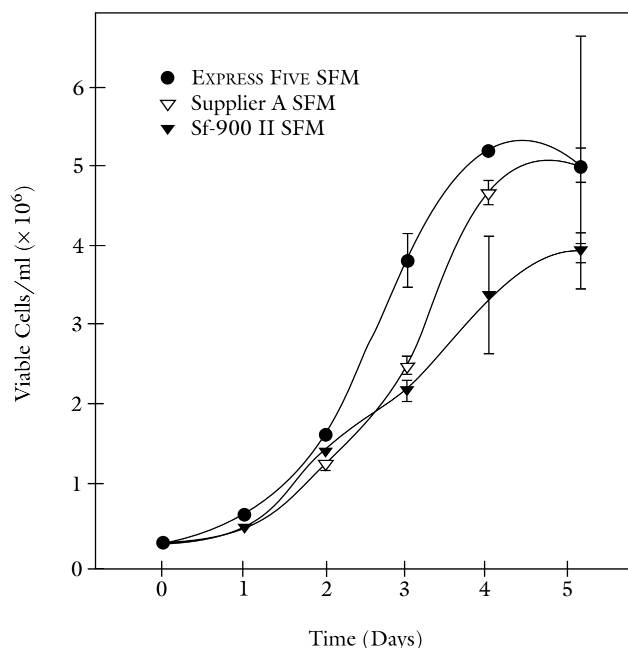


FIGURE 1. Growth of BTI-TN-5B1-4 cells. Cells adapted to EXPRESS FIVE SFM (●), Sf-900 II SFM (▼), or Supplier A SFM (▽) were seeded in 125-ml shake flasks (triplicate cultures). Samples were taken daily for estimation of viable cell density.

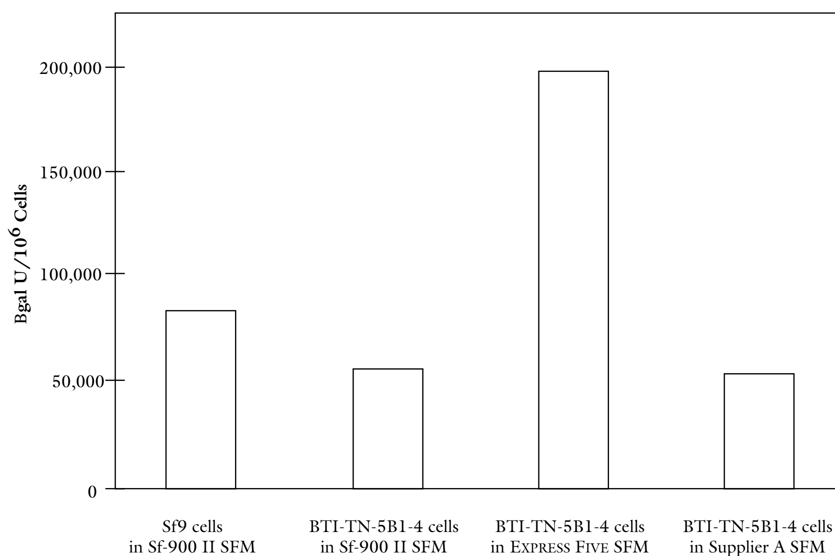


FIGURE 2. Recombinant β-galactosidase production in BTI-TN-5B1-4 cells. Cells adapted to each medium were seeded in 125-ml shake flasks (replicate cultures). Sf9 cells adapted to Sf-900 II SFM were similarly seeded in Sf-900 II SFM. Cells were infected with rAcNPV (Clone VL-941) at an MOI of 5.0 at a density of 1.5×10^6 viable cells/ml. Samples were analyzed 72 h post-infection for β-galactosidase.

with the BTI-TN-5B1-4 cells to express recombinant product. The infection strategy (MOI, infection density, time of recombinant product harvest) must be optimized for every rAcNPV clone following completion of plaque purification and baculovirus amplification.

Results and Discussion

Express Five SFM has been optimized for use with the BTI-TN-5B1-4 cell line and supported saturation cell densities of 4×10^6 to 5×10^6 viable cells/ml, with population doubling times of 20 to 24 h during exponential growth. Express Five SFM supported high peak cell densities and more rapid growth rates than other media (figure 1). For recombinant β-galactosidase expression, Express Five SFM had higher protein production than other media with BTI-TN-5B1-4 cells or Sf9 cells in Sf-900 II SFM (figure 2).

For applications where BTI-TN-5B1-4 cells are required, Express Five SFM is the preferred medium, supporting rapid growth rates, high saturation densities, and high levels of recombinant protein production.

References

1. Hink, W.F. and Bezanson, D.R. (1985) *Techniques in Life Sciences*, Vol. C111, Elsevier Scientific Publishers Ltd., Ireland, p. 1.
2. Hink, W.F. and Hall, R.L. (1989) in *Invertebrate Cell System Applications*, Vol. II (Mitsuhashi, J., ed.), CRC Press, Inc., p. 269.
3. Hink, W.F., Thomsen, D.R., Davidson, D.J., Meyer, A.L., and Castellino, F.J. (1991) *Biotechnol. Prog. 7*, 9.
4. Wickham, T.J., Davis, T., Granados, R.R., Shuler, M.L., and Wood, H.A. (1992) *Biotechnol. Prog. 8*, 391.
5. Davis, T.R., Wickham, T.J., McKenna, K.A., Granados, R.R., Shuler, M.L., and Wood, H.A. (1993) *In Vitro Cell. Dev. Biol. 29A*, 388.
6. Heeneman, S., Deutz, N.E.P., and Buurman, W.A. (1993) *J. Immunol. Methods 166*, 85.
7. Visek, W., Kolodny, G.M., and Gross, P.R. (1972) *J. Cell. Physiol. 80*, 373.

Sensitivity of Transfection Efficiency to Culture Age

Pam Hawley-Nelson
Po-Jen Shih
Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

Cationic lipid transfection is a simple procedure for nucleic acid delivery to a wide variety of cells (1–3). Occasionally, transfection efficiency changes from one experiment to the next. In many cases, the health and physiological state of the cells have changed in response to environmental factors (gradual or sudden). Here, the effect of culture age on transfection efficiency with NIH3T3 cells is investigated.

NIH3T3 cells (either freshly thawed or 8 passages in culture from the same stock) were maintained in Gibco BRL media and reagents—D-MEM with 10% calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. LipofectAMINE™ Reagent and pCMVβgal (4) DNA were diluted separately into Opti-MEM® I Reduced Serum Medium without serum and combined to form DNA-lipid complexes. The cells were rinsed with serum-free D-MEM, and DNA-lipid complexes were diluted into transfection medium consisting of D-MEM without serum and added to the cells for a 5-h exposure. Cultures were fixed 30 h after transfection to detect cells expressing β-galactosidase with X-gal (5).

Both freshly thawed and passaged cultures were growing vigorously (data not shown), but the freshly thawed culture was more competent for transfection (figure 1). This higher transfection efficiency of fresh cells does not decrease over the next few passages, so the effect was not attributed to the freeze/thaw process (data not shown). The nature of the change in these cells which caused this decreased efficiency is unknown.

In summary, fresh cells should be thawed when efficiency decreases over time. Also, maintaining healthy cell cultures will help decrease variability in transfection efficiency.

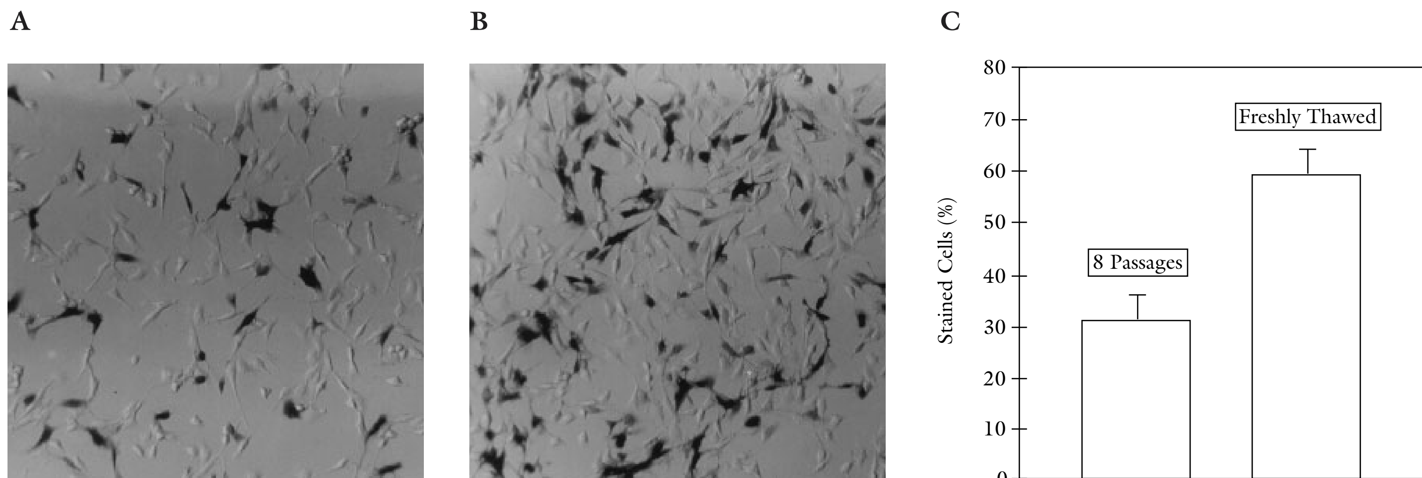


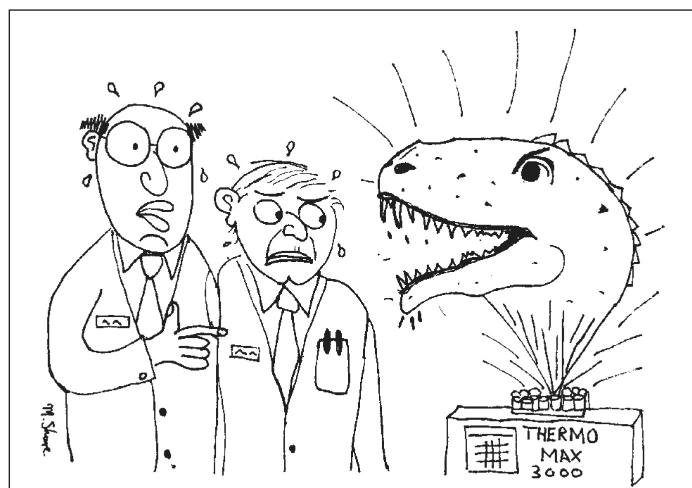
FIGURE 1. Transfection of NIH3T3 cultures. 8×10^4 NIH3T3 cells were plated per 35-mm well (6-well plate). The day after plating, cells were transfected with 1 µg pCMVβgal DNA and 3 to 15 µl LIPOFECTAMINE Reagent. Peak activity points (9 µl) from each dose-response were photographed. Panel A. NIH3T3 cells cultured 8 passages after thaw (several months in culture). Panel B. NIH3T3 cells freshly thawed. Panel C. Percent stained cells in peak activity well (mean ± SD for n = 3).

REFERENCES

1. Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987) *Proc. Natl. Acad. Sci. USA 84,* 7413.
2. Whitt, M.A., Buonocore, L., Rose, J.K., Ciccarone, V., Chytil, A., and Gebeyehu, G. (1991) *FOCUS 13,* 8.
3. Hawley-Nelson, P., Ciccarone, V., Gebeyehu, G., Jessee, J., and Felgner, P.L. (1993) *FOCUS 15,* 73.
4. MacGregor, G.R. and Caskey, C.T. (1989) *Nucleic Acids Res. 17*, 2365.
5. Sanes, J.R., Rubenstein, J.L.R., and Nicolas, J.-F. (1986) *EMBO J. 5*, 3133.

Does how I make the lipid-DNA complex affect my transfection efficiency?

Yes. For LIPOFECTAMINE Reagent, dilute the lipid in 100 µl of OPTI-MEM I and dilute the DNA in 100 µl OPTI-MEM I. Mix the 2 solutions and incubate for 15 min at room temperature. For LIPOFECTIN® Reagent, allowing the diluted LIPOFECTIN Reagent and medium to incubate for at least 30 min before adding the DNA solution increases efficiency ~3-fold. Be sure that the complex is formed in the absence of serum. After the 15-min incubation, serum-containing medium (800 µl) can be added to the complexes.



Maybe we should try reducing the number of cycles!

ELONGASE™ REAGENTS FOR AMPLIFICATION OF LONG DNA TEMPLATES

Barry Westfall
Kalavathy Sitaraman
Mark Berninger
Lawrence M. Mertz
Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

ABSTRACT

A system for amplification of long, as well as short, dsDNA and cDNA targets is presented. Amplification of targets from 1.3 kb to 29.9 kb is demonstrated from single-stranded cDNA, human genomic DNA, λ DNA, and supercoiled plasmid DNA. Substantial amplification was obtained from as little as 100 ng of human genomic DNA and 25 pg of λ DNA, as well as DNA directly from bacterial colonies and λ plaques.

Amplification with *Taq* DNA polymerase is generally limited to DNA templates <5 kb. This size limitation is presumably due to the inability of *Taq* DNA polymerase to correct dNTP misincorporations, since it lacks 3´ to 5´ exonuclease (proofreading) activity (1). In addition, the rate of primer elongation is significantly decreased from a mismatch (2), which results in greatly reduced amplification efficiency, especially for longer targets. Recently, enzyme systems that can amplify longer DNA templates have been described (3,4). These systems consist of a combination of two thermostable DNA polymerases, one of which contains proofreading activity. It is believed that this proofreading enzyme functions to correct nucleotide misincorporations so the other enzyme, which lacks this editing function, can subsequently elongate the nascent strand. In this report, we describe the use of the ELONGASE Enzyme Mix composed of *Taq* and *Pyrococcus species* GB-D DNA polymerases in the amplification of a variety of long DNA targets.

METHODS

Sample preparation. For direct amplification from λ plaques, 2 µl of water was placed over the plaque and then triturated to allow elution of the phage particles into the water. Pieces of top agar were liberated into the solution. The 2 µl was then placed in a tube containing 10 µl of water, followed by vortexing and a brief centrifugation to pellet the agar pieces. 10 µl of the supernatant was subjected to 20 cycles of amplification. Incubation of the agar pieces in water at room temperature for longer time periods led to greater amplification yields. For amplification from single colonies, each colony was removed from the agar plate with a sterile pipet tip and directly placed into 10 µl of water, followed by vortexing, addition of amplification reagents, and 20 cycles of PCR. Supercoiled plasmid DNA was prepared by the alkaline lysis method.

cDNA synthesis. First strand cDNA was synthesized from 500 ng total HeLa RNA with oligo(dT) and SUPERSCRIPT™ II RT from the GIBCO BRL Preamplification System in a 50-µl volume.

Primers. All cDNA, plasmid, λ, and human genomic DNA targets (β-globin, serum albumin, and Factor IX genes) were amplified using GIBCO BRL Custom Primers (table 1). The GIBCO BRL M13/pUC Forward and Reverse Amplification Primers were used for amplification of plasmid inserts. For amplification of cDNA, λ DNA, and plasmids, the concentration of each primer was at 400 nM, whereas for genomic DNA, each primer was at 200 nM.

Amplification. All reactions were performed using the GIBCO BRL ELONGASE Amplification System (Cat. No. 10481) in GeneAmp® thin-walled reaction tubes and consisted of 200 µM each dNTP and 1 U (1 µl) of ELONGASE Enzyme Mix in buffer A/B, which was mixed in different ratios to optimize magnesium. For many primer sets and targets, the optimal magnesium concentration was between 1.6 and 2 mM. For targets amplified by 1U of *Taq* DNA polymerase, comparable conditions were used and the reaction buffer was 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$. 50-µl volumes were used for cDNA, genomic, and λ DNA amplifications, and 25-µl volumes were used for amplification of plasmids from single colonies. In these 25-µl reactions, the concentrations of all amplification reagents were the same as in a 50-µl reaction, except for the enzyme, whose concentration was doubled.

TABLE 1. Primer sequences.

Template	Target size (kb)	Primer sequences
Single-stranded cDNA	5.3 (TSC-2)	ACT GAG CAT GGA ATG TGG CCT CAA CAA T
		CTC TAT GTC TGT GCA CTG GGG TCA GGA CTT TA
	6.8 (DNA Polymerase ε)	CGC CAA ATT TCT CCC CTG AA
		CCG TAG TGC TGG GCA ATG TTC
	8.9 (APC)	GCT GCA GCT TCA TAT GAT CAG TTG TTA
		ATA CCA ATT TTT CCC TGA TGT AAG TTT AGT CA
Human genomic DNA	1.3 (β-globin)	TTA GGC CTT AGC GGG CTT AGA C
		CCA GGA TTT TTG ATG GGA CAC G
	4.1 (β-globin)	GGT GTT CCC TTG ATG TAG CAC A
		CCA GGA TTT TTG ATG GGA CAC G
	7.5 (β-globin)	CTG CTG AAA GAG ATG CGG TGG
		GCA CTG GCT TAG GAG TTG GAC T
	12.4 (serum albumin)	AAA AAG GGG GCA AAT GAA ATG AG
		AGG ACA ATG GGC AAC ACT GAA AC
	15.1 (Factor IX)	GTT TCC CCC TTT TCC CTC ATT CT
		CCC AAC CTG CGT AAC CAA C
	20.0 (Factor IX)	GTT TCC CCC TTT TCC CTC ATT CT
		GGG CAA AGT GGA CAA AGC AGT G
λ DNA	12.7	GGT GCT TTA TGA CTC TGC CGC
		GGA CCT ATC TGC CCG TTC GT
	14.1	GCT GGG TCA GGT TGT TCT TTA GGA G
		TTG CCT CTT TGC CCG TCA TAC ACT T
	20.8	GCT GAA GTG GTG GAA ACC GC
		GCC TCG CAT ATC AGG AAG CAC
	23.4	CTC TGA TGC TTG AAC CCG CCT ATG C
		ATG ACA GAG GCA GGG AGT GGG ACA A
	29.9	GCT GAA GTG GTG GAA ACC GC
		GTC GGA CTT GTG CAA GTT GCC

Thermal cycling parameters consisted of a preamplification denaturation of 30 s at 94°C followed by 30 to 35 cycles of 94°C for 30 s, 55 to 65°C (annealing) for 30 s, and 68°C for 45 to 60 s per 1 kb of target length. The annealing temperature was variable depending on the primer Tm. For amplification of supercoiled plasmids, preamplification denaturation was extended to 1 to 3 min and 20 cycles of amplification were performed. For amplification of the 20-kb plasmid insert, the cycle denaturation time was increased to 45 s for the first 5 cycles, followed by a decrease to 15 to 30 s for the remaining 15 cycles. For amplification directly from bacterial colonies and λ plaques, extension times of 60 s per 1 kb of target length were used. Between 5 and 10 µl of amplification product was resolved on a 0.4 to 0.8% TAE-agarose gel containing ethidium bromide.

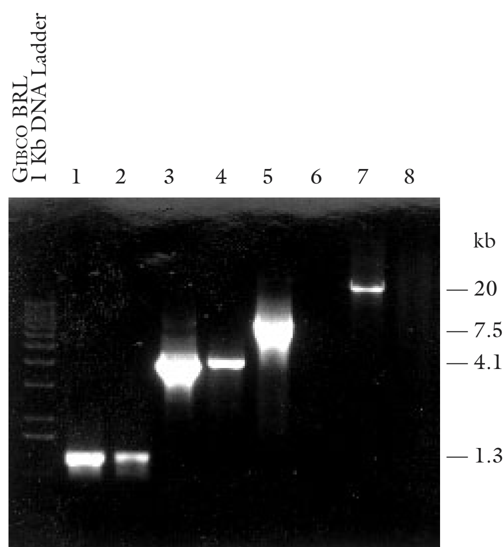


FIGURE 1. Enzyme comparison. Single-copy genomic targets were amplified using ELONGASE Enzyme Mix (odd-numbered lanes) or *Taq* DNA polymerase (even-numbered lanes) for 35 cycles using 100 ng of starting K562 human genomic DNA. 1.3-kb (2.0 mM Mg^{2+}; lanes 1 and 2), 4.1-kb (2.0 mM Mg^{2+}; lanes 3 and 4), 7.5-kb (1.6 mM Mg^{2+}; lanes 5 and 6), and 20-kb (1.9 mM Mg^{2+}; lanes 7 and 8) target.

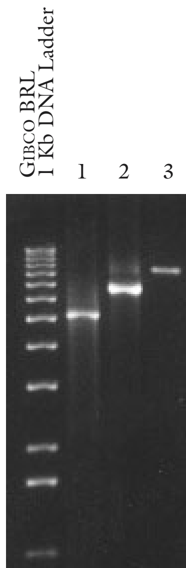


FIGURE 2. RT-PCR. PCR was performed with 2 µl of RT reaction, for 35 cycles with 1.7 mM magnesium, and products were electrophoresed on a 0.8% agarose TAE gel. Lane 1. 5.3-kb product from tuberous sclerosis II (TSC-II) cDNA. Lane 2. 6.8-kb product from polymerase ε cDNA. Lane 3. 8.9-kb product from familial adenomatous polyposis coli (APC) cDNA.

RESULTS AND DISCUSSION

Enzyme comparison. A comparison of ELONGASE Enzyme Mix to *Taq* DNA polymerase for single-copy genomic targets showed that even at 1.3 kb the ELONGASE Reagent produced more amplification product than *Taq* DNA polymerase alone (figure 1). No product was seen with *Taq* DNA polymerase alone at 7.5 kb or larger.

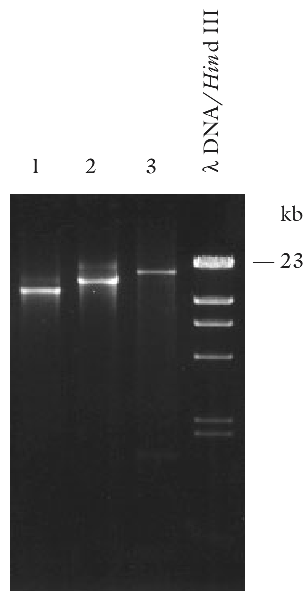


FIGURE 3. Genomic DNA. Samples were electrophoresed on a 0.8% agarose TAE gel. Lane 1. Amplification of a 12.4-kb (2.0 mM Mg^{2+}) serum albumin target. Lanes 2 and 3. Amplifications of 15.1-kb (2.0 mM Mg^{2+}) and 20-kb (1.9 mM Mg^{2+}) Factor IX sequences, respectively.

Long RT-PCR. ELONGASE Reagents amplified cDNA targets up to 8.9 kb (figure 2). Since the sizes of the TSC2, DNA polymerase ε, and APC mRNA coding regions are reported to be ~5.3 kb (6), 6.8 kb (7), and 8.9 kb (8), respectively, this represented amplification of the full-length cDNA. SUPERSCRIPT II was able to reverse transcribe to the 5´ end of these long mRNAs, and ELONGASE Reagents efficiently amplified these long cDNA targets. No amplification was detected from control reactions without RT (data not shown).

Human genomic and λ DNA. For single-copy human genomic DNA up to 20 kb, amplification products of the expected sizes were obtained from 100 ng of template (figure 3). Denaturation conditions were 94°C for 30 s. More extreme denaturation conditions or long preamplification denaturations led to reduced amplification, especially with the longer targets, which suggests template damage under extreme thermal conditions. For λ DNA sequences up to 29.9 kb, amplification products of the expected sizes were obtained from 25 pg of template (figure 4).

DNA from λ plaques and bacterial colonies. Amplification of long DNA targets contained within cruder biological samples was seen with λ plaques (figure 5). No amplification products were obtained with placement of agar plugs

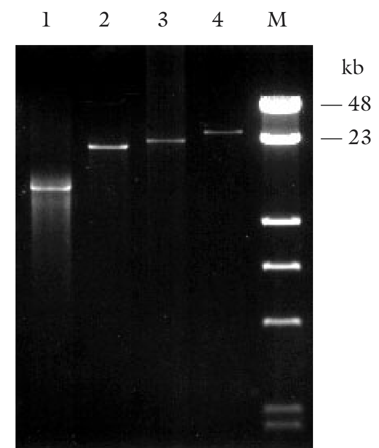


FIGURE 4. λ DNA. Lanes 1–4. Amplifications of 12.7-kb (1.6 mM Mg^{2+}), 20.8-kb (1.6 mM Mg^{2+}), 23.4-kb (1.3 mM Mg^{2+}), and 29.9-kb (1.6 mM Mg^{2+}) sequences, respectively. Lane M. The size standard is a mixture of intact λ DNA and λ DNA/*Hin*d III fragments (0.4% agarose-TAE gel).

directly into amplification reactions. For bacterial colonies, amplification up to 10.5 kb was observed (figure 6). Other colony-lyzing methods such as proteinase K treatment (10,11) and incubation of resuspended colonies at 37°C or 95°C in water followed by centrifugation and removal of the supernatant prior to amplification reactions were comparable to the method described here (data not shown). Treatment of the mini-prep DNA or lyzed colonies with RNase H or RNase A prior to amplification did not improve product yield (data not shown). When amplifying targets directly from plaques or bacterial colonies, elongation times of 1 min/kb led to a marked improvement in product yield, whereas for pure DNA samples, shorter elongation times were sufficient. Amplification of the 6.4-kb, 8-kb, and 10.5-kb inserts has been performed previously with *Taq* DNA polymerase alone (12). However, to achieve acceptable amplification yields, 25-µl reactions contained 4–8 ng of starting template and >5 U of *Taq* DNA polymerase. In this study, amplification was performed using 50 pg of purified plasmid or single bacterial colonies along with 1 U of ELONGASE Reagent.

In summary, the application of SUPERSCRIPT II RT and ELONGASE Reagents in RT-PCR demonstrates the ability to amplify large cDNA targets. Also, amplification of long DNA targets contained within complex genomic and episomal DNA is demonstrated. In addition, ELONGASE Reagents can be used to amplify long DNA sequences present within cruder samples such as bacterial colonies and λ plaques.

ACKNOWLEDGEMENTS

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REFERENCES

1. Tindall, K.R. and Kunkel, T.A. (1988) *Biochemistry 27*, 6008.
2. Innis, M.A., Myambo, K.B., Gelfand, D.H., and Brow, M.A.D. (1988) *Proc. Natl. Acad. Sci. USA 85*, 9436.
3. Barnes, W.M. (1994) *Proc. Natl. Acad. Sci. USA 91*, 2216.

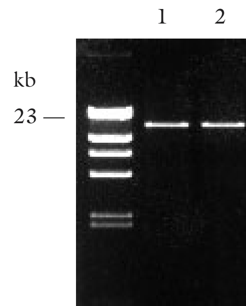

FIGURE 5. λ plaque DNA. Amplification of a 14.1-kb λ DNA sequence from 25 pg of pure λ DNA (lane 1) and from a single plaque (lane 2).

4. Cheng, S., Fockler, C., Barnes, W.M., and Higuchi, R. (1994) *Proc. Natl. Acad. Sci. USA 91*, 5695.
5. Mertz, L.M. and Rashtchian, A. (1994) *FOCUS 16*, 45.
6. The European Chromosome 16 Tuberous Sclerosis Consortium (1993) *Cell 75*, 1305.
7. Kesti, T., Frantti, H., and Syvaoja, J.E. (1993) *J. Biol. Chem. 268*, 10238.
8. Kinzler, K.W., Nilbert, M.C., Su, L-K., Volgelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M.S., Altschul, S.F., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I. and Nakamura, Y. (1991) *Science 253*, 661.
9. Wang, G., Berg, C.M., Chen, J., Young, A.C., Blakesley, R.W., Lee, L.Y., and Berg, D.E. (1993) *FOCUS 16*,55.
10. Young, A.C. and Blakesley, R.W. (1991) *FOCUS 13*, 137.
11. Young, A.C. and Blakesley, R.W. (1992) *FOCUS 14*, 56.
12. Sitaraman, K. and Rashtchian, A. (1993) *FOCUS 16*, 55.

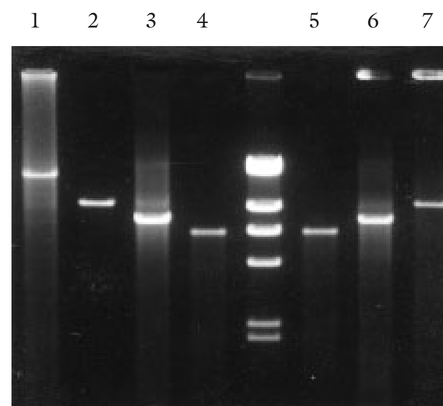

FIGURE 6. Amplification from bacterial colonies. Purified plasmid DNA with large inserts (lanes 1–4) and DNA directly from bacterial colonies (lanes 5–7) were amplified. The starting template amounts for reactions containing purified plasmid DNA were 1 ng for the 20-kb insert (lane 1), 100 pg for the 10.5-kb insert (lane 2), and 50 pg for the 8.0-kb (lane 3) and 6.4-kb (lane 4) inserts. Amplification from single bacterial colonies containing plasmids with 6.4-kb (lane 5), 8.0-kb (lane 6), and 10.5-kb (lane 7) inserts.

AFLP™: A Novel PCR-Based Assay for Plant and Bacterial DNA Fingerprinting

Jhy-Jhu Lin
Jonathan Kuo
Agricultural Biotechnology and Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

Abstract

DNA polymorphism of different ecotypes of *Arabidopsis thalianas* and different strains of *E. coli* were demonstrated using a novel PCR-based assay—Amplified Fragment Polymorphism (AFLP). In AFLP, the number of selective nucleotides in the *Eco*R I and *Mse* I primers for the selective amplification, as well as the complexity of the genomic DNA, determine the number of resulting amplified DNA fragments. The AFLP patterns were not affected by the amounts of genomic DNA (100 ng to 5 µg), but they were complicated by partially digested genomic DNA.

DNA fingerprinting with Restriction Fragment Length Polymorphism (RFLP) has been used by plant researchers to identify cultivars, to localize desirable genes, and to develop genetic maps (1). Recently, PCR-based techniques such as Random Amplified Polymorphic DNA (RAPD) (2), DNA Amplification Fingerprinting (DAF) (3), Single Strand Confirmational Polymorphism (SSCP) (4), and microsatellite/PCR (5) have been used to identify DNA markers. These techniques have improved identification of molecular markers, but these assays can be laborious and time consuming.

A novel PCR-based assay for plant DNA fingerprinting, AFLP, is easy to perform and can identify significant polymorphisms. In AFLP, genomic DNA is digested by restriction endonucleases such as *Eco*R I and *Mse* I, ligated to *Eco*R I and *Mse* I adapters, and amplified by PCR using primers that contain the common sequences of the adapters and one to three arbitrary nucleotides as selective sequences (figure 1). The polymorphism of these amplified DNA fragments is resolved by a sequencing gel. This paper describes AFLP analysis of *Arabidopsis* and *E. coli* strains and evaluates the effect of the complexity and amount of genomic DNA and the number of selective nucleotides in the amplified primers.

Methods

Genomic DNA isolation. A well-separated colony was resuspended in 5 ml LB broth for *E. coli* or in 5 ml YM broth for *Agrobacterium tumefaciens* EHA101. 1.0 ml of the cultured cells (37°C, overnight for *E. coli*; 30°C, 36 to 48 h for *A. tumefaciens* EHA101) was transferred into a microcentrifuge tube, and the cell pellets were collected by centrifuging at 11,000 × *g* for 5 min at room temperature. The pellets were resuspended in 1 ml TES-sucrose buffer [8% sucrose, 50 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA] and incubated at 25°C for 5 min with 1 mg/ml lysozyme. 100 µl 10% SDS was added and the tube was vortexed. The DNA was extracted with phenol-chloroform and precipitated with 0.3 M sodium acetate and 1 volume isopropanol. The pellet was washed with 70% ethanol and centrifuged at 11,000 × *g* for 10 min at room temperature. The DNA pellet was dissolved in 200 µl TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and digested with 1 µg/ml RNase A at 37°C for 10 min. The phenol-chloroform extraction and isopropanol precipitation were repeated. The final DNA pellet was dissolved in 200 µl TE. The growth and isolation of genomic DNA of *A. thalianas* and tomato used a modified CTAB method (7).

AFLP assay. AFLP assays were performed with the Gibco BRL AFLP Analysis System I (Cat. No. 10544) as recommended by the manufacturer. Briefly, 500 ng of genomic DNA was digested with *Eco*R I and *Mse* I, and the enzymes were inactivated at 70°C for 15 min. The DNA fragments were ligated with 5 µl of *Eco*R I and *Mse* I adapters. For preselective amplification, 5 µl of a 10-fold diluted ligation mixture was amplified for 20 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s using *Eco*R I + 0 primer and *Mse* I + 0 primer for *E. coli* and

A.t. EHA101 and *Eco*R I + A primer and *Mse* I + C primer for *Arabidopsis* and tomato. For selective amplification, *Eco*R I primers with 1 to 3 selective nucleotides were ^{32}P-labeled using the T4 polynucleotide kinase. 0.5 µl of ^{32}P-labeled *Eco*R I primers were mixed with 0.5 µl of 50-fold diluted preamplified DNA, PCR buffer, and the *Mse* I primers with 1 to 3 selective nucleotides. The mixtures were amplified for 1 cycle of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s, then lowering annealing temperature 0.7°C each cycle for 12 cycles; and then 23 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s. After adding 20 µl of sequencing loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue), the mixtures were heated at 90°C for 3 min, and 2 µl of the samples were electrophoresed in a 5% polyacrylamide sequencing gel with 8 M urea. The gel was dried and exposed to X-ray film.

Results and Discussion

A useful DNA marker generates reliable polymorphisms that are resolved by a gel electrophoresis. The resolution of polymorphisms using AFLP was demonstrated for genomic DNA purified from ecotypes of *A. thalianas* (Columbia and Landsberg), and 4 strains of *E. coli* (figure 2). Several unique bands of amplified DNA were easily seen among the common DNA fragments (50–100 bands).

The numbers of selective nucleotides in the *Eco*R I primers and *Mse* I primers are critical to AFLP analysis. For example, in *A. tumefaciens* EHA101, one selective nucleotide in both *Eco*R I and *Mse* I primers produced enough differences in amplified DNAs for determining polymorphism (figure 3). The number of different amplified DNAs decreased as the number of selective nucleotides of the *Mse* I primers increased. Interestingly, in *Arabidopsis*, which contains 50-fold more genomic complexity than *A. tumefaciens* EHA101, the demonstration of polymorphism in *Arabidopsis* required the combination of the *Eco*R I primer containing two selective nucleotides with the *Mse* I primer containing three selective nucleotides. Too many different amplified DNAs were observed with either *Eco*R I primers or *Mse* I primers containing only two selective nucleotides (figure 3). Similar results were also observed in *E. coli* and tomato (data not shown). Therefore, polymorphism using AFLP depends on the relationship between the number of selective nucleotides in the PCR primers and the complexity of the genome. If the complexity of the genome is not well defined, it is important to evaluate the number of selective nucleotides in the primers.

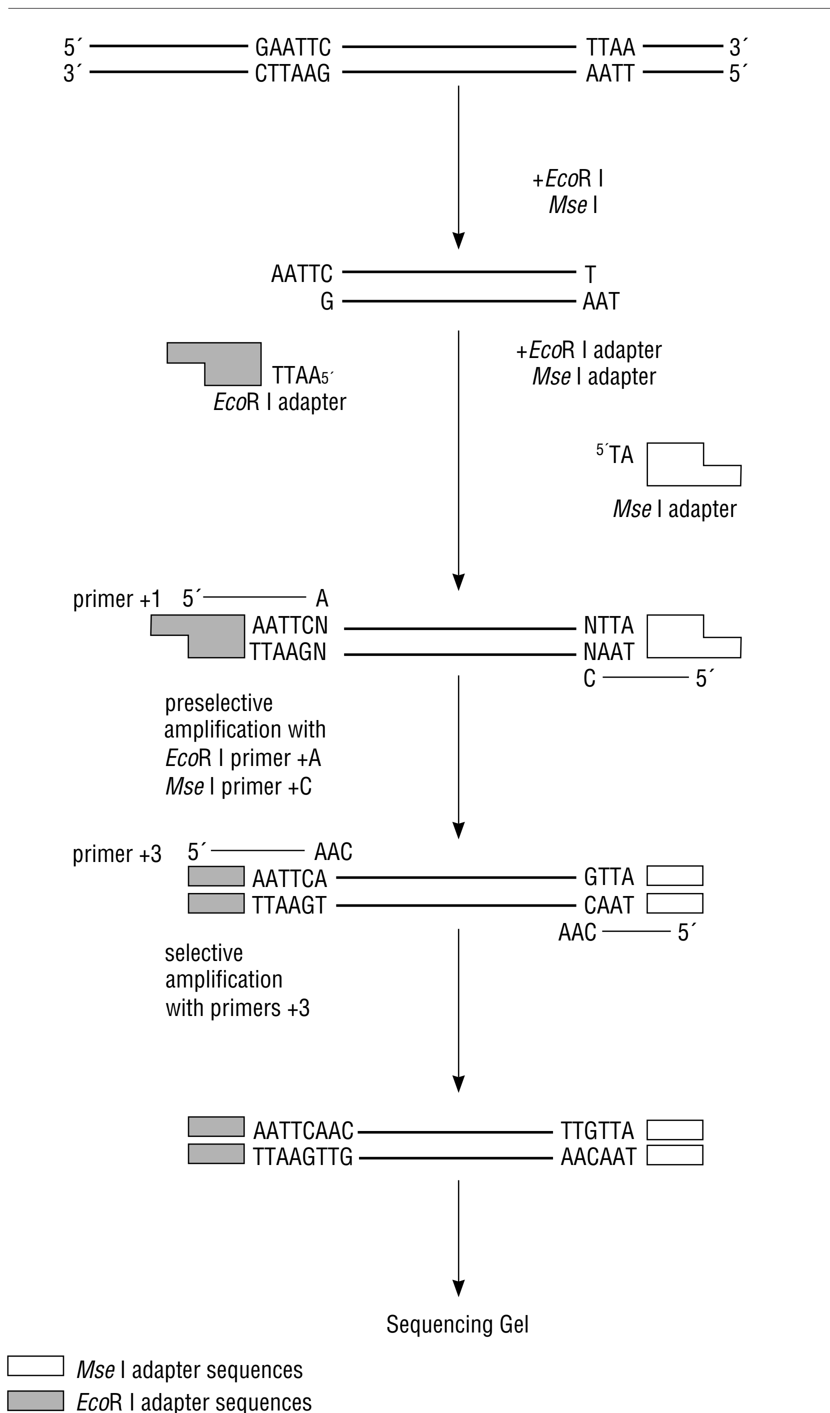

FIGURE 1. AFLP procedure with one primer pair.

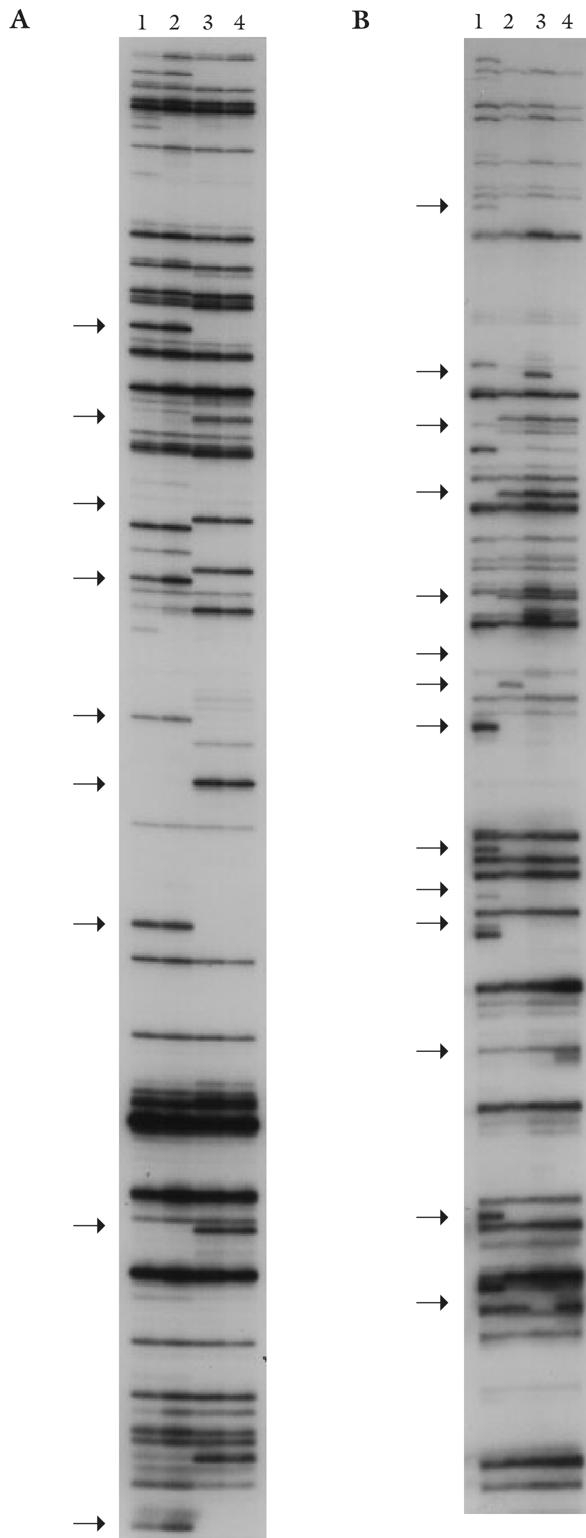

FIGURE 2. The polymorphism of *A. thalianas* ecotypes and *E. coli* strains using AFLP. AFLP was performed on 500 ng of genomic DNA, using selective primers (for *E. coli*: *Eco*R I+**A** and *Mse* I+**C**; for *Arabidopsis*: *Eco*R I+**CC** and *Mse* I+**AAA**). Panel A. *A. thalianas* ecotypes, lanes 1 and 2. Columbia; lanes 3 and 4. Landsberg. Panel B. *E. coli* strains BL21; HB101; DH5α; and DH11S; lanes 1–4, respectively.

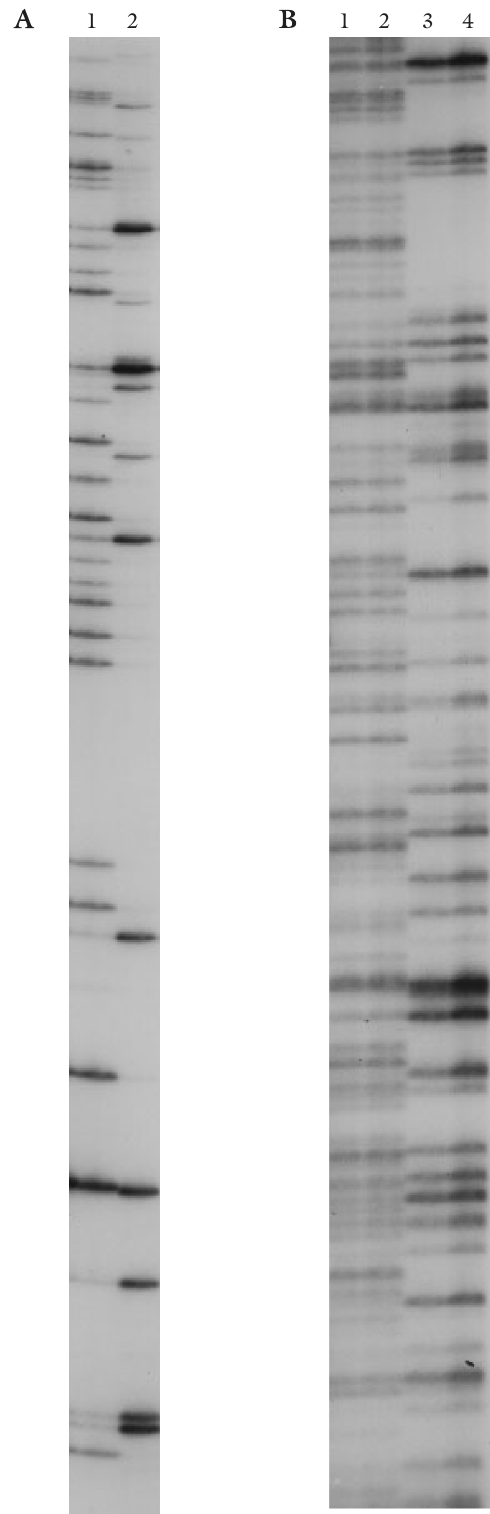

FIGURE 3. Effect of the number of selective nucleotides in the selective primers on AFLP. 500 ng of genomic DNA was digested and amplified by PCR with preselective *Eco*R I primers (*Eco*R I+0 in EHA101, and *Eco*R I+**A** in *Arabidopsis*) and *Mse* I primers. Then the ^{32}P-labeled samples were amplified with *Eco*R I selective primer and different *Mse* I selective primers. Panel A. *A. tumefaciens* EHA101, using *Eco*R I+**A** primer and *Mse* I primer+**A** (lane 1); *Mse* I primer+**AA** (lane 2). Panel B. *A. thalianas* Columbia, using *Eco*R I primer+**AC** and *Mse* I primer+**AA** (lanes 1 and 2); *Mse* I primer+**AAA** (lanes 3 and 4).

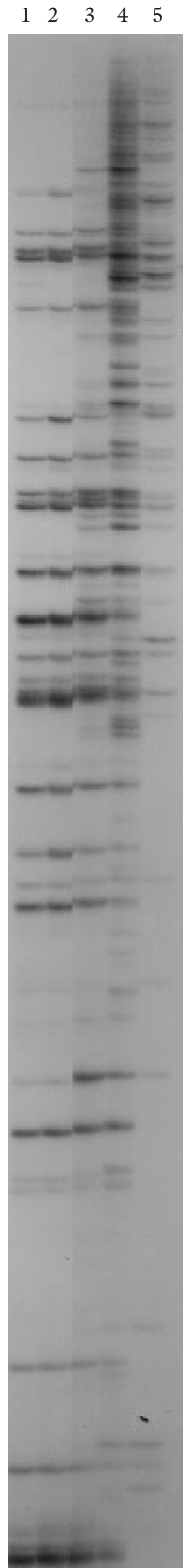

FIGURE 4. Effect of partial digestion on AFLP. Genomic DNA isolated *A. thalianas* Columbia was digested with different dilutions of *Eco*R I and *Mse* I, and AFLP was performed. The concentrations of the mixtures of *Eco*R I and *Mse* I were 5X, 1X, 0.1X, 0.01X, and 0.001X in lanes 1–5, respectively.

FIGURE 5. Effect of amount of DNA on AFLP. Different amounts of genomic DNA of *E. coli* DH5α were digested by *Eco*R I and *Mse* I. 100, 250, 500, 1,000, 2,500, and 5,000 ng in lanes 1–6, respectively.

In the preparation of large numbers of genomic DNA samples, the preparation may vary either in quality or in quantity from sample to sample. High-quality genomic DNA is necessary for AFLP to ensure complete digestion by the restriction endonucleases. DNA that is not digested completely by restriction endonucleases can be identified by gel analysis. To evaluate the effect of genomic DNA partially digested by a restriction enzyme on AFLP, DNA was digested by different dilutions of restriction endonucleases prior to AFLP. In comparison to the AFLP patterns of completely digested DNA, many additional lengths of amplified DNA, especially in the high-molecular-weight DNA, were observed in those genomic DNA samples digested by 10- to 100-fold dilution of the restriction endonucleases (figure 4). The presence of the bands resulting from partial digestions may be misinterpreted as false polymorphisms.

The effect of genomic DNA concentration on AFLP was examined. No differences in DNA patterns were observed using 100 ng to 5 µg genomic DNA (figure 5). This demonstrated that AFLP was achieved using a wide range of DNA.

AFLP is a reliable and reproducible molecular marker assay. The number of polymorphisms per reaction determined by AFLP are much higher than those performed by RFLP or RAPD. In addition, it is not necessary to predetermine genomic DNA sequences in order to perform AFLP, as is required by microsatellite marker assays. Moreover, identification of polymorphisms in both prokaryotic and eukaryotic organisms suggests that AFLP has the potential to become a universal molecular markers test.

References

1. Tanksley, S.D., Young, N.D., Paterson, A.H., and Bonierbale, M.W. (1989) *Bio/Technology 7*, 257.
2. Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. (1990) *Nucleic Acids Res. 18*, 6231.
3. Caetano-Anolles, G., Bassam, B.J., and Gresshoff, P.M. (1991) *Bio/Technology 9*, 553.
4. Ortia, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989) *Proc. Natl. Acad. Sci. USA 86*, 2766.
5. Health, D.D., Iwama, G.K., and Devlin, R.H. (1993) *Nucleic Acids Res. 21*, 5782.
6. Zabeau, M. and Vos, P. (1992) European Patent Application.
7. Lin, J.-J., Assad-Garcia, N., and Kuo, J. (1994) *Focus 16*, 72.

DNAzol™ Reagent for Rapid and Efficient Purification of Genomic DNA

Abdul H. Ally
Molecular Biology Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20884

Piotr Chomczynski
Molecular Research Center, Inc.
Cincinnati, Ohio 45212

Traditional methods of DNA purification are based on enzymatic and chemical methods to purify genomic DNA away from contaminating proteins, RNA, and other cellular components (1). The most popular method begins with lysis in a buffer containing proteinase K, followed by at least two phenol/chloroform extractions and ethanol precipitation to harvest genomic DNA. The isolation of DNA from fresh tissues often requires that the nuclear fraction be isolated prior to extraction of DNA.

DNAzol Reagent is a novel guanidine-detergent lysing solution that allows the rapid (10 to 30 min) and efficient purification of genomic DNA from different sources in a few steps and does not use phenol or chloroform. The use of guanidine salts for purification of DNA was proposed by Cox (2). DNAzol Reagent has been tested on a variety of animal and plant tissues as well as bacterial cells. We report the DNAzol Reagent purification of DNA from frozen rat liver tissue and HT29 cells growing in monolayer.

For frozen rat liver tissue, 0.3 ml of DNAzol Reagent were added to 11 mg of tissue and homogenized using a loosely fitted Teflon homogenizer, applying 10 strokes. The homogenate was centrifuged at 10,000 × *g* at 4°C for 10 min to pellet cellular debris and RNA. The supernatant was transferred to a clean tube using a wide-bore pipette, and 0.5 volume of 100% ethanol was added. The visible DNA precipitate was pelleted by centrifugation for 1 to 2 min at 1,000 × *g* and washed twice

with 95% ethanol. The air-dried pellet was dissolved in 8 mM NaOH. Spectrophotometric measurement of the DNA showed a yield of 3.7 µg/mg of tissue.

For HT29 cells, cells were trypsinized and the cell pellet was divided into 2 aliquots (2×10^6 cells per aliquot). 0.5 ml of DNAzol Reagent was added to one aliquot. The solution was pipetted to lyze the cells. 0.3 ml of 100% ethanol was added. The visible DNA pellet was collected by centrifugation at $1,000 \times g$ for 10 min. The supernatant was carefullly pipetted off, and the pellet was washed twice with 1 ml each of 95% ethanol. The DNA was air dried briefly and dissolved in 200 µl of 8 mM NaOH. The use of 8 mM NaOH to dissolve the genomic DNA facilitated the speed at which the DNA dissolved, but TE or water can be used as well. The yield of DNA was 33 µg, with an $A_{260/280}$ ratio of 1.74.

DNA was prepared from the second aliquot by a standard proteinase K/ phenol extraction procedure. Proteinase K (10 µg) was added to the cell pellet for 3 h at 50° C. After 4 extractions with phenol/chloroform, the sample was digested with 50 µg of RNAse A for 30 min at 37° C. The sample was extracted with phenol/chloroform, ethanol precipitated and the DNA pellet was dissolved in 200 µl of 8 mM NaOH. The yield was 6 µg, with an $A_{260/280}$ ratio of 1.74.

The DNA isolated by DNAzol Reagent was intact and was digested with *Eco*R I (figure 1). In addition, PCR amplification showed no differences between DNAzol Reagent-purified HT29 DNA and control DNA (figure 2). In conclusion, our data show that DNAzol Reagent is a useful tool for rapid isolation of high-quality DNA from animal cells and tissues.

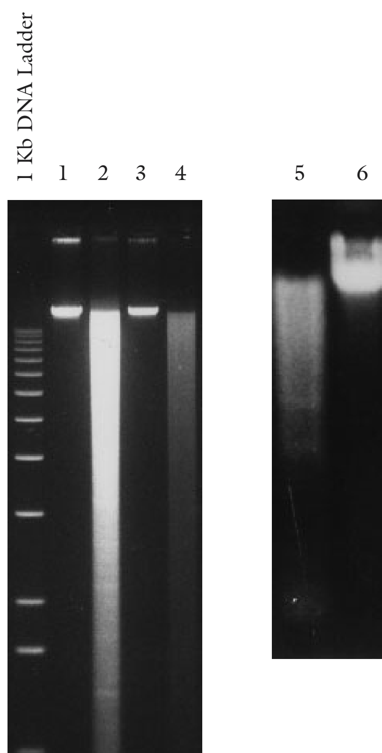

FIGURE 1. Comparison of genomic DNAs purified by DNAzol Reagent and the standard method. DNAs were digested with 3 units of *Eco*R I/µg DNA for 3 h at 37°C. Control HT29 cell DNA was purified by the standard proteinase K/phenol-chloroform/RNAase method. Lane 1. Undigested DNAzol-purified HT29 DNA (240 ng). Lane 2. *Eco*R I-digested DNAzol-purified HT29 DNA (800 ng). Lane 3. Undigested control DNA (240 ng). Lane 4. *Eco*R I-digested control DNA (800 ng). Lane 5. *Eco*R I-digested DNAzol-purified rat liver DNA (4 µg). Lane 6. Undigested DNAzol-purified rat liver DNA (4 µg).

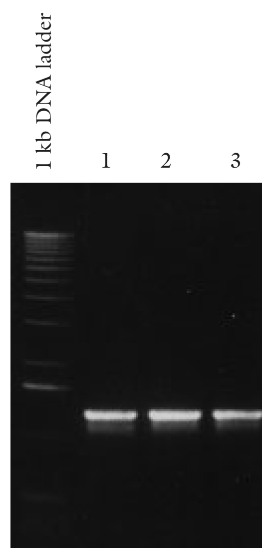

FIGURE 2. PCR of a 1.3-kb fragment of the β globin cluster. DNA (40 ng) was prepared by DNAzol Reagent treatment or the standard method from HT29 cells. 35 cycles of PCR were at 94°C for 3s, 55°C for 30 s, and 72°C for 90 s in 50-µl reactions, loading 5 µl per lane. Lane 1. Control DNA. Lanes 2 and 3. DNA purified by DNAzol Reagent.

References

1. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, 1st ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
2. Cox, R.A. (1968) in *Methods in Enzymology* (Grossmann, L. and Moldave, E., eds.), Vol 12, Part B, Academic Press, p. 120.

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Propagation of Plasmid Containing an Unstable Insert of Potato Virus Y^O Using Stbl2™ Competent Cells

Mathuresh Singh
Rudra P. Singh
Agriculture and Agri-Food Canada
Fredericton Research Centre
P.O. Box 20280
Fredericton, New Brunswick,
Canada

Potato virus Y (PVY), the type member of the genus Potyvirus, has a single-stranded RNA genome approximately 9.7 kb long with a genome-linked protein at the 5′ terminus and a poly A tail at the 3′ terminus (1). PVY has three distinct strains based on the symptomology in indicator plants. One strain, known as PVY^O (common strain), causes symptoms of mosaic in most of the commonly grown potato cultivars. We are cloning and sequencing various parts of the genome of one of the North American isolates of PVY^O. We have cloned and sequenced 4,500 bases from the 3′ terminus of the PVY^O genome using UDG cloning. While cloning sequences beyond 4,500 bases using RT-PCR, we obtained a 2,333-bp DNA fragment. We were unsuccessful in propagating this DNA fragment in DH5α™ cells. This indicated the instability of the plasmid-insert combination in DH5α cells. This paper describes the propagation of this unstable plasmid in Stbl2 cells.

Methods

RNA isolation. The PVY^O isolate 139 was multiplied in Samsun tobacco, and virus particles were purified (2). RNA was isolated by incubating 1 mg of purified virus with 50 µg/ml of proteinase K and 0.5% sodium dodecyl sulfate for 30 min at 37°C. The mixture was extracted once with TE-saturated phenol:chloroform (1:1) and once with chloroform (1:1). The viral RNA was recovered by ethanol precipitation.

PCR primer design and synthesis. The 32-base forward and 31-base reverse primers, including additional 12-base dUMP-containing sequence (CAU CAU CAU CAU) and (CUA CUA CUA CUA), respectively, to the 5′ end of PCR primers (3), were designed using a primer version 0.5 program (Whitehead Institute). The forward primer was homologous to the nucleotide 3007-3026, and the reverse primer was complementary to the nucleotide 5339-5319 in PVY genome (4).

cDNA synthesis and RT-PCR amplification. cDNA synthesis was carried out in 20 µl reaction mixture. Reagents were added to give the following final composition: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM each dNTP, 20 units of RNasin, 0.1 µg of reverse primer, 200 units of Gibco BRL M-MLV Reverse Transcriptase, and 0.2 µg viral RNA. Samples were incubated at room temperature for 20 min to allow the primer to anneal, then at 42°C for 1 h for reverse transcription. Aliquots (5 µl) of the reaction were transferred to tubes containing 45 µl of the PCR mixture, overlaid with one drop of mineral oil. The cycling conditions were as follows: 35 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min followed by one incubation at 72°C for 8 min. Amplification products were analyzed on a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide.

Cloning and transformation. A PCR product of 2,333 bp was recovered from the gel with the Gibco BRL GlassMAX® DNA Isolation System and cloned with the Gibco BRL CloneAmp® pAMP1 System, according to the manufacturer's instructions. Transformations were performed using Gibco BRL Subcloning Efficiency™ or Max Efficiency® DH5α or Stbl2 Competent Cells. The selection of transformants was performed at 30° or 37°C using either Luria-Bertani (LB) agar or S.O.B. agar.

Analysis of recombinants. Colonies were analyzed for the presence of the correct insert by growing them overnight in LB broth or Terrific Broth (TB) medium at 30° or 37°C. Plasmid DNA was isolated by an alkali lysis method (5). The oligonucleotide primers used for amplification of insert and PCR conditions were the same as above.

Sequencing. DNA sequencing of the cloned PCR product was carried out by the dideoxy-chain termination method (6) with the Gibco BRL M13/pUC Forward and Reverse Sequencing Primers.

Results and Discussion

The use of LB agar for selection of transformants at 30° or 37°C gave very high (58% blue colonies) vector background. The

TABLE 1. Plasmid stability.

Strain	Temperature	Medium LB[1]	S.O.B.[2]
DH5α	37°C	0% (0/24)	0% (0/44)
DH5α	30°C	0% (0/21)	0% (0/40)
STBL2	30°C	57% (16/28)	–

[1] LB agar was used for selection and LB broth for propagation of plasmid.
[2] S.O.B. agar was used for selection and TB for propagation of plasmid.

white colonies, propagated in LB broth overnight, contained only 0.7 to 0.9-kb inserts (figure 1). Thus, the propagation of plasmid containing a 2,333-bp insert of PVY^O in DH5α cells was unstable. Similar results were obtained using S.O.B. and TB media (table 1). It has been reported that the growth of plasmid at 30°C reduces the probability of deletions in retroviral clones (7) or other unstable sequences (8). In our case, reduction of temperature resulted in selection of transformants with only 0.7–0.9-kb inserts.

STBL2 cells have been used to propagate unstable or retroviral clones (9). These cells were used for the 2,333-bp PVY^O insert. Since these cells are not capable of blue/white selection, we screened 28 colonies after transformation. 57% (table 1) of the colonies yielded the correct insert (figure 1). Partial sequencing from both ends of the clone obtained from STBL2 cells showed that the clone indeed originated from the right position of the PVY^O genome. We do not know whether this insert has any direct repeats or any other abnormality. However, the published sequence of PVY^O does not indicate any repeats (4,10). Use of STBL2 cells allowed the propagation of an otherwise unstable plasmid.

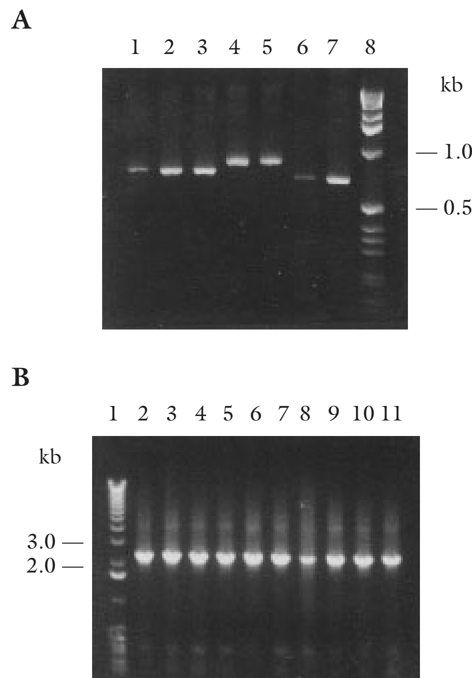

FIGURE 1. Amplification products of a 2,333-bp DNA fragment. DNA was cloned in pAMP1 and propagated in DH5α cells (panel A) or STBL2 cells (panel B).

REFERENCES

1. Riechmann, J.U., Lain, S., and Gracia, J.A. (1992) *J. Gen. Virol. 73*, 1.
2. Singh, R.P. and McDonald, J.G. (1981) *Am. Potato J. 58*, 181.
3. Rashtchian, A., Buchman, G.W., Schuster, D.M., and Berninger, M.S. (1992) *Anal. Biochem. 206*, 91.
4. Robagalia, C., Durand-Tardif, M., Trouchet, M., Bozadin, G., Astier-Manifacier, S., and Casse-Delbert, F. (1989) *J. Gen. Virol. 70*, 935.
5. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Vol. 1, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
6. Sanger, F., Nicklen, S., and Coulson, R. (1977) *Proc. Natl. Acad. Sci. USA 74*, 5463.
7. Joshi, A. and Jeang, K. (1993) *Biotechniques 14*, 883.
8. Kanahan, D., Jessee, J., and Bloom, F. (1991) *Meth. in Enzymol. 204*, 63.
9. Trinh, T., Jessee, J., Bloom, F.R., and Hirsch, V. (1994) *FOCUS 16*, 78.
10. Thole, V., Dalmay, T., Burgyan, J., and Balazs, E. (1993) *Gene 123*, 149.

How can I improve my plasmid yield from STBL2 cells?

Use fresh colonies for inoculation of medium for plasmid production. Colonies picked from agar plates after 4 days result in lower plasmid yields than fresh colonies.

Purification of YAC DNA Using the GlassMAX® System

John H. Riley
Department of Molecular Pathology
Glaxo Research and Development
Greenford, Middx
UB6 OHE

Yeast artificial chromosome (YAC) systems enable cloning of DNA 50 kb to 2,000 kb (see 1 for review). Mapping and size analysis of YACs are usually carried out using pulsed-field gel electrophoresis (PFGE) of DNA isolated from the yeast and embedded in agarose blocks. The agarose block not only contains the YAC but also contains yeast chromosomal DNA. The yeast DNA content of the agarose blocks is a problem for some methods, including: isolation of coding sequences from YACs, which is of major importance in determining candidate genes associated with particular disease loci; direct screening of cDNA libraries using the YAC as a hybridization probe (2); single-pass sequencing of sub-libraries made from the YAC; exon trapping (3–5); and cDNA selection protocols (6). All these methods require large amounts of YAC DNA purified away from the yeast chromosomal DNA.

After removal of the yeast chromosomal DNA by PFGE, the agarose has to be removed. Usually agarase is used to recover the DNA from low melting point agarose. The use of low melting point agarose with concentrated agarose plugs leads to a loss of resolution of the chromosomes, causing contamination of the YAC with yeast sequences. Here we describe a method that isolates large quantities of YAC DNA from PFGE gels in high melting point agarose using a fast, simple spin column.

Methods

YACs were subcultured on agar plates containing synthetic dextrose (SD) medium with casamino acids (7). The YAC was inoculated and grown for 48 h in 200 ml SD medium containing casamino acids. Yeast cells were harvested by centrifugation at 1,800 × *g* at 18°C for 5 min. The cells were washed with 100 ml double distilled water, harvested, and recombined to 50 ml with yeast resuspension buffer (YRB) (7). 1,000 U of lyticase and 2-mercaptoethanol to 14 mM was added. The cell suspension was incubated at 37°C for 1 h, until 95% of the cells were spheroplasted as determined by microscopy, (7). Cells were harvested by centrifugation at 1,800 × *g* at 20°C for 5 min, 2 ml of pre-warmed YRB (37°C) and 2 ml of 1.5% LMP agarose in YRB (45°C) was added to the pellet. The cell suspension was placed in the cooled plug former. Approximately 50 (120 µl) plugs were made. Plugs were removed from the former and placed in 25 ml of yeast lysis buffer YLB (7). After 1 h the YLB was changed and a fresh 25 ml was added and incubated at 45°C for 16 h. A further 25 ml of YLB was added and the plugs were stored in this at 18°C for up to 1 year. Prior to loading on a gel, the plugs were washed in 10 mM Tris-HCl containing 1 mM EDTA (pH 8.0) for 1 h at 18°C.

Separation of YACs from the yeast chromosomes was carried out (8) on a 350-ml 1% Gibco BRL agarose gel in 0.5X TAE. YACs that electrophorese very close to a yeast chromosome can be separated more effectively using a higher-percentage gel (1.5%). Plugs were inserted into the agarose gel and sealed in with 1% LMP agarose.

After separation, the gel was stained in 0.5 µg/ml ethidium bromide for 20 min. (If ethidium bromide inhibits any of the enzymes required for further processing of the YAC, stain the edges of the gel and carefully excise the unstained central portion.) The YAC band was excised and placed in a preweighed 7-ml plastic container, 4.5 volumes of Gibco BRL GlassMAX Binding Solution are added, and incubated at 60°C for 10 min until the agarose was dissolved. 0.5-ml fractions were placed into 2 GlassMAX Spin Cartridges and centrifuged as recommended. This was repeated until all the solution was loaded into the 2 columns. The columns were washed with 3 × 0.5 ml of wash solution and finally with 0.5 ml of 70% ethanol. Following a final centrifugation the column was air dried for 20 min at 18°C. The DNA was eluted from each column using 2 × 50 µl of double-distilled sterile water prewarmed (65°C). To determine the yield of isolated DNA from

the YACs, a small amount of DNA was electrophoresed on an agarose gel and compared to a standard DNA.

Results and Discussion

YACs of varying sizes have been purified from agarose using the GLASSMAX DNA Isolation Spin Cartridge System (table 1). The yield of YAC D was less than the others due to this YAC being unstable and having 3 separate deletion products when grown and analyzed by PFGE. The 820-kb band was the largest YAC of the three and was used to screen a cDNA library. The data presented show the usefulness of the GLASSMAX System for purifying large amounts of YAC DNA.

TABLE 1. DNA yields for several YACs.

YAC	Number of Plugs	Yield of DNA (μg)	Size of YAC (kb)
YAC A	20	1.2	380
YAC B	20	1.1	440
YAC C	20	1.0	700
YAC D	20	0.5	820

Acknowledgements

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References

1. *Techniques for the Analysis of Complex Genomes* (1992) (Anand, R., ed.), Academic, Harcourt Brace Jovanovich.
2. Elvin, P., Glynn, G., Black, D., Graham, A., Butler, R., Riley, J., Anand, R., and Markham, A.F. (1990) *Nucleic Acids Res. 18,* 3912.
3. Buckler, A.J., Chang, D.D., Graw, S.L., Brook, J.D., Haber, D.A., Sharp, D., and Housman, D.E. (1991) *Proc. Natl. Acad. Sci.USA 88,* 4005.
4. Nisson, P.E., Hadley, R.G., and Watkins, P.C. (1993) *FOCUS 15*, 2.
5. Nisson, P.E., Ally, A., and Watkins, P.C. (1994) *PCR Methods and Applications 4*, 524.
6. Lovett, M., Kere, J., and Hinton, L.M. (1991) *Proc. Natl. Acad. Sci. USA 88,* 9628.
7. Anand, R., Riley, J.H., Butler, R., Smith, J.C., and Markham, A.F. (1990) *Nucleic Acids Res. 18,* 1951.
8. Southern, E.M., Anand, R., Brown, W.R.A., and Fletcher, D.S. (1987) *Nucleic Acids Res. 15,* 5925.

Substituting Pyridoxine for Pyridoxal in the Formulation of D-MEM Media

Paul J. Price
Dale Gruber
Cell Culture Research and Development

Shawn R. Smith
Manufacturing
Life Technologies, Inc.
Grand Island,
New York 14072

A precipitate forms and sediments over time in the published D-MEM formulation and other chemically related cell culture formulations (*i.e.* D-MEM:F12) and can be a problem. These studies demonstrate that the precipitate that forms with time in the published formulation is most likely sulfur. The major catalyst for this precipitation was traced to the high concentration of pyridoxal in the medium. Many formulations contain this form of vitamin B6, but in D-MEM the concentration is 4 to 65X higher. The data presented here support the hypothesis that the precipitate is due to a chemical reaction involving cystine, pyridoxal, and ferric nitrate. Growth studies using MRC-5, SP2/0, COS 7, Vero, and HeLa cells demonstrated that D-MEM made with pyridoxine functioned as well as the same medium made with pyridoxal without formation of a precipitate.

Methods

HPLC analysis. In order to identify the role of pyridoxal in the precipitate that forms in D-MEM with time, several lots of D-MEM of different ages in 500-ml glass bottles were analyzed by HPLC. Five of the lots were made with pyridoxal and 3 with pyridoxine. The lots were coded and the code not broken until after analysis. Sulfur testing was by liquid-liquid extraction of D-MEM into n-hexane followed by HPLC analysis on an Alltech Adsorbosphere HS silica column. Sulfur was detected by absorbance of a 40-μl injection at 254 nm.

Identification of the precipitate in D-MEM.

TABLE 1. Growth comparison of D-MEM made with either pyridoxal or pyridoxine.

Cell Lines	MRC-5	SP2/0	Vero	COS 7	HeLa
Experiment 1					
Pyridoxine	48.2 ± 4.4*	26.5 ± 2.8	23.5 ± 1.1	29.0 ± 1.4	24.2 ± 3.4
Pyridoxal	47.5 ± 1.2	26.9 ± 4.0	21.4 ± 1.6	29.7 ± 1.2	23.9 ± 1.0
Experiment 2					
Pyridoxine	42.6 ± 5.0	24.2 ± 2	-	-	-
Pyridoxal	43.0 ± 1.1	23.5 ± 2.5	-	-	-

*Data are the mean population doubling times in hours over 5 subcultures in experiment 1 and 3 subcultures in experiment 2.

Different concentrations of pyridoxal or pyridoxine were incubated at room temperature with different concentrations of L-cystine with or without ferric nitrate at a pH of 7.0 ± 0.2 in either water or PBS. Samples were monitored for appearance of a precipitate.

The flocculent precipitate developing with age in standard D-MEM was separated out of solution by centrifugation and the chemical composition of the pooled samples identified using energy-dispersive X-ray analysis. Charring analysis was also used to help identify the precipitate. The D-MEM was analyzed after removal of the precipitate, by PTC amino acid HPLC.

Growth of cell lines in D-MEM formulated with either pyridoxal or pyridoxine. D-MEM was made from concentrated intermediates without either pyridoxine or pyridoxal. The medium was subdivided and either 4.00 mg/L of pyridoxine or pyridoxal hydrochloride was added. Cultures were monitored for 5 subcultures in the first experiment and 3 subcultures in the second. At each subculture, cultures were plated in duplicate, and the average number of cells/T-25 flask determined at the time of transfer (every 3 or 4 days). MRC-5 and COS-7 cells were plated at each subculture at a concentration of 5×10^5 cells/T-25 flask in D-MEM supplemented with 10% FBS. SP2/0, Vero, and HeLa cultures were plated at 5×10^5 cells/T-25 flask in D-MEM supplemented with 5% FBS. At each subculture, population doubling times were determined using the formula: population doubling time = $0.6931 \times$ number of days between transfer divided by natural log (final count/plating concentration) $\times$ 24.

Results and Discussion

HPLC analysis of D-MEM made with pyridoxal and D-MEM made with pyridoxine showed various concentrations of sulfur in all 5 lots made with pyridoxal and in none of the 3 lots made with pyridoxine. The accelerated shelf-life assay clearly indicated that the formation of sulfur was due to a chemical interaction between vitamin B6 aldehyde (pyridoxal) and the sulfur-containing amino acid L-cystine, with catalysis being provided by iron. There was no precipitate in the absence of cystine or with the substitution of pyridoxine for pyridoxal, and precipitation was significantly slowed in the absence of ferric nitrate.

The flocculent precipitate that developed with age in standard D-MEM was shown by energy-dispersive X-ray analysis to be sulfur. Sulfur was the only element detected. Charring analysis also indicated that the material was most likely elemental sulfur. Isolated residue from both the accelerated assay and manufactured lots was shown by spectrophotometric and HPLC analysis to be identical to results obtained with elemental sulfur. D-MEM analyzed by PTC amino acid HPLC after removal of a heavy precipitate showed a large reduction of cystine (39%) and small reductions in threonine, serine, valine, lysine, and phenylalanine from the "0" time analysis.

Table 1 shows the growth of SP2/0, MRC-5, Vero, HeLa, and COS-7 cells in both media formulations. The growth studies showed that in serum-supplemented media, pyridoxine can be substituted for pyridoxal. Any differences in growth were not statistically significant. As serum contains the enzymes needed to convert pyridoxine to pyridoxal, these results are not surprising. The utilization of the pyridoxine formulation as a base for serum-free conditions will have to be examined on an individual basis.

The substitution of pyridoxine for pyridoxal significantly increased the shelf life of the medium by slowing the formation of precipitate. D-MEM made with pyridoxine and stored refrigerated has shown no precipitation for up to a year. In contrast, some D-MEM made with pyridoxal has shown precipitation as early as 9 months, with the percentage of precipitated lots increasing with time.

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