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# HETEROLOGOUS PROTEIN EXPRESSION IN MAMMALIAN CELLS WITH THE SFV GENE EXPRESSION SYSTEM

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## ABSTRACT

The SFV Gene Expression System is a novel DNA expression system based on the Semliki Forest Virus (SFV) replicon that can produce recombinant protein in eukaryotic cells (1). DNA sequences are cloned directly into the pSFV1 vector from which mRNA is transcribed *in vitro*. The mRNA is transfected into eukaryotic cells or packaged into conditionally infective, recombinant SFV viral particles. Then, recombinant protein is produced by the cells from the mRNA. Since SFV has a broad host range, virtually any eukaryotic cell type can be infected.

Several expression systems have been used to study and overexpress cloned eukaryotic proteins. The baculovirus expression system (3) is limited to insect cells. With the baculovirus system, construction of recombinant virus stocks through homologous recombination can be difficult. Alternatively, the vaccinia virus system is a highly efficient mammalian expression system (4); however, it also requires generating recombinant viruses.

A new eukaryotic expression system has been described that overcomes some of the limitations of other systems. In the Semliki Forest Virus (SFV) system (1), the DNA of interest is cloned directly into an SFV vector that serves as a template for *in vitro* synthesis of recombinant RNA. The RNA is then transfected into cultured animal cells, or packaged into conditionally infective, recombinant SFV viral particles (2). Due to the broad host range of SFV, virtually any eukaryotic cell type can be infected with recombinant viral particles with ensuing heterologous protein expression. This allows expression with the appropriate post-translational modifications. The SFV RNA molecule has a positive polarity and thus functions directly as mRNA. The recombinant RNA encodes its own replicase, resulting therefore in very efficient replication and transcription of capped RNA in the cell cytoplasm. This results in effective inhibition of

host protein synthesis and efficient production of heterologous proteins.

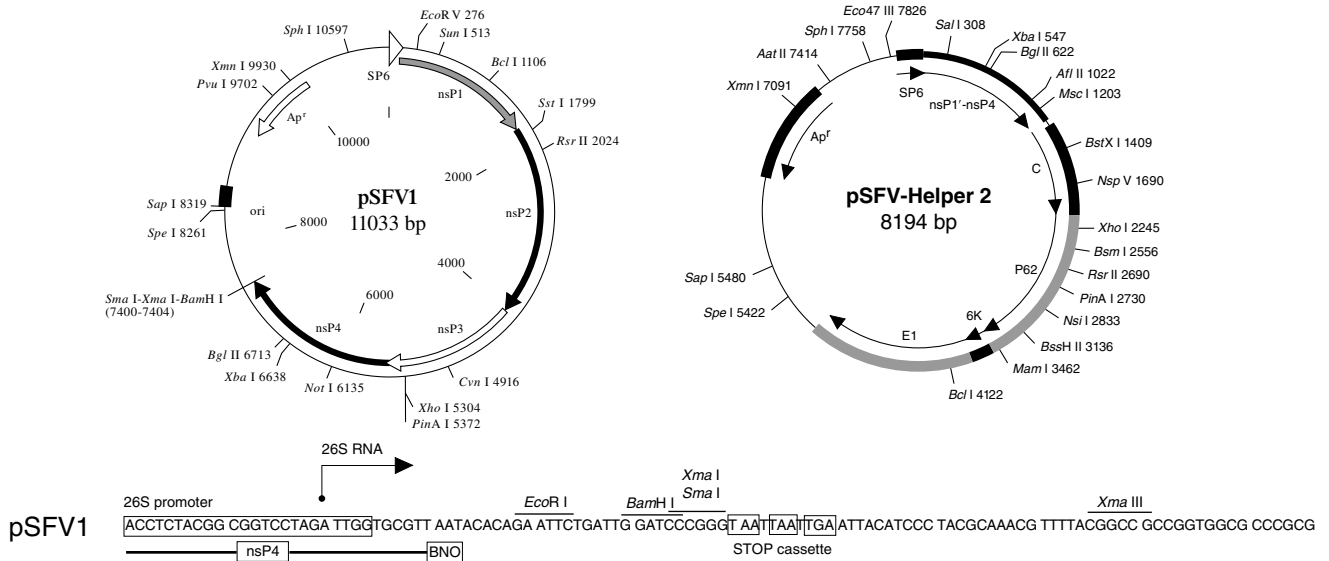
The SFV Gene Expression System has expressed several proteins in mammalian cells, including human transferrin receptor (membrane protein), mouse dihydrofolate reductase (cytoplasmic protein), chick lysozyme (secreted protein), and bovine terminal deoxynucleotidyl transferase (TdT) (nuclear protein), following transfection of SFV recombinant RNA or infection with SFV recombinant viral particles (1, 5). In addition, rab8 and VIP21 proteins were expressed in cultured rat hippocampal neurons (6); HIV-1 glycoproteins were expressed in BHK, HeLa, and Molt-4 CD4<sup>+</sup> T cells (7); rab12, 22, and 24 were expressed in BHK, HeLa, MDCK II, and NmuLi cells (8); and plasminogen-activator inhibitor type 2 was expressed in CHO cells (9) following infection with SFV recombinant viral particles.

The pSFV1 eukaryotic expression vector and its applications were previously described (5). In this report, we focus on the production and use of SFV viral particles for expression of *E. coli*  $\beta$ -galactosidase ( $\beta$ -gal) and bovine TdT. Previously, human TdT cDNA has been expressed in *E. coli* (10) and bovine TdT has been expressed in COS-7 cells, but with very low protein yields (11). Also, we show the importance of optimizing conditions for protein expression with the SFV Gene Expression System.

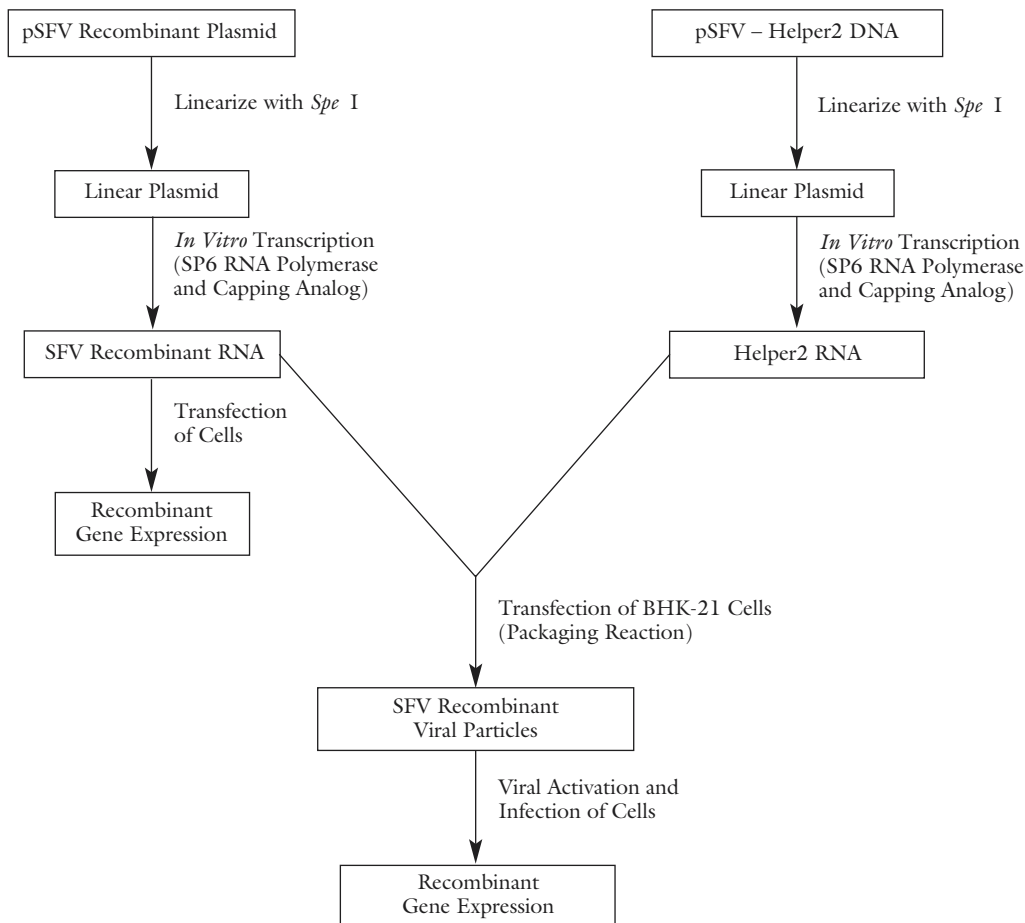
## METHODS

**Plasmid DNA.** The pSFV1 vector (figure 1) is for cloning the gene to be expressed. pSFV3-LacZ contains the *E. coli*  $\beta$ -gal gene and is used to optimize transfection, packaging, and infection conditions. The pSFV1-TdT recombinant plasmid was constructed by cloning the full-length bovine TdT cDNA into the *Sma* I site of pSFV1 in the proper orientation with respect to the viral genes in the vector.

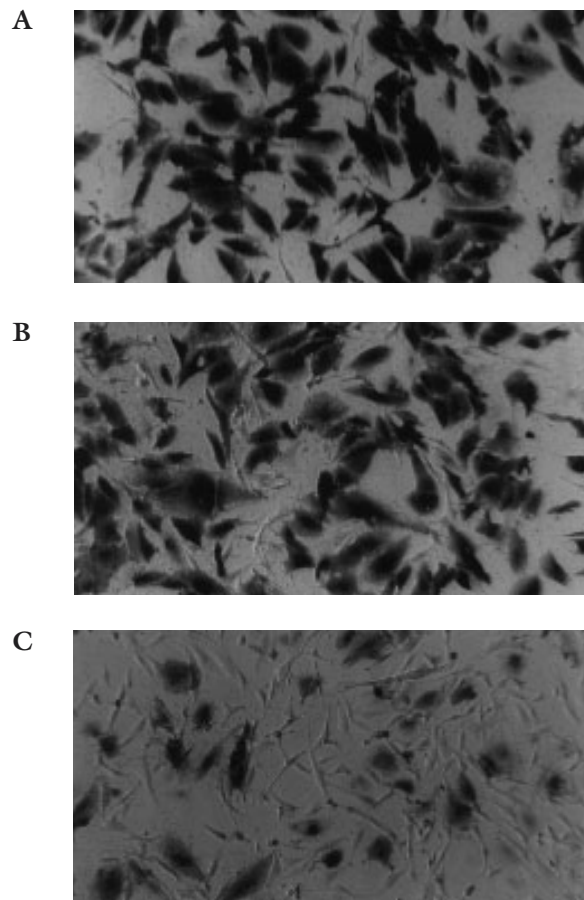
**Cell culture.** All cell culture reagents were from Life Technologies, Inc. BHK-21 cells (ATCC, C13, CCL 10) were cultured in Glasgow Minimum Essential Medium



**FIGURE 1. pSFV1 and pSFV-Helper2 DNA.** The pSFV1 vector was constructed from a full-length clone of SFV with a deletion in the coding region for the viral structural proteins. The multiple cloning site contains a *Bam*H I-*Sma* I-*Xma* I polylinker cassette. The region encoding the viral polyprotein that is cleaved into nonstructural proteins (nsP1-4) is indicated. The position of the promoter for the subgenomic 26S RNA and the first nucleotide transcribed *in vivo* is indicated by an asterisk. The three translation stop codons in all three reading frames following the polylinker are boxed. pSFV-Helper2 is also constructed from a full-length SFV clone with a deletion in the region coding for nonstructural proteins.



**FIGURE 2. Summary of the SFV Gene Expression System protocol.** Once an SFV-recombinant plasmid is constructed, the protein of interest can be expressed by transfection of cells with *in vitro*-transcribed RNA or by infection of cells with packaged SFV recombinant viral particles.



**FIGURE 3. Optimization of infection conditions.**  $2 \times 10^5$  BHK-21 cells were plated in 35-mm wells. The day after plating, cells were infected with 300, 30, or 3 µl of chymotrypsin-activated SFV-lacZ virus, panels A through C, respectively. At 16 h post-infection, cells were stained with X-gal.

(G-MEM) with 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, 10 mM HEPES (pH 7.3), and 2 mM glutamine. CHO-K1 (ATTC, CCL61) cells were cultured in MEM with 2 mM glutamine, 20 µg/ml proline, and 5% FBS. COS 7 (ATTC, CRL 1651) and HeLa (ATTC, CCL2) cells were cultured in D-MEM with 10% FBS. NIH 3T3 (ATTC, CRL 1658) cells were cultured in D-MEM with 10% calf serum. Human fibroblasts were isolated from neonatal foreskin tissue: rinsed, fresh tissue was exposed to 25 units/ml dispase overnight at 4°C, separated into epidermis and dermis, and the minced dermis was digested 10 min at 37°C in 0.25% trypsin, 1 mM EDTA. The reaction was stopped by a rinse and centrifugation in D-MEM with 10% FBS, in which cells were also cultured. All media were supplemented with 100 U/ml penicillin and 100 µg/ml strepto-

mycin. Cell cultures were maintained at 37°C, with 5% CO<sub>2</sub>.

**Production of viral particles.** The protocol is summarized in figure 2. Transcription of the recombinant plasmid and pSFV-Helper2 DNA, and co-transfection of the resulting RNAs with LIPOFECTIN® Reagent to make SFV recombinant viral particles, were performed as described in the SFV system manual. Cell supernatants containing SFV recombinant viral particles were collected 16 to 24 h post-transfection and stored in aliquots at -80°C.

**Infection of cultured cells with SFV recombinant virus.** Cells were infected with SFV-recombinant viral stocks as described in the product manual. Viral stocks were activated with 200 µg/ml chymotrypsin A4 and diluted in Dulbecco's phosphate-buffered saline (D-PBS), containing calcium and magnesium. Cells were infected with SFV recombinant virus at 37°C for 1 to 2 h in D-PBS. After removal of the virus, cells were rinsed briefly and incubated in growth medium for 12 to 72 h post-infection. 100 µg/ml PLURONIC® F68 (Cat. No. 24040) was added to the medium following infection to maximize cell viability. Attached as well as detached cells were collected for analysis of protein expression.

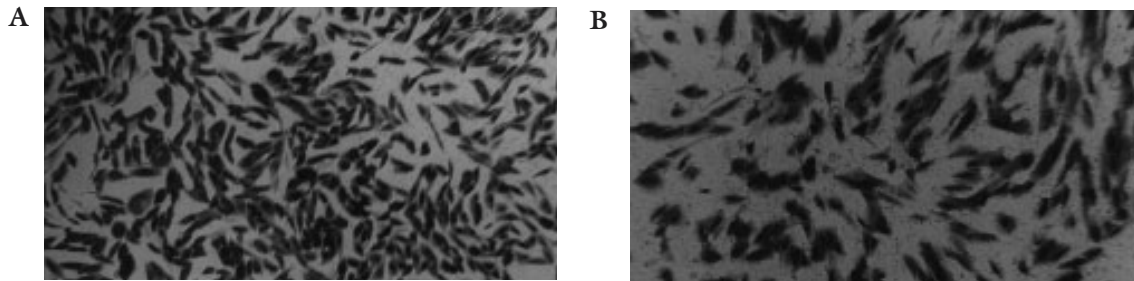
**In situ β-gal staining.** Cells were stained (12) 2 h to overnight with 0.1% GIBCO BRL X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub> in PBS and photographed using a 10X or 20X objective on a Nikon inverted microscope with Hoffman optics.

**Quantitation of β-gal.** To quantitate the amount of β-gal expressed by transfected or infected cells, cells were harvested, extracts were cleared by centrifugation and were assayed (13) using ONPG.

**SDS-PAGE protein analysis.** To extract total soluble protein, cells were lysed directly in 0.1 M Tris-HCl (pH 8.0) with 0.1% TRITON X-100 or 1% NONIDET P40, 2 mM EDTA, 1 µg/ml PMSF. Cell extracts were cleared by centrifugation and analyzed on acrylamide gels (14).

## RESULTS

For optimal protein expression with the SFV Gene Expression System, it is critical to optimize conditions for RNA transfection (both



**FIGURE 4. Expression of  $\beta$ -gal.**  $2 \times 10^5$  cells were plated in 35-mm wells so that they were  $\sim 80\%$  confluent on the following day at the time of transfection. Cells in each well were infected with chymotrypsin-activated SFV-lacZ recombinant viral particles. At 16 to 24 h post-infection, cells were stained. Panel A. CHO-K1. Panel B. Primary, passaged human fibroblasts.

the amount of RNA and LIPOFECTIN Reagent) and viral infection using pSFV3-lacZ. The effect of viral concentration on  $\beta$ -gal expression from SFV recombinant virus in BHK-21 cells is shown in figure 3. The highest percentage of  $\beta$ -gal-positive cells and the highest intensity of staining are seen when higher viral titers are used. The percentage of infected cells was increased from  $\sim 20\%$  to 98% by increasing the amount of viral stock from 3 to 300  $\mu$ l. Less than 1 in  $10^4$  cells express lacZ when the viral stock is not activated with chymotrypsin (data not shown).

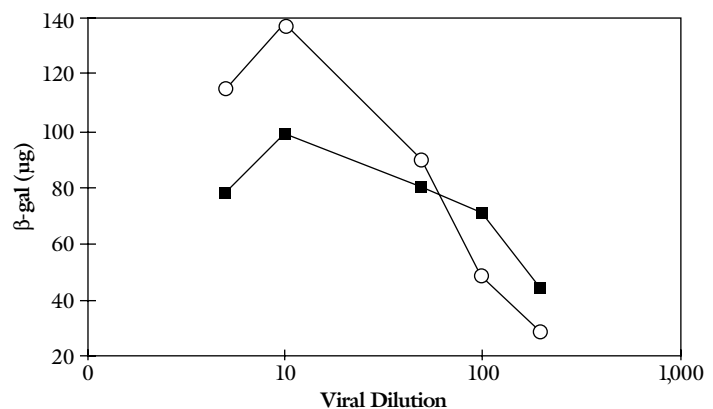
Recombinant SFV can infect a range of cell types. In CHO-K1 and primary, passaged human fibroblasts, approximately 98% of the cells expressed  $\beta$ -gal after infection (figure 4). BHK-21, NIH-3T3, COS-7, and HeLa cells also expressed  $\beta$ -gal at efficiencies of 80% to 98% (data not shown). These results illustrate that the SFV expression system can be used in a wide variety of cell types from different species and lineages, and in primary cell as well as established lines.

The amount of heterologous protein produced in infected BHK-21 cells was examined with several dilutions of SFV-lacZ recombinant virus. At 48 h post-infection,  $\sim 100 \mu$ g (per  $10^6$  cells) of active  $\beta$ -gal was expressed (figure 5). The bell-shaped curve shows that there was an optimal MOI for protein expression and that by optimizing this parameter both high-level protein expression and high specific activity (percent of total soluble cellular protein) were achieved. In this case, a 1:10 dilution of the virus stock (or 100  $\mu$ l of a packaging reaction in 1 ml of D-PBS) was optimal.

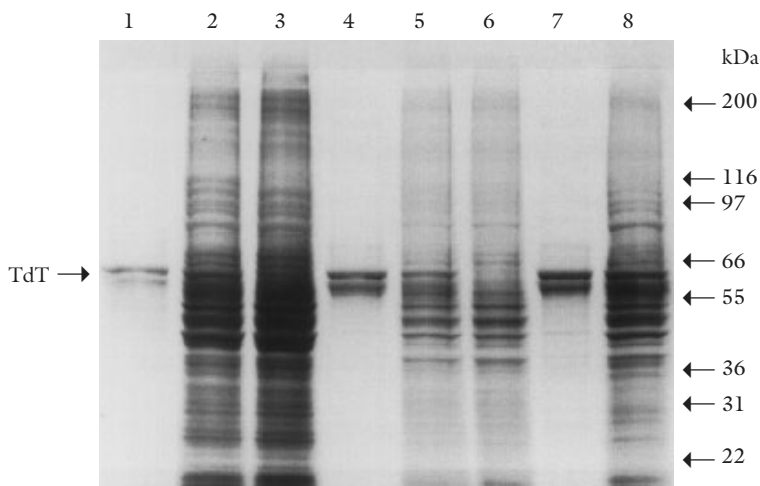
The expression of TdT was analyzed in several cell types following transfection or infection

with SFV-TdT recombinant RNA or virus (figure 6). A significant protein band is seen in transfected or infected cells corresponding to the molecular weight of TdT. The TdT band represents a higher percentage of the total cell protein in infected cells, probably due to the high efficiency of infection and also to viral inhibition of host protein synthesis. Western blots of cell lysates confirmed that the bands were TdT (data not shown). The infected cell extracts were also assayed for TdT enzymatic activity and found to contain active enzyme (data not shown). Optimal TdT expression was obtained when cells were infected with 30 to 300  $\mu$ l of viral stocks. Additionally, expression of TdT was higher at 24 h than at 48 h, emphasizing the importance of optimizing the time post-infection for evaluating gene expression.

In a recent report, it was shown that the rate of protein synthesis in Sindbis virus-infected cells could be increased by the addition

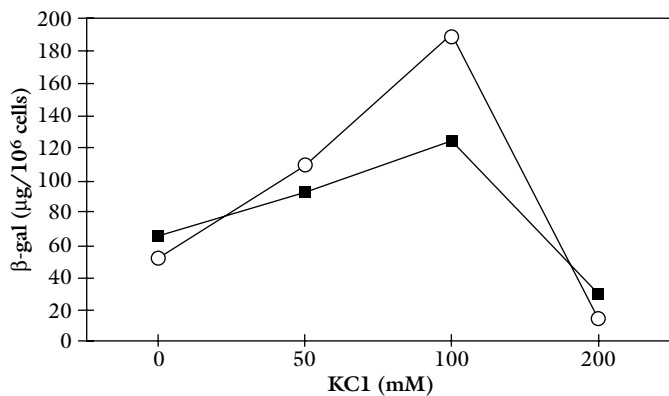


**FIGURE 5. Quantitation of  $\beta$ -gal expression in BHK-21 cells.**  $2 \times 10^5$  cells were plated per 35-mm well of 6-well plates. Cells were infected with chymotrypsin-activated SFV-lacZ recombinant viral particles at 1:5 to 1:200 dilutions of viral stocks. At 48 h post-infection, cell extracts were prepared and assayed for  $\beta$ -gal using ONPG. Total  $\beta$ -gal activity per  $10^6$  cells (■) and  $\mu$ g  $\beta$ -gal/mg of total soluble cell protein (○) are shown.



**FIGURE 6.** Analysis of TdT expression in BHK-21, CHO-K1, and COS-7 cells by SDS-PAGE.  $1-2 \times 10^5$  cells were plated in 35-mm wells of 6-well plates. The day after plating, cells were transfected with  $\sim 2.5 \mu\text{g}$  RNA transcribed from pSFV1-TdT using 10  $\mu\text{l}$  of LIPOECTIN Reagent, or infected with SFV-TdT recombinant virus. At 48 h post-transfection or infection, cell lysates were prepared and analyzed by SDS-PAGE on 14% acrylamide gels. Lanes 1, 4, 7: 10  $\mu\text{l}$  of cell extract from infected BHK-21, CHO-K1, and COS-7 cells, respectively. Lanes 2, 5: 10  $\mu\text{l}$  of cell extract from transfected BHK-21 and CHO-K1 cells, respectively. Lanes 3, 6, 8: 10  $\mu\text{l}$  of cell extract from untransfected BHK-21, CHO-K1, and COS-7 control cells, respectively.

of excess levels of KCl in the growth medium (15). Sindbis virus, like SFV, is a member of the Alphavirus genera. The effect of elevated extracellular KCl concentration on SFV-recombinant protein expression was examined in BHK-21 cells (figure 7). The addition of 100 mM KCl to the medium increased lacZ expression 2- to nearly 4-fold. Protein expression was higher at 48 than at 24 h. 200 mM KCl resulted in significant cytotoxicity,



**FIGURE 7.** Effect of KCl on SFV-lacZ expression.  $2 \times 10^5$  BHK-21 cells were plated in 35-mm wells of 6-well plates. Cells in each well were infected with chymotrypsin-activated SFV-lacZ recombinant viral particles, and 0–200 mM KCl was added to the medium 4 h post-infection. At 24 h (■) or 48 h (○) post-infection, cell extracts were prepared and assayed for  $\beta$ -gal using ONPG.

suggesting that the concentration of KCl and time of addition should be optimized for each cell type and specific protein.

#### EDITOR'S NOTE:

The SFV Expression System is only available in the United States.

#### ACKNOWLEDGEMENTS

We thank Deb Chatterjee for construction of the pSFV-TdT recombinant plasmid, Anna Chytil for plasmid DNA purification, and Pam Hawley-Nelson for the primary human fibroblasts.

#### REFERENCES

- Liljeström, P. and Garoff, H. (1991) *Bio/Technology* 9, 1356.
- Berglund, P., Sjöberg, M., Garoff, H., Atkins, G.J., Sheahan, B.J., and Liljeström, P. (1993) *Bio/Technology* 11, 916.
- Miller, L.K. (1988) *Annu. Rev. Microbiol.* 42, 177.
- Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W.A., and Fuerst, T.R. (1990) *Nature* 348, 91.
- Ciccarone, V., Jessee, J., Berglund, P., and Liljeström, P. (1993) *FOCUS* 15, 103.
- Olkkonen, V.M., Liljeström, P., Garoff, H., Simons, K., and Dotti, C.G. (1993) *J. Neuroscience Res.* 35, 445.
- Paul, N.L., Marsh, M., McKeating, J.A., Schulz, T.F., Liljeström, P., Garoff, H., and Weiss, R.A. (1993) *Aids Res. and Human Retroviruses* 9, 963.
- Olkkonen, V.M., Dupree, P., Killisch, I., Lütcke, Zerial, M., and Simons, K. (1993) *J. Cell Science* 106, 1249.
- Mikus, P., Urano, T., Liljeström, P., and Ny, T. (1993) *Eur. J. Biochem.* 218, 1071.
- Peterson, R.C., Cheung, L.C., Mattaliano, R.J., White, S.T., Chang, L.M.S., and Bollum, F.J. (1985) *J. Biol. Chem.* 260, 10495.
- Koiwai, O., Yokota, T., Kageyama, T., Hirose, T., Yoshida, S., and Arai, K. (1986) *Nucleic Acids Res.* 14, 5777.
- Sanes, J.R., Rubenstein, J.L.R., and Nicolas, J.F. (1986) *EMBO J.* 5, 3133.
- Sambrook, J., Fritsch, E.F., and Maniatis, T., eds. (1989) *Molecular Cloning, A Laboratory Manual*. Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Laemmli, U.K. (1970) *Nature* 227, 680.
- Garry, R.F. (1994) *J. Gen. Virol.* 75, 411.

## THE HYBRITUBE: A NOVEL MEMBRANE TREATMENT APPARATUS

**B**lotting involves the localization of specific target molecules on membranes using labeled ligands. The primary concern is to ensure that the whole membrane surface is evenly exposed to the ligand, minimizing background.

As an alternative to hybridization bags, ovens, or other devices, Life Technologies has introduced the Hybritube Apparatus for Southern, Northern, and Western blotting. This apparatus allows membranes to be evenly exposed to solutions with minimal volumes. Typically, 50  $\mu\text{l}/\text{cm}^2$  of probe solution is used, although as little as 30  $\mu\text{l}/\text{cm}^2$  may be used when three membranes are treated in one Hybritube. Incubations are performed vertically in standard laboratory water baths or incubators. Also, the design provides increased safety when handling radioactivity (about 10-fold lower radioactive activity is detectable at the surface of a Hybritube than a plastic container holding equivalent quantities of  $^{32}\text{P}$ -label). In this report, the Hybritube is used for nucleic acid detection with radiolabeled and biotinylated probes.

### METHODS

*Use of the Hybritube.* The Hybritubes (patent pending) have three components: an outer glass reaction tube, an inner polypropylene mandrel, and a melamine resin cap. The Hybritube 15 (Cat. No. 20116) processes membranes  $\leq 15 \times 15$  cm. The Hybritube 20 (Cat. No. 10117) processes membranes  $\leq 20 \times 25$  cm.

Membranes are pre-wetted, wrapped onto the mandrel, and inserted into the reaction tube containing prehybridization buffer (figure 1). The inserted mandrel displaces buffer, fully immersing the membrane. The membrane is applied to the inner wall of the reaction tube by reverse rotation of the mandrel. This allows easy removal of the mandrel, with the membrane still in place, to allow the addition of hybridization probe, when required. The probe solution is thoroughly distributed over the membrane, which is immersed in a thin annulus of liquid,

by pumping gently with the mandrel. With the mandrel in place, a minimal volume of solution is required to completely saturate the membrane.

Incubations are carried out by standing the Hybritube vertically in a water bath (with up to 7 cm of the Hybritube protruding above the water surface) or incubator at the appropriate temperature. It is not necessary for the assembly to be shaken, as the membrane is completely immersed in buffer, allowing free diffusion of probe. Any bubbles forming during the incubation float to the top of the tube and are not trapped against the membrane.

Membrane washing or stripping can be performed with the mandrel removed. During the washing steps, wash buffer (50 and 60 ml, respectively, for the Hybritube 15 and 20) is added, the cap is replaced, and the membrane is released from the walls by several inversions of the tube and incubated at the appropriate wash temperature. The membrane can easily be extracted from the Hybritube by fine-curved forceps.

*Radioactive detection.* The blots were hybridized in a Hybritube 15 with  $4 \times 10^6$  dpm/ml of probe (5 ng/ml), in 10 to 11 ml of 7% SDS, 1 mM EDTA, 0.5 M sodium phosphate (pH 7.0) after 1 h prehybridization at  $65^\circ\text{C}$  in the same buffer (1). Denatured, sonicated salmon sperm DNA was usually included, but this made little difference to the

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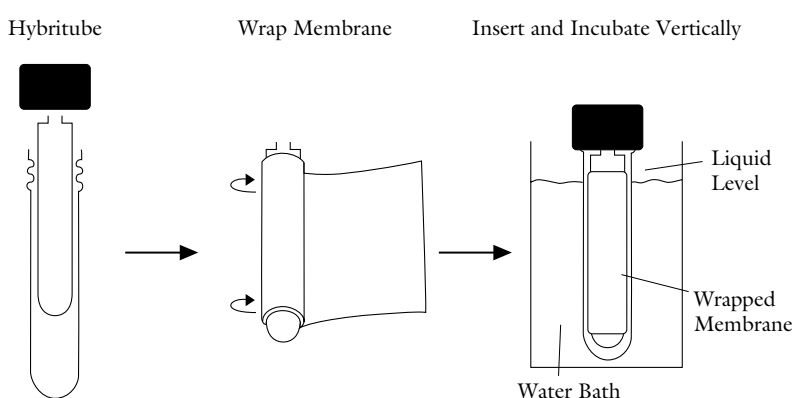
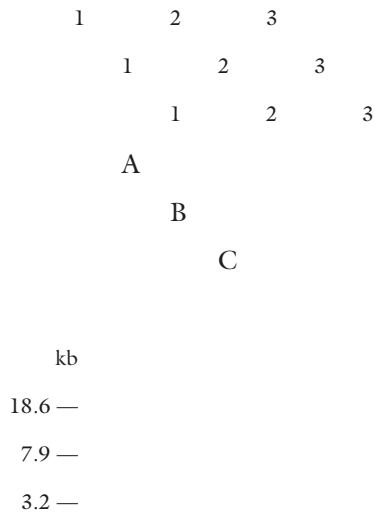
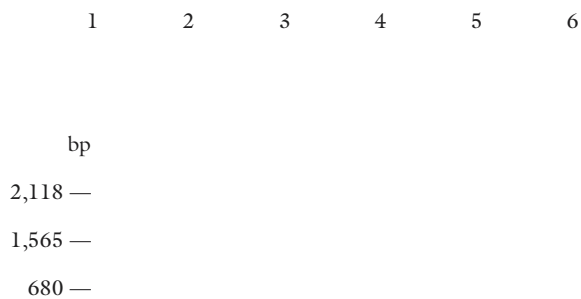


FIGURE 1. Using the Hybritube.



**FIGURE 2. Southern blot of genomic DNA from a Chinese Hamster Ovary cell line transformed with pSV2neo.** Aliquots of 10  $\mu$ g DNA digested with *Bam*H I (lane 1), *Eco*R I (lane 2), and *Hind* III (lane 3) were electrophoresed on 0.7% agarose gels, transferred to nylon membranes, and hybridized sequentially with the neo probe (Panel A), the Krev-1 probe (Panel B), and the GAPDH probe (Panel C). Between each analysis, the probe was removed from the membrane in 0.4 M NaOH at 45°C for 30 min.



**FIGURE 3. Southern blot with nonradioactive detection.** Dilutions of *Rsa* I-digested pBR322 (lanes 1–6: 10 ng, 1 ng, 100 pg, 10 pg, 5 pg, and 1 pg) were electrophoresed on a 1% agarose gel. After transfer to a 15  $\times$  15 cm PHOTOGENE™ membrane, the blot was hybridized and developed in the Hybritube. The membrane was removed, incubated with detection solution for 3 h, and exposed to Kodak XAR™ film for 15 min.

background, which was low without blocking DNA. Probes were either the combined three small *Pvu* II fragments from pSV2neo (2), the 2-kb *Bam*H I insert of Krev-1 (3), or the 1.3-kb cDNA of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4).

After overnight hybridization at 65°C, the membranes were washed 3 times in the Hybritube 15 without the mandrel with 50 ml 2X SSC, 0.1% SDS at room temperature for a total of 30 min; washed once in 2X SSC, 0.1% SDS for 30 min at 65°C; and finally washed in 0.1X SSC, 0.1% SDS for 15 min at 65°C. The membrane was sealed in plastic wrap and exposed to Kodak X-omat S™ film with intensifier screen at –70°C.

Replicas of  $\lambda$  plaques in 132-mm plates were prepared by blotting onto nylon membranes followed by denaturation, neutralization, and fixation of the DNA to the membranes by baking and UV irradiation.

**Nonradioactive detection.** Southern blots were hybridized in the Hybritube 15 with pBR322 labeled with biotin-14-dATP by the GIBCO BRL BIONICK™ Labeling System. Membranes were washed twice in the Hybritube with 50 ml of 5X SSC, 0.5% SDS at 50°C for 30 min; once with 0.1X SSC, 1% SDS at 50°C for 30 min; and finally with 2X SSC for 5 min at room temperature. The PHOTOGENE™ System was used to detect the biotinylated probe.

## RESULTS AND DISCUSSION

Using the Hybritube, Southern blots with consistently low levels of background were obtained. A Southern blot was reprobbed sequentially with three <sup>32</sup>P-labeled probes (figure 2). The neo and Kev-1 probes are single-copy genes, whereas the GAPDH gene is present in multiple copies in mammalian cells (5). Detection sensitivity was equivalent to that obtained with other hybridization devices (data not shown), allowing routine detection of single-copy genes in Southern blotting.

Nonradioactive detection with the Hybritube restricted the need to handle the membrane between hybridization, washing, and signal development steps and resulted in good signal detection with low levels of background (figure 3).

Multiple membranes may be treated simul-

A  
B  
C

**FIGURE 4. Plaque lifts of  $\lambda$  phage on multiple membranes.**  $\lambda$  phage containing a 16-kb insert (Panels A and C) or random inserts from a genomic library (Panel B) were used to infect bacteria and plated at appropriate dilutions. Plaque lifts were prepared on nylon membranes and the three 132-mm-diameter membranes were wound consecutively around the mandrel in the order A, B, C. The combined length would be equivalent to a single membrane 396 mm long. The membranes were hybridized in 12 ml with the  $^{32}\text{P}$ -labeled 16-kb insert.

taneously in a single Hybritube (figure 4). The middle membrane (figure 4B) was produced from a dish containing 27 plaques of  $\lambda$  phage not hybridizing with the probe. There was no cross-contamination between the individual membranes and the background was very low; neither the outlines of the membranes nor the negative plaques were detectable. This procedure has been used to screen a whole genomic library, processing 240,000 plaques at a time (6 membranes in 2 Hybritubes). The low background obtained allows easy recognition of positive signals.

The Hybritube allows the use of small reaction volumes to produce good signal sensitivity with low levels of background, even for multiple membrane processing and nonradioactive

detection. In addition, user safety is improved when compared to traditional techniques, while vertical incubations eliminate the possibility of tube leakage.

#### REFERENCES

1. Church, G.M. and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* 81, 1991.
2. Southern, P.J. and Berg, P. (1982) *J. Mol. Appl. Genetics* 1, 327.
3. Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., and Noda, M. (1989) *Cell* 56, 77.
4. Fort, P., Marty, L., Piechaczyk, M., El Sabrouly, S., Dani, C., Jeanteur, P., and Blanchard, J.M. (1985) *Nucleic Acids Res.* 13, 1431.
5. Piechaczyk, M., Blanchard, J.M., Riaad-El Sabrouly, S., Dani, C., Marty, L., and Jeanteur, P. (1984) *Nature* 312, 469.



#### What are the advantages of using formamide in the hybridization solution?

Formamide decreases the  $T_m$  of nucleic acid hybrids and the incubation temperature can be reduced to 42°C. This has several practical advantages: the probe is more stable at lower temperatures, there is better retention of noncovalently bound nucleic acids on the membrane, and nitrocellulose membranes are less likely to degrade at lower temperatures. Formamide also can be used to alter the stringency of the reaction conditions.

# INHIBITION OF SUPERSCRIPT™ II REVERSE TRANSCRIPTASE BY COMMON LABORATORY CHEMICALS

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Investigators frequently are concerned about the effect of various common laboratory reagents on enzymes used to modify nucleic acids. In the case of reverse transcriptase (RT), there is interest in knowing the effect of (a) reagents such as spermidine-(HCl)<sub>3</sub> and sodium pyrophosphate that have been used to stimulate RT (1); (b) chemicals such as formamide, EDTA, glycogen, or SDS that are used in the manipulation of nucleic acids and that might be carried over into RT reaction mixtures; (c) glycerol that is carried over into reaction mixtures from RT storage buffer; and (d) reagents such as DMSO or chaotropic salts that disrupt the structure of proteins. I report here on an investigation of the effect of some of these chemicals on SUPERSCRIPT II RT.

## METHODS

*cDNA synthesis from mRNA.* cDNA synthesis reaction mixtures (20 µl) contained 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM MgCl<sub>2</sub>; 10 mM dithiothreitol; 500 µM each of dTTP, dATP, dCTP, and [<sup>3</sup>H]dGTP (175 cpm/pmole); 25 µg/ml oligo(dT)<sub>12-18</sub>; 100 µg/ml GIBCO BRL 7.5 Kb Poly(A)-Tailed RNA (Cat. No. 15621); and 200 units of GIBCO BRL SUPERSCRIPT II RT (Cat. No. 18064). After incubation at 37°C for 10 min, total incorporation into acid-insoluble cDNA was determined.

## RESULTS AND DISCUSSION

Figure 1 shows the effect of a number of chemicals on the RNA-directed DNA poly-

merase activity of SUPERSCRIPT II RT. Concentrations of spermidine-(HCl)<sub>3</sub> (0.5 mM) and sodium pyrophosphate (4 mM) that stimulate AMV RT (1) inhibited SUPERSCRIPT II RT 70%. The use of either of these additives with SUPERSCRIPT II RT is therefore not recommended. EDTA at 2.5 mM inhibited >90% in reactions containing 3 mM MgCl<sub>2</sub>.

Substantial amounts of glycerol were tolerated by SUPERSCRIPT II RT (34%, v/v, inhibits 50%); however, the enzyme was much more sensitive to DMSO and formamide, being inhibited 50% at 17%, v/v, and 5%, v/v, respectively.

As might be expected, SUPERSCRIPT II RT was inhibited >90% by low concentrations of denaturing agents: 0.005%, w/v, SDS; 70 mM guanidine isothiocyanate; and 160 mM guanidine hydrochloride. The anionic polysaccharide, heparin, also inhibited at low concentrations (>90% at 30 µg/ml). In contrast, the uncharged polysaccharide, glycogen, had little effect on SUPERSCRIPT II RT until concentrations of >2 mg/ml were reached. Therefore, glycogen can be used as a carrier in RNA precipitations prior to copying RNA with SUPERSCRIPT II RT.

## REFERENCE

1. Krug, M.S. and Berger, S.L. (1987) *Methods in Enzymology* 152, 316.

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### What is the optimal temperature of incubation of SUPERSCRIPT II RT and AMV RT?

For AMV RT, incubation is optimal at 42°C. Both SUPERSCRIPT RT and SUPERSCRIPT II RT produce the best yield of full-length cDNA when the synthesis is performed at 42°C to 45°C, and good yields are obtained at 50°C.

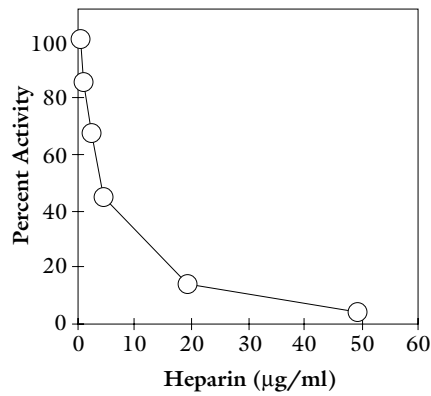
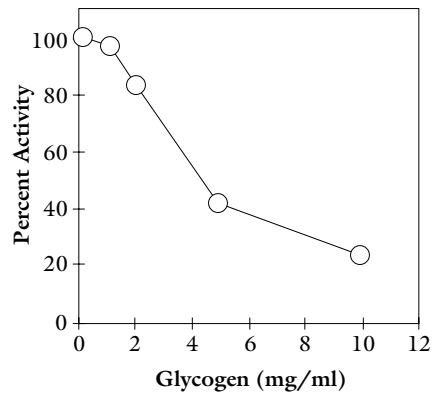
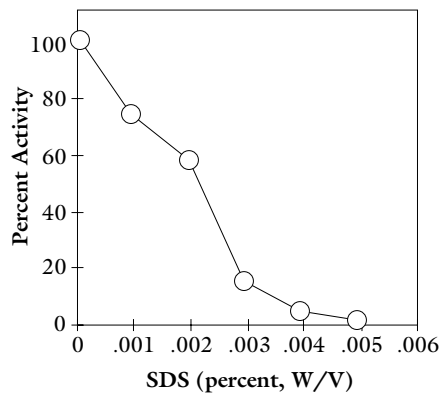
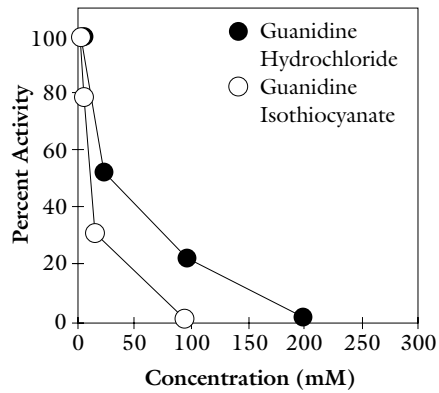
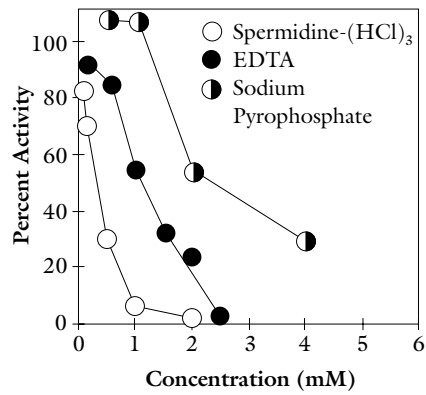
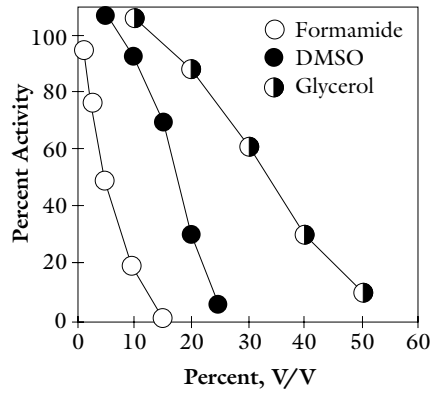


FIGURE 1. Effect of various chemicals on the activity of SUPERSCRIPT II RT.

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# URACIL DNA GLYCOSYLASE: MINIMIZING RESIDUAL ENZYME ACTIVITY AFTER NESTED PCR

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Carryover from previously amplified Polymerase Chain Reaction (PCR) products is often a source of contamination in subsequent PCR. Several preventive measures have been suggested to avoid this complication (1), including substituting dUTP for dTTP in the reaction to generate dU-containing PCR products (2). Single- and double-stranded DNA that contain uracil are substrates for Uracil DNA Glycosylase (UDG), which cleaves the N-glycosidic bond between the uracil base and the phosphodiester backbone of DNA. The resulting apyrimidinic DNA molecule becomes susceptible to hydrolysis at high temperatures (3). To avoid degradation of the intended PCR products, reactions are maintained at a soak temperature of 72°C to inactivate the UDG enzyme (4).

Nested PCR employs the use of "inner" or "nested" primers internal to an "outer" set of primers used in a previous PCR. Products from the first amplification serve as the template for the subsequent amplification (5). The technique is often utilized to increase both the sensitivity and the specificity of the assay. Such gains are complicated by the potential for contamination inherent in the method, which requires the transfer of an exponentially amplified target into a separate reaction.

The use of dUTP and UDG in nested PCR can prevent amplification of contaminants from the outer reaction, and it prompted us to examine if, after UDG dilution and a second PCR, there was detectable residual UDG activity sufficient enough to affect the product yield from the nested reaction.

## METHODS

Quantities of 5, 50, and 500 ng of total RNA were obtained from liver tissue (positive control), reverse transcribed for the positive strand in the 5' nontranslated region of the Hepatitis C virus, and amplified by PCR as previously described (6). RNA was heat denatured

at 70°C and reverse transcribed in a 10- $\mu$ l solution containing 2  $\mu$ l of 5X RT buffer [0.25 M Tris-HCl (pH 8.3), 0.375 M KCl, 15 mM MgCl<sub>2</sub>], 1  $\mu$ l of 10 mM dNTPs, 1  $\mu$ l of 0.1 M dithiothreitol, 1  $\mu$ l of GIBCO BRL M-MLV Reverse Transcriptase (200 U/ $\mu$ l), and 1  $\mu$ l of a 20- $\mu$ M specific primer. Actinomycin D (0.5  $\mu$ g) was included in samples used for sensitivity studies. Incubation was at 37°C for 1 h.

Calculated volumes of sterile, deionized water were added to reactions, while the buffer components for the master mix were placed in a separate microcentrifuge tube. cDNA used for the PCR was added to the final master mix containing a final concentration of 200  $\mu$ M of each dNTP, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), along with 0.4  $\mu$ l of *Taq* DNA polymerase (5 U/ $\mu$ l), 1  $\mu$ l of GIBCO BRL UDG (1 U/ $\mu$ l), and 0.2 pmoles/ $\mu$ l of primers chosen from the 5' region (sense primer, CAC TCC CCT GTG AGG AAC TAC TGT CT; anti-sense primer, TAC CAC AAG GCC TTT CGC GAC CCA ACA CTA CTC) for a total reaction volume of 100  $\mu$ l. The mixture was incubated at 37°C for 10 min and at 94°C for 10 min, and then was amplified for 30 cycles at 94°C for 45 s, 60°C for 90 s, and 72°C for 90 s with a final extension time of 6 min and a soak temperature of 72°C. Three 10- $\mu$ l aliquots of the products from the outer PCR were used as templates for the nested reaction using the identical master mix from the outer PCR without UDG. Nested primers used were GCA GAA AGC GTC TAG CCA TGG CGT TAG TAT (sense, nt 68–98), and CAG GCA TTG AGC GGG TTG ATC CAA GAA AGG (anti-sense, nt 192–222) (7).

Products from the second amplification were maintained at a soak temperature of either 4°C, 25°C, or 72°C for 24 h, visualized on a 2% agarose/ethidium bromide gel, and transferred to a nylon membrane for Southern blotting (8).

Hybridization was performed at 65°C for

18 h using a  $^{32}\text{P}$ -labeled probe (GTG AGT ACA CCG GAA TTG CCA GGA CGA CCG, nt 159–189) (9) with a specific activity of  $2.0 \times 10^7$  cpm/ $\mu\text{g}$  probe. The blots were exposed for 2 h. Band intensity was quantitated using a densitometer.

## RESULTS

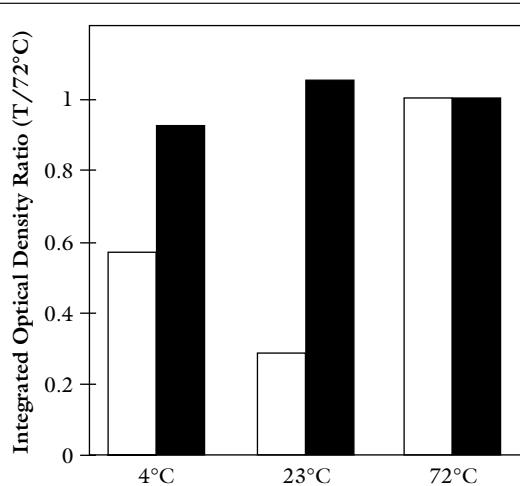
The appropriate 154-bp nested PCR product was visualized on agarose gels in all reactions using UDG and containing the RNA template (data not shown). No bands were detected in negative controls and in both single and nested PCR blanks. The signal intensities appeared similar, independent of the initial template concentration used (5–500 ng).

With Southern hybridization, product integrity was maintained best at an inactivating temperature (72°C) for UDG, while enzymatic degradation was present at 23°C (figure 1). Greater enzyme activity could be predicted had the soak temperature been at UDG's optimum temperature of 37°C. At a lower temperature (4°C) near the limits of most thermal cyclers, partial enzyme activity is still evident. Samples without UDG demonstrated no significant decrease in intensities after Southern hybridization.

## DISCUSSION

Previously, residual UDG activity after PCR was shown at an incubation or soak file temperature of 4°C or 25°C (4). This activity was neutralized by maintaining temperatures at 72°C after cycling. We have found that even after a 5-fold dilution of the enzyme, as well as repeated exposure to inactivating temperatures (during a second round of PCR), residual temperature-dependent UDG activity can still be detected with 4°C and 23°C soak files after nested PCR.

Longer radiographic exposure of a Southern blot will diminish the differences seen in signal strength. Although nested PCR is designed to overcome low product yields from nonnested amplification, less than optimal conditions may diminish yields of products adversely affected by residual UDG activity, resulting in borderline positives or false negatives. UDG activity may also compromise visualization of PCR products subjected to restriction endonuclease analysis during digestion with subsequent



**FIGURE 1. Quantitation of nested PCR products.** Densitometry profiles are expressed as a ratio of integrated optical densities for the given temperature over values obtained at 72°C. Template was 50 ng of total RNA. Reactions with (□) or without (■) UDG treatment.

exposure to increased temperatures for detection-probe hybridization.

Uracil DNA Glycosylase has been successfully used in diagnostic PCR assays (8,10,11). Our results demonstrate residual UDG activity after nested PCR. Thus, the use of UDG in a nested PCR should include a soak file of 72°C on both the outer and nested cycles to avoid partial degradation of intended PCR products.

## REFERENCES

1. Kwok, S. and Higuchi, R. (1989) *Nature* 339, 237.
2. Longo, M., Berninger, M., and Hartley, J., (1990) *Gene* 93, 125.
3. Lindahl, T., Ljunquist, S., Siebert, W., Nyberg, B., and Sperens, B. (1977) *J. Bio. Chem.* 252 (10), 3286.
4. Thornton, C., Hartley, J., and Rashtchian, A. (1992) *Biotechniques* 13 (2), 180.
5. Garson, J.A., Tedder, R.S., Briggs, M., Tuke, P., Glazebrook, J.A., Trute, A., Parker, D., Barbara, J.A.J., Contreras, M., and Aloysius, S. (1990) *Lancet* 335, 1.
6. Mateo, R., Faruki, H., Cooper, D., and Ehrlich, G. (1993) in *PCR-Based Clinical Diagnostics: A Laboratory Approach*. (Ehrlich, G.D. and Greenburg, S.J., eds.), Blackwell Scientific Publishing, Inc., Cambridge.
7. Choo, Q.L., Rickman, K.H., Han, J.H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P.J., Weiner, A.J., Bradley, D.W., Kuo, G., and Houghton, M. (1991) *Proc. Natl. Acad. Sci. USA* 88, 2451.

8. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, New York.
9. Mateo, R., Demetris, A., Sico, E., Frye, C., Wang, L.F., El-Sakhawi, Y., Reilly, M., Ehrlich, G.D., Cooper, D., and Fung, J.J. (1993) *Surgery* 114 (2), 442.
10. Pang, J., Modlin, J., and Yolken, R. (1992) *Molecular and Cellular Probes* 6(3), 251.
11. Wang, X., Chen, T., Kim, D., and Piomelli, S. (1992) *Am. J. of Hematology* 40(2), 146.



**Can dU-containing DNA be cleaved by restriction endonucleases?**

Recognition of the cleavage sites in dU-containing DNA by restriction endonucleases may be dependent on flanking target sequences as well as the restriction sites themselves. Each DNA and enzyme should be examined on an individual basis. [See Bebee, R.L., Thornton, C.G., Hartley, J.L., and Rashtchian, A. (1992) *Focus* 14, 53.]

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## RESIDUAL IS10 ELEMENTS DO NOT EXHIBIT TRANSPOSITIONAL MUTAGENESIS IN *ESCHERICHIA COLI RECA* MUTANTS: STUDIES WITH STRAINS DH5 $\alpha$ <sup>TM</sup> AND DH10B<sup>TM</sup>

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**T**he transposon Tn10 consists of a tetracycline resistance element flanked by two IS10 elements designated IS10-Left and IS10-Right (1). IS10-Right is a mobile genetic element itself, as it encodes a functional transposase protein. Alteration of chromosomal loci by Tn10-mediated intramolecular transposition followed by the use of fusaric acid selection for loss of tetracycline resistance (2,3) is a widely used technique in the construction of *Escherichia coli* strains (4,5). This technique leaves at least one IS10 element behind in the chromosome (6). We have recently shown that residual IS10 elements in *E. coli* are capable of transpositional mutagenesis (7). The technique of Tn10-mediated intramolecular transposition was utilized in the construction of DH5 $\alpha$  and DH10B strains. The existence of residual IS10 elements in these

strains and their potential to mediate genome rearrangements are of concern to researchers employing these bacterial strains. The results presented here show that transpositional mutagenesis is not a problem for DH5 $\alpha$  and DH10B cells.

### METHODS

The presence of residual IS10 elements in chromosomal DNA samples was determined by hybridization analysis, using nonisotopic detection of nylon-membrane-bound chromosomal DNA. A 1,338-bp *Cel* II fragment containing an entire IS10 element was isolated from plasmid pXT107 (7) and labeled by nick translation with biotinylated nucleotides (8). Target chromosomal DNA was isolated (9) from *E. coli* strains DH5 $\alpha$  and DH10B and transferred to a nylon membrane. The biotin-labeled IS10

probe was hybridized to the membrane-bound DNA and detected with a streptavidin and alkaline phosphatase system (10).

The strains to be tested for transpositional mutagenesis were transformed with pXT28 and plated on Vogel-Bonner media (11) containing 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 10 µg/ml indole acrylic acid (IAA), and 100 µg/ml ampicillin. Plasmid DNA was isolated from IAA-resistant, Lac<sup>+</sup> (blue) mutants and screened for IS10 insertion mutations by restriction analysis with *Cel* II as described (7).

## RESULTS

The only *E. coli* strains commercially available from Life Technologies that were subjected to Tn10-mediated intramolecular transposition during the course of their construction are DH5α and DH10B (personal communication from Fred Bloom, Life Technologies). Hybridization experiments showed positive signals for both DH5α and DH10B cells and thus these cells contain residual IS10 elements on their chromosomal DNA (data not shown).

DH5α, DH10B, DH5α-rec<sup>+</sup>, and DH10B-rec<sup>+</sup> were transformed with pXT28, which encodes a BST-β-galactosidase fusion protein under control of the *trp* promoter (7). When induced with IAA, these strains form inclusion bodies containing the BST-β-galactosidase fusion protein. Under these conditions, the consequent inhibition of cell division (12) renders the strains sensitive to IAA.

All strains tested were Lac<sup>-</sup>, making the plasmid pXT28 their only source of β-galactosidase. X-gal was included in the media to enable discrimination of IAA-resistant colonies that arose from deletion of part of the BST::lacZ fusion gene (white) from those that were still producing β-galactosidase (blue).

The portion of pXT28 that encodes the BST portion of the fusion protein contains a known insertion site for IS10. In the event of IS10 insertion into the plasmid at this site, the strains become IAA resistant (7). By plating on minimal media containing IAA and X-gal, and screening for Lac<sup>+</sup>, IAA-resistant colonies, potential transpositional mutants can be isolated.

A large-scale selection for IS10 insertion mutants was made with each strain. In the case

of both *E. coli* strains DH5α and DH10B, no transposition IS10 from chromosome to plasmid could be detected with the pXT28 selection scheme (frequency of transposition <1 × 10<sup>-10</sup>).

Since both DH5α and DH10B are *recA* mutants, the possibility that a functional *recA* gene is required for transpositional mutagenesis by residual IS10 elements was examined. Rec<sup>+</sup> versions of strains DH5α and DH10B, designated DH5α-rec<sup>+</sup> and DH10B-rec<sup>+</sup>, were obtained, transformed with pXT28, and subjected to the selection scheme for IAA resistance described above. For both strains DH5α-rec<sup>+</sup> and DH10B-rec<sup>+</sup>, transposition of IS10 elements to the plasmid pXT28 was readily detected at a frequency of about 2 × 10<sup>-7</sup>.

## DISCUSSION

*E. coli* strains, constructed by schemes in which chromosomal loci were altered by Tn10-mediated intramolecular transposition and fusaric acid selection, contain residual IS10 elements that are capable of causing genome rearrangements including transposition of IS10 elements to plasmids (7). We evaluated the two GIBCO BRL *E. coli* strains that had been constructed in this manner. The residual IS10 elements found in *E. coli* strains DH5α and DH10B were not capable of transpositional mutagenesis (as measured by the pXT28 selection technique) unless the strains were rendered rec<sup>+</sup>. The strains are sold as *recA* mutants, and they exhibited no detectable instability from their residual IS10 elements.

In more recent studies, we have observed that residual IS10 elements are the cause of very high frequency spontaneous mutations in chromosomal loci, usually representing essentially 100% of the mutations obtained (O'Neil and Bogosian, manuscript in preparation). This fact, along with our earlier observations and those described in this paper, should be kept in mind by those using rec<sup>+</sup> *E. coli* strains in which Tn10 had been employed during their construction.

## REFERENCES

1. Kleckner, N. (1989) in *Mobile DNA* (Berg, D.E. and Howe, M.M., eds.), American Society for Microbiology, Washington, D.C., p. 227.
2. Bochner, B.R., Huang, H.-C., Schieven, G.L., and Ames, G.N. (1980) *J. Bacteriol.* 143, 926.

3. Maloy, S.R. and Nunn, W.D. (1981) *J. Bacteriol.* 145, 1110.
4. Berg, C.M. and Berg, D.E. (1987) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., and Umberger, H.E., eds.), American Society for Microbiology, Washington, D.C., p. 1071.
5. Berg, C.M., Berg, D.E., and Groisman, E.A. (1989) in *Mobile DNA* (Berg, D.E. and Howe, M.M., eds.), American Society for Microbiology, Washington, D.C., p. 879.
6. Kleckner, N. (1983) in *Mobile Genetic Elements* (Shapiro, J.A., ed.), Academic Press, New York, p. 261.
7. Bogosian, G., Bilyeu, K., and O'Neil, J.P. (1993) *Gene* 133, 17.
8. Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977) *J. Mol. Biol.* 113, 237.
9. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) (1990), *Current Protocols in Molecular Biology*, Wiley Interscience, New York, p. 2.2.1.
10. Leary, J.J., Brigati, D.J., and Ward, D.C. (1983) *Proc. Natl. Acad. Sci. USA* 80, 4045.
11. Vogel, H.J. and Bonner, D.M. (1965) *J. Biol. Chem.* 218, 97.
12. Kane, J.F., Balaban, S.M., and Bogosian, G. (1990) in *Surface Reactive Peptides and Polymers: Discovery and Commercialization* (Sikes, C.S. and Wheeler, A.P., eds.), American Chemical Society Books, Washington, D.C., p. 186.

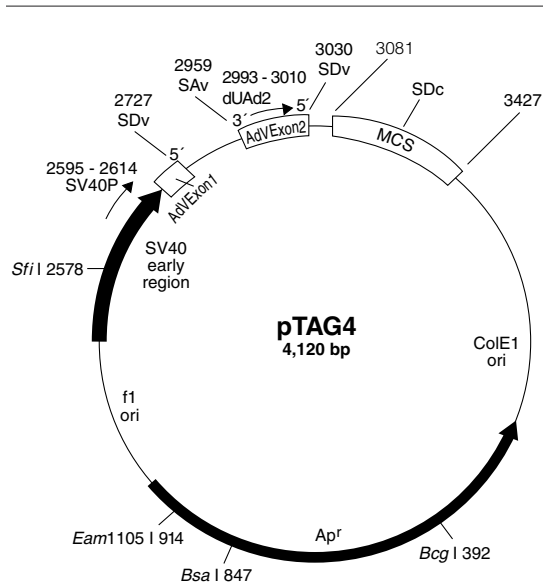
## TRAPPING 3'-TERMINAL EXONS FROM YACs USING THE 3'-EXON TRAPPING SYSTEM

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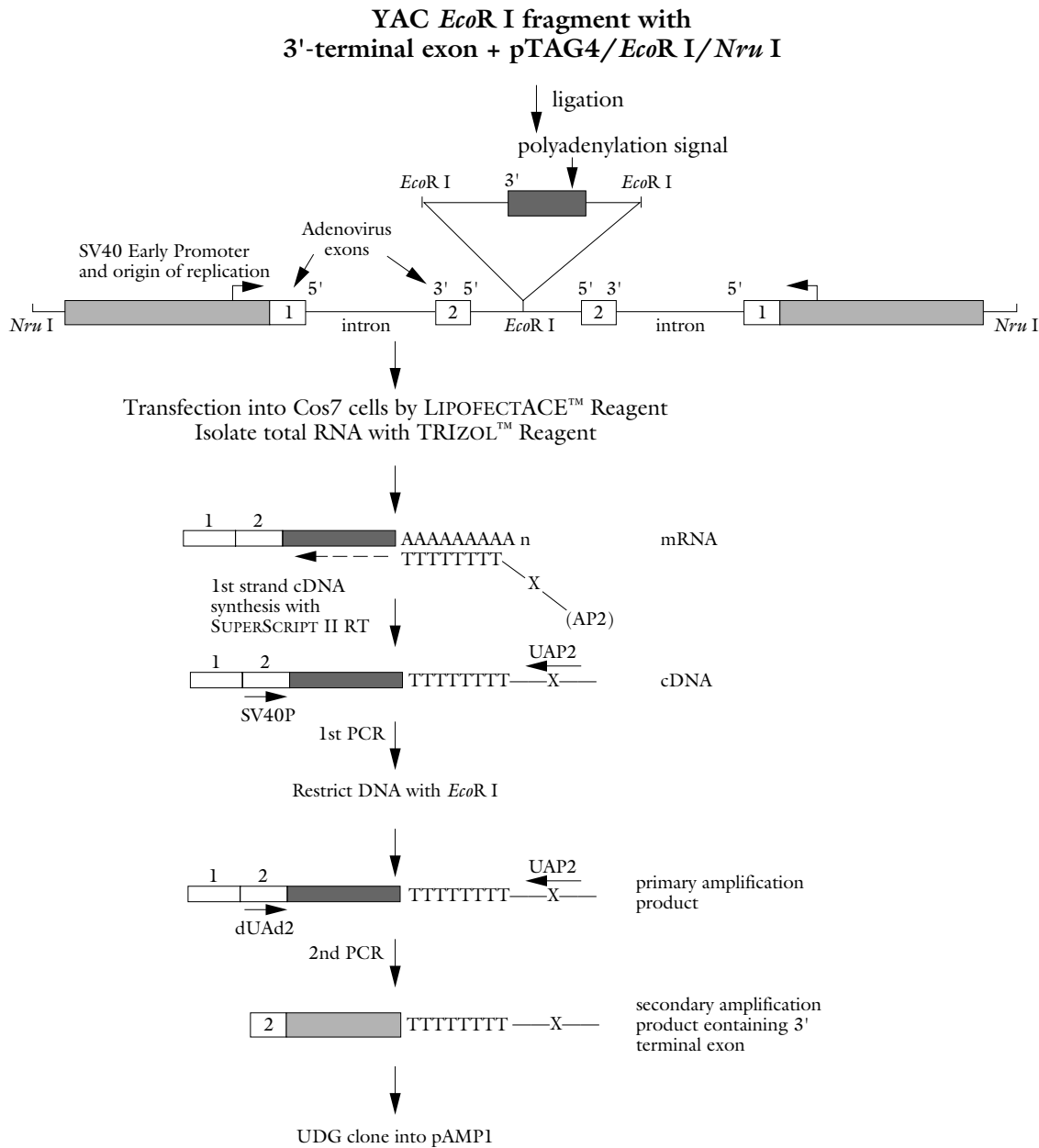
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The initial step of positional cloning traces a trait or phenotype representing a genetic locus and localizes it to a subchromosomal region (1). Genetic mapping is followed by the reconstruction of the surrounding region as overlapping clones (also known as a contig) of genomic DNA cloned in cosmids, yeast artificial chromosomes (YACs), or, more recently, PIs or PACs.

Once the region of interest is contiged, the clones are screened for expressed sequences or exons using either hybridization-based approaches such as direct cDNA selection or exon trapping (2,3). Direct cDNA selection requires knowing which cDNA source expresses the gene of interest (2). Exon trapping can be done directly on genomic DNA subcloned into one of several vectors available to target internal or 3'-terminal exons without knowing where or when a particular exon is expressed (3). After an exon is trapped, the full-length cDNA sequence is obtained by library screening and sequencing of candidate cDNAs. The detection of a mutation in affected individuals is evidence that a



**FIGURE 1. Map of pTAG4.** The intron and exon segments and the multiple cloning site (MCS) are shown along with their map coordinates. The MCS contains 38 unique restriction endonuclease sites. Depending on the restriction site the DNA is cloned into, one of two splice donors are used: SD<sub>v</sub>, if sites 5' to and including the *Cyn* I site are used; SD<sub>c</sub>, if sites 3' to and including the *Sma* I site are used. P = the SV40 early promoter.

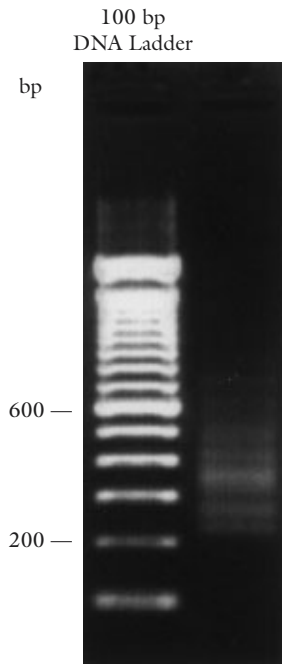


**FIGURE 2.** Schematic of 3'-exon trapping from YACs.

candidate gene is responsible for the phenotype observed. Recently, effort has increased to develop extensive physical maps of human and other model genomes using YAC vectors (4). Previously, exon trapping from genomic clones has used cosmid DNA subcloned into an exon trapping vector. Exon trapping directly from mapped YAC clones would offer a more efficient way of finding exons in YAC systems. This report describes a method for 3' exon trapping from YACs.

## METHODS

*Purification of YAC DNA.* A 400-kb YAC containing mouse genomic DNA was gel-purified on a 1.0% low melting point pulsed-field gel. The DNA pellet was dissolved in 30  $\mu$ l of TE [10 mM Tris-HCl (pH 8), 1 mM EDTA] and digested with *EcoR* I. The digested YAC DNA was phenol extracted, ethanol precipitated, and dissolved in TE to a final concentration of 0.5  $\mu$ g/ $\mu$ l. The trapping vector, pTAG4 (figure 1), was digested with *Nru* I and



**FIGURE 3.** Agarose gel of secondary PCR products following 3'-exon trapping of a mouse YAC. Following the 3'-exon trapping procedure, a portion of the secondary PCR was electrophoresed on a 1.2% agarose/1X TAE gel and stained with ethidium bromide.

*EcoR* I and purified in a 0.8% agarose gel. The purified linear vector was dissolved in TE to a final concentration of 0.5  $\mu\text{g}/\mu\text{l}$ .

**3'-Exon trapping.** Direct ligation transfection, an alternative subcloning method (figure 2), is described in the GIBCO BRL 3'-Exon Trapping System (Cat. No. 18439). The reverse transcription (RT) reaction was performed as follows: 100 ng of poly(A)<sup>+</sup> mRNA was mixed with 5 ng of the AP2 primer in water in a total volume of 20  $\mu\text{l}$  and heated to 70°C for 5 min. The mixture was incubated at 42°C for 5 min, and 30  $\mu\text{l}$  of the RT cocktail was added. The RT cocktail was 12  $\mu\text{l}$  of water, 10  $\mu\text{l}$  of 5X first strand RT buffer, 1  $\mu\text{l}$  of 25 mM dNTPs, and 5  $\mu\text{l}$  of 0.1 M DTT. 2  $\mu\text{l}$  of SUPERScript™ II RT was added after the mix was pre-warmed to 42°C for 3 min. The reaction was incubated at 42°C for 30 min, 55°C for 5 min, and then 2 units of RNase H were added and incubated at 55°C for 10 min. 5  $\mu\text{l}$  of the RT reaction was amplified in a final volume of 100  $\mu\text{l}$  using the primers SV40P and UAP2 at 0.4  $\mu\text{M}$  [1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200  $\mu\text{M}$

dNTPs-final concentrations]. The PCR was heated at 94°C for 5 min, and then, holding the temperature at 80°C, 1.25 U *Taq* DNA polymerase was added. The PCR was 20 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 1 min. 17  $\mu\text{l}$  of the first PCR was digested with *EcoR* I, and 1  $\mu\text{l}$  (at 1:10) was used as a template for the second PCR with UAP2 and dUAd2 primers. The second PCR product (6  $\mu\text{l}$ ) was shotgun cloned into GIBCO BRL pAMP1 (25 ng) with Uracil DNA Glycosylase (UDG) cloning following the manufacturer's recommendations.

## RESULTS AND DISCUSSION

A 400-kb mouse YAC was 3' exon trapped using the above protocol. After the secondary PCR, a number of bands were visible on an agarose gel (figure 3). A fraction of the secondary PCR was shotgun UDG cloned into pAMP1, and 29 randomly picked clones were sequenced. Twenty-five clones were considered to be potential 3' exons using the following criteria: 1) a splice event occurred between the splice donor of the pTAG4 adenovirus exon 2 and a novel sequence, and 2) the presence of one of the highly conserved poly(A) signal sequences AATAAA or ATTAAA 10 to 30 bp 5' to the 3' terminus of the novel sequence. The 25 clones that met these criteria identified 10 independent potential exons. Genbank was scanned using the blastn method (5) and, although 8 of 10 did not match any sequence in the database with significance, one clone did significantly align with a human gene and a second clone aligned significantly with a mouse long interspersed repetitive element. Among these 10 sequences there was no significant homology with the major rodent repetitive elements B1 or B2; the first Exon Trapping System vector, pSPL1, detected B1 elements (6). Parts of the pTAG4 vector were not trapped.

As the human and mouse genetic maps near completion and are integrated with the YAC physical maps, the efficiency of positional cloning will increase dramatically. We have presented a method for 3'-exon trapping directly from YACs that will expedite the identification of new genes from blocks of DNA several hundred kilobases to more than a megabase in size.

## REFERENCES

1. Collins, F.S. (1992) *Nat. Genet.* 1, 3.
2. Lovett, M., Kere, J., and Hinton, L.M. (1991) *Proc. Natl. Acad. Sci. USA* 88, 9623.
3. Krizman, D.B. and Berget, S.M. (1993) *Nucleic Acids Res.* 21, 5198.
4. Cohen, D., Chumakov, I., and Weissenbach, J. (1993) *Nature* 366, 698.
5. Altschul, S.F., Gish, W., Miller, W., Myers, E., and Lipman, D.J. (1990) *J. Mol. Biol.* 215, 403.
6. Gibson, F., Lehrach, H., Buckler, A.J., Brown, S.D.M., and North, M.A. (1994) *BioTechniques* 16, 453.

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# EXON TRAPPING VECTOR pSPL3-CAM: IMPROVED SHOTGUN SUBCLONING OF COSMID-DERIVED FRAGMENTS

The exon trapping method developed by Buckler *et al.* (1), and modified by Church *et al.* (2), is a rapid approach for screening large stretches of cloned DNA for expressed sequences. The method can be divided into three distinct steps: 1) shotgun subcloning of cloned genomic DNA (generally cosmids) into the exon trapping vector pSPL3; 2) introducing pools of either selected recombinant pSPL3 clones or entire shotgun minilibraries into COS cells; and 3) isolating and analyzing the resulting RNA for the presence of trapped exon sequences. While we have successfully employed this system to isolate and characterize gene sequences encoded within cosmids, our surveying efficiency is not maximal due to several intrinsic features of the current system.

One area for improvement in the present scheme is shotgun subcloning of fragments derived from cosmids that express  $\beta$ -lactamase (ampicillin resistance), since pSPL3 also relies on this antibiotic selection. Furthermore, most of the common cosmid vectors do not contain *Bam*H I or *Bgl* II sites (common cloning sites in pSPL3) between their origin of replication and the  $\beta$ -lactamase gene. Of the restriction endonuclease sites for subcloning into pSPL3, only *Pst* I interrupts the coding region of the  $\beta$ -lactamase gene in some common vectors. Consequently, the resulting subclones, selected based on resistance to ampicillin ( $Ap^r$ ), harbor either a circularized pSPL3 vector, a circularized cosmid vector containing fragment, or recombinant plasmids containing either type of vector. Optimized enzymatic dephosphoryla-

tion of the trapping vector will minimize the number of pSPL3-only clones but will have no impact on clones resulting from cosmid-vector-containing ligation products. The cosmid-vector-derived ligation products are the result of an intramolecular ligation event, in contrast to an intermolecular event that is required of all pSPL3-derived ligation products. In our hands, experiments revealed that ~80% of all transformants analyzed with minipreps as well as Southern blots were derived from recircularized cosmid vector and not pSPL3 and only ~5% were recombinant pSPL3.

To eliminate the propagation of clones harboring non-pSPL3-containing plasmids, we replaced the  $\beta$ -lactamase cassette of pSPL3 with one encoding chloramphenicol acetyltransferase. The pSPL3-CAM vector was engineered by removing a 1.6-kb *Bam*H I fragment from pUC18 CMR (3) (ATCC 37718), which was then partially end-filled to generate a single dG overhanging end. This 1.6-kb CAM-containing fragment was then ligated with a 5,023-bp *Bsp*H I fragment from pSPL3 that had been partially end-filled to generate a complementary dC overhanging end. *Bsp*H I digestion of pSPL3 cleanly removes the  $Ap^r$  cassette from the trapping vector. The trapping vector pSPL3-CAM is nearly the same size as the original pSPL3 (~6.6 kb) and confers chloramphenicol resistance ( $CAM^r$ ). As expected, bacterial cells harboring pSPL3-CAM are sensitive to ampicillin.

To demonstrate the utility of the new vector, two cosmids were digested with both *Bam*H I and *Bgl* II and then ligated to *Bam*H I

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TABLE 1. Plating efficiencies from ligation reactions of pSPL3-CAM with *Bam*H I/*Bgl* II-digested cosmids.

	Ampicillin Plates		Chloramphenicol Plates		Ratio of Colonies Ap <sup>r</sup> :CAM <sup>r</sup>
	Number of Colonies	Cloning Efficiency (colonies/ $\mu$ g)	Number of Colonies	Cloning Efficiency (colonies/ $\mu$ g)	
cosmid LA325A					
20 $\mu$ l	190	$9.5 \times 10^7$	4	$2.0 \times 10^6$	48:1
200 $\mu$ l	TNTC <sup>1</sup>	—	47	$2.5 \times 10^6$	38:1
cosmid LA2H2					
20 $\mu$ l	131	$6.5 \times 10^7$	3	$1.5 \times 10^6$	43:1
200 $\mu$ l	TNTC	—	39	$2.0 \times 10^6$	33:1
pSPL3-CAM only					
20 $\mu$ l	0	—	0	0	
200 $\mu$ l	0	—	4	$2.0 \times 10^5$	

Ligation ratios were 10:1, insert:vector. The ratio used was previously optimized. An additional ligation reaction was set up that just contained linearized pSPL3-CAM. Competent DH5 $\alpha$  cells were transformed with 1  $\mu$ l of each ligation reaction and plated onto LB agar plates containing either 50  $\mu$ g/ml ampicillin or 25  $\mu$ g/ml chloramphenicol. The resulting colonies were counted the following day. Efficiencies are based on micrograms of pSPL3-CAM.

<sup>1</sup> TNTC—Too numerous to count.

linearized pSPL3-CAM (table 1). The cloning efficiency of the cosmid-vector-derived plasmids, as judged by propagation on ampicillin, was 33- to 48-fold that of pSPL3-CAM on chloramphenicol-containing plates. Duplicate colony hybridization filters were prepared from each plate and hybridized with radiolabeled oligonucleotides specific for the cosmid vector or for pSPL3-CAM. All of the colonies present on the plates containing ampicillin hybridized with the cosmid-vector-specific oligonucleotide. Similarly, all of the colonies present on the plates containing chloramphenicol hybridized with pSPL3-CAM-specific oligonucleotide. If pSPL3 had been used instead of pSPL3-CAM for these studies, only 2–3% of the resulting Ap<sup>r</sup> colonies would harbor a trapping vector subclone. It follows that if the experiment was continued, then of the 10  $\mu$ g of plasmid DNA introduced into the COS cells, only 200 to 300 ng would contain pSPL3. This modest amount of pSPL3 subclones still may be sufficient for adequate transcription and subsequent RT-PCR based detection of some subcloned exons.

However, an increased likelihood of exon identification can be achieved by improving the subcloning efficiency 33 to 48 times such as described with pSPL3-CAM. We have successfully used the pSPL3-CAM vector, in conjunction with the GIBCO BRL Exon Trapping System, to isolate functional exons from a cosmid contig containing sequences from human chromosome 16.

## REFERENCES

1. Buckler, A.J., Chang, D.E., Graw, S.L., Brook, J.D., Haber, D.A., Sharp, P.A., and Hausman, D.E. (1991) *Proc. Natl. Acad. Sci. USA* 88, 4005.
2. Church, D.M., Stotler, C.J., Rutter, J.L., Murrell, J.R., Trofatter, J.A., and Buckler, A.J. (1994) *Nature Genetics* 6, 98.
3. Schweizer, H.P. (1990) *Biotechniques* 8, 612.

## EDITOR'S NOTE:

The pSPL3-CAM vector is available for field testing. Contact Dr. Paul Nisson at Life Technologies, Inc. In the U.S. call 800-828-6686, ext. 7774.



### What are alternative methods to subclone genomic DNA that is cloned in an ampicillin resistant vector?

1. Dephosphorylate the digested target DNA prior to ligating it to phosphorylated pSPL3. This will eliminate "donor" vector/insert religation.
2. Purify the host vector from the insert prior to digesting the insert with the cloning restriction endonuclease.
3. Cleave the host vector with a restriction endonuclease that will inactivate the ampicillin gene (*e.g.*, *Pvu* I). It is important that this enzyme does not have any sites in the insert.

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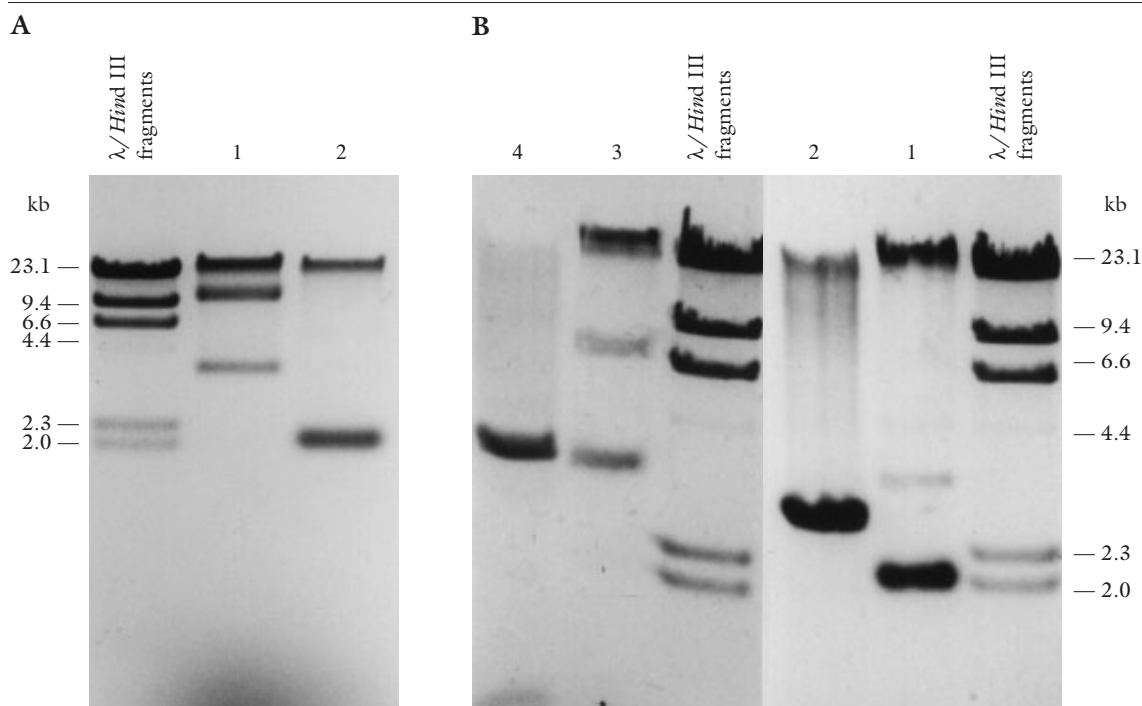
# A SHORT, SMALL-SCALE PREPARATION OF PLASMID DNA FROM *E. COLI* USING ORGANIC SOLVENTS

Lysis of bacteria by boiling or by alkali has been commonly used for the small-scale preparation of plasmid DNA. These methods involve either a large number of reagents, some of which are caustic, or a boiling step that has been reported to be deleterious to strains of *E. coli* expressing endonuclease A (1). Prolonged exposure of superhelical DNA to heat or alkali has been reported to result in irreversible denaturation (1). Moreover, both these methods suggest the use of phenol:chloroform as an optional step, particularly in those cases when the plasmid DNA obtained does not digest with restriction endonucleases (1). We present a simple, rapid, inexpensive method for preparing plasmid DNA on a small scale that is pure enough to be digested with restriction endonucleases.

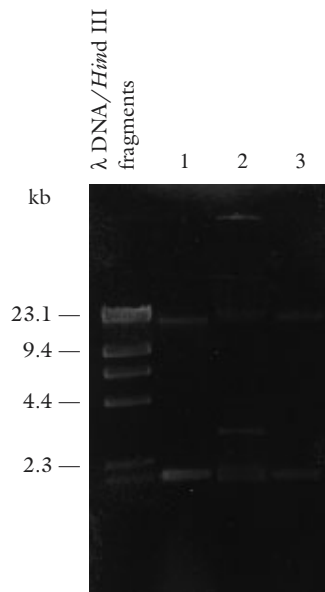
Our protocol involves the lysis of the bacteria harboring the plasmid of interest by suspending the bacteria in 70% ethanol. Addition of chloroform and phenol completes the lysis and simultaneously precipitates the cell lysate proteins. The supernatant obtained contains the plasmid DNA that is precipitated with excess of absolute ethanol.

Bacteria harboring the plasmids pUC18 and pB27 (an *ompA* expression plasmid) (2) were grown overnight at 37°C. An aliquot of the culture (1.7 ml) was centrifuged at 10,000 × *g* in a microcentrifuge for 30 s at 4°C. The supernatant was discarded and the pellet was suspended in 150 µl of distilled water. Then 350 µl of absolute ethanol followed by 500 µl each of chloroform:isoamyl alcohol (24:1) and buffer-saturated phenol were added and mixed

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**FIGURE 1. Analysis of plasmid DNA.** Plasmid DNA was prepared according to our method. Samples were electrophoresed on 0.8% agarose gels and stained with ethidium bromide. Panel A. Isolated pB27 DNA (lane 1) and pUC18 (lane 2). Panel B. Samples from Panel A were incubated at 37°C for 1 h with or without restriction endonucleases. Lane 1, undigested pUC18. Lane 2, pUC18 digested at its one cut site with *Hind* III. Lane 3, undigested pB27 DNA. Lane 4, double digestion of pB27 with *Bam*H I and *Hind* III.



**FIGURE 2. Comparison of plasmid preparation methods.** pUC18 DNA was prepared according to our method (lanes 1 and 3) or according to the boiling method of Holmes and Quigley (1) (lane 2).

well. The sample was centrifuged at  $10,000 \times g$  for 10 min. The plasmid DNA in the supernatant was precipitated with 2 volumes of absolute ethanol at  $10,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min. The pellet was dried, dissolved in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], and analyzed by restriction endonuclease digestion.

Isolated DNAs were electrophoresed with and without restriction endonuclease digestion (figure 1). Complete digestion was seen for both pB27 DNA and pUC18 DNA. Since undigested pB27 and pUC18 that had been

incubated at  $37^{\circ}\text{C}$  with the appropriate buffers resembled the respective plasmid DNAs shown in panel A, the preparations had no detectable endonuclease contaminants. Contamination with RNA was minimal and did not interfere with the enzyme digestions. The quality and yield of plasmid DNA obtained were comparable to those obtained with the boiling method (figure 2).

This method, which takes  $<30$  min, is quicker and simpler than the alkaline lysis and boiling methods and does not require the use of lysozyme or boiling. Moreover, both the alkaline lysis and boiling methods suggest the use of phenol:chloroform as an optional step, particularly in those cases when the plasmid DNA obtained does not lend itself to digestion with restriction endonucleases. The yield obtained with this method is comparable to the yield obtained with the boiling method of Holmes and Quigley. This method will be useful to screen large numbers of samples rapidly.

#### REFERENCES

1. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. Parker, K.C., Silver, L.S., and Wiley, D.C. (1992) *Mol. Immun.* 29, 371.

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# MICROWAVE ACTIVATION OF FLUORESCENCE *IN SITU* HYBRIDIZATION: A NOVEL METHOD FOR RAPID CHROMOSOME DETECTION AND ANALYSIS

**I**n *situ* hybridization provides a modern approach to identification of human chromosomes in many areas of biomedicine (1,2). The use of chromosome-specific DNA probes for fluorescence *in situ* hybridization (FISH) has become a routine approach to chromosome analysis. Chromosome-specific libraries and cloned  $\alpha$ -satellite DNA located in the pericentromeric regions of all human chromosomes are the most efficient chromosome-specific DNA probes (1,3,4). Usually FISH using chromosome-specific DNA probes takes 1 to 2 days (3,5). It has been proposed that the hybridization and detection times (*e.g.*, biotin-avidin-fluorescein) can be decreased by microwave activation (6). However, this has not been examined for *in situ* hybridization using chromosome-specific DNA probes. In this paper, microwave activation is used for FISH of alphoid DNA probes and produces good results in 11 min.

## METHODS

**Cells.** For examination of human interphase cells and metaphase chromosomes, preparations were obtained from lymphocyte and amniotic fluid cultures by standard methods and used 1 to 3 weeks after preparation (7,8). For lymphocyte cultures, GIBCO BRL Chromosome Medium 1A with phytohemagglutinin was used.

**Probes.** A set of  $\alpha$ -satellite probes specific for different human chromosomes was used (9). Probes were labeled by nick translation using the GIBCO BRL BIONICK™ Labeling System, precipitated by ethanol and resuspended in hybridization mixture (50% formamide, 2X SSC, 10% dextran sulfate). Probes can be stored at  $-20^{\circ}\text{C}$  for one year.

***In situ* hybridization and detection.** Hybridization used a modification of the proce-

dure previously described (10). Slides with fixed cells were denatured in 0.07 N NaOH for 30 s; dehydrated through ethanol 70%, 70%, 90%, and 100%, 1 min each; and air dried. The hybridization mixture containing 50 ng of biotinylated repetitive centromeric DNA probe in 10  $\mu\text{l}$  of hybridization solution (50% formamide, 2X SSC, 10% dextran sulfate) was denatured at  $90^{\circ}\text{C}$  for 4 min, applied to the slide, and sealed under a coverslip. To prevent uncontrollable, rapid heating of slides during microwave activation, 1 liter of water ( $20^{\circ}\text{C}$ ) in a glass jar was placed in the bottom of the oven. The slides were placed over the glass jar in the center of the microwave oven and microwaved for 5 min at 30% strength (position 1—lower power). The same results were obtained with an Emerson, Model AT736A, microwave oven with carousel and digital timer or a conventional microwave (Goldstar). After hybridization, the slides were washed in 0.2X SSC at room temperature for 1 min. Slides were treated with fluoresceinated avidin (10  $\mu\text{g}$  in 1 ml of 2X SSC containing 15% nonfat dry milk) in a microwave oven for 7 s at 100% strength. The slides were rinsed for a few seconds in 0.2X SSC, 0.2% TWEEN 20® and mounted in 0.2 M Tris-HCl:glycerol (pH 8.0) (1:9, v/v) containing the antifading reagent 1,4-di-azobicyclo-(2,2,2)-octane (DABCO) and propidium iodide (PI) as counterstain.

**Microscopy.** Slides were examined under a Leica epifluorescence microscope, equipped for the visualization of fluorescein isothiocyanate, PI, and DAPI fluorescence. Photographs were taken from slides using Kodak Ektachrome 400.

## RESULTS AND DISCUSSION

A FISH protocol was developed using microwave activation for hybridization and detection. In contrast with standard FISH, this

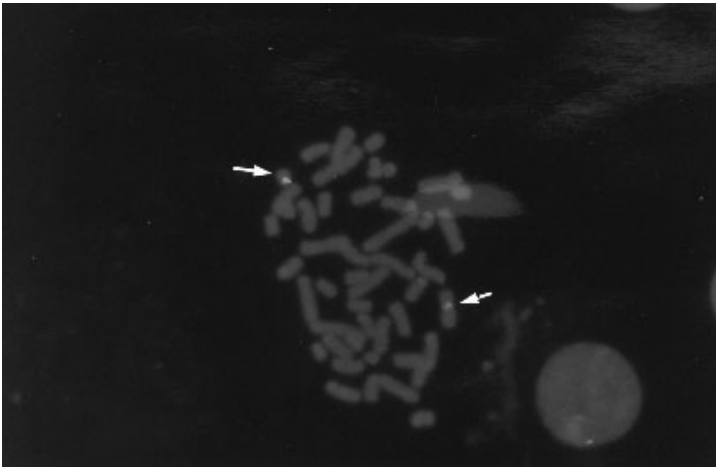
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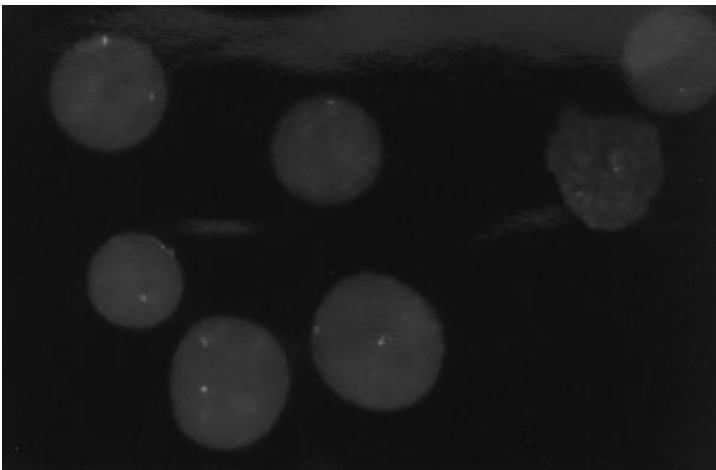
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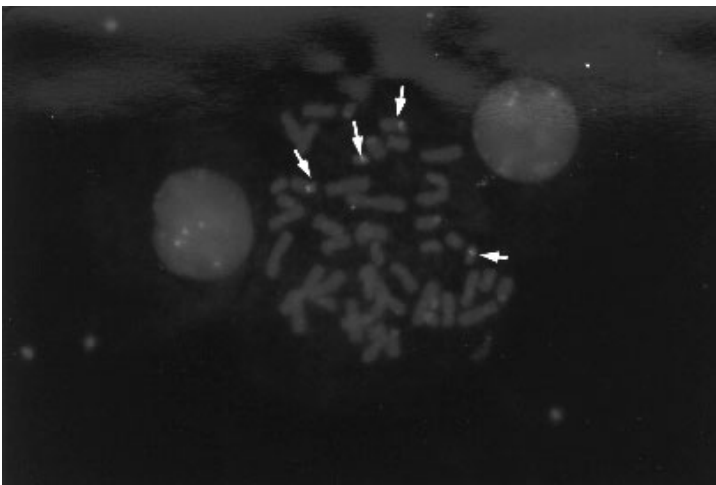
A



B



**FIGURE 1. FISH with microwave activation.** Hybridization signals from centromeric DNA probes X and Y (pYAM 10-40, gen-s-Y-class) on metaphase cells (Panel A) and interphase cells (Panel B) (karyotype 46, XY).



**FIGURE 2. FISH in the presence of cytoplasm.** Hybridization signals from centromeric DNA probes specific for chromosomes 13 and 21.

technique detected a hybridization signal within a few minutes, reducing the detection time 10- to 15-fold. No signal amplification was used to minimize the overlapping and nonspecific background of hybridization signals during analysis of chromosomal abnormalities in interphase nuclei (figure 1). Microwave activation gave a hybridization signal on cells containing cytoplasm (figure 2).

This technique was highly reproducible and applicable for many different chromosome-specific DNA probes. We have tested  $\alpha$ -satellite chromosome-specific DNA probes for 1, 3-19, 21, 22, X, and Y (9). This procedure has allowed for rapid chromosome detection in interphase and metaphase cells of peripheral blood and amniotic fluid for over 2 years in our laboratories. Chromosome-specific repetitive DNA probes with FISH microwave activation could be used for rapid diagnosis of common chromosomal syndromes, chromosome aneuploidies, fast sex determination in prenatal screening, and routine chromosome identification in post- and prenatal diagnosis.

#### REFERENCES

1. Devilee, P., Thierry, R.F., Kievits, T., Kolluri, R., Hopman, A., Willard, H.F., and Pearson, P. (1988) *Cancer Res.* 48, 5825.
2. Pinkel, D., Straume, T., and Gray, J.W. (1986) *Proc. Natl. Acad. Sci. USA* 83, 2934.
3. Darfler, M.M., Nisson, P.E., and Watkins, P.C. (1992) *FOCUS* 14, 58.
4. Willard, H.F. and Waye, J.S. (1987) *Trends Genet.* 3, 192.
5. Trask, B.J. (1991) *Trends Genet.* 7, 149.
6. Hopwood, D. (1993) *Eur. Microscopy and Analysis* 24, 13.
7. Hungerford, D.A. (1965) *Stain. Techn.* 40, 333.
8. Rooney, D.E. and Czepulkowski, B.H. (1992) *Human Cytogenetics: A Practical Approach*, Vol. 1, Oxford University Press, Oxford, p. 63.
9. Yurov, Y.B., Yakovlev, A.G., Alexandrov, I.A., Mitkevich, S.P., Rogaev, E.I., and Vorsanova, S.G. (1989) *Cytogen. Cell Genet.* 46, 1114.
10. Yurov, Y.B., Mitkevich, S.P., and Alexandrov, I.A. (1987) *Human Genet.* 76, 157.

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## TRANSIENT TRANSFECTION OF ENDOTHELIAL CELLS

All blood vessels and cavities of the heart are lined with a continuous monolayer of endothelial cells. Vascular endothelial cells play a vital role in many biological processes, including angiogenesis, thrombosis, wound repair, and functions of the immune and barrier systems. Because endothelial cells are in continuous contact with the bloodstream, they are a site for the delivery of therapeutic agents and for the modification of circulating substrates. When studying endothelial cell systems, *in vitro* cell culture is critical. The use of serum-free medium offers many advantages for *in vitro* cell culture (1).

Stable gene transfer into endothelial cells has enabled *in vitro* expression of a variety of proteins in both human and animal systems, including tissue plasminogen activator (2), pre-pro endothelin-1 (3), adenosine deaminase, and growth hormone (4). Cationic lipids are a proven method for the efficient transfer of DNA in a wide variety of eukaryotic cells for both stable and transient expression of DNA (5). Also, lipids offer advantages over the traditional calcium phosphate method (6).

In these studies, cationic lipid reagents were used for transient gene expression in several species of endothelial cells cultured in serum-free or serum-supplemented media. LIPOFECTIN<sup>®</sup> Reagent performed better than LIPOFECTAMINE<sup>™</sup> Reagent for the transient transfection of both human and nonhuman endothelial cells.

### MATERIALS AND METHODS

**Cell culture.** Unless otherwise indicated, all media, growth factors, cell culture, and transfection reagents were GIBCO BRL brand. Human umbilical vein (HUVEC) and human umbilical arterial endothelial cells (HUAEC) were cultured in Human Endothelial-Serum-Free Medium (SFM) or serum-supplemented Medium 199 as previously described (7,8). Porcine aortic endothelial cells (PAEC, Cell Systems Corporation) and ovine pulmonary

arterial endothelial cells (OPAEC) were established in Endothelial-SFM Plating Medium (Cat. No. 17602) and maintained in Endothelial-SFM Growth Medium (Cat. No. 17601). Controls were established and maintained in serum-supplemented Medium 199 (9). All cultures were maintained at 37°C in humidified air with 5% CO<sub>2</sub>.

**Transfections.** Plasmid pCMVβgal (10) was cesium chloride purified. LIPOFECTIN Reagent and LIPOFECTAMINE Reagent were used according to manufacturers' instructions. Briefly, lipid reagent and DNA were diluted separately in 100 μl OPTI-MEM I<sup>®</sup> Reduced Serum Medium (without serum), in polystyrene tubes. Solutions were combined, gently mixed, and incubated for 15 to 45 min at room temperature to allow formation of DNA-lipid complexes. Following incubation, 0.8 ml OPTI-MEM I were added to each tube and subsequently applied to cells that had achieved 50% to 70% confluence in 35-mm wells. Human endothelial cells were incubated for 3 h, while nonhuman cells were incubated for 6 h, at 37°C in humidified air with 5% CO<sub>2</sub>. Following incubation, the transfection mixture was removed and replaced with 2 ml/well serum-free or serum-supplemented medium. Transfected cells were stained for β-galactosidase (β-gal) expression 18 to 24 h after the start of transfection (11).

### RESULTS AND DISCUSSION

A variety of conditions were investigated as to their influence on the transfection efficiency of endothelial cells, including culture media, cell passage number, pooling cells, seeding density, transfection media, lipids, DNA concentrations, and incubation times. Results using optimal conditions for transfection of the β-gal plasmid with LIPOFECTIN Reagent are shown in figure 1. Cells that stained for β-gal were counted. LIPOFECTIN Reagent supported transfection efficiencies of >25% for the species of endothelial cells tested (table 1). Optimal lipid

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**TABLE 1. Optimal DNA and LIPOFECTIN concentrations for transient transfection of endothelial cells.**

Endothelial Cell Type	Optimal DNA Concentration	Optimal LIPOFECTIN Concentration	Percent Stained Cells <sup>1</sup>
Human Venous	1 µg/well	6 µl/well	25 ± 3
Human Arterial	1 µg/well	6 µl/well	28 ± 3
Ovine Arterial	1 µg/well	8 µl/well	25 ± 4
Porcine Aortic	1 µg/well	12 µl/well	33 ± 2

<sup>1</sup>Mean ± S.D. of 3 determinations from optimal transfection conditions for each endothelial cell type evaluated.

concentrations were determined by titrating LIPOFECTIN Reagent from 4 to 12 µl/35-mm well for human endothelial cells and 4 to 16 µl/35-mm well for nonhuman endothelial cells. DNA was titrated from 0.5 to 1.5 µg/35-mm well. The optimal DNA concentration was 1 µg for all endothelial cells tested. Similar experiments were performed with LIPOFECTAMINE Reagent. Peak efficiencies of ~5% for human endothelial cells and ~10% for porcine and ovine endothelial cells were obtained with 5 µl/well LIPOFECTAMINE Reagent (2 to 14 µl were tested) and 1 µg DNA/well (0.5 to 1.5 µg were tested).

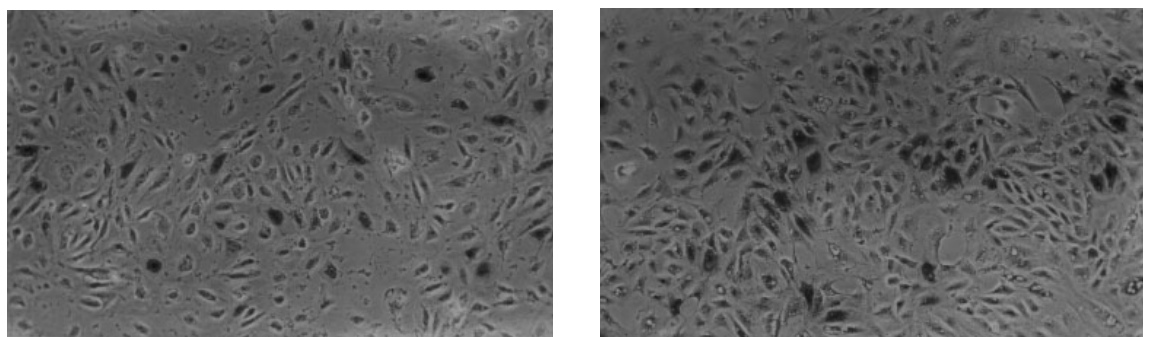
As endothelial cells have a finite *in vitro* lifespan, early passage endothelial cells consistently yielded maximum transfection efficiencies. Human endothelial cells subcultured <5 passages and nonhuman endothelial cells subcultured <10 passages yielded the best results. Also, pooling cells from different primary isolates was found to improve transfection efficiency. Endothelial cells should be 50% to 70% confluent at the time of transfection. Using an initial seeding density of 1 × 10<sup>4</sup> cells/cm<sup>2</sup>, endothelial cells were ready for transfection at 18 to 24 h postseeding. Lower

cell densities were associated with reduced viability, while higher cell densities resulted in efficiencies reduced by at least 25%.

Transfection efficiencies for cells cultured in SFM were similar to that obtained for cells cultured in serum-supplemented medium. However, the transfection medium was critical for high-efficiency transfection. When the lipid/DNA complex was prepared in OPTI-MEM I but incubated with 0.8 ml Human Endothelial-SFM, Endothelial-SFM, or serum-supplemented Medium 199, transfection efficiencies were decreased by ~50%. If cells were both complexed and incubated in any of these media, DNA uptake was <1% for all cells tested. Maximum efficiencies were obtained when OPTI-MEM I was used for both complexing and incubating, as suggested in the manufacturer's instructions.

Incubation times between 2 and 6.5 h were examined for both human and nonhuman endothelial cells. A 3-h exposure to the transfection mixture was optimal for human cells, while a 6-h exposure was optimal for nonhuman cells. Longer exposure times were associated with decreased transfection efficiencies due to cell death.

Many researchers are interested in using endothelial cells as a way to improve reconstructive arterial surgery and to introduce genetically modified cells into the body. Before this can be done, however, researchers need to thoroughly examine *in vitro* gene expression models. Cationic lipids offer a fast, easy, and efficient method of DNA transfer. When using LIPOFECTIN Reagent to transfect endothelial cells, researchers can obtain transfection efficiencies of ≥25%.



**FIGURE 1. Expression of β-gal following transfection of endothelial cells cultured in SFM.** Panel A. Human arterial endothelial cells cultured in Human Endothelial-SFM and transfected with 1 µg DNA and 6 µl LIPOFECTIN Reagent. Panel B. Porcine aortic endothelial cells cultured in Endothelial-SFM with 1 µg DNA and 12 µl LIPOFECTIN Reagent.

## REFERENCES

1. Jayme, D.W., Epstein, D.A., and Conrad, D.R. (1988) *Nature* 334, 547.
2. Dichek, D.A., Neville, R.F., Zwiebel, J.A., Freeman, S.M., Leon, M.B., and Anderson, W.F. (1989) *Circulation* 80, 1347.
3. Ryan, U.S., Zhong, R., Hayes, B.A., Visner, G., and Sauter, M.L. (1993) *J. Cardiovasc. Pharm.* 22, S38.
4. Zwiebel, J.A., Freeman, S.M., Kantoff, P.W., Cornetta, K., Ryan, U.S., and Anderson, W.F. (1989) *Science* 243, 220.
5. Hawley-Nelson, P., Ciccarone, V., Gebeyehu, G., Jessee, J., and Felgner, P.L. (1993) *FOCUS* 15, 73.
6. Murray, E.J., ed. (1991) *Gene Transfer and Expression Protocols*, Humana Press, Clifton, New Jersey.
7. Battista, P.J., Bowen, H.J., and Gorfien, S.F. (1994) *FOCUS* 16, in press.
8. Soderland, C., Veres, J.S., and Battista, P.J. (1994) *FOCUS* 16, in press.
9. Gorfien, S., Spector, A., DeLuca, D., and Weiss, S. (1993) *Exp. Cell Res.* 206, 291.
10. MacGregor, G.R. and Caskey, C.T. (1989) *Nucleic Acids Res.* 17, 2365.
11. Sanes, J.R., Rubenstein, J.L.R., and Nicolas, J.F. (1986) *EMBO J.* 5, 3133.



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