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At Invitrogen, we strive to develop innovative technologies that help you achieve your research goals faster, easier, and more efficiently. *Focus*® Journal will keep you up to date with these developments, introduce new uses for existing technologies, and present improved protocols for your work.

In this issue, we describe a few of the products and techniques recently developed to further enable your research efforts. For example, the SureScore™ Genotyping Kit (page 6) allows you to accurately identify single nucleotide polymorphisms (SNPs) using an ELISA-based assay. This method eliminates the need for expensive equipment, making SNP genotyping available to every lab. OptiPRO™ Serum-Free Medium (page 24) provides optimal cell growth in an environment completely free of any material derived from an animal source. Also featured are new polymerases for improved RT-PCR and PCR results and a time-saving method for performing mammalian two-hybrid studies.

At Invitrogen, we feel that we are making progress towards our goal of becoming the world's leading provider of innovative products that accelerate biological discovery. In June, BioInformatics, an independent Virginia-based market research firm, named Invitrogen the "top supplier in the drug discovery market". Read a summary of this survey and the results on page 29 of this issue of *Focus*®.

As new technologies are developed and existing methods improved, you'll read about them in each issue of *Focus*®. We look forward to helping you achieve your research goals.

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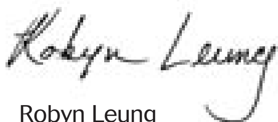

Robyn Leung
Editor

About The Cover:
Microarray analysis results. (See the article on page 14 for information on how the microarray was generated and screened).

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Focus® contains manuscripts describing techniques, improvements of common techniques, simplified protocols, and troubleshooting. "Instructions to authors" are available on the internet.

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Focus® is published triannually by Invitrogen Corporation

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FluoroScript™ RT: A Superior Method to Produce Fluorescently Labeled cDNA Targets for Microarray Screening

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Abstract

FluoroScript™ RT, an avian RNase H⁻ reverse transcriptase (RT), is more thermostable than other RTs, enabling its use at temperatures up to 55˚C. This results in reduced RNA secondary structure and increased full-length cDNA synthesis. FluoroScript™ RT was shown to incorporate fluorescently labeled nucleotides at least seven-fold more efficiently than SuperScript™ II. Here we demonstrate that FluoroScript™ RT can be used to effectively produce fluorescent targets directly from total RNA, mRNA, and amplified substrates for use in microarray applications.

Introduction

A microarray is a microscopic selection of nucleic acids immobilized on a solid matrix. Microarray technology has been employed in determination of transcript abundance differences between mRNA preparations, characterization of the differences in the mRNA population between diseased tissues and normal cells, and differentiation of nucleic acid sequences between cell types. Microarrays are expected to be the dominant technology for expression and genetic analysis in pharmaceutical development.

Many researchers use SuperScript™ II RT to produce fluorescently labeled cDNA for microarray hybridization. Although very popular, SuperScript™ II has a less than 2% incorporation rate of fluorescent nucleotides. To address this need, we have developed an avian RNase H⁻ reverse transcriptase, called FluoroScript™ RT, that incorporates fluorescent nucleotides (*e.g.*, Cy™3- or Cy™5-dNTP) at least seven-fold more efficiently than SuperScript™ II. In addition, FluoroScript™ RT is not greatly inhibited by ribosomal and transfer RNA. Therefore, total RNA can be used directly to produce fluorescently labeled cDNA target. FluoroScript™ RT also exhibits increased thermostability and can be used at temperatures up to 55˚C, which reduces RNA secondary structure and increases primer specificity. Here we demonstrate that the superior performance of FluoroScript™ RT results in a higher incorporation of fluorescent nucleotides into first-strand cDNA and longer labeled cDNA targets, generating high-quality array images.

Methods

First-strand cDNA synthesis. The FluoroScript™ cDNA Labeling System (Cat. no. L1013-01) and SuperScript™ II RT were used in first-strand cDNA synthesis reactions starting with 1 µg of 2.3 kb control RNA and 20-25 µg of HeLa total RNA. For the FluoroScript™ RT reaction, 1 µl (0.5 µg/µl) of oligo $(dT)_{12\text{-}18}$ primer and DEPC-treated water

Table 1 – Comparison of SuperScript™ II RT (SS II) and FluoroScript™ RT (FS) Cy™3-dUTP incorporation using 2.3 kb control RNA and HeLa total RNA

Conditions	cDNA synthesis yield (%)	Cy™3-dUTP incorporation (%)	Percent incorporation increased fold
SS II & 2.3 kb RNA	50.4	1 .0	—
FS & 2.3 kb RNA	47.7	10.3	10.3
SS II & HeLa total RNA	30.8	1.4	—
FS & HeLa total RNA	34.8	12.8	9.1

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Table 2 – Temperature evaluation using 2.3 kb control RNA as template

Temperature (˚C)	cDNA synthesis yield (%)	Cy™3-dUTP incorporation (%)
45	46.4	24.6
50	49.8	24.3
55	46.8	23.9

(to 5 µl) were added to each RNA template. The mixtures were heated in a 70˚C water bath for 5 min. and then quenched on ice for 10 min. While on ice, the following reagents were added: 4 µl 5X cDNA Synthesis Buffer, 2 µl 0.1 M DTT, 1 µl dNTPs (250 µM), Cy™3-dUTP to a final concentration of 100 µM, and DEPC-treated water to a final volume of 19 µl. After vortexing and a quick spin, 1 µl of FluoroScript™ RT (15 U/µl) was added to each reaction. Reactions were incubated in a 50˚C water bath for 1 hr. and then stopped by the addition of 2 µl of 200 mM EDTA. SuperScript™ II RT-mediated cDNA synthesis reactions were performed according to the supplied SuperScript™ II RT protocol. Competitor RT kits' ability to incorporate fluorescent nucleotides was also measured. All cDNA synthesis reactions were performed according to the recommended protocols.

*Fluorescently labeled cDNA target purification using the S.N.A.P.™ column "One-Step" method***.** To degrade the RNA, 2 µl of 1 N NaOH was added to each tube and incubated in a 70˚C water bath for 10 min. This was followed by the addition of 2 µl of 1 M HCl (for neutralization) and 1/10 volume of 1 M Tris buffer (pH 7.5). After vortexing and a quick spin, 500 µl of Loading Buffer (2.25 M guanidiniun HCl in 70% isopropanol) was added to each cDNA reaction mixture and mixed well. The mixture was then loaded into a S.N.A.P.™ spin cartridge, which was placed into a collection tube. Following a 2-5 min. room temperature incubation, the reactions were centrifuged at room temperature, 13,000 x g for 1 min. Flow-through was discarded and 500 µl of Wash Buffer (100 mM NaCl in 75% ethanol) added to the spin cartridge. Reactions were centrifuged at room temperature, 13,000 x g for 1 min; and the flow-through discarded. Sixty microliters of TE buffer was added to each spin cartridge and incubated at room temperature for 2-5 min. The cartridges were placed into new 1.7-ml centrifuge tubes and centrifuged at room temperature, 13,000 x g for 1-2 min. The eluate, containing the fluorescently labeled cDNA, was saved. The elution was repeated one more time. The two eluates were combined and completely dried with a speed vacuum.

Array hybridization. The DNA-spotted array slide was placed in a Corning hybridization chamber with the DNA-spotted side facing up. To maintain a high relative humidity in the hybridization chamber during hybridization, 25 µl of dd-H_2O was added to each chamber. The Cy™3- and Cy™5-labeled cDNA targets were each resuspended in a total of 3 µl of dd-H_2O followed by the addition of 30 µl hybridization solution (30% formamide, 5X SSC, 0.1% SDS, and 20 µg of human Cot1-DNA® and poly (dA)). To denature any secondary structure of the fluorescently labeled cDNA, the mixture was heated at 95˚C for 1 min. The mixture was applied to the DNA array slide, which was then covered with a 22 x 50 mm cover slip. The hybridization chamber was assembled and placed in a 65˚C water bath for 8-20 h.

Prior to washing, 50 ml of Wash Solution I (2X SSC, 0.5% SDS) was heated in a coplin jar in a 65˚C water bath to a temperature of 60˚C. The hybridization chamber was then disassembled and the slide dropped into Wash Solution I. After 30 s., the cover slip separated. The slide was dipped into Wash Solution I a couple of times and immediately dropped into a 50-ml conical tube containing 40 ml of Wash Solution II (1X SSC. 0.05% SDS) at room temperature. The conical tube was placed in a tube rack and shaken gently on a RotoMixer at room temperature for 5 min. Wash Solution II was replaced with 40 ml of Wash Solution III (0.5% SSC, 0.05% SDS) and shaken for 5 min. The slide was transferred from Wash Solution III to a clean 50-ml conical tube containing 40 ml Wash Solution IV (0.5X SSC) and shaken at room temperature for 1 min. This final wash step was repeated with fresh Wash Solution IV and shaken for 5 min. The washed slide was placed in a clean 50-ml conical tube and centrifuged at room temperature, 500 x g for 1 min. The array image was then scanned.

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Results and Discussion

Comparison of FluoroScript™ RT vs. SuperScript™ II RT using different RNA templates. One microgram of 2.3 kb control RNA and 20-25 µg of HeLa total RNA were used as RNA templates in first-strand cDNA synthesis using FluoroScript™ RT and SuperScript™ II RT. Cy™3-dUTP at a reaction concentration of 100 µM was used as the fluorescent nucleotide. From this study, the SuperScript™ II fluorescent nucleotide incorporation efficiency was shown to be between 1.0% and 1.4% (Table 1, page 2). The incorporation efficiency of FluoroScript™ RT was shown to be between 10.3% and 12.8%. This demonstrates that FluoroScript™ RT is superior to SuperScript™ II RT at directly incorporating fluorescent nucleotides.

Evaluation of FluoroScript™ RT reaction conditions. The 2.3 kb control RNA and oligo $dT_{12\text{-}18}$ were used as template and primer, respectively. Temperatures between 45° C and 55° C were evaluated using FluoroScript™ RT (Table 2, page 3). The data from this study showed that cDNA yield and fluorescent nucleotide incorporation remained equivalent over various temperatures. The 50°C reaction temperature was chosen as the optimal temperature because of a longer enzymatic half-life.

Various molar ratios between 1.6:1 and 16:1 of normal nucleotide vs. fluorescent-labeled nucleotide (Cy™3-dUTP) were studied (Table 3). The ratio of 2.5:1 was shown to yield the best incorporation efficiency. With a ratio of 4:1 or higher, the incorporation efficiency dropped significantly. FluoroScript™ RT incorporated the Cy™5-fluorescent labeled nucleotide with the same efficiency as the Cy™3-dUTP (data not shown).

We found that the FluoroScript™ RT units needed to be adjusted with different amounts of total RNA between 10 µg and 100 µg used in the first-strand cDNA synthesis. Two microliters of FluoroScript™ RT was required for 100 µg of total RNA, and 1 µl of FluoroScript™ was required for 25 µg of total RNA (data not shown).

Competitors' audit. Two competitors' kits were evaluated and compared with FluoroScript™ RT in cDNA size and fluorescent nucleotide incorporation

Table 3 – The fluorescent nucleotide molar ratio evaluation using 2.3 kb control RNA

Normal to fluorescent nucleotide molar ratio	cDNA synthesis yield (%)	Total Cy™3-dUTP incorporated, (pmole)
All unlabeled nucleotide	50.7	N/A
16:1	61.9	20
8:1	56.0	44
4:1	58.3	60
2.5:1	55.2	80
1.6:1	25.4	56

Table 4 – FluoroScript™ RT vs. Competitor A in percent incorporation and length

Conditions	Cy™3-dUTP incorporation (%)	cDNA size (kb)
FluoroScript™ RT- HeLa total RNA	12.8	0.5-7
Competitor A (kit)- HeLa total RNA	0.5	0.3-1.3
FluoroScript™ RT- 2.3 kb RNA	10.3	2.3
Competitor A (kit)- 2.3 kb RNA	0.4	0.3-0.7

Table 5 – FluoroScript™ vs. Competitor B in percent incorporation and length

Conditions	Cy™3-dUTP incorporation (%)	cDNA size (kb)
FluoroScript™ RT-HeLa total RNA	15.0	0.5-7
Competitor B (kit)- HeLa total RNA	10.8	0.5-2

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efficiency using the 2.3 kb control RNA or HeLa total RNA (Tables 4 and 5, page 4). In all cases, the vendors' recommended protocols were used. FluoroScript™ RT produced longer fluorescently labeled cDNA targets and demonstrated greater fluorescent nucleotide incorporation than the competitors' kit.

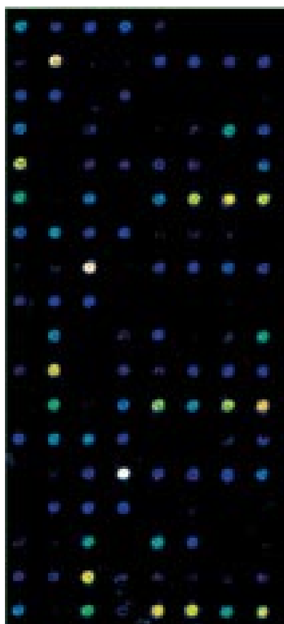
Microarray hybridization results. We evaluated he ability of fluorescently labeled cDNA targets produced using FluoroScript™ RT to generate signals in microarray hybridization experiments. Various RNA samples, between 5 µg and 50 µg of total RNA, were used as the starting material. The fluorescently labeled cDNA target was purified following the one-step S.N.A.P.™ spin-column method (see "Methods"). A scatter plot, generated by plotting the intensities of the control group against those of the experimental group, was used to evaluate the quality of the data. Better signal intensity and a tighter scatter-plot distribution were observed with 20-25 µg of total RNA (Figure 1).

In conclusion, we have shown that FluoroScript™ RT can effectively produce fluorescently labeled targets directly from total RNA, mRNA, and amplified substrates for subsequent microarray applications.n

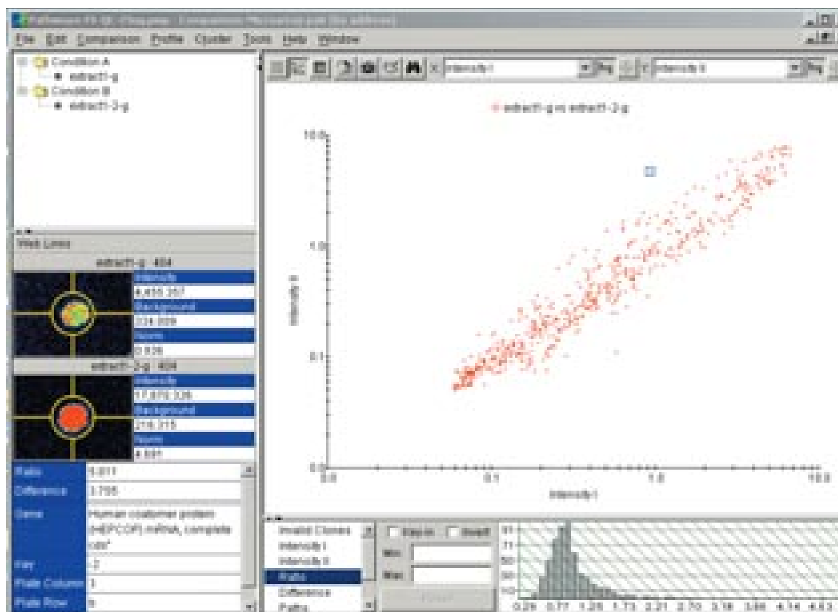
The FluoroScript™ cDNA Labeling System is subject to Limited Use Label License no. 13. Please refer to the Invitrogen web site or catalog for the Limited Use Label License corresponding to the number indicated.

Figure 1 – Microarray hybridization results using fluorescently labeled cDNA generated with FluoroScript™ RT and 25 µg total RNA

A. Signal intensity



B. Scatter plot



To generate Cy™3- and Cy™5-labeled cDNA, 25 µg total RNA was isolated from control and resveratrol-induced HCT116 cells following the FluoroScript™ cDNA Labeling System protocol. Labeled cDNA was co-hybridized to a glass microarray. Slides were scanned (A) and the image was analyzed using Pathways™ 4-Universal microarray analysis software (B). A signal indicating up-regulation was selected on the scatter plot for analysis. Details are shown on the left side of figure B.

SureScore™ SNP Genotyping Kit: A Single-Base Extension ELISA for Discrimination of Nucleotide Polymorphisms

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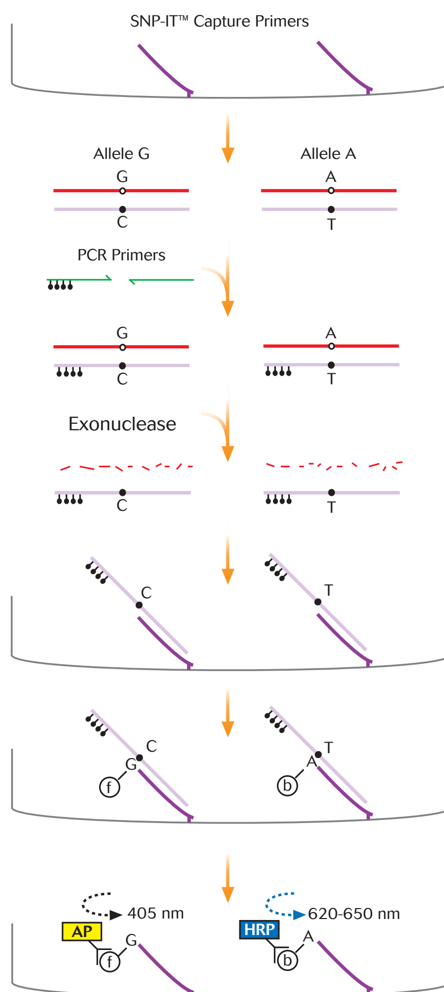
Abstract

Analysis of single nucleotide polymorphisms (SNPs) will significantly contribute to the elucidation of gene functions, therapeutic efficacy, predisposition to disease, and evolutionary linkage studies. In order to quickly evaluate multiple SNPs with minimal optimization and no specialized instrumentation, a simple, accurate, and flexible assay for nucleotide discrimination based on single-base extension and colorimetric detection has been developed (SureScore™ Kit, Invitrogen). A SureScore™ assay can be set up for any SNP or mutation of interest simply through the synthesis of three oligos; its high accuracy is derived from the inherent ability of DNA polymerase to incorporate the precise nucleotide in a single-base extension reaction; and the robustness of antibody-enzyme conjugates allows for the extended nucleotide to be scored in an ELISA format at similar reaction conditions for each SNP or mutation. In this study, the reproducibility, accuracy and flexibility parameters for the SureScore™ SNP Genotyping Kit (www.invitrogen.com/surescore) were validated.

Introduction

Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation, and therefore, are useful markers for understanding the relationship between genetic variation and biologic function (1). SNPs are distinguished from rare mutations by having a frequency of greater than 1% in the population and occur an average of 1 in every 1,000 bp

Figure 1 – The SureScore™ SNP Genotyping procedure



1. Attach the SNP-IT™ capture primer to the well bottoms of the SureScore™ 96 strip-well plate.

2. From genomic DNA samples, amplify the region surrounding the SNP of interest using a PCR reaction with one phosphorothioate-modified primer ( ) and one unmodified primer.

3. Digest the PCR product with exonuclease. Phosphorothioate modification protects one strand, generating a single-stranded template.

4. Hybridize the single-stranded template to the SNP-IT™ capture primer on the SureScore™ plate. The immobilized capture primer serves as the template for primer extension, and is designed to hybridize immediately adjacent to the polymorphic site.

5. DNA polymerase extends the 3´ end of the capture primer by one base using a biotin-labeled or fluorescein-labeled terminating nucleotide.

6. Detect the labeled nucleotide using an ELISA-based colorimetric assay. Antibody conjugates of alkaline phosphatase and horseradish peroxidase are used to determine the nature of the extended base.

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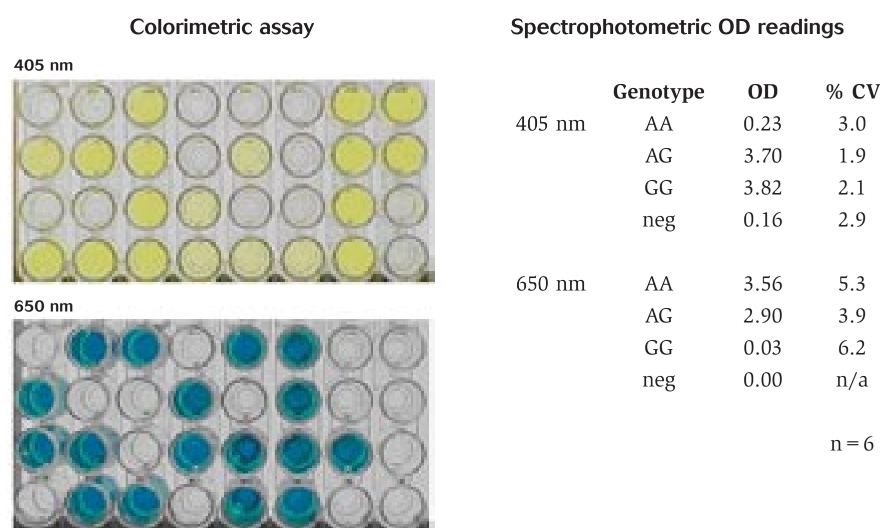
in the 2.9 billion bps of the human genome (1-4). Of the reported 2.1 million SNPs, roughly 20,000 SNPs may potentially alter gene expression or protein structure (3,4). Approximately two-thirds of all SNPs are G-A or C-T transitions (1,5), whereas the remaining third of SNPs are equally divided among the various possible transversion combinations.

Recent studies have shown that each gene has an average of 14 distinct and 2-3 common haplotypes (a combination of SNPs in a defined region), which segregate in linkage disequilibrium as variable length 10-100 kb blocks within the human genome (5-7). Consequently, the identification of roughly 10% of SNPs can reveal as much as 80% of the polymorphisms within a surrounding region (6). Hence, it is expected that a select few SNPs will have significant utility for: 1) the identification of common haplotypes, 2) comprehensive whole-genome association studies, and 3) the elucidation of gene functions and complex disease processes (1,5,6).

Toward these aims, the single-base extension method (8,9) is one of the simplest and most effective means of SNP typing by relying on the inherent ability of DNA polymerase to incorporate the precise nucleotide. The format is well established in the SNP Consortium (10) (Orchid Bioscience, 11) and has been adopted over several high-throughput platforms (9). This study evaluates the reproducibility, accuracy, and flexibility of the SureScore™ Kit in SNP genotyping.

Figure 2 – Evaluation of reproducibility



Spectrophotometric OD readings

	Genotype	OD	% CV
405 nm	AA	0.23	3.0
	AG	3.70	1.9
	GG	3.82	2.1
	neg	0.16	2.9
650 nm	AA	3.56	5.3
	AG	2.90	3.9
	GG	0.03	6.2
	neg	0.00	n/a

n = 6

Genomic DNAs (0.2-20 ng) of varied genotypes for SNP343 were PCR amplified as a 110 bp fragment spanning the polymorphic site in standard 15-50 µl reactions for 35 cycles. Replicates of the PCR products (15 µl) were directly scored in the assay; no additional purification was required.

Methods

The SureScore™ SNP Genotyping Kit (Cat. no. T6000-07) has been developed for low- to medium-throughput assays using a simple ELISA 96 strip-well format which does not require any additional instrumentation and may be scored visually right at the bench top. In this single-base extension method (Figure 1, page 6), a SNP-IT™ capture primer designed to hybridize immediately adjacent to the polymorphic site is passively attached to a 96 strip-well plate (step 1); a genomic DNA region spanning the polymorphic site is then PCR amplified using one phosphothioate primer and one standard PCR primer (step 2); the four 5´ phosphothioates protect one strand from exonuclease digestion, resulting in the generation of a single-stranded PCR template (step 3); the single-stranded PCR template is hybridized to the SNP-IT™ capture primer (step 4); the capture primer is extended with DNA polymerase by a single hapten-labeled terminating dideoxynucleotide that is complementary to the nucleotide at the polymorphic site on the PCR template (step 5); and the identity of the incorporated nucleotide is determined with antibody-enzyme conjugates, which produce a yellow and/or blue colorimetric reaction defining the genotype of the sample (step 6). Each kit includes all of the necessary components to conduct an assay, including exonuclease, control capture primer, 96 strip-well plate, 20X wash buffer, attachment buffer, hybridization solution, extension mix, control templates, detection complexes

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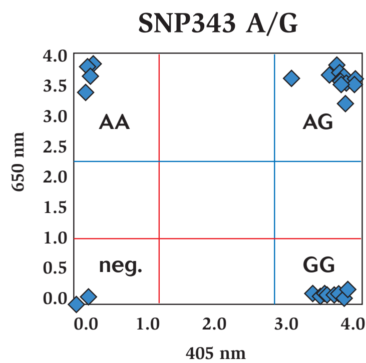
Figure 3 – Accuracy of the SureScore™ method



Genomic DNA samples blinded by an independent laboratory (11), were PCR amplified and scored using the SureScore™ method. 58/58 samples (SNP343, SNP3572, MBL2) were scored correctly with the SureScore™ Kit as compared with the confirmed genotype.

and detection substrates (see manual for formulations, www.invitrogen.com/surescore).

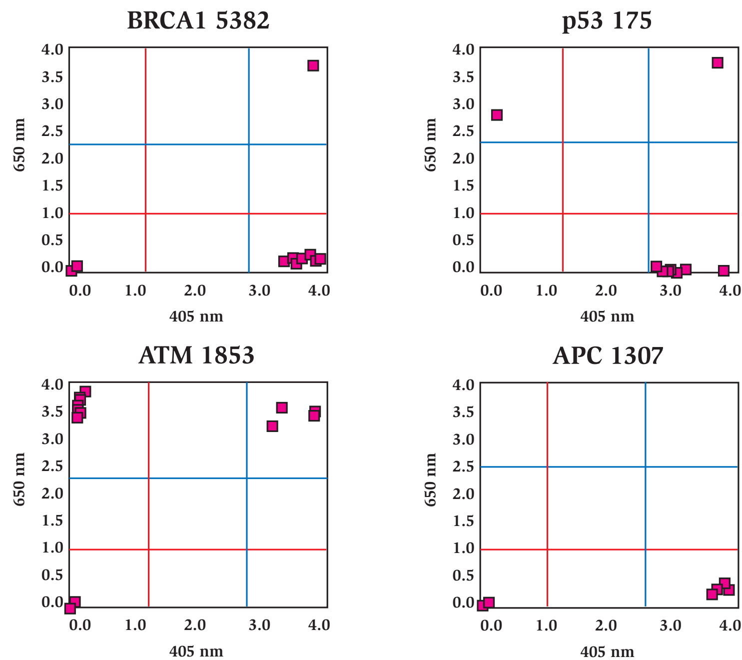
Results and Discussion

The single-base extension method is flexible for any SNP of interest through the synthesis of three gene-specific oligos. A web-based Primer Design Software is provided for efficient design of PCR primers and SNP-IT™ capture oligos, and selects the appropriate extension mix for each SNP of interest (www.invitrogen.com/surescore).

The reproducibility of the SureScore™ method was evaluated using replicates of PCR fragments of various genotypes for SNP343. As shown in Figure 2 (page 7), replicates of standard 15-µl PCR reactions from genomic DNA were directly assayed (no additional purification) for the A/G genotype. The assay was scored visually by a standard colorimetric reaction, whereby the heterozygous samples yielded a yellow color (allele G) following addition of antibody/substrate I and yielded a blue color (allele A) following the antibody/substrate II incubation. Reactions that yielded a single color identified either the wild-type or variant homozygous genotype. In each case, the genotypes were clearly discernible visually (left panels); and spectrophotometric OD readings yielded average discrimination ranges of 16-fold for the G allele (405 nm) and >90-fold discrimination for the A allele (650 nm). Moreover, the average percent coefficient of variation (%CV) for each genotype was 3.6%. The above results are consistent with a robust and reproducible criteria.

The accuracy of the SureScore™ method was determined in collaboration with an independent laboratory (11) by evaluating blinded genomic samples for three different SNPs–SNP343 A/G (11), SNP 3572 C/T (11), and MBL2 C/T (12). Shown in Figure 3 is a scatter plot of the SNP343 assay. In this study, 58 of 58 genotypes were correctly scored (100%), with heterozygotes easily discriminated

Figure 4 – Use of the SureScore™ method for genotyping tumor suppressor genes



The single-base extension method (SureScore™ Kit) was employed to quickly develop assays for four SNPs in tumor suppressor genes (13-16) and then used to screen DNA samples of diverse DNA origin (Coriell) and control samples.

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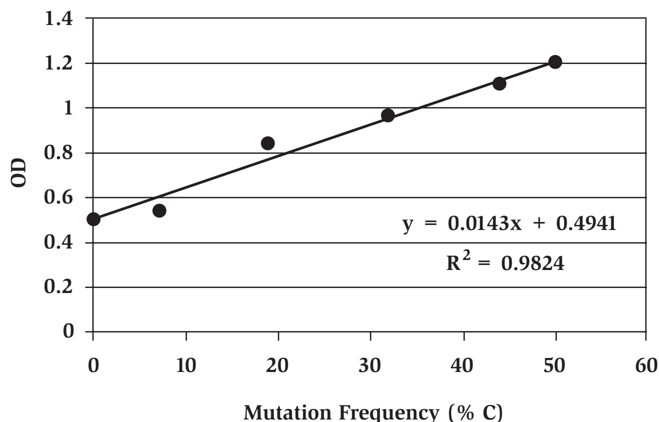
Figure 5 – Detection of somatic mutations and determination of allele frequency within mixed sample populations using the SureScore™ method



Heterozygous (G/C) and homozygous (G/G) genomic DNAs were mixed at varied proportions and the resultant PCR fragments were scored (SureScore™ method) for incorporation of the C nucleotide at ATM 1853 SNP-IT™ capture.

from homozygotes in this format.

To measure flexibility, four SNPs/mutations from the tumor suppressor genes BRCA1 (13), p53 (14), ATM (15), and APC (16), which identify either a common haplotype or have been associated with cancer, were quickly developed with the SureScore™ method. Shown in Figure 4 (page 8) are scatter plots of four SNPs using a publicly available panel of 8-24 ethnically diverse DNAs (Corriell Institute) and control samples. In each case, the SureScore™-designed assay clearly identified the wild-type and heterozygous genotypes present within the populations.

The SureScore™ method was also evaluated for the capability to detect somatic mutations present at low percentages within a mixed cell population and to determine allele frequencies in genomic DNA pools. Shown in Figure 5, genomic DNA containing the heterozygous C nucleotide was mixed at varied proportions with homozygous (G/G) genomic DNA and the resultant PCR fragments (ATM 1853) were scored (SureScore™ method) for the presence of the C nucleotide. The minor C nucleotide was consistently detected at levels as low as 10% within a mixed genomic DNA population. Thus, the SureScore™ method can easily and rapidly detect somatic mutations and determine allele frequencies within a mixed DNA population.

The single-base extension reaction (8) is becoming the most widely used method for SNP scoring because of its simplicity, robustness, accuracy, and flexibility. The basic procedure has been adopted over several platforms (9) and is advantageous over other technologies due to the inherent ability of DNA polymerase to incorporate the precise nucleotide in a single-base extension reaction. Moreover, the procedure allows for low- to medium-throughput SNP scoring without the need for expensive equipment and with little to no optimization. The format is well validated in the SNP Consortium (10) and has been quickly and successfully applied to a variety of SNPs and blinded genomic samples. SureScore™ studies will significantly contribute to the elucidation of gene functions and complex disease processes (www.invitrogen.com/surescore).n

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AccuPrime™ *Taq*: A next generation DNA Polymerase for PCR

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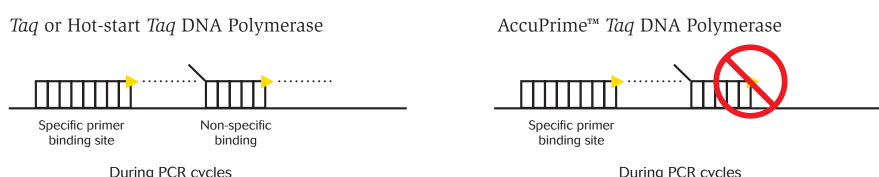
Introduction

PCR is a powerful tool widely used in molecular analysis of DNA and RNA. The drawback to PCR is that in addition to amplifying the target, it can also produce non-specific products. These undesirable by-products, resulting mainly from non-specific annealing or primer-dimer formations, decrease the yield of the target of interest and may cause reduced sensitivity in detection assays. They can also interfere with downstream processes, such as cloning or sequencing. In the past few years, a wide variety of methods have been developed to improve the specificity and sensitivity of PCR, most notably hot-start PCR (1-3). Hot-start PCR improves specificity by preventing non-specific priming during reaction set-up and the initial stages of PCR (3). However, hot-start PCR cannot eliminate non-specific products resulting from non-specific priming during PCR cycling. Currently these are dealt with by performing extensive reaction optimization procedures and/or redesigning primers, which waste time and incur additional expenses to an otherwise routine procedure.

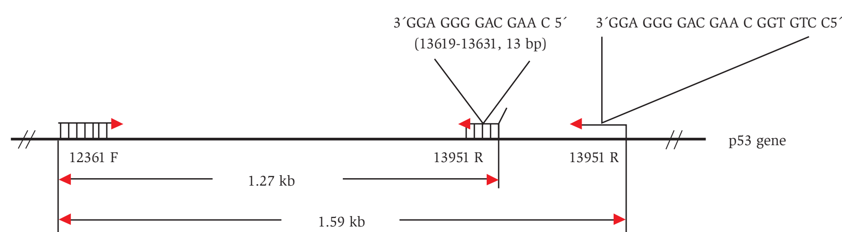
In this manuscript, we describe the introduction of a replication accessory protein (AccuPrime™ protein) to existing hot-start technology to produce a next generation polymerase, AccuPrime™ *Taq* DNA polymerase. The new technology improves specificity, sensitivity, and fidelity over automatic hot-start *Taq* DNA polymerase (4). In this platform, we find the most robust PCR enzyme, suitable for high-throughput screening and multiplex PCR.

Figure 1 – Specificity of AccuPrime™ *Taq* DNA polymerase

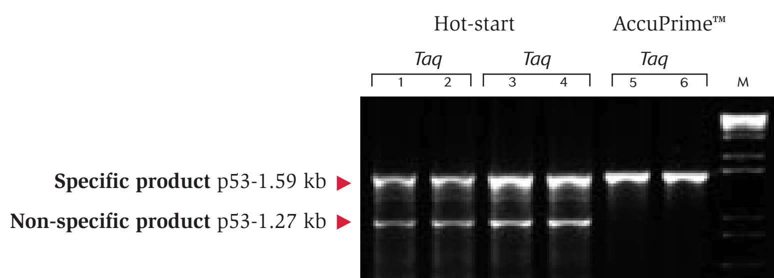
A. AccuPrime™ *Taq* mode of action



B. Schematic of amplified p53 region



C. Specificity of AccuPrime *Taq* vs. other *Taq* polymerases



1A. With AccuPrime™ *Taq*, extension does not occur at non-specific sites

1B. Schematic of defined 13 bp 3' homology p53 reverse primer sequence information and location. The 13 bp homology region located between nucleotides 12361 (p53 forward primer) and 13951 (p53 reverse primer). Detailed homology sequence and location are indicated.

1C. Specific PCR products obtained with AccuPrime™ *Taq* DNA polymerase using a defined 13 bp 3' homology primer. Twenty nanograms of K562 genomic DNA was used as template with p53 gene specific primers (Figure 1B) to amplify a 1.59 kb p53 gene fragment using *Taq*, automatic hot-start *Taq*, and AccuPrime™ *Taq* DNA polymerases. The arrows indicate the specific amplified products (1.59 kb) and non-specific amplified products (1.27 kb). Lanes 1 and 2. *Taq* DNA polymerase with room-temperature assembly. Lanes 3 and 4. Automatic hot-start *Taq* DNA polymerase with room-temperature assembly. Lanes 5 and 6. AccuPrime™ *Taq* DNA polymerase with room-temperature assembly. M: 1 Kb Plus DNA Ladder.

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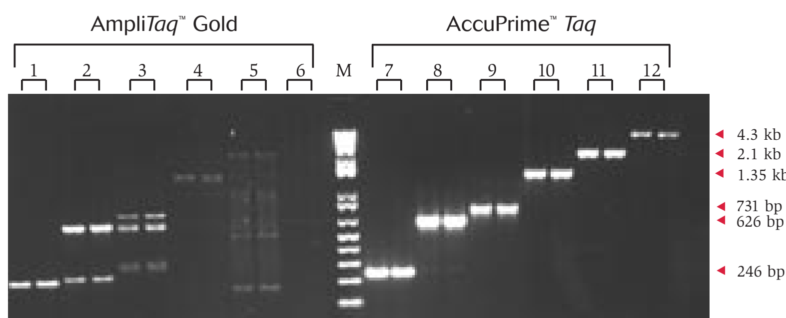
Figure 2 – Improved product specificity and yield with AccuPrime™ *Taq* DNA polymerase



Using 20-100 ng human genomic DNA as template, PCR reactions were set up at room temperature as described in Methods. Reactions were run in duplicate. The specific amplified products indicated by the arrows are as follows: Pr1.3 246 bp (lanes 1 and 7), Rhodopsin 626 bp (lanes 2 and 8), β-globin 731 bp (lanes 3 and 9), Hpfh-6 enhancer 1.35 kb (lanes 4 and 10), p53 2.1 kb (lanes 5 and 11), and p53 4.3 kb (lanes 6 and 12). Lanes 1-6 products were amplified using Ampli*Taq*™ Gold DNA polymerase with Gold buffer (Applied Biosystems, Inc.). Lanes 7-12 products were amplified using AccuPrime™ *Taq* DNA polymerase. M is the 1 Kb Plus DNA ladder. The same amount of enzyme units was used for each reaction. For Ampli*Taq*™ Gold DNA polymerase, the PCR reactions were heated to 95˚C for 10 minutes before PCR cycling.

Methods

PCR. All reagents and enzymes were obtained from Invitrogen. Amplification reactions were performed in 50 µl volumes using 2 units of recombinant *Taq* DNA Polymerase, automatic hot-start *Taq* DNA Polymerase, or AccuPrime™ *Taq* DNA polymerase (Cat. no. 12339-016). The buffer for all reactions was 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, and 200 nM of each primer. For the AccuPrime™ *Taq* reaction, the buffer also contained the thermostable AccuPrime™ protein. Reactions were assembled at room temperature, then incubated for 2 min. at 94˚C, followed by 28-35 cycles at 94˚C for 15 s., 55˚C-60˚C for 30 s., and 68˚C for 1 min/kb. PCR products (10 µl from each reaction) were analyzed on 0.8%-1.5% (w/v) agarose gels containing 0.4 µg/ml ethidium bromide.

rps*L fidelity assay*. pMOL 21 plasmid DNA (4 kb), containing the ampicillin resistance (Ap^r) and reporter (*rps*L) genes, was linearized with *Sca* I. Standard PCR was performed on the linearized product using biotinylated primers. Amplification was completed using 2 units of AccuPrime™ *Taq* DNA polymerase and 1 ng template for 25 cycles. PCR cycling parameters were 94˚C for 2 min., followed by 25 cycles at 94˚C for 15 s., 58˚C for 30 s., and 68˚C for 5 min. PCR products were purified using streptavidin-labeled magnetic beads to ascertain linearity. Purified PCR products were analyzed on an agarose gel and DNA concentration and template doubling time were estimated. The purified DNA was religated using T4 DNA ligase and transformed into MF101 competent cells. To determine the total number of transformed cells, cells were plated on LB plates containing 100 µg/ml ampicillin. To determine the total number of *rps*L mutants, cells were plated on LB plates containing 100 µg/ml ampicillin and 40 µg/ml streptomycin. Mutation frequency was determined by dividing the total number of mutations by the total number of transformed cells. The error rate was determined by dividing the mutation frequency by 130 (the number of potential mutation sites that cause phenotypic changes in the *rps*L gene) and the template doubling time.

Results and Discussion

Elimination of non-specific priming. PCR specificity is dependent on multiple factors, including primer design, magnesium concentration, and annealing temperature. Non-specific products are often prominent when the target gene is buried within an excess of other DNA, such as in genomic DNA templates. Extensive PCR condition optimization may be required to obtain specific products. Here we show that the unique combination of anti-*Taq* antibodies and the AccuPrime™ accessory protein in AccuPrime™ *Taq* DNA polymerase is able to amplify a specific target with minimal optimization (Figure 1A, page 10).

To demonstrate the enhanced specificity, a specific 13 bp 3' homology primer on the p53 gene was designed (Figure 1B, page 10). As a control, the same amount of recombinant *Taq* or hot-start *Taq* was used in side-by-side reactions. A clean, specific product of the expected size (1.59 kb) was generated using AccuPrime™ *Taq* (Figure 1C, lanes 5 and 6, page 10). Amplifications

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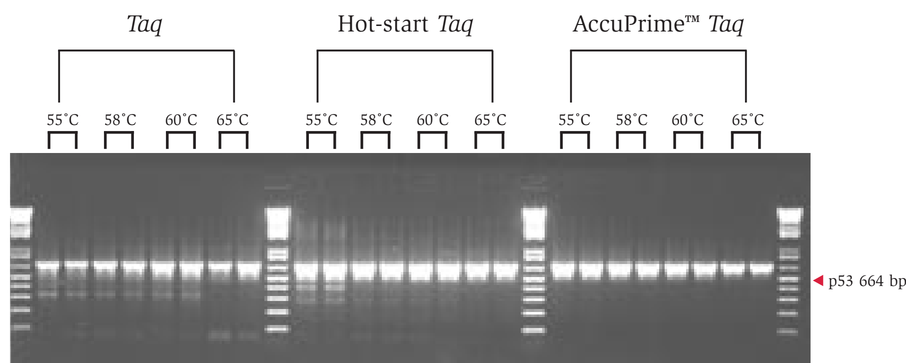
Figure 3 – Improved specificity and wide range of annealing temperature with AccuPrime™ *Taq* DNA polymerase



The arrow indicates the specific amplification product. *Taq*, automatic hot-start *Taq*, and AccuPrime™ *Taq* DNA polymerase, as well as the different annealing temperatures used, are indicated.

performed using *Taq* or automatic hot-start *Taq* produced a non-specific 1.27 kb product in addition to the 1.59 kb target (Figure 1C, lanes 1-4, page 10). The non-specific DNA fragment (1.27 kb) was gel purified and the sequence confirmed by double restriction digest assays (data not shown). These assays indicated that the 1.27 kb product was generated from a 13 bp homology sequence at the 3' end of the reverse primer. This result suggests that the AccuPrime™ accessory protein assists in specific primer-template hybridization during PCR cycles.

Statistical analysis of specificity enhancement. To demonstrate that AccuPrime™ *Taq* retains this enhanced specificity in a wide range of reactions, we compared the activities of AccuPrime™ *Taq*, recombinant *Taq*, and hot-start *Taq* in over 300 PCR amplifications. Reactions were performed using different randomly designed genomic primer sets and plasmid and cDNA, as well as linear templates. Overall, AccuPrime™ *Taq* produced higher specificity and yield than *Taq*. In addition, about 40% of the AccuPrime™ *Taq* reactions showed significant improvement and 35% showed minor improvement in specificity and/or yield compared to the hot-start *Taq*-mediated reactions (data not shown). AccuPrime™ *Taq* performance in PCR was compared to another commercially available *Taq* preparation, Ampli*Taq*™ Gold DNA polymerase with Gold buffer (Applied Biosystems, Inc.). The standard protocols for each polymerase, as indicated on product profile sheets, were followed. The same amount of enzyme, 1.5 mM Mg^{2+}, and the same annealing temperature were used in PCR. For Ampli*Taq*™ Gold DNA polymerase, the PCR reactions were heated to 95°C for 10 min. before the PCR cycling. The data in Figure 2 (page 11) clearly shows higher yields of more specific product were obtained using AccuPrime™ *Taq* than Ampli*Taq*™ Gold DNA polymerase. The non-specific products produced in the Ampli*Taq*™ reaction can be reduced or eliminated by increasing the annealing temperature and/or changing Mg^{2+} concentration. But with AccuPrime™ *Taq*, specific products are obtained without modifying or optimizing PCR conditions. We also tested the functionality of AccuPrime™ *Taq* over a wide range of annealing temperatures (Figure 3).

rpsL fidelity assay. The fidelity of AccuPrime™ *Taq* DNA polymerase was determined using the *rps*L fidelity assay. The *rps*L fidelity assay is based on the forward mutation rate of the *rps*L gene by the polymerase of interest, developed by Sekiguchi and his colleagues (5), and successfully applied to thermostable polymerases (6). Since the assay utilizes positive selection for mutation, it can detect infrequent mutations among a very large population of wild-type copies of the gene (7). The spontaneous mutation rate, a major cause of background, of the gene was measured to be 50 times lower than the mutation rate of the highest fidelity enzyme, a replicative enzyme of *E. coli* (7). *Taq* DNA polymerase (Invitrogen) was used as a control for the *rps*L assay. The error rate of

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AccuPrime™ *Taq* DNA polymerase was determined to be 1.72 x 10^{-5}. Over the course of three independent fidelity runs, AccuPrime™ *Taq* DNA polymerase showed nearly a two-fold improvement in fidelity over *Taq* DNA polymerase (Table 1). Mutant colonies were PCR amplified with *rps*L primers to verify results.

Conclusion

AccuPrime™ *Taq* DNA polymerase has automatic hot-start capabilities, as well as exceptional ability to prevent non-specific primer annealing during each PCR cycle, dramatically improving PCR specificity. Yet, AccuPrime™ *Taq* DNA polymerase is a very robust and user-friendly DNA polymerase, requiring almost no optimization compared to all other PCR polymerases tested for target sizes up to 4.4 kb. The performance of AccuPrime™ *Taq* DNA is equal to, or better than, other hot-start *Taq* DNA polymerases in every aspect examined. Based on the above features, AccuPrime™ *Taq* DNA polymerase is an ideal enzyme for demanding PCR reactions such as high-throughput applications and multiplex PCR.n

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Table 1 – Fidelity improvement using AccuPrime™ *Taq* DNA Polymerase

Enzyme	Expt.	(Colonies on Amp plate)/(Total Colonies)	(Colonies on Amp + Strep Plate)/(Volume)	TD	mf (%)	Er (E-6)	Relative Fidelity
Taq DNA polymerase	1	999 (3,996)	235 (1.2 ml)	12.3	5.88	36.8	1X
	2	906 (9,060)	420 (600 µl)	12.3	4.64	29.0	
	3	731 (14,260)	616 (600 µl)	11.6	4.32	28.6	
	Avg.			12.1 ± 0.3	4.95 ± 0.62	31.5 ± 3.57	
AccuPrime™ *Taq* DNA polymerase	1	732 (7,320)	234 (1.2 ml)	11.6	3.20	21.2	1.8X
	2	942 (28,260)	633 (600 µl)	11.6	2.24	14.9	
	3	798 (15,960)	330 (600 µl)	10.2	2.07	15.6	
	Avg.			11.1 ± 0.6	2.5 ± 0.47	17.2 ± 2.63	

TD: template doubling time, mf: mutation frequency, Er: error rate. See "Methods" for an explanation of calculations.

Generating Microarrays for Retinal Studies Using a High-throughput DNA Isolation System

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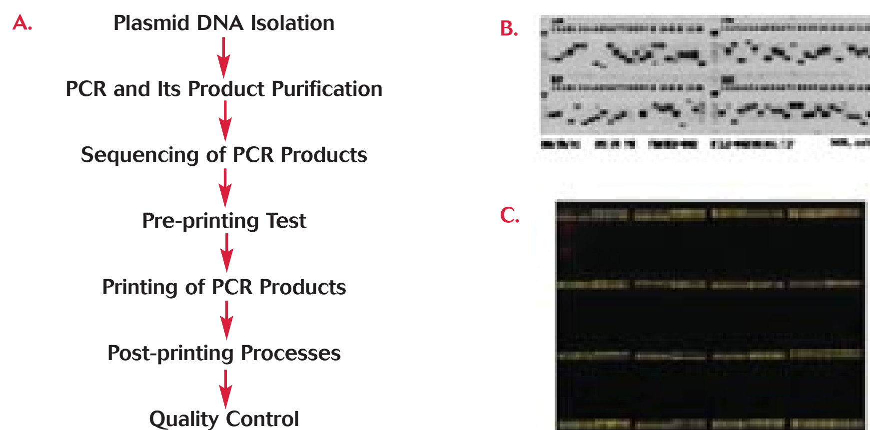
Macular Degeneration is a blinding disease that afflicts over 10 million people in the United States, and 30% by the time they are 75 years old. At the moment, we have a poor understanding of its causes and no effective treatments (1). To further our knowledge of the progression of this disease, we have generated a custom microarray starting with 9,216 sequenced mouse retinal cDNAs (2). One of the most critical steps in microarray generation is the isolation of high-quality plasmid DNA as a substrate for PCR amplification. After considering several options, we chose the Concert™ 96 system to isolate high-copy number plasmid DNA using the 96-well plate format. The system produces high-quality DNA in 45-60 minutes. In addition, the DNA performed well in subsequent steps of microarray production.

Figure 1 – Generation of PCR products for cDNA microarray printing



A. Schematic for generation of cDNA microarray. B. Image of agarose gel electrophoresis after purification of PCR products. C. Pre-printing of cDNA microarray using PCR products analyzed in Figure 1B.

Methods

Figure 1A shows the process for generating a cDNA microarray.

Starting material. A non-redundant panel of 9,216 sequenced mouse retinal cDNAs was used as starting material for microarray generation.

Plasmid isolation. DH10B cells containing plasmid DNA were grown in 96-well plates in 1.25 ml 2XYT medium containing 50 µg/µl of ampicillin. The wells were inoculated by sterilizing a pin replicator with 100% ethanol and transferring frozen stocks of each clone from a 384-well plate. After growing overnight at 37˚C (18-22 hours) at 250 rpm, each plate was harvested using Protocol B of the Concert™ 96 Plasmid Purification System (Cat. no. 12263-018, Invitrogen, Carlsbad, CA). Cells were harvested and resuspended in 200 µl Cell Suspension Buffer. They were transferred to a filter plate sitting atop a receiver plate containing 200 µl isopropanol per well. Cells were lysed with 100 µl Lysis Buffer containing RNase A for 10 min. at room temperature. The stacked plates were spun at 3,000 x g for 15 min at 4˚C. The filter plate was discarded and the filtrate poured off. The receiver plate was briefly dried with a stack of paper towels. Each well was washed with 200 µl 70% ethanol and incubated at room temperature for 2 min. The liquid was again poured off and the receiver plate spun briefly inverted to 35 x g. The plate was then allowed to air dry for 5 min. Pellets were resuspended in 25 µl TE buffer.

PCR. PCR was performed in a 96-well format utilizing 100-µl reactions. The PCR master mix consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTPs, 1 µg each primer and 2.5 units Promega *Taq* Polymerase (Promega, Madison, WI). The primers were M13/pUC forward (CGC CAG GGT TTT CCC AGT CAC GAC) and M13/pUC reverse (AGC GGA TAA CAA TTT CAC ACA GGA). Template DNA was 3 µl of Concert™ 96-isolated plasmid. PCR reactions were heated at 94˚C for 4 min., followed by 40 cycles of 94˚C for 30 s., 58˚C for 30 s., and 72˚C for 1.5 min. in a 96-well plate. The final extension was 4 min. at 72˚C.

Microarray generation. PCR products were purified and run on a 1% agarose gel stained with ethidium bromide

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(Figure 1B). After transferring the clones back into a 384-well format, slides were printed by the Yale Keck Facility on in-house poly L-Lysine coated slides utilizing a GeneMachines Omnigrid robotic arrayer (GeneMachines, San Carlos, CA) (Figure 1C, page 18).

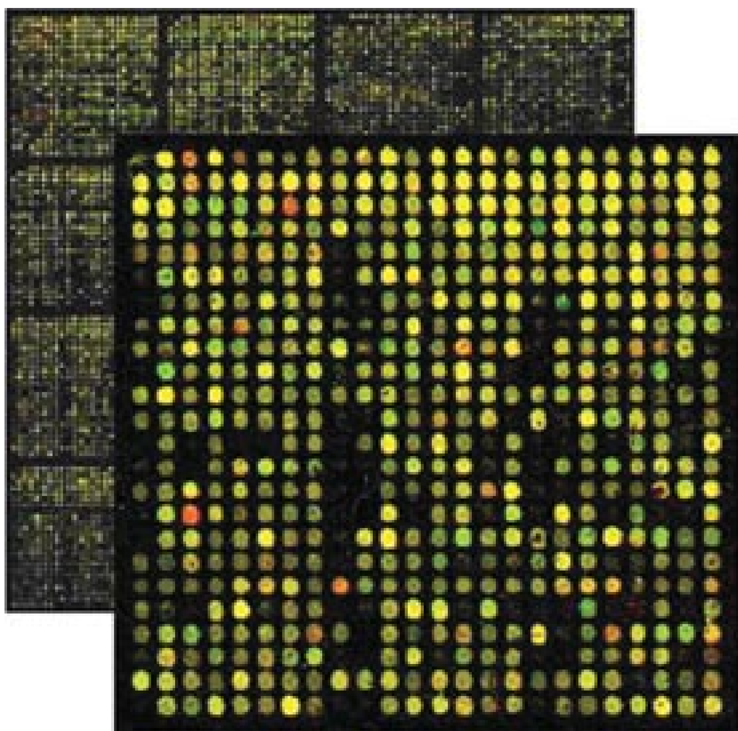
Microarray analysis. Microarrays were hybridized with probes made from mouse retina using the Genisphere cDNA Array Kit (Genisphere Inc, Hatfield, PA). Slides were scanned on a GenePix 4000A scanner (Figure 2) and the data manipulated with GenePix software (Axon Instruments, Union City, CA).

Results and Discussion

Microarray analysis is a complex process that is prone to technical difficulties if reagents and input material are not of suitable quality. Our lab has found that the quality of the input plasmid DNA can dramatically affect results. Any data that is to be analyzed downstream by microarray analysis must start with quality plasmid DNA. The Concert™ 96 Plasmid Purification System is a good choice for our microarray project because of the efficiency of the system and the quality of the DNA produced. In isolating 9,216 clones, the reproducibility of high throughput DNA isolation was imperative for our work. Since the Concert™ 96 system procedure contains no mixing or vacuum filtration steps, cross contamination was held to a minimum. This was judged at a gross level by sequence analysis of a random selection of clones and at a finer level by PCR amplification and gel analysis.

Our retinal microarrays have already given insights into changing patterns of gene expression during embryonic development. In addition to detecting the appearance of known genes at expected times in development, we have found numerous novel genes that show similar expression patterns and may thus be expressed by the same cell types or used in the same pathway. The microarrays are also helping us understand changes that can occur in retinal aging. Genes whose expression changes markedly during aging are good candidates for risk factors for degenerative diseases such as Macular Degeneration.n

Figure 2 – Retinal cDNA microarray analysis



This image shows hybridization of two probes from postnatal day 1 (PN1) and postnatal day 28 (PN28) of mouse retina. PN1 was labeled with Cy3 and PN28 was labeled with Cy5 (Genisphere Inc, Hatfield, PA). Image was scanned by Axon GenePix 4000A scanner and analyzed by GenePix Pro3 software (Union City, CA).

The Concert™ 96 Plasmid Purification System is subject to Limited Use Label License no. 47. Please refer to the Invitrogen web site or catalog for the Limited Use Label License corresponding to the number indicated.

Acknowledgements
We thank the Yale DNA Microarray Core lab for their support in this project.

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Green Fluorescent Protein as a Transcriptional Reporter in Epithelial Cells: Real-Time Studies of the Human Involucrin Promoter

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Abstract
Green Fluorescent Protein (GFP) is a popular reporter for numerous uses in mammalian cells because its activity can be measured directly without cofactors or reagents. We show that an improved GFP variant, the cycle-three GFP gene in the pGlow-TOPO® vector is useful as a reporter of mammalian promoter activity. The 5´ regulatory region from the human involucrin gene was cloned upstream of the cycle-three GFP gene and the resultant constructs transfected into C33-A epithelial tumor cells and normal keratinocytes. Fluorescence analysis whole cells showed that cycle-three GFP in pGlow-TOPO® is reliable for reporting mammalian promoter function under different conditions. GFP activity was comparable to that of CAT, indicating that this method can be used in transcriptional studies for numerous mammalian promoters.

Introduction
Transcriptional regulation studies have been greatly simplified by the development of reporter genes coding for proteins that possess a unique activity, or produce a signal, that allows the protein to be easily discernable within a complex mixture of other proteins (1). Desirable characteristics in a reporter protein include detection with high sensitivity, wide dynamic range of response, and ease of use. The Green Fluorescent Protein (GFP) was originally isolated from the jellyfish *Aequorea victoria* (2,3). The protein has been extensively used as an *in vivo* marker and for monitoring dynamic processes inside living organisms (4,5,6). The fluorescence of GFP is the result of an internal chromophore formed by the autocatalytic post-translational cyclization of three amino acids, Ser65-Tyr66-Gly67 (7,8). GFP has several characteristics that make it an excellent reporter protein. Unlike other reporter proteins, GFP does not require substrates or cofactors to emit light (8). GFP retains its fluorescence capability upon exposure to mild denaturants, heat, detergents, and proteases because of the protected location of the chromophore inside the β-barrel of the protein (8). Most importantly, GFP mutants with different fluorescent properties, including increased fluorescence intensity and shifted wavelengths of excitation and emissions have been obtained. In the present study we used the pGlow-TOPO® vector, containing the cycle-three GFP mutant described by Crameri *et al.*, 1996 (9), to establish a novel reporter system for transcriptional analysis and promoter studies in eukaryotic cells. This novel system was used to monitor transcriptional activity driven by the human involucrin 5´ non-coding region in cultured epithelial cells. The system proved to be reliable in detecting activity changes under external signals affecting involucrin promoter activity. Cycle-three GFP fluorescence correlated with the transcriptional activity of the promoter and with the number of positively transfected cells. Finally, the GFP system was compared with the CAT reporter gene. Similar sensitivity and response range were noted, making cycle-three GFP and the pGlow-TOPO® vector a useful system for future transcriptional studies.

Materials and Methods
Plasmids. The pGlow-TOPO® vector (Invitrogen, Cat. no. K4830-01) contains the cycle-three GFP mutant obtained after optimization of codon usage in *E. coli* and three cycles of DNA shuffling. This GFP variant shows a significant improvement in whole cell fluorescence signal over wild-type GFP (9). Additionally, this allele can be easily detected with UV light in a wide range of assays (4,9). pGlow-TOPO® provides a highly efficient, one-step cloning strategy for the direct insertion of promoter sequences amplified by *Taq* polymerase upstream of the cycle-three GFP gene. *Taq* polymerase has a non-template dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. pGlow-TOPO® has a single, overhanging 3´ deoxythymidine (T) residue that allows efficient ligation of PCR products. pCAT-Basic (promoterless) and pCAT-Control vectors (Promega) were used as controls in the transfection and co-transfection assays. pCAT-Control contains the CAT gene cloned upstream of the SV40 promoter and enhancer. p220CAT contains the amplified fragment from nt -156 to +43 cloned in pCAT-Basic (10).

Promoter cloning. The 5´ non-coding region from the human involucrin gene (2456 nt), previously cloned into the

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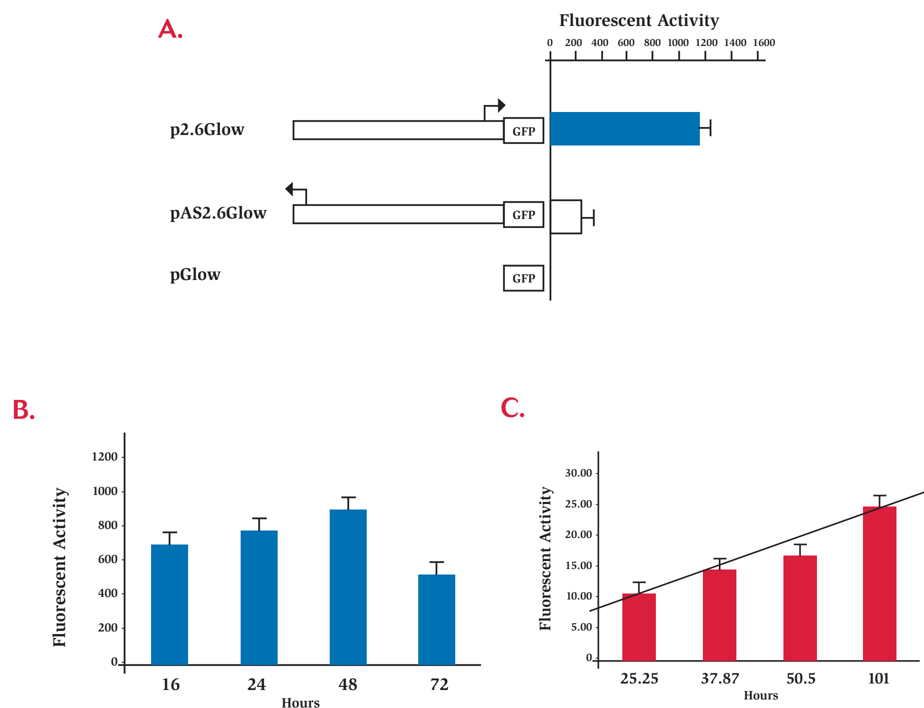
Figure 1 – Expression of cycle-three GFP from pGlow-TOPO® containing the human involucrin promoter.



A. C33-A cells were transfected with 3 µg of plasmid DNA from the sense and antisense constructs containing the involucrin promoter region (maps on the left) and harvested 48h post-transfection. B. Changes in fluorescence were recorded over time after C33-A cells were transfected with 2.5 µg of p2.6Glow plasmid and harvested at 16, 24, 48, and 72 hours. C. Correlation between the fluorescent signal and the number of GFP expressing-cells. 10^6 C33-A cells were transfected with 5 µg of p2.6Glow, harvested 48 hours post-transfection, counted, and divided in samples. In all cases, fluorescence was measured in a fluorimeter. Fluorescent activity was set by subtracting the fluorescence of the promoter less plasmid (pGlow) from the fluorescence in cells transfected with each construct and normalized by protein content. The fluorescent activity was obtained from four independent experiments.

p2.6CAT plasmid (10), was PCR amplified using the following oligonucleotides INVO2: 3´-GGG TCT AGA CAG ACT CAG AG-5´ and ENH1: 5´-CCC AAG CTT CTC CAT-3´. The oligonucleotides 220 linker 5´CCCAAGCT-TCGTACGGGGCC TAAAGGGTTGC-3´ and INVO2 were used to amplify a 220 nt fragment (-156 to +43) containing the core promoter. Amplified fragments were ligated directly into the pGlow-TOPO® vector. The resulting constructs were transformed into chemically competent DH5α™ cells. Picked colonies were cultured overnight in LB medium containing ampicillin (100 µg/ml) followed by plasmid DNA isolation. Clones were analyzed for insertion and orientation by restriction analysis. Selected constructs were sequenced using the Sequenase system (USB Corp.).

Cell culture and transient transfection assays. The cervical carcinoma cell line C33-A was cultured in D-MEM/F12 (1:1) medium plus 10% fetal bovine serum and gentamicin, penicillin, and streptomycin (final concentration 100 µg/ml, each) at 37˚C in a 5% CO_2 atmosphere. Normal multiplying human keratinocytes (HK) from human foreskins were cultured in Gibco™ Keratinocyte-SFM medium (with 50 µg/l of bovine pituitary extract, 5 µg/l Epidermal Growth Factor (EGF), 100 µg/ml each penicillin, streptomycin, and gentamicin, in a 5% CO_2 atmosphere. Differentiation-induction in confluent cultures was performed with 2 mM $CaCl_2$ Keratinocyte-SFM lacking epidermal growth factor and bovine pituitary extract. C33-A cultures, seeded in 24-well plates and 80% confluent were transfected with different plasmid DNA concentrations and/or different plasmids using the calcium phosphate method described by Ausubel *et al.* (11). Normal human keratinocytes cultures, 70% confluent in 24-well plates were transfected with 6 µg of p2.6Glow or p2.6CAT using Lipofectamine™ (Invitrogen) according to the manufacturer's protocol (10). Transfection efficiency was determined in all experiments by fluorescent microscopy using a Nikon Eclipse E600 fluorescence microscope and filter with an excitation range between 480 to 510 nm and emission in 510 nm. Fluorometric and CAT determi-

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nations were done only when transfection efficiency did not vary more than 10%.

Fluorometric assays. For GFP assays, cells were harvested at different times post-transfection in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). Whole cell pellets were re-suspended in 50 µl PBS buffer and transferred to a 96-well plate containing black wells (Labsystems). Fluorescence was measured using the Fluoroskan Ascent 374 (Labsystems) and the following filter settings: excitation at 380 nm and emission at 510 nm. Fluorescence was corrected for background activity shown by cells transfected with the pGlow-TOPO® vector alone and for the cell number between individual experiments by cell protein content determined using the Bio-Rad protein assay system.

CAT assays. CAT assays were carried out as described in López-Bayghen, *et al.* (10). Cells were harvested 48 hours post-transfection in TEN buffer (40 mM Tris-HCl pH 8.0, 1 mM EDTA, 15 mM NaCl). Protein lysates were obtained by freeze/thaw methods followed by precipitation in 0.25 M Tris buffer. Standardized amounts of protein lysates were incubated with 0.25 µCi of [^{14}C]-Chloramphenicol (50 mCi/mmol, USB corp.) and 0.8 mM Acetyl-Co-A at 37˚C. Acetylated forms were separated by thin-layer chromatography and quantitated using an AMBIS 4000 radioactive image analyzer (Scanalytics). CAT activities were expressed as the acetylated fraction corrected for activity in pCAT-Basic.

Results

To characterize the level of fluorescence produced by the cycle-three GFP/in pGlow-TOPO® reporter, we selected the previously characterized involucrin promoter (10). The cervical carcinoma cell line C33-A was transfected with the following constructs containing the regulatory region from the human involucrin gene upstream of cycle-three GFP: p2.6Glow (5´-3´, sense), pAS2.6Glow (3´-5´, antisense), and the pGlow-TOPO® empty vector (without promoter). Initially, cells were transfected with 3 µg of plasmid DNA and harvested 48 hours post-transfection. Total fluorescence activity was determined by subtracting the background fluorescence produced by cells transfected with the promoterless plasmid (pGlow-TOPO®) from that produced by cells transfected with a promoter-containing construct. Only the p2.6Glow construct, with the promoter inserted in the sense orientation, demonstrated significant transcriptional activity (Figure 1A, page 17). Activity from antisense pAS2.6Glow was clearly lower, indicating that the fluorescent activity is dependent on the functionality of the promoter. A time-course study was conducted to determine when fluorescence activity peaked. C33-A cells were transfected with 3 µg of p2.6Glow plasmid (Figure 1B, page 17). Fluorescence peaked 48 hours post-transfection and decreased after 72 hours, as is usual in transient experiments. In order to determine the minimal number of cells needed to yield a measurable fluorescent signal, cell cultures in a 60 mm dish format were transfected with 5 µg of p2.6Glow, counted with a hemocytometer (Neubauer), and resuspended in PBS buffer prior to loading into a 96-well plates to measure fluorescence (Figure 1C, page 17). The lower detection limit was determined to be about 252,500 cells. The fluorescent signal was plotted against the cell number and it proved to be linear. Under the same transfection efficiency (determined by fluorescence microscopy), an increase in GFP expressing cells correlated with fluorescent measurements.

To determine the optimal DNA concentration needed to detect fluorescent activity and to make a comparison between the CAT reporter and this novel reporter system, we co-transfected C33-A cells with different concentrations of the p2.6Glow and pCAT-Control vectors. Cells were harvested 48 hours post-transfection and assayed for fluorescence and CAT activity. The results (Figure 2A, page 19) show that DNA amounts between 3 and 5 µg of DNA are sufficient to produce fluorescence. Both reporters demonstrated increased activity as DNA concentration increased. We also tested the same promoter directing both reporters under the same conditions (Figure 2B, page 19). For this purpose we transfected normal human keratinocytes with p2.6Glow, p2.6CAT, p220Glow, and p220CAT constructs under multiplying and calcium-induced differentiation conditions. Only plasmids containing the complete involucrin regulatory region (2.6 kb) displayed similar 2-fold activation once the cells were induced to

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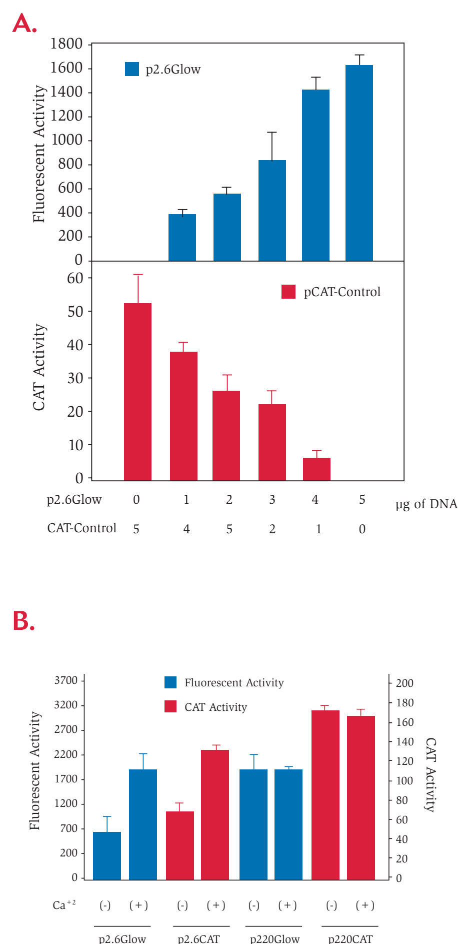
Figure 2 – Comparative activity of GFP and CAT reporters in transfection assays



A. C33-A cells were co-transfected with p2.6Glow and pCAT-control plasmids. Cells were harvested 48 hours post transfection. Average fluorescence and CAT activity were obtained from three independent experiments.
B. The activity of GFP and CAT genes under the control of the same promoter (the involucrin promoter) is shown. Normal human keratinocytes cultures were transfected with 6 µg of p2.6Glow, p220Glow, p2.6CAT or p220CAT plasmids. Calcium-induced differentiation in keratinocytes was done switching to 2 mM $CaCl_2$ medium 12 hours after transfection. Cells were harvested 48 hours post-transfection and fluorometric and CAT assays were carried out. Average fluorescence was obtained from four independent experiments and for CAT activity from six independent experiments.

differentiate by switching to 2 mM $CaCl_2$. This condition has been reported before as a stimulus for involucrin transcription (10), proving that the cycle-three GFP in pGlow-TOPO® reported here is equally sensitive as CAT for registering transcriptional changes in a defined promoter.

Discussion

We established a novel transcriptional reporter system using the cycle-three GFP gene as a reporter of relative promoter activity in quantitative assays. Through a number of experiments we have proven that cycle-three GFP displays sensitivity and dynamic range equivalent to CAT. The fluorescence assays developed here are rapid, simple, and sensitive. They allow quantitation of relative promoter strength over an activity range comparable to that of CAT for assessing promoter activity. Fluorometric detection of GFP does not require the use of substrates or cofactors to emit light. Furthermore, GFP can be detected in a non-destructive manner. We show here that it is not necessary to prepare cell extracts, making GFP useful for the study of tissue-specific gene expression in intact tissues or in organotropic cultures. A simple fluorometric assay is used to measure GFP fluorescence in whole cells. Since mammalian cells do not possess an activity similar to that of GFP, the sensitivity of the system is only limited by the "background noise" of the fluorometer. This assay permits the use of a small number of cells because of the increased sensitivity, being less expensive and less time-consuming than other systems and allowing data acquisition in a matter of minutes rather than hours or days. One drawback of this reporter gene system is that a fluorometer is a relatively expensive and uncommon laboratory instrument. Fortunately, fluorescence can be visualized by confocal and fluorescence microscopy, providing a useful method for determining transfection efficiency as well.

The cycle-three GFP in the pGlow-TOPO® reporter system is also useful for determining the level of promoter response to external stimulus in the same range shown in a traditional CAT assay. We think all of the advantages of this novel system, as described in this article, will improve transcriptional regulation studies. n

Acknowledgments

This work was supported by a grant from CONACyT-Mexico (30679-M) to E.L.B. A.G.C was supported by a CONACyT fellowship. We thank Dr. Luis Alvarez-Salas for critical reading of the manuscript and Gerardo Marmolejo for technical assistance.

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Mammalian Two-Hybrid System With TOPO® Tools Technology: A Fast Method for Generating Expression Cassettes for Protein-Protein Interaction Assays

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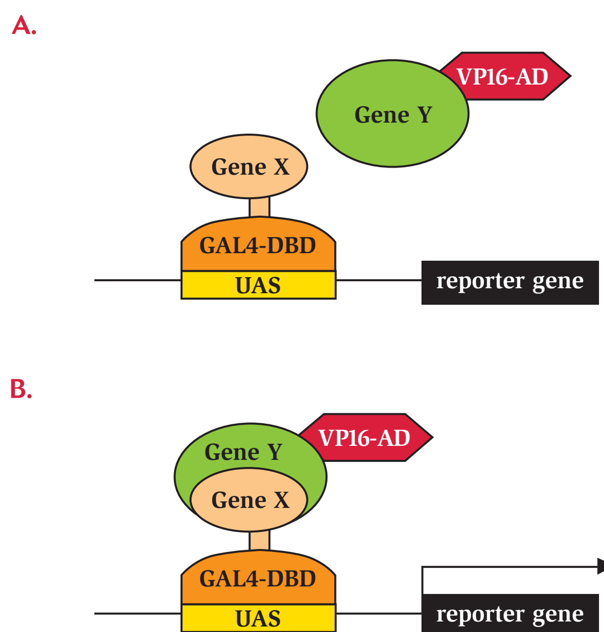
Abstract

TOPO® Tools enables rapid construction of linear nucleic acid cassettes for use in a variety of applications ranging from RNA generation to the production of native and fusion proteins. To use the system, gene sequences are first PCR-amplified with primers that introduce a topoisomerase recognition site and linker-specific sequences upstream and downstream of the coding sequence. The PCR products are then mixed with TOPO® Tools elements, which are functional nucleic acid sequences (promoters, fusion tags, termination sequences, etc.) containing bound topoisomerase. The topoisomerase enzyme catalyzes joining of the functional elements to the gene sequence in a rapid (5-10 min.) reaction at room temperature. The resultant joined products are then amplified in a second PCR reaction to produce functional linear cassettes that are directly used for downstream applications. This novel approach circumvents the need to pass the recombinant constructs through a bacterial host, greatly reducing the time required to perform expression analysis. Here, we present the assay data for mammalian two-hybrid studies performed with TOPO® Tools-generated expression cassettes and with supercoiled plasmids. We show that comparable data are obtained from both versions of DNA.

Figure 1 – The principle behind mammalian two-hybrid systems



Gene X is cloned downstream of a DBD to form a bait construct. Gene Y is cloned downstream of an AD to form a prey construct. Bait, prey, and reporter constructs are co-transfected into a mammalian cell line. If protein X does not interact with protein Y, the reporter gene will be silent (A). If protein X interacts with protein Y, the AD is brought together with the DBD, which will turn on the reporter gene (B).

Introduction

Protein-protein interactions play a central role in various aspects of the structural and functional organization of the cell. The two-hybrid system has been one of the most powerful genetic techniques in identifying and characterizing protein-protein interactions (1). In this system, a transcription factor is functionally and physically divided into a transcriptional activation domain (AD) and a DNA binding domain (DBD). If these domains are reconstituted *in trans*, their activity is restored. To study a protein-protein interaction, one protein of interest is expressed as a fusion to the AD, and another protein is expressed as a fusion protein to the DBD. The vectors that express these fusion proteins are cotransfected into the designated host with a reporter plasmid. If these fusion proteins interact, there will be a significant increase in the expression of the reporter gene (Figure 1).

Historically, two-hybrid experiments have been performed in yeast. However, there are times when it is advantageous to perform studies in mammalian cells (2,3). In mammalian cells, mammalian proteins are more

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likely to retain their native conformation, and the results are more likely to mimic the *in vivo* situation.

Standard techniques for two-hybrid systems require cloning genes of interests into vectors downstream of a DBD and/or an AD. After transformation, plasmid constructs are purified from bacteria. These procedures are time-consuming and labor-intensive. Therefore, there is a continued need for improved methods to generate expression cassettes. Here, we present the Mammalian Two-Hybrid System with TOPO® Tools Technology, a new method for constructing expression cassettes without cloning. The entire procedure takes less than one day. In this article, we present mammalian two-hybrid assay data with TOPO® Tools-generated expression cassettes.

Materials and Methods

TOPO® Tools procedure. The procedure for TOPO® Tools is illustrated in Figure 2. Briefly, bait and prey genes were first amplified with primers containing gene-specific sequences and TOPO® Tools-specific overhangs. The PCR products were mixed with the topoisomerase-charged or P_{SV40}/GAL4 (bait) or P_{SV40}/VP16 (prey) 5´elements, or the SV40 pA 3´ element from the Mammalian Two-Hybrid Kit with TOPO® Tools Technology (Cat. no. T501-100). Topoisomerase catalyzes joining of the functional elements to the gene sequences in a rapid (10 min.) reaction at room temperature. The resultant joined products were amplified with

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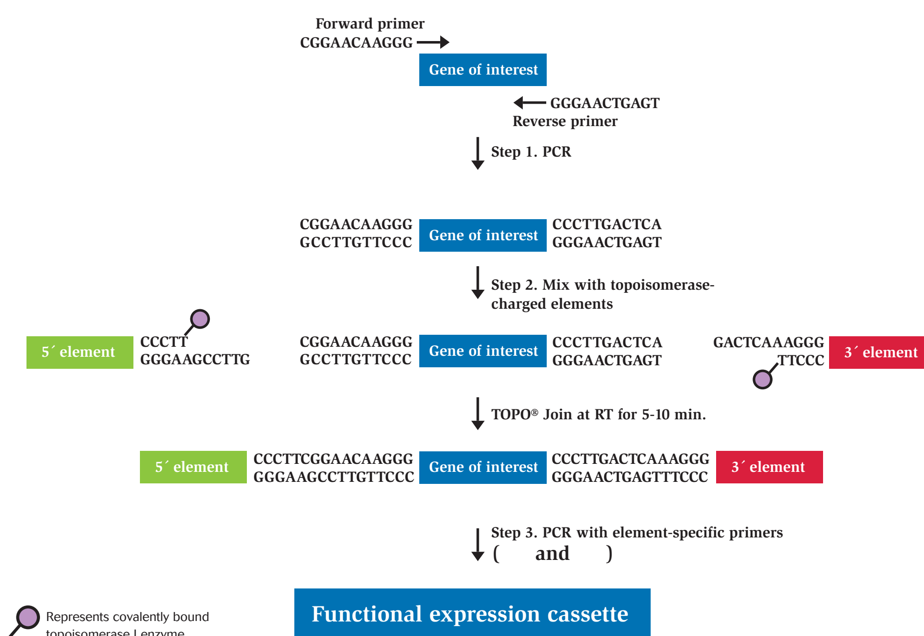
Figure 2 – Flowchart outlining the construct of expression cassettes with TOPO® Tools



Step 1. A gene of interest is PCR amplified with primers containing gene-specific sequences and an 11 nt sequence at each 5´ end for topoisomerase binding and directionality. Step 2. The PCR-generated fragment is mixed with topoisomerase-charged 5´ and 3´ elements at RT for 5-10 min. Topoisomerase directionally joins these three fragments together. Step 3. Joined products are used as templates to generate functional expression cassettes with a 5´ element-specific forward primer and a 3´ element-specific reverse primer.

Table 1 – Cell number and DNA concentrations used in the mammalian two-hybrid assay

	6-well	12-well	24-well	96-well
cell/well	$2.0x10^5$	$1.0x10^5$	$0.5x10^5$	$3.0x10^3$
plasmid prey/well	0.5 µg	0.25 µg	0.125 µg	15 ng
plasmid bait/well	0.5 µg	0.25 µg	0.125 µg	15 ng
reporter/well (for plasmid bait/prey)	0.4 µg	0.2 µg	0.1 µg	12.5 ng
TOPO® Tools prey/well	0.3 µg	0.15 µg	0.075 µg	10 ng
TOPO® Tools bait/well	0.3 µg	0.15 µg	0.075 µg	10 ng
reporter/well (for TOPO® Tools bait/prey)	0.8 µg	0.4 µg	0.2 µg	30 ng

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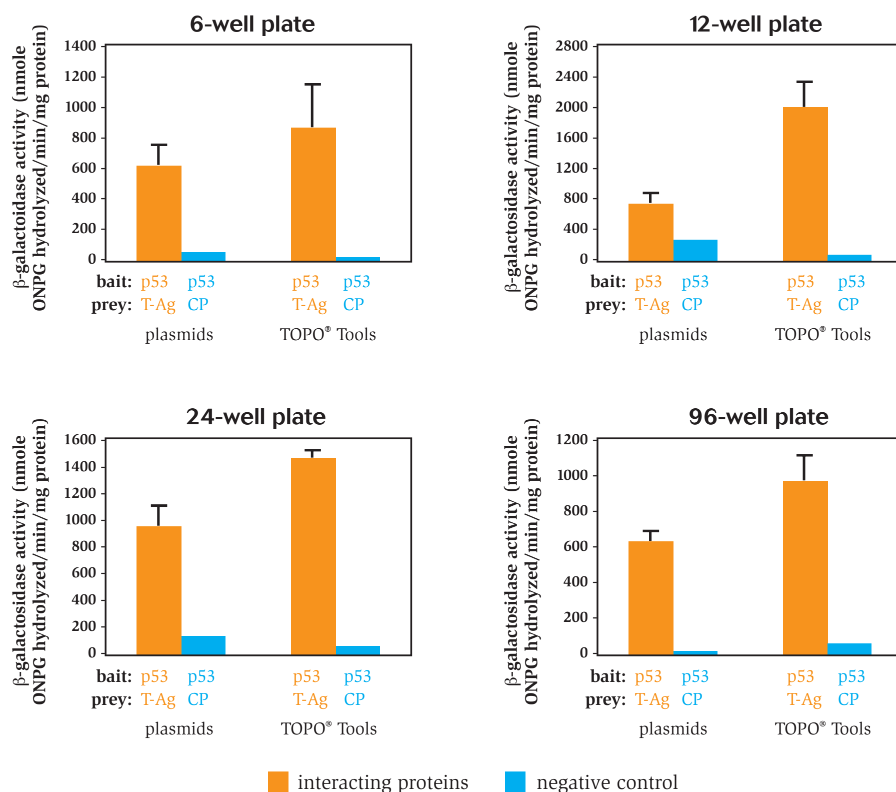
Figure 3 – Comparison of mammalian two-hybrid assay data obtained with TOPO® Tools-generated expression cassettes and with supercoiled plasmids in 6-, 12-, 24-, and 96-well format



element-specific primers to generate expression cassettes.

TOPO® Tools expression cassettes construction. Genes and expression cassettes were amplified in 100 µl volumes containing 5 units of Platinum® *Taq* DNA polymerase High Fidelity (Cat. no. 11304-011), 1X PCR buffer, 2 mM $MgSO_4$, 200 µM dNTP, 100 ng of each primer under the following conditions: 94˚C for 4 min. followed by 30 cycles of 94˚C for 30 s., 55˚C for 30 s., 68˚C for 1 min./kb. PCR-amplified DNA was purified using the S.N.A.P.™ MiniPrep Kit (Cat. no. K1910-01). DNA was electrophoresed on a 1.2% E-Gel® gel (Cat. no. G5018-01) to monitor the amplification efficiency.

Mammalian two-hybrid assay. Cell number and prey/bait/reporter molar ratio used for mammalian two-hybrid assays are listed in Table 1 (page 21). Chinese hamster ovary (CHO) cells were seeded into cell culture plates (Corning) one day prior to the experiment, resulting in 50-80% confluence at the time of the transfection. Transfection was performed with Lipofectamine™ 2000 (Cat. no. 11668-027). Lysates were prepared 48 hrs. after transfection. The β-Gal Assay Kit (Cat. no. K1455-01) was used for the reporter activity assay according to the manufacturer's instructions. Briefly, cells were scraped from the wells in lysis buffer. Supernatant (10 µl) was incubated with 50 µl of 1X Cleavage Buffer with β-mercaptoethanol and 17 µl of ONPG for 5 minutes at room temperature. Following the addition of 125 µl of STOP Buffer, the signal was measured at 420 nm. Data were normalized with protein concentrations measured using the standard Bradford method.

Results and Discussion

Vaccinia topoisomerase I can nick and religate single-stranded DNA in a DNA duplex. The 3´ fragment of the cleaved strand will dissociate from the DNA duplex if it contains less than seven base pairs (4). These properties were used to develop TOPO® Tools technology to create functional cassettes (Figure 2, page 21). The fragment upstream of the gene of interest (GOI) was named the 5´ element and the fragment downstream of the GOI was designated the 3´ element. These elements can be promoters, polyadenylation signals, detection tags, etc. To generate enough functional expression cassettes for downstream applications, the joined DNA was used as a template for PCR amplification.

In this study, we generated bait and prey expression cassettes using the

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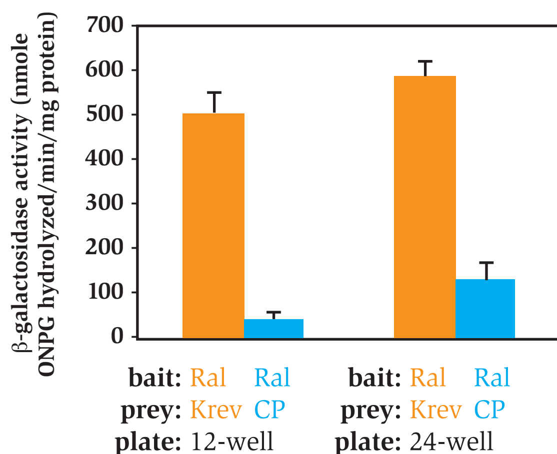
Figure 4 – Krev-RalGDS interactions with TOPO® Tools generated expression cassettes



TOPO® Tools Technology. The bait cassette was generated by joining PCR-amplified p53 gene (or RalGDS) to an SV40 promoter + GAL4 DBD 5´ element and a SV40 pA 3' element. The prey cassette was obtained by joining PCR-amplified large T antigen (or Krev) to an SV40 promoter + VP16 AD 5´ element and an SV40 pA 3´ element. To show that TOPO® Tools-generated elements perform equivalently to vector-based systems, the p53 and large T antigen genes were cloned into a vector-based mammalian two-hybrid system. As a negative control, we generated a prey cassette with a viral coat protein (CP) that does not interact with p53 or RalGDS.

Prey and bait DNA were cotransfected into CHO cells with a reporter plasmid containing the *lacZ* gene. β-gal activity was measured from lysates 48 hrs. after transfection. The signals obtained with TOPO® Tools expression cassettes were compared to the signals obtained with supercoiled plasmids. In both cases, the same transfection conditions were used. Prey/bait/reporter molar ratios were optimized individually for supercoiled plasmids and TOPO® Tools expression cassettes (data not shown). As shown in Figure 3 (page 22), the reporter gene expression in cells containing the positive interactors (p53-T antigen) was at least several fold higher than cells containing non-interacting p53 and CP. A second known protein-protein interaction, Krev-RalGDS, was also tested. We generated Krev bait and RalGDS prey cassettes with TOPO® Tools. As shown in Figure 4, positive interactions were also detected with TOPO® Tools expression cassettes.

Compared to traditional mammalian two-hybrid systems using supercoiled plasmids, TOPO® Tools has several advantages. First, no cloning, transformation into *E. coli*, and DNA preparation steps are needed. Second, using a pool of DNA for downstream applications avoided concern that particular PCR products carry mutations. The TOPO® Tools technology is a flexible method to generate expression cassettes. We have used this technology to generate constructs for mammalian expression with different tags, mRNA synthesis, and *in vitro* protein synthesis by joining the same PCR product to different 5´and 3´elements. In addition, the TOPO® Tools procedure yields results in a few days versus one or more weeks following traditional cloning procedures The Mammalian Two-Hybrid System with TOPO® Tools Technology enables quick generation of constructs for use in testing protein-protein interactions in mammalian cells.n

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A Versatile Serum-Free Medium For Kidney Epithelial Cell Growth and Virus Production

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The major discovery that poliovirus could be grown in cell cultures of non-neural origin, including kidney epithelial cells (1), changed the methodology for producing viral vaccines. As kidney cell lines were introduced and virus growth demonstrated, the use of primary cells gradually gave way to the far less expensive and more easily manipulated kidney epithelial cell lines (2,3). Traditionally, these cell lines were grown in basal media supplemented with 5% to 15% animal serum. An improvement in cell growth systems was the introduction of Opti-MEM® I, which allowed for a reduction in serum supplementation to <2% (4).

The challenge was, therefore, to develop a serum-free cell culture medium (SFM) that greatly reduced the risk of viral or prion contamination by eliminating all components of animal or human origin. Protein-free and ultra-low protein media containing no animal-derived proteins typically have exhibited relatively narrow utility in that such formulations have been optimal for the growth of only one or two cell lines (2) and have (with the exception of VP-SFM) been limited to the growth of attachment-independent cells (5). OptiPRO™ SFM is a unique medium that supports the growth of multiple attachment-dependent cell lines without the addition of attachment proteins to the medium or pretreatment of the attachment surface. The medium has also been demonstrated to support virus growth equivalent to titers seen with serum-supplemented media.

Materials and Methods

Cell lines. All cell lines were originally from the ATCC and were tested and found to be free of mycoplasma, bacteria, or fungal contamination. Stock cultures were originally grown in MEM w/Earle's salts (EMEM) supplemented with 10% FBS. The cultures were adapted for growth in OptiPRO™ SFM (Cat. no. 12309-019) by sequential reduction of serum over 1 to 6 subcultures (cell line dependent) and subsequent stock cultures were maintained in OptiPRO™ SFM. Some cell lines, such as canine kidney (MDCK) and the SV40-transformed African Green monkey kidney (COS-7), adapted immediately to the new medium, while others, such as bovine kidney (MDBK), baby hamster kidney (BHK-21), African Green monkey kidney (VERO), and porcine kidney (PK-15), required more extensive sequential adaptation.

Procedure for preparing Cell Factories. Stock cultures were grown in 162 cm^2 plastic flasks in the respective test and reference media. Stock cultures and Cell Factories (Nunc) were harvested when confluency reached 70-80%. A 2X volume of Soybean Trypsin Inhibitor (Gibco™) was used to neutralize the activity of the trypsin:EDTA (Gibco™) and the cell suspensions were centrifuged at 100 x g for 5 minutes. The cell pellet was washed once in basal media and the wash medium was discarded. The cells were resuspended in their respective media and the total cell count determined using a Coulter Z_2. Viability was determined by trypan blue exclusion. One day prior to cell inoculation, duplicate Cell Factories were washed with DPBS (Gibco™) and then preconditioned with 200 ml of respective media overnight at 37˚C in 5% CO_2 in air. The Cell Factories were then inoculated with 1.2-1.5 x 10^4 viable cells/cm^2 (7.6-9.6 x 10^6 cells/Cell Factory). When the cultures were 60% to 80% confluent

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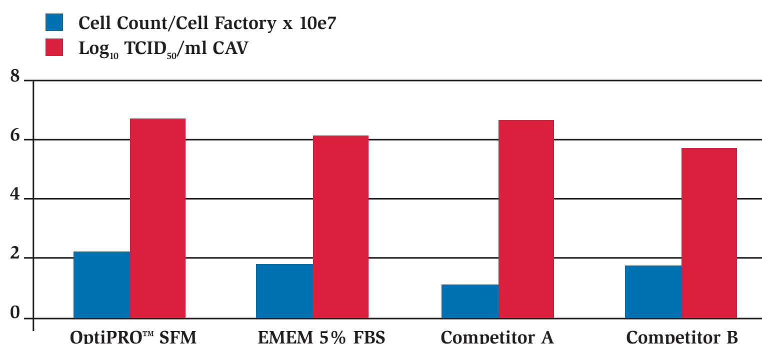
Figure 1 – MDCK cells grown in Cell Factories



The bar graph compares the growth of MDCK cells in OptiPRO™ SFM and other media formulations in Cell Factories and the log_{10} of the $TCID_{50}$ for CAV in the various cell/media systems.

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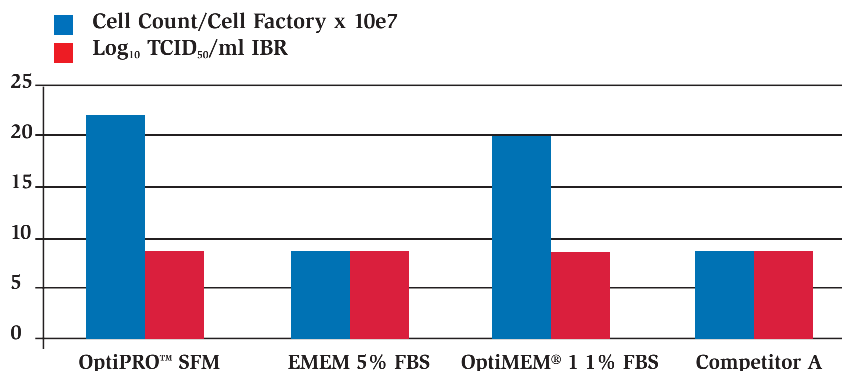
(2 to 4 days later), one of each pair was harvested for cell count and the second Cell Factory inoculated with virus.

Procedure for preparing shaker flasks for BHK-21 cells. BHK-21 cells adapted to suspension growth were grown in duplicate in 125 ml plastic shaker flasks (Corning) at a plating concentration of 1.2 x 10^5 cells/ml and 30 ml per flask (3.6 x 10^6 viable cells per flask). The test (OptiPRO™ SFM) and serum-supplemented control (EMEM supplemented with 5% FBS) media were further supplemented with 4 mM L-glutamine and 0.2% of a 10% solution of Pluronic F68 (Gibco™). The caps were left loose and the cultures agitated at 125 rpm on an orbital shaker in an 8% CO_2 in air, humidified incubator. Cultures were inoculated with virus on the third day.

Protocol for virus titrations. Virus stocks were originally received from either the Diagnostic Laboratory, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York, or the National Veterinary Services Laboratory, USDA, APHIS, Ames, Iowa. The Cell Factories were rinsed 3X with respective media. Cultures were then inoculated with virus at a multiplicity of infection (MOI) of 0.1 (1 virus per 10 cells) in ≅40% of original volume and placed on a rocker platform for 2 hours. The suspension cultures (BHK-21) were spun down at 800 rpm for 8 minutes and the cells reconstituted in the shaker flasks at half volume, inoculated at 0.1 MOI, and the flasks placed back on the shaker and agitated for 2 hours. The medium was then added back to original volume (both with the Cell Factories and shaker flasks) and the cultures observed daily for cytopathic effect (CPE). When the serum control showed 90% CPE, the cultures were frozen and thawed 3X and the supernatant centrifuged at 300 x g for 10 minutes at +4°C and passed through a 0.45 µm filter. Viruses were titered using 10-fold serial dilutions with 7 wells per dilution in 96-well microtiter plates in the respective cells grown in EMEM with 5% FBS. The time of incubation was dependent on the virus/cell system used. $TCID_{50}$ is defined as the dilution of virus required to kill 50% of the inoculated cultures and was calculated using a standard procedure for determining the endpoint (6).

Figure 2 – MDBK cells grown in Cell Factories



The bar graph compares the growth of MDBK cells in OptiPRO™ SFM and other media formulations in Cell Factories and the $\log_{10}$ of the $TCID_{50}$ for IBR in the various cell/media systems.

Results

The results presented compare cell growth in a single-tier Cell Factory (632 cm^2) or 125 ml shaker flasks (for BHK-21 cells) to the virus titer. Cell growth is expressed as number of cells x 10^7 and virus titer as the $\log_{10}$ $TCID_{50}$/ml.

Growth rate of MDCK cells. Figure 1 (page 24) compares both MDCK cell growth and Canine Adenovirus (CAV) production in cells grown either in OptiPRO™ SFM, a serum-supplemented control, and two commercially available formulations (an MDCK specialty medium containing no animal derived proteins and an MDCK specialty SFM containing serum protein). As shown in Figure 1, the growth rate of MDCK cells in the OptiPRO™ SFM exceeded that of the protein-free SFM, the serum control, or the SFM containing serum protein. The titer of CAV at day 2 was equivalent to, or better than, the serum or the other control media.

Growth rate of MDBK cells. The growth rate of the MDBK cells in OptiPRO™ SFM significantly exceeded that of both the serum control and a commercially available MDBK specialty SFM containing serum protein. The growth rate of the MDBK cells in OptiPRO™ SFM was only

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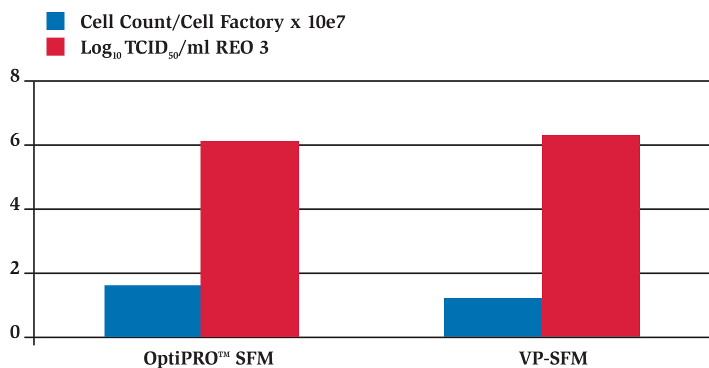
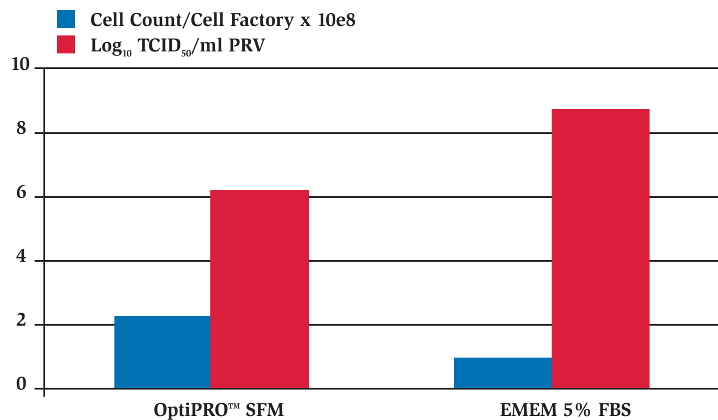
Figure 3 – VERO cells grown in Cell Factories



The bar graph compares the growth of VERO cells in OptiPRO™ SFM and VP-SFM in Cell Factories and the $\log_{10}$ of the $TCID_{50}$ for REO 3 in both cell/media systems.

Figure 4 – PK-15 cells grown in Cell Factories



The bar graph compares the growth of PK-15 cells in OptiPRO™ SFM and the serum control in Cell Factories and the $\log_{10}$ of the $TCID_{50}$ for PRV in both cell/media systems.

slightly better than the same cells grown in Opti-MEM® I with 1% FBS. Infectious Bovine Rhinotracheitis (IBR) titers at day 6 were equivalent (Figure 2, page 25). The high cell counts are due to the lack of contact inhibition with MDBK cells.

Our MDBK cells did require an adaptation period to select away from cell clumping. This was accomplished by plating the cells in OptiPRO™ SFM and incubating the culture for about 2 hours at 37˚C in a 5% CO_2 in air incubator, removing poorly adhering cells by the gentle agitation of the medium and then replacing the medium with fresh medium. This was repeated for three subcultures.

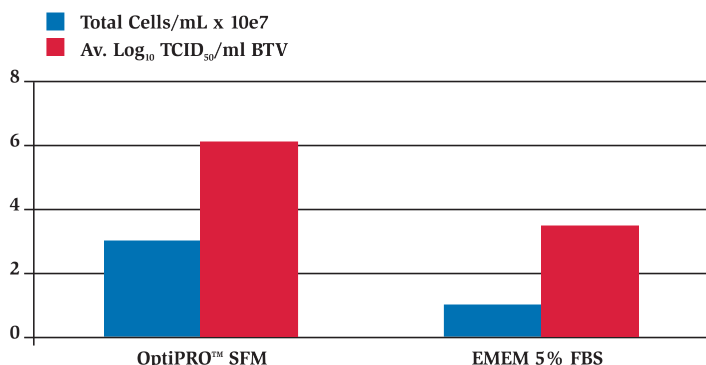
Growth rate of VERO cells. With VERO cells, VP-SFM (5) was used as the control medium. VP-SFM is a specialty medium for the growth of VERO cells and is also free of any components of animal or human origin. In this case, the growth rate of the cells and titer of REO 3 at day 2 were equivalent (Figure 3). In experiments using flask cultures, both cell growth and virus titer for OptiPRO™ SFM and VP-SFM were equivalent to, or slightly lower than, cell growth and virus titer in the VERO cells grown in EMEM supplemented with 5% FBS (data not shown). VERO cells required a short adaptation period (1-2 subcultures) for growth in OptiPRO™ SFM from VP-SFM and longer (4-6 subcultures) if being adapted away from serum-supplemented media.

Growth rate of PK-15 cells. In contrast to the other cell lines grown in the

continued on page 27

continued from page 26

Figure 5 – BHK-21 cells grown in shaker flasks



The bar graph compares the growth of BHK-21 cells in OptiPRO™ SFM and the serum control in shaker flasks and the $\log_{10}$ of the $TCID_{50}$ for BTV in both cell/media systems.

Cell Factories, the virus titer of Pseudorabies Virus (PRV) in PK-15 cells lagged behind that of the serum control. In this case, however, cell growth was significantly higher (Figure 4, page 26). Flask experiments (data not shown) showed equivalent virus titers of PRV in PK-15 cells grown in either OptiPRO™ SFM or the serum control medium. It is important, therefore, to develop your production protocol to optimize both cell growth and virus production with respect to the specific cell/virus system being utilized.

Growth rate of BHK-21 cells. Both the growth rate for BHK-21 cells and the titer of Blue Tongue Virus (BTV) in OptiPRO™ SFM in the shaker flasks significantly exceeded that of the serum control (Figure 5).

Virus Production. MDCK cells grown in 25 cm^2 flasks in duplicate were inoculated with CAV stocks that had been grown in either EMEM with 5% FBS or in OptiPRO™ SFM. Both cultures grown in EMEM with 5% FBS and those that had been grown in OptiPRO™ SFM were inoculated with either virus stock and the cells again harvested on days 3, 4, 5, and 6 post-inoculation and the virus titered (Figure 6, page 28). Virus stocks grown in OptiPRO™ SFM performed equivalent to stocks of the same virus grown in serum-supplemented media.

Discussion

Numerous problems plague the use of cell culture media containing components of animal origin (7,8). Not the least of these is the difficulty in proving that products produced in cell culture are free of mammalian viruses such as FMD virus and prions such as BSE (Bovine Spongiform Encephalopathy). Outbreaks of BSE or "Mad Cow Disease" in Europe and the association in humans of CJD (Creutzfeldt-Jakob disease) from ingestion of beef contaminated with BSE, have heightened the awareness of the risk to biologicals, such as recombinant proteins and viral vaccines, produced in living cells grown in the presence of animal-derived proteins. This in turn has increased regulatory pressure to ensure that nutrient media and other cell culture reagents are indeed free of these contaminants (9). One solution would be to produce the proteins or vaccines in systems completely free of any animal-derived components.

As serum plays multiple roles in cell growth, including cell attachment, the ability to replace serum with non-animal derived components is not trivial. The ability of OptiPRO™ SFM to allow for the growth of multiple attachment-dependent cell lines in an ultra-low protein medium that is free of animal origin components, and at a growth rate comparable to the same cells grown in a serum-supplemented medium, may be unique. OptiPRO™ SFM contains only 7.5 µg/ml of recombinant protein and does not require the addition of attachment proteins or manipulation of surface charge. This requirement was accomplished by designing the medium to allow the cells to rapidly produce their own attachment proteins. The growth of each cell line over a specific time course is, therefore, cell-specific and dependent on the rate of synthesis for that cell line for their attachment proteins. As cell attachment may lag behind that seen with serum-

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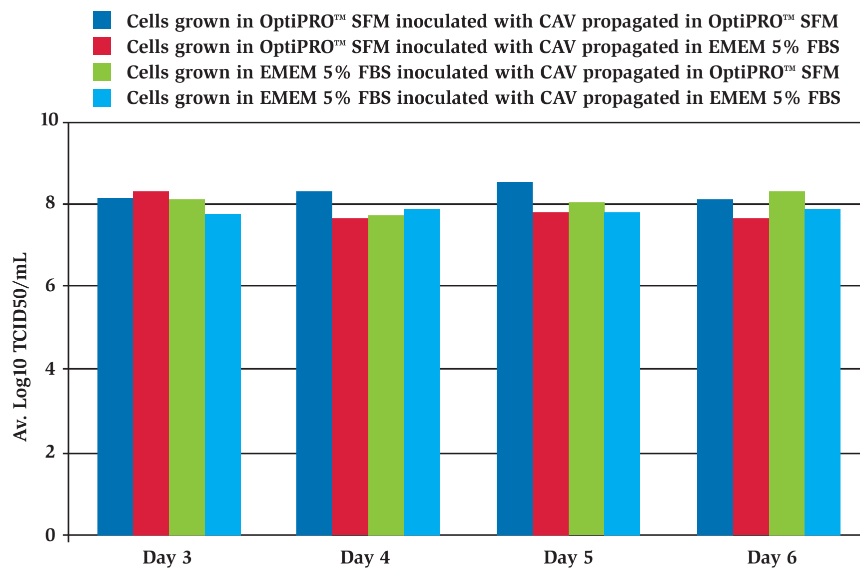
supplemented media, the timing for virus inoculation and harvest must be determined for each virus/cell system.

In summary, the studies presented demonstrate that a medium devoid of any components of animal or human origin has been developed, which supports the growth of numerous attachment-dependent cell lines without the need for the addition of attachment proteins to the medium or pre-treatment of the attachment surface. The growth of four of the kidney cell lines studied at either laboratory scale and pilot/production scale (MDCK, PK-15, BHK-21, and MDBK) in OptiPRO™ SFM exceeded that of the growth in the serum-supplemented control medium over the same incubation period. The growth of a fifth cell line (VERO) was equivalent to that of the specialty SFM control medium. With the exception of PK-15, virus production in the Cell Factory was equal to or exceeded that seen with the same cells grown in serum-supplemented media. Model virus titers were obtained from these cells for Canine Adenovirus (CAV), Infectious Bovine Rhinotracheitis (IBR), Pseudorabies Virus (PRV), Blue Tongue Virus (BTV), and REO 3. Since the medium contains no components of animal (including human) origin, the risk of culture contamination by viruses or prions is greatly reduced.n

Acknowledgements

We thank Mark Plavsic, Mark Seep, Mark Stramaglia, Jim Mecca, Eric Cornavaca, Maureen Cook, Susan Genier, and John Lowery for their roles in the development of OptiPRO™ SFM.

Figure 6 – Viral growth kinetics with MDCK, CAV propagated in OptiPRO™ SFM vs. CAV propagated in EMEM with 5% FBS



MDCK cells in 25 cm^2 flasks in duplicate were inoculated with CAV stocks that had been grown in either EMEM with 5% FBS or in OptiPRO™ SFM. Both the cultures grown in EMEM with 5% FBS and those that had been grown in OptiPRO™ SFM were inoculated with either virus stock and the cells again harvested on days 3, 4, 5, and 6 post-inoculation and the virus titered. The bar graph shows the daily titers as the average log_{10} $TCID_{50}$/ml.

OptiPRO™ SFM is for research use only and is not intended for human or animal diagnostic, therapeutic, or other clinical uses.

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