

# Postings Winter 1998

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*“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.”*

*Sir William Bragg*

## Welcome

Welcome to the first issue of PanVera's new newsletter – *Postings*. You will find scientific information that you can use in your everyday work as well as reports on new discoveries and products from PanVera. We hope *Postings* will be a publication you look forward to receiving. If you have questions, comments or contributions, please contact us at (608) 233-5050 or e-mail at [info@panvera.com](mailto:info@panvera.com).

*James E. Hagstrom, Lisa J. Hanson and Jon A. Wolff*  
Mirus Corporation

### Introduction

The delivery of exogenous genes into cells in culture has become the primary means by which researchers study both gene product function and promoter and enhancer function. In many cases a reporter gene such as luciferase or beta-galactosidase is used to quantitatively assay gene expression. For these type of studies the efficiency of gene transfer into the recipient cells is usually the most critical parameter. In recent years cationic lipid mediated transfection has become the method of choice because of its ease of use, reproducibility and relative efficiency. While the current generation of cationic lipids are able to transfect a variety cell lines efficiently, there are still problems such as cellular toxicity and transfection cost. Mirus *TransIT* LT (Low Toxicity) reagents have been designed to minimize cellular toxicity while transfecting at the highest level of efficiency.

### Principle

The mechanism by which cationic liposomes deliver DNA into the nucleus of cells is still poorly understood. It is now believed that the majority of cationic liposomes deliver DNA through the plasma membrane via an endocytosis pathway but exactly how the DNA escapes these endosomes and travels to the nucleus is unclear. In many cases the endosomal acidification that occurs during endosomal maturation triggers the DNA release and specific inhibitors that block this acidification process also inhibit transfection (Budker, *et al.*, 1996; Zhou and Huang, 1994).

Mirus *TransIT* 100 cationic lipid transfection reagent is a pH sensitive reagent in that endosomal acidification is required for transfection. This reagent consists of a 1:1 mixture of the cationic lipid DPlm (Budker, *et al.*, 1996) and DOPE. Mirus *TransIT* LT-1 and LT-2 reagents consist of a mixture of a 3:1 wt:wt ratio of protein and cationic polyamines. The LT-1 and LT-2 reagents are also believed to use the endosomal pathway for cellular entry but cause much lower cellular toxicity following transfection than conventional cationic liposomes. The mechanistic reasons for this are still unclear.

### Results

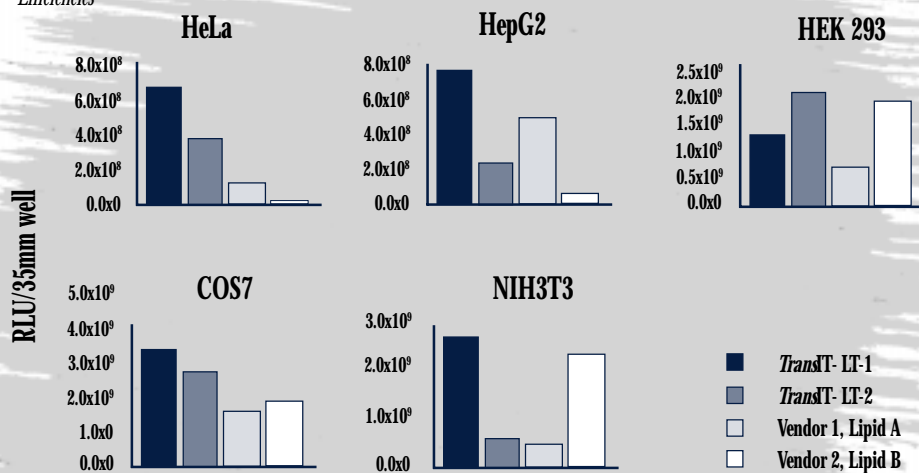
#### High transfection efficiency:

To compare the transfection efficiency of the *TransIT* LT-1 or LT-2 reagents with a leading competitor's cationic liposome reagents, we transfected a variety of common cell lines with the luciferase reporter plasmid pCILuc. After transfection the cells were incubated for 40 hours before being harvested and assayed for luciferase activity. The results indicate that either *TransIT* LT-1 or LT-2 was more efficient than reagents from a leading competitor in

*(continued on next page)*

Figure 1

Comparative Transfection Efficiencies





# TransIT™ Transfection Reagents

(continued from front page)

5 common cell lines (HeLa, NIH3T3, COS-7, HEK293, HepG2) (see Figure 1). In four out of five cell lines *TransIT* LT-1 was the better reagent based on luciferase activity but in one cell line (293) the *TransIT* LT-2 had higher activity.

## Low Cellular Toxicity:

Second generation cationic lipids (i.e. DOSPA/PE) are more efficient at transfecting a variety of cell lines than previous formulations (DOTMA/PE) but in most

naive cell so any effect from the transfection agent would be highly undesirable. While many insults are not lethal to a cell directly, they can activate a cascade of intracellular second messengers that can stimulate the expression of both endogenous cellular genes and exogenously introduced genes. Thus even sub-lethal insults are undesirable. In contrast to the current second generation cationic liposomes, *TransIT* LT reagents are formulated with a combination of a non-toxic cellular protein and a small amount of a novel polyamine. This much reduced amount of polyamine, as compared to

cationic lipid content in the current liposome formulations, is one possibility for the much reduced toxicity.

## Materials and Methods Complex Formation

Transfection competent complexes were prepared according to manufacturer's recommendations. Briefly, complexes were formed by mixing several different ratios of transfection reagent (*TransIT* LT or cationic lipid) to plasmid DNA in 150  $\mu$ l of Opti-MEM (Gibco BRL, Life

Technologies, Inc., Grand Island, NY). Two micrograms of plasmid DNA (pCILuc) were used for each 35 mm well. pCILuc was constructed by removing the luciferase cassette (*NheI*-Luc-*EcoRI*) from pSPLuc+ (Promega, Madison WI) and ligating it into *NheI*-*EcoRI* digested pCI expression vector (Promega, Madison WI).

## Transfections and Reporter Gene Assays

NIH3T3 (mouse fibroblast), HeLa (human cervical carcinoma), 293 (human embryonic kidney), HepG2 (human hepatoma) and COS7 cells (monkey kidney, SV40 T antigen transformed) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and split one day prior to transfection. At transfection, 50-60% confluent cultures were washed once with 2 ml Opti-MEM followed by addition of 2 ml of Opti-MEM to each 35mm well. Pre-formed complexes (2 micrograms pDNA/well in 150  $\mu$ l Opti-MEM) were then added dropwise to each well and dishes were placed at 37°C in 5% CO<sub>2</sub>. After a 4 hour incubation, complexes were removed and 2 ml of fresh growth medium was added.

Cells were harvested after 40-48 hours as previously described (Wolff, *et al.*, 1992; Wolff, *et al.*, 1992) and assayed for luciferase expression.

## Measurements of Cellular Toxicity

NIH 3T3 cells were grown on glass coverslips in 35 mm wells prior to staining with the fluorescent dye propidium iodide (Molecular Probes). Ten micrograms of propidium iodide (1 mg/ml) was added to each well and the cultures were incubated at 37° C for another 15 min. The plates were removed and washed two times with PBS. The coverslips were then removed and mounted on glass slides using Gel/Mount (Biomedica Corp., Foster City, California). Using a Leitz Orthoplan fluorescent microscope, a total of five fields were randomly selected and the numbers of cells with nuclear red staining (propidium iodide) were noted.

Nuclear staining with propidium iodide indicates a cell with a compromised plasma membrane and hence a non-viable cell. For each transfection reagent, the staining pattern in a total of over 300 cells (in the five fields) was noted.



James E. Hagstrom

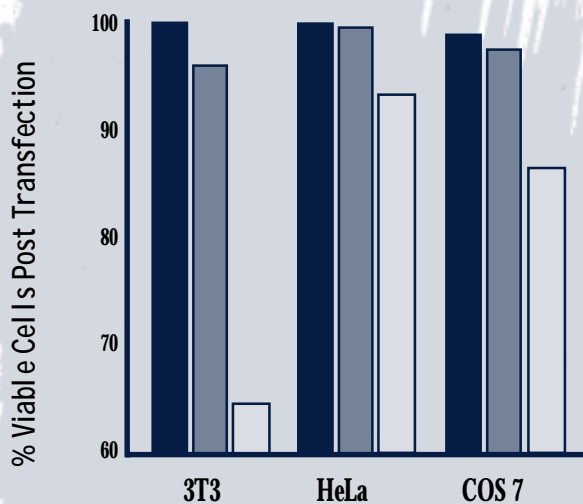


Figure 2

Comparative cellular toxicity

Mock Transfection  
 *TransIT* LT-2  
 DOSPA/PE

cases there has been a tradeoff with increased cellular toxicity (see Figure 2). Direct cellular toxicity is an obvious detriment to transfection but there are more subtle reasons for not wanting to alter the state of the cell with your transfection agent. Many transfection experiments are designed to study how the introduction of an exogenous gene affects the function of a

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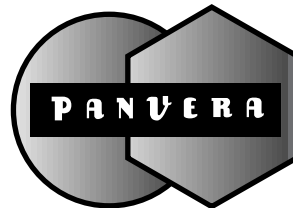
PanVera POSTINGS 

# PostIT

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| Fluorophore   | Excitation Wavelengths, nm | Emission Wavelengths, nm |
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| 1,5 IAEDANS   | 337                        | 520                      |
| Pyrene  | 345                        | 378                      |
| Fluorescamine   | 370                        | 488                      |
| Hydroxycoumarin   | 385                        | 445                      |
| Lucifer Yellow  | 425                        | 540                      |
| NBD (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole)             | 470                        | 554                      |
| DTAF (Dichlorotriazinylamino-fluorescein dihydrochloride) | 489                        | 513                      |
| Fluorescein   | 492                        | 520                      |
| TMR (Tetramethylrhodamine)                                | 543                        | 566                      |
| Cy3™  | 552                        | 568                      |
| Lissamine Rhodamine B                                     | 570                        | 590                      |
| Texas Red® (Sulforhodamine 101 acid chloride)             | 587                        | 620                      |
| Cy5™  | 650                        | 667                      |

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## TaKaRa RNA PCR Specials!

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| TAK 6122       | RACE Core Set, 5'-Full                        | 10 reactions      | \$280          | \$140       |
| TAK R019A      | RNA PCR Kit, Version 2.1                      | 50 reactions      | \$300          | \$250       |
| TAK 6607       | Human $\beta$ -Actin Competitive PCR Set      | 20 reactions      | \$180          | \$90        |
| TAK 6125       | Competitive RNA Transcription Kit             | 10 transcriptions | \$150          | \$90        |
| TAK RR017      | Competitive DNA Construction Kit              | 10 constructions  | \$150          | \$90        |
| TAK RR023A     | BcaBEST™ RNA PCR Kit                          | 50 reactions      | \$270          | \$185       |
| TAK 6608       | Rat Cytochrome P450<br>Competitive RT-PCR Set | 20 reactions      | \$250          | \$165       |
| TAK RR012A     | RNA LA PCR Kit, Verson 1.1                    | 50 reactions      | \$500          | \$325       |



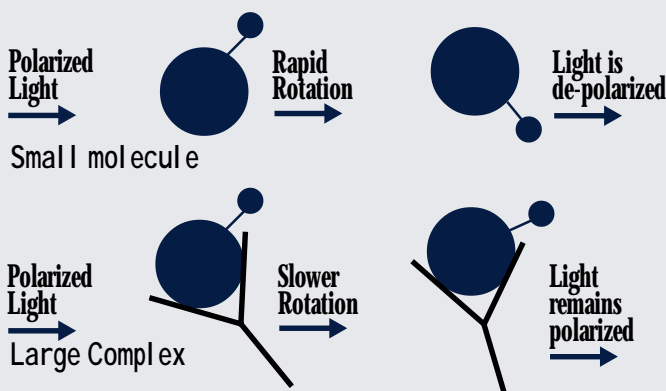
# What is FP?

Fluorescence Polarization (FP) is an increasingly popular technique that can be used to observe molecular interactions at equilibrium, in a homogeneous format. The binding of a fluorescent molecule to another molecule can be quantified by its speed of rotation and FP is a measure of that speed or tumbling rate.

When plane-polarized light is used to excite a solution of fluorescent molecules, only those molecules whose excitation dipoles are in the same plane will become excited. If the molecules remain stationary during the period of excitation (4 nanoseconds for fluorescein) the emitted light will remain highly polarized. If the molecules tumble during the period of excitation the emitted light will be random and therefore depolarized.

FP is a measure of the tumbling rate of the fluorescent molecule and is directly related to its molecular volume. Therefore, an increase in the volume of a fluorescent molecule (due to receptor-ligand binding, antibody-antigen binding, DNA hybridization, or DNA-protein binding) or a decrease in molecular volume (due to dissociation or enzymatic degradation) can be measured directly by FP.

The observed FP value (see figure below) is a weighted average of the FP values of the individual bound and free molecules and is therefore a direct measure of the fraction bound. The data are manipulated in the same way as a conventional radioligand-binding assay. Polarization is plotted against receptor concentration to obtain the familiar saturation-binding isotherm.



Schematic representation of fluorescence polarization differences between small and large complexes.

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# Use of Fluorescence Polarization

Connie Rickey and Randy Bolger  
PanVera Corporation

There has been increasing interest lately in characterizing environmental compounds for their effects as estrogenic agonists or antagonists. The classic estrogen receptor (ER- $\alpha$ ) is a 66 kDa transcription factor that regulates expression of genes involved in tissue growth and differentiation. The binding of agonists activates ER- $\alpha$ , resulting in several changes in the

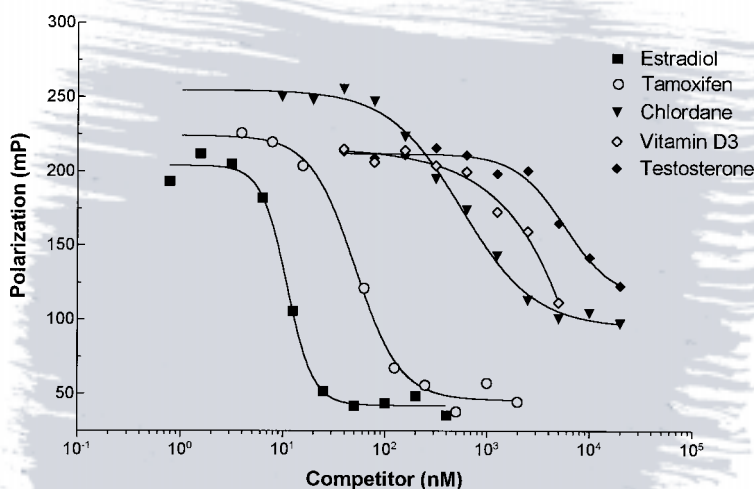


Figure 1

**Fluorescent Estrogen Competition Assay.** Several compounds were investigated for their ability to compete with FES1 binding to purified ER. Competitors were serially diluted in buffer. FES1 and ER were then added to each tube to a final concentration of 1 nM and 5 nM, respectively. Polarization readings were taken with the Beacon® 2000 FP instrument (PanVera Corporation). Estradiol is the most potent competitor ( $IC_{50}$  = 8 nM), followed by tamoxifen ( $IC_{50}$  = 50 nM). Vitamin D3 and testosterone were both poor competitors, with  $IC_{50}$  values > 5  $\mu$ M.

# Polarization in Studying Endocrine Disruption

receptor, including altered conformation, dimerization, and new interactions with other proteins. Recently, a second estrogen receptor, ER- $\beta$ , has been cloned (Paech *et al.*, 1997). Studies indicate that the two receptors differ both in their affinities for ligand, and in their response to ligand binding. Fluorescence polarization provides an easy method for the rapid screening of compounds for estrogen receptor binding capacity, either in the search for endocrine disruptors or for new therapeutics for diseases such as breast cancer and osteoporosis.

A human recombinant estrogen receptor (hrER) produced with baculovirus in insect cells, and an intrinsically fluorescent estrogen (FES1, Hwang *et al.*, 1992) with high-affinity to ER- $\alpha$ , were employed in developing a competitive FP assay. The binding affinity of a panel of compounds was measured by their abilities to replace the FES1 in the FES1:hrER complex (Figure 1). The competitors are characterized by their  $IC_{50}$ s, that is the concentration of the compound necessary to displace 50% of the fluorescent estrogen from the complex (inflection point of the semi-logarithmic binding curve).

A similar FP competition assay was performed to determine the effects of increasing the amount of estradiol to an FES1:ER- $\beta$  complex (Bolger *et al.*, 1998). The results using this second estrogen receptor are shown in Figure 2.

The applicability of the competition assays with the two human estrogen receptors can be extended to studies of androgen receptor, glucocorticoid receptor, and thyroid receptor. Indeed, the versatility of the technique has led beyond receptor-ligand studies to applications for antibody-antigen binding, DNA hybridization, DNA-protein binding and proteases, DNases or RNases.



Connie Rickey

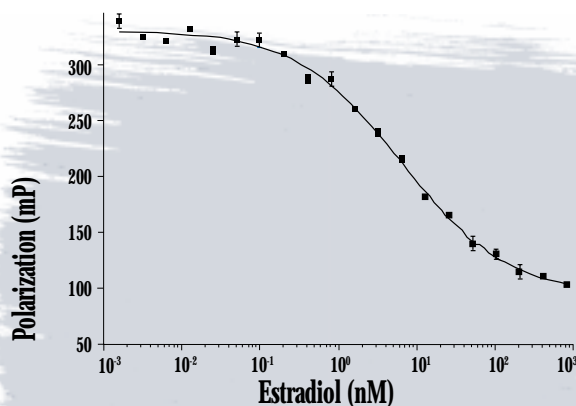


Figure 2

**Estradiol Competition using ER- $\beta$  and FES1.** *The  $IC_{50}$  for estradiol was 6 nM, leading to a calculated  $K_D$  for the FES1:hrER- $\beta$  binding of 0.15 nM. This compares favorably to the  $K_D$  observed in direct binding studies with  $^3H$ estradiol and hrER- $\beta$  (Clark *et al.*, 1992).*

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PanVera Corporation manufactures and offers the purified recombinant human ER- $\alpha$  and ER- $\beta$  proteins. We also offer convenient FP-based Estrogen Competitor Screening Kits in 96-well formats or for use with the Beacon 2000 System. Please contact us for additional information.

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| P2466    | ER- $\beta$   | 750 pmol (40 $\mu$ g) | \$250    |
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| Part No. | Product Description   |                       | Price    |
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\* Larger sizes are available for each of these products.

\*\* This kit will be available March 1998.

† Beacon Systems are also available with 500  $\mu$ l chambers and in 220/240 V versions.

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| MIR 3125 | <i>LabelIT</i> <sup>TM</sup> Rhodamine Labeling Kit-Trial Size<br>(for labeling 25 µg nucleic acid)   | \$99  |
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| MIR 3225 | <i>LabelIT</i> <sup>TM</sup> Fluorescein Labeling Kit-Trial Size<br>(for labeling 25 µg nucleic acid) | \$99  |
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| TAK 6125   | Competitive RNA Transcription Kit, 10 reactions      | \$90*  |
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| TAK E406   | LA PCR Buffer II (Mg <sup>++</sup> -free), 30 ml     | \$50   |

\*Sale prices apply until March 31, 1998

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| MBL 0313  | Anti-TRADD Antibody, monoclonal, 100 µg                    | \$210 |
| MBL 0323  | Anti-FLICE (Caspase 8) Antibody, clone 5F7, 100 µg         | \$210 |
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| MBL 4700  | MEBCYTO Apoptosis Detection Kit, Annexin-V-FITC, 100 tests | \$400 |
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LabelIT Kits are available for biotin, digoxin, rhodamine, and fluorescein labeling applications.

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Available now, human recombinant Estrogen Receptor- $\beta$  (hrER $\beta$ ) joins our Estrogen Receptor- $\alpha$  and Vitamin D<sub>3</sub> Receptor in our growing family of human nuclear receptors. Like our other receptors, hrER $\beta$  is active and highly purified after baculovirus-mediated expression in insect cells. In equilibrium binding experiments, hrER $\beta$  bound tritiated estradiol with a K<sub>D</sub> of approximately 0.3nM.

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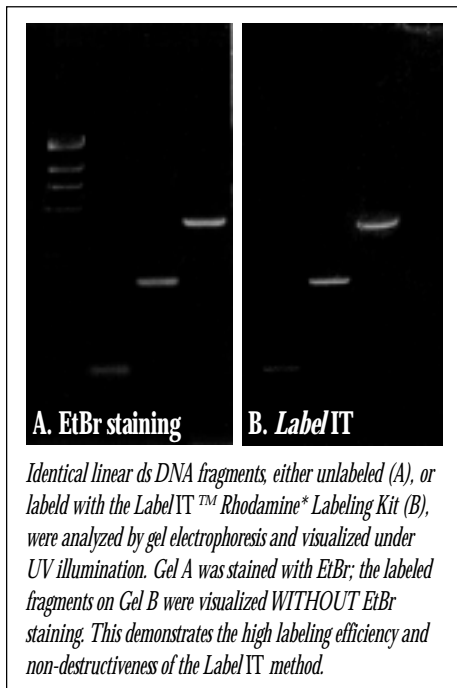
PanVera also offers our human recombinant PKC isoforms in a sample-pack format. Each isoform is expressed by a baculovirus-mediated system and purified to >95% while retaining activity. The panel contains 1 $\mu$ g each of PKC  $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ,  $\epsilon$ ,  $\zeta$ , and  $\delta$ . The cost of the panel is \$250.

## ApoE Polyclonal Antibody (Part #P2213)

PanVera's ApoE antibody is sensitive multi-use antibody that recognizes all three of PanVera's human recombinant ApoEs (E2, E3, and E4) and serum ApoE from human, rabbit, rat, mouse, and dog. While ideal of immunoblotting, the antibody has also been tested by use in direct ELISAs and immunoprecipitations. The antibody was raised in goats.

## MAbs for Apoptosis Research

PanVera announces the availability of several new monoclonal antibodies for use in apoptosis research. These new antibodies, manufactured by Medical and Biological Laboratories (MBL) of Japan, include: anti-TRAF1, anti-TRAF6, anti-TX (Caspase 4), anti-TRADD, anti-FADD, anti-Bag-1, anti-RIP, and anti-FLICE (Caspase 8). The MBL line also includes antibodies to several other Bcl-family proteins, as well as the MEBCYTO (Annexin V) and MEBSTAIN (TUNEL) Apoptosis Detection Kits. Anti-Fas (mouse and human) apoptosis-inducing and -inhibiting antibodies, and sFas and Fas Ligand ELISA Kits are also available.



**A. EtBr staining** **B. LabelIT**  
Identical linear dsDNA fragments, either unlabeled (A), or labeled with the LabelIT™ Rhodamine\* Labeling Kit (B), were analyzed by gel electrophoresis and visualized under UV illumination. Gel A was stained with EtBr; the labeled fragments on Gel B were visualized WITHOUT EtBr staining. This demonstrates the high labeling efficiency and non-destructiveness of the LabelIT method.



# Q & A

## **PanVera offers several ApoE's. What is the difference between ApoE2, E3, and E4?**

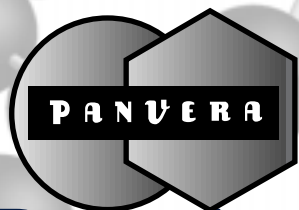
The three common isoforms of ApoE differ by a single amino acid substitution at two locations. ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), ApoE4 (Arg112, Arg158). These differences are best visualized by two-dimensional gel electrophoresis - each isoform exhibiting a distinct pI. Each ApoE also exhibits multiple minor isoforms that each share the same amino acid sequence, but probably differ in terminal glycosylations and non-specific deaminations. These differences result in complicated multi-band isoform patterns on two-dimensional gels. All of these bands are considered to be authentic forms of ApoE and are found in any isolation of ApoE from human plasma. Indeed, the pattern of human ApoE secondary modifications by way of baculovirus-mediated expression is more similar to human plasma ApoE modifications than ApoE expressed in bacterial systems.

## **Do PanVera's ApoE's require special treatment prior to use?**

PanVera's human recombinant ApoE's (hrApoE) can be used as they come, but many researchers prefer to reconstitute them in lipid particles. ApoE is an amphipathic protein and will quickly associate with lipid particles. The questions become 'which lipids should I use?', 'what size particles should I make?', and whether to make vesicles or liposomes. The answers depend on your research interest (cardiovascular or neuroscience); ApoE-containing lipoproteins in the CNS and circulation differ in lipid composition and buoyant density.

## **Do you have to add exogenous NADPH-P450 reductase to PanVera's Cytochrome P450 BACULOSOMES™ in order to get biotransformation activity?**

No, not at all. Cytochrome P450 BACULOSOMES are prepared from insect cells infected with recombinant baculovirus containing a cDNA insert for a human P450 *AND* the NADPH-P450 reductase. Therefore, BACULOSOMES contain a complete, functional P450 enzyme system. The activity of these insect microsomes is generally higher than that seen in human liver microsomes (HLMs) and is due not only to the overexpression of the individual P450 enzyme but also to the stoichiometric excess of the NADPH-P450 reductase to the P450 enzyme. The opposite is true of HLMs where the NADPH-P450 reductase to P450 ratio is considerably lower and is believed to be the limiting factor of functional P450 activity. Cytochrome *b<sub>5</sub>* can also boost activity in those P450 systems known to be affected by its presence (i.e. 3A4).



## Postings

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