Biotechnology Division Overview
FY 2004

The Biotechnology Division is the focus of the NIST effort addressing critical measurement and data needs for other government agencies, and the rapidly developing biotechnology industry.

MISSION

The mission of the Division is to provide the measurement infrastructure necessary to advance the commercialization and application of biotechnology. This is achieved by developing a scientific and engineering technical base along with reliable measurement techniques and data to enable U.S. industry to produce biochemical products, and to enable the government to apply advances in biotechnology to the benefit of societal needs. The Division has established a variety of long-range research projects to maintain critical expertise needed for the development of Standard Reference Materials, Standard Reference Databases, and advanced measurement methods. The Division fosters collaboration among NIST scientists conducting biology-related research, and strives to reach the goals set by the NIST Chemical Science and Technology Laboratory strategic plan.

Division scientists participate in scientific meetings, topical workshops, and numerous national and international organizations such as: Biotechnology Industry Organization (BIO), IUPAC Commission on Biophysical Chemistry, ASTM Committee E-48 on Biotechnology, the International Measurement Standards Consultative Committee for the Amount of Substance (CCQM), Bioanalytical Working Group. Division members were also active as reviewers for the NIST Advanced Technology Program (ATP), for several NSF and DOE programs, NIH study section panels, for the Department of Homeland Security on issues related to bioterrorism defense, and with the Department of Justice on issues related to forensics and human identification.

The staff of the Biotechnology Division consists of 36 NIST employees and a comparable number of contract researchers, guest scientists, and post-doctoral fellows. The Division is organized into four groups: (1) DNA Technologies; (2) Bioprocess Measurements; (3) Structural Biology; and (4) Cell and Tissue Measurements. Brief descriptions of technical highlights from each Group are given below.

Selected Program Highlights:

DNA TECHNOLOGIES

The DNA Technologies Group has research projects that are included in the Program Areas of Health and Medical Products, Forensics and Homeland Security, and Food and Nutritional Products. Advanced mass spectrometry measurements of DNA damage are used to describe the cellular accumulation of two major oxidative stress-induced DNA lesions in cells of Cockayne syndrome (CS) patients after exposure to ionizing radiation. As a disease with implications for understanding the human aging process, these studies are undertaken as a collaborative effort with scientists at the National Institute of Aging. Projects in the area of DNA diagnostics for the detection of human disease include the NIST-National Cancer Institute Biomarkers Validation

Laboratory (BVL), the NIST component of the Early Detection Research Network which serves to refine recently discovered cancer biomarkers, and to format new research tests for field trials in EDRN clinical laboratories. Another area is the study of cellular biomarkers that can be used for quality assurance of tissue-engineered medical products in terms of genetic damage. In the human identity/forensic science project, the group focuses on new methods for DNA profiling, ranging from developing well-characterized DNA standards for restriction fragment length polymorph-isms (RFLPs) to performing research for rapid determination of DNA profiles by polymerase chain reaction (PCR) amplification and automated detection of fragments. New methods were developed for identification of victims of the World Trade Center (WTC) disaster of September 11, 2001 where the high degree of DNA fragmentation due to the severe environmental conditions has meant that only about 50% of the specimens yielded results with standard DNA testing methods.

BIOPROCESS MEASUREMENTS

The Bioprocess Measurements Group (http://www.cstl.nist.gov/div831/bioprocess/) is concerned with the development of measurement methods, databases, and generic technologies related to the use of biomolecules and biomaterials. The results are directed at the biomanufacturing, agbiotechnology, and pharmaceutical industries and, most recently, to Homeland Defense, where there are needs for the detection and quantification of very small amounts of biological materials. The effort is organized into four project areas that are part of the CSTL Pharmaceuticals and Biomanufacturing, Food and Nutritional Products, and Forensics and Homeland Security Programs. In the spectroscopy of biological systems project, one study is directed at investigating the mechanism of fluorescence resonance energy transfer (FRET) when it is used to quantify the extent of a polymerase chain reaction (PCR). In the figure, FRET efficiency is seen to decline by five-fold as a function of fluorophore separation, counted as number of nucleic bases between the fluorophore labeling sites. These results are relevant to an ongoing project done in collaboration with the USDA to detect and measure genetic modifications in grain, sometimes referred to as GMOs. In the biocatalysis project, enzyme characterization is being carried out to address industrially important biotransformation problems such as those found in hydroxylation and aromatic amino acid metabolic pathways. The methods used include site-directed mutagenesis, circular dichroism, ellipsometry, spectroelectro-chemistry, and X-ray diffraction to characterize several key steps along metabolic pathways. In the biothermodynamics project, chromatography and microcalorimetry are used with chemical equilibrium analysis of complex reacting systems to develop thermodynamic data for industrially important biotransformations that are included in the NIST Standard Reference Database "Thermodynamics of Enzyme-catalyzed Reactions." A new project, microbial forensics, has recently started to develop standard methods, materials and data related to the national efforts to defend against threats of biological warfare.

STRUCTURAL BIOLOGY

The Structural Biology Group at the Center for Advanced Research in Biotechnology (CARB) is focused in key areas of industrial biotechnology, especially in the Pharmaceuticals and Biomanufacturing Program. These areas are supported at CARB through a highly interactive group of scientists, from both the University of Maryland Biotechnology Institute (UMBI) and NIST. In the structure-function of biological macromolecules, a recent study of the dimerization of two homologous strands of genomic RNA reveals an essential reaction in the replication of

retroviruses such as HIV-1 (see figure). A new effort has been launched in structural genomics that capitalizes on existing expertise macromolecular structure determination by X-ray crystallography. This project area also supports the NIST activity related to the Protein Data Bank (**http://pdb.nist.gov/**). Results from the physical, molecular and cellular biochemistry studies of key recognition elements in G coupled protein receptors suggest new, quantitative models for signal transduction pathways in vision and viral infection. The energetics of enzyme-catalyzed reactions are being studied by differential stopped flow microcalorimetry. A recent highlight was completion of a study of the binding interactions in the model system composed of a well-characterized enzyme-inhibitor pair, namely bovine carbonic anhydrase II (CA II) and 4-carboxybenzenesulfonamide (CBS). Under the initiative of The Molecular Interactions Research Group (MIRG) of the Association of Biomolecular Resource Facilities (ABRF), this model system for monitoring complex formation was distributed to a panel of analytical ultracentrifugation (AUC), isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR). operators. The study participants were asked to measure one or more of the following: 1) the molecular mass, homogeneity, and assembly state of CAII by AUC; 2) the affinity and thermodynamics for complex formation by ITC; and 3) the affinity and kinetics of complex formation by SPR. The results of the study showed excellent agreement between the ITC and SPR results on the binding thermodynamics for complex formation. The AUC results showed that the enzyme exists as a monomer in solution in agreement with the ITC results. The results from this study provide a benchmark for comparing the capabilities of individual laboratories and defining the utility of the different instrumentation.

From the bioinformatics for disease markers project, an HIV structural database has been developed that was highlighted in Science magazine's Netwatch (Science, May 30, 2003; srdata.nist/hivdb). A project on gene expression is just underway in collaboration with platform developers, reagent makers, clinical users and other government agencies (Nature, January 8, 2004, p. 91; http://www.cstl.nist.gov/biotech/workshops/ERCC2003/).

CELL AND TISSUE MEASUREMENTS

Over the last fifteen years, new technologies have enabled scientists to look inside cells and image individual molecules at work. Today we are beginning to understand life processes at a cellular and molecular level and the need for new analytical techniques to study living cells and molecules is ever greater. To address those needs, the Cell & Tissue Measurements Group contributes to the new post-genomic era of medical science by developing measurement tools and standards for the biotechnology and pharmaceutical industry wanting to capitalize on the potential rewards of understanding cellular processes. In quantitative cell biology, advanced measurement tools, indicator cells that express green fluorescent protein to report a cellular response, standard protocols and biomaterials are being developed for quantifying cell response using automated fluorescence microscopy and imaging and other measurement methods. Methods to quantitatively assess biomarker expression in live cells as well as to predict and evaluate the influence of the extracellular milieu on selected biomarkers in a statistically relevant number of cells in an unbiased fashion are being developed. In order to reproducibly control the adhesion substrate for cells, series of biomimetic materials based on highly-reproducible ultra-thin films of biologically relevant extracellular matrix (ECM) proteins adsorbed onto alkanethiol self-assembled monolayers have been developed. The physical properties of these thin films and the cellular response to these films have been extensively evaluated to validate their biomimetic nature and their reproducibility in generating a cell response. The goal of the proteomics project

is to address needs of the proteomics communities as outlined in a September 2002 workshop and to develop general protocols for handling and characterizing membrane associated proteins in gel platforms and mass spectrometry. In the area of 2-D gels, as its ability to be used along with complementary technologies such as liquid chromatography (LC) is expected to encourage their uptake, one project is addressing the need for universal internal gel standards that can permit precise measurements between different gel runs. Concomitantly, as proteomics is one of the application area making giant strides in the mass spectrometry market, another project is exploring the production of quantitative mass spectrometry and the use of stable isotopes as internal standards. The goal of the bioinformatics project is to develop adaptive, automated method of processing and presenting biological and chemical data using connection tables that are sufficiently flexible and easy-to-use and allow users to find, with confidence, information for the most structurally-relevant data used in structure-based drug design. Web-based databases, started and maintained by NIST staff, are used to test these methods. These techniques allow interoperable annotation, query, and analysis across diverse data; a plug-and-play scalable annotation and adoptive query tool environments that facilitates seamless interplay of tools and data; and versatile user interfaces that allows researchers to annotate, visualize and present the results of analysis in the most intuitive and user-friendly manner.

Current databases include the 1) HIV Structural Reference Database (compounds targeting HIV protease), 2) human Mitochondrial Protein Database, and 3) Enzyme Thermodynamics Database.

The Short Tandem Repeat DNA Internet Database:
http://www.cstl.nist.gov/biotech/strbase/
Protein Data Bank:
http://rcsb.nist.gov/
Thermodynamics of Enzyme-Catalyzed Reactions:
http://wwwbmcd.nist.gov:8080/enzyme/enzyme.html
The Biological Macromolecule Crystallization Database:
http://wwwbmcd.nist.gov:8080/bmcd/bmcd.html
HIV Protease Database:
http://srdata.nist.gov/hivdb/

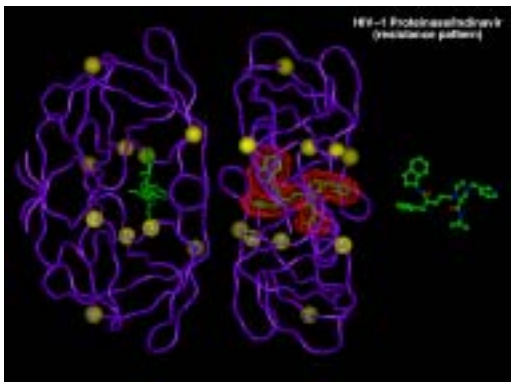
SELECTED TECHNICAL ACTIVITIES

Title: NIST Launches New HIV Bioinformatics Database

Authors: T. N. Bhat (831) and A. Wlodawer (NCI, NIH)

Currently, much of the research for the treatment of AIDS is directed either towards vaccine development or towards drug development. Although several promising leads on vaccine have been reported (Barough et al., Nature 2002;415 (6869):335-339) no effective vaccine has been developed at this time. Drugs provide the only proven method for the treatment of AIDS. However, despite the availability of several web resources (http://www.niaid.nih.gov/daids/dtpdb/intro.htm, http://www.hiv.lanl.gov/content/index) on AIDS related drug development, structural information on proteins that are potential targets for AIDS are scattered and data exchange between these resources is difficult causing technical barriers to drug development researchers. Structural data play a crucial role in the development of drugs and in understanding drug resistance as evidenced by the fact that a large fraction of the current drugs for the treatment of AIDS were developed using structure based approaches.

Despite the wide and expanding availability and use of physical, chemical, and biochemical data collections, the ability to organize and retrieve structure data remains antiquated. While it is possible to query compounds whose physical structures are known in advance, the ability to query compounds in large, complex structural collections is unsatisfactory. To enable the bioinformatics community to have a more efficient means of accessing complex data, NIST researchers have developed a standardized techniques to annotate, index and present structural data (in press, Proteins Structure Function and Bioinformatics). This method establishes metadata, ontology and data standards to express structures in terms of standard fragments of chemical and structural significance.



Some drug resistance mutant locations (left); drug, Indinavir, in its bound state (middle); molecular structure of drug (right). *Munshi et al. Acta Crystallogr D Biol Crystallogr. 2000 Apr;56 (Pt 4):381-8.*

NIST, in collaboration with NCI/NIH, unveiled a new online database that contains the structures of HIV protease and compounds targeted against this enzyme (HIVSDB http://xpdb.nist.gov/hivsdb/hivsdb.html). HIVSDB, a specialized resource for a very important healthcare topic- AIDS, permits faster and better reliable access of standardized data related to the design and development of compounds against HIV. It will also provide improved resources for analyzing drug resistance of drugs that are currently used to treat AIDS. The availability of such a resource to industry is anticipated to foster the development of new and better drug products. NIST has taken an active role in developing standards and databases in many technology areas. This effort outlines how NIST plans to extend its leadership role in standardization into a new area of great importance to the biotechnology industry, namely bioinformatics/computational biology and AIDS.

HIVSDB plans to annotate and distribute structural data on AIDS related molecules with special emphasis to technological growth and drug development. The plan is to focus on all aspect of the data and information interoperability of structural data. Data standards, data uniformity, data quality, data archival, distribution are just a few of these issues. HIVSDB will receive, annotate, archive, and distribute structural information about AIDS-related proteins and the compounds that inhibit them. These proteins play a crucial role in the maturation of the AIDS virus, and some of the compounds (inhibitors) are the most effective drugs thus far developed against AIDS. The NIST HIVSDB provides a crucial platform for AIDS research efforts directed towards the development of new drugs and treatments. This effort will also entail the expansion of the content of the data, the enhancement of the quality and uniformity of the data, the development of unique navigation and structural analysis tools, and the offering of comprehensive downloadable data from the resource. Efforts are currently underway to use this opportunity to establish collaboration between NIST and NIAID in the area of data and informatics for AIDS.

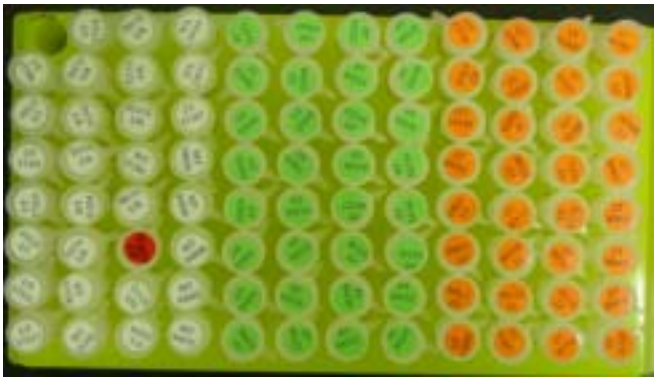
Title: Evaluation of Genetic Variation in Major U.S. Population Groups using Human Identity Testing Markers

Authors: John M. Butler, Margaret C. Kline, Peter M. Vallone, Janette W. Redman, Amy E. Decker, and Michael D. Coble, DNA Technologies Group, Biotechnology Division

The purpose of this work is to examine the ability of commonly used and new genetic markers to differentiate between samples of U.S. populations. A set of approximately 650 anonymous population samples from U.S. Caucasians, African Americans, and Hispanics (self-declared ethnicities) have been purchased from a commercial blood bank. Over the past two years, these samples have been characterized across a variety of genetic loci used in human identity testing. Results from these samples are being used to evaluate performance of individual markers and various combinations of loci to enable differentiation of the samples. Concordance studies have also been performed with these samples between in-house multiplex polymerase chain reaction (PCR) assays and commercial kits to verify the absence of allelic dropout due to PCR primer binding site mutations. Information collected from these samples is being made available over the Internet through the NIST STRBase website: http://www.cstl.nist.gov/biotech/strbase/NISTpop.htm.

To-date a total of 8 manuscripts has been published or submitted describing our results across these samples. Over 85,000 allele calls have been made so far on these samples. These population samples will likely become some of the most well characterized samples in the world. Decisions are being made about useful loci to pursue in future assays that are developed at NIST based on variation observed in these samples. These samples have also been useful in a beta-test of a new commercial kit for Y-chromosome short tandem repeat (Y-STR) amplification being released in December 2004 by Applied Biosystems.
In future, we will continue testing new loci in this set of samples.

Below is a picture showing a subset of the sample tubes. The tops of 95 sample tubes are shown each at a concentration of approximately 1 ng/µL with different color labels representing the various ethnicities.



Title: Metrology Tools for Quantitative Cell Biology

Authors: John T. Elliott and Kurt J. Langenbach (831.04)

Cell based assays are utilized extensively in the biotechnology and pharmaceutical industry during multiple phases of product and drug development. Despite the wide spread use of these assays, there has been little effort in developing metrics and standards to validate cell lines and the culture conditions before their use in an experimental setting. The absence of these validation procedures can cause complications when intra- or inter-laboratory data comparisons are required to fully interpret experimental results. NIST is leveraging our expertise in material and biological sciences towards meeting the metrology needs for these industries. The program for quantitative cell biology contains three parts: 1) development of indicator cells that express green fluorescent protein to report a cellular response, 2) development of highly reproducible extracellular matrix protein thin film substrates for use as reference cell culture substrates and 3) automated fluorescence microscopy and image analysis methodologies for quantifying a cellular response.

Indicator cells being developing contain an artificial gene encoding Green Fluorescent Protein (GFP), causing it to generate its own fluorescent signal if a property in the culture/material induces a specific cell response. As proof of principle, a mammalian fibroblast cell line has been engineered to fluoresce during proliferation using an artificial GFP-tenascin-C reporter gene. Rigorous biochemical studies designed to validate cellular biomarker response and to ensure the robust nature of the engineered cell line have confirmed the activation of intracellular events associated with proliferation in these cells correlate with induction of cytoplasmic GFP.

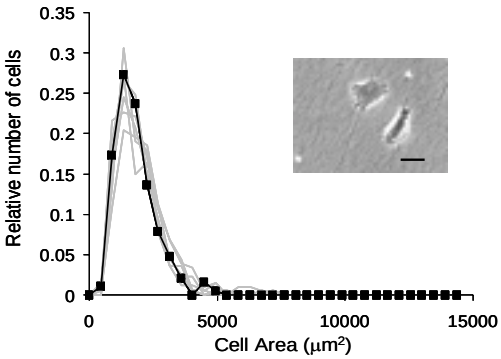


Figure 1. Cell morphology on reference fibrillar collagen films. Black-prepared 2003; Grey- prepared 2004.

In order to reproducibly control the adhesion substrate for cells, NIST has developed a series of biomimetic materials based on highly-reproducible ultra-thin films of biologically relevant extracellular matrix (ECM) proteins adsorbed onto alkanethiol self-assembled monolayers. The physical properties of these thin films and the cellular response to these films have been extensively evaluated to validate their biomimetic nature and their reproducibility in generating a cell response. Figure 1 shows the population distribution of cell morphology on the reference fibrillar collagen films prepared one year apart (black vs. grey). This precise level of validation is critical to stakeholders for establishing the utility of the collagen thin films as reference cell growth substrates.

NIST has adopted automated fluorescence microscopy and image analysis as the primary tool for rapidly quantifying how indicator cells respond to changes in culture conditions. Cells are stained with a novel two-color staining method developed in our laboratory which allows measurement of cell density, cell morphology and level of response (GFP signal intensity) in cells. The identification of this high-contrast and robust staining procedure for fixed cells will be useful to any research groups to address how culture conditions, extracellular matrix

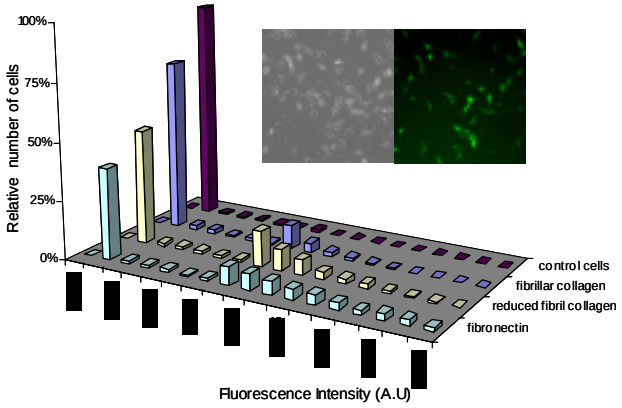


Figure 2. Histograms of GFP intensity from indicator cells on different reference ECM surfaces. The highest level of GFP expression occurred on fibronectin surfaces. Inset- phase and fluorescent pictures of GFP expressing cells.

modification and biomaterial substrates influence cellular behavior. Histograms of indicator cell GFP intensity on different reference ECM substrates (inset: phase contrast and fluorescent image of indicator cells on reference ECM surface) are shown in Figure 2. The highest level of GFP expression occurs when the cells are cultured on a fibronectin surface.

Future Directions: Critical issues such as how cryopreservation and prolonged culturing influence cell behavior are being evaluated. In addition, future emphasis will involve the development of additional reference materials and protocols for standardizing measurements of cell-material interactions with the hope of bridging the gap between knowledge generation by basic scientists and product development in industry as well as addressing FDA concerns about quantitative cell measurements.

Title: Reference Material 8640 for the calibration of flow cytometers

Authors: A. Gaigalas and L. Wang (831)

We have completed the development of a Reference material (RM 8640) for the calibration of flow cytometers, which are used to detect and measure the number of surface receptors on living cells. The type and number of receptors is an indicator of the state and health of the cells and is of critical importance to health care and biothreat detection.

The release of RM 8640 represents an important step in an effort to quantitate fluorescence intensity in flow cytometer measurements. The RM consists of six vials each containing a suspension of microspheres with different amount of immobilized fluorescein isothiocyanate (FITC). Each vial of RM 8640 contains a suspension of microspheres with an assigned value of "molecules of equivalent soluble fluorophore"(MESF). A significant effort was made in developing an original methodology for assigning the MESF values to the microspheres. The assignment is based on the comparison of fluorescence yield of the microsphere suspension with the fluorescence yield of a solution of SRM 1932. The SRM 1932 is a fluorescein solution with a certified concentration of fluorescein. The graph below shows the relationship between the assigned values of MESF and the flow cytometer response given by the channel number and is a typical calibration of a flow cytometer. The "channel number" refer to the digital number associated with the average height of the fluorescence pulse arising from the passage of the microsphere through the flow cytometer. This calibration can be used to assign MESF values to cells with labeled antibodies.

The work leading to RM 8640 has resulted in the development of a scientific basis for quantitative fluorescence intensity measurements for flow cytometry. Extension of the work to quantitation of multicolor flow cytometer measurements is underway.

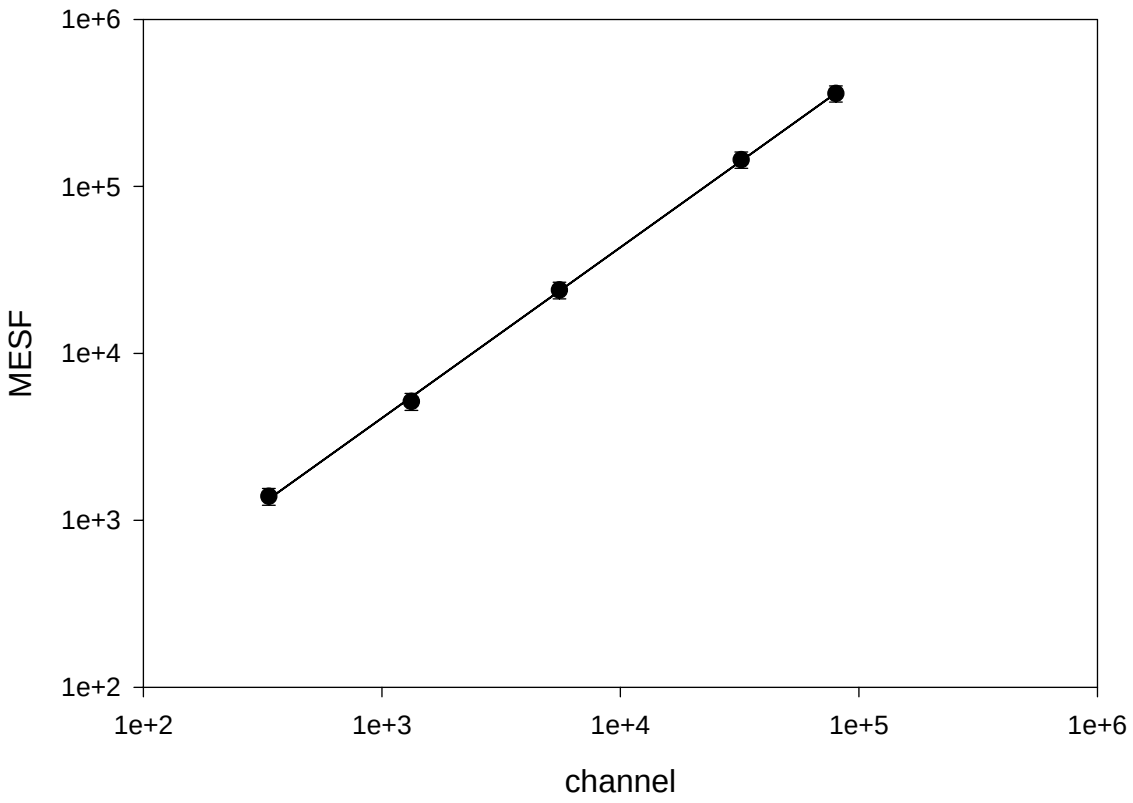


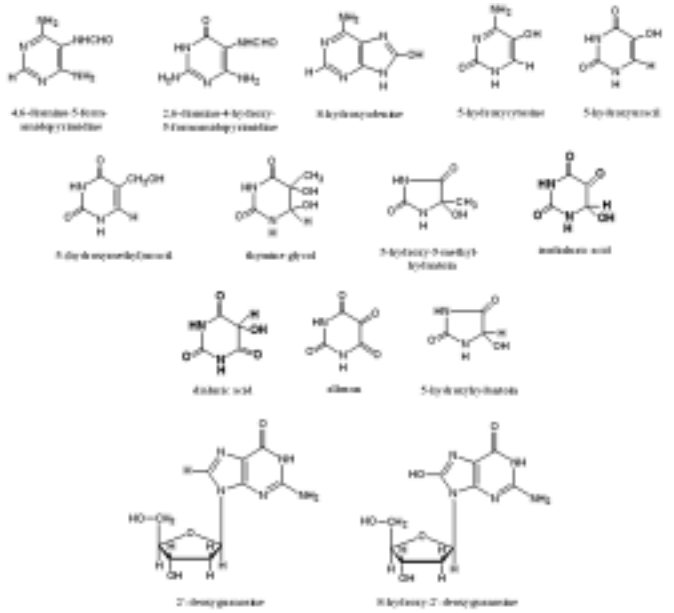
Figure caption: The six suspensions were mixed and passed through a cytometer. The graph shows the relationship between the assigned values of MESF and the response of the flow cytometer given by the channel number. The result for the microsphere with no immobilized FITC is not shown.

Title: Standard Reference Material for Measuring DNA Damage Related to Disease and Aging

Authors: Henry Rodriguez, Pawel Jaruga and Miral Dizdar, DNA Technologies Group, Biotechnology Division

Every living cell produces free radicals as part of its normal metabolism. Free radicals are known to cause damage to DNA resulting in oxidative DNA damage. This type of DNA damage has been associated with numerous age-related diseases such as cancer, atherosclerosis, and Parkinson's and Alzheimer's diseases. Free radicals can take over as the body's antioxidant defense mechanisms weaken, a condition referred to as oxidative stress. Additionally, external sources of free radicals—air pollution, radiation, ultraviolet light and certain drugs - can tip the balance in the wrong direction. Oxidative DNA damage can be repaired by enzymes in normal cells, but for a variety of reasons the cellular repair process may fail or slow down, resulting in elevated levels of oxidative DNA damage that may lead or contribute to disease process and aging.

NIST scientists pioneered the development of methods for detecting and quantifying oxidative DNA damage at levels approximately one modified base per million DNA bases. NIST's analysis techniques positively identify and accurately quantify numerous DNA base lesions caused by free radicals. Other techniques generally measure only one modification and present no structural evidence for verification; such results might be misleading and might not reflect the overall rate of DNA damage.



The structures of the components of SRM 2396. Alloxan and 5-hydroxyhydantoin result from oxidation in aqueous solution and acidic treatment of dialuric acid, respectively.

A new Standard Reference Material (SRM 2396) from NIST will help scientists better measure oxidative DNA damage implicated in the progression of cancer and other diseases, as well as in the aging process. The new SRM can be used to calibrate methods for measuring oxidative damage in a DNA sample by techniques that use a combination of chromatography and mass spectrometry. Free radicals produce more than twenty different types of lesions in the nitrogen-containing heterocyclic compounds or "bases" of DNA.

SRM 2396 contains the stable-isotope labeled analogues of eleven of the most-studied DNA bases modified by free radicals, and that of an intact nucleoside for DNA quantification shown in the figure. These compounds are used as internal standards for the measurement of analogous unlabeled lesions in DNA. Users can also calibrate their methods and equipment by using the

components of this SRM. SRM 2396 is intended for use in the measurement of oxidative DNA damage by gas chromatography/mass spectrometry (GC/MS), and liquid chromatography/mass spectrometry (LC/MS), using the isotope-dilution technique for quantification in both cases. The SRM is expected to help establish measurement accuracy and consistency among different laboratories as well as traceability to the NIST standard.

Title: Development of ICP-OES determination of phosphorus as a primary measurement tool for quantitation of plant deoxyribonucleic acid (DNA)

Authors: M. J. Holden (831), M. Winchester (839), J.R. Blasic, Jr. (831), Miral Dizdar (831), P. Jaruga (831), Y. Tewari (831)

The successful detection and quantitation of biotech crop material in grain or food is highly dependent on the acquisition of pure and non-degraded DNA in a quantity that is appropriate for the limits of detection and quantitation of the measurement methods. The most important measurements, related to U.S. export of biotech commodity crops and prepared foodstuffs, are the ones relevant to the detection of trace amounts of biotech material. Thus the amount of DNA that is used for the detection becomes critical. Laboratories currently use spectroscopic methodologies to quantitate DNA preparations, for example, DNA absorbance at 260 nm or fluorescent dye binding. The values obtained can be seriously compromised by impurities in the DNA preparations or the state of the DNA itself.

Development of primary methods, that provide accurate and traceable measurement of total plant DNA, will support the development of standard reference materials for the calibration and validation of plant DNA measurements using other methods that are appropriate for testing and research laboratories. In this project the primary measurands are phosphorus and the four-nucleotide bases that comprise DNA. A substantial effort has been directed during FY04 toward the development of a high-performance inductively-coupled plasma optical emission spectrometric (HP-ICP-OES) method for determining the total mass of phosphorus present within a given sample of DNA. HP-ICP-OES employs a clever experimental design, a well-chosen internal standard, and an innovative drift correction technique to enable expanded uncertainties on the order of a few parts per thousand. A methodology for measuring phosphorus has been developed, and several determinations using phosphorus spectrometric solution standards as 'mock' DNA samples have been demonstrated. As a more realistic test, a sample of corn DNA has been successfully digested, and suitable phosphorus spectra have been acquired. Quantitation of real DNA samples using the HP-ICP-OES approach is forthcoming.

A remaining challenge concerns instabilities within the microconcentric nebulizer that is required for the combination of high sensitivity and small sample volumes. High Performance Liquid Chromatography (HPLC) is a second unrelated technique for the quantitation of phosphorus. We have developed a suitable digestion protocol to release phosphorus and measured the phosphorus mass in DNA preparations. This methodology will provide an independent validation of the phosphorus content. Nucleotide analysis is the other critical component. This analysis is accomplished using both gas chromatography and liquid chromatography coupled with mass spectroscopy. Our investigations has shown that DNA from corn kernels and soy beans responds differently to the digestion and analysis protocols that work with human DNA. Work is continuing to find the best protocol suitable for use with plant DNA.

The importance of this work has been highlighted recently in experiments we conducted to compare dye binding properties of plant DNA with that of animals and microbes. Significant differences in response were observed with two commonly used fluorescent dyes which highlight

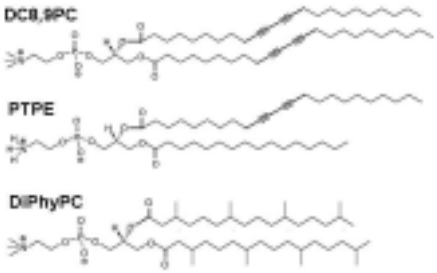
the inappropriate use of mammalian and microbial DNA as calibrants for plant DNA measurements and the necessity of new plant DNA standard reference materials.

Figure caption: DNA quantitation plays an important role in commerce, for example in detecting the amount of DNA in genetically modified crops.

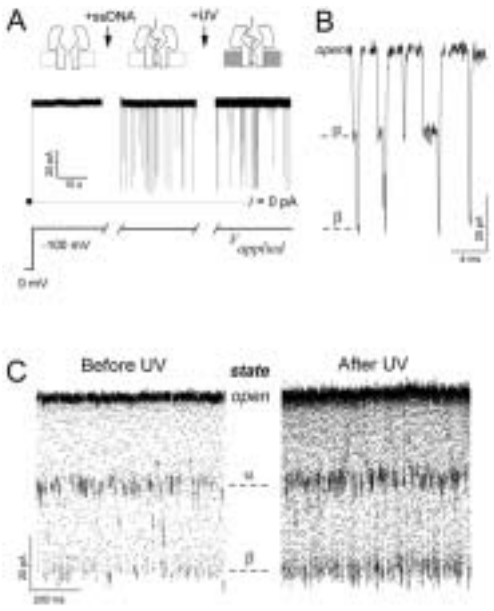
Title: Functional Reconstitution of Ion Channels in Polymerizable Lipid Membranes

Authors: D.K. Shenoy (NRL), W. Barger (NRL), A. Singh (NRL), R.G. Panchal (USAMRIID), M. Misakian (817), V.M. Stanford (894) and J.J. Kasianowicz (831)

For the past four decades, the art of making planar lipid bilayer membranes has enabled the study of ion and macromolecular transport through single or multiple nanometer-scale pores. It is in such matrices that nanopore ion channels, which are the molecular basis of many cellular functions (e.g., nerve and muscle activity), have been shown to be capable of detecting and characterizing different ions, polynucleotides and particular proteins. Because only weak intermolecular interactions stabilize liquid-crystalline phospholipid membranes (i.e. the membranes are too fragile), nanopore-based applications would benefit if ion channels could be functionally reconstituted and immobilized in robust ultra-thin films. Towards that goal, this study demonstrates that two different protein ion channels (formed by *Bacillus anthracis* protective antigen 63 and *Staphylococcus aureus* α-hemolysin) are fully functional in two different polymerizable lipid membranes in the liquid crystalline state. In addition, one of these channels functions even after the membrane is partially polymerized.



Both the anthrax channel and the α-hemolysin channel spontaneously formed highly conducting nanopores in membranes formed by either of two polymerizable lipids (DC8,9PC and PTPE) and the nonpolymerizable control phospholipid diphytanoyl phosphatidylcholine (DiPhyPC). Exposure of the polymerizable lipids to UV light causes diynes in the hydrocarbon chains to covalently link with others in like neighboring lipid molecules.



The α-hemolysin ion channel remained completely functional in a PTPE bilayer membrane before and after the matrix was polymerized. The recordings in panel A show that in the absence of single-stranded DNA, the ionic current through a single α-hemolysin channel is quiescent. Adding 50-nucleotide long poly(thymidine) causes transient current blockades that occur at random intervals. Following UV illumination of the PTPE membrane, the polynucleotide-induced current blockades persist. Five of the characteristic poly[dT]-induced blockades that occurred after UV illumination are shown in panel B. Note that there are three predominant states (fully open, α and β). These states predominate before and after UV irradiation as is shown in the time series in panel C.

More recent work suggests that the anthrax pore might

prove useful for the rapid screening of anthrax therapeutics. Thus, the ability to functionally reconstitute such channels into matrices that can be made robust is promising.

Title: Chorismate Pathway Enzymes: Structural Studies

Authors: J.E. Ladner(831), E. Eisenstein (CARB/UMBI), J. Parsons (CARB/UMBI), K. Calabrese (CARB/UMBI)

Purpose: Aromatic hydrocarbons are difficult to produce synthetically. The study of how these compounds are produced enzymatically in bacteria can be of great benefit industrially and pharmaceutically. In particular, the phenazines are difficult to synthesize but are important potential drugs.

Context: This work benefits pharmaceutical and chemical companies. The elucidation of natural biochemical pathways makes it easier to alter and utilize these pathways to make these and similar chemicals. In particular, the phenazines, which are produced by a branch of the chorismate pathway in some bacteria, are difficult to synthesize but are important potential drugs. Only when the detailed three-dimensional structures are known for the enzymes can the precise enzymatic mechanisms and relationships between the structure and the physical properties be predicted.

Several species of *Pseudomonas*, including the human pathogen *P. aeruginosa*, produce secondary metabolites known as phenazines. Dozens of naturally occurring phenazines have been described, all of which share the characteristic tricyclic heteroaromatic ring system. Phenazines are redox active compounds that participate in reactions yielding superoxide and peroxide ions, and hydroxyl radicals. These toxic molecules are thought to control the growth of other microorganisms, provide *Pseudomonas* with a competitive growth advantage, and may enhance the ability of these pathogens to colonize human and other tissue.

Two operons in *Pseudomonas aeruginosa,* each containing seven genes, are involved in the biosynthesis of phenazine (*phzA-G*). A similar, single operon has been described in *P. fluorescens* 2-79. Each of these operons encode all of the genes required to produce phenazine-1-carboxylic acid (PCA) from chorismate.

Major accomplishments: The work this year has yielded a better understanding of the phenazine pathway. We have solved the structures of the products of genes phzF and phzG and have pursued both biochemical and crystallographic methods to determine their roles, substrates, and products. Previously, we had solved the structure of phzD of this pathway.

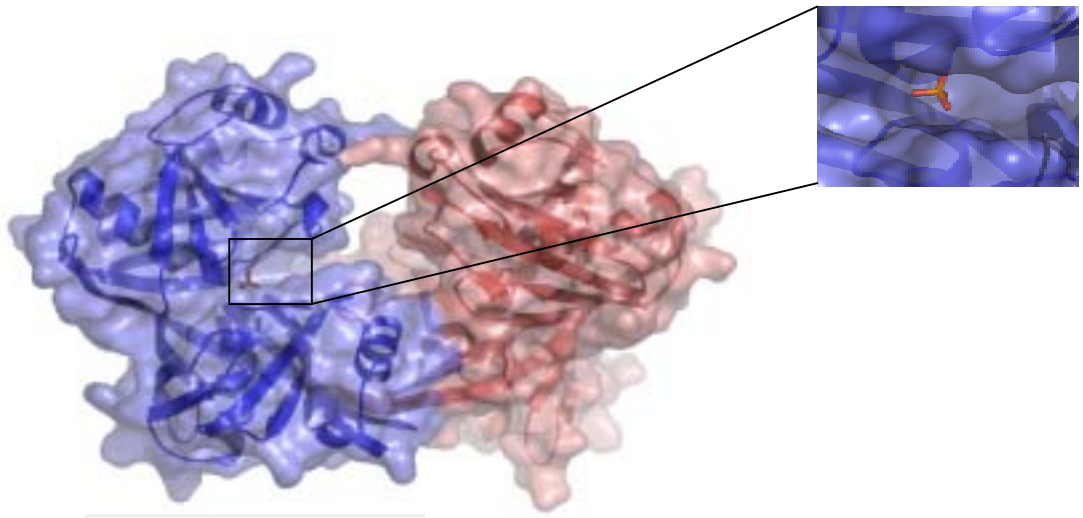
Future plans: We plan to continue our studies of the enzymes along the chorismate pathway. In particular, we are continuing to look at other enzymes in the phenazine pathway in *Pseudomonas* in order to more fully elucidate the mechanisms involved in the production of these biologically active products. We are also looking at enzymes which are homologues of these enzymes from other related pathways to try to understand the details of how the enzymes perform their specialized tasks.

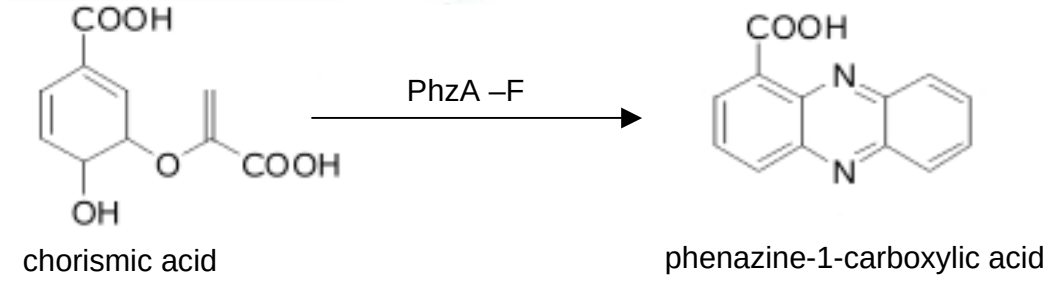
Publications:

Parsons, JF, Song, F, Parsons, L, Calabrese, K, Eisenstein, E, Ladner, JE (2004) Structure and Function of the Phenazine Biosynthesis Protein PhzF from *Pseudomonas fluorescens 2-79*. *Biochemistry* 43, 12427-12435.

Parsons, JF, Calabrese, K, Eisenstein, E, Ladner, JE (2004) Structure of the Phenazine Biosynthesis Enzyme PhzG, *Acta Crystallographica* D60, 2110-2113.

PhzF from *Pseudomonas fluorescens*





chorismic acid

phenazine-1-carboxylic acid

On the left is a dimer of phzF, one monomer blue and one monomer salmon. The secondary structure is shown inside a transparent molecular surface. The monomer has two domains and there is a cleft at the junction of the domains. In the crystal structure, a sulfate ion is positioned at the bottom of this cleft. An enlargement of this cleft with the sulfate ion is shown on the right. We hypothesize this as the probable active site. The substrate for the phenazine pathway is chorismic acid and the end product is phenazine-1-carboxylic acid. The phenazine operon codes for seven enzymes, phzA-F.

Title: The Development of Two Standard Reference Materials: Heteroplasmic Mitochondrial DNA Mutation Detection Standard (SRM 2394) and Fragile X Human DNA Triplet Repeat Standard (SRM 2399)

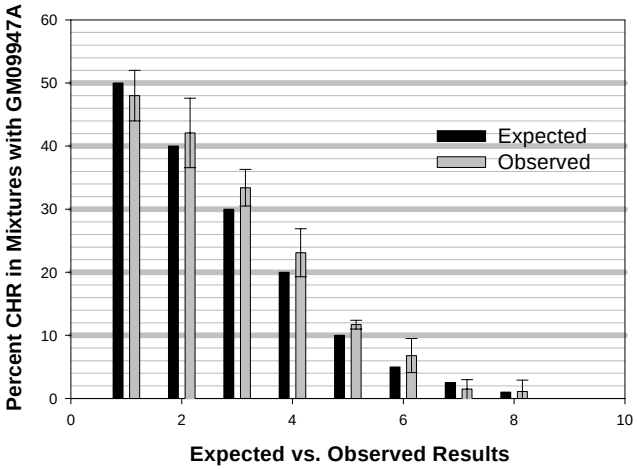
Authors: Barbara C. Levin, Diane K. Hancock, and Kristy L. Richie, DNA Technologies Group, Biotechnology Division

SRM 2394: Human mitochondrial DNA (mtDNA) mutations are important for forensic identifications and mitochondrial disease diagnostics. If a mutation is present in every mtDNA molecule, detection is routine; however, low-frequency mutations, heteroplasmies (the existence of two nucleotides at the same site), or single nucleotide polymorphisms (SNPs), scattered throughout the DNA in the presence of a majority of mtDNA with the Cambridge Reference Sequence are almost impossible to detect. Therefore, NIST has developed heteroplasmic human mtDNA Standard Reference Material (SRM) 2394 to provide quality control to forensic, medical, and DNA scientists who wish to determine their sensitivity in detecting low-frequency mutations, single nucleotide polymorphisms (SNPs) in either mtDNA or in pooled nuclear DNA samples, or in heteroplasmic sites in mitochondrial DNA (mtDNA). SRM 2394 is composed of mixtures of two 285 base pair (bp) PCR products from two cell lines (CHR and GM09947A) that differ at one nucleotide position. The CHR cell line designated polymorphic has a T at np 6371 and the GM09947A cell line containing the Cambridge Reference Sequence has a C at that site. SRM 2394 is composed of 10 tubes, one tube containing only the DNA with the polymorphism, one tube containing the DNA whose sequence agrees with the Cambridge Reference Sequence and 8 tubes containing different percentages of the polymorphic/CRS mtDNA mixtures (in which the mass % polymorphic levels are 1%, 2.5%, 5%, 10%, 20%, 30%, 40% and 50%). Before the final SRM was prepared, twelve laboratories including NIST participated in an Interlaboratory Evaluation (ILE) of a prototype of SRM 2394. This ILE was a blind study in which the investigators could use any mutation detection method of their choice. The methods included automated DNA sequencing with three different chemistries and different sequencers; denaturing gradient gel electrophoresis (DGGE)**;** the use of a designer peptide nucleic acid (PNA); the Luminex 100 system: the LigAmp procedure; and denaturing high performance liquid chromatography. Most of these procedures were unable to detect the heteroplasmy if present below 20%; an indication that, in many real life cases, low-frequency mutations remain undetected and that more sensitive mutation detection techniques are urgently needed.

SRM 2399: Fragile X syndrome is the most common form of inherited mental retardation and affects approximately 1/4000 to 1/6000 males. Symptoms range from mild to severe mental retardation, hyperactivity, autism-like characteristics, and distinctive physical attributes. This genetic disease has been associated with the expansion of an unstable CGG repeat in the FMR1 gene on the X chromosome. Fragile X Human DNA Triplet Repeat SRM 2399 is intended to provide clinical diagnostic laboratories with the quality control and quality assurance that they are correctly and accurately determining the number of triplet repeats in fragile X patient families (those individuals with normal and pre-mutation numbers of repeats). This SRM provides the fragile X positive control required by the American College of Medical Genetics Guidelines for any genetic testing. Late-onset of neurological symptoms has recently been shown in male carriers of pre-mutation alleles, a range covered by this SRM. SRM 2399 consists of 9 vials of polymerase chain reaction (PCR) products generated from DNA obtained from fragile X

cell lines or patient samples. Each vial of PCR product contains a different number of CGG repeats. An ILE with nine laboratories was completed. Both of these SRM have been completed and are waiting approval by the Office of Standard Reference Materials.

HETEROPLASMY DETECTION OF SRM 2394 USING LUMINEX100 SYSTEM



Heteroplasmy mixtures: 50, 40, 30, 20, 10, 5, 2.5, 1%

Sequencing sensitivity required 20% to be detected above noise.

Luminex beads designed w/ capture oligos:
5' GGTGTCTCCTCTATCTTAG
5' GGTGTCTCTTCTATCTTAG

Mutant was detected down to 1%

Title: Software for phyloinformatics: nextool, nexplot and a NEXUS API in Perl

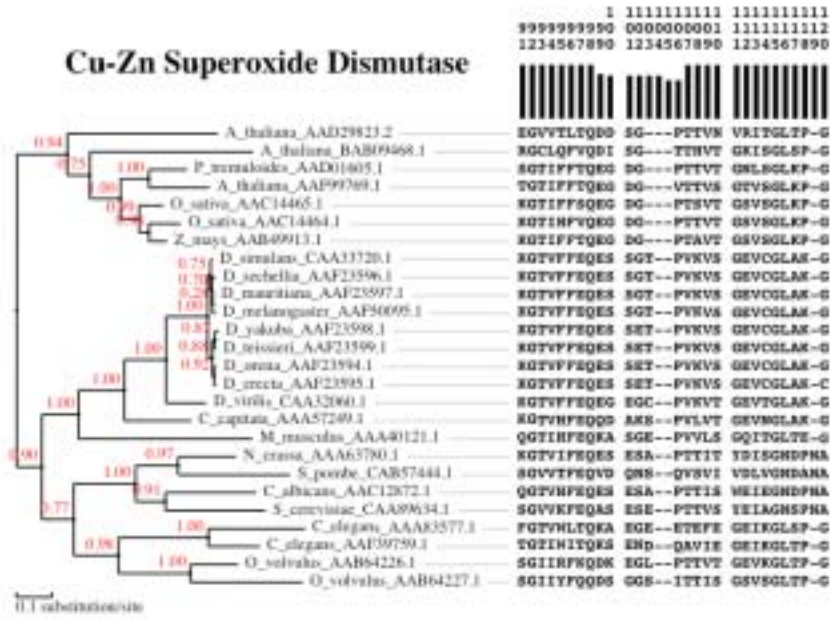
Authors: C. Liang (CARB), W. Qiu (Hunter College, CUNY, NY), P.J. Yang (CARB), B. O'Brien (Hunter College, CUNY, NY), and A. Stoltzfus (CARB; NIST Div. 831)

Vision: Develop procedures, software tools, and databases for analysis of genomic data that combine the rigor of evolutionary analysis with the scope of bioinformatics.

Context: Biological data make more sense when analyzed comparatively, in a historical (evolutionary) context, though it is often technically difficult to do so. Genome annotation, drug target discovery, biomolecule engineering, and medical genetics have come to rely increasingly on relatively crude forms of comparative sequence analysis. The success of a more robust approach will depend on software tools that facilitate the storage, exchange, processing and visualization of genomic data together with phylogenetic trees. Ongoing work by Stoltzfus and collaborators (CARB, Division 831) is focused on developing and applying "phyloinformatics" tools that combine the rigor of evolutionary analysis with the scope of bioinformatics. These tools include procedures for data analysis, software applications, and database systems.

Major accomplishments: For over a decade, evolution researchers have relied successfully on a little-known data exchange format for comparative analysis called NEXUS, which stores data sets, such as sequences to be compared, together with trees. To adapt this format, and render it more accessible to the genome analysis industry, we have developed a NEXUS Applications Programming Interface (API) in Perl, the most commonly used computer language in bioinformatics. The utility of this software tool-box is demonstrated by two applications, nextool and nexplot. Nextool is a scriptable editor designed to automate tasks such as extracting subsets of data from a NEXUS file. Nexplot is a visualization tool that produces a customizable PostScript plot that combines sequence data (or other data) with a tree (see Figure).

Future plans: These tools continue to be developed with input from users, and also are used in our ongoing research on the evolution of genome structure. In the future they will be incorporated into a server that provides, not only tools, but also sets of data based on thousands of families of protein-coding genes.



Title: A proton-coupled dynamic conformational switch in the HIV-1 dimerization initiation site kissing complex

Authors: M.-R. Mihailescu (UMBI) and J. P. Marino (831/CARB)

Vision: RNA and RNA-protein (RNP) complexes represent attractive targets for new drug therapies aimed at treating retroviral and bacterial infection. For instance, modulation of RNA-protein interactions involved in retroviral gene expression could provide novel ways to combat viral infection, or enhance the effectiveness of existing antiviral agents.

Purpose: Overall, our research focuses on the development of general approaches for detecting and quantifying RNA-protein and RNA-small molecule interactions, which can be employed in high-throughput screens (HTS) and for obtaining rapid structural information to guide rational drug design.

Context: Measurement technology developed in our studies of RNA-protein and RNA-small molecule interactions will have an impact on the biotechnology and pharmaceutical industries, as well as the Structural Biology program at NIST. The research is also responsive to ATP's interest in projects directed at developing novel approaches for manipulating protein-nucleic acid interactions for possible therapeutic benefits or medical diagnostic purposes.

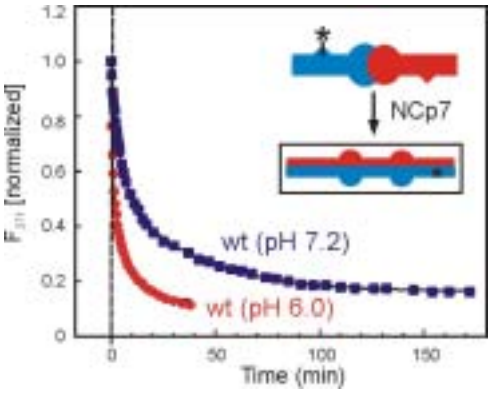


Fig. 1: pH dependence of NCp7 catalyzed DIS maturation. Plot of the normalized fluorescence decay as a function of time after NCp7 protein was added to a DIS kissing complex at pH 6.0 (red circles) and 7.2 (blue squares). Inset is a schematic of the NCp7 catalyzed structural isomerization of the kissing dimer. Asterisks indicate the position of 2-AP in the DIS24(GA)-4ap stem-loop.

Major Accomplishments: In the human immunodeficiency virus type 1 (HIV-1), the dimerization initiation site (DIS) is the sequence primarily responsible for initiating the non-covalent linkage of two homologous strands of genomic RNA during viral assembly. In a structural rearrangement catalyzed by the HIV-1 nucleocapsid protein (NCp7) and suggested to be associated with *maturation* of the budded viral particle, the DIS converts from a metastable kissing dimer to an extended duplex. Using fluorescence and NMR methods, we have demonstrate that the DIS kissing dimer displays localized conformational dynamics that result from the specific protonation of the N1 base nitrogen of the DIS loop residue A272 at near physiological pH. The rate of NCp7 catalyzed maturation of the DIS kissing dimer has also been shown to directly correlate with the observed proton-coupled conformational dynamics, where NCp7 is found to convert the dynamic A272 protonated state with a faster rate. Taken together, these results reveal a novel role for base protonation in modulating local RNA structure and demonstrate a mechanism for promoting the chaperone mediated structural rearrangement of a kinetically trapped RNA conformational state.

Impact and Future Plans: The RNA conformational 'switch' identified in the HIV-1 DIS dimer represents a possible new target for antiviral drugs. Through collaboration with researchers at the HIV-1 Drug Resistance Program at the National Institute of Cancer, we plan to screen large public domain libraries of low molecular weight compounds that target DIS and inhibit its functional role is genome dimerization and maturation.

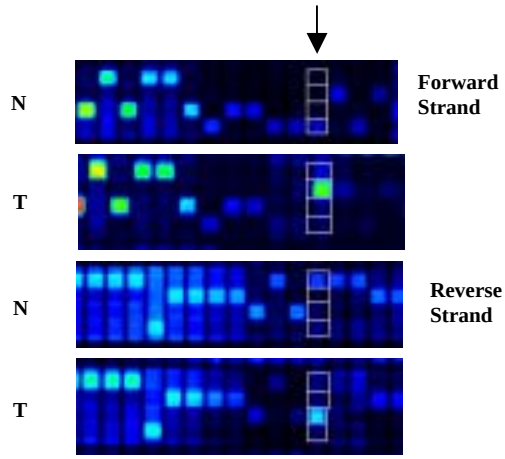
Reference: M.R. Mihailescu and J. P. Marino (2004) A proton-coupled dynamic conformational switch in the HIV-1 dimerization initiation site kissing complex, *Proc. Natl. Acad. Sci., USA.*, **101(5)**, 1189-1194.

Title: Validation of Mitochondrial Changes in Cancer

Authors: Catherine D. O'Connell and John P. Jakupciak, DNA Technologies Group, Biotechnology Division

In the past several years, homoplasmic changes have been reported in the DNA sequence of the mitochondrial genome in patient tumors. Changes can be identified by comparing tumor sequence to that of normal tissue or blood from the same individual. Lung cancer, responsible for more cancer related deaths than any other tumors in both men and women, remains a difficult problem for the early detection of cancer, and biomarkers for this purpose are not currently available. As a validation laboratory for the National Cancer Institute's Early Detection Research Network (EDRN), NIST scientists are measuring the frequency of mutation and specific mutations associated with lung cancer.

We have successfully completed a pilot study to develop a robust, high throughput protocol to directly sequence the entire mitochondrial genome (16.5 kb) to detect these mutations. This protocol was used to validate reported lung cancer mutations in previously analyzed clinical samples. In this preliminary study, we detected mutations in the tumor tissue from 5 of 11 (45%) lung cancer patients. Further, the protocol provided 100% sequence coverage where DNA quality was high (19/22 mitochondrial genomes). This protocol demonstrated that lung cancers could be detected at increased sensitivity compared to other biomarkers under consideration, and was sufficiently robust to fully sequence both clinical materials with a propensity for degradation, and which are large in size. The protocol was labor intensive, with the need for robotics to provide the necessary throughput for clinical laboratory use. To address this concern, a mitochondrial sequencing chip (MitoChip) was developed during the course of the pilot study.



MitoChip detection of a T>C homoplasmic mutation in a lung cancer patient. Both forward and reverse DNA strands are sequenced on the MitoChip. N: normal blood, T: tumor

NIST scientists are evaluating the MitoChip capabilities for the detection of both homoplasmic and heteroplasmic mutations. Initial studies are promising, with mutations detected in 6/8 lung tumors with respect to the patient's blood. Further studies are underway to address the metrics critical for reproducible, sensitive detection of tumor specific mutations, and to validate the MitoChip in other body fluids useful for early tumor detection. This program is expected to provide a measurement base for the clinical use of resequencing (genotyping) microarrays.

J.P. Jakupciak, W. Wang, M.E. Markowitz, D. Ally, S. Srivastava, A.Maitra, P.E. Barker, D.Sidransky and C.D. O'Connell. Mitochondrial DNA as an Early Detection Cancer Biomarker. Manuscript submitted.

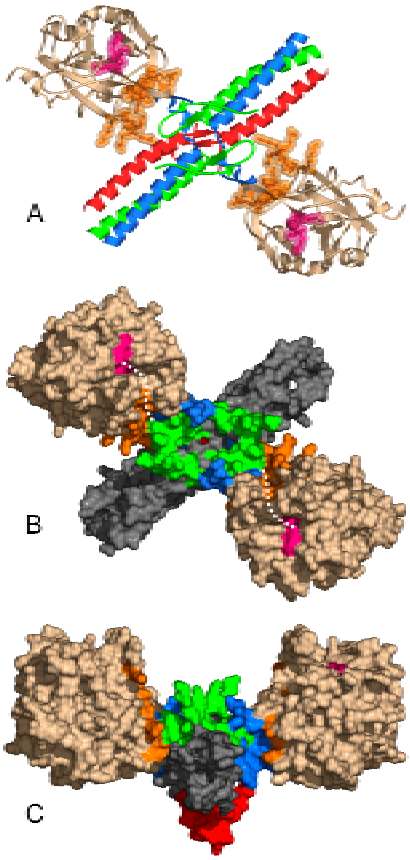
Title: Crystal Structure of the Complex between Thrombin and the Central 'E' Region of Fibrin

Authors: I. Pechik (American Red Cross and CARB), J. Madrazo (American Red Cross), L. Medved (American Red Cross), and G. L. Gilliland (CARB, 831.03)

Vision: A new frontier area in structural biology, particularly X-ray crystallography, is providing details of the interactions of biological macromolecules.

Purpose: As structural genomics efforts ramp up, structures of many components of complex macromolecular systems will be determined. The next stage will be the structures of assembles in configurations that provide biological relevance. In preparation for larger-scale efforts, CARB and American Red Cross scientists have begun investigating structural complexes that are essential for the formation of blood clots.

Major Accomplishments: A structural investigation of the nonsubstrate interactions of thrombin with fibrin, which play an important role in modulating its procoagulant activity, was undertaken. To establish the structural basis for these interactions, inhibited thrombin in complex with a fragment, E_{ht}, corresponding to the central region of fibrin, was crystallized, and the structure of the complex was determined at 3.65 Å resolution. This was a challenging effort that used X-ray data from twinned crystals and a phasing procedure that employed molecular replacement. The structure reveals a complex consisting of two thrombin molecules bound to opposite sides of the central part of E_{ht} in a way that seems to provide proper orientation of their catalytic triads for cleavage of fibrinogen fibrinopeptides (Fig.1).



(vi. picture) Figure (1). *Panels A* and *B* represent respectively ribbon diagram and solvent accessible surface of the complex viewed along a non-crystallographic 2-fold symmetry axis perpendicular to the plane of the page. *Panel C* represents the complex viewed along the axial projection of the coiled coil domains. Aα, Bβ, and γ chains in *panel A* are in blue, green, and red, respectively. NH_2-terminal portions of the Aα and Bβ chains and that of γ chain forming the funnel-shaped and the γN-domains, respectively, in *panels B* and *C* have the same color scheme as in *panel A*, while their remaining portions forming the coiled coil domains are in

Impact: The structure was consistent with a large body of biochemical data that mapped these binding sites. This work had identified a large number of charge-charge interactions, which were evident in the structure, but the presence of a central region rich in hydrophobic contacts was not predicted. These findings are of significant value to future healthcare studies developing drugs and therapies for diseases and for blood clotting.

Future Plans: It should be noted that this structure determination took advantage of an "engineered" fragment and an inhibited thrombin. These were carefully constructed to facilitate complex formation. Thus, this

work sets the stage for large-scale structure determination efforts at CARB which will be directed at other systems involving protein-protein/protein-nucleic interactions.

Publication Pechik, I., Madrazo, J., Mosesson, M.J., Hernandez, I., Gilliland, G.L. and Medved, L. 2004. Crystal Structure of the Complex between Thrombin and the Central 'E' Region of Fibrin. *Proc. Natl. Acad. Sci. USA* **101**, 2718-2723.

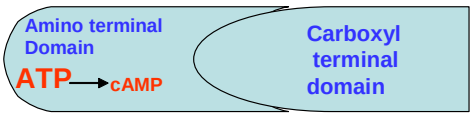
Title: Characterization of the Catalytic Domain of Class I Adenylyl Cyclase from *Yersinia pestis*

Authors: S-K. Kim, S. K. Reddy, and P. Reddy (831)

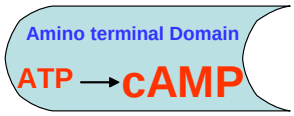
Yersinia pestis bacterium is the causative agent of bubonic and pneumonic plague. Because of its potential for major public health impact and as a bioterrorism agent, *Y. pestis* has been classified as a Category A pathogen by the Center for Disease Control and Prevention. Adenylyl cyclase (AC) is one of the important virulence factors in the pathogenesis by bacterial pathogens such as *Bacillus anthracis*. Our study was aimed at understanding, on a molecular basis, the connection between the fundamental genetic information that defines *Y. pestis* and the presumptive virulence factor adenylyl cyclase. This type of understanding could lead to a basis either for therapeutic intervention or for a vaccine for this biothreat agent.

The *Yersinia pestis* chromosome has two genes for adenylyl cyclase, *cya*A and *cya*B. The *cyaA* gene encodes 850 amino acids (aa) Class I adenylyl cyclase (YpAC1) and the *cya*B gene encodes 179 aa Class IV adenylyl cyclase (YpAC2). YpAC1 is the subject of this study. YpAC1 has discrete amino terminal and carboxyl terminal domains. These domains interact with each other to produce a low activity form of the enzyme. Removal of the carboxyl terminal domain by genetic manipulation resulted in a free amino terminal catalytic domain with enhanced adenylyl cyclase activity. The activity of the amino terminal domain was found to be four fold higher than that of the full length holoenzyme. This result suggests that the carboxyl terminal domain is inhibitory to the catalytic function and that this carboxyl terminal domain regulates the activity of the amino terminal domain. Mutagenesis studies revealed important amino acid residues for catalysis in the catalytic domain. Mutagenesis of Asp-114 and Asp-116 resulted in a complete loss of activity. This Asp-x-Asp catalytic signature is conserved in the infamous *Bacillus anthracis* adenylyl cyclase toxin. Adenylyl cyclases have been definitively shown to be virulent factors in bacterial pathogenesis. For these reasons, the adenylyl cyclase gene is an important target for gene knockout experiments and is also a useful working model to produce a vaccine against *Y. pestis.* Gene knockout experiments are planned with other Federal Laboratories having a Biosafety Safety Level 3 capability.

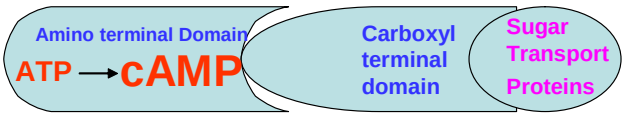
Insert: The highly active adenylyl cyclase utilizes ATP, the primary source of energy in cells, and produces a supraphysiological concentration of cyclic AMP (cAMP). In anthrax disease, an elevated concentration of cyclic AMP inhibits the activity of the gene transcription factor NF-kB, a protein that would normally stimulate the production of several inflammatory factors that coordinate immune response. Cyclic AMP thus disables the proteins in the signaling cascade. In turn, this leads to the cell death of the host.



Yersinia pestis **adenylyl cyclase has discrete amino terminal and carboxyl terminal domains. These domains interact with each other and the protein complex exhibits low adenylyl cyclase activity.**



Removal of carboxyl terminal domain by genetic manipulation results in a free amino terminal catalytic domain with enhanced adenylyl cyclase activity and thereby produces high concentration of cAMP in the cell.



Interaction of carboxyl terminal domain with sugar transport regulatory proteins in the cell results in a weaker interaction between the amino and carboxyl terminal domains resulting in a free amino terminal domain with enhanced adenylyl cyclase activity with concomitant high cAMP concentration.

Title: Thermodynamics of the redox reaction for fatty acid desaturase

Authors: Vytas Reipa (831), John Shanklin (Brookhaven National Laboratory), and Vincent L. Vilker (831)

A proper ratio of saturated to monounsaturated fatty acids contributes to cell membrane fluidity. Alterations in this ratio have been implicated in various disease states including cardiovascular disease, obesity, non-insulin-dependent diabetes mellitus, hypertension, neurological diseases, immune disorders, and cancer. Since desaturase enzymes play a critical role in these processes, they are potential targets for therapeutic intervention. Fatty acid biosynthesis in higher organisms has also attracted increased interest because of the possible use of plant oils as renewable sources for reduced carbon and for environmental cleanup.

The mechanistic details of these reactions are just beginning to emerge. Our research addresses the absence of the thermodynamic property data for the desaturase cycle. The absence of this data hampers further progress in desaturase research and its applications.

We have, for the first time, measured the redox potential E'° for the redox reaction of the only structurally characterized member of the desaturase family of enzymes. Our measurements used a methodology developed in the Biotechnology Division of NIST. By using nano-crystalline metal oxide electrodes and spectroscopic monitoring we were able to follow enzyme redox state in the presence of its cofactors.

We plan to apply our methodology to elucidate the role of the different fatty acid substrates in desaturase activity.

Publication: "Substrate binding and the presence of ferredoxin affect the redox properties of the soluble plant Δ^9-18:0-acyl carrier protein desaturase," V. Reipa, J. Shanklin, and V.L. Vilker, *Chemical Communications* **21** (2004) 2406.

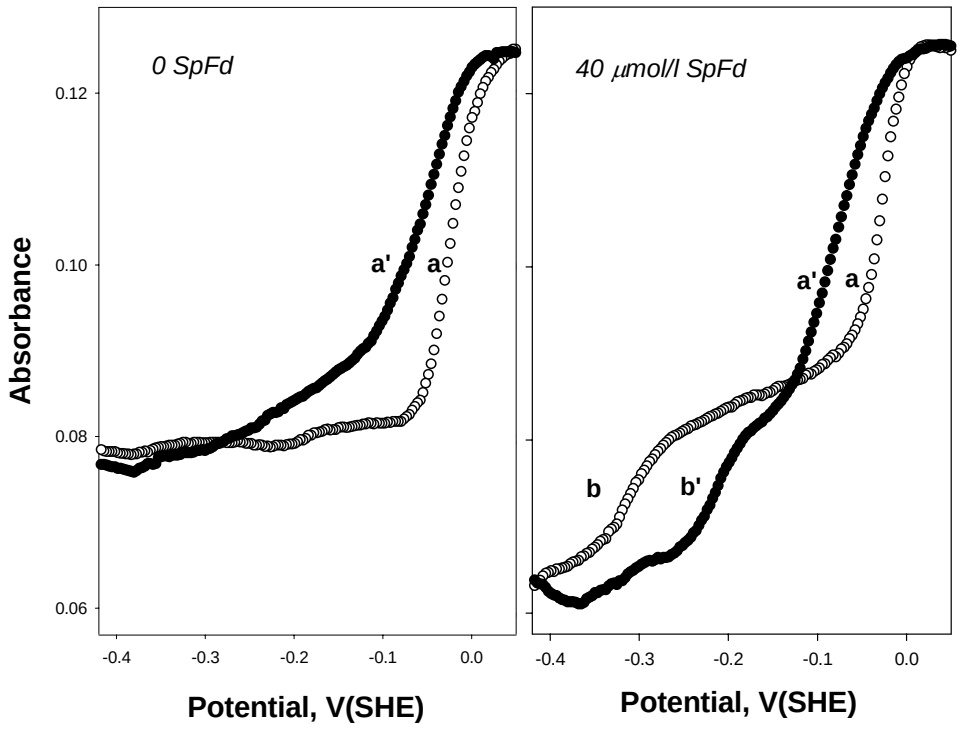


Figure caption: Absorbance at 340 nm vs. the potential of substrate-free (○) and 18:0-ACP bound (●) Δ^{9}-18:0-ACP desaturase. The left panel curves were recorded in solutions without spinach ferredoxin (SpFd); the right panel results were obtained in the presence of spinach ferredoxin.

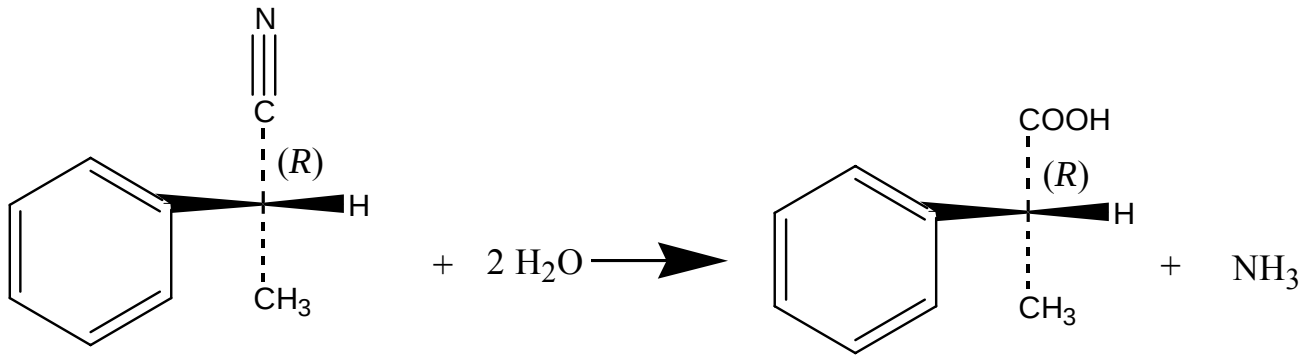
Title: Thermodynamics of the Hydrolysis Reactions of Nitriles

Authors: Y. B. Tewari and R. N. Goldberg (831)

The nitrilase enzymes catalyze the direct hydrolysis of organic nitriles to the corresponding carboxylic acids, which are potentially useful as intermediates in the agricultural and pharmaceutical industries. The use of nitrilase enzymes has attracted substantial interest in the biotechnology community because conventional chemical methods for nitrile hydrolysis entail the use of severe conditions such as the use of concentrated acids or bases and high temperatures. Such harsh conditions are generally not useful when sensitive complex molecules or chiral compounds are involved. Most importantly, the chemical methods do not permit the asymmetric synthesis of chiral compounds. In contrast, the nitrilase-catalyzed reactions proceed under mild conditions and produce a high yield of a stereospecific product. The importance of nitrilase enzymes has led recently to the development of a library consisting of over 200 new forms of this enzyme.

Several representative (model) nitrilase catalyzed reactions were selected for this investigation, which used HPLC and calorimetry combined with equilibrium modeling calculations. In all cases the reactions proceeded to completion. Specifically, the HPLC results (forward and reverse reactions) gave values of the apparent equilibrium constant *K*' having a lower limit of 350. A thermochemical cycle calculation showed that a value of the equilibrium constant for a typical ionic reference reaction for this class of reactions is on the order of 10^{11}. Values of the standard molar enthalpy of reaction $\Delta_r H_m^\circ$ for the ionic reference reactions ranged from -77 to -99 $kJ \cdot mol^{-1}$. The thermodynamic results obtained in this study provide quantitative data that can be used for the bioprocess engineering of these enzyme-catalyzed reactions. The results are the first to be reported in the literature.

Publication: "Thermodynamics of the hydrolysis reactions of nitriles," Y. B. Tewari and R. N. Goldberg, The Journal of Chemical Thermodynamics, in press.



α-Methylbenzylcyanide

α-Methylbenzeneacetic acid

Figure caption: The nitrilase catalyzed hydrolysis of α-methylbenzylcyanide to α-methylbenzeneacetic acid and ammonia

Title: Using flow cytometry to assess the utility of the biomarker CD20 for the diagnosis of B-cell chronic lymphocytic leukemia

Authors: L. Wang (831), A. Gaigalas (831), G. Marti (FDA), F. Abbasi (FDA)

B-cell chronic lymphocytic leukemia (B-CLL) is the most common hematologic malignancy in adults and accounts for 30% of all leukemias. Flow cytometry has been used to establish the phenotyping profile (identity) of B-CLL by detecting a variety of surface antigens CD5, CD19, CD20, and CD23 (note: CD stands for "cluster of differentiation"). It has been found that fewer CD20 cell receptors are present in B-CLL patients than in the normal state; this is referred to as "down regulation." However, the number of CD20 cell receptors reported in the literature for the disease state varies by more than one order of magnitude. Because of this, the potential biomarker CD20 has limited use for the diagnosis of B-CLL.

We have assessed the utility of CD20 as a critical biomarker for the diagnosis of B-CLL. We found that signals from lymphocytes stained with CD20 that had been labeled with either fluorescein isothiocyanate (FITC) or with R-phycoerythrin (PE) were lower in B-CLL patients than in healthy blood donors. This finding required the use of carefully designed and executed control experiments. The fluorescence measurements were performed on lymphocytes stained with CD20 FITC, the MESF (Molecules of Equivalent Soluble Fluorophore) values were assigned to the stained lymphocytes based on calibration curves. The down regulation of CD20 in B-CLL patients was observed by comparison with values obtained from normal donors. Because PE has a much larger fluorescence signal than fluorescein, quantifying CD20 expression using CD20 with PE may give a tighter distribution and a smaller coefficient of variation and more accurate results. The work using PE as the fluorophore is in progress.

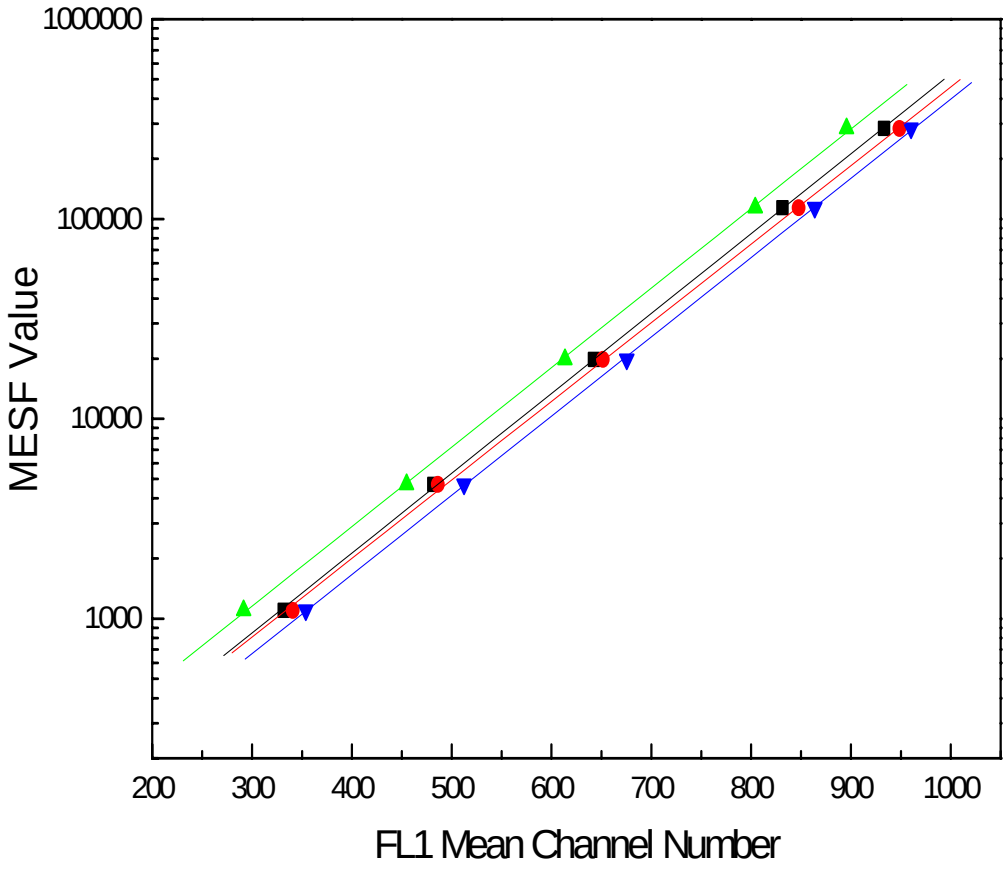


Figure caption: The figure shows calibration curves of a FACScan flow cytometer (BD Biosciences) involving four separate experiments using NIST reference material RM 8640. MESF (Molecules of Equivalent Soluble Fluorophore) values are shown as a function of the fluorescence channel number.