OMB No. 0925-0195
Expiration Date 2/28/98

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Biosensor for Rapid Screening of Galactosemia

2. SOLICITATION NO. **SBIR 96-2**

3. PRINCIPAL INVESTIGATOR ■ New Investigator

3a. NAME *(Last, first, middle)*: Wang, C.-Y.

3b. DEGREE(S): Ph.D. | B.S. |

3c. SOCIAL SECURITY NO.: 529-91-1621

3d. POSITION TITLE: Research Scientist

3e. MAILING ADDRESS *(Street, city, state, zip code)*:
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 801-583-9301
FAX: 801-583-9301

BITNET/INTERNET Address: ---

4. HUMAN SUBJECTS ■ NO ☐ YES
4a. If "Yes," Exemption no. or IRB approval date ☐ Full IRB or Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS ■ NO ☐ YES
5a. If "Yes," IACUC approval date
5b. Animal welfare assurance no.

6. DATES OF PROJECT PERIOD
From: 7/1/97 Through: 12/31/97

7. COSTS REQUESTED
7a. Direct Costs: $ 71,800
7b. Total Costs: $ 94,480

8. PERFORMANCE SITES *(Organizations and addresses)*
Protein Solutions, Inc.
391 G Chipeta Way, Suite 320
University Research Park
Salt Lake City, UT 84108

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093

10. ENTITY IDENTIFICATION NUMBER: Fed. Tax # 87-0451813
Congressional District: 2

11. SMALL BUSINESS CERTIFICATION
■ Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

12. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages _____ of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ■ YES ☐ NO

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: J.D. Andrade
Title: President and CEO
Address:
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093

Telephone: 801-581-4379
FAX: 801-583-9301
BITNET/INTERNET Address: Joe.Andrade@m.cc.utah.edu

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*: C.-Y. Wang
DATE: 12/12/96

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*: J.D. Andrade
DATE: 12/12/96

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093
Phone: 801-583-9301

YEAR FIRM FOUNDED	NO. OF EMPLOYEES *(include all affiliates)*
1987	6

TITLE OF APPLICATION

Biosensor for Rapid Screening of Galactosemia

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
C.-Y. Wang, Ph.D.	Protein Solutions, Inc.	Principal Investigator
R. Scheer, Ph.D.	" " "	Research Scientist
R. Van Wagenen, Ph.D.	" " "	Research Scientist
J.D. Andrade, Ph.D.	" " "	Technical Advisor

ABSTRACT OF RESEARCH PLAN: State the application's broad, long-term objectives and specific aims, making reference to the health-relatedness of the project. Describe concisely the research design and methods for achieving these goals and discuss the potential of the research for technological innovation. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. ***Therefore, do not include proprietary or confidential information.*** DO NOT EXCEED 200 WORDS.

Newborns are regularly screened for inborn errors of metabolism during their first days of life. Typical screenings target galactosemia, phenylketonuria, maple syrup urine disease, and homocystinuria. These screenings require that a blood sample be drawn and sent to a clinical chemistry lab with definitive results often not being available for three to four days. This has the potential to delay and sometimes eliminate the possibility for patient compliance with doctor prescribed positive intervention. This research is designed to demonstrate the feasibility of detecting clinically relevant amounts of galactose (an indicator of galactosemia) in urine using a disposable dipstick-type of biosensor based upon ATP fueled and luciferase catalyzed bioluminescence. The quantitative indication from the biosensor is presented as a spatial distribution of light which can be directly determined by visual analysis. Future development will broaden the range of analytes detected and extend the analyses to blood. Such a method for point of care screening for galactose and many other biomolecules linked to inborn errors of metabolism could significantly reduce the incidence of retardation and delayed development by offering simple, accurate, rapid and less costly analyses which will increase the extent of screening and compliance.

Provide key words (8 maximum) to identify the research or technology.

ATP , biosensor, bioluminescence, luciferase, galactose, galactokinase, galactosemia

Provide a brief summary of the potential commercial applications of the research.

A need exists for simple, inexpensive, rapid and definitive analyses of numerous biochemicals whose absence or presence may lead to inborn errors of metabolism. Ideally, such analyses can be made with disposable biosensors at the point of care.

Budget Justification

Using continuation pages if necessary, describe the specific functions of the personnel and consultants. Read the instructions and justify costs accordingly.

Dr. C.-Y. Wang, Research Scientist and Principle Investigator, will devote 50 percent of his time to this six month project. He will manage and coordinate all of the activities as well as contribute technically. He has extensive experience with the firefly luciferase reaction for ATP analysis. He will assist on all five of the objectives and his expertise will be especially useful in characterizing galactokinase (Objective 1) and in designing the microtiter plate gradients for Objectives 2, 3, and 4. His experience in luciferase stabilization and preservation will be used in Objective 5 to preserve the ATP and galactokinase.

Dr. Rick Van Wagenen, VP of Research and Development, will devote 20 percent of his time in the areas of optics and operation of the CCD camera system, data analysis and interpretation, materials issues, and design of the front end sensor for future integration into the ATP sensor platform currently under development at PSI.

Dr. Rob Scheer, Research Scientist will devote 50 percent of his time to this project. He will assist with experimental design and conduct of experiments in Objectives 2 through 5.

A laboratory technician will be appointed to conduct much of the basic laboratory chemistry and to assist Dr.'s Wang and Scheer in performing the luminescence studies.

Dr. J.D. Andrade, although not budgeted in the proposal, will be available to provide technical support, assistance and advice as needed.

Total direct costs are projected to be $71,800. Total direct personnel costs are $31,500 and fringe benefits are based on 20 percent of direct personnel costs. The companies current indirect cost rate of 60 percent was established recently with the National Science Foundation on a Phase II STTR grant.

No consultants, subcontracts, patient care costs, or fees are proposed for this work. Requested funds for equipment, supplies, travel, and "miscellaneous other" expenses are all below the limits which require detailed itemization.

Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Include laboratory, clinical, animal, computer, and office facilities at the applicant small business concern and any other performance site listed on the FACE PAGE. Identify support services such as secretarial, machine shop, electronics shop, and the extent to which they will be available to the project. Use continuation page(s) if necessary.

Protein Solutions, Inc. (PSI) occupies 1,200 square feet of research laboratory and office space located in the Research Park adjacent to the University of Utah. This space includes a chemistry lab, a biology-cell culture lab, an optics lab, and two offices. The space and equipment are adequate for the work proposed with the addition of the equipment funding requested in this proposal. Office equipment is standard and is adequate to address the administrative aspects of the project.
The company has a Technology Transfer Agreement with the University of Utah which allows for the transfer of jointly developed technologies to PSI. The company is also a member of The Center for Biopolymers at Interfaces (CBI) a state-university-industry consortium which is one of the Centers for Excellence in the Utah State System of higher education. CBI membership provides a number of key benefits including: (1) fee for service access to many analytical services at the University of Utah at a very low rate, e.g. the SEM/TEM facility, the surface analytical facility (XPS, SIMS, etc.), (2) access to specialized lab equipment in the departments of Bioengineering and Chemistry, and (3) access to faculty members who can provide expertise on a consulting basis (although no consulting funds are requested for this work). Finally, the close proximity to the University of Utah makes it easy to utilize machine shop and electronic shop capabilities at rates that are competitive for the Wasatch front.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Basic laboratory equipment at PSI includes analytical balance, stirrers, hot plates, fume hood, oven, water bath, some cell culture facilities, steam autoclave, reverse osmosis water, luminometers, optics and refrigerator. A charge coupled device (CCD) array camera and associated computer and controller are also available for the recording and quantification of luminescence experiments.

TABLE OF CONTENTS

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Chung-Yih Wang
Research Scientist

Current Employment

Protein Solutions, Inc., 391 G Chipeta Way, Suite 320, Salt Lake City, Utah 84108

Birth Date: November 7, 1962 **SSN:** 529-91-1621

Education

Ph.D., Bioengineering, University of Utah, 1996.
B.S., Chemical Engineering, National Taiwan University, Taipei, Taiwan, 1985.

Professional Experience

Research Scientist, Protein Solutions, Inc. Salt Lake City, Utah 1996 - present.

Research Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah 1989 - 1996.

Teaching Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah, 1990 - 1992.

Research Chemist, Taiwan Power Company, Taipei, Taiwan, 1987 - 1989.

Honors

University of Utah Graduate Research Fellow, 1991-1992.
Deluca Prize, 1996.

Representative Patents and Publications

C. - Y. Wang and J.D. Andrade, "Denaturation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Current Status*, P. Stanley and E. Kricka, eds. Wiley, p.427, 1991.

C. - Y. Wang and J. D. Andrade, "Interfacial Behavior of Firefly Luciferase" in *Bioluminescence and Chemiluminescence: Status Report*, A.A. Szalay, ed., Wiley, pp.99-103, 1993.

C. - Y. Wang and J.D. Andrade, "Purification and Preservation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka and P.E. Stanley, eds., Wiley, pp. 494497, 1995.

D.J. Min, C.-Y. Wang and J.D. Andrade, "Air/Water Monolayer Studies of Bioluminescent Enzymes" in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka and P.E. Stanley, eds, Wiley, pp. 494-497, 1995.

C.-Y. Wang, S. Hitz, J.D. Andrade, and R. Stewart, "Biotinylation of Firefly Luciferase in vivo: A recombinant Protein with a Special Immobilization Site" submitted to Anal. Biochem., 1996.

XI. RESEARCH PLAN

A. Specific Aims

The critical objective of this phase I research is to assess the feasibility of a direct reading quantitative, dipstick type sensor for the measurement of galactose in urine (an indicator galactosemia). The sensor design is given in figure 1. The device can be divided conceptually into three or more "zones". A liquid sample is placed in a specificity zone. In this zone, the galactose in the sample is reacted with ATP and its associated kinase (galactokinase, enzyme class (EC) 2.7.1.6). This reaction results in analyte ATP which is then quantitatively detected by the ATP sensor platform. Both the spatial modulation and transduction zones comprising the ATP sensor platform were previously developed at Protein Solutions, Inc. and will be described in more detail later. The purpose of this proposal is to develop the galactose specific "specificity zone" of the sensor. Development of this specificity zone will require enzyme concentration and substrate concentration optimization, pH buffering, and elimination of "cross-talk" from other urine constituents.

1. Characterize the available galactokinases with respect to cost, specificity, pH and temperature stability, and sensitivity.

Several types and sources of galactose are available. We will determine which one performs best for our expected conditions.

2. Study galactose in water solution using the luciferase based ATP detection system.

Samples of galactose in distilled water will be incubated with mixtures of ATP and galactokinase and applied to the luciferase based ATP detection system. (All experiments in objectives 1-4 will be performed in the liquid state.) Our approach to this objective as well as the next two will be to utilize a system of evaluating the signal as a function of ATP and galactokinase concentration using microtiter plates and a CCD electronic camera system. In this way, several series can be run simultaneously. We are regularly using this system and have found it to be both efficient and effective. Using this system we will determine the concentrations of ATP and galactokinase for optimal signal generation. We will also establish a baseline readout with which we can compare the signals received from the more chemically complex objectives.

3. Study the singular effect of individual urine components on the luciferase-based ATP detection system, including: glucose, lactose, fructose, maltose, pentose, chlorine, sodium, urea, creatinine, uric acid, and ketone bodies.

Urine has many sugars, proteins and salts which may affect the sensor readout. We will screen our system to determine how other chemicals beside galactose will affect the readout by incorporating important urine constituents such as urea, glucose, etc. into the water/galactose solutions tested in objective 1. This screening will be done using the microtiter arrays and compared to the baseline readout developed in objective 1. Preliminary studies suggest that, because of their low concentrations, urine's constituents will not be a serious problem (1).

4. Quantitate galactose in commercial urine standards using the luciferase based ATP detection system.

We will apply commercial urine standards containing known amounts of galactose to our optimized liquid state specificity zone. The analyte ATP signal will then be applied to our luciferase-based ATP detection system (transduction zone). Data from this objective will be compared to the baselines established in objectives 2. and 3.

FIGURE 1 Schematic illustration of various zones comprising the galactose biosensor.

5. Study device stability and reliability with emphasis on the preservation of galactokinase and ATP.

The stability and preservation of the basic ATP sensor platform is well understood. We will apply the trehalose/sucrose preservation methods we have already successfully applied to luciferase/luciferin (2) to preserving ATP and galactokinase for up to four months.

Objectives 1 through 4 will be performed in the liquid state using microtiter plates and our CCD camera sensing system. Phase II work will apply this knowledge to the development of an actual "dry" dipstick type device.

B. Significance and Background

Galactosemia:

Reported frequencies of galactosemia range from 1:148,000 to 1:63,000 (3-5). Clinical signs of the disease include bilateral cataracts, speech abnormality, ovarian dysfunction, growth retardation, and developmental delay. The acute effects can be controlled very effectively through implementation of a galactose free diet in the early stages of maturation (6). Galactosemia is considered a good choice for universal screening because 1) it occurs frequently, 2) it can be detected and 3) it can be treated effectively (7). Delays in detection of only a few days, however, can be fatal (8,9).

Newborns in the United States are regularly screened for inborn errors of metabolism during their first days of life. If the incidence of the disease in the newborn population is high enough that if one missed case will result in state expenditures larger than the cost of screening all newborns, then it is cost beneficial to screen all newborns (10). Typical screenings cover phenylketonuria (PKU), galactosemia, maple syrup urine disease (MSUD) and homocystinuria (3). All states screen for PKU and hypothyroidism, 26 states screen for galactosemia, 20 screen for MSUD, 19 screen for homocystinuria, and three screen for histidinemia (11). These screenings require that a blood sample be drawn and sent to a clinical chemistry laboratory, with results taking 2-3 days. This may be too long a time. Infant metabolic screening costs range from $30-$50/test. Current newborn screening programs for inborn metabolic errors include screening of umbilical cord blood, newborn blood, and urine, 40% of funds and about 80% of requests for repeat specimens are devoted to the urine screening (Massachusetts, 1976-1977)(12).

Currently there are three methods for detecting galactosemia in newborns (8). One method of screening for galactosemia directly measures the activity of galactose-1-phosphate uridyl transferase (GALT), the deficiency of which is the most common form of galactosemia. This test is highly specific to GALT and will not detect either galactokinase or epimerase deficiency, less common but still important causes of galactosemia. Since the enzyme (GALT) is thermally unstable, the test requires "fresh" blood samples. As a result, a relatively large number of tests give a false positive reading. A second method measures blood galactose levels using a mutant *E. coli* strain which relies on galactose for survival. Growth of the bacteria indicates the presence of galactose in the blood. This is a difficult test requiring expertise in the field of culturing bacteria, not to mention the delays cause by waiting the required time for bacterial growth. A third method measures galactose by monitoring its oxidation by galactose dehydrogenase with the detection of NADH by fluorescence.

Recently, a shift toward point-of-care testing – testing done at or near the patient's bedside, has taken place. This type of testing can reduce cost while decreasing delays in treatment. It reduces pre-analytic errors due to collection, storage, transportation, and reporting. Results are ready in minutes, leading to faster diagnosis and faster treatment implementation. The net results are decreased hospitalization, lower costs and improved quality of care (13). Dipsticks will reduce medical costs due to labor savings, time savings, and misdiagnosis (18).

During the course of this Phase I research project, we will show the feasibility of detecting clinically relevant amounts (0-100mg/dl) of galactose (an indicator of galactosemia) in urine using "dipstick" technology now in development at Protein Solutions, Inc. Future work will lead to the

detection of galactose in the blood samples normally collected in newborns for detection of other metabolic diseases. Our method of screening for galactose will improve current methods in the categories of speed, accuracy, cost, simplicity and will lead to an increase in the compliance/screening rate.

Galactose Sensor:

There are two very special molecules that play unique and central roles in biology, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) and its phosphate form (NADPH). NADH is a ubiquitous electron donor and ATP is generally recognized as one of the key energy currencies in biology. The two molecules act in a cyclic manner and can be regenerated or recharged. They are the basic coupling agents of cellular metabolism. A very large number of biochemical enzyme processes involve one of these two molecules.

There is a large body of literature on the development of biosensors for ATP and ATP-dependent processes and for NADH and NADPH-dependent processes, using the firefly and bacterial luciferase enzymes, respectively. Such biosensors generally employ fiber-optic or other wave guided means for delivering the luminescence to a device which can accurately measure light intensities (14,15). Although one of the most portable and most sensitive photon detectors available to the scientist is his or her own eye, it is notoriously difficult to calibrate for accurate measurements of even relative light intensity. Our eyes can, however, reliably and accurately measure changes in spatial position of light.

Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests (14,16). Since the firefly luciferase reaction is dependent on an ATP co-factor, it has been extensively used in the development of biosensors for the measurement of ATP. Until very recently such applications were frustrated by the instability of luciferases and the difficulties encountered in attempts to incorporate them into commercial devices exhibiting required reliability, accuracy, and shelf life (17).

Our sensor concept is based on the fact that, for a given luciferase/luciferin concentration, a minimum concentration of ATP is required to produce a detectable light output. Above that concentration, light is visible; below that ATP concentration, no light is detected. By filtering or consuming the ATP (with an ATP consumase such as apyrase) before it reacts with the luciferase we control the intensity of the light at each position along the sensor. A high concentration of ATP will still produce light even at the higher consumase concentrations because enough ATP remains after consumption to produce a measurable light output. A low concentration of ATP will produce light only at the lowest consumase concentrations because at higher consumase concentrations all of the ATP is consumed before it reacts with the luciferase.

To measure an ATP concentration, a sample containing ATP is distributed to the luciferase/apyrase gel at which time the ATP is consumed by the two competing enzymes. Because the turnover rate of apyrase is two orders of magnitude higher than that of luciferase, the apyrase quickly moderates the ATP concentration. The amount of ATP which reacts with the luciferase to produce light depends on both the initial ATP concentration and the concentration of apyrase.

With high initial concentrations of ATP, a large concentration of apyrase is required to eliminate emission of a detectable light signal. With low initial concentrations of ATP, only small concentrations of apyrase are required to prevent emission of light at a detectable signal. The goal of modulating the light signal with apyrase is to generate a specific light cut off point indicative of a specific concentration of ATP in the analyte solution.

Figure 2 depicts a prototype galactose sensor discussed in terms of its various functional zones: a sampling region, containing galactose analyte; a pH adjustment region; a zone which provides the ATP needed for the galactose specific reaction; a zone containing galactokinase which, in the presence of ATP and galactose, phosphorylates galactose, consuming ATP; a zone containing an apyrase gradient which modulates the ATP signal for direct visual detection; and a portion containing luciferin and luciferase which tranduces the ATP gradient to an easily viewed photon gradient. Conceptually, the galactose sensor can be split into a sensor front portion comprising the ATP containing region and the galactokinase containing region, and an ATP sensor

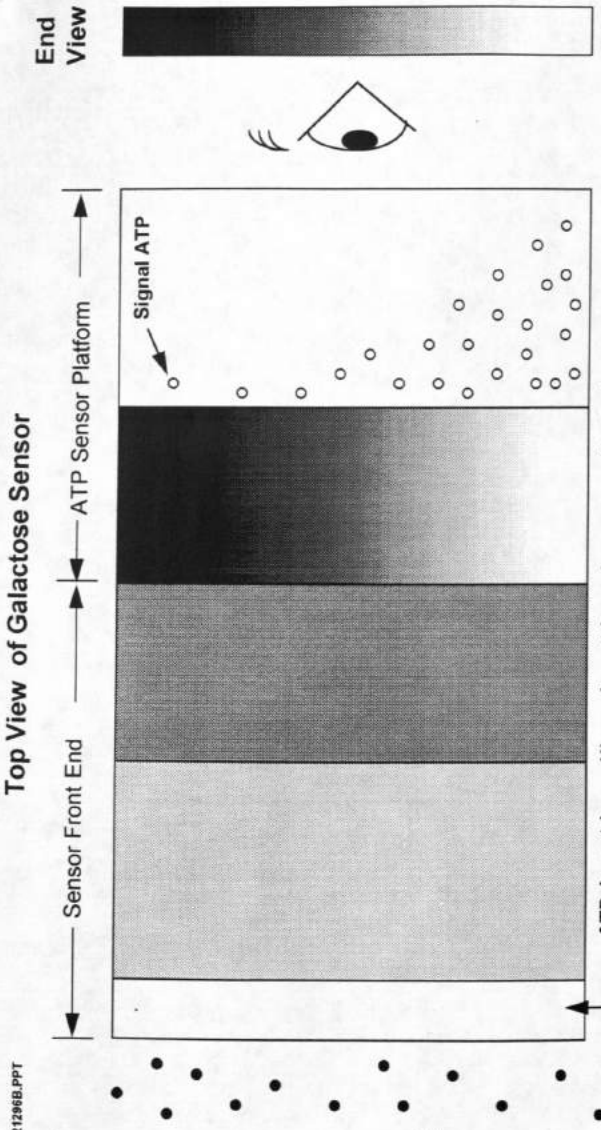


FIGURE 2 Schematic illustration of a spatial luminescence galactose biosensor.

platform end comprising the apyrase containing region and luciferin and luciferase containing region. The sensor is based on the partial consumption of ATP which in turn is modulated by an ATP "consumase" gradient (in this case apyrase) and converted to light in the transduction region by luciferase and luciferin. The resultant signal photons are spatially distributed to yield an indication of the original galactose concentration.

Operation of the sensor begins when the sensor contacts a solution containing galactose. The solution is drawn into the pH adjustment region, the ATP-containing region, and the galactokinase-containing region. The galactose is phosphorylated, and ADP is produced, thus depleting the ATP concentration. The remaining ATP, called "analyte ATP", is then drawn into the apyrase region where there is a well-defined concentration gradient of apyrase. Analyte ATP is partially converted to ADP by the apyrase leaving a residual amount of "signal ATP" which is spatially distributed. This signal ATP is then transported into a region of uniform luciferin and luciferase. The luciferase catalyzes the reaction of ATP with luciferin to produce a spatial distribution of light which can then be directly visually correlated with the amount of signal ATP and, in turn, analyte ATP, and thus the galactose concentration in the original sample.

To demonstrate and test the dependence of light output as a function of signal ATP and apyrase, we produced two dimensional matrices with gradients of ATP concentration ($2x10^{-4}$ to $1x10^{-7}$ mol/l ATP) versus apyrase concentration (2 units/ml to 0 units/ml). The gradients were produced as serial dilutions of ATP and apyrase, and pipetted in the appropriate patterns into test wells. At the bottom of the test wells we fabricated gels containing luciferase. A light intensity surface profile was made immediately after ATP was applied to the test wells. The image was made with a CCD camera and appears as Figure 3. Each of the eight rows shown in figure 3 has the same apyrase gradient and, consequently, this assay is composed of eight identical biosensors. Each of eight different ATP concentrations is applied to one of the eight identical biosensors and the resultant luminescent patter after five minutes was noted visually in the dark by an observer and then photographed with a cooled, CCD camera. The actual quantitative determination of ATP is made by looking at the spatial illumination pattern of each of the eight sensors and noting the position of the last visible luminous circle, e.g. positions A, B, C, D, and E in figure 3 would correspond to ATP analyte concentrations of 0.2mM, 0.01mM, 2.0µM, and 1,0µM ATP, respectively.

In this proposal the intent is not to determine ATP concentration (since this is accomplished in the ATP sensor platform section,) but rather to use its concentration as an indicator of the concentration of galactose. This is accomplished by reacting the ATP with galactose in the presence galactokinase. The phosphorylation of the sugar and the dephosphorylation of the ATP occur before the ATP reaches the apyrase/luciferase gel layer where light emission is initiated. Thus, the kinase serves as a mediator of ATP concentration by catalyzing its reaction with galactose. Figure 4 schematically depicts the consumption of ATP using galactose and galactokinase, note that one ATP molecule is consumed for every one molecule of galactose present in solution.

The depleted ATP "produces" a light signal, once it reaches the luciferin/luciferase transducer zone, which is inversely proportional to the concentration of galactose in the test sample. The luminescence is imaged or waveguided onto photographic film for detection, or detected by the unaided eye. Comparison with an empirically derived table correlating galactose concentration with obtained light pattern will allow for a rapid estimation of the concentration of galactose in the sample. The final sensor will include a scale along the light output zone which will permit the operator to read out the galactose concentration by correlating the scale with a sharp inflection region between light and no light.

PSI was recently awarded a STTR Phase II grant from NSF entitled, "Direct Reading Quantitative Biosensors for ATP" which focuses on enhancing the sensitivity of our existing ATP-based bioluminescent spatial sensor by several orders of magnitude. We therefore have the commitment and resources to fully develop the direct reading, quantitative ATP sensor. Allowing this proposal to focus on the galactose specific portion of the device.

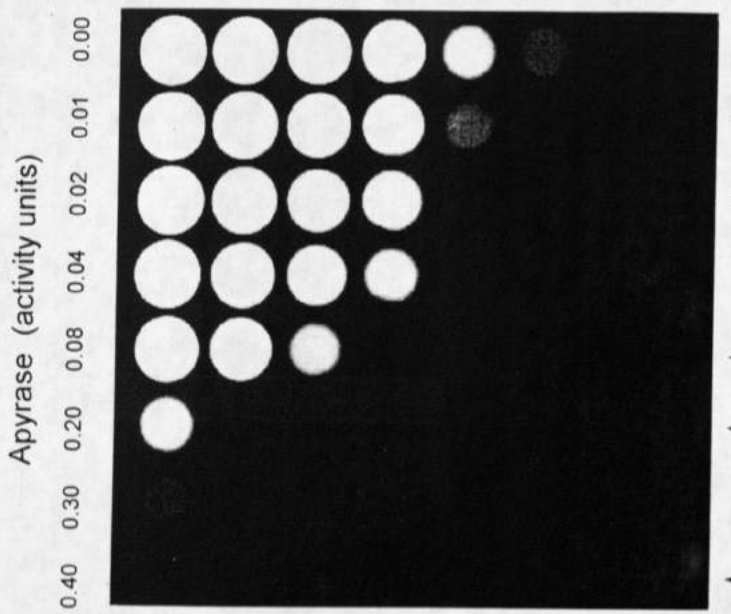
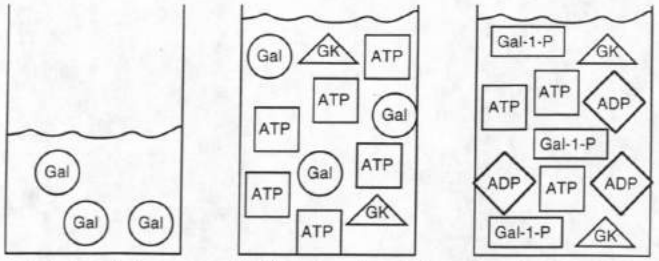


FIGURE 3 Luminescence pattern from an 8 X 8 multi-well plate experiment. See text for details.



Galactose to be detected in Solution

Addition of analyte ATP and Galactokinase

Reaction forms ADP and Galactose-1-Phosphate with signal ATP

A B C

FIGURE 4 A) a hypothetical solution with three molecules of galactose, B) galactokinase and six molecules of ATP are added, C) galactose is phosphorylated using up three molecules of ATP.

Phase II :

Expected work to be addressed during phase II will include dipstick device design, longer term stabilization studies of device, incorporation of both front-end and back-end into a single test strip, and refining reproducibility and accuracy. The phase II work will generate a device as described above and shown schematically in Figure 2.

C. Relevant Experience

1. Principle Investigator

Dr. C.Y. Wang, Research Scientist, recently completed his Ph.D. studies under Joe Andrade's supervision at the University of Utah. Dr. Wang has worked on the Firefly luciferase system for five years. He recently received the M. DeLuca Award at the 9th International Symposium on Bioluminescence and Chemiluminescence in Woods Hole Massachusetts for the studies of recombinant luciferase. He is an expert on enzyme immobilization and stabilization. He has developed a technique of immobilizing luciferase with agarose and dehydrating the system with the protection of disaccharides. Such gels can be desiccated and successfully rehydrated with full enzymatic activity. This technique supported the success of the ATP sensing platform.

Dr. Wang is also knowledgeable in carbohydrate screening and dip-stick type biosensors. He successfully screened glucose with the newly developed ATP measuring platform. He is now working as a Research Scientist at PSI and supervising a team that is continuing to develop the bioluminescence based direct reading quantitative biosensor for ATP. He will serve as the Principle Investigator for this research project. His biosketch is included.

2. Other Key Personnel

Dr. R. Van Wagenen, Ph.D., Vice President of R and D, is a bioengineer with considerable product research, design, and development experience in the medical device industry. Before joining PSI in 1996, he spent ten years working on biomedical instrumentation as VP of R & D and Director of R & D for Albion Instruments and then Ohmeda Medical Devices, respectively.

During this time, Rick and his co-workers developed a unique Raman spectroscopy respiratory/anesthesia gas monitor. His background in Materials Science, and his earlier research work dealing with the characterization of surfaces for biomedical applications are also directly relevant particularly to the design and characterization of the gels and the supports for the sensors. His product development background will enable this concept to be effectively developed into commercial products. His bio-sketch is included.

Dr. Robert Scheer, Research Scientist, received his Ph.D. in Materials Science and Engineering in 1993 and was Principal Investigator of PSI's NSF-STTR Phase I grant on the development of ATP-based biosensors using firefly luciferase. He has had considerable experience with the handling of native firefly luciferase and its stabilization in agarose gels and fiber matrices. He has worked and will continue to work closely with Dr. C.Y. Wang. Rob's background is in polymers, polymer structure and morphology, and the modeling and testing of polymeric materials. His biosketch is included.

Dr. Joseph Andrade is founder, President, and CEO of Protein Solutions, Inc. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. Five years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. Joe will be available to assist and consult in the areas of interfacial biochemistry, bioluminescence, and biosensor expertise when required.

D. Experimental Design and Methods

We have devised a series of simple liquid-phase experiments that enable us to rapidly assess the feasibility of our sensing methods. They consist of preparing a series of dilutions of the reactants and evaluating their performance in all combinations to arrive at optimal concentrations. We react the constituents in question with the firefly luciferase system in microtitration test wells, and determine the relative light output from the wells using a CCD camera. The light profiles give us a graphic record of which combinations are successful (see Figure 3), and also yield information about the sensitivity of the assay.

1. Characterize the available galactokinases with respect to cost, specificity, pH and temperature stability, and sensitivity. (weeks 1-4)

Dr. Wang will lead the study to determine galactokinase suitability. Several enzyme types and sources will be reviewed. The process will include electrophoresis studies to determine maximum purity, cross-reactivity studies with other sugars to determine maximum specificity for galactose, intrinsic fluorescence studies over a range of operating temperatures to determine maximum temperature stability, measurement of kinase activity over a range of operating pH values to determine maximum pH stability, and monitoring of ATP consumption over time to determine maximum activity.

2. Study galactose in water solution using the luciferase based ATP detection system. (weeks 1-6)

We will perform a series of experiments to determine the optimum concentrations of galactokinase (GK) and ATP for detecting galactose in the concentration range of 0 to 100mg/dl. The corresponding molar concentration range is from 0 to 5.5mM. Because each mole of galactose will theoretically consume one mole of ATP (as depicted in figure 4), the corresponding ATP detection range is 0 to 5.5mM. See figure 3. Our ATP detection system is sensitive to 10^{-7}M ATP so there should be little difficulty in detecting these amounts of galactose.

For these studies, the amount of ATP in solution should be nearly equal to the largest amount of galactose we wish to detect. Less ATP would adversely affect sensitivity at high levels of galactose, more ATP would adversely affect sensitivity at low levels of galactose. The amount of galactokinase will affect the required incubation time. More galactokinase will decrease the incubation time, less will increase the time in the specificity zone. Optimum values of both ATP and galactokinase will be determined. These studies will be performed using the microtitration plates described above and shown in figure 3. Figure 5 depicts the expected luminescent pattern after the galactose, galactokinase and ATP have incubated for the required period of time (time is dependent on amount of analyte and galactokinase) and the firefly luciferase system has been added and had time to react. No consumase (apyrase) gradient is present here. The left hand side is fully luminescent because all the ATP in solution reacts with the luciferase and luciferin to create light. The right hand side is completely dark because all of the ATP was used to phosphorylate the galactose and none is left to create light.

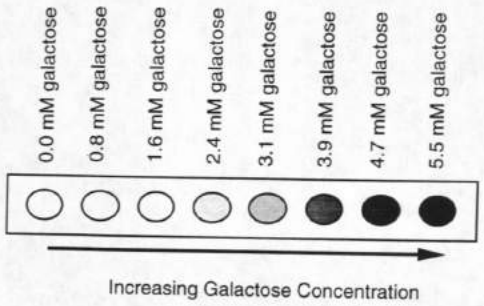


Figure 5 For a single concentration of galactokinase and 5.5 mM ATP, a one dimensional gradient of galactose is studied

The optimum concentration of ATP and GK will be the one which most efficiently phosphorylates the galactose (how well it consumes ATP.) The signals obtained here will serve as the baseline for future work. Dr. Wang has collected preliminary data similar to that shown in figure 5 to suggest that the concept may work.

3. Study the singular effect of individual urine components on the luciferase-based ATP detection system, including: glucose, lactose, fructose, maltose, pentose, chlorine, sodium, urea, creatinine, uric acid, ketone bodies, and pH. (weeks 6-12)

These screenings will be performed with the ATP/galactokinase solution optimized in objective 2 using the same concentrations of galactose previously tested. A two dimensional microtiter arrangement will be used to individually screen the carbohydrates, pH and the salts found in urine. One axis will be a galactose gradient, the other will be the urine component gradient. See figure 6. A series of dilutions ranging from zero to maximum expected concentration will be used.

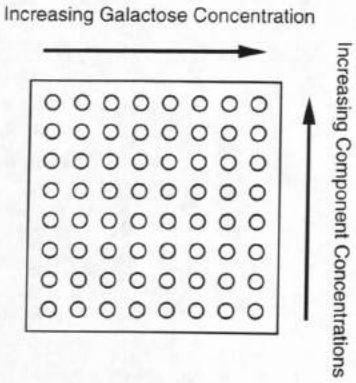


Figure 6 For a series of galactose dilutions, a two dimensional array of urine component dilutions (including a blank) will be arranged as shown.

In addition to these chemicals, we will arrange an array of pH values common to urine (4.8-8.5). Additional buffer solution will likely have to be added to restore a pH of 7.8 (optimal for the light producing luciferase-luciferin reaction.) Different enzymes perform optimally at different pH values, ultimately, the sensor will incorporate the appropriate buffering system to ensure optimum pH values for each enzymatic reaction (pH adjustment region on figure 2.)

4. Quantitate galactose in commercial urine standards using the luciferase based ATP detection system. (weeks 12-22)

We will incubate commercial urine standards containing known amounts of galactose in our optimized specificity solution (ATP/galactokinase). The result of this incubation will be applied to the signal transduction solution (luciferase/luciferin) and compared to the baseline values obtained in objectives 2. and 3. Once different amounts of galactose are detectable, we will apply the incubated samples to the spatial modulation solution (apyrase gradient) prior to detection by the signal transduction solution. This last step will allow quantification of galactose in liquid urine samples.

We will quantify clinically relevant amounts of galactose in commercial urine standards using our luciferase-based ATP detector system.

5. Study device stability and reliability with emphasis on the preservation of galactokinase and ATP. (weeks 4-24)

During the first weeks of this grant period Dr.'s Wang and Van Wagenen will initiate preservation studies of galactokinase and ATP. Studies will first focus on incorporating the enzyme and substrate into a trehalose/agarose sol, which will be gelled and dehydrated under low particulate and partially sterile conditions to minimize contamination. The sols will be of varying agarose and trehalose concentrations to determine optimum conditions. Several drying condition will also be studied including vacuum drying, air drying, cold drying and inert gas drying. Various storage conditions will also be studied. The dehydrated gels will be stored in vacuum sealed foil packs at room temperature, 4°C, and -20°C for periods ranging from one day to 20 weeks (or until the end of the grant period).

Dr. Wang has successfully preserved luciferase/luciferin gels for periods of up to two years using a similar system (2). We don't anticipate any difficulty with this objective.

E. Human Subjects (none)

F. Vertebrate Animals (none)

G. Consultants/Advisors

We will not have any paid consultants for this Phase I research. The following individuals, however, frequently serve in an advisory and consulting capacity with reimbursement from other sources. Dr. Larry Kricka will be particularly helpful for the proposed research.

Scientific Advisory Board:

Dr. Larry Kricka, Director of the General Chemistry Laboratory and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania, Philadelphia. Dr. Kricka is internationally recognized for his work on applying bio- and chemi- luminescence to clinical chemistry. He is editor of the *Journal of Bioluminescence and Chemiluminescence* and editor/author of many books on Bio- and Chemi-luminescence in clinical biochemistry. We interact with Dr. Kricka several times each year. He will provide appropriate advice and guidance to this project as part of his service on the Scientific Advisory Board.

Dr. Jerry Nelson, microbiologist and President of Nelson Labs, a nationally recognized lab providing a wide range of biological testing and compliance monitoring testing for industry.

Dr. Russell Stewart, Assistant Professor of Bioengineering at the University of Utah. Dr. Stewart is an expert in recombinant techniques for the study of luciferases and motor proteins.

Dr. Vladimir Hlady, Associate Professor of Bioengineering at the University of Utah. Dr. Hlady is an expert in interfacial fluorescence studies of proteins at surfaces.

Dr. Woody Hastings, Professor of Biology at Harvard University. Dr. Hastings is internationally recognized for his basic scientific studies in bioluminescence.

Dr. Henry Kopecek, Professor of Pharmaceutics and Bioengineering at the University of Utah, President of the Controlled Release Society. Dr. Kopecek is internationally recognized for his work in hydrogels and related polymers for drug delivery and biocompatibility.

Dr. Don Johnson, former new biotechnology product manager for DuPont. Now, Dr. Johnson runs an independent consulting firm and is the Chairman of CBI's (Center for Biopolymers at Interfaces) Industrial Advisory Board.

H. Contractual Arrangements (none)

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