OMB No. 0925-0195
Expiration Date 2/28/96

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)*
Direct Reading Biosensor for GALT (E.C.2.7.7.12)

2. SOLICITATION NO. PHS 97-2

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

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

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

3c. SOCIAL SECURITY NO. *Provide on Personal Data Page*

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
BITNET/INTERNET Address:

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 801-583-9301
FAX: 801-583-4463

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: September 30, 1998 *Through:* March 30, 1999

7. COSTS REQUESTED
7a. Direct Costs: $ 70,435
7b. Total Costs: $ 98,896

8. PERFORMANCE SITES *(Organizations and addresses)*
Protein Solutions, Inc.
391 G Chipeta Way, Suite 320
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 84518-0093

10. ENTITY IDENTIFICATION NUMBER: Fed. Tax # 87-045-1813
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.

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

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

Telephone: 801-583-9301
FAX: 801-583-4463
BITNET/INTERNET Address:

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.)*: Clarence Yih Wang
DATE: 12-12-97

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.)*:
DATE: 12-12-97

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: 1987
NO. OF EMPLOYEES *(include all affiliates)*: 5

TITLE OF APPLICATION
Direct Reading Biosensor for GALT (E.C.2.7.7.12)

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
C.-Y. Wang, Ph.D.	Protein Solutions, Inc.	Principal Investigator
R. Van Wagenen, Ph.D.	" " "	Research Scientist
R. Scheer, Ph.D.	" " "	Research Scientist
J. D. Andrade, Ph.D.	" " "	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. Typically, screenings are done for galactosemia, phenylketonuria, maple syrup 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 a day or more. This has the potential to delay and sometimes eliminate patient compliance with doctor prescribed positive intervention. This research is designed to demonstrate the feasibility of detecting clinically relevant GALT (a critical enzyme responsible for most galactosemia cases) activity in blood using a disposable, dipstick-type biosensor based on ATP fueled and luciferase catalyzed bioluminescence. This sensor has the advantages of being fast, instrument free, low cost, and easy to use. Future development will broaden the range of analytes to include a complete screening panel for galactosemia. Such a method for point of care screenings for GALT could significantly reduce the incidence of retardation and delayed development by offering simple, accurate, less costly analyses which will increase the effectiveness of screening and patient compliance.

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

GALT, ATP, bioluminescence, galactose-1-phosphate, luciferase, galactosemia, galactokinase

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

A need exists for simple, inexpensive rapid and definitive analyses of numerous bio-molecules whose presence or absence are related to inborn errors of metabolism. Ideally, such analyses can be made with disposable biosensors at the point of care and used to manage these chronic diseases.

Budget Justification

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

Personnel: Dr. C.-Y. Wang will serve as Principal Investigator on this project. He has served as PI on other SBIR grants. He is an expert in the application of firefly luciferase to biosensor development and applications. His technical contribution will be primarily in the protein, enzymatic assay and biochemistry aspects of this work, primarily as this relates to the luciferin-luciferase system. **Dr. R. Van Wagenen** has an extensive background in interfacial phenomena, optics and spectroscopy instrumentation. His background in luminescence and device development will address the hardware and materials aspects of the biosensor development. He will also assist with planning and coordination. **Dr. Robert Scheer**, Research Scientist, serves both part time with the company and is also a member of the faculty in the Department of Materials Science and Engineering at the University of Utah. Given Rob's background in materials science, he will make his technical contributions in the area of materials science and surface chemistry. **Dr. J.D. Andrade** is not an employee of the company, however, he does actively serve as a technical advisor in a wide range of areas related to biomaterials, proteins at interfaces, adsorption, device development and polymer science. Mara Hammer has a Biology Degree and serves as a Lab Technician.
Financial: Total direct personnel costs are $47,435. Fringe benefits are 20% of salary and indirect costs are 60% of total direct personnel costs. No consultants, subcontracts, patient care costs or fees are requested for this work.
Equipment: A relatively inexpensive HPLC system is requested for protein purification work. A thin film spreading device is requested for preparation of well defined thin films on our prototype sensor substrates.
Supplies: Funds are requested for general lab supplies and biochemical supplies such as proteins, enzymes, etc., as well as analytical supplies related to the HPLC, i.e., chromatography sorbent materials, buffers, etc.
Travel: Travel funds are requested to attend a conference related to GALT (E.C. 2.7.7.12) biochemistry or clinical chemistry such as the annual meeting of the AACC, or a conference addressing inborn errors of metabolism.
Other: Funds are requested for external clinical laboratory analyses of some samples. These analyses will be conducted by Associated Regional University Pathologists, Inc. (ARUP) a major clinical chemistry laboratory serving the western U.S. Funds are also requested for a partial annual membership fee for the Center for Biopolymers at Interfaces, a state-university-industry consortium at the University of Utah which provides us with full and extensive access to research resources and interactions with professors, students and staff at the University of Utah.

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 1,400 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 lab, an optics lab, a conference room and two offices. The space and laboratory equipment (noted below) 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 proposed project, I.e., computers, laser printer, copy machine, FAX machine, etc. 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 a very low rates, e.g. the SEM/TEM facilty, (2) the Surface Analytical Facility (XPS, SIMS, etc.), (3) access to specialized laboratory equipment in the departments of Bioengineering (Optics Laboratory), and Chemistry (analytical biochemistry labs), and (4) access to faculty members who can provide expertise on a consulting basis. Finally, there are a wide range of technical consulting and fee for service machining and electronic design companies located in the immediate area.

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 balances, stirrers, hot plates, a fume hood, oven, pH meter, water baths, HEPA filtered laminar flow air work areas, steam autoclave, reverse osmosis - filtered water, light microscopes, Turner Designs luminometer, cameras, vacuum system, a gel electrophoesis system, -85 C freezer, and a lap top computer. A charge coupled device (CCD) array camera and associated computer and controller are also available for the recording and quantification of luminescence experiments to supplement the luminometer. A Beckman UV-Visible spectrophotometer is available for spectrophotometric characterization of proteins.

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, 1997.
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.

RESEARCH PLAN

A. Specific Aims

The critical objective of this phase I research is to access the feasibility of a direct reading (instrument free), dipstick type threshold sensor for galactose-1-phosphate uridyltransferase (GALT, E.C.2.7.7.12) deficiency. This disease is known as Type II galactosemia. The sensor concept and the chemistry are given in Fig.1. The device can be conceptually divided into three zones: a sample pretreatment zone, a GALT reaction zone, and a galactose-1-phosphate sensing zone. All the reaction zones will be described in detail in section B. We propose to:

1. Derive a mathematical model and simulate the reaction system. The results of simulation will provide information for the solution phase chemistry study.

2. Study the chemistry in solution phase. The feasibility and optimal reaction conditions (enzyme concentrations, substrate concentrations and reaction time) will be determined.

3. Study the interference of other components in typical blood samples. Possible components that may affect the performance of the sensor include: free adenosine-5'-triphosphate, glucose-1-phosphate, UDP-galactose, and galactose. The results of these studies will feed back to the design of the blood pretreatment region.

4. Study the preservation and immobilization of both enzymes and substrates. Two methods will be investigated: physical entrapment by agarose/trehalose (sucrose), and lyophilization.

Objectives 2 and 3 will be performed in solution phase using luminometer and CCD camera sensing systems. GALT will be purchased from commercial sources. No blood samples will be used in this Phase I research.

Phase II work will apply this knowledge to an actual dipstick type sensor design. In phase II, we plan to:

a. Design the prototype sensor (geometry, sample transportation)
b. Study the feasibility of combining reaction zones and simplify the geometry
c. Design the blood pretreatment region
d. Develop a quantitative sensor using a specially designed, low cost, and compact sized instrument as a readout device. This instrument is currently under development at Protein Solutions, Inc. in another research project.

B. Significance and Background

Galactosemia

Galactose is normally metabolized to glucose through the coordinated activities of three enzymes: galactokinase, galactose-1-phosphate uridyltransferase (GALT), and

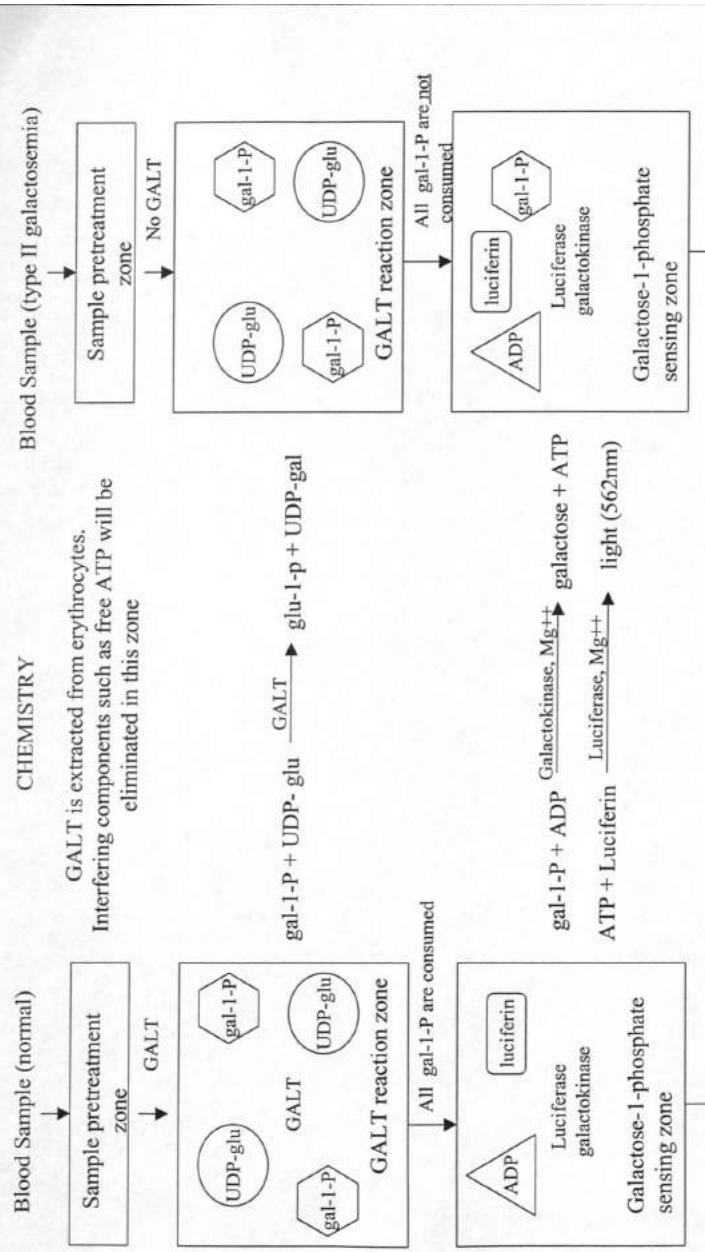

Fig.1 A schematic illustration of GALT sensor. ***gal-1-P***, galactose-1-phosphate; ***UDP-glu***, uridine diphosphoglucose; ***glu-1-P***, glucose-1-phosphate; ***UDP-gal***, uridine diphosphogalactose; ***ATP***, adenosine-5'-triphosphate; ***AMP***, adenosine-5'-monophosphate.

uridine diphosphate 4-epimerase (1). Deficiency of any of these enzymes is diagnosed as galactosemia (see Fig.2 and Table.1).

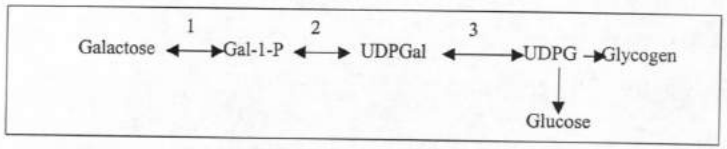

Fig.2 Simplified galactose metabolism. ***Gal-1-P***, galactose-1-phosphate; ***UDPGal***, Uridine diphosphogalactose; ***UDPG***, uridine diphosphoglucose. ***1***, galactokinase; ***2***, galactose-1-phosphate uridyltransferase; ***3***, UDP-galactose 4'-epimerase.

Table.1 Different types of galactosemia (2-4).

Galactosemia Type	Deficient Enzyme	Incidence
Type I	Galactokinase	1/168,000
Type II	Galactose-1-phosphate uridyltransferase (GALT)	1/62,000
Type III	UDP-galactose 4'-epimerase	< 1/168,000

High concentration of galactose and its metabolites are toxic to mammals. Clinical signs of the disease include bilateral cataracts, speech abnormality, ovarian dysfunction, growth retardation, and developmental delay (1). The acute effects can be controlled very effectively through the implementation of galactose free diets in the early stages of maturation (5). 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. Delays in detection of only a few days, however, can be fatal or can lead to severe physical and mental retardation (7,8).

Newborns in the United States are regularly screened for inborn errors of metabolism during the first days of life. If the incidence of the disease in the newborn population is high enough that one missed case will result in state expenditure larger than the cost of screening all newborns, then it is cost beneficial to screen all newborns (9). Typical screenings cover phenylketonuria (PKU), galactosemia, maple syrup urine disease (MSUD) and homocystinuria. Currently, 47 states screen for galactosemia (10). These screenings require a blood sample be drawn and sent to a clinical chemistry laboratory, with results often taking 2-3 days. This may be too long a time.

Currently there are three major methods for screening galactosemia in newborns (11,12). The first screening method (Beutler's Test) is to assay the GALT activity directly by coupling the GALT reaction with two other enzymes (7). This method requires an UV source to visually detect the fluorescence from NADPH and can only be performed in a clinical laboratory environment. Beutler's test can only detect transferase deficient galactosemia (type II), however, it is the most common screening method used in the United States. Two reasons explain its popularity in clinical chemistry laboratory: 1) it is faster than Paigen's test (described later), 2) it cost-effective to screen for GALT

deficient galactosemia only since the incidence of type II galactosemia is much higher than type I and type III (see Table.2). A second method (Paigen's Test) measures blood galactose and galactose-1-phosphate levels using a mutant *E.coli* strain which cannot survive in the presence of galactose and galactose-1-phosphate (11,13). No cell growth indicates a positive test. This is a difficult test requiring expertise in the culture of bacteria, not to mention the delay cause by waiting the required time for bacteria growth. A third method (Hill's Test) measures galactose by monitoring its oxidation by galactose dehydrognase with the detection of NADH by fluorescence (11). This method requires an UV source and again can only be carried out in clinical laboratory environment.

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 diagnosis and treatment. It reduces pre-analytic errors due to collection, storage, transportation, and reporting. Results are often ready in minutes, leading to faster diagnosis and faster treatment implementation. The net results are decreased hospitalization, lower costs and improved quality of care. Dipstick-like sensors will reduce medical costs due to labor savings, time savings, and misdiagnosis (18).

A good example would be the early release of newborns and mothers. It is common that a newborn be released from hospital within 24 hours in order to reduce insurance cost. 24 hours is too short a time to show any manifestation of most of the metabolic diseases. Pediatricians will not be able to give any treatment in such a short hospital stay. There is need for a fast and accurate screening technology, or the screening procedure is so simple that it can be performed by parents in home environment. A dipstick-like sensor seems most appropriate for these purposes.

Dipstick devices generally have a bad name in clinical chemistry and medical circles because they are normally based on colorimetric technologies and color matching processes, whose reading depends on the observer's color perception. Such devices are difficult to calibrate and standardize. More recently, the development of linear reading devices, more or less "thermometer-like", such as immunochromatographic (14) drug assays and the over the counter cholesterol test, have started to improve the reputation of dipstick analytic devices. A diagnostic GALT sensor which can accurately measure GALT activity is scheduled in Phase II research. For the purpose of screening, a "threshold" type design will fit the purpose. A comparison of the sensor we proposed and traditional screening methods are summarized in Table.2.

1. Blood Sample Application Region

2. Blood Pretreatment Region (GALT will be extracted from erythrocytes, interfering components such as free ATP will be eliminated in this region)

3. Water Soluble Barrier (Reaction time will be controlled by the thickness of the barriers)

4. GALT Reaction Zone (GALT from blood sample will react with galactose-1-phosphate and UDP-glucose)

5. Galactose-1-phosphate Sensing Zone (galactose-1-phosphate concentration is determined in this zone by the intensity of bioluminescence)

6. Reaction Completion Indicator (Chemical will change color when react with water)

Fig.2 One possible configuration for GALT sensor. The reaction regions are engraved into the sensor. This "channel" will guide the sample into different reactions zones. A "+" image on the film represents a positive test. In actual use, the sensor will be mounted on a holder with certain tilt angle, so the sample will be driven by gravity.

sensor we proposed here is one of the channels. Such panel will be able to provide all the information a pediatrician needs. This panel can also be modified into a quantitative panel by coupling with simple instrumentation.

A Complete Galactosemia Screening Panel			
	Channels	Target Analyte	Diagnostic Information
1	Galactose Channel	Galactose	High galactose concentration ---- Type I or Type II
2	Galactose-1-phosphate Channel	Galactose-1-phosphate	High galactose-1-phosphate ---- Type II or Type III
3	Galactokinase Channel	Galactokinase Activity	Low galactokinase activity ---- Type I
4	GALT Channel	GALT activity	Low GALT activity ---- Type II

Fig.3 Schematic illustration of a galactosemia screening panel. If channel 1 and/or channel 2 detect high concentration of galactose and/or galactose-1-phosphate but channel 3 and channel 4 show normal activity, then it is Type III galactosemia.

Sample pretreatment zone

Protein Solutions, Inc. is currently developing a thin layer chromatography based device, which is able to extract and separate proteins from erythrocytes under another project. This technology can be applied to the sample pretreatment zone study in phase II.

GALT reaction zone

GALT extracted from erythrocytes will be transported to this region. Galactose-1-phosphate and uridine diphosphoglucose need to be built into this reaction zone. Concentration of both substrates should saturate the enzyme. Galactose-1-phosphate concentration need to be optimized so it can be consumed in a few minutes by normal GALT activity sample but still has enough driving force to produce detectable ATP in galactose-1-phosphate sensing zone (see Fig.1).

Galactose-1-phosphate sensing zone

There are two very special molecules that play unique and central roles in biology, adenosine-5'-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 and recharged. They are the basic coupling agents in 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 NADH-dependent processes, using firefly luciferase and bacteria luciferase, respectively. Such biosensors generally employ fiber-optic or other wave guided methods for delivering the bioluminescence to a device, which can measure light intensities (15,16). However, the most portable and sensitive photon detector to a scientist or a physician is his/her own eyes that are reliable in differentiating large difference in light intensity.

Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests (15,17). Since the firefly luciferase reaction depends on an ATP co-factor, it has been extensively used in the development of biosensors in measuring 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 (18,19).

The firefly luciferase reaction requires ATP, luciferin (a benzothiazole compound) and the presence of molecular oxygen:

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow{\text{luciferase, Mg++}} \text{AMP} + \text{oxyluciferin} + CO_2 + \text{PPi} + \text{light(562 nm)}$$

When luciferase is saturated with luciferin and oxygen, the bioluminescence intensity is proportional to ATP concentration up to approximately 1 uM ATP (20). The key to measure galactose-1-phosphate concentration by using the firefly luciferase is to couple luciferase with galactokinase reaction:

$$\text{Galactose-1-phosphate} + \text{ADP} \underset{}{\overset{\text{galactokinase, Mg++}}{\rightleftharpoons}} \text{galactose} + \text{ATP}$$

Although the equilibrium of galactokinase reaction favors the formation of galactose-1-phosphate, the reaction is reversible (21,22). By adding high concentration of galactose-1-phosphate and adenosine-5'-diphosphate (ADP), the equilibrium will be shifted towards the formation of ATP which can be measured by firefly luciferase. system. High galactose-1-phosphate concentration will result in high ATP concentration thus high intensity of bioluminescence. The consumption of ATP by luciferase also facilitates the galactokinase reaction shift to the right hand side. Luciferase system is very sensitive to ATP, 10^{-8} M ATP can produce visually detectable light. Preliminary studies of a galactose-1-phosphate sensing platform is presented in Fig4a and 4b.

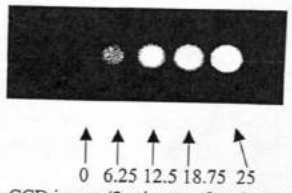

Fig. 4a CCD image (2 minutes after the reaction started) of the intensity gradient resulted from varied galactose-1-phosphate concentration

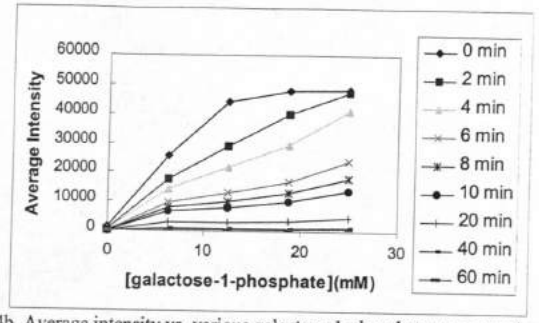

Fig.4b. Average intensity vs. various galactose-1-phosphate concentration. [ADP]=2.5mM, [galactokinase]=3U/ml,[luciferase]=4.4U/ml.

In Fig.4a and 4b, luciferase and galactokinase were mixed in a homogeneous solution. The reaction was initiated by the addition of galactose-1-phosphate, the four bright spots in Fig.4a remained visible for one hour and the intensity increases as galactose-1-phosphate concentration increases. In Fig.4b, the light intensity seems to be proportional to galactose-1-phosphate of the observation window between 2 minutes and 10 minutes. Mathematical models need to be applied to confirm this relationship. Fig.4b also demonstrates the potential to modify the sensor into a quantitative system by using a simple instrument as readout device.

C. Relevant Experience

1. Principle Investigator

Dr. C.Y. Wang, Research Scientist, recently completed his Ph.D studies under Joe Andrade's supervision at 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 development of recombinant luciferase. He is also an expert on enzyme immobilization and stabilization. He has developed a technique to of immobilizing luciferase with agarose and the dehydrating the system with the protection of disaccharides. Such gels can be desiccated and successfully rehydrated with full enzyme activity. This technique supported the success of the galactose-1-phosphate sensing platform.

Dr. Wang is also knowledgeable in carbohydrate screening and biosensors. He has successfully screened glucose with ATP/luciferase system. He is now working as a Research Scientist at PSI and supervising a team that is continuing to develop the bioluminescence based direct reading biosensor for galactose. He will serve as the Principle Investigator for this 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 coworkers developed a unique Raman spectroscopy respiratory/anesthesia monitor. His background in Materials Science, and his earlier research work dealing with the characterization of surfaces of biomedical applications are also directly relevant to the design and characterization of the gels and supports for the sensors. His product development background will enable this concept to be effectively developed into commercial products. His biosketch is included.

Dr. Robert Scheer, Research Scientist, received his Ph.D. in Materials Science and Engineering in 1993 and was the Principle Investigator of PSI's NSF-STTR Phase I grant on the development of ATP-based biosensors using firefly Luciferase. Her has had considerable experience with the handling of native firefly luciferase and its stabilization in agarose gels and fiber matrices. He has worked closely with Dr. C. Y. Wang. Rob's background is in polymers, polymer structure and morphology, and the modeling and testing of polymer 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 access the feasibility of our sensing methods, they consists of a series of dilutions of the reactants and evaluating their performance in all combinations to arrive optimal concentrations. Most of the experiments will be performed in microtitration test wells. 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 (see Fig.4a). The gradient in Fig.4a can be expanded to two-dimensional. A typical experiment layout is given in Fig.5. For the study of kinetics aspects, which needs to follow the time course closely, our highly sensitive luminometer will suit the purpose.

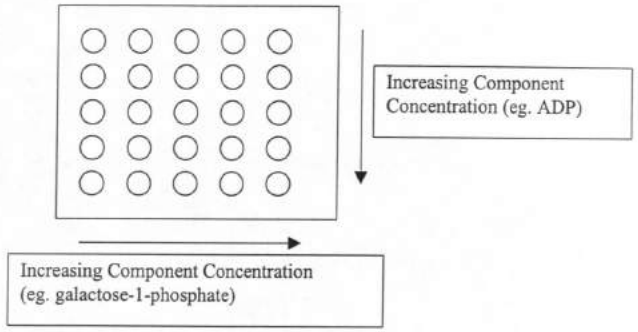

Fig.5 A typical experiment layout, each circle represents one microtitration test well, concentrations of other substrates and enzymes are fixed. Bioluminescence images will be recorded as a function time CCD camera sensing system.

1. Kinetic model derivation and simulation. (week 1-3)

   Based on the kinetics constants and normal GALT activity range in blood, the reactants depletion and products formation concentration–time profile of GALT reaction can be determined. The required incubation time for the reaction to consume most of the galactose-1-phosphate can also be calculated. Use the results from GALT reaction simulation, the bioluminescence profile and reference galactose-1-phosphate concentration can also be calculated. Simulations will be carried out using mathematical software such as Maple and Mathematica. Information deduced from the simulation can serve as our initial guess in solution phase chemistry studies.

2. Measure GALT activity in solution phase using luciferase/gakactokinase detection system. (week 4-12)

   Besides ATP/luciferase system, a standard protocol to access the GALT activity system needs to be established. This will serve as a reference assay of our sensor. GALT standard will be purchased from commercial sources. The optimal reaction condition for GALT will be determined, this include: pH, ionic strength, and buffer system.

   We will perform a series of experiments similar to Fig.5 for determining optimal concentrations of reference galactose-1-phosphate, ADP, galactokinase, and luciferase (these four reactants are considered the most critical) experimentally. Since the two reaction zones we propose to study are interacting sequentially, the solution phase study will also run theses reactions in two steps. ADP and galactose-1-phosphate will incubate with GALT for a certain time, then sample will be transferred to galactokinase/luciferase system to produce biluminescence.

The concentration ratio between luciferase and galactokinase will determine most of the kinetic behavior of the sensor, this ratio will be optimized to produce a steady light intensity.

For a 100ul blood sample with normal GALT activity in erythrocytes, the total activity ranges from 0.26U to 0.45U. Reference galactose-1-phosphate should be consumed within a few minutes for normal activity sample but still provide enough driving force to produce certain amount of ATP for detection for galactosemic sample (see Fig.1).

3. Study the singular effect of individual blood components on the reactions. (week 12-16)

   Possible interfering molecules include:

   1) free ATP (if free ATP concentration is high enough, it will result a lot of false positive tests)
   2) UDP-galactose (inhibit GALT reaction)
   3) glucose-1-phosphate (inhibits GALT reaction)
   4) galactose (inhibit galactokinase reaction)
   5) proteins (some proteins are present in blood at high concentration levels such as hemoglobin which may affect the activity of other enzymes)

   We will introduce different concentrations of interfering components in question into the system. The bioluminescence will be recorded by CCD camera sensing system (see Fig.5). Results of these studies will apply to design of sample pretreatment zone in Phase II.

4. Preservation and immobilization of enzymes and substrates. (week 12-24)

   Two possible methods will be investigated: physical entrapment by agarose gel system and lyophilization. In physical entrapment part, two disaccharides (trehalose or sucrose) will be used as protectants. Different agarose gel concentrations will also be tested. We have already successfully applied both methods to the preservation and immobilization of firefly luciferase (19). These methods will be applied to galactokinase as well. The activity of galactokinase will be determined as a function of preservation time. We have already successfully developed an efficient assay to determine the galactokinase activity by ATP/luciferase system.

   At least four substrates need to be pre-deposited to the sensor: ADP, galactose-1-phosphate, UDP-glucose, and luciferin. We have accumulated considerable experience in preserving luciferin in agarose/trehalose (sucrose) system. All four substrates will be preserved in both methods, the concentration change will be recorded as a function of preservation time. Different preservation conditions such as inert gas atmosphere and preservatives will also be tested according to their chemical and physical properties.

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 this proposal.

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 also the editor of the Journal of Bioluminescence and Chemiluminescence and editor/author of many books on Bio- and Chemi-luminescence in clinical biochemistry. 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. Russel 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. Hasting 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 biocompatability.

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) Industry Advisory Board.

H. Contractual Arrangements (none)

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SUMMARY STATEMENT
(Privileged Communication)

Application Number: 1 R43 DK54539-01

DUAL PROGRAM CLASS CODE: MRDD DUAL: HD
ZRG3 SSS-2 (1)

Review Group: CHEMISTRY AND RELATED SCIENCES SEP

Meeting Dates: IRG: FEB/MARCH 1998 COUNCIL: MAY 1998 7B-2
Requested Start Date: 09/30/1998

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PROTEIN SOLUTIONS, INC
PO BOX 58093
SALT LAKE CITY, UT 84158-0093

Project Title: DIRECT READING BIOSENSOR FOR GALT (E C 2 7 7 12)

IRG Action: **
Human Subjects: 10-NO HUMAN SUBJECTS INVOLVED
Animal Subjects: 10-NO LIVE VERTEBRATE ANIMALS INVOLVED

GENDER, MINORITY, & CLINICAL TRIAL CODES NOT ASSIGNED

PROJECT YEAR	DIRECT COSTS REQUESTED
01	70,435
TOTAL	70,435

Wang ✓
Rick ✓
Rob ✓
Chris ✓
Joe

NOTE TO APPLICANT FOLLOWS SUMMARY STATEMENT.

CRITIQUE 1: The investigators have extensive experience with enzymatic assays and ATP sensors. However, this reviewer is very concerned about their ability to eliminate several potentially strong interferents such as ATP, glucose -1-phosphate,... The investigators did not offer any possible means to eliminate interferents. This reviewer has also a problem with the concept of performing screening test for genetic metabolic disorders with serious implication at home or in the pediatrician's office. Testing in these setting will be done by non-laboratory trained personnel. History has shown that when testing is done in these settings chances of error increase. These tests are currently done in one laboratory in each state (State Lab). In addition to the ability of providing high quality testing , the fact the testing is done in one site under the control of the state will enable tracking of changes in disease incidence and outcome. The loss of a centralized entity for such testing will mean a loss of this information. Therefore I anticipate great problem in commercializing this product.

CRITIQUE 2: This is a Phase I SBIR application for support to develop a direct-readout assay for galactose-1-phosphate uridyltransferase deficiency (Type II galactosemia) which causes various diseases of tissue differentiation.

Date released: 04/14/98 Date Printed: 04/14/98

The largest market is in new-born screening which requires extraction of blood for a three-day turn-around assay. The proposed assay, in the form of a test strip, is expected to give answers within a day.

The PI expects to study the feasibility of an indicator stick format for this assay, using technology that has previously been used in the lab for other assays. While the initial goal is a dip-stick assay for Type II galactosemia, the PIs intend to make a similar assay that can test for all galatosemia types. Prior to initiating the actual construction of the prototypical dipstick, studies on the various liquid phase reactions that will be part of the test stick will be performed, including kinetics and incipient effects. The proposed study aims (p. 11 and 20-22) are consistant at this stage of the project, with many of the items that could impact shelf life, assay reproducibility, and assay sensitivity being studied. However, this reviewer is not convinced that the format can be adapted to generate a test-strip which will detect for all galactosemias. More fundamentally, the proposal is derivative of other assays that Protein Solutions has generated, but it appears the applicant is trying to force the proposal to fit earlier technology.

A strength of the proposal, in addition to the systematic studies on enzyme kinetics and incipient effects, is the consortium of consultants (Drs. Kricka, Nelson, Stewart, Hlady, hastings, Kopecek, and Johnson) that the PIs have put together for the project.

Investigator(s): Dr. Wang received his Ph.D. in bioengineering in 1997 from the University of Utah; he is a research scientist at Protein Solutions in Salt Lake City since 1997. Dr. Van Wagenen received his Ph.D. in materials science and engineering in 1976 from University of Utah; he is vice-president for research and development at Protein Solutions. Both investigators are qualified to perform various aspects of the proposed research.

**NOTE TO APPLICANT: As part of the initial scientific merit review process, reviewers were asked to identify those applications with the highest scientific merit, generally the top half of applications that they customarily review. At the study section meeting, those applications were discussed and assigned a priority score. All other applications, including this application, did not receive a score. Provided is a compilation of reviewers' comments prepared prior to the meeting, without significant modification or editing by NIH staff.