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Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

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Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Creatinine Biosensor for Renal Transplant Monitoring

2. SOLICITATION NO. **PHS 98-2**

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)*
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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
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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: January 1, 1999 *Through:* June 30, 1999

7. COSTS REQUESTED
7a. Direct Costs $ 71,062
7b. Total Costs $ 99,815

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
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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

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.)*
Clemy Yi-Ts Wang
DATE: 4-13-98

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: 4-13-98

Principal Investigator *(Last, first, middle):* Wang, C. - Y.

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

Protein Solutions, Inc.
P.O. Box 58093
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YEAR FIRM FOUNDED	NO. OF EMPLOYEES *(include all affiliates)*
1988	5

TITLE OF APPLICATION

Creatinine Biosensor for Renal Transplant Monitoring

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
John Holman, Jr., M.D.	" " "	Consultant

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.

This proposal addresses the feasibility of developing an easy-to-use, low cost, personal biosensor for serum creatinine, a critical indicator of renal function. A personal creatinine sensor would enable kidney transplant patients to monitor the health of their transplanted kidney in the home environment. The sensor design consists of two parts; a test strip which contains all the biochemistry in a dry format and a compact, inexpensive instrument to read and store the test results. The instrument is currently under in house development for application to other analytes including ATP, galactose, and phenylalanine. This Phase I proposal focuses on the biochemistry of the creatinine bioluminescence-based biosensor. The test strip can be conceptually divided into two "reaction zones", i.e., a sample pre-treatment zone and a signal transduction zone. The biochemistry involves the conversion of creatinine to creatine. Creatine is then phosphorylated by the enzyme creatine kinase with the simultaneous consumption of ATP. Unused ATP is then quantified via the firefly-luciferase reaction which produces light that is measured in the instrument. The result is a two channel device which measures both creatine and creatinine and will be applicable to both blood and urine samples.

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

creatinine, creatine, biosensor, kidney, transplant, blood, urine, bioluminescence

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

Routine, creatinine monitoring is a highly desirable capability to offer kidney transplant patients. An increasing trend in serum creatinine is an early predictor of transplant rejection and subsequent kidney failure. There are fifty to sixty thousand transplant patients who would benefit from weekly monitoring of their serum creatinine. At $10 per assay this is a $25 - $30 million gross annual market.

A. Specific Aims

1. Enzymes and Reactants: Select, obtain, characterize, and evaluate the enzymes and reactants needed. Consider cost, availability, kinetic constants ,stability, and interferents. Characterize the enzymes as to their suitability for use in dry reagent applications.
2. Modeling and Simulation: Model and simulate the reactions using published and estimated kinetic constants and appropriate concentration ranges. Estimate the concentrations and conditions needed for preliminary sensor studies in urine and blood environments.
3. Creatine Sensor: Optimize the creatine reaction and its detection via ATP-firefly luciferase using the data of Specific Aims 1 and 2. Assess the feasibility of a creatine sensor based on the ATP depletion bioluminescence detection principle. Optimize these reactions
4. Creatinine Sensor: The data of Specific Aims 1 and 2 and the creatine sensor prototype of Specific Aim 3 permit the feasibility studies on a complete creatinine sensor. Optimize the creatinine prototype channel and independent control channels to monitor endogenous creatine and ATP.
5. Preliminary Urine Studies: Evaluate the prototype sensors, with both creatine and creatinine channels, in a simulated urine environment with particular attention to interferents, including ATPases and endogenous creatine and creatine phosphate. Evaluate optimum reaction times/conditions and optimum detection conditions.
6. Preliminary Blood Studies: Evaluate the prototype sensors, with both creatine and creatinine channels, in a simulated blood environment with particular attention to interferents, including ATPases and endogenous creatine and creatine phosphate. Evaluate optimum reaction times/conditions and optimum detection conditions.

Phase II Specific Aims (tentative)

1. Implement Phase I pre-prototype sensors as a test strip device for urine and/or blood application. Compare and validate against traditional measurement methods.
2. Modify existing CCD luminometer device to accommodate prototype creatinine sensor. Modify/optimize test strip device if necessary.
3. Perform more extensive testing in urine and blood experiments. Evaluate potential interferents, including any possible effects of common immunosuppressive drugs.
4. Work with kidney transplant surgeons and patients on optimum design and application of the creatinine sensing system.
5. Perform limited trial and validation with small patient/surgeon pool and compare with existing standard means for creatinine measurement.
6. Enhance luminometer instrument to facilitate individual patient data storage, presentation, and assessment; incorporate means to transfer such information to patients' physician/nurse via normal telephone line access. Perform preliminary test and evaluation of prototype instrument.

Phase III

Production of ten instruments for limited patient trial (12 months). Begin FDA submission process.

B. Identification & Significance of Problem (1, 3, 4)

Creatinine is a low molecular weight, cyclic compound which is one of the nitrogenous end products of metabolism. It is excreted in the urine at roughly 10 mM. The normal circulating plasma level of creatinine is roughly in the 100 microM range (3, 5-6). Creatinine is a product of muscle metabolism where it is usually formed spontaneously and irreversibly from creatine. Creatine in the creatine phosphate form is used to recharge ATP in muscle. Creatinine is not protein bound and is freely distributed throughout the total body water. The amount of creatinine excreted per day for an individual is approximately constant and is directly proportional to muscle mass. Creatinine is thus used as a reference for the quantitation of other constituents in urine and is a key measure of renal function. A typical adult excretes slightly over one gram per day of creatinine in about one liter of urine (about 10 mM).

The most useful index of overall renal function (6, 32) is the glomerular filtration rate (GFR) (32), normally determined by creatinine clearance measurements. Significant decreases in urinary creatinine excretion result in an increase in the plasma creatinine level and is indicative of serious renal dysfunction. Significant reduction in renal function over a short period of time (less than six or so weeks) is defined as acute renal failure. Chronic renal failure is defined as a permanent and significant reduction in GFR and a consequent significant increase in serum or plasma creatinine eventually leading to end stage renal disease (ESRD). Patients suffering from ESRD are generally treated by hemo or peritoneal dialysis or by renal transplantation.

Renal transplantation is the most effective therapy for treatment of end stage renal disease. A functioning renal transplant removes endogenous waste products more effectively than dialysis and provides a physiologic source of endocrine products produced by the kidney. ESRD patients have a better quality of life with a functioning renal transplant as opposed to dialysis therapy. Currently 85-90% of renal transplants survive at least one year with a survival half time of 8-15 years after that, depending on the kidney donor source.

Long term survival of renal transplants is however limited by the host immune response to the donor organ. Hyperacute rejection, generally mediated by preformed host antibody, is generally avoided today by good lymphocyte cross matching techniques. Acute rejection is a cell-mediated event occurring most commonly in the first few months after transplant. It can, however, occur at any time following transplantation. Chronic rejection is mediated in part by antibodies to the donor organ vascular endothelium and is poorly understood. It occurs to some extent in all patients. The rapidity with which it develops and progresses is highly variable. One of the risk factors for developing chronic rejection is having had an acute rejection episode (even a successfully treated acute rejection).

Patients must take medications to suppress the host immune response. Because of the multiple pathways that the immune system can take in responding to the allograft, the patient usually takes a "cocktail" of medications each of which works to inhibit a different immune pathway. There are a variety of immunosuppression drug protocols. However, the majority of protocols include a steroid and cyclosporine or tacrolimus with or without azathiaprine or mycophenolate. Each of these medications has a different effective therapeutic level and side effect profile. Therefore therapeutic blood monitoring is used to tailor the patient's medication individually. The most critical drugs to monitor are cyclosporine or tacrolimus.

Monitoring for acute rejection usually is done by observing renal function. Although many modalities have been used (ultrasound, nuclear medicine renal scan), serum creatinine is the more specific. A rise in serum creatinine of 30 microM from baseline is generally significant and prompts more detailed investigation into the possibility of rejection (3). Similarly blood samples are taken for determination of immunosuppressive drug level. The optimal drug level is different for each patient and depends on the length of time post transplant, higher drug levels required immediately following transplant (0-3 months).

Compliance with monitoring limits to some degree its effectiveness at detection rejection episodes. In the early post transplant period (0-3 months) the host immune response requires more medicatin for suppression and breakthrough rejection is more likely. During this period, creatinine and drug levels are often measured 2-3 times per week. After this time (3-12 months) measurement is less frequent as the risk of rejection becomes less and drug levels stabilize. After 12 months, acute rejection is much less common (90% of all acute rejection episodes occur in the first year after transplant). The reduced frequency of renal function and drug monitoring with time of transplant is a compromise between the need for monitoring to detect possible rejection and the inconvenience of going to the lab for blood draws.

Acute rejections can occur late following transplant. If renal function is being monitored regularly, irreversible transplant damage can be avoided.

What is needed is a simple, convenient, accurate method of determining renal function that can be performed by patients at home. Whole blood creatinine is the classic measure of renal function used in transplant monitoring. Ideally the methodology for determining creatinine would include the ability to store the result for later "downloading" to the transplant center patient files.

The more than fifty thousand individuals in the United States with functioning renal transplants would save a great deal of time and effort if a simple and effective home test for creatinine were available. The over two million creatinine determinations per year for this patient population at an average cost of twenty to thirty dollars per measurement, leads to a significant overall cost to the patients and to the health care system. A simple lancet-based test strip-type test, analogous to the glucose test used regularly by millions of diabetics, would dramatically improve the present situation. Not only would it reduce costs, greatly improve the convenience and quality of life for the patients, but most importantly would lead to earlier diagnosis of transplant rejection and/or dysfunction and thereby earlier treatment to minimize the possibility of eventual rejection. In addition, a creatinine sensor would have an application far greater than the renal transplant population.

C. Relevant Experience (4, 17, 18, 24)

Protein Solutions, Inc. (PSI) is committed to the development and eventual marketing of "simple," easy-to-use, inexpensive, and highly quantitative and reliable test strip-type sensors for use in point of care testing and in the home environment.

The synthesis and degradation of practically all biochemicals are dependent on the two key molecules of bioenergetics: ATP (adenosine triphosphate) and NADH (nicotinamide adenine dinucleotide). These molecules are the fundamental basis of bioenergetics and are involved in all life as we know it. PSI has applied ATP and NADH-specific bioluminescence reactions to the monitoring and measurement of a range of common metabolites.

Although the firefly luciferase (ATP-specific) and bacterial luciferase (NADH-specific) enzymatic reactions are well known and have been applied as highly specific and sensitive detection methods in clinical and research laboratories (15, 16), they have not been utilized for the monitoring of important metabolites in a home or other non-laboratory environment. It is quite suprising that there are no simple and inexpensive means by which to measure the key metabolites of living systems (4). The only significant exception is the glucose test strip and its companion glycometer which permits the quantitative measurement of glucose in a small drop of blood using reflectance colorimetry or electrochemistry. It is the high incidence of diabetes in the relatively affluent part of the world which has encouraged many companies to invest millions of dollars in the development and manufacture of simple, inexpensive, high performance analytical instruments focused almost exclusively on glucose.

Although there is considerable interest in the monitoring of specific carbohydrates, amino acids, and other "nutrients" important to the biochemical process and biotechnology industries, the instruments required generally cost several thousand dollars or more, and each analyte of interest requires a special sensor, probe, electrode, etc., generally costing several hundreds of dollars.

Our goal is to provide means for the simple, quantitative, direct analysis of carbohydrates, amino acids, vitamins, and other low molecular weight molecules of interest to metabolism, metabolic abnormalities, nutrition, sports and physical performance, and related areas, including the biotechnology and bioprocess industries.

Our approach is based on a relatively well known but little used curiosity of biology: bioluminescence (15, 16). The bioluminescence in fireflies is based on an enzyme catalyzed oxidation reaction utilizing ATP as a highly specific co-reactant. The bioluminescence of marine bacteria is closely coupled to an NADH dependent enzyme reaction. Thus, mother nature has literally handed to us two unique, different, ultra sensitive and highly specific reactions for the measurement and monitoring of ATP and/or NADH. The readout is photons, green and yellow in the case of the firefly reaction, and blue in the case of the bacteria process. The reactions are highly sensitive to and quantitative for ATP or NADH over a five or more order of magnitude concentration range (15). Since all of biochemistry depends on ATP or NADH, practically all biochemical reactions can be monitored via bioluminescence.

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 the firefly and bacterial luciferase enzymes, respectively (4, 15, 20). Such biosensors generally employ

fiberoptic or other wave guided means of delivering the luminescence to a device which can accurately measure light intensities.

For many of the analytes of interest (such as urine creatinine), the bioluminescence is intense enough that the unaided eye can serve as the analytical instrument. In most other cases, including plasma creatinine, a relatively inexpensive luminometer will suffice. For very low concentrations of analytes, in the nano to picomolar range, a more sophisticated photon counting luminometer is usually required.

We are now in the process of developing "dollar devices" (4) for the analysis of galactose (24) and phenylalanine (18).

A "Simpler" Approach to Bioluminescence-Based Analysis: Although bioluminescence analysis is well known and has been used regularly in research, in analytical laboratories, and clinical laboratories (15, 16), it has not been widely applied outside of those specialty areas for several reasons:

1. The exquisite sensitivity for very low ATP concentrations has encouraged the application of the technique to those problems where such sensitivity is indeed needed. Thus, it has acquired the reputation of an ultra sensitive technique and has not been seriously considered for the measurement of analytes in the micromolar to millimolar range.
2. The luciferases and other reagents involved have developed a reputation of being somewhat labile, unstable, and perhaps difficult to utilize.
3. The nature of the bioluminescence reaction, and in particular its complex kinetics, made it necessary to develop rapid mixing techniques and to utilize an instrument capable of sensing a flash or short pulse of light. Application of trace concentrations required, in addition, a highly sensitive, and therefore relatively expensive, luminometer. Thus, the technique evolved a reputation for requiring an expensive instrument, and a precise and somewhat sophisticated analysis protocol.
4. The wide spread application of the firefly luciferase reaction to the monitoring of very low concentrations of ATP released from bacterial and other cells in hygiene monitoring applications lead to a mysterious or "black magic" reputation because of the "cocktails"—the surfactants, detergents, and other agents required to disrupt cell membranes—needed to release the ATP. Those same reagents, of course, denatured and inactivated the luciferase involved, thus these processes always involved a delicate balance, a careful optimization, and were often difficult to carry out in a reliable and reproducible manner.

About five years ago, we became convinced that ATP-based firefly luminescence and NADH-based bacterial bioluminescence could serve as a highly specific and sensitive means of monitoring metabolism. We began to develop an ATP detection platform which would obviate or minimize the problems noted above. This platform has been under development for the past several years. We are now in the process of developing an NADH detection platform. Our approach is based on the following considerations:

1. The biotechnology community knows how to express, produce, and purify proteins via simple organism cultures and processes. Indeed, recombinant firefly and bacterial luciferases have been known for several decades now, and recombinant firefly luciferase is commercially available.
2. The biotechnology and protein pharmaceutical industries have learned how to formulate, passivate, store, and reconstitute proteins and enzymes with considerable retention of activity (31, 33). We addressed the instability of firefly luciferase using our experience, understanding, and control of the denaturation of proteins at interfaces and in solution (17).
3. A reaction which actually produces photons has many advantages. One does not have the problems associated with color perception, as in the case of reflectance colorimetry. One does not require a light source, as in the case of fluorescence spectroscopy. One does not require electrodes and their tendencies to become contaminated or to participate in side reactions, as in the case of much of analytical electrochemistry.

Practically all scientists, laboratory technicians, and even patients come equipped with two ultra sensitive, portable, reliable, and inexpensive photon detectors: their own eyes. We realized, however, that most bioluminescence analysis is dependent on the measurement of an intensity (15), although the total number of photons, the integral of the intensity-time curve, can also be used. We know that the human eye is incapable of integrating photons. The eye is also a highly variable and therefore unreliable detector of photon intensity. The human eye's incredible ability to accommodate, to adjust its sensitivity to photon flux, makes it very difficult to calibrate and use as an analytical or quantitative measure of photon flux. Also, the human eye's exquisite photon sensitivity is really only manifested under dark adaptation conditions, which require twenty to thirty minutes of accommodation time for maximal sensitivity.

We appreciated, however, that the human eye is unsurpassed as an imaging device—as a position sensitive contrast detector—with sophisticated and sensitive means to perceive changes or differences in relative photon yields as a sensitive function of position. We, therefore, undertook a means to transform the quantitative analytical signal from one relying on relative intensity to one relying on relative spatial position. Our current work involves both approaches, the more or less conventional intensity-based approach, and a spatial or position approach.

We thus have two different ways of measuring ATP. One is by measuring the absolute intensity of the glow, which can be made proportional to substrate concentration. This is the standard approach. It generally requires an analytical instrument, a type of luminometer which can objectively measure intensity. The second means, the spatial position of the glow, can be detected by an imaging device, such as a diode array, a CCD, or by the human eye. These two different approaches can be coupled in the same sensor/device for increased reliability and sensitivity.

Substrate Specific Sensors: The simplest substrate-specific sensor is one in which the enzyme reaction produces ATP:

$$\text{substrate} + \text{ADP} \xrightarrow{E} \text{product} + \text{ATP}$$

A good example is the transformation of phosphoenolpyruvate (PEP) to pyruvate. The ATP sensor then measures the ATP produced, which directly correlates with substrate concentration.

Another typical reaction involves the consumption of ATP:

$$\text{substrate} + \text{ATP} \xrightarrow{E} \text{product} + \text{ADP}$$

In this case the ATP sensor measures the decrease in ATP concentration, thus light intensity correlates inversely with substrate concentration. A good example is the phosphorylation of galactose (Gal):

$$\text{Gal} + \text{ATP} \xrightarrow{\text{Galactokinase}} \text{Gal-1-P} + \text{ADP}$$

The galactokinase reaction can be carried out for some time, and the resulting ATP concentration then measured by the firefly luciferase reaction. This is a sequential or series sensor. The two enzyme reactions can also be used at the same time (a homogeneous or parallel sensor), with both enzymes competing for ATP. Because galactokinase is a much faster enzyme than luciferase, the simpler homogeneous approach is appropriate for a Gal sensor. Although the results are not linear, a simple function allows the integrated intensity to relate to Gal concentration.

The sensors are designed for discrete samples. The concentration is measured using a simple disposable device. Both the device and the sample are then discarded. For medical and clinical purposes, the sample of choice is generally blood, usually derived from a simple lancet-based fingertip, earlobe, or heal prick. Modern micro-lancets are almost painless and can readily generate a one hundred to two hundred microliter droplet, adequate for the devices described, even for a multi-channel device or panel.

Common analytes in the millimolar range can be detected and measured using a disposable analytical device which can be directly read by the operator or patient. The devices have the appearance of a glowing "thermometer," with the length of the glow either directly or inversely proportional to the concentration of the analyte of interest. In the micromolar range, the same approach utilizes a simple luminometer, somewhat analogous in application and cost to the present generation of sophisticated glycometers for the measurement of blood glucose. Analyte

concentrations in the nanomolar to picomolar or below range require a more sensitive analytical instrument, expected to cost in the range of one to two thousand dollars.

Our goal is to design and produce disposable analytical devices in the dollar range (4). We also expect to develop and produce multichannel, multianalyte devices appropriate to the monitoring and management of various metabolic diseases, sports and physical performance assessment, and nutrition assessment.

The Creatinine Connection: PSI's work on a galactose sensor for the management of galactosemia (17, 24) and a phenylalanine sensor for the management of PKU (18) came to the attention of Dr. John Holman, Jr., a renal transplant surgeon at the University of Utah College of Medicine. Dr. Holman is very interested in more effectively and less expensively monitoring his renal transplant patients via the analysis of plasma creatinine, as noted earlier. PSI had been seriously considering the development of creatine and creatinine sensors, but had not taken those interests and concepts forward to the proposal stage. The collaboration with Dr. Holman makes such a project effective and appropriate at this time (3).

A thorough search of the literature has shown that creatinine is usually measured by complex enzymatic reactions which lead to colorimetric products (7-10, 26-27). It has also been monitored by bioluminescence (21, 22). This early work has not been further developed and has not been employed in a test strip-type, patient-based format.

Mr. Chris Eu has conducted a preliminary feasibility analysis using a multiple enzyme reaction simulation program and appropriate values of the enzyme constants and substrate concentrations (24). This simulation supported the earlier published work. He then did a preliminary study which conclusively demonstrated that the assay of creatinine via the ATP-dependent phosphorylation of creatine is indeed feasible and practical (24).

D. Experiments & Methods

Specific Aim 1: Enzymes and Reactions

Historically, creatinine has been measured using non-specific complexation resulting in a red-orange chromophore (the Jaffe Reaction) (25). Most modern methods for creatinine analysis use specific enzymatic methods (7-12, 26-27), often culminating in an NADH/NAD reaction monitored by quantitative UV absorbance. Hydrogen peroxide outputs have also been used, followed by reaction to a chromophore. Ammonia-based reactions have been utilized, followed by ammonia analysis reactions which involve a peroxide output which can then be detected electrochemically. Peroxide products can also be detected via chemiluminescent reactions (7-12, 19-22).

The method of choice for our purpose is to enzymatically transform creatinine to creatine, followed by the phosphorylation of creatine via creatine kinase. The phosphorylation reaction results in consumption of ATP, which is then detected by our ATP detection platform, noted briefly earlier (4).

The various enzyme reactions are summarized in Table 1. Several kits involving colorimetric readouts and an enzyme electrode-based device are commercially available (11). The enzymes needed are summarized in Table 2, including their sources and general properties.

In this first Specific Aim, we will obtain and characterize those enzymes derived from various sources. The activity of creatininase can be determined by the formation of creatine which can be measured by the α-napthol-diacetyl method or the Jaffe reaction (27). The activity of creatine kinase can be measured through two other enzymatic reactions: hexokinase and glucose-6-phosphate dehydrogenase. The activity of creatine kinase can be determined from the NADH/NAD^+ method (Table 1). In principle, the activity of creatine kinase can also be determined by bioluminescence. The consumption of ATP by creatine kinase can be measured by firefly luciferase. The activity of firefly luciferase is routinely measured at PSI. The activity of firefly luciferase is calculated from the flash light intensity when the required substrates are saturated. All enzyme concentrations will be measured spectrohotometically.

homogeneous { pH, buffer, enzyme ratio, Biosensor } → { activity of freeze/thaw lyophilized, Time Storage Conditions } → Specific Aims 3 & 4

Table. 1 Major enzyme-based methods used to determine serum (or urine) creatinine.

Method	Chemistry	Enzymes	Chemical being detected	Detection method	Ref
Jaffe Reaction	Picric acid + creatinine $\longrightarrow$ orange-yellow complex	Non-enzymatic reaction	Formation of orange-yellow complex	Colorimetry	25
Ammonia Method	Creatinine + $H_2O \xrightarrow{1}$ creatine Creatine + $H_2O \xrightarrow{2}$ sarcosine + urea Urea + $H_2O \xrightarrow{3}$ CO_2 + 2 NH_3 2 2-oxoglutarate + 2 NH_3 + 2 NADPH $\xrightarrow{4}$ 2 glutamate + 2 $NADP^+$ + $2H_2O$	1. creatininase 2. creatine amidinohydrolase 3. urease 4. glutamate dehydrogenase	Depletion of NADPH	Spectrophotometry	9
Peroxide Method	Creatinine + $H_2O \xrightarrow{1}$ creatine Creatine + $H_2O \xrightarrow{2}$ urea + sarcosine Sarcosine + H_2O + $O_2 \xrightarrow{3}$ glycine + formaldehyde + H_2O_2 H_2O_2 + 3,5-dichloro-2-hydroxybezenesulfonic acid + 4-aminophenazone $\xrightarrow{4}$ quinone-monoimine dye + $2H_2O$ + HCl	1. creatininase 2. creatine amidinohydrolase 3. sarcosine oxidase 4. peroxidase	Formation of H_20_2 or quinone-monoimine dye	Colorimetry or Electrochemistry or Chemiluminescence	7
NADH/NAD+ Method	Creatinine + $H_2O \xrightarrow{1}$ creatine Creatine + ATP $\xrightarrow{2}$ creatine phosphate + ADP ADP + phosphoenolpyruvate $\xrightarrow{3}$ ATP + pyruvate Pyruvate + NADH + H+ $\xrightarrow{4}$ lactate + NAD+	1. Creatininase 2. Creatine kinase 3. Pyruvate kinase 4. Lactate dehydrogenase	Depletion of NADH	Spectrophotomery	8
Bioluminescence Method	Creatinine + $H_2O \xrightarrow{1}$ creatine Creatine + ATP $\xrightarrow{2}$ creatine phosphate + ADP ATP + O_2 + luciferin $\xrightarrow{3}$ AMP + PPi + oxyluciefrin + CO_2 + light	1. Creatininase 2. Creatine kinase 3. Firefly luciferase	Depletion of ATP	Luminometry (Bioluminescence)	21, 22

Table. 2 Source and properties of enzymes for bioluminescence based creatinine sensor

Enzyme name	Creatine kinase (E.C.2.7.3.2)	Creatininase (E.C.3.5.3.3)	Firefly luciferase (E.C.1.13.12.7)
Source	Rabbit muscle	Pseudomonas Species	Photinus Pyralis (recombinant)
Vendor (example)	Sigma	Sigma	Promega
M.W.	80 kd	175 kd	62 kd
Kinetic constants	$Km_{,creatine}$=16 mM $Km_{,ATP}$=0.48 mM	$Km_{,creatinine}$=26 mM	$Km_{,ATP}$=0.25 mM $Km_{,luciferin}$=2 μM
Optimal pH	8.8~9	7~9	7.8
Thermal stability	Retained 70% activity after incubation at 30°C for 56 hours	Retained 75% activity after incubation at 75°C for 30 minutes	Retained 7% activity after incubation at 4-8°C for 20-28 hours
Price	$0.86/ mg protein	$30/ mg protein	$20/ mg protein
Reference	26	27	17

As the goal is to integrate all of the chemistry onto a small test strip, each of the enzymes and substrates will be quickly evaluated as to their suitability for preservation and use in dry reagent form by lyophilization. We have evolved a set of lyphiliztion protocols for our existing studies involving firefly luciferase and galactokinase. Galactokinase is a particularly labile enzyme but can be very effectively lyophilized and reconstituted with little loss of activity using the appropriate protectants and protocols (17, 31, 33).

As part of the lyophilization process, we include several preservatives: Polyethylene glycol (8000 M.W.) is included as a freezing protectant (50 mg/ml). Trehalose is included as a dehydration protectant (120 mg/ml). Bovine serum albumin (0.3 mg/ml) is included as a denaturation protectant. DTT (3 mM) is included as a sulfide bond linkage protectant. The lyophilization or freeze-drying process follows protocols prescribed in pharmaceutical literature (31, 33). After mixing the preservatives with the reagents necessary for the biosensor application, the solution is pipetted into individual test wells. The sample is then frozen at -70° for 12 hours. The final step in the preservation process involves removing the water from the reagent solution at high vacuum and various temperatures using a lyophilizer. We will evaluate various moisture removal protocols. Variables include shelf temperature, vacuum pressure, and time.

The overall goal of this Specific Aim is to provide well characterized, reliable, robust reagents for the entire project. We will also evaluate the availability and suitability of recombinant sources of creatininase.

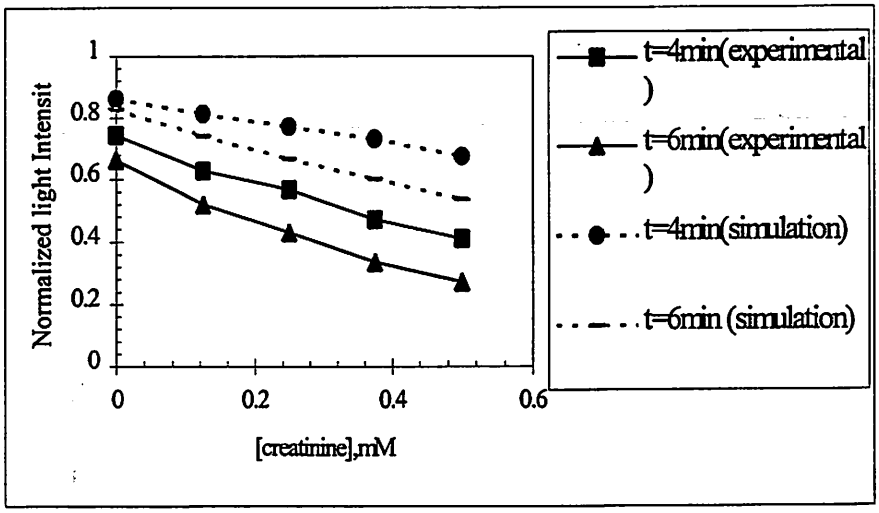

Fig.1 Preliminary simulation of a homogeneous creatine assay based on bioluminescence. Simulation conditions: [ATP]=10 μM, [luciferin]=25 μM, [luciferase]=18 nM, [creatininase]=0.16 μM, and [creatine kinase]=0.5 μM. Experimental data under the same conditions were also plotted.

Specific Aim 2: Modeling And Simulation

The proposed sensor contains three different enzymes: creatininase, creatine kinase, and firefly luciferase (Table 2). The kinetic behavior of these enzymes has been extensively studied (26, 27, 28). Based on the reaction mechanism of each enzyme and its kinetic constants, the bioluminescence response of the sensor to the concentration of creatinine in the sample can be simulated. The simulation can be done by a general mathematical package, such as Maple, or by enzyme kinetic simulation software like KINSIM (23). Since the signal output can be obtained in the time domain, the optimal conditions can be determined. The effect of the four most critical factors will be studied: creatininase concentration, creatine kinase concentration, firefly luciferase concentration, and reference ATP concentration. The optimal observation window will also be simulated. Information obtained from simulation will be used as initial conditions in the solution phase chemistry studies.

Figure 1 shows a preliminary simulation and the result of a preliminary solution experiment (24). Note that the experimental results are actually "better" than the model from the perspective of a creatinine assay. Clearly both the simulation conditions can be enhanced (such as by increasing the concentration of both enzymes) in order to more effectively plan and conduct the optimization experiments.

Specific Aim 3: Creatine Sensor

Our proposed sensor for creatinine can be thought of as two sensors. It incorporates a creatine sensor in which the phosphorylation of creatine (bottom panel of Table 1) results in the depletion of ATP, which is detected by the firefly luciferase reaction. The work reported over ten years ago (21, 22) and our own simulation and preliminary studies (previous paragraph and Figure 1) has verified the feasibility of this approach (24). Although our simulation dealt with a homogeneous mixture of the enzymes, we are interested in characterizing the creatine portion of the process independently. We expect that we will need to have a separate creatine channel as a control channel for the possible presence of exogenous creatine. Historically, creatine has been measured indirectly by converting it to creatinine by heating the sample with acid in the presence of heavy metal. The creatinine was then measured before and after the addition of the reagent and creatine calculated as the difference (30).

We will assess creatine directly via our ATP sensor. A creatine sensor is important in its own right, as there is considerable interest in monitoring creatine for other purposes (30).

The basis for a bioluminescence based creatine sensor is that the consumption rate of ATP depends on the initial concentration of creatine in the sample. Higher concentration of creatine will consume more ATP during a certain time interval. In the bioluminescence approach, ATP is consumed by two enzymes: firefly luciferase and creatine kinase. In order to simplify the kinetics of the whole sensor, it is always desirable that the consumption of ATP depends on creatine kinase only. The bioluminescence intensity resulting from the firefly luciferase reaction can be adjusted to a constant light output if certain reaction conditions are met: a) ATP concentration is less than 10μM; b) ATP is in great excess compared to firefly luciferase. Under the aforementioned conditions, the ATP consumed by firefly luciferase will be negligible. In order to create an observable change in ATP concentration (light intensity), the concentration of creatine kinase needs to be kept high (above 0.1μM), since the turnover rate of creatine kinase in the reverse direction is lower.

Specific Aim 4: Creatinine Sensor

Optimal conditions for the prototype creatinine sensor will be determined (Table 3). In order to simplify the actual design of the sensor, all three enzymatic reactions will proceed simultaneously in a homogeneous solution. A homogeneous assay can avoid sample transport and reduce liquid handling. The concentrations of three enzymes (firefly luciferase, creatine kinase, and creatininase) and reference ATP concentration are the most critical factors in the assay. The optimal range of these factors and incubation time will be studied using our CCD camera sensing system. For the reaction of firefly luciferase, luciferin concentration is generally set to saturation. Other factors such as pH, ionic strength, and buffer system will also be optimized (Table 3). Most

of the experiments in this proposal will be similar to the layout in Figure 2. The large sampling area of our CCD camera (Santa Barbara Group Instruments Model ST6) makes it very efficient to simultaneously measure the bioluminescence from 96 samples (12x8 matrix) in microtiter wells. Figure 2 is an example for pH optimization as a function of creatinine concentration.

Table. 3 Parameters and ranges for creatinine sensor optimization

Parameter	Range
[creatinine], serum	0.005~0.2 mM
[creatinine], urine	1~1.5 mM
[creatine], serum	0.005~0.2 mM
[creatine], urine	0.4~0.8 mM
Sample volume	10~200 uL
pH range	7~9
Buffer	Tris, glycine-glycine, phosphate
Reaction time	0~15 minutes
[firefly luciferase]	0.01~1 μM
[creatininase]	0.1~5μM
[creatine kinase]	0.1~5 μM
Reference [ATP]	0.1~10 μM

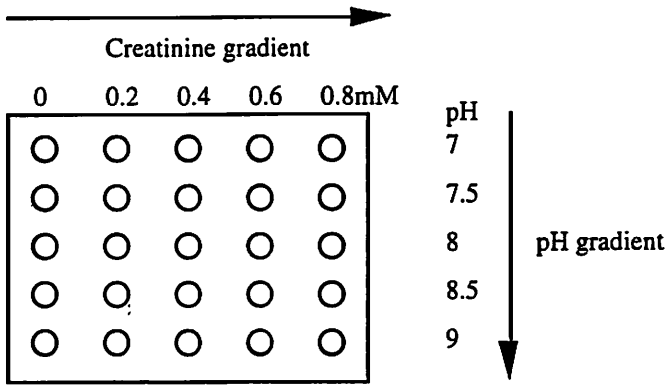

Figure 2. A multiwell plate used in a two-dimensional step gradient mode for rapid screening and optimization. Each circle represents a microtiter well. Concentrations of other components are fixed. Bioluminescence images will be recorded as a function of time by CCD camera sensing system.

Figure 2 only uses a 5x5 matrix as an example. Our instrumentation allows us to use a 12x8 matrix. Our CCD camera sensing system can record one image every two minutes, since the image processing software requires about 90 seconds. For a more riorous kinetic study of the sensor, where the time course needs to be followed more rapidly, we will use our high sensitivity luminometer (Turner Designs Model TD-20/20). The luminometer can record light intensity every 0.2 seconds.

Table 4 summarizes possible serum components that may affect the accuracy of the sensor. Free ATP can be removed by exposure to immobilized apyrase if necessary. ATP converting enzymes can be removed, if necessary, by exposure of sample to a suitable adsorbent. Endogenous creatine will be monitored in a parallel creatine channel.

Table 4. Possible Interfering Components In Serum

Component	Reason	Expected Effect
ATP converting enzymes	The concentration of the reference ATP will be changed.	For ATP-producing enzymes: measured creatinine < actual creatinine For ATP-consuming enzymes: measured creatinine > actual creatinine
Creatine	The reaction rates of creatininase and creatine kinase will be altered, as a result, the consumption of ATP will be changed.	Need experimental determination
Free ATP	Increase the concentration of reference ATP.	Measured creatinine < actual creatinine

The extent of the possible interferences can be determined using a gradient matrix as in Figure 2. Interference from other compounds will also be investigated. Components in serum that may need to be removed by a sample pretreatment zone will be identified.

We do not propose to develop the test strip device in this Specific Aim, but rather to develop the homogeneous solution conditions which would facilitate an effective assay. These are the conditions which might be used in a commercial clinical laboratory analyzer where the reagents can be appropriately mixed and the reaction then monitored in time, in our case using a CCD-based luminometer. The actual design and development of the test strip will be reserved for the early part of Phase II.

Although we cannot give any specifics at this stage because the optimal conditions are yet to be determined, the device is likely to be similar to the test strip that we are developing for the measurement of galactose (top of Figure 3). This particular strip is monitored in the intensity mode using the CCD luminometer. As the project develops, we will also consider the spatial or glowing thermometer mode (4). We will select the mode that is the most appropriate for the analytical needs and requirements of the creatinine assay. A recent study on "within person" and "between person" variability for the assay of creatinine in the mean range of about 100 microM was about ± 10%. The "within person" and methodological variability was about ± 5% (14). This means that at this stage in the development of the device, a target specification of ± 5 microM is appropriate.

Specific Aim 5: "Urine" Studies

The optimum analysis conditions for creatine and creatinine derived in Specific Aims 3 and 4 will then be used with creatine and creatinine standards in simulated and control urine. The appropriate concentration ranges are in Table 3. Standardized urine is available from commercial sources (34).

Because of the very high concentration of creatinine in urine, we will do some preliminary experiments to determine the possibility of direct visual detection. Although the major goal for this project is plasma analysis of creatinine, the analysis of creatinine in urine is also very important. We will also assess the various interferents present in urine, including endogenous creatine, ATP, and enzyme activities which might interfere with the reactions. These can be addressed with an appropriate control channel and probably will be fully accounted for by the creatine control channel in the final sensor.

Specific Aim 6: Preliminary "Blood" Studies

The optimum homogeneous reaction conditions for creatinine and creatine will be applied to a simulated blood environment. These studies will be performed with our scientific and clinical collaborators in the Center for Biopolymers at Interfaces (CBI), an industrial consortium of the University of Utah's Department of Bioengineering. PSI is a corporate member of CBI.

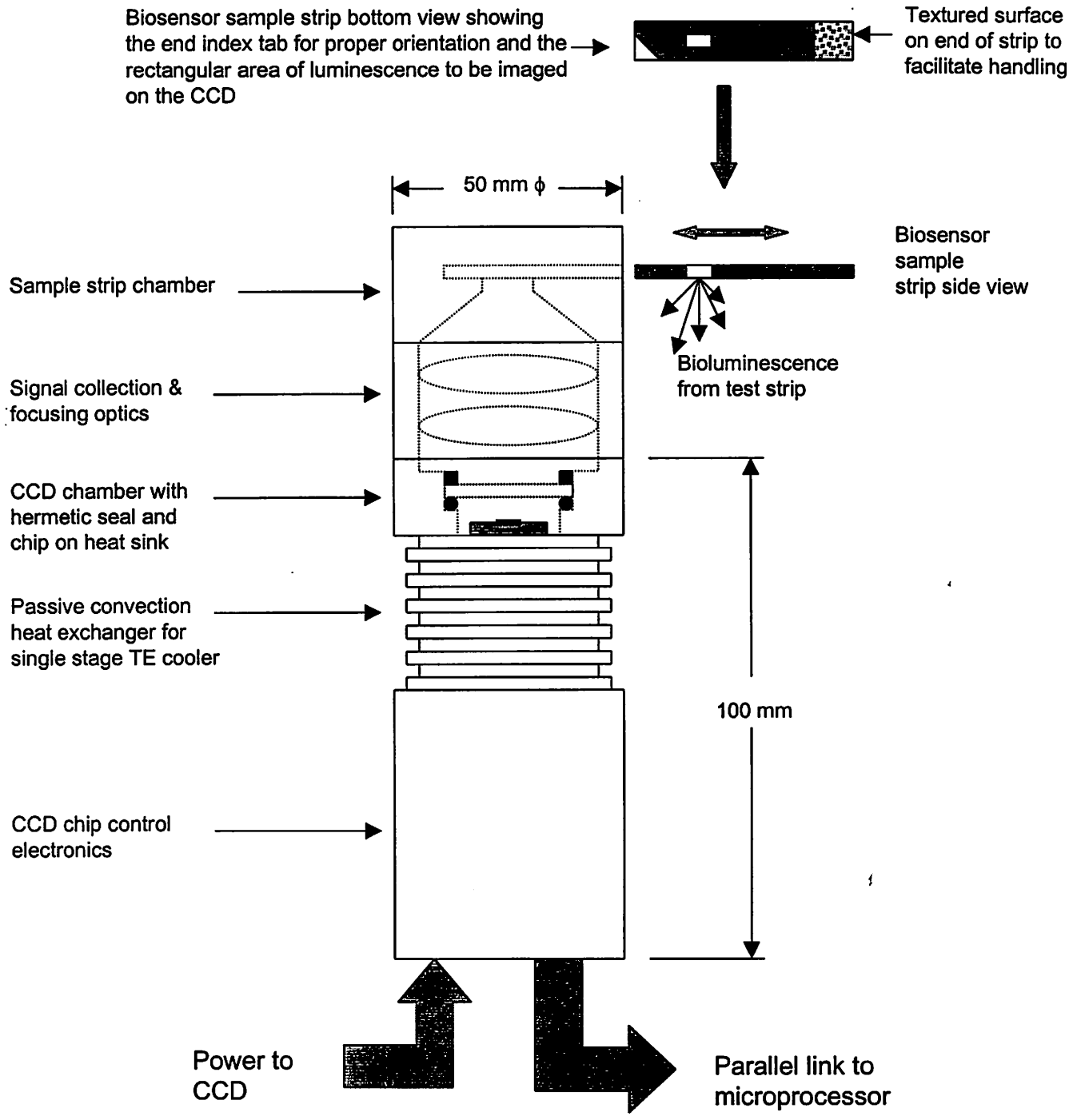

FIGURE 3 Schematic illustration of biosensor instrumentation based upon a charge coupled device array detector (CCD). A portion of the bioluminescence is collected by a macro-lens and imaged onto the CCD. Control of the CCD and single stage thermoelectric (TE) cooler is managed by a remote microprocessor. The microprocessor also processes data from the CCD and provides a user interface via the computer display. A bottom view of the test strip is shown at the top of this figure.

Phase II

The purpose of this Phase I application is to assess the feasibility of a test strip-based creatinine sensor utilizing bioluminescence readout and inexpensive CCD detection. Given the work in the literature, our own preliminary simulation, and our own preliminary experiments, we are quite confident that the approach will be deemed feasible and practical as a result of these Phase I studies.

In the Phase II application we will address in detail the design of the test strip and the modification if necessary of the prototype luminometer system. Figure 3 presented our very preliminary, pre-prototype test strip now under development for the analysis of galactose. The pre-prototype of the inexpensive CCD luminometer designed to read that test strip is in Figure 3. The CCD luminometer now under development does not have the capability to store and process multiple readings. The use of plasma creatinine measurements for the monitoring of renal transplant patients will be greatly augmented by means to store and process the data to establish trends. Given the slight variability associated with all measurements, each individual measurement is not of great importance, unless it is significantly out of range. It is the trends in the numbers over time that will signal to the physician that the patient needs attention. We therefore expect the Phase II application to include a task on data collection, processing, and presentation, including means to routinely deliver that data to the patient's physician.

Other components of the Phase II application will include a thorough evaluation of potential interferences in both blood and urine (see Table 4 again), including the various drugs and therapeutics appropriate to renal transplant patients. We will also more thoroughly and exhaustively evaluate our approach against more standard and established means for the measurement of creatinine. We will work closely with Dr. Holman and with his collaborators in a limited clinical study in a small pool of patients. These studies will be done by subcontracting to the University of Utah College of Medicine.

Significance and Market

PSI is striving to be The Personal Chemistry Company. Our goal is to provide simple inexpensive, easy-to-use means for the monitoring of important analytes in blood, urine, and sweat. Our work to date has focused on two analytes which might be called orphan diagnostics, a metaphor to orphan drugs. Galactosemia and PKU are two different inborn errors of metabolism, both of relatively low frequency, although PKU afflicts about one in 10,000 in the general population. These two diseases are "treated" by the careful control of dietary intake, by minimizing galactose and by tight control of phenylalanine, respectively. Although there are extensive and relatively inexpensive technologies for the measurement of urine and blood glucose in the home environment, only recently has attention been given to the possibility of such monitoring for galactose and phenylalanine, hence our term orphan diagnostics.

The National Kidney Foundation recently issued a press release arguing that all Americans should have their blood urea nitrogen and creatinine levels monitored. The release said "two simple blood tests can help determine if your kidneys are functioning normally." Unfortunately those "two simple blood tests" are not readily available to the average person. You must go to your physician or health care provider and have such a test ordered. It is inconvenient, time consuming, and relatively expensive.

There is also the problem of test compliance. Patients are not particularly eager to go to a physician or the laboratory to have blood drawn for a test if they are feeling fine, even if they have a kidney transplant. We are confident that patients can and should be empowered to be part of their personal health care team. A renal transplant patient can be expected to have the motivation to perform a truly simple blood test on a regular basis to help assess the functioning of his kidney and to minimize the possibility of its potential rejection.

There are currently over 50,000 patients in the United States with transplanted kidneys. Assuming that each patient should perform a home measurement weekly results in 2.5 million determinations per year. Assuming the test strips could be sold for approximately $10 each and the instrument required to read and process the strip would cost about $500, leased or rented to the patient via the test strip purchases, this relatively small niche market would be in the vicinity of 25 million dollars per year. Obviously all patients would not participate, but also obviously there are many other applications for a creatinine sensor. Other applications include the monitoring of patients on hemo and peritoneal dialysis, particularly if the sensor was coupled with several other channels which would more completely monitor the state of the patient prior to and after dialysis.

The National Kidney Foundation has warned that kidney disease affects more than 20 million Americans. It is likely that a significant subset of that population could and would utilize a convenient, easy to use, assessment of kidney function. The creatinine test strip is likely to be the first component of an eventual multi channel test strip which could include urea, uric acid, and total protein in the case of urine samples. The test strip for renal transplant patients could also include a channel for the analysis of cyclosporine by bioluminescent imuno assay.

In addition to the existing 50,000 renal transplant patients there are about 10,000 transplants performed each year with an average one year survival of about 85%. Given the increasing number of patients and increasing survival times, the market estimates above are likely to be on the low side.

Since nearly half of renal transplant patients today are also diabetics and their end stage renal disease was in part a complication of their diabetes, it is likely that a significant proportion of the diabetic population would also want to be screened regularly for renal function. Indeed, one might imagine in the near future a test strip which would include a wide range of analytes appropriate to diabetes, to include glucose and hemoglobin A1C, as well as creatinine and other analytes important in assessing the complications and status of diabetes.

E. Human Subjects—none

F. Vertebrate Animals—none

G. Consultant

Our consultant is Dr. John Holman Jr., Associate Professor of Surgery and Surgical Director of Renal Transplantation at the University of Utah. We have budgeted Dr. Holman at one day per month for the six months of this Phase I grant at a consulting rate of $440 per day x 6 days = $2,640. Dr. Holman will consult and advise in the design and conduct of the Phase I studies and will play a major role in the preparation of the Phase II application. His biosketch and letter of participation are enclosed.

Three key advisors are Dr. Larry Kricka, Dr. Russell Stewart, and Dr. Joseph Andrade. Larry and Russell serve on the Scientific Advisory Board.

Dr. Larry Kricka is 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. He will provide appropriate advice and guidance to this project as part of his service on the Scientific Advisory Board.

Dr. Russell Stewart is Assistant Professor of Bioengineering at the University of Utah and PI of our University of Utah STTR Phase II subcontract on recombinant firefly and bacterial luciferase. He will advise and assist in areas of protein engineering, production, and characterization as needed.

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