OMB No. 0925-0195
Expiration Date 4/30/01

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase II Grant Application

Follow instructions carefully.

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

PSI-PKU (1)

1a. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Quantitative, Direct Reading Phenylalanine Biosensor

1b. Phase I Grant No.
1R43MH57591-1

2. PRINCIPAL INVESTIGATOR ☐ New Investigator

2a. NAME *(Last, first, middle)*
Van Wagenen, Richard A.

2b. DEGREE(S)
B.S. | Ph.D.

2c. SOCIAL SECURITY NO.
Provide on Personal Data Page.

2d. POSITION TITLE
Research Scientist and Principal Investigator

2e. MAILING ADDRESS *(Street, city, state, zip code)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093

2f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 801-583-9301
FAX: 801-583-4463

BITNET/INTERNET Address:
biolight@concentric.net

3. HUMAN SUBJECTS
☒ NO ☐ YES
3a. If "yes," Exemption no. or IRB approval date
☐ Full IRB or ☐ Expedited Review
3b. Assurance of compliance no.

4. VERTEBRATE ANIMALS
☒ NO ☐ YES
4a. If "Yes," IACUC approval date
4b. Animal welfare assurance no.

5. DATES OF ENTIRE PROPOSED PHASE II PERIOD	6. COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD		7. COSTS REQUESTED FOR ENTIRE PROPOSED PHASE II PERIOD	
	6a. Direct Costs	6b. Total Costs	7a. Direct Costs	7b. Total Costs
From: 04/01/00				
Through: 03/30/02	$ 248,320	$ 385,002	$ 462,216	$ 740,072

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 84158-0093

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

Congressional District
2

11. INVENTIONS AND PATENTS
☒ NO ☐ YES If "Yes," ☐ Previously reported *OR* ☐ Not previously reported

12. SMALL BUSINESS CERTIFICATION
☒ Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

13. 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.

15. 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

14. 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:
biolight@concentric.net

16. 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 2a *(In ink. "Per" signature not acceptable.)*
Richard A. Van Wagenen

DATE
August 12, 1999

17. 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 15 *(In ink. "Per" signature not acceptable.)*

DATE
August 12, 1999

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

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

YEAR FIRM FOUNDED	NO. OF EMPLOYEES *(include all affiliates)*
1988	3

TITLE OF APPLICATION

Quantitative, Direct Reading Phenylalanine Biosensor

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
R. Van Wagenen, Ph.D.	Protein Solutions, Inc.	Principal Investigator
C. Eu, Ph.D.	Protein Solutions, Inc.	Research Scientist
D.J. Min, Ph.D.	Protein Solutions, Inc.	Research Scientist
J.D. Andrade, Ph.D.	Protein Solutions, Inc.	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.

It is now generally recongnized that phenylketonuria (PKU) patients should be on a phenylalanine (Phe) controlled diet and that their Phe levels should be monitored regularly. Although monitoring by the patient or care provider in the home environment is now recommended, no suitable tests, kits, or devices are available for this purpose. The Phase I effort demonstrated the feasibility of an NADH based bioluminescence detection system for the determination of Phe. The Phase I work and subsequent studies have shown that a simple bioluminescence based enzyme assay for Phe is likely to be successful in a dry reagent, dipstick type sensor format. This Phase II application addresses the development of a Phenylalanine/tyrosine sensor for use in the home by PKU patients or by their caregivers. The sensor will require about 50 microliters of blood, a little more than the volume used in the current generation of home glucometers. All necessary reagents are incorporated into the dipstick device. The device will be read by a small handheld luminometer now under development. In addition to the design and development of the biosensor, the Specific Aims include the development and production of recombinant Phe dehydrogenase, means to enhance the dynamic range of the device, means to enhance the bioluminescence output and reagents stability, calibration and validation studies against established methods, and preliminary evaluation in a clinical environment.

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

phenylketonuria, PKU, phenylalanine, tyrosine, biluminescence, NADH, home healthcare

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

Home based, self monitoring of phenylalanine and tyrosine for the management of PKU is of interest to the 25,000+ Americans afflicted with phenylketonuria. Weekly monitoring provides information on the medical status of the disease and the degree of dietary compliance. Weekly monitoring with a $10 per test cost is a $13 million annual U.S. market and approximately a $30 million world market.

Introduction to the Revised Application

Since the completion of the Phase I grant and the original Phase II submission, we have learned much more about PKU, its monitoring, its management, and its treatment. We have worked closely with the University of Utah's Cost Reducing Health Care Technologies (CRHCT) program, sponsored by the Whitaker Foundation and by the National Science Foundation, which has had considerable interest and activity on PKU (see www.healthtechcost.med.utah.edu).

We have continued working on the project after the termination of the Phase I grant using internal funds. These studies are reflected in the expanded progress report (Section 3) in this proposal. We have significantly redesigned the sensor, done additional studies to demonstrate the feasibility of the approach, and carefully considered the sensitivity and accuracy of the analytical methods. We have thoroughly modeled the multiple enzyme reactions, compared the simulation against experimental data, and used the model to optimize sensor design.

We have placed a stronger emphasis on recombinant production of especially engineered phenylalanine dehydrogenases, bacterial luciferase, and the needed oxidoreductase, including setting up the appropriate facilities in our own laboratories.

We have obtained a modern, semi-production scale, instrumented lyophilizer and have obtained far more experience with the formulation and lyophilization of enzymes and enzyme cocktails, thus permitting our reagent preparation and stabilization studies in this grant to be far more productive and effective.

The original Principal investigator, Dr. C.-Y. Wang is not longer with the company. Our new PI is Dr. Richard A. Van Wagenen, Vice President for Research and Development. Richard has an extensive background in interfacial phenomena, optics, spectroscopy instrumentation and medical device development. He has had more than fifteen years of project management experience and has served as Principal Investigator on several Phase II and numerous Phase I SBIR grants. He currently serves as the PI on two SBIR grants which address the Bio-Light instrument development and the creatinine biosensor development. Dr. Van Wagenen is a full time employee of the company.

Our responses to the specific critique and comments in the Summary Statement for the Phase I and original Phase II submission follow:

Phase I critique:
There was some concern raised as to potential problems with the development of the spatial gradient required for the direct instrument less detection of the bioluminescence signal. There was also concern with the lateral sensor design, which depends on fluid transport via capillarity and wicking processes. There was also concern with the containment of the alkyl aldehyde reagent, as well as with means to produce the local pH microenvironment needed for the dehydrogenase reaction.

Phase II critique:
One of the reviewers expressed concern with the possible effect of variations in hematocrit as well as interferents in plasma. There was concern with liquid transfer issues -- the reproducibility and reliability of capillarity-based wicking processes; reagent mixing was also a concern. There was also a question regarding the reproducibility of enzyme reaction rates, particularly given the time gradient approach used in the first Phase II submission. One reviewer also noted that there needs to be a means to ensure adequate blood sample volume applied to the sensor.

In the past year we have interacted much more extensively with the metabolic disease community in general and the PKU community in particular. Although our original goal was the development of a simple dipstick device which would not require an instrument (instrument less biosensor) (1,2), our interactions with the clinical community have taught us that they prefer an instrument be used in order to provide an objective number, to record data, to process and analyze data and establish trends, and to transfer that data and

those trends to the health care provider. Although we are still interested in instrument less biosensors, it is also clear that having an instrument with which to read the signal produced from such sensors greatly expands their analytical capabilities. The remarkable sensitivity of bioluminescence (3,4), coupled with the sensitivity of a modern handheld, CCD- based luminometer, makes possible the measurement of micromolar concentrations in small samples. Given that we will have access to BioLight, our own small, inexpensive, handheld luminometer to objectively and quantitatively measure bioluminescence intensity, we no longer require a gradient approach for direct visual detection -- neither the spatial gradient from the Phase I application nor the time gradient in the first Phase II submission. The direct measurement of bioluminescence intensity by a small, inexpensive instrument allows the sensor to be much simpler than originally envisioned. This instrument will be designed to accommodate a range of bioluminescent dipstick assays for specific analytes, including the first generation PKU sensor (this proposal) and a second generation sensor to include measurement channels for other important amino acids (5,6) (future work and funding). Our PKU-Direct sensor design is now much simpler, facilitating the development of multichannel sensors

Although we can design and develop the sensor to measure blood and/or plasma Phe/Tyr concentrations, we have chosen to measure plasma levels at this time. Most Phe determinations are made by a filter paper spot collection system, followed by extraction of Phe and other analytes from the spot, followed by conventional laboratory analysis (7,8,20), resulting in a blood level rather than a plasma or serum level. However, reference values for Phe and other amino acids are usually determined from a venipuncture sample as a plasma or serum analysis.

The concern with the reproducibility of enzyme reaction rates is now greatly alleviated by the new design and by the use of an instrument which can measure and record light intensity as a function of time and of position.

The concern with stability of the alkyl aldehyde reagent is an important one, and is specifically addressed in this revised application. We have also more extensively studied possible interferents during the past year; this is addressed both in the progress report and in the experimental section.

The sensor now includes a measure of the adequacy of the blood volume applied by the user.

The involvement of a group of distinguished clinical consultants will help ensure that the sensors will be tested in relevant environments and will be calibrated and validated against accepted clinical analytical procedures.

As noted above, most of the proposal has been rewritten. These rewritten sections are indicated by a vertical line in the right margin. Not much of the original proposal remains! We thank the reviewers for their insightful and helpful critique.

Research Plan

1. Background, Introduction, and Specific Aims

Phenylketonuria (PKU) is an inborn error of metabolism of moderate incidence (~1 in 10,000). About 99% of PKU patients have a recessively inherited deficiency of Phe hydroxylase, an enzyme important in the conversion of phenylalanine (Phe) to tyrosine (Tyr); Figure 1 (9).The defect results in excessive concentrations of Phe (normal range is about 100 microM), ranging to 800 microM or even higher. Such high Phe levels lead to severe mental and growth retardation. Fortunately, most states and developed nations now routinely screen infants for PKU within a few days of birth. PKU patients are placed on a Phe-restricted diet (9,14,15). If the diet is started early and maintained for life, treated patients can lead normal lives with minimal mental or physical problems.

The management of PKU is undergoing constant reevaluation. It was once thought that the very high circulating concentrations of Phe were only damaging in infancy, due to neurotoxicity effects on the rapidly developing brain. Later it was recognized that a Phe restricted diet was required throughout childhood, although clinicians and other caregivers would often give in to adolescent teenagers who rebelled against their bland, tasteless, Phe restricted diets. It is becoming increasingly recognized that the circulating Phe level must be maintained at near normal values in order to minimize neuro psychological effects (9,37,38). The metabolic pathways normally presented for Phe and Tyr (Figure 1) tell only a small part of the story. Figure 2 provides a different perspective by emphasizing the role of Tyr as a precursor to several key neurochemicals, including L-DOPA, dopamine, and the epinephrines.

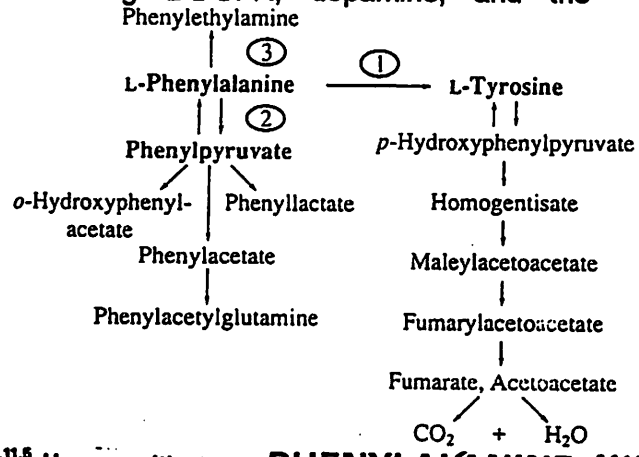

Figure 1. Phe/Tyr Metabolic Pathways (9) Pathway 1 is the principle enzyme defect in PKU, leading to a buildup of Phe and the enhancement of the "minor" pathways 2 and 3.

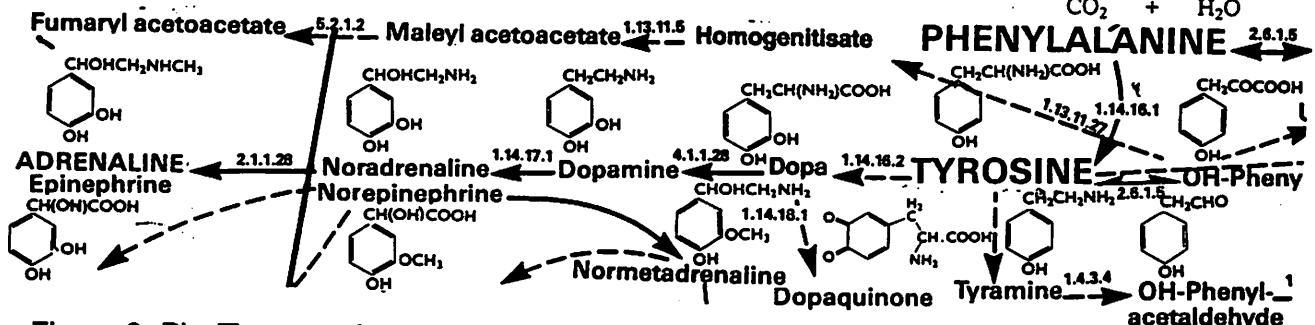

Figure 2. Phe/Tyr part of a more extensive Metabolic Map (40), emphasizing the pathways leading from Tyr to key neurochemicals.

It has also become recognized that elevated Phe levels result in a decrease in brain permeability to other large neutral amino acids, due to competition for a common amino acid

transport mechanism (22,26,28). Thus there is growing concern about the bioavailability of other essential amino acids in PKU. This has led to the development of more advanced or refined PKU diets, in order to properly manage Phe restriction and nutritional needs. There is growing discussion of the role of valine, leucine, isoleucine (see www.angelfire.com/ct/lrp regarding a clinical trial), and even methionine and tryptophan, as well as the more established Tyr and Phe, in the management of PKU (5,6,22,28). Recent studies have also indicated that lysine may need to be added to this growing list (22). Although this Phase II proposal deals only with a device for the measurement of Phe and Tyr, it is clear that subsequent generations of the sensor may need to include these other important amino acids.

As if that increased complexity were not enough, recent studies have shown that there is a significant fluctuation in Phe and Tyr levels during the course of the day (10,11,26). This behavior is reminiscent of the situation in diabetes in which the glucose levels can fluctuate dramatically as a result of dietary intake and activity (21). The PKU community may require a means of measurement of the integrated or average Phe concentration over time, much as glycosylated hemoglobin (HbA1c) has become a marker of the longer term effectiveness of glucose management in diabetes (21).

There is now a much stronger realization of the need for an inexpensive Phe/Tyr test for home use (12-14). A year ago in the original Phase II submission we were estimating biweekly or even monthly use of such a home test. Given the brief discussion above, and the activity in the literature just over the past year, it is likely that the community will slowly move towards a weekly testing frequency. The availability of a simple test will also make the study of Phe and Tyr fluctuations (10,11,26,27) simpler and less traumatic to the patients involved.
A major problem with a low incidence disease is that there are not many patients, meaning that most studies involve only small numbers. Given the rapid growth of the Internet, and the very rapid evolution of patient support and disease specific organizations (see www.pkunews.org), studies PKU will become more widespread, particularly if there are simple, inexpensive, home-based means to facilitate the biochemical measurements required.

Figure 3 shows our dipstick type PKU-Direct! sensor. Fig 3 (upper) is a top view (not to scale). Fig 3 (bottom) shows a cross-section of the sensor. A small blood droplet is placed on a central depression, migrates down into the strip through a blood separation membrane. The plasma is wicked into a set of spatially distinct 'chambers" in which the analytical reagents are predeposited. The bioluminescence is read at the bottom by the Bio-Light! CCD luminometer (Table 1). In this general drawing 15 analyte chambers are shown, each requiring 1-2 microliters of plasma. The first generation PKU-Direct! sensor (this proposal) consists of only two chambers, each requiring 10 microliters of plasma.

The sensor strip will be inserted into the BioLight! portable, handheld luminometer (Table 1). The production version of the BioLight! instrument will likely be a little larger than a typical home glucometer. Although the initial price will likely be in the $500 range, the final price may be as low as $100 by using photon detection chips based on CMOS technology (30).

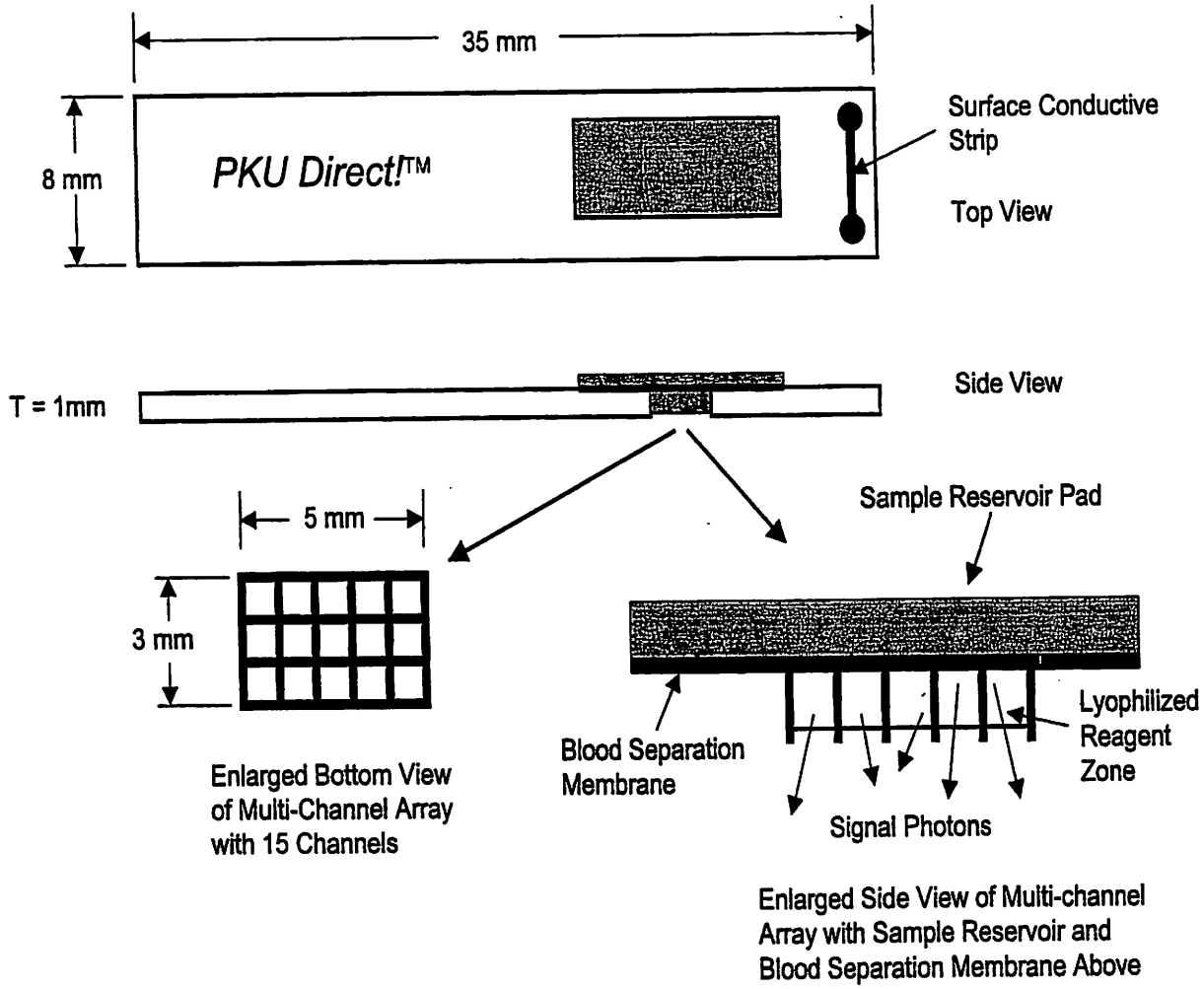

Figure 3: The Bio-Light! Multi-Analyte Sensor.

$$\textbf{Phenylalanine} + NAD^+ + H_2O \xrightarrow{\text{Phenylalanine dehydrogenase}} \text{Phenylpyruvate} + \textbf{NADH} + NH_4^+$$

$$\textbf{NADH} + FMN + H^+ \xrightarrow{\text{Oxidoreductase}} NAD^+ + \textbf{FMNH}_2$$

$$\textbf{FMNH}_2 + RCHO + O_2 \xrightarrow{\text{Bacteria luciferase}} FMN + RCOOH + H_2O + \textbf{Light}$$

Figure 4: The Biochemical Reactions involved in the Phe/Tyr PKU Sensor: the phenylalanine dehydrogenase (PheDH), oxidoreductase, and luciferase reactions.

TABLE I. Device Specifications for CCD Multi-Channel Bio-Light! Analyzer and Associated Biosensor

Maximum Dimensions:	Length: 125 mm, Width: 75 mm, Thickness: 25 mm
Maximum Weight:	250 grams (8.8 oz)
Power Supply:	Disposable batteries with sufficient capacity to conduct one test per day for one year.
Operating Temperature:	50°F to 95°F (10°C to 35°C)
Storage Temperature:	-20°F to 130°F (-29°C to 54°C)
Operating Humidity:	10 to 90 % RH (non-condensing)
Memory:	Sufficient to store 100 test results and associated dates and times.
User Interface:	51 mm x 51 mm monochrome liquid crystal display. Audible beeper/alarm Three buttons (On/Off, Scroll/Select, and Enter).
Mechanical Robustness:	Capable of surviving a 30 G deceleration (on board accelerometers).
Communications:	Modem or wireless communications for data download, new software updates and instrument remote diagnostics.
Disposable Size:	Approximately 45 mm long, 10 mm wide and 1 mm thick with a minimum of four analyte capability.
Sample:	A minimum 100 μL whole blood sample which yields plasma via a polymeric blood separation membrane.
Hematocrit Range:	25 to 60 %
Calibration:	Plasma equivalent concentration values for all analytes. Calibration to be accomplished with individual channels on the biosensor or if necessary with a small liquid sample of calibration solution.
ATP Analysis Range:	10 nano-Molar to 10 milli-Molar.
Accuracy:	± 10 percent of full scale over any single decade range and this being applicable over the complete analysis range.
Reliability:	No moving mechanical parts, i.e., no fans, shutters, gears, etc. Minimum MTBF of six years with twenty minutes of daily operation.
Warranty:	Three years except for batteries and obvious mechanical abuse (in excess of 30 G's deceleration) or exposure to liquids.
Materials Cost:	$350 with individual part volume quantities of 10,000.

Phase II Specific Aims:

Specific Aim 1: Sensor Optimization and Construction (Fig. 3).

The base and top of the sensor is designed to minimize wave-guiding of ambient light into the luminometer and to provide a strip which is easily handled and inserted; only the bottom sensing region is transparent. The patient produces a lancet generated 50 microliter drop of capillary blood, generally from a finger tip. The droplet is touched to the sample application region on the top of the strip and is instantly drawn by capillarity to fully fill the zone. A plasma separation membrane contains the red cells and permits the passage of plasma into the reaction chambers. The plasma separation membranes are widely used by the in vitro diagnostics industry, particularly the Whatman PlasmaSep and the Pall/Gelman Hemasep series. The plasma reconstitutes the lyophilized and predeposited analytical reagents in the the reaction "chambers", one each for Phe and Phe+Tyr, each requiring 10 microliters of plasma.

The main tasks here are to evaluate the available plasma separation membranes and to optimize filling time, sample volume (analysis area), and luminometer detection parameters. The dynamic range will be addressed under Experimental Methods, after presentation of the Progress Report.

The periphery of the plasma wetted region (outside of the reaction "chambers") will contain predeposited ATP, luciferin and firefly luciferase, which, when activated by the plasma, bioluminesces. This signal is also measured and indicates that the strip is fully filled with blood/plasma. A task here is to optimize the detection region and conditions for this function.

The strip also contains spacer regions to facilitate its insertion into the luminometer and alinement within the luminometer. The far right end of the strip may also contain an electrically conductive strip to facilitate proper insertion and placement, functioning as a switch which activates the luminometer display to indicate that filling and insertion of the strip has been performed correctly. The strip insertion housing of the luminometer will be removeable for regular cleaning as needed.

Specific Aim 2: Optimize the Biochemical Reactions involved in the Phe and Tyr Sensing Channels, including Alkyl Aldehyde Production and Immobilization and the Preservation and Stability of all Reagents—(see Figures 3 and 4).

In sequential reaction sensors, the PheDH reaction is facilitated by a high pH environment. Our Phase I studies have shown that a homogenous reaction, i.e., running the PheDH reaction and the luciferase—oxidoreductase reaction simultaneously in the same volume, permits a suitable quantitative output. The consumption of the NADH produced by the oxidoreductase reaction helps drive the Phe reaction to partial completion (Fig. 4). This allows for a much simpler sensor design, allowing all three reactions to proceed simultaneously in the same volume (the reaction "chamber", Fig. 3). This region of course includes the NAD+ and the alkyl aldehyde needed for the bioluminescent reaction. The Phe channel utilizes a PheDH which is highly specific for Phe. The Tyr channel used a Phe/TyrDH, which acts on both amino acids; thus the Tyr channel is really a Tyr+Phe channel. The Tyr value is obtained by subtraction of the two signals.

Dry reagents are needed to enhance the stability and shelf-life of the biosensor. Lyophilization generally yields the best result for long term storage (32). We will determine the optimum conditions (combination and concentration of preservatives, temperature and vacuum control) for the lyophilization process. Various lyophilization approaches will be evaluated in short term (120 day) stability tests. The most promising conditions and formulations will then be evaluated in longer term (one year) stability studies. The effect of various storage conditions on reagent preservation will also be evaluated. Preservation success will be evaluated by comparing the stored samples with a fresh standard multi-enzyme bioluminescence reference assay.

There is some concern that the alkyl aldehyde (decanal or dodecanal) required in the luciferase reaction (Fig. 4) may be unstable to oxidation and too volatile. We will address this concern in three ways:

- Each individual strip will be sealed in a metallized, impermeable packet, opened just prior to blood sample application. We will study and optimize the application of the alkyl aldehyde after the lyophilization process, and just prior to the packaging of each strip.

- We will also prepare a polyethylene glycol (PEG)-derivatized alkyl aldehyde from an amino dodecanoic acid precursor, resulting in a more soluble, low volatility, and likely highly active alkyl aldehyde reagent (42).
- The third approach is more exploratory and involves utilizing the enzyme complex in bioluminescent bacteria which is responsible for the local synthesis of the alkyl aldehyde from the fatty acid (43).

We will also use the results of Specific Aim 3 to further optimize the reactions and overall sensor performance.

Specific Aim 3: Produce Recombinant Enzymes, Enhance Enzyme Immobilization, and Design and Produce Organized Multi-Enzyme Complexes;

The PheDH is expensive, as is bacterial luciferase and oxidoreductase, and they will account for a considerable fraction of the biosensor cost. Less expensive and more reliable sources are highly desirable. Recombinant protein engineering, expression, purification and characterization are proposed in order to generate all the needed enzymes in house.

Through our University of Utah collaborator, Dr. R. Stewart, we have already expressed and regularly produce engineered firefly luciferase, bacterial luciferase, and oxidoreductase which can be immobilized and purified in one step using small poly-histidine and biotin segments (33, 34 – see Appendix). We propose to develop that technology in house (the work to date has been done in Dr. Stewart's lab and under his direction at the Univ. of Utah).

We propose to produce engineered PheDH with the specificities, stabilities, and substrate concentration dependence (Km values) needed for enhanced sensing applications. The availability of the three key enzymes with biotin-expressing domains will facilitate the preparation of unique di- and tri-enzyme nanocomplexes. The availability of such hybrids will permit less expensive, more reproducible, reliable and probably higher activity sources of our key biochemical reagents.

Specific Aim 4: Calibrate, Validate, and Evaluate Prototype Sensors.

The prototype biosensors will be evaluated for accuracy, precision and linearity for the ranges of Phe and Tyr encountered in blood and plasma under PKU conditions. These studies will use standard assays and methods of analysis, including receiver operating characteristic (ROC) plots (8) the Clarke Error Grid analysis commonly used to evaluate glucose analysis strips (29), and methods relevant to Phe (20,51).

Specific Aim 5: Begin Preliminary Design of the Second Generation Sensor for PKU.

The vertical design of the sensor facilitates the incorporation of many analyte channels (15 are shown in Fig. 3). The problem is one of available plasma volume and sensitivity of the analytical method. As one goes from a 10 microliter plasma sample to, say, a 1 microliter sample, the analytical method must be 10 times more sensitive for the same analyte concentration. That is possible and probably practical with bioluminescence, particularly if the bioluminescence output can be significantly enhanced via multi-enzyme nanocomplexes which obviate much of the FMNH2 autooxidation problem (discussed later) and provide some crude substrate channeling properties (44). A two fold enhancement would allow a sample volume of 5 microliters per analyte. Thus, even the present design could sense 4 different analytes; a 3 microliter volume would permit 6 to 7 analytes, etc., assuming comparable plasma concentrations.
All of the amino acids can be individually analyzed via specific reactions which couple to bioluminescence (35,36). The Bio-Light! luminometer is being designed to accomodate a

strip with up to 15 analytical channels. The CCD chip contains roughly 400 x 600 individual sensing elements, which can be combined or imaged in almost any configuration. Assuming the enhancement activities of Specific Aim 3 prove successful, we will then, in the last 6 months of the Phase II program, begin the design of a second generation multi-analyte sensor for PKU and allied conditions.

2. Significance

There has been growing awareness that PKU requires regular self-monitoring to ensure dietary compliance and facilitate diet adjustments (12). The management and treatment of PKU has many similarities to diabetes. The problem is that there are no simple, inexpensive means to measure Phe in the home environment. The availability of a home test device would greatly improve the monitoring and management of PKU.

"...A satisfactory diet compliance with ideally low blood Phe (Phe) concentrations can only be obtained if the principle of frequent monitoring...of blood Phe by the patient himself (self-monitoring) is realized." (12)

Although McCabe and co-workers (13) proposed a home device for Phe "estimation" some ten years ago, they were disappointed that there was no commercial/industrial interest in manufacturing and selling such a device. They noted that:

"A simple, portable monitoring system would provide families and their local physicians with an estimate of the blood Phe concentration within an hour of obtaining the specimen. This might be especially useful in attempting to moderate rising Phe concentrations during intercurrent illnesses. Home or office monitoring should not replace the clinical laboratory, but would supplement the traditional process by providing more rapid and frequent Phe estimates. Another use...would be to provide information for the maintenance of metabolic control in pregnant women with PKU... maintain strict metabolic control throughout the pregnancy."

The "Quantase" technology, used in the study by Wendel and Lagenbeck (12) and by others (8) has not been developed for direct home use. One reason for the lack of commercial interest is the relatively low incidence of PKU (roughly 25,000 patients and perhaps 1,000,000 tests per year in the USA). One could, therefore, call a Phe dipstick device for PKU an "orphan diagnostic" (an analogy to "orphan" drugs). There is a market, but it is not a large one.

A major first step in meeting the analytical needs is this Phase II application. The development and availability of a dipstick-like device to measure blood Phe and Tyr (the first generation of our PKU-Direct! sensor) will provide improved disease management, patient/provider empowerment and much needed data for research. We expect to follow this work with the development of a second generation sensor (PKU-Direct! II) which will include the analysis of other important amino acids of growing interest to the clinical PKU community (5,6,22), providing biochemical information for research and, hopefully, for improved treatment.

There may be other applications for these sensors. There is growing realization that there are considerable interindividual differences in the circulating level of many biochemicals, metabolites, and nutrients. We have called this *biochemical individuality* (18,19). The normal concentration distribution for individual amino acids can be very broad (27); a normal physiological variation in adults is generally at least +/- 25 % of the mean value and can be as high as +/- 50%, depending on the amino acid measured (27). In the case of Tyr about 15 % of a normal population have circulating Tyr values nearly two times that of the mean (24-

26). The situation is comparable in the case of Phe (24-27). We really do not know what is the medical and health significance of significantly low or significantly elevated levels of essential or near essential amino acids. The experience with extensive monitoring of PKU populations indicates that average values three times normal can result in measurable changes in performance on neuropsychological tests (37,38). What this means for our purposes is that the monitoring of hyper Phe levels, even those below the normal PKU diagnosis threshold, may become of increasing interest and possibly importance. It may well be that the relatively limited market projected for our PKU-Direct! sensor (25,000 patients in the USA x weekly monitoring = roughly one million tests per year) may indeed be far too small.

It is difficult today for the biochemical and clinical research communities to make rapid progress due to the difficulty in accessing suitable numbers of patients. Both funding and patients are greatly limited, typical problems with rare diseases. The patient issue could be partially alleviated by the availability of a simple, minimally invasive, inexpensive assay which patients and their care-givers could routinely use to monitor and manage their disease and provide a substantive increase in the amount of biochemical data for the research community. The availability of the internet has allowed much of the PKU community to organize and interact (www.pkunews.org). This will enhance the involvement and empowerment of this community in participation in research that will hopefully lead to better understanding, treatment and outcome for this insidious, orphan disease.

3. Phase I final report SBIR Phase I grant NO. 1 R43 HD36148-01
Project period: from 4/1/1998 through 9/30/1998

This report summarizes two periods of activity: (1) the work done on the Phase I project itself, which concluded on 09/30/98 and (2) additional work done using internal funds through 08/13/99. The latter work was, in part, based on the critique to our Phase II proposal. The present proposal is a highly augmented and revised submission of the original Phase II proposal.

Table 2. Key Personnel and Effort

Title	Name	Dates of service	% FTE	% Effort
Principal Investigator	Chung-Yih Wang, Ph.D.	4/1/98-9/30/98	100	50
Research Scientist	Robert J. Scheer, Ph.D.	4/1/98-8/10/98	33	50
Research Scientist	Rick Van Wagenen, Ph.D.	4/1/98-9/30/98	100	20
Technical Advisor	Joseph D. Andrade, Ph.D.	4/1/98-9/30/98	25	10
Lab Technician	Mara Hammer	4/1/98-9/30/98	50	50

Phase I Results by Specific Aim:

1. Evaluation of PheDH

We have reviewed six different PheDH (Table 3). Although the optimum pH of the reaction is species specific, in general the deamination of Phe requires a high pH to ensure the forward reaction. The amination of phenylpyruvate dominates at lower pH. PheDH also oxidizes

Table 3 Review of phenylalanine from different species

Species	Molecular weight	Km	Specificity (Phenylalanine= 100%)	Optimum pH and Stability	References
Sporosarcina ureae	305,000 (octamer)	For phenylalanine: 0.096 mM For NAD: 0.14 mM For phenulpyruvate: 0.16 mM	Tyrosine: 5.4% Leucine: 2.3% Methionine: 4.1% Tryptophan: 5.0%	For oxidative deamination: 10.5 For reductive amination: 9.0 Thermostability: 40 °C	Y. Asano et al., J. Biol. Chem., 262, 10346-10354, 1987
Rhodococcus Sp	69,000	For phenylalanine: 0.16 mM For NAD: 0.12 mM For phenulpyruvate: 0.16 mM	Tyrosine: - Leucine: - Methionine: - Tryptophan: -	For oxidative deamination: >10 For reductive amination: 9.25 Thermostability: -	W. Hummel et al., Home-monitoring and screening of phenylketonuria. S. Girotti et al., Talanya, 40, 425-430 (1992).
Rhodococcus maris	70,000 (dimer, 35,000/subunit)	For phenylalanine: 3.8 mM For NAD: 0.25 mM For phenulpyruvate: 0.5 mM	Tyrosine: 2.0% Leucine: 2.0% Methionine: 5.4% Tryptophan: 7.5%	For oxidative deamination: 10.8 For reductive amination: 9.8 Thermostability: 35 °C	H. Misono et. al., J. Bacteriology, 171, 30-36, 1989
Bacillus badius	310,000 (octamer)	For phenylalanine: 0.088 mM For NAD: 0.15 mM For phenulpyruvate: 0.11 mM	Tyrosine: 9.0% Leucine: 3.0% Methionine: 8.0% Tryptophan: 4.0%	For oxidative deamination: 10.4 For reductive amination: 9.4 Thermostability: 65 °C	Y. Asano et al., Eur. J. Biochem., 168, 153-159, 1987
Bacillus sphaericus	340,000 (octamer)	For phenylalanine: 0.22 mM For NAD: 0.17 mM For phenulpyruvate: 0.4 mM	Tyrosine: 72% Leucine: 1.3% Methionine: 3.0% Tryptophan: 1.2%	For oxidative deamination: 11.3 For reductive amination: 10.3 Thermostability: 55 °C	Y. Asano et al., J. Biol. Chem., 262, 10346-10354, 1987
Thermoactinomyces intermedius	270,000 (hexamer, 41,000/subunit)	For phenylalanine: 0.078 mM For NAD: 0.045 mM For phenulpyruvate: 0.045 mM	Tyrosine: 0% Leucine: 3.9% Methionine: 0% Tryptophan: 0%	For oxidative deamination: 11 For reductive amination: 9.2 Thermostability: 70 °C	T. Ohshima et. Al. J. Bacteriology, 173, 3943-3948, 1991