APPENDIX B

NATIONAL SCIENCE FOUNDATION
PROPOSAL COVER SHEET
STTR Phase II Proposal

KRUCKA

2 copy

TOPIC	ANALYTICAL CHEMICAL INSTRUMENTATION
PROPOSAL TITLE	Direct Reading Quantitative Biosensors for ATP-Dependent Processes
NAME OF PROPOSING SMALL BUSINESS CONCERN	Protein Solutions, Inc.
NAME OF RESEARCH INSTITUTION	University of Utah
ADDRESS (Including ZIP CODE)	6009 Highland Dr. Salt Lake City,UT 84121
ADDRESS (Including ZIP CODE)	Dept. of Bioengineering BPR Bldg. Rm 506C Salt Lake City, UT 84112
REQUESTED AMOUNT	$ 349,407
PROPOSAL DURATION	24 months
REQUESTED STARTING DATE	April 1, 1996

THE SMALL BUSINESS CONCERN CERTIFIES THAT:	YES	NO
1. It is a small business as defined in this solicitation.	X	
2. It qualifies as a socially and economically disadvantaged business as defined in this solicitation. FOR STATISTICAL PURPOSES ONLY		X
3. It qualifies as a women-owned business as defined in this solicitation. FOR STATISTICAL PURPOSES ONLY		
4. It will exercise management direction and control of the performance of the STTR funding agreement.	X	
5. The primary employment of the principal investigator will be with this firm at the time of award and during the conduct of the research.	X	
6. It will permit the government to disclose the title and technical abstract page, plus the name, address and telephone number of the corporate official if the proposal does not result in an award to parties that may be interested in contacting you for further information or possible investment.	X	
7. It will comply with the provisions of the Civil Rights Act of 1964 (P.L. 88-352) and the regulations pursuant thereto.	X	
8. It will perform 72 percent of the work and the collaborating research institution will perform 28 percent of the work as described in the proposal.	X	
9. NSF is the only Federal agency that has received an equivalent or overlapping proposal from the small business concern. If not, you must disclose all equivalent or overlapping proposals as required by the STTR Program Solicitation.	X	

PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR

NAME	Robert J. Scheer
SOCIAL SECURITY NO.	253-35-1321
TELEPHONE NO.	(801)583-9301

RESEARCH INSTITUTION INVESTIGATOR

NAME	Russell Stewart
SOCIAL SECURITY NO.	501-78-4766
TELEPHONE NO.	(801)581-8581

OTHER INFORMATION

PRESIDENT'S NAME	YEAR FIRM FOUNDED	NUMBER OF EMPLOYEES
Phil Triolo	1988	AVERAGE PREVIOUS 12 MO.: 6 CURRENTLY: 5

PROPRIETARY NOTICE See Section 7.4 for instructions concerning proprietary information. Proprietary information is contained on page number(s)_____ of the proposal.

NOTE: The signed Certification Page must be submitted immediately following this Cover Sheet with the original copy only.

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APPENDIX C

NATIONAL SCIENCE FOUNDATION
STTR-Small Business Technology Transfer Program

PROJECT SUMMARY

NSF AWARD NO.

NAME OF FIRM

Protein Solutions, Inc.

ADDRESS (Including ZIP CODE)

6009 Highland Drive
Salt Lake City, UT 84121

PRINCIPAL INVESTIGATORS (NAME and TITLE)

Robert J. Scheer, Project Director

TITLE OF PROJECT

Direct Reading, Quantitative Biosensors for ATP-Dependent Processes

TECHNICAL ABSTRACT (LIMIT TO 200 WORDS)

This NSF Phase II research project is undertaken in response to the need for an inexpensive, direct reading sensor, capable of real time measurement of bacterial contamination in sensitive areas, such as food and pharmaceutical facilities and water treatment plants. During phase I of this project we demonstrated the feasibility of detecting ATP in quantities as small as 10^{-9} g ($2x10^{-12}$ moles) both visually and on photographic film. The technology employs the firefly luciferase catalyzed light producing reaction between luciferin and ATP. By mediating the ATP concentration with the "ATPase" apyrase, we are able to produce a spatial light pattern which indicates the concentration of ATP present in a sample.

In this project we focus on increasing the sensitivity of our direct reading ATP sensor to levels more suited to bacterial detection (10^{-12} to 10^{-13} g). This increase in sensitivity will be achieved by three primary means:

1) Increasing the intensity and/or duration of the luminescence for a given quantity of ATP,
2) increasing the amount of light that reaches the detector (film or otherwise), and
3) optimizing the non-instrumented detector for the system.

Each of these objectives should contribute at least one order of magnitude increase in absolute ATP sensitivity.

Rapid, simple, inexpensive and reliable measurement of bacterial contamination will facilitate industrial compliance with safe food and dairy practices. The sensors can also be applied to a range of medical and pharmaceutical environments and products. Eventually, marketing efforts will lead to consumer use in the home for the sanitary monitoring of kitchen and bathroom surfaces.

KEY WORDS TO IDENTIFY RESEARCH OR TECHNOLOGY (8 MAXIMUM)

Microbe, ATP, Detection, Luciferase, Direct-reading, Bacteria

POTENTIAL COMMERCIAL APPLICATIONS OF THE RESEARCH

Food, beverage and water processing industries to sense levels of cleanliness. Monitoring of pharmaceutical and medical device cleanroom areas, hospitals, and other biological laboratories for microbial contamination.

SYNOPSIS OF PHASE I RESEARCH RESULTS

Identification and Significance of the Problem or Opportunity: A national trend toward improving the preparation conditions of foods, beverages, and drugs is driving research toward an inexpensive and easy-to-use sensor for the detection of bacteria.

We have developed an ATP measurement device whose primary use will be to quantify bacterial contamination in sensitive environments. This sensor incorporates two fundamental technologies,

1) an enzyme-based detection of ATP (luciferase/luciferin) and
2) a spatial light intensity readout proportional to the amount of ATP in the sample.

Currently, real-time bacterial monitoring is carried out using expensive luminometers and ancillary sampling devices. Besides being expensive, these luminometers are bulky and require calibration and maintenance. Other methods of detection require long incubation times (up to 72 hours) to allow colonies to grow to detectable sizes for enumeration.

Our real-time sensor technology does not require preconditioning of the sample (i.e. concentration or culturing), but will match the bacterial sensitivity of methods currently available which require either 24 hour culture systems or sophisticated luminometer systems costing thousands of dollars. Further, by using high speed film as a detector, one can both increase the sensitivity of the assay and obtain a hard copy record of the monitoring procedure.

Therefore, our system will save both time and money for industries that monitor their work areas for bacterial contamination.

Background and Technical Approach: Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests (1-3). Since the firefly luciferase/luciferin reaction is dependent on an adenosine triphosphate (ATP) co-factor, it has been extensively used in the development of biosensors for the measurement of ATP. Using luminometers and photomultiplier tubes, sensitivities to 10^{-13} g are commercially available (4).

Under a contract between Protein Solutions, Inc. and the University of Utah's Center for Biopolymers at Interfaces (CBI), Mr. C.Y. Wang has been working on the trehalose stabilization of firefly luciferase for the past two years. Mr. Wang and coworkers have succeeded in stabilizing firefly luciferase in agarose gels for periods up to 6 months (5). These gels maintain their optical clarity in the dehydrated state, and can be rehydrated after extended periods with high levels of activity. With the completion of the Phase I research project, Mr. Wang, and Dr.'s Scheer and Andrade have extended this gel preservation technique to apyrase and have developed a device for measurement of ATP concentration based on the spatial position of light output.

Our unique approach to ATP detection is to produce a spatial distribution of the emitted light proportional to the analyte (ATP) concentration. The human photon detector can then be used to assess spatial position rather than absolute light intensity. This positioning is achieved by carefully controlling the ATP concentration using an ATP "filter", a second ATP consuming enzyme, apyrase. In PSI's system, apyrase, with a turnover rate approximately 100 times faster than luciferase, serves as an ATP concentration regulator to the sensor.

Measurements of enzyme and substrate activity after prolonged storage were performed for different gel preparation and storage conditions. These measurements gave us preliminary information necessary to select enzymes and substrates based on their stability, and aided with the selection of the appropriate method of preservation.

After learning that both luciferase and apyrase could be stored for prolonged periods in a dehydrated state, refinement of the luciferase and apyrase concentration levels for appropriate readout were performed. Appropriate concentration levels were determined through a systematic examination of light output relative to the concentrations of luciferase, apyrase, and ATP. Each of the gel components including the luciferase and apyrase enzymes, and the analyte components including the luciferin substrate and coenzyme A were reviewed. We were looking for the highest light output for a given ATP concentration.

Finally, wicking, capillarity, and optical studies to determine proper device geometry for rapid delivery of analyte to the sensor gel spots were performed. These studies gave us the information necessary to decide on a likely test strip geometry. We were looking for a geometry which gave us a bright readout as well as fast analyte delivery.

These experiments have resulted in the following prototype design. The luciferase/apyrase enzymes are deposited as circular spots along one dimension of the device, each dot of increasing apyrase concentration. The physical support for the device is a strip of filter paper sandwiched between two polyester films with an agarose coating. An opening is made in the top film to allow the delivery of the analyte. (See figure 1.)

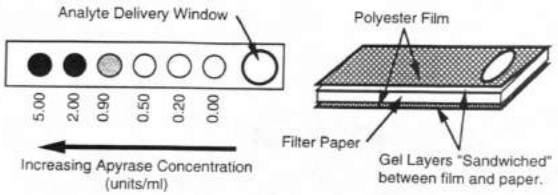


Figure 1. Diagram of increasing apyrase concentration in consecutive sensor dots and schematic of device design.

The analyte (containing the ATP sample to be measured, luciferin, and coenzyme A) is delivered to the analyte delivery window of the direct reading sensor and readout takes place in three minutes. This design takes advantage of both the wicking properties of the filter paper and the capillary action of the gel film sandwich. The test strip geometry allows sample delivery to each gel "dot" within one second of analyte delivery. The time limiting factor of the reaction is the rehydration of the enzyme gel.

The concept is based on the fact that, for a given luciferase/luciferin concentration, a minimum concentration of ATP is required to produce a "measurable" light output. Above that concentration, light is visible; below that ATP concentration, no light is measured. If a uniform concentration of ATP is delivered to a series of sensors (dots), they will either produce a measurable signal or an immeasurable signal. By filtering or consuming the ATP before it reacts with the luciferase we control the intensity of the light at each position along the sensor.

To determine an ATP concentration, a sample containing ATP is introduced to the luciferase/apyrase gel. The ATP is consumed by the two competing enzymes. Because the turnover rate of apyrase is two orders of magnitude higher than that of luciferase, the apyrase quickly moderates the ATP concentration. The amount of ATP which reacts with the luciferase to produce light depends on both the initial ATP concentration and the concentration of apyrase. A high concentration of ATP will produce light even at the higher apyrase concentrations because enough ATP remains after consumption to produce a measurable light output. A low concentration of ATP will produce light only at the lowest apyrase concentrations because at higher apyrase concentrations all of the ATP is consumed before it reacts with the luciferase.

To demonstrate and test the dependence of light output on both of these concentrations, we produced 2-D (two dimensional) gradients of ATP ($1x10^{-5}$ to $1x10^{-8}$ g ATP) versus apyrase concentration (2 units/ml to 0 units/ml). Figure 2 is a positive surface profile of this 2-D gradient, the height is directly related to light intensity. The image was captured with a CCD camera. With high initial concentrations of ATP, a large concentration of apyrase is required to modulate the light intensity. With low initial concentrations of ATP, only small concentrations of apyrase are required to modulate the light intensity. The goal of such modulation is to generate a specific light cutoff point indicating a specific concentration of ATP in the analyte solution.

The measurement of ATP concentration itself is of commercial interest as an indicator of the presence of bacteria or other microorganisms based on the release of intracellular ATP (1,4,6-9). It has applications in the food and dairy industry for rapid measurement of total microbial contamination. Other uses include household use for detection of bacteria and other microbes on eating surfaces, in the bathroom and in drinking water. The advantages of this technology over existing methods include a lower initial investment (no luminometer or spectrophotometer to buy) and simple and immediate bacterial counts. A more complete analysis of commercial potential is contained in the Commercialization Plan at the end of this report.

The other is the time delay required for pre-test amplification methods (centrifuging, filtering or culturing the sample).

Our phase II research project will increase the absolute sensitivity of our direct reading sensor to at least 10^{-12} grams of ATP (4000 average bacteria) per sample. Research efforts will focus on the four primary objectives outlined in Table 1.

Table 1. Outline showing research objectives and responsibilities.

Primary Objectives	Secondary Objectives	University of Utah	Protein Solutions, Inc.
Maximize luminescence	Optimize concentrations	Wang	Stewart
	Increase duration	Wang	Stewart
	Evaluate gel versus solution		Wang/ Scheer
	Evaluate stability	Wang	
	Prepare summary analysis		Scheer
Optimize detection	Decrease sample to sensor distance		Scheer
	Concentrate luminescence into small area		Triolo/ Hlady
	Utilize the optimum film type and exposure		Andrade/ Scheer
	Design for visual reading		Andrade/ Triolo
	Minimize light leakage		Triolo
	Evaluate detection		Scheer
Prototype Design	Optimize shelf life of enzymes	Sohn	Wang
	Optimize shelf life of substrates	Wang/Sohn	Wang/ Triolo
	Evaluate shelf life		Wang/ Triolo
	Minimize Costs		Triolo/ Scheer
	Select final configuration		Triolo
Prototype evaluation	Detect microorganisms		Kricka
	Determine absolute sensitivity		Nelson Labs
	Suggest uses in other markets		Kricka

1. Maximize luminescence.

Our first step in improving the sensitivity is to increase the luminescence for a given amount of ATP. This can be achieved in two ways, light intensity can be increased, or the duration of luminescence can be increased.

Increases in light output will be achieved by adjusting the enzyme/substrate concentrations to their optimum values. Optimization will be evaluated by coupling computer simulation studies with real experiments. Faster production of light will mean an increased intensity. The University of Utah subcontract, under the direction of Russell Stewart, will include these simulation studies. By correlating the experimental data with the simulated results, an optimum concentration of the enzymes will be determined and utilized for all subsequent studies.

A second study will be performed to increase the *duration* of the luminescence using enzyme amplification. Enzyme amplification refers to the process of "recycling" the adenosine monophosphate (AMP), produced by the luciferase catalyzed bioluminescent reaction, to ATP. Again, computer simulations will be used to identify the best enzymes and relative concentrations for maximum enzyme amplification (at least three and up to four enzymes may be involved in simultaneous reactions). Early studies in our lab have demonstrated that enzyme amplification will improve both the duration and the output of luminescence for a given concentration of ATP over that of a system not employing enzyme amplification (12,31-36).

We expect, and it will be shown that, amplification enzymes can be used with our detection system to both increase and prolong the light output from the luminescent reaction. Direct

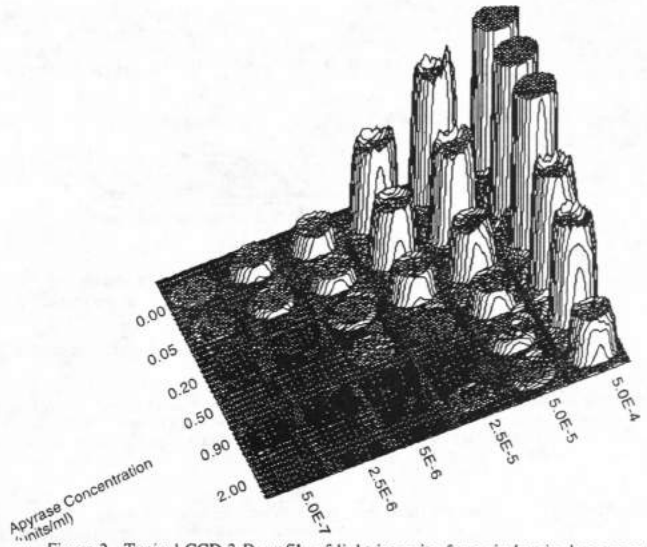


Figure 2. Typical CCD 3-D profile of light intensity for a six by six detector array.

Since submission of the Phase I Final Report, we have successfully extended sensitivity for ATP from 10^{-8} to 10^{-9} grams using both visual and photographic detection. Thus, we are on our way to achieving the sensitivity required for microbial detection.

PHASE II RESEARCH OBJECTIVES

With minimum optimization efforts, our Phase I feasibility studies have shown that we can monitor (using both direct visual and photographic film) ATP amounts as small as 10^{-9} grams, a sensitivity equivalent to the best photographic detectors on the market or referred to in the literature (6,10-13). Detection of smaller amounts of ATP currently requires expensive electronic detection devices such as luminometers or spectrophotometers, which are typically sensitive to 10^{-13} grams of ATP (14-26).

A 1994 review of automated luminometer systems available for measuring bacterial ATP reported sensitivities in the range of 10^3 to 10^4 cells/ml (each cell contains an average of 2.5×10^{-16} g of ATP) (20). However, many of these tests rely on sample "enrichment" (such as concentration or culturing) prior to detection. Other methods of bacterial detection include cell culture readings such as the 3M product using Petrifilm™ which has the ability to detect very low concentrations of bacteria (1 cell/25 ml) and even differentiate between cell types. However, Petrifilm™ requires 18-22 hours of culturing at 42°C (27-29).

A recent EPA summary report outlined a study of bacterial sensing of drinking water (21). The report stated that an acceptable level of sensing capability would be 500 bacteria per milliliter of water. To achieve this sensitivity required a 200-600 fold concentration of the water prior to assay by PMT.

Drawbacks to current methods, then, are two-fold. One is the initial investment required to purchase a luminometer or a spectrophotometer as well as the training required to use them (20,30).

comparison between systems incorporating recycling enzymes and those without recycling enzymes will be made using a CCD camera and a spectrophotometer. Further studies will involve photographic film detection.

These studies will first be conducted in liquid solutions then transferred to gel or preservation studies.

The combined refinements of enzyme concentration and substrate concentration along with development of substrate recycling should result in an increased luminescence of at least one order of magnitude for a given amount of ATP.

2. Optimize detection of luminescence.

To optimize device sensitivity and make the most of the luminescence provided by the ATP/enzyme reaction the generated light must be efficiently and completely detected. To optimize the sensitivity of the device we must, therefore, increase the number and efficiency of photon capture by the detector, be it photographic film or the human eye.

Our group has gained considerable experience over the years in the development of fiber-optic biosensors based on fluorescent technologies. These developments have included application of cylindrical optical fibers and planar waveguides. Dr. Joe Andrade, founder and Chief Scientific Officer of PSI, and Dr. Vladimir Hlady, consultant on this project and a member of PSI's Scientific Advisory Board, have worked closely together for many years on optical sensors for protein detection projects. They will both advise and assist in our optical enhancement and collimating approaches to optimizing device design and photon collection efficiency.

Our goal is to produce enough photons at the ATP sensitivity limit to generate a detectable light emitting event. This can be at least partially accomplished by focusing or directing all the available photons into the smallest possible area and then (in the case of film) integrating for an extended length of time.

One way of improving the efficient collection of luminescence is to minimize photon loss, by decreasing sample to sensor distance, then all of the photons contained within the solid angle of emission will strike the detector. This method of enhancement will also work with direct visual detection, although to a lesser extent because the eye cannot get as close to the sample as the film can.

Another technique that will be used to increase the amount of light that reaches the detection device is to concentrate the luminescence into a small area. A minimization of the display area for a given quantity of sample will project the same number of photons over a smaller area, thereby increasing the exposure per unit area.

The goal is to increase the number of photons that strike a given area of the detector, thereby increasing the intensity. It is expected that an order of magnitude increase in intensity will be achieved by incorporating these device configuration modifications.

Quantification of results will be made by relative light units using the CCD camera, optical density using photographic film, and a 5 member review panel of uninterested individuals for direct visual reading.

3. Prototype Development.

Prototype development will be divided into several studies. These include the preparation and preservation of the reactive components as well as the development of device configuration and mode of operation.

In order to arrive at an acceptable device, the shelf life of all the components needs to be increased to at least 6 months. The stability of luciferase in solution and dry gel has been studied by Wang et al. These studies will be extended to other enzymes and substrates to be included in the device.

We intend to improve enzyme shelf life by immobilizing the enzymes in gels. Chemical and mechanical immobilization methods of the enzymes will be evaluated.

To improve the shelf life of the luciferin, a complete and rapid dehydration process is preferred. We will evaluate two methods to increase luciferin stability: 1) incorporate luciferin in high concentration agarose-luciferase gels (or other suitable medium) and rapidly dehydrate it. Then, maintain the gels in a, moisture-free, oxygen-free, light-free environment until they are needed. Luciferin stability studies will be performed for periods of at least one year.

2) Encapsulate the devices in packets, protecting them from light, oxygen, and moisture until use. The packet is simply opened when required, and the device introduced to the analyte solution of interest. A vacuum seal package will also be studied. These methods of preservation will be

evaluated and compared with dehydration. Final optimization of luciferin shelf life may require a combination of these methods.

The sensing device itself is composed of several basic elements:

- A mechanism for introducing a sample to the device,
- a mechanism for ensuring that the sample is distributed to all reactants of the device,
- a means of optimizing conditions so that the analyte reacts efficiently and completely with the reagents per design,
- a mechanism for relating the amount of ATP in the sample to a measurable light signal employing firefly luciferase and luciferin,
- optical components that focus and guide the emitted light to the detector, and
- the detector itself, which is either the human eye or photographic film

Evaluation and refinement of these elements will continue during this Phase II research project.

4. Prototype Evaluation.

At least once a year, both Dr. Larry Kricka and Dr. Vladimir Hlady will visit our labs to evaluate of project progress and prototype designs. It is likely that our corporate partner will also visit our lab at least twice a year to discuss progress made toward reaching our milestones and goals.

PHASE II RESEARCH PLAN

Our research effort focuses on increasing the device sensitivity and convenience. It is based on the four objectives outlined below.

1. Maximize luminescence.

Our phase I research project established the feasibility of determining the amount of ATP in a given sample based on the spatial distribution of luminescence. We did not, however, maximize the device sensitivity for ATP. Our first step in improving sensitivity will be to increase the luminescence for a given amount of ATP. There are two ways to achieve this goal. One is to increase the number of photons emitted per unit time (increase intensity). Another is to increase the duration of photon production. Both will increase the exposure of photographic film. Because of the eye's short integration capability (0.1 seconds) only the increase in intensity will improve direct visual reading capability.

These optimizations will be achieved by optimizing the concentrations of all reactants, we will couple computer simulation studies with real experiments to arrive at optimum conditions for light emission. First, enzyme kinetic simulations will be performed. These simulations use data from enzyme turnover rates and enzyme/ substrate concentrations to calculate the quantities of reaction byproducts, which in our case is light. Faster production of light translates as an increased intensity. The University of Utah subcontract to Dr. Russell Stewart, includes funding for these simulation studies.

Once the simulation studies are underway and preliminary results obtained, we will begin actual experiments using the optimized concentrations indicated by the simulations. Experiments will be initiated to maximize the luminescence from the luciferase/luciferin/ATP reaction. An optimum concentration of the enzymes will be determined and utilized for all subsequent studies.

Several additives are reported to enhance luminescent intensity and duration (37,38). Neutral surfactants, such as Triton X-100 and Tween 20 are reported to increase luminescence by several fold. Some polymers, such as PVP and PEG have also been shown to enhance bioluminescence. Some combination of these additives should provide for optimum enhancement. We expect to begin studies with these additives once the required enzyme and substrate concentrations are optimized.

A second study, to increase the *duration* of the luminescence using enzyme amplification and allow longer exposure times, will follow the effort to maximize of luminescent intensity. Enzyme amplification refers to the process of "recycling" the adenosine monophosphate (AMP) produced from the luciferase catalyzed bioluminescent reaction back to ATP through appropriate phosphorylation reactions. Again, computer simulations will be required to identify the best enzymes and relative concentrations for maximum enzyme amplification (at least three, and up to four

luminescent intensity and/or duration are established, photographic film detection will be used for comparison.

The combined refinements of enzyme concentration, substrate concentration, additive concentration and substrate recycling should result in an increased luminescence of at least one order of magnitude for a given amount of ATP.

Another benefit of using the enzyme amplification systems described above is that they can utilize all of the adenosine compounds available from the ruptured bacteria cell. Bacteria contain ATP, ADP and AMP in various ratios. At different times in the reproductive cycle, the relative amounts of these compounds will vary. Stress can also severely alter the ratio, rapidly depleting the ATP stores. A system which senses total adenosine content (not only ATP but also ADP and AMP) will be better suited to detect bacteria than a sensor which only measures ATP. Not only because there will be more adenosine to detect but also because the total amount of adenosine per bacteria is a more accurate measure of cell number than total ATP content. (39).

These studies will be computer simulated and conducted in solution. A summary study of this optimization will incorporate a system for storage of the sensor mixture. This preservation system may incorporate lyophilized enzymes/substrates or dehydrated gels (see section on prototype development).

Close coordination of these luminescence optimization efforts is essential and will involve continual communication and comparison between research personnel. It will be necessary to monitor the compatibility of enzymes on a weekly basis to insure that each modification contributes to the overall improvement of the sensor's sensitivity.

Quantification of results will be made by relative light units using the CCD camera, optical density using photographic film, and a 5 member review panel of uninterested individuals for direct visual reading.

2. Optimize detection of luminescence.

To optimize device sensitivity and make the most of the luminescence provided by the ATP/enzyme reaction the generated light must be efficiently and completely detected. To optimize the sensitivity of the device we must, therefore, increase the number of photons and efficiency of photon capture by the detector, be it photographic film or the human eye.

It is important to note that the human eye can detect high contrast features only a millimeter or two apart. The detector should, then, focus and direct all of the available photons from each analysis into a relatively small detection area, whether that detector is the human eye or photographic film. In the case of photographic film, the exposure needs to be above the photon sensitivity threshold for that film. This exposure is more readily attained by focusing all of the photons on a small area than on a large area. The same is true for visual detection, although the integration time for visual detection is only about 0.1 seconds.

Our problem is to produce enough photons at the low levels of ATP that we wish to detect to insure that the film is exposed. This is partially accomplished by focusing or directing all the available photons into the smallest possible area and then integrating for an extended length of time.

One way of improving the efficient use of luminescence is to maximize the angle of collection (minimize photon loss). The closer the detector is to the light source, the greater the solid angle. See figure 4.

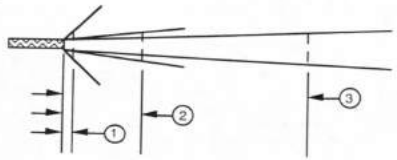


Figure 4. The effect of distance on solid angle. At distance 3 the angle is smallest, at distance 2 the angle is larger, at distance 1 the angle is nearing 180°. At the farthest distance very few of the available photons are collected. At the closest distance most of the photons are collected.

enzymes may be involved in simultaneous reactions). Preliminary studies in our lab have demonstrated that enzyme amplification will improve both the duration and the output of luminescence for a given concentration of ATP. (Also see reference 32)

The firefly luciferase catalyzed bioluminescence system utilizes one ATP and generates one AMP (see equation 1). Adenylate kinases which convert AMP to ADP can be coupled with pyruvate kinase to recycle AMP back to ATP (equations 2 and 3).

Three types of adenylate kinases are reported (33). Types I and II are myokinases, which convert AMP to ADP (adenosine diphosphate) by consuming one ATP. The two ADP molecules produced in this reaction are converted by pyruvate kinase to ATP by consuming phospho(enol)pyruvate (PEP). Type III is also known as GTP-AMP phosphotransferase (EC 2.7.4.10) which consumes GTP (guanosine triphosphate) instead of ATP in the recycling process (equation 4).

$$\text{ATP} + \text{luciferin} \xrightarrow{\text{luciferase}} \text{AMP} + \text{oxyluciferin} \quad (1)$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2\text{ADP} \quad (2)$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate} \quad (3)$$

$$\text{AMP} + \text{GTP} \xrightarrow{\text{GTP-AMP phosphotransferase}} \text{ADP} + \text{GDP} \quad (4)$$

Hawronskyj et al. used the amplification system of myokinase and pyruvate kinase to enhance their ATP detection limit (32). They suggest that the detection limit of ATP can be improved 2000 fold. In our study of appropriate enzyme amplification systems we will look to utilize the type III adenylate kinase as the recycling enzyme. This system avoids the consumption of ATP, a substrate of luciferase, which could diminish the optimum luminescence. The GTP utilized in reaction (4) is *not* a substrate of luciferase and, therefore, will not decrease the luminescent reaction. (See figure 3.)

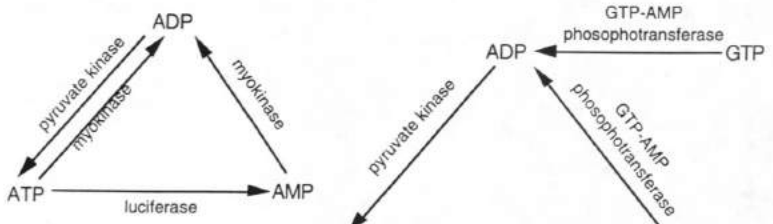


Figure 3. Energy transfer cycle of luciferase and various amplification enzymes.

We expect, and it will be shown that, amplification enzymes can be used with our detection system to both increase and prolong the light output from the luminescent reaction. Direct comparison between systems incorporating recycling enzymes and those without recycling enzymes will be monitored by a CCD camera and a spectrophotometer (PMT). Once an increase in

During the Phase I research project, photographs were taken at a distance of 15 centimeters from the luminescent source. For a 1.0 centimeter diameter exposure area, this distance translates to a collection angle of 4 degrees or a loss of 99.9% of the photons produced. We estimate that by reducing the sample to detector distance to 0.5 centimeters we can increase the collection angle to nearly 90°, thereby reducing the loss by 99%, resulting in a 100-fold increase in the number (concentration) of photons striking the film per unit time.

It can be seen from figure 4 that by decreasing sample to sensor distance the detector will utilize more of the available photons. This method of enhancement will also work with direct visual detection, although not to the same extent.

To evaluate these detection systems we will design and build a light-tight film holder with a mechanism for adjusting the detector to sample distance from 0 to 15 centimeters. Using a constant luminescence standard in the range of 10^4–10^8 photons/sec•ster-radian (available from Biolink Technology), the sample distance will be adjusted to provide the greatest film exposure while still providing adequate resolution of individual spots. Effectiveness of decreasing the sample to detector distance will also be quantified using the CCD camera and direct visual detection with the human eye.

Another technique used to increase exposure to the detection device is to concentrate the luminescence into a small area. Two methods are explored:

1) Minimize the "display" area with respect to analyte volume, and
1) collimate or "focus" the luminescence to a small point (1-2mm diameter).

If we disregard scattering and absorption in the liquid for the moment, a minimization of the display area for a given quantity of sample will project the same number of photons over a smaller area, thereby increasing the exposure per unit area. See figure 5.

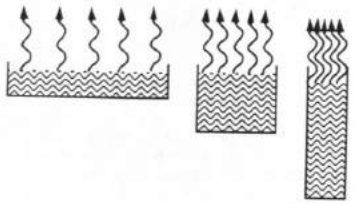


Figure 5. Once the maximum luminescence is achieved for a given sample, the efficient detection of those photons is important to further increasing sensitivity. For a given number of photons (represented as wavy lines) a smaller area of projection will increase both intensity and detectability.

To explore the benefits of decreasing surface area to sample size, we will examine several sampling geometries (some of which were explored during Phase I research for their wicking/capillarity properties) including microtitration plates, capillary sampling tubes, wicking fibers, "dipstick" sampling plates, and test tubes. By maintaining a constant sample volume and reagent concentration the overall light emission should not change. However, by changing the geometry of the sampling device, the *intensity* of the light emission will change. During our evaluation of different geometries we will incrementally adjust the surface (projection) area for a constant volume and concentration of sample, until a maximum intensity is found. Evaluation will be made with a CCD camera. The goal is to increase the number of photons that strike a given area of the detector, thereby increasing the exposure/intensity. Here we must keep in mind that some of the light will be lost (scattered and/or absorbed) as it travels through the sample volume, and a longer travel distance will mean more loss.

Another way to make the best use of available light is to *focus* or *channel* all of the generated light into one direction and small area. (See figure 6.)

Another method of capturing light is to use a system of total internal reflection. To do this requires a transparent medium with a higher index of refraction than the medium with which it shares an interface. Such is the case with an enzyme support such as a glass tube and air. (See figure 7)

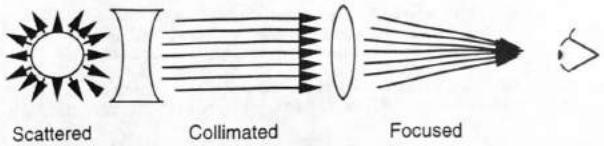


Figure 6. Light generated at the source is diffused in 360°. A series of focusing lenses can capture much of the diffused light and project it toward the detector.

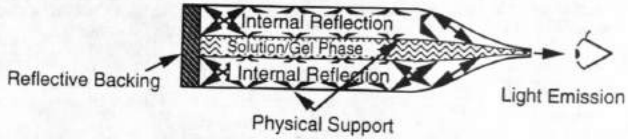


Figure 7. Light is generated within the solution/gel phase and transmitted to the physical support (glass capillary or polymer film) which then "collects" the light and transmits it to the detector.

We will examine both focusing and channeling of the emitted light using lenses and appropriate "sandwiching" materials (films and capillaries). Effectiveness of the designs will be determined by sensing the intensity of emitted light with a CCD camera. Follow-up evaluations will utilize photographic film and direct visual detection. It is expected that an order of magnitude increase in exposure will be achieved by incorporating these device configuration modifications.

Once the maximum amount of light is directed to the smallest reasonable area (1-2 mm diameter), an optimization of photographic film type and exposure will be necessary. Photographic film is generally accepted to be the most inexpensive, and among the most sensitive integrating means of sensing photons. The activation of a silver grain with a photon to produce a latent image (which is then developed) results in a billion fold magnification of transformed silver atoms. This is accomplished in a thin film format and, in the case of the Polaroid films, one in which the results are obtained almost instantly.

We are already collecting data on film exposure characteristics. We will be looking for films with a very steep exposure density curve or "threshold exposure level". This will provide the "on-off" signal recognition necessary to differentiate between two different amounts of ATP (i.e. the difference between 10^{-10} and $5x10^{-11}$ grams of ATP). Polaroid's 612 type film (20,000 ASA) is reported to have favorable exposure characteristics (11,40). See figure 8. It is also used in several commercial ATP luminescent detectors.

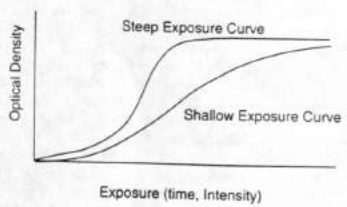


Figure 8. For the steep exposure curve, if the exposure falls below the minimum required for film development, then a no reading is recorded; if the exposure falls above the minimum then a positive reading is recorded. For the shallow exposure curve, shades of gray will be recorded, making positive determination of exposure difficult.

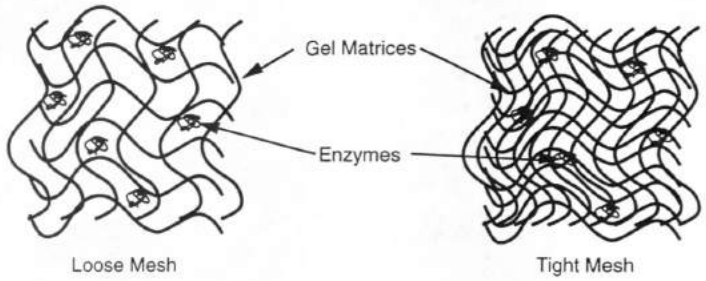


Figure 9. A tight mesh gel will physically hinder enzyme migration out of the preservation medium, therefore, physically immobilizing the enzymes.

Table 2. Comparison of commercial gel systems for entrapment of low molecular weight proteins.

Properties/Gel	NuSieve GTG (47)	SeaPlaque (47)	LM2,3 (48)	TreviGel 500 (49)	TreviGel 5000 (49)
Gelling Temp.	≤35°C (4%)	26-30°C (1.5%)	24-28°C (1.5%)	≤36°C	–
Gel Strength	>500 gr/cm^2	>200 gr/cm^2	>400 gr/cm^2	–	–
High concentration	6%	2.5%	–	3%	2%
Sizes able to pass through gel	8-50 bp	50-2000 bp	–	4-400 bp	100-2000 bp

Shelf life of the luciferin is also a concern. D-Luciferin is a substrate of firefly luciferase. Three degradation products of D-luciferin, dehydroluciferin, oxyluciferin, and L-luciferin, are competitive inhibitors of luciferase. D-Luciferin forms dehydroluciferin in the presence of ultraviolet light and/or moisture. For this reason it is usually stored desiccated, in the dark at -20°C. Also, in alkaline solution a slow racemization of the D-isomer to the L-isomer occurs. An acidic solution (pH 6.0-6.3) can help minimize the racemization. However, the luminescent reaction requires a pH of 7.8 for optimum performance, so that storage of luciferin in an acid is an unlikely solution. The degradation of luciferin to oxyluciferin without the catalyzation of luciferase is not reported (probably because of the high energy barrier), so it seems that the role of oxygen in D-luciferin degradation is less important (13).

To improve the shelf life of the luciferin, a rapid and complete method of dehydration is used. The air-drying process investigated in the Phase I research project was too slow, resulting in both an extended exposure to water and incomplete drying. A vacuum or freeze drying step (in the absence of UV light) is recommended and will be evaluated for storage periods ranging up to at least one year.

It is useful to note that a variety of luciferin esters have been synthesized, making the luciferin more membrane-permeable. The esters are hydrolyzed to active luciferin intra-cellularly by indigenous cell enzymes. Other luciferin derivatives include luciferin galactocide in which the galactose moiety is removed by galactosidase action, resulting in active luciferin (50). All of these chemistries suggest that luciferin is actually quite robust and reasonably stable, *if* it is protected from light, water and oxygen.

We will improve luciferin shelf life in two ways. We will incorporate it in the high concentration agarose-luciferase gel, (or other suitable medium), rapidly dehydrate it, and maintain it in a, moisture-free, oxygen-free, light-free environment. Prepared and stored in this fashion, we expect that luciferin will be stable until reconstituted with the analyte solution. Luciferin stability studies will be performed for periods of at least one year.

In contrast with the integrating capabilities of photographic film, the human eye only samples photons for about 0.1 seconds before it proceeds to process the image. The human eye is a photometric device ideally suited for detection of motion, rather than for ultra-low light level static imaging. We know that low light level detection can be enhanced by up to five orders of magnitude through suitable dark adaptation. We will assess the ability of the naked eye to detect light emitted by the device after different periods of adaptation to optimize the conditions for direct visual interpretation of sensor results.

Close coordination of these detection optimization efforts is essential and will involve continual communication and comparison between research personnel. It will be necessary to monitor the compatibility of device modifications on a weekly basis to insure that each component of the enhanced detection capability contributes to the improvement of the sensor's sensitivity.

Quantification of results will be made by relative light units using the CCD camera, optical density using photographic film, and a 5 member review panel of uninterested individuals for direct visual reading. All readings will be standardized using Biolink's luminescent standard.

3. Prototype Development.

Prototype development will be divided into several studies. These include the preparation and preservation of the reaction components as well as the development of device configuration and method of operation.

Shelf life of the enzymes must be increased to at least 6 months. Stability of luciferase in solution and dry gel has been studied by Wang et al. (41-43). For the enzyme amplification system two additional enzymes, most likely an adenylate kinase and pyruvate kinase, will be used. All enzymes will likely be stabilized in gels.

The gels should be uncharged and hydrophilic to increase compatibility with the enzymes. Preparation should involve a low temperature technique to avoid protein denaturation. The medium should be low in viscosity during preparation to ease transfer. Finally, the support should have high optical clarity and no air bubbles to minimize light scattering. The preparation will likely involve dehydration, so the medium must be easy to dehydrate and rehydrate and should prevent leakage of the enzyme into any excess rehydrating fluid.

One area of study, therefore, will be to improve enzyme shelf life by immobilizing the necessary proteins. This may be accomplished either by chemical or mechanical immobilization of the enzymes in the support medium.

Our lab has experience immobilizing enzymes using the 3M Emphaze activated biosupport medium. Emphaze is a preactivated bead composed of a bis-acrylamide/azlactone copolymer. In a preliminary study we attempted immobilization using avidin as a bridge between luciferase and the beads. This study demonstrated the feasibility of immobilizing biotinylated luciferase on the avidin coupled Emphaze beads with a high degree of retained activity (44). Studies of this and other immobilization techniques will continue during Phase II research.

Another method to avoid leakage of the enzymes from the storage medium is to trap them within a tight and stiff matrix (physical immobilization). Dr. Sohn, a visiting professor from Korea, will cooperate with the University subcontract to head this portion of the study (his salary is paid through Hallym University, Korea). To develop a "tighter" gel structure, a high concentration gel (probably near 6% w/v) is necessary. (During Phase I studies we utilized 1% w/v gels.) This will benefit gel storage capability through reduced porosity, minimized gel shrinkage, and enhanced gel strength. (See figure 9 and Table 2.) Difficulties may arise from the inherent increase in gelling temperature, lower transparency, higher viscosity, and increased aeration, which accompany increases in gel weight percent. Most of these factors can be adjusted by utilizing special "low temperature gelling point" agarose and gels. Several companies produce such systems, see Table 2. Trehalose additives have also been shown to increase gel clarity (41-43).

Gel materials other than polysaccharides (agarose) will also be evaluated. Gels made from water-soluble synthetic polymers such as polyacrylamide and polyvinylalcohol have been shown to immobilize enzymes in the hydrated state (45,46). To fit our requirements, which need dehydrating and rehydrating gels, we can alter and optimize the conditions of preparation. As for the final support matrix material, every possibility is available. It is possible that a blend of different matrices will be needed for a given enzyme, or that different enzymes may be immobilized in different gels.

Second, a variety of technologies are available to encapsulate the device in packets, protecting it from light, oxygen, and moisture until it is ready to be used. The packet is simply opened when required and the device introduced to the analyte solution of interest. A vacuum seal package will also be studied. These will be studied in a manner similar to that for the dehydrated luciferin. In fact, the final optimization of the luciferin shelf life study will likely be a combination of these methods.

The sensing device itself is composed of several basic elements:

- A mechanism for introducing a sample to the device,
- a mechanism for ensuring that the sample is distributed to all reactants of the device,
- a means of optimizing conditions so that the analyte reacts efficiently and completely with the reagents per design,
- a mechanism for relating the amount of ATP in the sample to a measurable light signal employing firefly luciferase and luciferin,
- optical components that focus and guide the emitted light to the detector, and
- the detector itself, which is either the human eye or photographic film

These elements are discussed individually below. Several configurations of the individual elements are then presented, any one of which may display advantages during evaluation that permit its development into a commercially acceptable product.

A critical element of the entire sensing process is the collection of the sample, elimination of extraneous ATP from non-bacterial (usually referred to as "somatic") sources, and extraction of ATP from the bacteria. Various methods have been employed to yield a relatively pure sample of ATP representative of the cellular contents of the original microbe population for analysis (13,22-26,51).

PSI's intent is not to develop an entirely new sample collection mechanism, but to adapt our sensor for use with existing sampling vehicles.

One product that is particularly adaptable to our sensing technology is 3M's Petrifilm™. This product already employs a gel in the sample collection film. It may be possible to build our gel layers containing luciferase, trehalose, and apyrase, along with appropriate cell lysing agents, into the current gel, or laminate our gels as thin films on top of those existing in Petrifilm™. (See Figure 10.) If needed, a physical barrier could be employed to separate the layers which is breached by the user during the analytical procedure. In order to couple our sensor device with Petrifilm™, a vertical lamellar configuration should be constructed and evaluated.

The remainder of the products we reviewed use luminometers to detect light emission from the ATP-sensitive firefly bioluminescent reaction. Samples are introduced as aqueous solutions. This form of sample is ideally suited for detection by our device. The reagent layers can be stacked vertical films, or consist of a series of layers that are adjacent to one another on a binding/wicking substrate that provides product integrity and assists in the delivery of the sample to the different levels of interactive gels. Eventually, the sample preparation elements can be combined with the sample *processing* (elimination of somatic ATP and extraction of bacterial ATP), *transduction* (utilization of ATP to produce light), *guiding* (optical focusing elements) and *detection* (film) elements of the device.

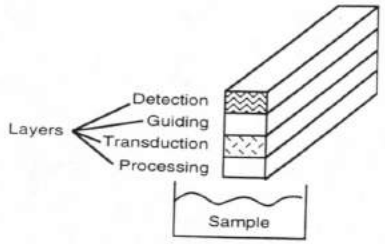


Figure 10. Possible layers of bacterial detection device.

In this development project we propose a system to accept aqueous samples. This will permit us to move directly from the analysis of control ATP solutions to solutions extracted from microbes by conventional commercial preparations. Total incorporation of our transduction/ detection system into an existing commercial sampling system will be accomplished in phase III using funds from interested commercial parties.

Functional Elements to employ in a prototype device:

Sample extraction, elimination of extraneous ATP

Existing techniques and commercial sample swabs will be used to obtain an aqueous sample of ATP from microbes (52).

Sample Introduction

The aqueous sample preparation will be added to the preserved reactants.

Luciferin Introduction

Luciferin is currently the most unstable element incorporated into the device, and it may, therefore, be necessary to prepare a fresh luciferin solution on a weekly basis and store it shielded from light in the refrigerator between uses. An alternative to be investigated will be to package the luciferin so that it is not exposed to those environmental conditions (light, heat, oxygen, moisture) that render it a luciferase inhibitor. Packaging options were previously discussed.

Sample Transport and Distribution

These have been addressed in Phase I studies. The designs that produced the best results are shown in figure 11.

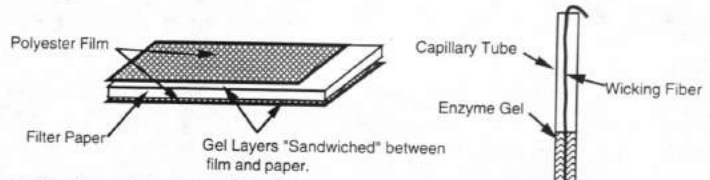


Figure 11. Two possible device designs, filter paper sandwich and fiber in tube.

The benefit of these designs is that they provide a suitable physical support for the protein gel and a simple mechanism for rapidly delivering sample to the active device layers. Analyte is delivered to the filter paper sandwich at the "open" end of the support, and to the cotton fiber tube at the lower end, near the gel. Delivery consisted of placing the respective delivery ends of the devices in a shallow tray of the analyte and allowing delivery by capillary and wicking action.

A Means Of Optimizing Conditions So That The Analyte Reacts Efficiently And Completely With The Reagents Per Design

Optimization of reaction conditions and reactant concentrations were explored in the section on maximizing luminescence. The optimal concentrations will be incorporated in the support matrix (currently a trehalose/agarose gel), which will be distributed on preferred surfaces of the device as required for the different configurations.

A Mechanism For Optimally Converting The Concentration Of Analyte To A Light Signal Employing Firefly Luciferase And Luciferin

The objective of the early phases of this project is to increase light production by optimizing the reactant concentrations and conditions, and to employ enzyme systems to recycle ADP and AMP to ATP so that light emission is prolonged. Once the enzymes are selected, attempts will be made to preserve them in trehalose gels.

The recycler enzymes will be incorporated into the support matrix containing the luciferase and apyrase (and possibly luciferin), as the AMP and ADP will be in high concentrations in this layer. Studies will be undertaken to determine the compatibility of all of these enzymes in one layer. If

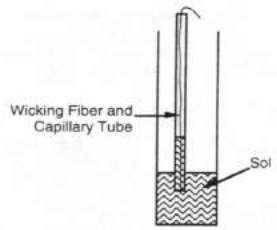


Figure 13. Construction of fiber in capillary tube configuration.

4. Prototype Evaluation.

At least once a year, both Dr. Larry Kricka and Dr. Vladimir Hlady (consultants) will visit our labs for evaluation of project goals and prototype designs. It is likely that our corporate partner will also visit our lab at least twice a year to discuss progress and goals.

Evaluation will occur monthly to insure compliance with expected goals. The first three semi-annual reviews will include reports of absolute sensitivity to ATP. We expect to reach a detection sensitivity of 10^{-12} grams of ATP within the first 18 months of this Phase II research project. It is likely, particularly with photographic integration of luminescence, that a greater sensitivity can be achieved.

The device designs will be thoroughly evaluated by the end of the third report period. Evaluations will include a measure of the reproducibility of results achieved with developed prototypes, and a measure of achieved sensitivity. Major market areas will be identified for targeting and licensing of the product at the conclusion of the Phase II research project.

At this stage, the expertise of our consulting crew as well as our corporate partner, will be most useful.

By periodically checking that certain goals are met (such as sensitivity and reproducibility) we will insure that all goals are met and that development proceeds in an orderly, controlled fashion.

Conclusion

Reports in the *Journal of Food Protection* and other biosensor type journals have pointed to the need for a rapid, simple, sensitive, and inexpensive bacterial screening device (15-21,28,29,33,43-55). Such a device would find use in the restaurant, the home, regulatory agencies, food packaging industries, drink packaging industries, pharmaceutical industry, and anywhere bacteria are not wanted (56).

We will develop an ATP measurement device whose primary use will be to quantify bacterial contamination in sensitive environments. Our real-time sensor technology does not require preconditioning of the sample (i.e. concentration or culturing), but will match the bacterial sensitivity currently achievable using 24 hour culture systems or sophisticated and expensive luminometer systems. Use of high speed film as a detector will both increase the sensitivity of the assay and yield a hard copy record of the monitoring process.

Therefore, our system will save both time and money for industries that monitor their work areas for bacterial contamination.

COMMERCIAL POTENTIAL

PSI's Interests and Business

Protein Solutions, Inc., was founded in 1988 in Salt Lake City by Drs. Peter Gerity, James McRea, and Joe Andrade. The company's original objective was to develop science education tools for school and home use.

Government and private funding was sought for three distinct product lines; personal sensors; educational kits employing bioluminescent organisms; and Labless Labs®, a collection of specially selected and engineered materials to accompany telecourses and other courses where hands-on

incompatibilities exist, it may be possible to form a multilaminate gel, consisting of a series of very thin gels of varying composition. Another possibility is to fabricate a gel which is an interpenetrating polymer network, each constituent containing different enzymes.

Elements That Focus And Guide The Emitted Light To The Detector

These elements have been discussed above. Inexpensive, disposable plastic lenses are available as focusing elements, but their expense may still preclude use in the final device. Lenses will still be incorporated in the prototypes, however, if they have been proven to significantly increase the sensitivity.

Waveguides, on the other hand, can be manufactured quite inexpensively, especially in micromachined devices. Micromachining will not be incorporated unless other prototype configurations are proven inadequate. Waveguiding materials do not have to be micromachined, however. Waveguiding occurs when light encounters two media of greatly different optical densities. If all light can be trapped in the waveguide and directed at the sensor, then the intensity of light incident on the detector can be amplified, and sensitivity increased.

Waveguiding can occur in either the gel-in-capillary configuration, or the polyester sandwich configuration by choosing appropriate materials for the capillary and external membrane layer of the sandwich, respectively.

The Detector Itself, Which Is Either The Human Eye Or Photographic Film

Optimization of the detector is discussed above. For photographic detection, a contact print will most probably be used, or the film will be located at the focal point of a focusing element.

General Characteristics of the Fabrication Procedure

Each gel layer can be thought of as a self-contained module. By stacking the individual modules in the proper order, one can create a device with appropriate characteristics for assay of the analyte in question.

Methods of Fabrication

The filter paper sandwich configuration is made by depositing two rows of enzyme gel dots with their respective concentrations of apyrase on the film support. Once the gels are dehydrated, they are pressed into the filter paper, thus adhering to both the film and the filter paper. The wicking capillary tubes are made by first threading a cotton fiber through the capillary tube and securing one end to avoid pulling the fiber out. The other end of the thread is trimmed to ease delivery of the sol. The gel is delivered into the capillary tube by simply dipping the trimmed end into the enzyme sol and allowing capillarity and wicking to pull the sol into the tube. See figures 12 and 13.

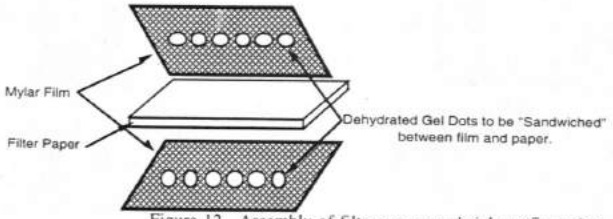


Figure 12. Assembly of filter paper sandwich configuration.

laboratory experience is lacking. The common denominator to these ambitious projects is that they all centered on the development of experience-based learning tools.

Recently, the company has focused its research and development efforts on the development of bioluminescent sensors, as these have the greatest immediate potential for commercialization.

Potential Applications of Anticipated Results

The sensors employ a novel configuration that utilizes firefly luciferase and luciferin to sense ATP in nanogram quantities. This type of assay is the most sensitive known for ATP, and the ultimate goal of PSI's sensor development is to produce an assay that enables direct *visual* determination of the picogram quantities of ATP present in a sample. This is accomplished by spatially distributing the light emitted by the assay so that its position is indicative of the amount of light present in a sample. Phase I research, funded by the NSF, demonstrated feasibility of the concept, and PSI is seeking funding (this proposal) to proceed with increasing the sensitivity of the assay so that it can be used for assaying microbial contamination.

Markets for Microbial Assays

Currently, microbial assays are primarily employed in the food, beverage, and water processing industries to test sanitation levels. Additional markets include sensitive areas that must be kept relatively microbe free, including pharmaceutical and medical device cleanroom areas, hospitals, and other biological laboratories. We even anticipate a military need for the sensors in areas where the use of biological warfare agents is suspected.

Commercially Relevant Innovations

Through application of this technology, there exists the possibility of employing extremely sensitive bioluminescent assays without an expensive luminometer. The technology can be optimized and incorporated into a small dipstick-type device to rapidly detect microbial contamination on surfaces, or it could be incorporated into a collection device as a rapid assay for airborne microbial contamination, or it could be combined with appropriate up front enzymes, to selectively determine the quantities of selected analytes.

Under a PSI funded subcontract, Dr. Russell Stewart's lab developed a method for producing recombinant luciferase. This has reduced our cost for luciferase by a factor of 100, and enabled us to accelerate our investigations with this enzyme, as well as made many of our technologies much more commercially attractive from a financial perspective. PSI is again funding a subcontract with Dr. Russell Stewart's lab to develop and characterize the myokinase and pyruvate kinase required for substrate recycling.

To our knowledge, there are no technologies currently available that allow for the sensitive detection of ATP by non-instrumented means. The possibility exists for moving sensing assays that normally require sophisticated laboratory equipment and trained personnel in the laboratory environment to the consumer market. An analogous shift was that of pregnancy tests, which were once solely performed in hospital and clinical laboratories, and have moved into the consumer market as a result of advances in technology.

Patent Status

PSI has filed a preliminary patent application for the technology. The patent has been disclosed through the University of Utah, with whom Protein Solutions has a technology transfer agreement. Through this agreement, PSI has rights to all technologies developed at the University with PSI funding in exchange for a royalty payment to the University for any technologies that are developed commercially.

Competition

Existing rapid techniques for microbial detection all necessitate the use of a luminometer, the least expensive of which costs $3500. Although portable, the luminometers can be bulky and are not easily carried about the facility. Additionally, they require periodic calibration and maintenance. A brief summary of competitive products follows in table 3.

In addition to the luminometer, existing products include a special sampling accessory. These assemblies range from simple ATP-free cotton swabs to fairly complex configurations including the sampling swab, a solution to release ATP, and a means of delivering the ATP to the

reaction portion of the apparatus. The cost of the collection ancillaries ranges from $1.78 to $3.20, and one swab is required for each sample collected.

Table 3. Description of commerial availability of bacterial detection systems.

Company	Luminometer list price ($)	Sample Swabs list price ($)	Comments
Celsis	7500 -35,000	1.78	
Lumac	N/A	N/A	
Bio-Orbit	5700	3.20	
Promega	N/A	N/A	
Charm Sciences	3000	2.30	Not yet available
Analytical Luminescent Labs	10,000	1.26	
Idexx Labs	3500	2.60	
Lightning Systems	N/A	N/A	

Less rapid microbial assays, although more sensitive than these rapid tests, require culturing times up to 72 hours before results can be determined.

Competitive Advantages

The advantages of PSI's product are that it will:

1) obviate the need for an expensive and cumbersome luminometer,
2) permit direct, real-time assessment of microbial contamination and rapid detection of samples that otherwise require plating, and
3) provide a hard copy record (photograph) of the cleanliness of areas for documentation purposes to help meet government and internal requirements.
4) The technique can be expanded to a wide range of analytes.
5) Once the marketplace becomes familiar with the use of the initial product, the concept can be easily developed into a family of products to address sensing needs in parallel markets.
6) The device will be small and disposable.
7) The cost of the device will be comparable to existing sampling ancillaries.
8) It will be simple to use.

Production Plan

The production plan at this stage in the product development cycle is necessarily vague. Our goal of this proposed Phase II research project is to demonstrate that a device can be configured with the appropriate concentrations of necessary analytes and enzymes to accurately indicate concentrations of ATP down to at least 10^{-12} grams, permitting the detection of microbial contamination in real-time either visually or with photographic film. We will have a prototype that demonstrates this feasibility by the end of the Phase II research period.

Subsequent development will be undertaken with the assistance of our corporate partner. At this time it is fairly certain that, if feasibility is shown, 3M will help develop the appropriate final package for the device, quite possibly incorporating it into its currently marketed Petrifilm™ product line. In fact, 3M was approached as a potential corporate sponsor, in part, because of the good match between their existing, and our innovative, technologies.

Regardless of the identity of the corporate partner, development of a final pre-production prototype will take 1 to 2 years. PSI plans to remain actively involved in the research and development activities that support product development and manufacturing, but has not made a final decision on whether to manufacture the items internally or have a corporate partner manufacture the final product, although the latter is more likely.

PSI intends to retain ownership of the technology and license it to companies that are established leaders in distinct market segments. In addition, we will continue to contact corporations that may have an interest in supporting development of the products for their applications. In all cases we are seeking funding of in-house development through the pre-production prototype phase, with final production decisions to be based on the capabilities we have at that time and the needs of our corporate partners for their licensed applications.

Phil Triolo
350 West 800 North, Suite 218
Salt Lake City, Utah 84103
(801) 596-2675

EXPERIENCE:

1994-current Protein Solutions, Inc., **President.** Direct research in the application of bioluminescence for sensing applications in the health care industry.

1983-1994 (interrupted) Independent Contractor to several local medical device companies. Projects have included the design, evaluation, and development of cardiovascular and heparin-releasing catheters, angioplasty devices, nerve and tracheal prostheses, an implantable catheter for the delivery of insulin, evacuated polymeric test tubes for blood collection, and a heparin sorbent system. Also wrote major portions of successful SBIR grant applications and business plans.

1992-1993 Research Medical, Inc., SLC. **Sr. Product Development Engineer.** Responsible for evaluation and modification of sorbent system for the removal of heparin at the conclusion of bypass surgery.

1990-1991 Merit Medical Systems, SLC. **Director of Engineering.** Supervised four staff responsible for implementing new product introductions and product improvements of high pressure syringes and tubing for angioplasty product line.

1980-1983 Abbot Critical Care Systems, SLC. **Manufacturing and Product Design Engineer** (1980-'81). Responsible for cost reductions and product improvements on $4 MM annual hemodialysis product line.

EDUCATION:

1988 Ph.D., Bioengineering, University of Utah. Dissertation, "The Controlled Release of Macromolecules from Biodegradable Poly(lactide) Matrices," completed under the direction of Prof. S.W. Kim.

1980 M.S., Bioengineering, University of Utah. Completed thesis, "Surface Modification and Evaluation of Catheter Materials," under the direction of Prof. J.D. Andrade.

1976 B.S., Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY. Minors in Psychology and Philosophy.

APPOINTMENTS & HONORS:

University of Utah. University of Utah Research Fellow (1978-'79). Chairperson, Bioengineering Student Advisory Committee and student chapter of Biomedical Engineering Society (1978-'79).

Rensselaer Polytechnic Institute. Graduate *cum laude*. Dean's List, all semester. RPI Alumni Scholarship (1972-'76). President, Rushing Chairman, Theta Chi Fraternity. Member, Tau Beta Pi.

AFFILIATIONS:

Adjunct Instructor, Dept. of Bioengineering, University of Utah. Member, Biomedical Engineering Society, Intermountain Biomedical Association, Center for Biopolymers at Interfaces

PATENTS:

J.D. Andrade, P.M. Triolo, L.M. Smith, RFGD Plasma Treatment of Polymeric Surfaces to Reduce Friction, U.S. Patent 4,508,606, issued to the University of Utah.

L.J. Stensaas, F.J. Todd, P.M. Triolo, Prosthesis and Methods for Promoting Nerve Regeneration and for Inhibiting the Formation of Neuromas, Issued to Research Medical, Inc.

R.H. Hoffer, J.L. Orth, P.M. Triolo, Implantable Device for the Administration of Drugs or Other Liquid Solutions. Issued to Biosynthesis, Inc.

P.M. Triolo, A. Nelson, D. Staplin, Coupler for High Pressure Medical Tubing, U.S. Patent Application 10928.28.1, to be issued to Merit Medical Systems, Inc.

W.M. Padilla, P.M. Triolo, Locking Syringe with Thread Release Lock, U.S. Patent Application 10927.12.2, to be issued to Merit Medical Systems, Inc.

PUBLICATIONS:

P.M. Triolo, J.D. Andrade, "Surface Treatment and Characterization of Some Commonly Used Catheter Materials. I. Surface Properties, *J. Biomed. Mater. Res.* **17** (1983) 129-147.

P.M. Triolo, J.D. Andrade, "Surface Treatment and Characterization of Some Commonly Used Catheter Materials. II. Friction Characterization," *J. Biomed. Mater. Res.* **17** (1983) 149-165.

Marketing Plan

PSI is not a marketing organization, and we do not intend to develop a strong marketing function. We therefore plan to provide devices on OEM basis, or, as described above, develop the technologies for specific applications, and then license the technology to interested corporations for production and distribution. It is our intent to leverage off the marketing expertise of our corporate partners to introduce the product and distribute it through existing channels to their customers, especially in the food and beverage industries. It is anticipated that the features of our device will allow greater penetration into these markets, as well as expansion of market share in other relevant segments.

PRINCIPAL INVESTIGATOR(S) AND SENIOR PERSONNEL

Dr. Robert Scheer, Principle Investigator (PI), received his Ph.D. in Materials Science and Engineering in 1993 and has since been working for PSI. He was the PI on this NSF STTR Phase I Research Project. Rob's background is in polymers, polymer structure and morphology, and the modeling and testing of polymeric materials. As a result of his coordination of the Phase I research project, he also has considerable experience with the handling of firefly luciferase and its stabilization in agarose gels and fiber matrices. His abbreviated vita and letter from the University of Utah describing his non-research position is appended to this section. Note that Rob is no longer teaching at Salt Lake Community College.

Dr. Joseph Andrade is founder, and Chief Scientific Officer of PSI. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, particularly with their behavior at surfaces and interfaces. About 5 years ago he became quite interested in bioluminescence and particularly in the firefly and bacteria luminescent systems. He will provide much of the interfacial biochemistry, bioluminescence, and biosensor expertise required. Joe is a three quarter time Professor of Materials Science and Bioengineering at the University of Utah. His abbreviated vita is also attached.

Dr. Phil Triolo is the president and a senior scientist of PSI. He is a bioengineer with considerable product development experience in the medical device industry. Until joining PSI in 1994, he spent seven years as a contractor, working on various medical product and drug delivery development projects. All of the projects involved the selection and evaluation of appropriate materials for blood contact or drug delivery purposes, or the design and execution of experiments in order to demonstrate the safety and efficacy of devices to meet FDA requirements. His industrial background will enable this concept to be effectively developed into a commercial product. See attached vita.

Mr. C-Y Wang is a graduate student working under Joe Andrade's supervision at the University of Utah. Mr. Wang has worked on the firefly luciferase system for nearly four years and will be completing his Ph.D. studies on this system in 1995. He will be available as a post doctoral assistant during the Phase I award period to conduct the firefly luciferase-based studies and to transfer his rich, comprehensive expertise on that system to the others involved in the project.

CONSULTANTS AND SUBCONTRACTS

Larry Kricka is a member of Protein Solutions' Scientific Advisory Board and an expert in the field of ATP dependent biosensors and their application. He has agreed to serve as a consultant for this STTR Phase II research project. We expect he will make two or three trips to Salt Lake City to review and critique our device technology. See attached letter.

University of Utah. Sub-contract with the University of Utah as per STTR Guidelines. See separate sub-contract budget (Section P) and Table 1 in Research Plan.

Vladimir Hlady is a member of Protein Solutions' Scientific Advisory Board and an expert in the use of optical techniques to probe molecular and surface interactions. He is extremely familiar with the very sensitive light detection devices required for this work. A letter of agreement and his CV are included.

Russell Stewart is an assistant professor of Bioengineering at the University of Utah, and a member of PSI's scientific advisory board. He will serve as the principle investigator of the University subcontract as well as an independent consultant. A letter of agreement and his CV are included.

UNIVERSITY OF PENNSYLVANIA MEDICAL CENTER

University of Pennsylvania School of Medicine
Hospital of the University of Pennsylvania

Larry J. Kricka, D.Phil., F.R.C.Path.
Director, General Chemistry Laboratory
Professor of Pathology and Laboratory Medicine

September 11, 1995

Dr. Rob Scheer
Protein Solutions, Inc.
6009 Highland Drive
Salt Lake City, UT 84121

Dear Rob:

I was pleased to learn that Protein Solutions, Inc. is submitting Phase II proposal, "Direct Reading, Quantitative Biosensors for ATP-Dependent Processes." This is an area in which I am deeply interested. I would be glad to serve as a consultant during the research and development stages of this device.

I understand that you have budgeted at least two trips to Salt Lake City for me so that I might review your progress, and that there will be a consulting fee in addition to travel allowance.

Yours Sincerely,

Larry J. Kricka, D.Phil., F.R.C.Path.

Robert J. Scheer, Project Director
Protein Solution, Inc. (PSI)
6009 Highland Dr.
Salt Lake City, UT 84121

September 12, 1995

Dear Rob,

I am very excited about the potential of your ATP detection projects. I agree to serve as a consultant in support of your NSF STTR Phase II proposal entitled: "Direct Reading, Quantitative Biosensor for ATP-Dependent Processes". I understand that you will have in your budget funds to reimburse me at the rate of $400 per day for 5 total days of consultation and evaluation.

I hope that my experience in sensor design and optical sensing will prove valuable to your research project.

Sincerely,

Center for Biopolymers at Interfaces
Biomedical Polymers Research Building
Salt Lake City, Utah 84112
(801) 581-[illegible]

THE UNIVERSITY OF UTAH

Vladimir Hlady, D.Sc.
Associate Professor

THE UNIVERSITY OF UTAH

Sept. 8,1995

Protein Solutions Inc.
6009 Highland Drive
Salt Lake City, UT 84121

Dear Dr. Scheer:

I am writing to confirm my willingness to serve as a consultant to Protein Solutions Inc. on matters regarding genetic manipulation, production, and application of luciferase proteins. A recent c.v. is enclosed to illustrate my background and qualifications in the area of genetic engineering of proteins. Beyond my formal graduate education in Biochemistry and Molecular Biology I have worked for the last six years on the structure and function of cytoskeletal proteins using the techniques of molecular biology and protein chemistry. As we discussed, a consultancy fee of $400 per day is acceptable to me.

Sincerely,

Russell Stewart

Department of Bioengineering
2480 Merrill Engineering Building
Salt Lake City, Utah 84112
(801) 581-8528
FAX: (801) 585-5361

9. P.J. Worsfold and A. Nabi, "Bioluminescent Assays...," *Anal. Chim.Acta* **179** (1986) 307.
10. K. Green and L.J. Kricka, "Rapid Assays based on Immobilized Bioluminescent Enzymes and Photographic Detection of Light Emission," *Talanta* 31 (1984) 173-176.
11. R.A. Bunce, G.H.G. Thorpe, J.E.C. Gibbons, P.R. Killeen, G. Ogden, L.J. Kricka, and T.P. Whitehead, "Camera Luminometer for Use with Luminescent Assays," *Analyst* **110**, (1985) 657-663.
12. F. Scheller and F. Schubert, *Biosensors*, Elsevier, 1992.
13. Analytical Luminescence Laboratory (Ann Arbor, Michigan) 1995.
14. "Handbook of ATP-Hygiene Monitoring", Bio-Orbit, Turku, Finland.
15. Roy Betts, "The separation and rapid detection of microorganisms," in *Rapid Methods and Automation in Microbiology and Immunology*, Intercept, pp.107-119.
16. K.J. Littel, S. Pikelis and A. Spurgash, "Bioluminescent ATP Assay for Rapid Estimation of Microbial Numbers in Fresh Meat," *Journal of Food Protection* **49**, *1* (1986) 18-22.
17. J.M. Jay, *Modern Food Microbiology*, Van Nostrand Reinhold, 1986, pp.140-143.
18. J.W. Avis and P. Smith, "The Use of ATP Bioluminescence for the Analysis of Beer in Polyethylene Terephthalate (PET) Bottles and Associated Plant," in *Rapid Microbiological Methods for Foods, Beverages and Pharmaceuticals*, Blackwell Scientific Publications, 1989, pp.1-11.
19. D.Y.C. Fung, "Historical Development of Rapid Methods and Automation in Microbiology," *Journal of Rapid Methods and Automation in Microbiology* **1**, (1992) 1-14.
20. B. Swaminathan, "Rapid Detection of Food-Borne Pathogenic Bacteria," *Annu. Rev. Microbiol.* **48** (1994) 401-426.
21. G.L. Picciolo, E.W. Chappell, J.W. Deming, R.R. Thomas, D.A. Nibley and H. Okrend, "Firefly Luciferase ATP Assay Development for Monitoring Bacterial Concentrations in Water Supplies," United States Environmental Protection Agency Project Summary (EPA-600/S2-81-014) April 1981.
22. IDEXX Laboratories, Inc. (Westbrook Maine) 1995.
23. Promega Corporation (Madison Wisconsin) 1995.
24. BioOrbit Oy (Turku, Finland) 1994.
25. Charm Sciences Inc. (Malden, Massachussets) 1995.
26. Celsis (Monmouth Junction, New Jersey) 1995.
27. 3M Microbiology Products (St. Paul, Minnesota) 1995.
28. M.L. Calicchia, J.D. Reger, C.I.N. Wang and D.W. Osato, "Direct Enumeration of *Escherichia coli* O157:H7 from Petrifilm™ EC Count Plates Using the Petrifilm™ Test Kit — HEC Without Sample Pre-Enrichment," *Journal of Food Protection*, **57** (1994) 859-864.
29. A.J.G. Okrend, B.E. Rose and R Matner, "An Improved Screening Method for the Detection and Isolation of *Escherichia coli* O157:H7 From Meat, Incorporating the 3M Petrifilm™ Test Kit - HEC - for Hemorragic *Escherichia coli* O157:H7," *Journal of Food Protection*, **53**, (1990) 936-940.
30. D.Y.C. Fung, "What's Needed in Rapid Detection in Foodborne Pathogens," *Food Tech.* June (1995) 64.
31. D. Pfeiffer, et al., "Cascade-like Exponential Substrate Amplification in Enzyme Sensors," *Biosensors & Bioelectronics* **10** (1995) 169-180.
32. J-M Hawronskyj, R.S. Chittock, C.W. Wharton and J. Holah, "Low Level Bacterial Contamination Measured Using a Novel Biluminescent Assay," in A.K. Campbell et al. eds., *Bioluminescence and Chemiluminescence*, Wiley, 1994, pp. 411-414.
33. U. Wollenberger, et al., "Enhancing Biosensor Performance Using Multienzyme Systems," *TIBTech* **11** (1993) 255-262.
34. N.N. Ugarova and O.V. Lebedeva, "Immobilized Bacterial Luciferase..." *Appl Biochem Biotech* **15** (1987) 35-51.
35. L.Y. Brovko, N.A. Romanova and N.N. Ugarova," Bioluminescenct Assay of Bacterial Intercellular AMP, ADP, and ATP ..." *Analytical Biochemistry*, **220** (1994) 410-414.
36. N.N. Ugarova, et al., "Bioluminescent Microassay of Various Metabolites Using Bacterial Luciferase Co-immobilized with Multienzyme Systems," *Analytical Biochemistry* **173** (1988) 221-227.
37. W.J. Simpson and J.R.M Hammond, "The Effect of Detergents on Firefly Luciferase Reactions," *Journal of Bioluminescence and Chemiluminescence*, **6** (1991) 97-106.

Jerry Nelson, although not an official consultant on this research project, is a member of PSI's scientific advisory board. He is a microbiologist and the head of Nelson Laboratories, a Salt Lake City-based microbiology testing facility. His expertise in quantifying bacterial contamination will be valuable during the microbial evaluation stage of this research project. He has agreed to provide expert advice at no charge to the project.

EQUIPMENT, INSTRUMENTATION, COMPUTERS, AND FACILITIES

PSI rents 800 square feet of research space at 391 Chipeta Way in the University of Utah Research Park. Our lab is equipped with a reverse osmosis water supply, a dark room, clean lab and storage space, etc. In addition, it houses secretarial and office areas equipped with computers. Enzyme and substrate preparations as well as detection evaluations will be performed in these laboratories. We will require purchase of a CCD camera and a devoted computer platform to complete our equipment needs (see budget). Any special, additional equipment or facilities which we might require are available through our membership with the University of Utah's Center for Biopolymers at Interfaces (see letter of support from Karin Caldwell) and our relationship with Utah State University's food and diary program (see letter of support from Pete Gerity).

The University of Utah has excellent facilities for their portion of this work including cell culture facilities and computing facilities for the studies of enzyme kinetics.

CURRENT AND PENDING SUPPORT OF PRINCIPAL INVESTIGATOR AND SENIOR PERSONNEL

Pending support for Rob Scheer is from an NSF SBIR Phase I Proposal (DMI-9561228, A Quantitative, Modular Exploration System for Chemistry) submitted in June 1995. It does not overlap with the technology proposed here.

Pending support for Phil Triolo is from an EPA SBIR Phase I Proposal (EPA No. 310-95, Continuous, Real-time Enumeration of Microorganisms) submitted in January 1995 and a USDA SBIR Phase I (Rapid, Direct Reading Devices for Carbohydrate Assay) submitted in September 1995. They do not overlap with the technology proposed here.

EQUIVALENT OR OVERLAPPING PROPOSALS TO OTHER FEDERAL AGENCIES

None.

CITED REFERENCES

1. L.J. Kricka, et al., *Anal. Applic. of Bioluminescence and Chemiluminescence*, Acad. Press., 1989.
2. T.O. Baldwin, et al., "...Immunoassay...Using Bacterial Luciferase," *Meth.Enzym.* **133** (1986) 248-264.
3. D. Griffiths and G. Hall, "Biosensors -- What Real Progress is Being Made?," *TIBTech* **11** (1993) 122-130.
4. A. Lundin, "ATP Assays in Routine Microbiology: From Visions to Realities in the 1980's," in *ATP Luminescence, Rapid Methods in Microbiology*, P.E. Stanley, B.J. McCarthy, and R. Smither, eds., Blackwell Scientific, 1989, pp. 11-30.
5. C.Y. Wang and J.D. Andrade, "Interfacial Behavior in Firefly Luciferase," in A.A. Szalay, et al., eds., *Bioluminescence and Chemiluminescence: Status Report*, Wiley, 1993, pp. 99-103.
6. P.E. Stanley, "A Concise Beginner's Guide to Rapid Microbiology Using Adensoine Triphosphate (ATP) and Luminescence," in *ATP Luminescence, Rapid Methods in Microbiology*, P.E. Stanley, B.J. McCarthy, and R. Smither, eds., Blackwell Scientific, 1989, pp.1-10.
7. L.J. Blum, et al., "Design of Luminescence Photobiosensors," *J. Biolum. Chemilum.* **4** (1989) 543-550.
8. P.R. Coulet, "Luciferase-based Sensors," in Proc. Second World Congress on Biosensors, *Biosensors 92*, Elsevier Adv. Tech., pp. 2-9.

38. L.J. Kricka and M. DeLuca, "Effect of Solvent n the Catalytic Activity of Firefly Luciferase," *Archives of Biochemistry and Biophysics*, **21** 1982) 674-681.
39. P.C. Brookes and D.S. Jenkinson, "ATP and Adenylate Energy Charge Leve the Soil Microbial Biomass," in P. Stanley and L. Kricka, eds.*Biolumine ce and Chemiluminescence: Current Status*, Wiley, 119-127 (1991).
40. R.K. Ferncase, "Film and Exposure" in *Basic Lighting Worktext for Film and Video*, Focal Press, 1992, pp. 7-10.
41. C.Y. Wang and J.D. Andrade, "Denaturation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Current Status*, P. Stanley and L. Kricka, eds. Wiley, 427-432 (1991).
42. C.Y. Wang and J.D. Andrade, "Purification and Preservation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Cambell, L.J. Kricka, and P.E. Stanley eds. Wiley, 423-426 (1994).
43. Chung-Yih Wang, Ph.D. Dissertation in preparation, "Firefly Luciferase at Model Interfaces: Activity, Stability and Sensor Application", expected December 1995.
44. C.Y. Wang, R. Stewart and J.D. Andrade, "Purification, Stability and Applications of Biotinylated Recombinant Luciferase", in preparation (1995).
45. W. Höbel, A. Papperger, and J. Polster, "Penicillinase Optodes: Substrate Determinations Using Batch, Continous Flow and Flow Injection Analysis Operation Conditions," *Biosensors and Bioelectronics* **7** (1992) 549-557.
46. S.M. Gautier, P.E. Michel, and L.J. Blum, "Reagentless Bioluminescent Sensor for NADH," *Anal. Lett.* **27** (1994) 2055-2069.
47. FMC BioProducts, Inc. 1995.
48. Hispanager, Inc., (Spain) 1995.
49. Trevigen, Inc. 1995.
50. P.E. Stanley, B.J. McCarthy and R. Smither, eds. *ATP Luminescence, Rapid Methods in Microbiology*, Blackwell (1989).
51. F.F. Craig, A.C. Simmonds, D. Watmore, F. McCapra and M.R.H. White, " Membrane-permeable luciferin esters for assay of firefly luciferase in live intact cells," *Biochem. J.* **276** (1991) 637-641.
52. S.E. Hoffner, C.A. Jimeniz-Misas and A. Lundin, "Improved Extraction of Mycobacterial ATP," in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Cambell, L.J. Kricka, and P.E. Stanley eds. Wiley, 1994, pp. 442-445.
53. D.Y.C. Fung, "Rapid Methods and Automation in Food Microbiology", *Food Reviews Int.* **10** (3) (1994) 357.
54. J.Y. D'Aoust, "Salmonella and the International Food Trade", *Int. J. Food Microbiol.* **24** (1994) 11.
55. A.N. Sharpe, "Preparing samples for rapid detection of microbes," in *Rapid Methods and Automation in Microbiology and Immunology*, Intercept, pp. 97-105.
56. C.J. Stannard, S.B. Petitt and F.A. Skinner, *Rapid Microbiological Methods for Foods, Beverages and Pharmaceuticals*, Blackwell (1989).

BIBLIOGRAPHY

Arne Lundin, "Applications of Firefly Luciferase," in *Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry*, M. Serio and M. Passagli, eds., Raven Press, 1982, pp. 29-52.

K. Wood, Ph.D. Dissertation, Department of Chemistry, University of California, San Diego, 1989.

J. Giese, "Rapid Microbiological Testing Kits and Instruments," *Food Technology* July (1995) 64-71.

J.D. Andrade, et al., "Proteins at Interfaces: Principles, Problems, and Potential," chapter in *Interfacial Behavior of Bioproducts*, J. Brash and P. Wojciechowski, eds., Dekkar, 1994, in press.

J.D. Andrade, et al., "Using Bioluminescence for Integrated Science Education," in A.A. Szalay, et al., eds., *Bioluminescence and Chemiluminescence: Status Report*, Wiley, 1993, pp. 69-73.

J.D. Andrade, et al., "Immuno-Biosensors: The Clinical Chemistry and Coagulation Laboratory on a Chip," in Y. Sezai, ed., *Artificial Heart: Biomation in the 21st Century*, Saunders, 1992, pp. 89.

Special issue, "Bioluminescence in the Sea," *Naval Research Reviews* **45** (1993) (#2).
T. Arakawa, S.J. Prestelski, W.C. Kenney, and J.F. Carpenter, "Factors Affecting Short-Term and Long-Term Stabilities of Proteins," *Adv. Drug Delivery Reviews* (1993), in Press.
J.F. Carpenter and J.H. Crowe, "Modes of Stabilization of a Protein by Organic Solutes During Desiccation," *Cryobiology* **25** (1988) 459.
J.H. Crowe, L.M. Crowe, D. Chapman, "Preservation of Membranes in Anhydrobiotic Organisms: The Role of Trehalose," *Science* **223** (1984) 701.
C. Colaco, et al., "Extraordinary Stability of Enzymes Dried in Trehalose," *BioTech.* **10** (1992) 1007.
B.P. Gaber, et al., "Interaction of Trehalose..." Molecular Modeling," in A.C. Leopold, ed., *Membranes, Metabolism, and Dry Organisms*, Cornell Univ. Press, pp. 231.
J.H. Crowe, et al., "Anhydrobiosis," *Ann. Rev. Physiol.* **54** (1992) 579.
J.D. Andrade, C.Y. Wang, V. Hlady, P. Triolo and R.J. Scheer, "Method of Measuring Chemical Concentration and/or Light Intensity Based on Spatial Separation and Resolution", Preliminary US Patent Application (*1995).
R.F. Hess, et al. eds. *Night Vision*, Cambridge University Press: UK (1990).
M.H. Pirenne and E.J. Denton, "Accuracy and Sensitivity of the Eye," *Nature* **170** (1952) 1039.
A. Rose, "Quantum effects in Human Vision," *Adv. Biol. Med. Physics* **5** (1957) 211.
S.M. Gautier, et al., "Bioluminescence-Based Fibre-Optic Sensor With Entrapped Co-Reactant...," *Analytica Chimica Acta* **243** (1991) 149-156.
D.J. Min, C.Y. Wang and J.D. Andrade, "Air/Water Monolayer Studies of Bioluminescent Enzymes", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Cambell, L.J. Kricka, and P.E. Stanley eds. Wiley, 423-426 (1994).
A. Roda, et al., "Coupled Reactions for the Determination of Analytes and Enzymes Based on ... Luminescence," *J. Biolum. Chemilum.* **4** (1989) 423-435.

BUDGET JUSTIFICATION

Post Doctoral Associate. Mr. C.Y. Wang is expected to graduate with his Ph.D. from the Department of Bioengineering, University of Utah this fall, so that he will be available full-time by the start of this research project.

Graduate Student. Mr. Dong Min will continue his Ph.D. work on bacterial luciferase and assist in small projects as needed.

Permanent Equipment: CCD Camera from SBIG Astronomical Instruments, Santa Barbara, CA. Price $2750
Image Capturing and computer interfacing software, $990.
MacIntosh Powerbook computer with image capturing capacity, $3800.
Biolink permanent luminescent standards, $ 700.

Travel: Expected attendance at two trade shows and technical seminars related to microbial detection.

Materials and Supplies: Photographic Film ($20/8exposures)
Lab consumables
Research reagents
Maintenance of ATP-free areas

Consultant Services: $ 10,500 to Dr. Russell Stewart (fees)
$ 2,000 to Dr. Vladimir Hlady (fees)
$ 3,000 to Dr. Larry Kricka (travel and fees)

Subcontract: University of Utah subcontract

Indirect Costs: 60% of Salaries, Wages and Benefits

The University of Utah
Department of Materials Science and Engineering

Gerald B. Stringfellow
Department Chair

304 EMRO
Salt Lake City, Utah 84112
801-581-8387 Fax 801-581-4816
stringfellow@ee.utah.edu

September 6, 1995

Grant Official STTR/DMII Program
National Science Foundation
4201 Wilson Boulevard
Arlington, VA 22230

To Whom It May Concern:

This is to advise you that Robert J. Scheer will be teaching one course each quarter during the academic year 1995-96 in the Department of Materials Science and Engineering. Dr. Scheer is an Adjunct Instruction in the Department.

Dr. Scheer will not seek research funding through the University of Utah while seeking or already under the support of the STTR Program.

If you need further information, please feel free to call.

Sincerely,

Gerald B. Stringfellow
Chairman

Dr. Rob Scheer, Project Director
Protein Solutions, Inc. (PSI)
P.O. Box 58093
Salt Lake City, UT 84158

September 7 1995

Dear Rob:

I am pleased to confirm that CBI's facilities are available to you and your co-workers at member rates.

I am pleased to learn of your STTR submission to the National Science Foundation, Direct Reading, Quantitative Biosensors for ATP Dependent Processes. CBI is pleased to acknowledge that you will have access to labs and facilities related to chemical and materials characterization.

Faculty affiliated with CBI and its technical staff have the experience and skills which will greatly aid you and your co-workers in the conduct of this innovative research.

Sincerely,

Karin D. Caldwell
Professor, Department of Bioengineering
CBI Director

Center for Biopolymers at Interfaces
108 Biomedical Polymers Research Building
Salt Lake City, Utah 84112
(801) 581-3867

THE UNIVERSITY OF UTAH

Dr. Philip Triolo, President
Protein Solutions, Inc.
6009 Highland Drive
Salt Lake City, UT 84121

September 9, 1995

Utah State UNIVERSITY

VICE PRESIDENT FOR RESEARCH OFF
Logan, Utah 84322-1450
Telephone: (801) 797-1180
FAX: (801) 797-1367
INTERNET: (pgerity@champ.usu.edu)

Dear Phil:

We were pleased to learn that Protein Solutions, Inc. is focusing its unique and exciting bioluminescence assay research and development to food and dairy applications.

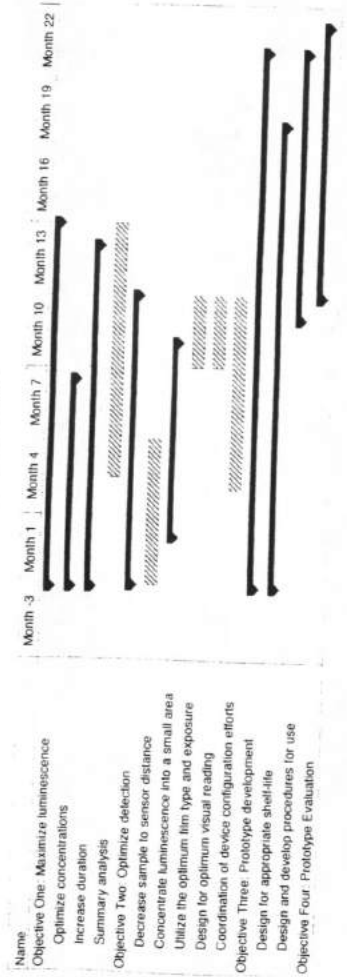
Utah State University, as the state's land grant institution, has strong and active programs in all aspects of food and dairy science. We are, of course, particularly interested in working with you in any way we can to study, develop, and apply modern, direct reading, luminescence-based biosensor technologies. We will continue to coordinate closely with you to schedule meetings with a number of our key faculty who have expressed interest in collaborating with you on these innovative projects.

Sincerely,

Peter F. Gerity

Peter F. Gerity, Ph.D.
Vice President for Research

Project Milestone Chart



Effort	6	12	18	24	Cumulative
Key Personnel	3 months	4 months	7 months	8 months	22 months
Consultants	15 days	8 days	7 days	3 days	33 days
Subcontracts	30%	36%	17%	17%	100%
Estimated Expenditures					
Key Personnel	$17,100	$17,100	$29,400	$29,400	$93,000
Consultants	$6,000	$4,000	$3,500	$2,000	$15,500
Subcontracts	$20,000	$24,000	$11,260	$11,260	$66,520
Permanent Equipment	$8,240	$0	$0	$0	$0

NATIONAL SCIENCE FOUNDATION
4201 WILSON BOULEVARD • ARLINGTON, VIRGINIA 22230

Award Date February 13, 1997
Grant No. DMI-9531303
Amendment No. 001

Dr. J. D. Andrade
President/CEO
Protein Solutions, Inc.
6009 Highland Drive
Salt Lake City, UT 84121

Dear Dr. Andrade:

By letter dated September 20, 1996, the sum of $349,430 was awarded to Protein Solutions, Inc. for support of the project entitled "STTR Phase II: Direct Reading Quantitative Biosensors for Adenosine Triphosphate (ATP) Dependent Processes" under the direction of Robert J. Scheer and Russell J. Stewart, University of Utah.

Effective with this amendment, this project is now under the direction of Richard A. VanWagenen and Russell J. Stewart, University of Utah.

Except as modified by this amendment, the grant conditions remain unchanged.

The cognizant NSF program official for this grant is Darryl G. Gorman (703) 306-1391. The cognizant NSF grants official is Loretta Hemsley (703) 306-1218.

Sincerely,

Graciela L. Narcho

Graciela Narcho
Grants Officer

1. **Title:** NATIONAL SCIENCE FOUNDATION STTR PHASE II PROPOSAL APPENDIX II

2. **Program Official/Organization:** Michael Crowley (Darryl Gorman)/DMII

3. **Program Name:** STTR/DMII

4. **PI/PD Name:** Robert J. Scheer

5. **Company, Address, Phone Number, and FAX Number:**

(Old Address)
Protein Solutions, Inc.
350 West 800 North, Suite 218
Salt Lake City, UT 84103
Phone: (801)596-2675
FAX: (801)596-2675

(New Address)
Protein Solutions, Inc.
6009 Highland Drive
Salt Lake City, UT 84121
Phone: (801)583-9301
FAX: (801)583-9301

6. **Phase II Proposal Number:** DMI-9531303

7. **Project Title:** Direct Reading, Quantitative Biosensors for ATP-Dependent Processes.

This material is based upon work supported by the National Science Foundation under award number DMI-9413561. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the National Science Foundation.

Table of Contents:

1. Company

Protein Solutions, Inc., was founded in 1988 in Salt Lake City by Drs. Peter Gerity, James McRea, and Joe Andrade. The company's original objective was to develop science education tools for school and home use.

Government and private funding was sought for three distinct product lines; personal sensors; educational kits employing bioluminescent organisms; and Lableess Labs® (see section on prior support), a collection of specially selected and engineered materials to accompany telecourses and other courses where hands-on laboratory experience is lacking. The common denominator to these ambitious projects is that they all centered on the development of experience-based learning tools.

Recently, the company has focused its efforts on the development of bioluminescent sensors, as these have the greatest immediate potential for commercialization.

2. STTR Project

This NSF Phase II research project is undertaken in response to the need for an inexpensive, direct-reading sensor, capable of real-time measurement of bacterial contamination in sensitive areas, such as food and pharmaceutical facilities and water treatment plants. During phase I of this project we demonstrated the feasibility of detecting ATP in quantities as small as 10^{-9} g (2×10^{-12} moles) both visually and on photographic film. The technology employs the firefly luciferase-catalyzed light producing reaction between luciferin and ATP. By mediating the ATP concentration with the "ATPase" apyrase, we are able to produce a spatial light pattern which indicates the concentration of ATP present in a sample.

In this project we focus on increasing the sensitivity of our direct reading ATP sensor to levels more suited to bacterial detection (10^{-12} to 10^{-13} g, ≈1,000–100 bacteria). This increase in sensitivity will be achieved by three primary means:

1) increasing the intensity and/or duration of the luminescence for a given quantity of ATP,
2) increasing the amount of light that reaches the detector (film or otherwise), and
3) optimizing the non-instrumented detector for the system (film).

Each of these objectives should contribute at least one order of magnitude increase in absolute ATP sensitivity.

Table 1. Outline indicating research objectives.

Primary Objectives	Methods
1) Increase luminescence	Optimize enzyme and substrate concentrations
	Increase duration of light emission
	Evaluate gel versus solution light emission
2) Increase detection	Decrease sample to sensor distance
	Concentrate luminescence into smaller area
	Utilize the optimum film type and exposure
	Design for visual reading
	Minimize light leakage
	Evaluate detection
3) Optimize detector	Optimize shelf life of enzymes
	Optimize shelf life of substrates
	Evaluate shelf life
	Minimize Costs
	Select final configuration

1) Increase luminescence

Our first step toward improving sensitivity is to increase the luminescence for a given amount of ATP. This can be achieved in two ways. Light intensity can be increased, and/or the duration of luminescence can be increased. (Increases in the duration of light emission will yield a greater total light output for integration purposes. Film will be used to integrate the prolonged light signal.)

Increases in light intensity will be achieved by adjusting the enzyme/substrate concentrations to their optimum values. We will use the software technology at the University of Utah's Center for Biopolymers at Interfaces (CBI) which is capable of simulating enzyme reaction rates for multiple enzymes systems, to optimize our system.

A second study will be performed to increase the *duration* of the luminescence using enzyme amplification of substrates. Enzyme amplification refers to the process of "recycling" the adenosine monophosphate (AMP) produced from the luciferase catalyzed bioluminescent reaction back to ATP through appropriate phosphorylation reactions. Preliminary studies in our lab have demonstrated that enzyme amplification will improve both the duration and the output of luminescence for a given concentration of ATP. (Also see references 1-3)

The firefly luciferase-catalyzed bioluminescence system utilizes one ATP and generates one AMP (see equation 1). Adenylate kinases which convert AMP to ADP can be coupled with pyruvate kinase to recycle AMP back to ATP (equations 2 and 3).

Three types of adenylate kinases are reported (2). We will focus on types I and II. These are myokinases which convert AMP to ADP (adenosine diphosphate) by consuming one ATP. The two ADP molecules produced in this reaction are converted by pyruvate kinase to ATP by consuming phospho(enol)pyruvate (PEP). (See figure 1.)

$$\text{ATP} + \text{luciferin} \xrightarrow{\text{luciferase}} \text{AMP} + \text{oxyluciferin} \quad (1)$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2\text{ADP} \quad (2)$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate} \quad (3)$$

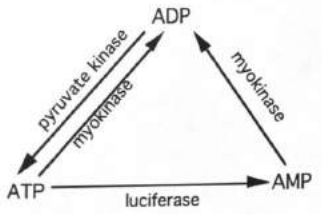


Figure 1. Energy transfer cycle of luciferase and various amplification enzymes.

We expect, and it will be shown that, amplification enzymes can be used with our detection system to both increase and prolong the light output from the luminescent reaction. By using high speed film as a detector, the increased duration of light emission can be integrated – that is, collected over time to acquire a greater exposure (≈1 minute is practical). The human eye integrates for approximately 0.1 seconds, so that film can represent a 100-1,000 fold increase in sensitivity.

The combined refinements of enzyme and substrate concentration optimization along with development of substrate recycling with enzymes should result in an increased luminescence of at least one order of magnitude for a given amount of ATP.

2) Increase (optimize) detection of luminescence

To optimize device sensitivity and make the most of the luminescence provided by the ATP/enzyme reaction the generated light must be efficiently and completely detected. This can be achieved by increasing the efficiency of photon capture by the detector, be it photographic film or the human eye. This can be at least partially accomplished by focusing or directing all the available photons into the smallest possible area and then (in the case of film) integrating for an extended length of time.

One way of improving the efficient collection of luminescence is to minimize photon loss by decreasing sample to sensor distance, thereby increasing the number of photons striking the detector. A contact print is most effective in this regard. This method of enhancement will also work with direct visual detection, although to a lesser extent because the eye cannot get as close to the sample as the film.

Another technique that will be used to increase the amount of light that reaches the detection device is to focus the luminescence into a small area. A minimization of the display area for a given quantity of sample will project the same number of photons over a smaller area, thereby increasing the exposure per unit area.

3) Optimize detector

Prototype development will be divided into several projects. These include the preparation and preservation of the reactive components as well as the development of device configuration and mode of operation.

In order to arrive at an acceptable device, the shelf life of all the components needs to be increased to at least 6 months. The stability of luciferase in solution and dry gel has been studied by C.Y. Wang et al. (4-6) These studies will be extended to other enzymes and substrates to be included in the device.

The sensing device itself is composed of several basic elements:

- a mechanism for introducing a sample to the device,
- a mechanism for ensuring that the sample is distributed to all reactants of the device,
- a means of optimizing conditions so that the analyte reacts efficiently and completely with the reagents per design,
- a mechanism for relating the amount of ATP in the sample to a measurable light signal employing firefly luciferase and luciferin,
- optical components that focus and guide the emitted light to the detector, and
- the detector itself, which is either the human eye or photographic film

We envision the device having a 1-2 mm diameter capillary feed, capable of providing a readout of ATP concentration similar to a thermometer's readout of temperature.

3. Commercial Applications

By far the largest existing application area for ATP-based bioluminescent sensors is hygiene monitoring of food processing facilities. Microbial contamination of foods, particularly fish, meat, and dairy products, is of increasing concern to the consumer and the governmental and regulatory bodies that regulate food processing.

The most widely accepted method for sensing microbial contamination is traditional culture plating. Critical working surfaces are swabbed to collect a sample which is then applied to appropriate growth media, incubated, and examined for the presence of colonies. The major drawback to this approach is the lag time between sampling and availability of results, which is 24

to 48 hours. Thus, food found to be contaminated may already have been shipped to a distributor, or worse, bought and consumed by the public.

Therefore, recent efforts have centered on obtaining real-time data on the cleanliness of food preparation areas. The Hazard Analysis and Critical Control Point (HACCP) approach is beginning to gain widespread acceptance as a method that can prevent the distribution of contaminated foods. HACCP emphasizes the identification and monitoring of specific hazards in the food production, distribution, and preparation chain and the incorporation of appropriate measures for their prevention. The monitoring procedure must provide the information in time for corrective actions to be taken. Culture plating techniques are not useful in HACCP because they require too much time. Nearly instantaneous methods must be employed at the critical points in order for the monitoring to be effective.

Current methods of choice for identifying microbial and organic contamination of surfaces are ATP-based. Low-cost luminometers are used to detect light emitted from samples collected from control points. Samples are treated to release both microbial and non-microbial ATP. The effectiveness of the cleaning procedure is indicated by the absence of ATP. PSI's technology eliminates the need for a luminometer, thus allowing rapid, on-site semi-quantitative assessments of microbial contamination via an easy-to-use, disposable sensor.

Currently, microbial assays are primarily employed in the food, beverage, and water processing industries to test sanitation levels. Additional applications include the monitoring of sensitive areas that must be kept relatively microbe free, including pharmaceutical and medical device cleanroom areas, hospitals, and other biological laboratories. The Army has also expressed interest in developing a sensor to detect microbial contamination of field rations.

4. Patent Status

A preliminary patent application has been filed for the spatial detection technology (the subject of the Phase I grant)(7). The patent, entitled "Method for Measurement of Chemical Concentration and/or Light Intensity Based on Spatial Separation and Resolution", has been disclosed through the University of Utah, with whom Protein Solutions has a technology transfer agreement. Through this agreement, PSI has rights to all technologies developed at the University with PSI funding in exchange for a royalty payment to the University for any protected technologies that are developed commercially.

5. Innovation

PSI's ATP detection technology has a number of unique features and advantages:

1) Using Phase I funding we conclusively demonstrated the feasibility and practicality of obtaining quantitative ATP measurements without the use of a luminometer or other electronic photo-detection equipment.

2) We have developed a method for the low-cost production of luciferase using recombinant technology.

3) We have conceived and will develop in the Phase II project a number of novel optical designs which further enhance the light directing and collecting properties of the device. These optical enhancements, together with the enzyme recycling, will lead to a hundred-fold increase in photon detection.

Through application of this technology, extremely sensitive bioluminescent assays can be performed without an expensive luminometer. The technology will be optimized and incorporated into a small dipstick-type device to rapidly detect microbial contamination on surfaces, and it will be combined with appropriate up front enzymes, to selectively determine the quantities of selected analytes. Another possible application is a collection device as a rapid assay for airborne microbial contamination.

Under a PSI-funded subcontract, Dr. Russell Stewart's lab at the University of Utah has developed a method for producing recombinant luciferase. This has reduced our cost for luciferase by a factor of 100, and enabled us to accelerate our investigations with this enzyme, as well as made many of our technologies much more commercially attractive from a financial perspective. PSI is again funding a subcontract with Dr. Russell Stewart's lab to develop and characterize the myokinase and pyruvate kinase required for substrate recycling.

To our knowledge, there are no technologies currently available that allow for the sensitive detection of ATP by non-instrumented means. The possibility exists for moving sensing assays that normally require sophisticated laboratory equipment and trained personnel in the laboratory environment to the consumer market. An example of such a shift is the pregnancy test, which was once performed solely in hospital and clinical laboratories and has now moved into the consumer market as a result of advances in technology.

6. Markets

Hygiene Monitoring- It has been estimated that 1.4 billion tests are performed annually to detect microbial contamination at an annual cost of over $1 billion (8). A rough estimation of the geographic breakdown of these tests suggests that some 550 million are performed in Europe, 550 million are performed in the US with the remaining 300 million performed in the Pacific Rim and Oceania.

The majority of these are tests are performed utilizing standard culture plate methods. A recent survey taken in Europe, where luminometer-based technologies have gained wider acceptance than in the US, indicated that some 20% of tests are performed using ATP-based monitoring techniques. In the US, only 10 to 15% of evaluations are performed by ATP-based technologies, and probably 5% of microbial assays are performed by ATP-based technologies in other geographic areas. Thus, the total number of ATP-based tests now performed world-wide annually is roughly 200 million.

Allowing for the fact that the 1.4 billion estimate for the number of microbial contamination determinations performed annually may be high, the current number of luminometer-based assays performed probably is in the range of 50 to 100 million, which, at an average price for disposables of $2.00, represents a world-wide market of $100 to $200 million annually for disposables, and an additional $10 million for the purchase of luminometers. Currently the market is growing at a robust 20% per year, spurred by the decreasing price of luminometers and the increasing adoption of HACCP methodologies. Growth would be rapidly accelerated in the US market if legislation were adopted requiring real-time monitoring and process control in the food industry. Although this is not likely in the current political climate, a few cases of contamination that result in fatalities could bring about quick adoption of the HACCP legislation that has already been drafted in the US.

Ophthalmic Applications Contact lenses are worn be roughly 20 million people in the US and 40 million people world-wide. There is growing concern that bacterial contamination of such lenses may lead to chronic inflammation in a growing patient population. Currently, no hygiene testing of contact lenses and solutions is made by consumers, but, if performed on a bi-yearly basis when lens wearers visit their ophthalmologist or optometrist for a check-up, the potential market is for 40 million tests in the US, and 80 million tests worldwide. At an average selling price of $2 per test, this represents potential sales of $80 and $160 million in the US and worldwide, respectively.

PSI's goal is to capture 20% ($20 million annually) of the rapid assay market in existing commercial markets (hygiene monitoring), and 50% ($40 million annually) of the ophthalmic market within 5 years of the introduction of its products.

7. Competition

Existing rapid techniques for microbial detection all necessitate the use of a luminometer, the least expensive of which costs $3000. Although portable, luminometers can be bulky and are

not easily carried about the facility. Additionally, they require periodic calibration and maintenance. A brief summary of competitive products follows:

Table 2. Summary of companies currently marketing rapid detection systems

Company	Luminometer list price ($)	Sample Swabs list price ($)	Comments
Celsis	7500 -35,000	1.78	
Lumac	N/A	N/A	
Bio-Orbit	5700	3.20	
Promega	N/A	N/A	
Charm Sciences	3000	2.30	Not yet available
Analytical Luminescent Labs	10,000	1.26	
Idexx Labs	3500	2.60	

In addition to the luminometer, existing products include a special sampling accessory. These assemblies range from simple ATP-free cotton swabs to fairly complex configurations including the sampling swab, a solution to release ATP, and a means of delivering the ATP to the reaction portion of the apparatus. The cost of the collection ancillaries ranges from $1.78 to $3.20, and one swab is required for each sample collected.

Less rapid microbial assays, although more sensitive than these rapid tests, require culturing times up to 72 hours before results can be determined.

8. Competitive Advantages

PSI's technology in ATP sensing will:

1) obviate the need for an expensive and cumbersome luminometer,
2) permit direct, real-time assessment of microbial contamination and rapid detection of samples that otherwise require plating, and
3) provide a hard copy record (photographic) of the cleanliness of areas for documentation purposes to help meet government and internal requirements.

In addition:

4) the technique can be expanded to monitor a wide range of analytes that participate in reactions that either phosphorylate or dephosphorylate ATP,
5) once the public becomes familiar with the use of the initial product, the sensors can be readily developed into a family of products to address specific sensing needs in high volume consumer markets,
6) the device will be small and disposable,
7) the cost of the device will be comparable to existing sampling ancillaries, and
8) trained personnel will not be required to perform analyses.

9. Production Plan

Our Phase II research will demonstrate that a device can be configured with the appropriate concentrations of necessary analytes and enzymes to accurately indicate concentrations of ATP down to 10^{-12} grams, permitting the detection of microbial contamination in real-time either visually or with photographic film. We will have a prototype by the end of the Phase II research period.

Subsequent development will be undertaken with the assistance of our corporate partners with the goal of incorporating the features required for specific market applications. Development of a final pre-production prototype will take 1 to 2 years. PSI plans to remain actively involved in

the research and development activities that support product development and manufacturing, but has not made a final decision on whether to manufacture the items internally or have a domestic (preferably local) corporate partner manufacture the final product, although the former is more likely.

PSI intends to retain ownership of the technology and license it to companies that are established leaders in distinct market segments. In addition, we will continue to contact corporations that may have an interest in supporting development of the products for their specific applications. In all cases we are seeking funding of in-house development through the pre-production prototype phase, with final production decisions to be based on the capabilities we have at that time and the needs of our corporate partners for their licensed applications.

10. Marketing Plan

The competitive advantages and innovations outlined above make this technology suitable for a very wide range of markets and applications, as indicated in Figure 2.

Our initial focus is in food processing and handling, with particular attention to hygiene monitoring. For this reason we are focusing our corporate interactions on those firms who are already involved in that market and, therefore, have the presence and marketing strength to assist us in that endeavor.

We are focusing the discussions and negotiations with firms who are not now major players in rapid detection, but who *are* major players in the slower culture plate or culture media approach to hygiene monitoring. These include those major firms that sell bacteria culture media, including those that sell it in a dry film/plate format. As indicated earlier, manufacturers of culture plate media control more than 80% of the U.S. market. These firms realize that the trend is towards rapid detection. Therefore, they are eager to collaborate on a rapid detection approach which meets their rigorous quality control requirements. These firms have large, microbiology products divisions and have the skills and expertise with which to assist PSI in device design and development.

The focus on food hygiene monitoring also lends itself to the even larger consumer market, including restaurants, fast food and cafeteria outlets, as well as consumer kitchens and eating places.

PSI is not a marketing organization, and we do not intend to develop a strong marketing function. We therefore plan to provide devices on OEM basis, or, as described above, develop the technologies for specific applications, and then license the technology to interested corporations for production and distribution. It is our intent to leverage off the marketing expertise of our corporate partners to introduce the product and distribute it through existing channels to their customers, especially in the food and beverage industries. It is anticipated that the features of our device will allow greater penetration into these markets, as well as expansion of market share in other relevant segments.

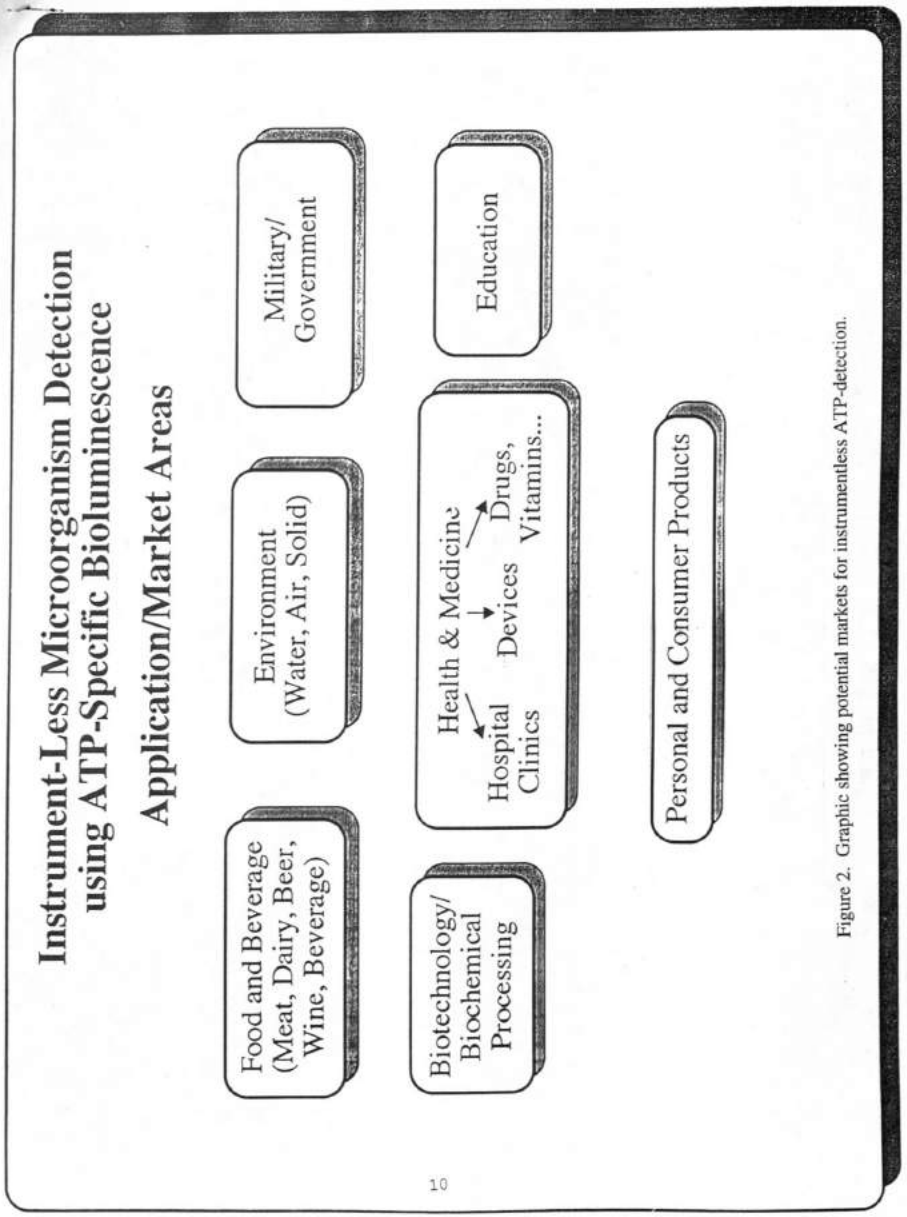


Figure 2. Graphic showing potential markets for instrumentless ATP-detection.

References

1. J-M Hawronskyj, R.S. Chittock, C.W. Wharton and J. Holah, "Low Level Bacterial Contamination Measured Using a Novel Bioluminescent Assay," in A.K. Campbell et al. eds., *Bioluminescence and Chemiluminescence*, Wiley, 1994, pp. 411-414.
2. U. Wollenberger, et al., "Enhancing Biosensor Performance Using Multienzyme Systems," *TIBTech* **11** (1993) 255-262.
3. C.Y. Wang, R. Stewart and J.D. Andrade, "Purification, Stability and Applications of Biotinylated Recombinant Luciferase", in preparation (1995).
4. C.Y. Wang and J.D. Andrade, "Denaturation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Current Status*, P. Stanley and L. Kricka, eds. Wiley, 427-432 (1991).
5. C.Y. Wang and J.D. Andrade, "Purification and Preservation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Cambell, L.J. Kricka, and P.E. Stanley eds. Wiley, 423-426 (1994).
6. Chung-Yih Wang, Ph.D. Dissertation in preparation, "Firefly Luciferase at Model Interfaces: Activity, Stability and Sensor Application", expected December 1995.
7. Andrade, J., Wang, C.Y., Hlady V., Triolo, P.M., Scheer, R.J. "Method for Measurement of Chemical Concentration and/or Light Intensity Based on Spatial Separation and Resolution." Provisional Patent Application Filed, 1995.
8. Stratton Research Center. "The Challenge: From Retrospective Testing to Real-time Monitoring", Celsis International, Cambridge, UK.

Commercialization of Prior Support

NSF SBIR ISI–9161158, "Light Crawlers: Bioluminescence-Based Discoveries for Science Education." (2/1/92-6/30/92) Phase II Proposal was not funded due to extenuating circumstances which prevented completing Phase I objectives (worms were not available due to drought conditions in their natural habitat). The technology has not been pursued.

NSF SBIR III-9361652, "The Labless Lab: Polymer Materials." (2/1/94-8/1/94) Phase II Proposal was not funded due to safety concerns. These concerns have been addressed; a prototype version of the Labless Lab® in Polymers will be sent to 30 reviewers across the country in early January, 1996. PSI is using internal funding to continue the development of this product

NSF STTR DMI-9413561, "Direct Reading, Quantitative Biosensors for ATP-Dependent Processes." (8/15/94-7/31/95) The following plan is part of the Phase II proposal for this project. See body of appendix for current commercial interest.

Phase II Cooperation and Phase III Follow-on Funding Commitments.

PSI is currently in the final negotiating stages with potential corporate partners in the four marketing areas shown in Figure 2. We have also attracted some local investments to complete the $200,000 Phase III Follow-on Funding requirement. See attached letter.

Joseph D. Andrade
6009 Highland Drive
Salt Lake City, Utah 84121

December 12, 1995

Dr. Phil Triolo, President
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, Utah 84158-0093

Dear Phil:

It is a pleasure to confirm my oral discussions with you and Dr. R. Scheer regarding your direct reading bioluminescence-based ATP sensing technologies.

I am very impressed with this innovative technology and with the progress made in your NSF Phase I STTR project demonstrating feasibility. Your Phase II proposal convinces me that you can obtain the sensitivity and reliability which will make this approach the method and product of choice for hygiene monitoring in several major industries, including food/dairy and medical device production. I personally feel it has enormous opportunities in the general consumer market as well. Everyone is becoming increasingly concerned with cleanliness in cafeterias, restaurants, and private kitchens, and this technology can be developed into a device that can assess cleanliness in these areas.

I understand PSI's stock is now selling at 50¢/share. I wish to continue to invest in PSI, with the understanding that funds provided by my investment will be used for your Phase III product commercialization activities, including seeking patent protection and licensing agreements. This, of course, assumes the funding of your NSF Phase II to provide the resources to complete the R & D required for enhanced sensitivity -- as described in the Phase II proposal.

The investment schedule and conditions are as follows:

- **$15,000 - November, 1995 - 30,000 shares**: These funds were invested -- and are being used -- to prepare your Phase II Commercialization Plan and to initiate preliminary discussions with potential corporate partners and licensees.
- **$20,000 - July, 1996 - 40,000 shares:** This and all subsequent investments are conditional on the Phase II award.
- **$15,000 - March, 1997 - 30,000 shares:** To continue licensing, partnering, and patent activities.

Cont/...

Joseph D. Andrade
6009 Highland Drive
Salt Lake City, Utah 84121

- **$50,000 -- December, 1997 -- 100,000 shares:** To initiate the Phase III Effort -- assuming the sensitivity targets noted in the Phase II proposal are met and that the technology in economically viable as evidenced by a signed contract with a corporate partner in the food/dairy monitoring area.

- **$50,000 -- December, 1998 -- 100,000 shares:** For continued manufacturing and marketing of first product -- conditional on a corporate partner with appropriate marketing skills, specific market experience, and an existing distribution/marketing system.

- **$50,000 -- December, 1999 -- 100,000 shares:** For prototype development and corporate partnering for a consumer (home) product version, conditional on an agreement with a major corporate partner with consumer product skills and activities.

Total Investments through 1998: **$200,000 -- 400,000 shares**

I understand NSF requires the following statement, to which I agree:

I certify to the best of my knowledge that this funding commitment will be used by NSF in evaluating the commercial potential of the firm's innovation and, therefore, will be a significant factor in determining if the STTR Phase II proposal will be funded. I further understand that willfully making a false statement or concealing a material fact in this commitment or any other communication submitted to the NSF is a criminal offense (U.S. Code, Title 18, Section 1001).

As you know, I am not a rich man, but I am convinced that you have a unique and valuable technology which will be successfully utilized in a range of bio-sensing products -- with enormous commercial potential. I will sell stock and other investments to generate the funds for the PSI stock purchases.

I have no doubt you will secure the needed corporate partnerships and other agreements -- your existing negotiations with four major firms in the food/dairy, consumer, health, and biotechnology industries should prove successful.

I look forward to continuing to serve on your Board of Directors and in doing all I can to assist the development and commercialization of your unique technologies.

Merry Christmas!

Sincerely

J.D. Andrade

cc: R. Scheer

Protein Solutions, Inc. *Sensing Innovators*

January 24, 1997

Darryl Gorman
National Science Foundation
4201 Wilson Blvd
Arlington, VA 22230
FAX: 703-306-0337

Dear Darryl,

Thank you for the opportunity to talk with you by telephone the other day regarding Protein Solutions, Inc. and particularly its Phase II NSF STTR DMI 95-31303. Thank you also for your recent telephone response. I am pleased to report that we have been making very rapid progress since the implementation of this Phase II award on October 1, 1996. We expect to have a functioning pre-prototype device for inclusion with our six month progress report prior to April 1, 1997.

I will be in Washington DC on April 28 and 29 and, if your schedule permits, Rick Van Wagenen and I would like to meet with you and to personally demonstrate this unique technology. Rick joined us earlier this year as Vice President of Research and Development. He has extensive experience in the medical device industry, having served as Director of Instrumentation Development at Biomaterials International and then V.P. of Research and Development at Albion Instruments, both Salt Lake companies. When Albion was acquired by Ohmeda Medical, Rick stayed on as Director of Research and Development and managed a group of twenty-four scientists and engineers with an annual budget of several million dollars. His group developed Rascal Raman spectroscopy based anesthesia and respiratory gas monitoring systems. He also has extensive experience in bioengineering and biomaterials. His post doc was done in a clinical pathology group. He is very knowledgeable in optics and low light level detection. He thus has an ideal set of skills and interests which match PSI's research and development and product development goals.

That is all good news. The bad news is that Dr. Robert Scheer, Principal Investigator on the Phase II STTR grant, has been offered a teaching position in the Department of Materials Science at the University of Utah. Rob, as you may recall, had been working part time at the University for a number of years. He is an absolutely outstanding teacher and interacts and works very well withstudents and faculty. Although he has done an outstanding job with PSI, for his own career development goals, I have not discouraged him from accepting the job at the University. That is a two thirds time position; he would spend the remaining third of his time continuing to work as a research scientist at Protein Solutions on the Phase II STTR and related projects. Unfortunately, since he would be less than half time with PSI, he would no longer qualify to serve as a principal investigator.

I, therefore, request a change in principal investigator of DMI 95-31303 from Dr. Robert J. Scheer to Dr. Richard A. Van Wagenen, effective as soon as possible. Rick Van Wagenen's biographical sketch and vita are enclosed.

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Protein Solutions, Inc. *Sensing Innovators*

The good news is that Rob will continue to work closely with work and other members of the team on this project. Although his level of effort will certainly be less than if he were to continue to serve as PI, it will still be very substantial. Rob's expertise and his background will continue to be applied to the project.

The other good news is that Rick Van Wagenen has an array of experience and skills almost ideally suited to running this project. In many respects he, frankly, is even more competent and qualified than Robert Scheer, particularly in light of his extensive industrial experience. Rick's signature is included on this letter so that it can also serve as the document that attests to his willingness to accept principal investigator responsibilities on the ongoing Phase II grant. Rob Scheer has also signed the letter attesting to his need to resign as PI , but to continue to serve in a part time capacity.

In light of these suggested personnel changes, changes are needed in the Personnel Salaries and Wages component of the budget, reflecting primarily the change in PI and the percentage efforts of Scheer and Van Wagenen (see below).

Please let me know if you need any further information. Rick and I look forward to meeting with you in late April assuming your schedule permits.

We hope and trust that you have a healthy and pleasant new year.

Sincerely,

J.D. Andrade
President/CEO
SS# 565-52-0772

Read and approved by:

Robert J Scheer

R.J. Scheer
Research Scientist
SS# 253-35-1321

Richard A. Van Wagenen

R. Van Wagenen
V.P. Research & Development
SS# 528-64-5667

Encl: Van Wagenen Bios

Budget Addendum:

	cal months	funds
A. Total Senior Personnel	11.9	49,500
Van Wagenen, PI	7	28,000
Scheer		Total: 77,500

All other categories are unchaned

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BIOSKETCH

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Professional Experience

Vice President of Research and Development, Protein Solutions, Inc., Salt Lake City, UT 1996 - present.

RVW Consulting, Salt Lake City, UT 1995-1996.

Director of Research and Development, OHMEDA Medical Systems, Salt Lake City, UT 1990-1994.

Vice President Research and Development, Albion Instruments, Salt Lake City, UT 1987-1990.

Director of Instrumentation, Biomaterials International, Inc., Salt Lake City, UT 1981-1987.

Research Assistant Professor, Department of Bioengineering, University of UT, Salt Lake City, UT 1978-1985.

Post Doctoral Fellow, University of British Columbia, Vancouver, B.C. 1976-1977.

Education

Ph.D., Materials Science and Engineering, University of Utah, 1976.
B.S., Mechanical Engineering, University of Utah, 1971
Associate in Science, Engineering Major, College of Eastern Utah, 1968.

Honors

Member of Pi Tau Sigma, National Honorary Mechanical Engineering fraternity.
Winner of the NASA Innovation Award, 1981.
Recipient of the R & D Award for Innovative Design and Development of the RASCAL II; a laser based respiratory/anesthetic gas monitor utilizing Raman light scattering spectroscopy, 1993.

Representative Patents and Publications:

J. D. Andrade and **R.A. Van Wagenen**, "Process for Conducting Fluorescence Immunoassays Without Added Labels and Employing Attenuated Internal Reflection", January 11, 1983, United States Patent 4,368,047.

R. A. Van Wagenen, J.D. Geisler, D.E. Gregonis and D.L. Coleman, "Multi-Channel Molecular Gas Analysis by Laser-Activated Raman Light Scattering", November 15, 1988, United States Patent 4,784,486.

R.E. Benner, J.D. Andrade, **R.A. Van Wagenen** and D.R. Westenskow, "Molecular Gas Analysis By Raman Scattering In Intracavity Laser Configuration", December 29, 1992, United States Patent Re 34,153.

R.A. Van Wagenen, J.D. Andrade and J.B. Hibbs, Jr., "Streaming Potential Measurements of Biosurfaces", J. Electrochemical Society, 123 1438 (1976).

R.A. Van Wagenen and J.D. Andrade, "Flat Plate Streaming Potential Investigations: Hydrodynamics and Electrokinetic Equivalency", J. Colloid and Interface Science, 76 305 (1980).

R.A. Van Wagenen, D.L. Coleman, R.N. King, P. Triolo, L. Brostrom, L.M. Smith, D.E. Gregonis and J.D. Andrade, "Streaming Potential Investigations: Polymer Thin Films", J. Colloid and Interface Science, 84 155 (1981).

R.A. Van Wagenen and J.D. Andrade, "Potential Sensor Applications of Total Internal Reflection (TIRF) Spectroscopy", Federation Proceedings, 41 1483 (1982).

S.A. Rockhold, R.D. Quinn, **R.A. Van Wagenen**, J.D. Andrade and M. Reichert, "Total Internal Reflection Fluorescence (TIRF) as a Quantitative Probe of Protein Adsorption", J. Electroanalytical Chemistry, 150 261 (1983).

J.D. Andrade, **R.A. Van Wagenen**, D.E. Gregonis, K. Newby and J.-N. Lin, "Remote Fiber-Optic Biosensors Based on Evanescent-Excited Fluoro-Immunoassay: Concept and Progress", IEEE Transactions, Ed-32 1175 (1985).

V. Hlady, **R.A. Van Wagenen** and J.D. Andrade, "Total Internal Reflection Intrinsic Fluorescence (TIRIF) Spectroscopy Applied to Protein Adsorption" in Surface and Interfacial Aspects of Biomedical Polymers Vol. 2 J.D. Andrade, ed. Plenum Press, (New York, N.Y.) 1985, pp 81-119.

J.D. Andrade, **R.A. Van Wagenen**, D.E. Gregonis, K. Newby and J.-N. Lin, "Remote Fiber-Optic Biosensors Based on Evanescent-Excited Fluoro-Immunoassay: Concept and Progress", IEEE Transactions, Ed-32 1175 (1985).

R.A. Van Wagenen, D.R. Westenskow, R.E. Benner, D.E. Gregonis, and D.L. Coleman, "Dedicated Monitoring of Anesthetic and Respiratory Gases by Raman Scattering", J. Clinical Monitoring, 2 215 (1986).

D.R. Westenskow, D.L. Coleman, D.E. Gregonis, K.W. Smith and **R.A. Van Wagenen**, "Laboratory and Clinical Evaluation of a Raman-Scattering, Multiple-Gas Analyzer", J. Clinical Monitoring, 3 312 (1987).

D. R. Westenskow, K.W. Smith, D.L. Coleman, D.E. Gregonis, and **R.A. Van Wagenen**, "Clinical Evaluation of a Raman Scattering Multiple Gas Analyzer for the Operating Room" Anesthesiology, 70 350 (1989).

D. Gregonis, **R. Van Wagenen**, D. Coleman, and J. Mitchell, "A Commercial Anesthetic Respiratory Gas Monitor Utilizing Raman Spectroscopy", J. SPIE 1336 247 (1990).

Protein Solutions, Inc.

391 G Chipeta Way Suite 320 Salt Lake City, UT 84108 Phone/FAX 801-583-9301/801-583-4463

September 30, 1997

Dr. Darryl G. Gorman
National Science Foundation
4201 Wilson Blvd. , Room 590
Arlington, VA 22230

Dear Dr. Gorman:

Enclosed for your review are three copies of our second semi-annual report detailing the progress we have made on the Phase II NSF STTR "Direct Reading Quantitative Biosensors for ATP-Dependent Processes" (Grant No. DMI-9531303). Also enclosed are three copies of the annual commercialization report for this grant.

I would very much appreciate your prompt review and acceptance of these reports. We are a small company and a two week delay in receipt of funds could have a significant impact to our cash flow.

Thank you for your assistance and if you have any questions please feel free to give me a call at 801-583-9301. If I am out of the office please call Dr. Joe Andrade at 801-581-4379.

Sincerely,

Rick Van Wagenen

Rick Van Wagenen, Ph.D.
Principal Investigator

Encl.

cc: J. D. Andrade

031197C.DOC

SMALL BUSINESS TECHNOLOGY TRANSFER (STTR) PHASE II REPORT COVER SHEET

NSF Award Number: DMI-9531303
Project Title: "Direct Reading Quantitative Biosensors for ATP Dependent Processes"
Date: September 20, 1996
Period Covered by This Report: 4/1/97 to 9/30/97
Grantee Name: Protein Solutions, Inc.
P.I. Name: Richard A. Van Wagenen
Grantee Address: P.O. Box 58093 Salt Lake City, UT 84158-0093
Phase I Award No.: DMI-9413561

Please check as appropriate:

■ Semi-annual Report* ☐ Final Report*

* Report content requirements are identified in Article 5 of the SBIR Phase II Grant General Conditions (9/95). This Cover Sheet is required for submission of all reports. Reports should be attached to this Cover Sheet.

Certifications:

I certify that the Principal Investigator currently is ■ , is not ☐ , "primarily employed" by the grantee organization as defined in the STTR Solicitation.

I certify that the work under this project has ☐, has not ■, been submitted for funding to another Federal agency and that it has ☐ , has not ■, been funded under any other Federal grant, contract, or subcontract.

I certify that to the best of my knowledge the work for which payment is hereby requested was performed in accordance with the award terms and conditions and that payment is due and has not been previously requested.

I certify that to the best of my knowledge (1) the statements herein(excluding scientific hypotheses and scientific opinions) are true and complete, and (2) the text and graphics in this report as well as any accompanying publications or other documents, unless otherwise indicated, are the original work of the signatories or individuals working under their supervision. I understand that the willful provision of false information or concealing a material fact in this reprot or any other communication submitted to NSF is a criminal offense (U.S. Code, Title 18, Section 1001).

Authorized Grantee Representative: _____ Date: 9-30-97

P.I. Signature: Richard A. Van Wagenen Date: 9-30-97

NSF Form 1372 (STTR 2/96)

A. Reporting Period: From: April 1, 1997 To: September 30, 1997

B. Total Estimated Expenditures this reporting period: $61,555

C. Cumulative Estimated Expenditures: $126,399

D. Principal Investigator and Key Personnel:

Name and Title	Estimated Level of Effort
1. Richard A. Van Wagenen, P.I.	3.0 person months
2. C. Y. Wang, Research Scientist	6.0 person months
3. Robert J. Scheer, Research Scientist	2.5 person months
4. J. D. Andrade, Consulting Scientist	1.0 person months

E. Subcontractor(s) Utilized and Services Provided:

During this first year period, $38,307 has been billed to date against our subcontract to Dr. Russell Stewart at the University of Utah. The two primary purposes for the subcontract are: (1) provide Protein Solutions, Inc. with a well characterized source of recombinant luciferase and (2) assist in the optimization of all reactants and designs via computer simulation studies in conjunction with lab experimentation. A Turner Model 2020 luminometer in Dr. Stewart's lab was modified such that rapid injection of reagents could be employed to study rates of key bioluminescent reactions relevant to our research and development. The quality and quantity of the recombinant luciferase received continues to meet our expectations.

F. Consultant(s) Utilized and Services Provided:

1. **Dr. Vladimir Hlady**: Dr. Hlady has helped us model the anticipated light flux from our bioluminescent sensors and has utilized his CCD based instrumentation to validate our models and measured results obtained with company equipment. He has continued to assist Mr. Chris Eu in doing the modeling of the luciferase reaction rates.

2. **Dr. Russell Stewart**: Dr. Stewart continues to play an active role in this project by assisting us in properly interpreting results of experiments and in diagnosing problems associated with the purification and characterization of the recombinant luciferase which we utilize in almost all of our studies. He is also playing an active role in assisting Mr. Chris Eu formulate his kinetic analysis of both enzyme reaction rates and mass transport rates in our biosensors. Dr. Stewart has also conducted some preliminary work to validate the use of a silicon PIN photodiode to measure the bioluminescence from our sensors. This work will be continued and expanded during the next funding period at the company's facilities.

G. Identification of Equipment Purchased

During the last semiannual period no equipment was purchased for this project. All of the equipment funds allocated for this grant were expended in the first semiannual period.

H. Technical Progress Report

Project Goal: Develop an inexpensive, direct-reading, disposable ATP biosensor which does not require expensive or sophisticated instrumentation for analysis of analyte ATP. The quantification of the ATP concentration is determined via the spatial distribution of luminescence which is created by controlling the ATP analyte concentration via an ATP filter, i.e., a second ATP consuming enzyme (apyrase) which is spatially distributed in or on the biosensor. The empirical goal is to extend the Phase I ATP sensitivity of 1×10^{-9} grams ATP (1.82×10^{-12} moles ATP) to at least 1×10^{-12} grams ATP (1.82×10^{-15} moles ATP).

1. Maximize Luminescence

a. **Luminescence Enhancers:** The surfactants and polymers: Triton X-100, PEG 5000, Tween 20, and PVP 40000 in conjunction with coenzyme A (at 0.1 mM) were evaluated as enhancers of bioluminescent intensity. The enhancement results are shown in Table I below. Enhancement is significant for relatively high ATP concentrations greater that 1 micro molar, but ineffective below that concentration. We have concluded that the enhancement is not effective for the low concentrations of ATP that need it the most, i.e., extremely low ATP levels involved with bacterial and hygiene testing.

TABLE I. Cooperative Enhancement Effects of Various Surfactants and Polymers

	10^{-4} M ATP	10^{-6} M ATP	10^{-8} M ATP
No enhancers	1	1.0	1.0
Co-enzyme A*	7	1.1	1.0
Co-A + Triton X-100	23	5.4	1.0
Co-A + Tween 20	20	4.7	1.0
Co-A + PEG 5,000	12	1.9	1.0
Co-A + PVP 40,000	19	2.3	1.0

* Coenzyme A added to each test was 0.1 mM. The assay without additives used for luminescence enhancement was normalized to 1.0

b. **Optimize Apyrase Gradient:** The Apyrase gradient for the ATP concentration range of 1 micromolar to 1 millimolar has been optimized. The optimal apyrase gradient for effective spatial resolution is specified in Table II below.

TABLE II Range of apyrase gradient for the milli- and micro molar range of ATP

Apyrase gradient in U/ml for development of optimal ATP spatial resolution

Apyrase (U/ml)	0.1	0.025	0.0025	0.00005
milli molar ATP	1	0.1	0.01	0.001

c. **Minimize Reagent Activity Loss During Gel Drying:** Efforts to minimize reagent loss during the actual gel drying process have been unsuccessful to date. Evidence suggests that the luciferase remains stable during both gel drying and storage and that overall activity loss is due primarily to oxidation of luciferin. During the next phase efforts will be focused upon lyophilization of the gel and other reagents to more effectively minimize activity loss. We will also be drying selective mixtures of the gel and reagents and then adding either luciferin or luciferase to more definitively determine which component is contributing to the overall activity loss. During this period gel drying studies were also carried out to optimize the efficient drying of the agarose gel matrix under various conditions of temperature and humidity. Results indicate that our standard forced air, connective drying at room temperature and 40%-50% RH removes 90 % of the gel water in four hours of drying followed by and additional 8 % of the gel water with an additional 24 hours of room temperature drying.

d. **Kinetic and Mass Transport Analyses:** These studies were limited to solution studies of luciferase kinetics in a rapid injection luminometer because the actual design of the biosensor has not yet been finalized. A comprehensive literature review of luciferases was conducted that addressed the variation in reaction kinetics and stability for all luciferases published to date. It was determined that both more stable luciferases and luciferases with different spectral emission maxima exist, however, there does not seem to be much choice in alternative luciferases having a higher reaction rate towards luciferin.

e. **Evaluation of Alternative Luciferases:** We have evaluated a commercially available monomeric (61 K Daltons) recombinant luciferase recently introduced by Promega. It is provided in 25 mM Tris-acetate buffer (pH 7.8) with 1 mM EDTA, 1 mMDTT, 0.2 M ammonium sulfate, 15% glycerol, and 30% ethylene glycol. The batch evaluated showed a very high protein concentration of 13.33 mg/ml (about a factor 10 X that of our BCCP-luciferase) and unlike ours it is apparently not histidine-tagged. The specific activity as determined by our in house assay suggests that it has about twice the activity of the luciferase produced for us by the Russell Stewart subcontract. It is, however, somewhat more expensive that the luciferase we utilize, but it may certainly be a more cost effective alternative in any commercial biosensor.

f. **ATP Recycling - Increase Luminescence Duration:** No effort was directed to this topic during the last phase. This will receive attention during the fourth semi-annual funding period. We do, however, know from our monitoring studies with the CCD camera that once the aqueous ATP sample is added to the dried gel bioluminescence begins within about a minute (as the gel rehydrates) and continues with considerable intensity for more than one hour. Modeling studies suggest that for our standard gel and reagents and even micro molar concentrations of ATP, the total number of photons produced over a one hour period (as all of the ATP is consumed) is extremely large. Visual observation and even photographic recording captures only a small fraction of the total bioluminescent photons available.

2. Optimize Detection

We continue to utilize luminescence standards from Biolink Ltd. in the U.K. These standards provide discrete luminous emissions from each 4 mm diameter channel (five different light intensity channels per standard block) which are stable and well quantifiable over the range of $1 \times 10^{+4}$ to $1 \times 10^{+11}$ photons/second/sterradian.

a. **Human Visual Perception Testing:** This study was designed and conducted to determine the light intensity variation necessary for someone to differentiate between a visual perception of light versus no light emanating from each of ten channels of two Biolink light standards. Figure 1 shows the two Biolink Standards, No. 82 and No. 70 used in the study along with the quoted photon flux from each of the ten channels. Twenty subjects ranging in age from 19 to 52 were surveyed. Three ambient environment light flux preconditionings were also studied, i.e., (1) bright, sunny outdoor lighting (about 20,000 lux), (2) typical office environment (approximately 100 lux) and a dimly illuminated room (about 1 lux). All subjects were placed in each of three preconditioning environments for five minutes prior to observing the luminescent standards (sealed in a light tight housing with face mask interface) in a room environment of about 100 lux.

The results are summarized in Table III. All subjects could detect channels 82-1 and 82-2 with five minutes or less of dark adaptation. While no one was able to detect channels 82-5, 70-4 or 70-5 with 5 minutes of dark adaptation. Since everyone tested could see channel 82-2 and no one could see channel 82-5 and the intensity range between these two channels as measured with the CCD camera was 1,200 one may conclude that over the entire range of ages and preconditions evaluated to date a three order of magnitude difference in light intensity is necessary for all subjects to identify one position as "on" and another as "off". Our literature review of human visual response to low light fluxes has indicated that there is a very wide range in detection of low light flux. The primary factors are: physical health, diet, age, time taken for dark adaptation (full dark adaptation requires about 30 minutes) and the flux of ambient light exposure prior to attempting low light flux detection. The latter two variable being the most dominant and most variable in the population at large.

b. CCD Camera Characterization

During this period the CCD camera has been characterized in terms of response linearity, dark background noise and minimum detection threshold limit (MDL) for ATP. For ATP samples analyzed in 350 microliter wells of a 96 well white polystyrene microtitre plate the linearity range was found to be 10^{-8} to 10^{-3} molar ATP which corresponds to 10^{-13} to 10^{-7} moles of ATP with a signal integration time of 300 seconds being necessary at the lowest ATP concentrations. Significant signal non linearity sets in below 10^{-13} moles ATP due to the ubiquitous presence of ATP as a universally present contaminant. Since the CCD seemed to be capable of a lower MDL for ATP we attempted to remove the residual ATP contamination. We have developed a means of immobilizing apyrase onto 3M Emphaze beads (copolymer of vinyldimethyl azlactone and methylene-bisacrylamide) in a column. Thus far about 20% of the apyrase from a 500 microliter apyrase solution (1 mg/ml) can be immobilized on an ATP purification column which can in turn remove up to 80% of the ATP in a 10^{-10} molar ATP test solution.

The CCD camera exhibits very good linearity in the 0 - 100 micro molar range and the 0 - 100 milli molar range of ATP. This is encouraging for ATP based sensor platforms that can in turn be coupled to other biochemical reactions linking metabolism of sugars, amino acids, vitamins and hormones.

c. Comparisons of Visual, Photographic, and CCD Detection of Bioluminescence

A film evaluation study was conducted to determine: (1) the range of light intensities appropriate for film exposure and (2) the resolution capabilities of commercially available Polaroid film which could be incorporated into a biosensor, i.e., Polaroid 20,000 ASA and 3,000 ASA. As with the visualization study above Biolink standards # 82 and # 70 were used as reference light sources. The two film types were exposed to the Biolink standards for increasing periods of time in an attempt to detect the lower intensity channels in the standards. The dimmest standard detected with ASA 3,000 was 82-5 which required an integration time of about 25,000 seconds (7 hours). Channel 70-4 was detectable with ASA 20,000 and an integration time of about 72,000 seconds (20 hours). Such long times are not practical for a disposable, easy to use dipstick type biosensor. We have concluded that a carefully controlled exposure time would allow differentiation between a narrow range of light intensities and thus a narrow range of ATP analyte concentrations which could be detected.

Table III summarizes the comparative results of CCD, Film and human visual detection for the ten Biolink channels. Also, note that the corresponding ATP sensor configuration is included. The overall conclusion is that human visual detection is only feasible in the high micro molar to milli molar ATP range while signal integration for several minutes on commercial Polaroid film is practical down to the low micro molar ATP range. CCD detection is much more sensitive and quantitative and extends down to at least the 10^{-8} molar range of ATP.

TABLE III Comparative CCD Signal, Visual and Photographic Response to Biolink Luminescence Standards.

Biolink Channel	Normalized* CCD Response	3000 ASA Polaroid Film	Visual Response	ATP Sensor Configuration
82-1	111,000	< 10 seconds	Visible to all	High milli molar range
82-2	12,000	<10 seconds	Visible to all	Low milli molar range
83-3	2,060	<10 seconds	Visible to some	High micro molar range
70-1	569	~10 seconds	Visible to some	Low micro molar range
82-4	114	~200 seconds	Visible to some	Low micro molar range
70-2	103	~200 seconds	Visible to some	sub micro molar range
70-3	10	~25,000 sec	Visible to some	" " " "
82-5	9	~25,000 sec	Not visible	" " " "
70-4	1	Not detected	Not visible	" " " "
70-5	0.1	Not detected	Not visible	" " " "

* Note that the CCD was able to detect signal in all ten channels of the two Biolink standards. Channel 70-5 was the lowest intensity but was detectable using a 100 second integration with the CCD camera.

3. Design, Construct and Evaluate Sensor Prototype

a. Current Prototype Design: Shortly after the beginning of the second semiannual period we refocused our sensor development efforts away from a cellulose paper base material and onto a glass based material. We have utilized a commercially available substrate made by Cel-Line, Inc. The substrate is a 1" x 3" or a 2" x 3" glass microscope slide which employs a hydrophobic ink pattern deposited on one side of the slide. See Figure 2. The pattern can be designed by the user and may consist of any arrangement of hydrophilic glass surface patterns defined by hydrophobic ink in a wide variety of colors. The format of the evaluation slides used thus far consists of a blue hydrophobic mask which defines 12 hydrophilic glass circles (4 mm or 6 mm diameter). The agarose gel plus reagents are deposited on the circular hydrophilic areas where they are allowed to gel at 4 C and then air dry.

b. Optical Signal from Current Prototype: The signals from the ATP platform sensor prototype have been evaluated for a range of ATP in the micro molar to milli molar range. The sensor design has been evaluated for white, black and a mirrored background below the microscope slide. The white background was most appropriate in terms of reflecting bioluminescence back up toward the CCD camera, film or eye. The black background is most appropriate for minimizing signal cross talk between the different dots. In the next phase the overall glass slide platform and hydrophobic pattern and background will be optimized to eliminate a small amount of cross talk which still exists while maximizing signal.

c. Prototype Stability Studies: A more rigorous, second round of long term stability studies was initiated during the second six month period to determine the best storage conditions for our luciferase-luciferin, glass slide based ATP biosensor. The reagent-agarose gel system is the same as specified in the previous report with the exception that trehalose has been replaced by 10% sucrose as the protein stabilizer. Thirty micro liter droplets of reagent-gel solution were deposited on the surface of our glass slide biosensor, gelled at 4 C for 30 minutes and then dried for four hours under a laminar flow hood using fan forced air flow. There are five storage conditions being evaluated: (1) dry, desiccated nitrogen gas at 4 C (N4), (2) dry, desiccated nitrogen gas at 25 C (N25), (3) dry, desiccated air at 4 C (A4), (4) dry, desiccated air at 25 C (A25) and (5) a negative control - room air at a room temperature of about 25 C (O). Samples are stored for the following storage times: 0 hours, 24 hours, one week, two weeks, 4 weeks, 8 weeks, 16 weeks, 6 months, 8 months, 10 months, and 1 year. As in the previous studies there is an initial activity loss of about 50% during the first two weeks of storage followed by a stabilization of activity. The best condition of storage is desiccated nitrogen at 4 C and the worst is the negative control. These results are consistent with our expectations.

I. Research and Development Priorities for the Third Semiannual Period

1. Maximize Luminescence

a. Minimize reagent activity loss during drying via lyophilization of gel support matrix. Compare results to current convective air drying.

b. Incorporate both coenzyme A and Triton X-100 detergent into the sensor design. The detergent may be difficult to do since it will probably wet the surface of the hydrophobic ink which currently restrains the spread of our hydrophilic gel drops.

c. Design the gel matrix such that it wets rapidly and liberates the bioluminescence early in the signal transduction process.

d. Continue to evaluate novel recombinant luciferase enzymes should they become available. Enhanced kinetics, lower cost and higher stability are desirable.

2. Optimize Detection

a. Continue work on interfacing photographic Polaroid film to the ATP biosensor designed to function in the low micro molar to high milli molar range.

b. Evaluate the use of a silicon PIN photo diode and imaging optics as an alternative to the CCD for detecting the bioluminescence.

c. Continue to optimize the apyrase removal column. Success in this will allow us to then test the linearity and threshold sensitivity of detection for ATP to concentrations much lower than 10^{-13} moles.

d. Investigate the possibility of light to photocurrent transduction approach where the cummulative photocurrent could generate an electrochemical reaction product visually discernable in room light, thus integrating many more bioluminescent photons.

3. Design, Construct, and Evaluate Sensor Platform

a. Finalize the design of the glass microscope slide based ATP platform for optimal signal, no channel cross talk and good linearity.

b. Determine signal levels and linearity with film and CCD.

c. Test the concept of spatial distribution of light with the optimized apyrase gradient in terms of CCD, film and human visual response in the dark.

Biolink Standard and Channel #	Company quoted luminous flux (photons/s/sterradian)
82-1	8.17×10^{10}
82-2	7.78×10^{9}
82-3	1.21×10^{9}
70-1	0.88×10^{8}
82-4	7.54×10^{7}
70-2	1.51×10^{7}
70-3	5.15×10^{6}
82-5	1.44×10^{6}
70-4	1.37×10^{5}
70-5	1.38×10^{4}

Standard # 82 Standard # 70

Figure 1. Biolink standards #80 and #72 used for calibration and comparative studies between visual, photographic and CCD detection.

Figure 2 Current ATP platform sensor design based on a hydrophobic blue ink pattern deposited on a 1" x 3" microscope slide.

SMALL BUSINESS TECHNOLOGY TRANSFER (STTR) PHASE II REPORT COVER SHEET

NSF Award Number: DMI-9531303
Project Title: "Direct Reading Quantitative Biosensors for ATP Dependent Processes"
Date: September 20, 1996
Period Covered by This Report: 4/1/97 to 9/30/97
Grantee Name: Protein Solutions, Inc.
P.I. Name: Richard A. Van Wagenen
Grantee Address: P.O. Box 58093 Salt Lake City, UT 84158-0093
Phase I Award No.: DMI-9413561

Please check as appropriate:

■ Semi-annual Report* ☐ Final Report*

* Report content requirements are identified in Article 5 of the SBIR Phase II Grant General Conditions (9/95). This Cover Sheet is required for submission of all reports. Reports should be attached to this Cover Sheet.

Certifications:

I certify that the Principal Investigator currently is ■, is not ☐, "primarily employed" by the grantee organization as defined in the STTR Solicitation.

I certify that the work under this project has ☐, has not ■, been submitted for funding to another Federal agency and that it has ☐, has not ■, been funded under any other Federal grant, contract, or subcontract.

I certify that to the best of my knowledge the work for which payment is hereby requested was performed in accordance with the award terms and conditions and that payment is due and has not been previously requested.

I certify that to the best of my knowledge (1) the statements herein(excluding scientific hypotheses and scientific opinions) are true and complete, and (2) the text and graphics in this report as well as any accompanying publications or other documents, unless otherwise indicated, are the original work of the signatories or individuals working under their supervision. I understand that the willful provision of false information or concealing a material fact in this reprot or any other communication submitted to NSF is a criminal offense (U.S. Code, Title 18, Section 1001).

Authorized Grantee Representative: [signature] Date: 9-30-97

P.I. Signature: Richard A. Van Wagenen Date: 9-30-97

NSF Form 1372 (STTR 2/96)

National Science Foundation
Phase II STTR DMI 95-31303

Direct Reading, Quantitative Biosensors for ATP-Dependent Processes

Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, Utah 84158-0093

Year 1 (12 month) Commercialization Progress Report

September 30, 1997

Contents

Technology Status

A bioluminescence spatial gradiant technology was conceived and very preliminarily developed by the University of Utah several years ago. This technology served as the basis for this STTR program. PSI acquired an exclusive license to this technology prior to the submission of the Phase I STTR application. Since then a formal patent application has been filed by the University of Utah, December 1996, and is now pending. Foreign country applications include Europe, Canada, and Japan.

During the past year PSI has made considerable progress in the development of this unique concept to a viable, commercializable technology. Please refer to the Phase II, 12 month, Technical Progress Report for details.

In summary:

1. We have demonstrated the feasibility and effectiveness of the spatial gradient approach as a generic ATP detection platform which should be suitable for a wide range of biochemical analytes.
2. We have applied the technology to a shallow multiple well format which lends itself to application in laboratories and point of care testing facilities that are today almost exclusively based on multi well format devices.
3. We have optimized and enhanced the spatial gradient design to provide reliable, unambiguous readouts in a variety of concentration ranges.
4. We have evaluated the suitability of the human eye, of photographic film, and of electro-optical detection for the detection part of the sensors and have established which ATP analyte concentration range is suitable for each of those detection modalities. For example, direct visual detection is appropriate in a millimolar range; photographic detection, from submillimolar to micro molar range; and CCD or related electro optical detection, from micro molar ATP on down.

We have continued to evaluate the stability of air dried enzyme/gel preparations in order to design a device with appropriate shelf life. In year two of the Phase II grant, we are extending those studies to include lyophilization.

We have evaluated one commercial recombinant luciferase as well as continued to produce and develop our own recombinant luciferases via our subcontract with the University of Utah.

We have evaluated and optimized a range of low molecular weight, soluble, enhancers of bioluminescence as part of our objective of optimizing and enhancing the bioluminescence signal.

We have looked at the potential interactions between the multiple enzymes involved in our sensor prototypes, as well as substrate cross reactivity. Although these studies are on going, the results show that by appropriate selection of enzymes, the cross reactivity issue is greatly minimized.

We are now expanding our studies on the NADH-based sensing platform, in collaboration with the University of Utah using other funds.

During year two we will be extending our sensor tests to biological samples, particularly urine, as we move towards the prototype and extensive testing stage of the work.

We have studied the patent situation dealing with recombinant luciferases, concluding that it may be more cost effective to simply purchase commercial recombinant luciferase for our purposes. In addition, some new and novel recombinant E coli. technology has now become available for licensing, which may indeed lead to such extensive over expression of luciferase and ease of purification that the costs of this critical reagent will likely be negligible.

We are continuing to evaluate the patent status of the various analytes we are targeting.

In summary, during the past year we have established that the technology is indeed robust, sensitive, reliable, and applicable to a very wide range of analytes of interest in medicine, health, and wellness evaluation.

Market Analysis and Assessment

The activities of this STTR are focused on the development and prototyping of a generic ATP sensing platform, using our unique spatial distribution technology.

ATP, adenosine triphosphate, is the ubiquitous energy currency for all of life on the planet. When we talk of life on other worlds and use the phrase "life as we know it," we are basically referring to life which utilities two key energy transduction/transformation molecules, ATP and NADH. ATP is the basis of the firefly luciferase bioluminescence reaction. NADH is readily coupled to the bacterial luciferase, bioluminescent reaction. Thus, these two molecules can be directly monitored by highly enzyme specific bioluminescence process.

PSI is, to our knowledge, the only company committed to using these two unique processes as the basis for a range of sensors for diagnostic and clinical chemistry for direct consumer use.

The figure (next page) presents PSI's "problem." The figure shows a smiling, empowered patient. Overlaying this future customer is most of the markets in which PSI's sensor products could be distributed.

The left half of the diagram represents blood as the sample solution. Blood is the sample of choice for most clinical laboratory and point of care testing and even home monitoring today. There is growing interest, however, in utilizing non-invasively derived samples, especially urine, but now also to include saliva and sweat, for those tests where such samples can be shown to be suitable. They are represented on the right side of the diagram.

The upper half of the diagram represents those applications highly regulated by the FDA's invitro diagnostics (IVD) activities. Here we represent all tests and analyses which are important in medical diagnosis and treatment, ranging from the neonate and newborn, particularly inborn errors of metabolism, to the monitoring of critical metabolites and analytes in the aged. Age related problems roughly move clockwise from the top center towards the right, in the case of non-invasive samples, and counter clockwise from the top center to the left for blood samples. In-between the newborn and aged are the diseases and problems which are found in childhood, teenage , middle age, and so on.

Many of these analytes are not amenable to PSI's ATP/NADH based detection technologies. Many are more appropriate analyzed by immuno assay, which is a well developed and highly competitive field. But as we have noted before, those very analytes which are most amenable to ATP/NADH based sensing are those which are absolutely basic to metabolism and those for which immuno assay is generally impractical or impossible.

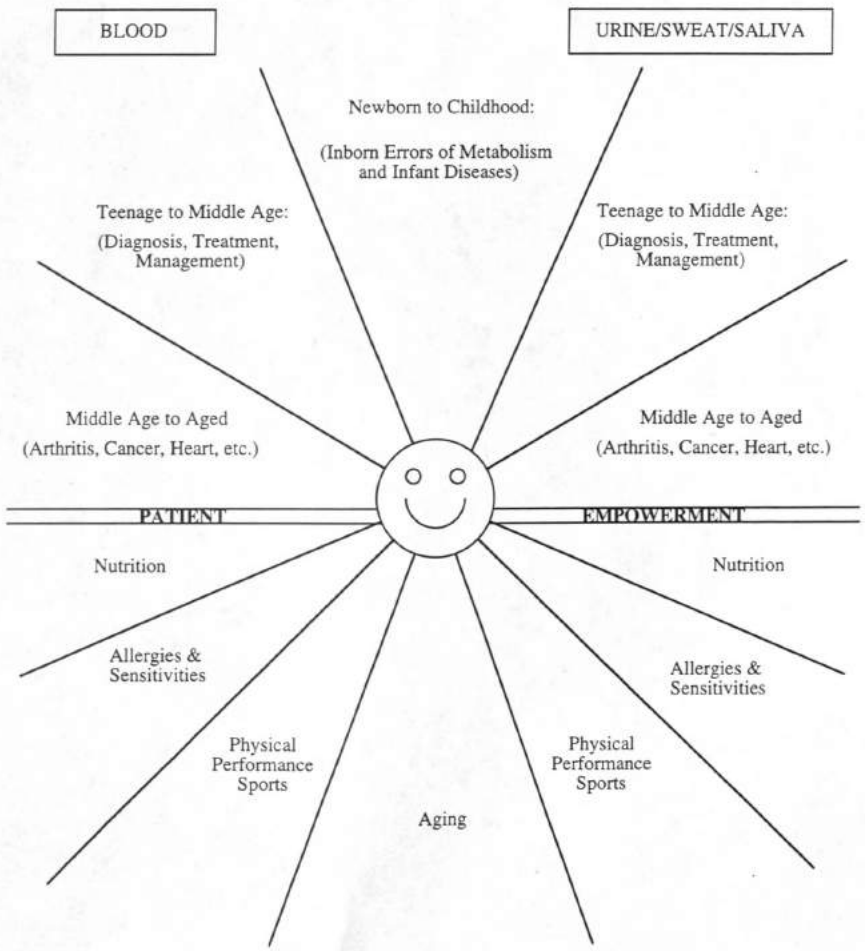
The bottom half of the diagram represents those opportunities not directly connected to diagnosis and medicine, and therefore minimally regulated by the FDA and related agencies. This represents the so called "wellness" arena, as well as sports and physical performance. In this bottom half, the representation is not based on age, but rather on specific application areas, including nutrition, food allergies and sensitivities, the issue of enhancement and physical performance, and finally, those applications related to aging and particularly the metabolic changes requiring monitoring and perhaps supplementation in the aged.

We are now defining the key analytes in each of the sectors of the figure with an emphasis on urine, a secondary emphasis on sweat, and a tertiary emphasis on blood. The markets are hard to estimate, largely because the patient empowerment/consumer market has only been developed for the monitoring and maintenance of diabetes. Patient monitoring and management of other chronic metabolic diseases, such as phenylketonuria (PKU) and galactosemia, two important errors of metabolism, has not developed due to the unavailability of any home tests for the appropriate analyte. This can all change dramatically with PSI's sensors.

Working with our own advisors and consultants and in close collaboration with the University of Utah's new program on cost effective health care technologies, funded by the Whitaker Foundation, we are now working with a wide range of clinicians and health care providers to identify those analytes and application areas which are most effective for patient empowerment and for the enhancement of cost effective health care.

During the next six months we will work closely with nutrition experts, with food allergy and sensitivity experts, with the physical performance and sports community, and with the gerontology community do develop market focus and estimates. All of this significantly effects PSI's decisions as to potential corporate partners and its own specific manufacturing and marketing directions.

DIAGNOSIS AND MEDICINE (FDA)



WELLNESS & PERFORMANCE (less FDA)

Partnering Analysis and Commercialization Plans

1. Cardiac Monitoring:

There is growing interest in chronic markers or predictors of heart disease. One of the most important and popular today is the amino acid metabolite homocysteine. Homocysteine can be measured and monitored in clinical laboratories, using liquid chromatography and mass spectrometry techniques. It is not particularly amenable to immuno assay and no simple test is available. Admittedly, one can develop a colorimetric enzyme-based assay for homocysteine, but the concentrations involved and the precision required make this a difficult route.

Elevated homocysteine levels correlate with a significantly increased incidence of cardiovascular disease in the later years. This elevated homocysteine level can often be corrected by appropriate vitamin supplements, hence there is a need to not only measure homocysteine, but to also measure the various vitamins and co-factors involved in homocysteine chemistry. Because most of the existing cardiac markers are proteins or protein fragments analyzed by immuno assay, those companies now involved in this field have little or no experience in low molecular weight metabolite analysis, such as homocysteine.

We have entered into very preliminary discussions with two local immuno assay manufacturers of cardiac marker panels. Our detection technology for homocysteine is based on a methionine ATP assay. Therefore, we have focused our efforts on obtaining access to the methionine enzymes and demonstrating feasibility of methionine analysis by ATP depletion using our bioluminescence ATP platform. Such feasibility has already been demonstrated.

During the remaining twelve months of the Phase II project, we will focus part of our effort on the homocysteine "front end" of the methionine sensor. If this goes as expected, we will have demonstrated feasibility and indeed have produced a prototype of a direct reading homocysteine sensor based on ATP bioluminescence.

In anticipation of this success, we will continue our discussions and negotiations with companies who can serve as a corporate partner in this area.

Although homocysteine for adult, chronic, cardiovascular monitoring is a very important problem and market opportunity, homocysteine is also an inborn error of metabolism. For this newborn application, the sensing requirements are much less stringent because the detection level in a newborn with homocysteinuria are ten times that of an adult interested in homocysteine as a chronic cardiac marker. That thus leads to a second market opportunity for the homocysteine sensor, as part of a panel for the diagnosis and monitoring of in born errors of metabolism. We are seeking SBIR and corporate partner support for the development of sensors for each of the major metabolic diseases found in newborns. These will involve our existing ATP detection platform as well as our developing NADH platform.

2. Infant Metabolic Disease Management

Although the detection and diagnosis of inborn errors of metabolism is not a problem in most of the United States and indeed in most of the developed world, the chronic monitoring and management of the diseases requires home based technologies. The two such applications targeted by PSI are phenylketonuria (PKU), which requires the analysis of phenylalanine and its metabolites in blood and/or urine.

A second problem of much lower incidence is galactosemia which requires the analysis of galactose and its metabolites in blood or urine.

We are now working closely with obstetricians and pediatricians to assess the practicality of monitoring the status of these diseases using analytes present in urine.

The beauty of urine is that it is easy to obtain in large volume and very efficient collection systems are available, particularly for infants. The diaper industry is incredibly competitive, quite technological, and is the simplest and most effective way to obtain routine urine samples.

We have entered into preliminary discussions with a very major diaper manufacturer about the possibility of developing a "intelligent" diaper which would actually have our direct reading sensors incorporated into a region of the diaper, somewhat remote from, but connected via capilarity fluid transport, to the urine source. Given the analyte concentrations involved and the problems in handling and "reading" a diaper, we are considering different means of presenting the concentration signal, although in all likelihood it will still use spatial presentation, which has been the heart of PSI's sensor development efforts.

Although PSI would likely develop and market more conventionally applied urine dipstick sensors which can utilize the direct bioluminescence readout, a diaper version would be so significant, practical, and profitable that it merits early consideration at this time.

3. Urinary Tract Infection

ATP analysis is a sensitive indicator of biomass. It is most widely used today for the measurement of total bacterial populations for hygiene monitoring. It has been used to diagnosis urinary tract infection by appropriate pre treatment of somatic cells as part of our urine analysis and potential diaper sampling interests and activities. We expect to further develop our capabilities for the use of the ATP sensor to detect bacterial concentrations in urine.

PSI is now negotiating with a major multi-national corporation for a significant equity investment which would permit continued funding of growth and bacterial sensing technologies. We are also working closely with Dr. Russell Stewart at the University of Utah (the PI on the Phase II STTR subcontract involved with producing and characterizing luciferases and the other enzymes needed for the sensors) on a number of new ideas related to significant enhancement and augmentation of the signal to permit direct bacterial detection.

4. Nutrition

There is growing interest in the nation in vitamins, food supplements, organic foods, and other approaches to health and wellness. The rapid growth of health food stores and natural (or organic) forms of alternative medicine has been dramatic. In spite of all these interests in food supplements, there appears to be little or no interest in the monitoring of the concentrations of the vitamins and other components taken regularly by millions of Americans. The major reason for this lack of interest is the lack of an available technology. These are people who somewhat distrust highly technological medicine and are not particularly interested in having blood or urine samples analyzed via physicians in clinical laboratories. These are people who want to assume personal responsibility for much of their health and wellness. PSI is just beginning discussions with several major providers of vitamins, of herbs, and other food supplements, again in the Utah area, with the hope and expectation that they will indeed be interested in investing, in marketing, and on distributing dipstick urine based sensors for several of their key products, probably several of them very common vitamins, as well as possibly those vitamins involved in homocysteine metabolism.

5. Sports and Physics Performance

PSI is helping to support a lactate sweat analysis sensor activity at the University of Utah using the unique spatial distribution technology. This particular sensor is based on the evolving NADH platform. Given the relatively high concentration of lactate in sweat and the growing interest, especially among the sports/physical performance community, in enhancing personal training and performance, we expect in about six months to seriously enter into discussions with those companies that provide so called power diets or power supplements to enhance physical performance and muscle mass. The best place to get an introduction to this area is by visiting a sports equipment store to look at the "power diet" section of the store.

A lactate sensor based on blood sampling is already commercially available and is used by coaches and trainers to optimize the training of athletes.

Investment and Resources

Although PSI is still very small and largely dependent on SBIR/STTR funds for its on going research activities, the rapid development and uniqueness of our bioluminescence based spatial sensing technologies has begun to attract considerable attention. Some of this was discussed in the above section.

Having a strong technology now in hand, and having a dramatic prototype for demonstration, we expect to generate significant equity investment of several private investors as well as from a number of companies. The companies alluded to above are now being approached with respect to equity investment, as well as for contract R & D support.

PSI is privately held and its existing investors are committed to continuing to provide the resources needed for its ongoing development and expansion. PSI has kept its development efforts and activities relatively quiet during this technology development phase. We expect to have a significant presence next year in major national and international meetings appropriate to the dissemination and application of our unique sensors and sensing technologies.

Further Information

PSI does not yet have a web site but expects to be on-line by late 1997.

For further information regarding PSI's plans, directions, and products contact J.D. Andrade, President and CEO, at 801-583-9301.