Appendix A

U.S. ENVIRONMENTAL PROTECTION AGENCY
SMALL BUSINESS INNOVATION RESEARCH PROGRAM
SOLICITATION NO. D500001M1
PROPOSAL COVER SHEET

COPY

Joe - FYI - pls rtn to file. thx!

EPA USE Only

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Proposal Title: Continuous, Real Time Enumeration of Airborne Microorganisms

Firm Name: Protein Solutions, Inc.

Mailing Address: 350 West 800 North, Suite 218

City: Salt Lake City State: Utah Zip Code: 84103-1441

Amount Requested: $64,820, Proposed Duration (Phase I): 6 months
(Not to exceed $65,000)

____ A. Drinking Water Treatment
____ B. Municipal and Industrial Wastewater Treatment and Pollution Control
____ C. Prevention and Control of NOx, VOC's SO2 and Toxic Air Emissions
____ D. Solid and Hazardous Waste Diposal
____ E. In-Situ Treatment of Hazardous Waste at Superfund Sites
____ F. Removal of Heavy Metals at Superfund Sites
____ G. Indor Radon Mitigation
____ H. Pollution Prevention
X I. Continuous Monitoring of Processes for Compliance and Control Effectivity Determination
____ J. Prevention and Control of Indoor Air Pollution

CERTICATIONS AND AUTHORIZATIONS

Answer Y (Yes) or N (No)

Y 1. The above concern certifies that it is a small business concern and meets the definition as stated in the program solicitation.
Y 2. The above concern certifies that a minimum of one-half of the research and/or analytical effort will be performed by the proposing firm.
Y 3. If the proposal does not result in an award, is the Government permitted to disclose the title and technical abstract page of your proposed project, and the name, address, and telephone number of the official of the proposing firm to any inquiring parties?
N 4. The above concern certifies that it is a woman owned small business concern and meets the definition as stated in the program solicitation. *
N 5. The above concern certifies that it is a socially and economically disadvantaged small business concern and meets the definition as stated in the program solicitation. *
N 6. Do you plan to send, or have you sent, this proposal or a similiar one to any other Federal Agency? If yes, which one? Use acronym(s) for each agency (e.g.) DOE, NIH, NSF, DOD, NASA etc. ____

* For information purposes

ENDORSEMENTS

Principal Investigator (See Requirements in Sec. I, Part C.) Type Name, Indicate Mr., Ms., or Dr.	Corporate/Business Official Type Name, Indicate Mr., Ms., or Dr.
Dr. Philip M. Triolo	Dr. Joseph D. Andrade
Title: President, PSI	Title: C.E.O., PSI
Telephone: (801) 596-2675	Telephone: (801) 581-4379
Signature of Principal Investigator [signature]	Signature of Corporate/Business Official [signature]
Date: 1/18/95	Date: 1/19/95

PROPRIETARY NOTICE

For any pupose other than to evaluate the proposal, this data shall not be disclosed outside the Government and shall not be duplicated, used or disclosed in whole or in part, provided that if a funding agreement is awarded to this offeror as a result of or in connection with the submission of this data the Government shall have the right to duplicate, use, or disclose the data to the extent provided in the funding agreement. This restriction does not limit the Govenment's fight to use information contained in the data if it is obtained from another source without restriction. The data in this proposal subject to this restriction is contained on pages 4, 6, 16 of this a proposal.

CONTINUOUS, REAL TIME ENUMERATION OF AIRBORNE MICROORGANISMS

Bioluminescent assays for the rapid estimation of microorganisms in collected aerosol samples are inherently inaccurate because the amount of ATP detected is not directly proportional to the number of organisms present in the sample. ATP is rapidly degraded after cell death (1), and organisms killed during sampling are not counted if ATP is inactive. In addition, the ATP content of cells can vary by orders of magnitude (2,3). ATP content is substantially reduced during periods of stress, and also fluctuates as a function of the cells' growth phase (2,3,4) and other factors (5). Consequently, it is impossible to establish an exact correlation between ATP content and cell numbers or biomass.(1) (N.B.: Biomaass, in this context, refers to the total amount of living matter present in the sample.)

This proposal suggests a means for eliminating these sources of error when employing a bioluminescent technique to quantifying the total microbial content of sampled air. Instead of determining total ATP content to obtain an estimate of biomass, the release of ATP and subsequent localized light generation by each individual organism will be detected. Each detectable light generating event represents the presence of one organism, assuming no interferences from non-microbial soources of ATP. Thus, there is no need to make assumptions about the average ATP content of a typical bacterium under specified collection conditions in order to arrive at an estimation of biomass. Each detectable emission of light- regardless of its intensity (representative of ATP content)- represents the presence of one organism.

Additionally, collection and sampling will occur simultaneously. Continuous, nearly real time monitoring is possible, and damage to cells incurred during storage and transport of samples to the microbiology lab will be eliminated.

The firefly luciferase reaction will be used to monitor ATP released from cells as they impact the collection media. The media will contain all of the reactants for bioluminescence except ATP, which will be provided by the incoming organism. As organisms strike the media (a gel to minimize migration of the impacted cells' released ATP), their released ATP will react with firefly reagents to form a discrete, localized source of light. Each light-producing event will be enumerated, and directly correlated with the existence of matter containing ATP, which, initially, will be considered to be one viable life form.

High-speed film (6) will be used to record the light emitting events, which will be counted to yield microbial content. Each "average" bacterium contains 10^{-15} g ATP (2,3) and the reported detection limit for ATP using high-speed photographic film (Polaroid Type 612, ASA 20,000) with the firefly luciferase-luciferin reaction is 2 pmol ATP(7). ATP contains roughly 500 g/mol, so a cursory analysis of the sensitivity of photographic film for ATP would indicate that the detection limit is 1000 pg, or 10^{-9} grams. This is the ATP content of roughly 1,000,000 average bacteria, and, therefore, the analysis would conclude much greater sensitivity is required in order to detect individual organisms.

However, this analysis may not be valid. It is worthwhile to critically examine the original papers that describe the methods that were used to arrive at a detection limit of 2 pmol for ATP (7,8). The first article (8) describes the experimental arrangement used for the investigation of solid-phase bioluminescent reagents (See Fig. 1.). This configuration was used for the determination of the minimum quantity of ATP that could be detected by high speed photographic film by experiments described in a subsequent article(7).

APPENDIX B

U.S. ENVIRONMENTAL PROTECTION AGENCY
SMALL BUSINESS INNOVATION RESEARCH PROGRAM
SOLICITATION NUMBER D500001M1
PHASE I - FY 1995
PROJECT SUMMARY

Firm Name, Address, Telephone Number:
Protein Solutions, Inc.
350 West 800 North, Suite 218
Salt Lake City, Utah 84103-1441
(801) 596-2675

Title of Proposal: Continuous, Real Time Enumeration of Airborne Microorganisms

Topic Letter (A-J): I

Name and Title of Principal Investigator/Project Manager: Philip M. Triolo

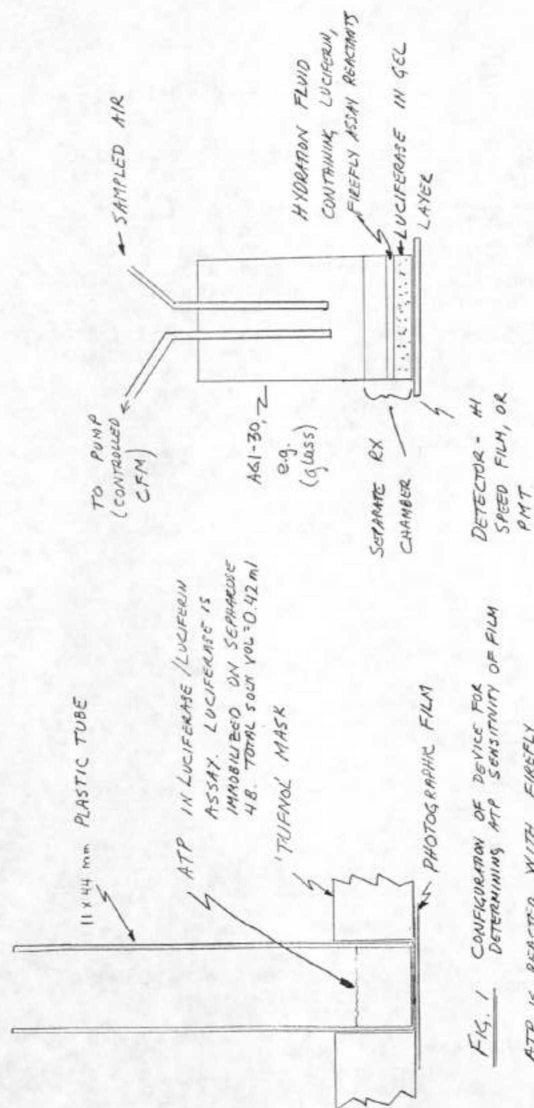
Technical Abstract (Limited to 200 words. Must be PUBLISHABLE):

Current bioluminescent assays for the quantitation of ATP to estimate microbial content of air samples are plagued by several inherent problems. The correlation between ATP detected and the number of bacteria or other microorganisms present is inexact because ATP content varies according to cell type, bacterial strain, and the growth cycle of the organism. Further, under stress, ATP levels decrease, and cell death is followed by ATP inactivation. The aerosol sampling process often kills or damages cells so that their ATP is not detected and, therefore, organisms viable before sampling are not counted.

We propose a method whereby the firefly luciferase bioluminescent assay for ATP is used to enumerate cells as they are collected. Impact of the cells with the collection media is used to destroy cell membranes and the ATP released is immediately reacted with luciferase and luciferin present in the media. The light emitted exposes high speed film. Each exposed area is representative of one microorganism, so a direct count of sampled microbes is obtained.

Anticipated Results/Potential Commercial Applications (both Phases I and II) as Described by the Awardee. (Limited to space provided):

There is considerable interest in and need for rapid, accurate monitoring of airborne microbial concentrations in health care facilities, food, and pharmaceutical preparation areas, microbiology and biotechnology laboratories, incineration sites, waste processing facilities and wastewater treatment facilities. The development of a bioluminescent technique to monitor changes in microbial content of sampled air in real time will be a valuable tool that will find great utility in these, and other, sensitive areas.



To determine the sesitivity of the film for ATP (7), the reaction between 10 ul of a suspension of firefly luciferase *immobilized on CNBr-activated Sepharose 4B* beads and a 10 ul sample of known concentration of ATP in glycylglycine buffer (25 mM, pH 7.8) was initiated by the addition of 400 ul of stock assay solution containing 8 ml of glycylglycine buffer (25 mM, pH 7.8), 1.0 ml magnesium chloride solution (0.1 M, pH 7.8), and 0.8 ml of aqueous luciferin solution (1mM). The reaction vessel was a 11x44 plastic tube (assumed to be polystyrene), whose bottom was in intimate contact with ASA 20,000 Polaroid film (type 612). Thus, 0.42 mls of solution containing reactants were placed in a plastic cuvette, whose bottom surface (0.95 cm^2) was in intimate contact with high speed film.

An analysis of the geometry of the set-up reveals that the height of the 0.42 mls of reaction solution in the 11x44 tube was 4.4 mm. It is doubtful that the light emitted from the upper layers of the solution reached the film, as quenching in the solution would severely diminish this possibility, as would collision with the beads in suspension. Further, the effect of the plastic cuvettes on the transmission of light to the film is unknown. Other losses could occur if the local pH of the solution were changed due to the presence of unreacted CNBr moities present on the Sepharose.

A comparison of the concentration of ATP in this solution with that in a cell supports the possibility that ATP from single organisms could react with luciferase to emit enough light for photographic detection. If one assumes a bacteria has the shape of a sphere with a diameter of 5 u, its volume is 1.56×10^{-11} cm^3. The average ATP content per cell is 1×10^{-15} grams, or 2×10^{-18} moles. The concentration of ATP in a bacterial cell is therefore 1.28×10^{-7} moles / cm^3.

In contrast, the concentration of ATP in the 11 x 44 tube is $2x\ 10^{-12}$ moles (2 pmol) in .42 cm^3, or 4.76×10^{-12} moles / cm^3. The concentration of ATP in the bacterial cell is 25,000 times greater than it is in the solution used to determine that the sensitivity of film for ATP is 2 pmol.

Further, the light from the ATP solution is shed on an area equal to the bottom of the 11 mm dia. test tube, or 0.95 cm^2. The light from the bacterium could be readily visualized if it exposed an area 0.2 mm in dia, or 3.14×10^{-4} cm^2. Thus, the potential light intensity (photons per unit area) from a 5u spherical bacterium whose light is concentrated on a .2 mm dia area of film is 25,000 (concentration factor) times 3000 (area factor) or 7.5×10^7 times greater for the cell when compared with the solution. This intensity is in the range of detectability. (Admittedly, the distribution of light from the bacteria would probably not behave so nicely. However, even if it were dispersed over an area 10 times as great as the 0.2 mm dia circle, the potential for detection by film remains quite high.)

Each cell contains 10^{-15} g ATP, or roughly 10^6 molecules. Therefore, if these molecules can all be reacted with the firefly reagents, there is the theoretical potential of producing 10^6 photons. It appears that there are ample opportunities to improve the efficiency of the bioluminescent reaction and detection so that enough photons strike the film to create an image.

The success of the proposed method depends on reacting as much of the ATP as is possible with the firefly reagents (possibly by interfering with the action of ATPases) and localizing the ATP and the emitted light so that light intensity at the film surface is great,

especially pp 1-72) NRB appears to be one of the better surfactants available, and it will be employed in initial experiments (12).

With improvements of the signal-to-noise ratio of photomultiplier tubes (60,61), the light emitted will be detected in real time by a photon detection system located at the base of the collection apparatus. Each emitting event will be counted as one viable organism. Non-emitting collisions created by particles will not be counted. It is anticipated that high-intensity pulses associated with "somatic" cells, having ATP concentrations 1000 times those found in bacterial cells, will be obvious and can be screened during the counting process. The same screening can occur with visual examinations; exceptionally bright exposure areas can be assumed to originate from eukaryotic cells, and simply not tallied.

The successful completion of this project will guarantee that the greatest numbers of photons are available to the improved PMT's or other photosennsitive device. It represents a parallel effort to those conducted on improving PMT signal-to-noise ratio and sensitivity.

It is anticipated that a good correlation can be made between the number of viable organisms present in a collected sample and the number of light pulses detected. Thus, a method will be available to enumerate microorganisms present in a sampled volume of air in real time at the sampling location. The time lag between collection of organisms and enumeration will be determined by the length of time the film is exposed to the sample chamber, the time required to develop the film, and the time required to count the points of light on the developed film.

Continuous sampling is possible with this method if appropriate measures are made to advance the film through the detection area and to replenish firefly reagents and luciferase gel. Such a continuous system might also require the addtion of an agent to suppress the activity of ATPases. In this scheme, spots would be transformed to short streaks across the film, which could still be enumerated after processing, and automated counting techniques could provide a continuous readout of the numbers of organisms detected.

The advantage of this technique over existing culture methods is that highly efficient air samplers can be employed (cell viability is not a critical issue), results can be obtained at the sampling location in real time, and the analysis can be made at low cost. Further, it is adaptable to continuous monitoring and is not subject to the inaccuracies inherent in existing bioluminescent assays that attempt to correlate ATP content with biomass.

The proposed method is to be used as a screening technique to establish the presence of microbial contamination or changes in microbial concentrations in air samples. It can also be employed to yield real counts of microbes present in a sample. Once the presence of contaminating organisms is established, other methods can be applied for verification and identification of the organisms involved (2,15,17).

2. Phase I Technical Objectives

The specific technical objectives of this project are to:

1) Optimize the concentration of luciferin, luciferase, buffers, and cell lysing agents in the sample chamber to maximize light emission for detection by high-speed photographic film.

thereby ensuring exposure. The above analysis of available data indicates that this is feasible. Further, the purity of reagents has increased since the study was performed, and this will undoubtedly contribute to the possibility of success. It is also highly likely that the immobilized luciferase used to determine sensitivity (7) is not as active as free luciferase, and that the efficiency of the reaction will be higher for luciferase physically immobilized in a gel than for the luciferase chemically immobilized to the Sepharose particles.

The degree of exposure of photographic film is a measure of the total emitted light (7). Lengthening exposure times beyond the thirty seconds employed to determine the reported sensitivity will increase exposure of the film. The kinetics of light emission from the firefly luciferase reaction are governed by the concentration of reactants (9). Increasing the concentration of ATP increases the initial rate of reaction, but, provided other interfering reactions are not present, does not alter the total amount of light emitted by the reaction. The area under the Intensity-time curve remains constant, and increasing the amount of time of exposure increases the total amount of light incident on the film.

Other means of increasing the possibility of producing film exposures that can be detected visually are employing lenses to focus the light from small regions in the sample chamber onto the gel and employing a light-induced enzymatic cascade whereby the emission of one photon from the ATP reaction initiates the further release of photons as a consequence of an enzyme-catalyzed bioluminescent reaction (10). It may also be possible to increase the concentration of intracellular ATP by increasing the oxygen content of the sampling environment (5). These will be investigated in Phase II of the project.

The foundation of the media will be a luciferase-containing gel. PSI has experience fabricating these gels, and has demonstrated that luciferase in the gels is capable of light emission when combined with luciferin-containing reagent and ATP after storage at four degrees C for periods of up to 6 months (11). The rehydration solution for the luciferase gel, which will be added immediately before sampling, will consist of luciferin, magnesium chloride and appropriate buffer to yield a pH of 7.7, where intensity of luminescence is greatest.

Initial experiments will be carried out utilizing an AGI-30 aerosol collector (all-glass impactor with jet located 30 mm from surface of collection media) (16). It will be modified to accommodate the firefly gel and housed in a dark enclosure. In Phase II the sampler will be further modified, or other samplers will be investigated, to optimally damage cell membranes/walls and distribute organisms over the entire surface of the collection media so the organisms and light pulses can be spatially distributed for ease of enumeration on exposed film.

In addition to employing the collection process and impaction of microorganism against the media to release cellular ATP, a suitable surfactant (e.g. NRB, Lumac bv, Landgraff, The Netherlands,(12)) may be employed in the media to release cellular contents. Both mechanical and chemical means of ATP liberation will be investigated.

As the ATP is released it will become available for interaction with the luciferase and luciferin present in the media. The firefly reagents will, of course, compete for reaction with the ATP with ATPases released from the cytoplasm, as well as those present on the exterior cell walls (13). It is desirable to minimize the capacity of ATPases to inactivate ATP. However, as luciferase is itself an ATPase, it is difficult to inhibit the unwanted action of ATPases while not interfering with the action of luciferase. This difficulty is associated with all bioluminescent techniques that attempt to assay ATP from cellular sources, and has been the subject of many investigations (See, e.g., ref. 14,

2) Vary thickness of gel, volume of rehydration fluid, air flow, and distance between inlet jet and surface of media of AGI-30 air sampler to maximize ATP release on impact of cells with media, and provide for reproducible counts of organisms. Released ATP must be confined to a specific area in order that its release and subsequent light emission is highly localized and can be associated with one organism.

3) Determine sensitivity of film and existing PMT's for highly localized, concentrated ATP solutions added to a luciferase gel hydrated with luciferin and appropriate buffer solution.

4) Make suggestions on how the technique should be modified in order to make it more sensitive and acceptable for specific commercial applications. The suggestions will form the basis of a Phase II proposal.

3. Phase I Work Plan

Task 1. *Optimize concentrations of reactants to provide for optimum detection of light emission from individual cells*

Parameters to be varied include concentration of luciferase in the gel, concentration of luciferin, composition of gel to increase optical clarity, thickness of gel. In addition, an appropriate metal ion will be selected. The sensitivity of the film to different wavelengths of incident light is not known. Different ions cause the emission spectrum of the firefly luciferase reaction to shift to different wavelengths, one of which may be an appropriate wavelength to effect maximum film exposure.

Initial experiments will be performed to determine the sensitivity of the film for ATP when it is reacted with luciferase and luciferin in solution. High concentrations of ATP will be injected into solutions, and the optimal concentration of reactants will be the one that allows for the lowest concentration of ATP to be detected by ASA 20,000 film located at the bottom of the reaction chamber (See Fig. 2. A much smaller version of the set up will be used to avoid wasting reactants. Initial studies will be performed in small dia glass tubes.)

Once the appropriate ratio of reactants is determined, the volume of solution will be decreased until a decrease in sensitivity is observed. The height of the reactants in the reaction vessel where loss of sensitivity is detected will indicate the approximate distance over which emitted photons are detected by the film. This information will be used to design gel thickness.

Concurrently, the composition of the agarose gels will be varied to increase their optical clarity. Gels will be prepared in test tubes, and their transparency to different wavelengths determined in a scanning spectrophotometer (at CBI). Transparency is required so that emitted light is optimally transmitted to the film. Normal media utilize agar and are translucent rather than transparent. PSI has extensive experience with agarose and has developed the capability to control its via a modification of agarose molecular weight and other molecular characteristics and selection of processing temperature to obtain a structure that is more transparent than agar. Our experience will permit us to rapidly develop an agarose-based gel that is relatively transparent.

After the optimal concentration of firefly reagents is determined in solution, gels will be fabricated containing a range of appropriate luciferase concentrations. Luciferin

concentration will be varied in the hydrating medium, consisting of Mg as well as appropriate buffer.

Identical volumes of ATP solutions varying in concentration will be injected into the gel/reactants having different compositions, and the film will be exposed for periods of time up to 10 minutes. Sensitivity of each combination will be determined from the results from the serial dilutions of ATP. All experiments will be performed in the dark.

Task 2. *Vary thickness of gel, volume of rehydration fluid, air flow, and distance between inlet jet and surface of media of AGI-30 air sampler to maximize ATP release on impact of cells with media, and provide for reproducible counts of organisms.*

This portion of the research will be performed at Nelson Laboratories. A non-pathogenic strain of *E. coli* will be selected for this study after consultation with Nelson Laboratory personnel. It will be incorporated into an ultrasonically generated aerosol in the lab and sampled by the AGI-30. The media will be appropriate for the growth of the bacteria. After the organisms have been sampled, the plates will be cultured. Efficiency of destruction will be inversely proportional to the number of cfu's present in the cultured media.

After conditions have been optimized to release ATP from *E. coli*, the experiment will be repeated with mammalian or yeast cells. However, the collecting media will be the luciferase gel with appropriate hydrating solution of buffered luciferin solution containing MgCl. Film will be employed to sense light output. Mammalian or yeast cells will be used because they contain roughly 1000 times the ATP of bacterial cells, and it is anticipated that film will be sensitive to detect their presence. An estimation of the localization of the mammalian cells in the ATP can be made by observing the size of the exposed areas of film.

Mechanical properties (hardness, bulk elasticity) of the gel can be adjusted to achieve localization of impacting organisms by varying crosslink density and water content.

Task 3 Determine sensitivity of film and existing PMT's for highly localized, concentrated ATP solutions added to a luciferase gel hydrated with luciferin and appropriate buffer solution.

Gel components will be optimized when task 1 is completed. The mechanical properties of the gel required to localize the ATP concentration to a small, confined area will be optimized at the completion of Task 2.

Optimized gels will be fabricated employing the information gathered. Highly concentrated ATP solutions will be intrduced into the gels by a micro syringe, and the location of the film and photomultiplier tube in relation to the gel will be optimized for maximum sensitivity.

When the optimal configuration is established, the sensitivity of the technique will be determined as the minimum quantity of highly concentrated ATP that can be added to the gel that yields an exposure that can be distinguished from a control by the unaided eye for film sensitivity.

PMT sensitivity will be defined as the minimum quantity of ATP that yields an output twice that of background (7). For PMT sensing applications, it is desirable to have a spike in the reaction rate. Excess luciferase will be added to the gels to generate an initially rapid rate of lightr emission.

Task 4 *Make suggestions on how the technique should be modified in order to make it more sensitive and acceptable for specific commercial applications.*

The suggestions will form the basis of a Phase II proposal.

4. Related Research

The firefly luciferase/luciferin reaction is the method of choice to assay for ATP. ATP serves as a cofactor in this very sensitive assay which has been reported to detect ATP in concentrations as low as 10 e-11 moles/ liter (2,14,18-36).

The reaction is specific for ATP. Other nucleotides are not detected, nor do they interfere with the reaction. Reagents for the measurement of ATP levels by bioluminescence have become readily available, highly reliable, and inexpensive. Because of its sensitivity, speed, and decreasing costs, use of the technique is growing rapidly.

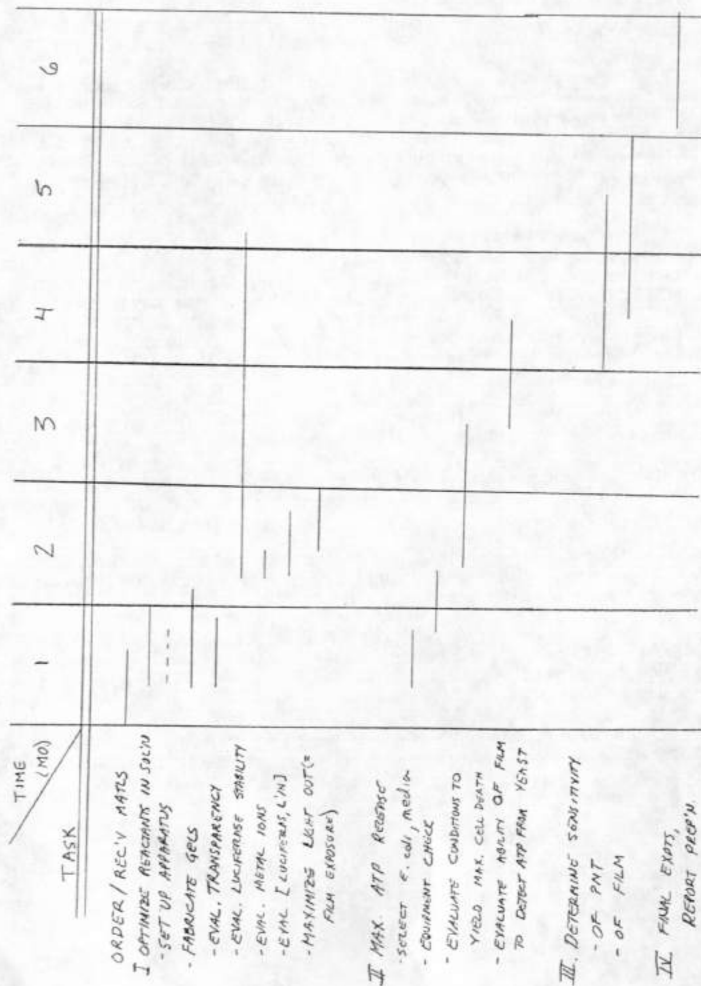
However, the current techniques for assaying ATP to estimate biomass or total microbial content or aerosol samples are not very accurate. This limitation, associated with the variation of ATP content in cells as a function of organism type, growth cycle, and environment, is discussed above (2-5)

In addition, cells may be damaged or even fully disrupted during the removal process releasing ATP. ATP has a relatively short half-life in these conditions, probably because bacterial ATP-ases are released along with the ATP which function to degrade the ATP almost immediately (20,22,37).

The steps for the bioluminescent assay of ATP from cells in suspension are as follows (30,31) :

1) A non-ionic detergent is added to the sample to selectively destroy non-bacterial cells. Unfortunately, some strains of bacteria have cell walls which are also destroyed in this process, but, in general, it is an effective method for releasing non-bacterial ATP so that it can be eliminated in the next procedural step.
2) An ATPase is added to the suspension to destroy the free ATP.
3) The ATPase is neutralized or removed by filtration. Any remaining ATPase can catabolize luciferase or bacterial ATP, the species of interest, released in the following step. Potato apyrase is typically employed for this task, and, if used in small quantities doesn't significantly interfere with the bioluminescent assay. However, the lack of interference is paid for in the requirement that the reaction be allowed to proceed for twenty minutes. In order to shorten the reaction time, higher concentrations of the apyrase are required, but these will destroy both ATP and luciferase, necessitating a neutralization or filtering step to eliminate this possibility (16).
4. An ionic surfactant is added to the suspension to destroy the cell walls of the bacteria that are to be enumerated. The surfactant can degrade luciferase and/or ATP.
5. The liberated bacterial ATP is assayed by the bioluminescent luciferase reaction.

The technology of producing bioluminescent reagents, adding them to the appropriate solution or suspension, and detecting the light output has dramatically advanced over the last few decades. Compact, even portable luminometers are now available (16,



31,38), and we have proposed a detection device that could even eliminate the need for costly detection systems (39).

A recent review (40) lists the current status of rapid microbiological techniques that might compete with the bioluminescent reaction sensitive for ATP. Included are flow cytometry for the screening of *Cryptosporidium* in water samples, Pyrolysis mass spectrometry for the initial screening of samples during suspected outbreaks of infection, and impedance instrumentation suitable for the detection of the rate of growth of bacterial cultures. However, flow cytometry is optimal for rapidly detecting large numbers of organisms, and requires staining techniques (41). It is, therefore, not readily adaptable to continuous monitoring of airborne samples which have relatively low concentrations of organisms.

Several books have been written which describe the various methods currently under investigation, or employed, to rapidly detect microorganisms (14,42,43). They confirm that currently the firefly luciferase reaction is one of the most sensitive means of detecting microorganisms. No existing technology can match its sensitivity, potential for rapid analyses, and relatively low cost.

5. Key Personnel and Bibliography

Phil Triolo, Ph.D., Principal Investigator and President of PSI, 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 device 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.

Dr. Triolo is not a microbiologist. However, he is accustomed to working in new research areas, enjoys the challenges presented by new technologies, and he learns quickly. His industrial background will enable this concept to be effectively developed into a commercial product.

Phil will be assisted by *J.D. Andrade, Ph.D., Chief Scientific Officer* of Protein Solutions, who has been working on bioluminescence research projects for the past 5 years and has supervised PSI's work to date in the area of bioluminescent dinoflagellates and the stabilization of luciferase in agarose gels. Joe is an accomplished scientist who has authored 5 books and over 100 peer-reviewed publications. Dr. Andrade has twice served as Chairman of the Dept. of Bioengineering at the University of Utah, and also held the position as Dean of the College of Engineering at that institution.

Joe is currently Director of the University of Utah's Center for Integrated Science Education (CISE) where he has conducted numerous courses for inservice elementary and high school teachers. Although Joe is on the faculty of the University of Utah, he spends 25% of his time at Protein Solutions, Inc., and will advise and assist on this project as needed. No funds are budgeted for his services.

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 semesters. RPI Alumni Scholarship (1972-'76). President, Rushing Chairman, Theta Chi Fraternity. Member, Tau Beta Pi.

AFFILIATIONS:

Adjunct Instructor, Dept. 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, R.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 Structure for Containing Substances for Delivery to a Body, U.S. Patent 5,324,518, 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.

6. Relationship of proposed work with future research and development activities

The successful completion of this phase I study will allows us to determine the sensitivity of high speed film for ATP present in high concentrations in a relatively small volume. We'll also be able to determine if mechanical means of cell destruction can be achieved employing a standard AGI-30 air sampler. This information will allow for an assessment of the feasibility of this method for the detection of individual microbes. (With the exception of viruses, of course, which contain no ATP).

Phase II will focus on the elimination of light emission of non-biologic origin. Filtering or separation by size might be employed, as with an Andersen sampler. The extremely large light output of eukaryotic cells should allow for easy identification and elimination of these cells from total cell counts. Separation of signals from eukaryotic and bacterial cells is not possible with detection techniques that correlate microbial counts with light intensity.

It is anticipated that the sensitivity of film for the detection of high concentrations of ATP will be much greater than is generally believed to be the case, but perhaps slightly lower than is required for routine detection of single bacteria. Therefore, Phase II studies will focus on the development of techniques for the amplification of the light output before it reaches the sensing device(10), the elimination of interference with the light that decreases its intensity before reaching the detector, chemical means of inactivating ATPases, orienting luciferase films so that light is emitted preferrentially in the direction or the sensor, and improving the coupling of the film with the sample chamber/bioluminescent reactor.

Additionally, air samplers other than the AGI-30 will be investigated in Phase II (62). For example, an Andersen sampler might offer some advantages because of its ability to segregate particles on the basis of size, and a cyclone sampler might be desirable because it can be modified to more effectively damage cell membranes to release ATP.

There will be no need to pursue further funding of this project if the sensitivity of the technique varies by orders of magnitude from the sensitivity required. However, this finding would be quite surprising.

7. Facilities

PSI rents a 1200 sq. ft. laboratory space equipped with balances, microscopes, and a fume hood for standard wet and dry chemistry experiments. In addition, it houses secretarial and office areas equipped with computers and a cell culture laboratory for the culturing of bioluminescent marine phytoplankton which it distributes in sealed microenvironments to educators in order to generate an interest in science among high school students. Preparation of luciferase-containing gels and optimization of the detection of ATP (from non-living sources) by the firefly luciferase bioluminescent reaction (as described in above experiments) will be performed in these laboratories.

PSI is not equipped to perform studies of airborne microbes. Experiments involving the production and assay of aerosolized microorganisms will be carried at Nelson Laboratories, a local microbiology test laboratory. Two aerobiology containment laboratories are located at this facility, where routine challenge tests of face masks, filters, etc. are performed. The laboratories are operated under negative pressure, and HEPA filters are employed to remove microbes from air before it is returned to the ventilation system. All tests of aerosols will be performed in these facilities under the supervision of

Jerry Nelson, director of the lab, who will serve as an advisor on this project . Dr. Nelson serves as a member of PSI's Scientific Advisory Board.

The photomultiplier tubes necessary for some of the described experiments are housed at the University of Utah's Fluorescence Spectroscopy Facility. The facility is operated under the auspices of the University's Center for Biopolymers at Interfaces. PSI, as a member of this university/industry consortium, has access to the full range of analytical equipment housed at this facility at reduced rates. Dr. Vlado Hlady directs the activities in the fluorescence lab and has significant experience in the detection of light from fluorescent systems. He will serve as a consultant on this proposal (See CV.)

8. Consultants and Advisors

Dr. Jerry Nelson is a microbiologist who is President of Nelson Laboratories, a Salt Lake City-based microbiology testing facility. He has extensive experience with general microbiology techniques and aerobiology challenge tests. He serves on PSI's Scientific Advisory Board and has offered to provide as-needed advice at no cost.

Dr. Vlado Hlady is an expert in the use of fluorescent techniques to probe molecular and surface interactions. He is extremely familiar with the very-sensitive light detection devices required for this work. His CV is included.

9. Potential Commercialization

The increasing prevalence of antibiotic-resistant strains of bacteria, the evolution of new strains of bacteria (44) and proliferation of the number of genetically engineered organisms with as-yet unknown potential as pathogens or producers of molecules with potential pharmacologic or allergenic effects (45) underscore the need for improved monitoring of air for the presence of microbes, especially in sensitive, high-risk environments.

There is considerable interest in and need for rapid, accurate monitoring of airborne microbial concentrations in health care facilities (46-49), food (42, 50,51) and pharmaceutical (52) preparation areas, microbiology (53) and especially biotechnology (59) laboratories, waste processing facilities (54) and wastewater treatment facilities. The proposed development of a bioluminescent technique to rapidly monitor changes in microbial content of sampled air or determine relative airborne microbial content will be a valuable tool that will find great utility in these, and other, indoor work areas (55-57).

Further, if the technique is feasible, it can be modified so that a count of any bacterial sample can be made. That is, the luciferin and luciferase can be added to gels in petri dishes. Bacterial samples plated on the gels can be enumerated employing bioluminescence and high speed film, providing a suitable means of releasing ATP is utilized. It is even possible that some identification of the plated organisms can take place by pre-exposing samples of the organisms to chemicals or conditions that selectively kill specific species or classes of organisms.

10. Submission of Similar Proposals

-NONE-

11. Prior SBIR Phase II Awards

-NONE-

M. L. Pierce, J. K. Stuart, A. Pungor, P. Dryden and V. Hlady: *Specific and non-specfic adhesion force measurements using AFM with a linear position sensitive detector*, Langmuir, **10** (1994) 3217 - 3221.

Y.-S. Lin and V. Hlady: *Human serum albumin adsorption onto octadecyldimethylsilyl-silica gradient surface*, Colloids and Surfaces B: Biointerfaces, **2** (1994) 481 - 491.

V. Hlady, J. D. Andrade, C.-H. Ho, L. Feng and K. Tingey: *Plasma Protein Adsorption on Model Biomaterials Surfaces*, J. Clin. Materials, **13** (1993) 85 - 93.

J. D. Andrade, V. Hlady, A.-P. Wei, C.-H. Ho, A. S. Lea, S. I. Jeon, Y.-S. Lin and E. Stroup: *Proteins at Interfaces; Multivariate Aspects; Protein Resistant Surfaces; Direct Imaging and Manipulation of Adsorbed Proteins*, J. Clinical Materials **11** (1992) 67 - 84.

Y.- S. Lin, V. Hlady and J. Janatova: *Adsorption of complement proteins on surfaces with a hydrophobicity gradients*, Biomaterials **13** (1992) 497 - 504.

A. S. Lea, A. Pungor, V. Hlady, J. D. Andrade, J. N. Herron and E. W. Voss Jr.: *Manipulation of proteins at mica using atomic force microscope*, Langmuir, **8** (1992) 68 - 73.

V. Hlady, P.-Y. Yeh and J. D. Andrade: *Adsorption of firefly luciferase at interfaces studied by total internal reflection fluorescence spectroscopy*, J. of Fluorescence, **1** (1991) 47 - 55.

V. Hlady: *Spatially-resolved adsorption kinetics of immunoglobulin G onto the wettability gradient surface*, Applied Spectroscopy, **45** (1991) 246 - 252.

C.-H. Ho, V. Hlady, G. Nyquist, J. D. Andrade and K. D. Caldwell: *Interaction of plasma proteins with heparinized gel particles studied by high resolution 2-D gel electrophoresis*, J. Biomed. Mat. Sci., **25** (1991) 423 - 441.

D. Horsley, J. Herron, V. Hlady and J. D. Andrade: *Fluorescence quenching of adsorbed human and hen lysozymes*, Langmuir, **7** (1991) 218 - 222.

P. Suci and V. Hlady: *Fluorescence lifetime components of Texas Red-labelled bovine serum albumin: comparison of bulk and adsorbed states*, Colloids and Surfaces, **51** (1990) 89 - 104.

C.-G. Gölander, V. Hlady, J. D. Andrade and K. Caldwell: *Adsorption and adsorbate activity of human lyzozyme as quantified by means of TIRF, 125-I labeling and ESCA*, Colloids and Surfaces, **50** (1990) 113 - 130.

C.-G. Gölander, Y-S. Lin, V. Hlady and J. D. Andrade: *Wetting and plasma protein adsorption studies using surface with hydrophobicity gradient*, Colloids and Surfaces **49** (1990) 289 - 302.

E. Brynda, V. Hlady and J. D. Andrade: *Energy Transfer of Fluorescence in Adsorbed Bovine Serum Albumin Layer*, J. Colloid and Interface Science, **139** (1990) 374 - 380.

V. Hlady, J.-N. Lin and J. D. Andrade: *Spatially Resolved Detection of Antibody-Antigen Reactions at Solid-Liquid Interfaces Using Total Internal Reflection Excited Antigen Fluorescence and Charge-Coupled Device Detection*, Biosensors and Bioelectronics, **5** (1990) 291 - 301.

V Hlady and J. D. Andrade: *A TIRF titration study of 1-anilinonaphthalene-8-sulfonate binding to silica-adsorbed bovine serum albumin*, Colloids and Surfaces, **42** (1989) 85 - 96.

V. Hlady, J. Rickel and J. D. Andrade: *Fluorescence of Adsorbed Protein Layers. II. Adsorption of Human Lipoproteins Studied by Total Internal Reflection Intrinsic Fluorescence*, Colloids and Surfaces, **34** (1989) 171 - 183.

V. Hlady, C. G. Gölander and J. D. Andrade: *Hydrophobicity Gradients on Silica Surfaces. A Study Using Total Internal Reflection Fluorescence*, Colloids and Surfaces, **33** (1988) 185 - 190.

G. A. Peeters, V. Hlady, J. H. B. Bridge and W. H. Barry: *Simultaneous measurements of calcium transients and motion in cultured heart cells*, Am J. Physiology, Heart and Circ. Physiology, **253** (1987) H1400 - H1408.

V. Hlady and J. D. Andrade: *Fluorescence emission from adsorbed bovine serum albumin and albumin-bound 1-anilinonaphthalene-8-sulfonate studied by TIRF*, Colloids and Surfaces, **32** (1988) 359 - 369.

L. K. Koopal, V. Hlady and J. Lyklema: *Electrophoretic study of polymer adsorption: Dextran, PEO, PVA on Silver Iodide*, J. Colloid Interface Sci. **121** (1988) 49 - 62.

LJ. Brecevic, V. Hlady, and H. Füredi-Milhofer: *Influence of Gelatin on the Precipitation of Amorphous Calcium Phosphate*, Colloids and Surfaces, **28** (1987) 301 - 313.

W. M. Reichert, J. T. Ives, P. A. Suci and V. Hlady: *Excitation of Fluorescence Emission from Solution at the Surface of Polymer Thin-Film Waveguides*, Appl. Spectr. **41** (1986) 636 - 640.

V. Hlady, D. R. Reinecke and J. D. Andrade: *Fluorescence of Adsorbed Protein Layers. I. Quantitation of Total Internal Reflection Fluorescence*, J. Colloid Interface Sci., **111** (1986) 555 - 569.

J. D. Andrade and V. Hlady: *Protein Adsorption and Materials Biocompatibility: Tutorial Review and Suggested Hypotheses*, Adv. Polym. Sci., **79** (1986) 1 - 63.

12. References

1. Brolin S, Wettermark G,"Biomass determinations in microbiology and cell research," sect. 8.2 in Bioluminescence Analysis, V C H, Weinheimm, Germany, 1992. pp. 104-111.
2. Lundin A, Hasenson M, Persson J, Pousette A In: De Luca MA,McElroy WD, eds., Methods in Enzymology. Academic Press, New York, 1986: 27-42.
3. Harber MJ In: Kricka LJ, Carter TJN, eds. Clinical and Biochemical Luminescence, Marcel Decker, New York, 1982: 212-220.
4. A. Lundin, "Analytical applications of bioluminescence: The firefly system," in Kricka LJ, Carter TJN, eds. Clinical and Biochemical Luminescence , Marcel Decker, New York, 1982, pp 43-74.
5. Stanley PE, "Extraction of adenosine triphosphate from microbial and somatic cells," *Methods in Enzymology* **133:** 14-22 (1986).
6. Kricka LJ, Thorpe GHG, "Photographic detection of chemiluminescent and bioluminescent reactions," *Methods in Enzym.*, **133**: 404-420 (1986).
7. Green K, Kricka LJ, Thorpe GHG, Whitehead TP, "Rapid assays based on immobilized bioluminescent enzymes and photographic detection of light-emission,"*Talanta***3 1**: 173-176 (1984).
8. Carter TJN, Whitehead TP, Kricka LJ, Investigation of a novel solid-phase chemiluminescent analytical system incorporating photographic detection for the measurement of glucose," *Talanta*, **29**: 529-31 (1982).
9. Brolin S, Wettermark G,"Principles of analytical applications of luminescence," ch.2 in Bioluminescence Analysis, V C H, Weinheimm, Germany, 1992. pp. 19-24.
10. Porter NA, Bruhnke JD, Koenigs P, "Photoregulation of Enzymes," ch. 4 in H Morrison (ed.), Bioorganic Photochemistry Volume 2: Biological Applications of Photochemical Switches, John Wiley and Sons, New York, 1993, pp. 197-241.
11. Wang CJ, Andrade JD, unpublished data.
12. Simpson WJ, Hammond JRM, "Cold ATP extractants compatible with constant light signal firefly luciferase reagents," in PE Stanley, BJ McCarthy, R Smither (eds), ATP Bioluminescence: Rapid Methods in Microbiology, Blackwell Scientific Publications, Oxford, 1989, pp. 45-52.
13. Bridger WA, Hendersonn JF, Cell ATP, John Wiley and Sons, New York, 1983, pp. 110-111.
14. PE Stanley, BJ McCarthy, R Smither (eds.), ATP Bioluminescence: Rapid Methods in Microbiology, Blackwell Scientific Publications, Oxford, 1989.
15. J.G.M. Hastings, "Applications of Luminescence in Clinical Microbiology", in Ref. 25, pp. 421-425.
16. Tyler ME, Shipe EEEL, "Bacterial Aerosol Samplers. I. Development and evaluation of the all glass impinger," *Appl. Microbiol.*, **7**: 337 (1959).
17. G.C. Salzman and C.T. Gregg, "Current and Experimental Methods of Rapid Microbial Identification", Bio/Technology (1984), pp. 243-248.
18. Neufield HA, Pace JG, Hutchinson RW, "Detection of microorganisms by bio and chemiluminescence techniques," in WH Nelson (ed.), Instrumental Methods for Rapid Microbiological Analysis, VCH Publishers, United States, 1985, pp 51.-66.
19. M.J. Harber, R. MacKenzie, and A.W. Asscher, "Rapid Bioluminescence Method for Quantifying Bacterial Adhesion...", J. General Microbial 129 (1983) 621-632.
20. A.A. Mafu, D. Roy, L. Savoie, J. Govlet, "Bioluminescence Assay for...Bacteria...", Appl. Environ. Microbiol. 57 (1991) 1640-1643.
21. P.L. Brezonik, F.X. Browne, J.L. Fox, "Application of ATP to...Biomass... Studies", Water Res. 9 (1975) 155-162.
22. D.M. Karl, "Cellular Nucleotide Measurements and Applications in Microbial Ecology," Microbio. Rev. 44 (1980) 739-796.

23. P.S. Senior and B.J. McCarthy, "Bioluminescent Assessment of Fungal Growth on Plastic Packaging Materials," in Biodeterioration 7, ed. D.R. Houghton, R.N. Smith, H.O.W. Eggins, Elsevier, pp.507-510.
24. P.S. Senior, et al., "Bioluminescent Assessment of Microbial Contamination on Plastic Packaging Materials," in Ref. 14, pp. 137-143.
25. A.Lundin, A. Rickardson, and A. Thore, "Continuous Monitoring of ATP...by...Luciferase," Anal. Biochem., 75 (1976) 621-633.
26. P.E. Stanley and L.J. Kricka, Bioluminescence and Chemiluminescence: Proc. 7th Int. Symp., Cambridge, UK, Sept. 1990, Wiley, 1991. Section 6: Firefly Luciferase-ATP: Applications and Assays, pp. 407-528.
27. Deans JS, Stewart IW, Salisbury TT, "Rapid enumeration of airborne microorganisms by bioluminescent ATP assay," in WF Nelson (ed.), Physical Methods of Microorganism Detection, CRC Press, Boca Raton, 1991, pp. 140-149.
28. E. Schram, "Evolution of Bioluminescent ATP Assays", in Ref. 26, pp. 407-412.
29. J. Scholmerich, et al., eds., Bioluminescence and Chemiluminescence, Wiley, 1987. Part 4: Luminescence Applications, pp. 453-594.
30. P.E. Stanley, "A Beginner's Guide to Rapid Microbiology using ATP and Luminescence", Ref. 14, pp. 1-10.
31. A. Lundin, "ATP Assays in Routine Microbiology", Ref. 14, pp. 11-30.
32. Picciolo GL, Chappell EW, Deming JW, Thomas RR, Nibley DA, Okrend H., "Firefly luciferase ATP assay development for monitoring bacterial concentrations in water supplies," U.S. Environmental Protection Agency, Center for Environmental Information, Cincinnati, Ohio, pub. EPA-600/s2-81-014, April, 1981. pp 1-6.
33. E. Schram and A. Weyens Van Witzenburg, "Improved ATP Methodology..." J. Biolum Chemilum. 4 (1989) 390-8.
34. S. Girotti, et al., "Methodological Problems of Direct Bioluminescent ATP Assay...", J. Biolum Chemilum. 4 (1989) 594-601.
35. E. W. Chappelle and G. L. Picciolo, "Method of Detecting and Counting Bacteria...", U.S. Patent 3745090, 7/10/73.
36. W.J. Simpson, et al., "Highly Sensitive Assay for ATP...", Letters in Appl. Microbiol. 11 (1990) 208-210.
37. R.T. Storey, "Cell Energetics", Amer. Biol. Teacher 54 (1992) 161-166.
38. Turner Designs, Mt View, CA. (415) 965-9800 Anal. Luminescence Lab., San Diego, CA. (800) 454-7050
39. Scheer, R. (P.I.), Direct Reading, Quantitative Biosensors for ATP-dependent Processes, NSF STTR Proposal, 1994.
40. Silley P, "Rapid Microbiology- is there a future?" , *Biosensors and Bioelectronics*, **9**: 15-21, 1994.
41. Hadley WK, Waldman F, Fulwyler M, "Rapid microbiological analysis by flow cytometry," ch. 3 in WH Nelson (ed.), Instrumental Methods for Rapid Microbiological Analysis, VCH Publishers, United States, 1985, pp. 67-86.
42. WH Nelson (ed.), Instrumental Methods for Rapid Microbiological Analysis, VCH Publishers, United States, 1985.
43. RC Tilton (ed.), Rapid Methods and Automation in Microbiology, American society for Microbiology, Washington, D.C., 1981.
44. Plouffe JF, "New microorganisms and their health risk," in PR Morey, JC Feeley, JA Otten, (eds), Biological Contaminants in Indoor Environments, ASTM STP 1071, American Society for Testing Materials, Philadelphia, 1990, pp176-189.
45. Salisbury, TT, Deans, JS, Stewart, IW, "Rapid Detection of Airborne Micro-organisms by ATP Assay," in PE Stanley, BJ McCarthy, R Smither (eds), ATP Bioluminescence: Rapid Methods in Microbiology, Blackwell Scientific Publications, Oxford, 1989, pp. 109-117.
46. PW Smith (ed.) , Infection Control in Long-term Care Facilities, Delmar Publishers, Inc., Albany, New York, 1994.

47. Berardi BM, Leoni E, "Indoor air climate and microbiological airborne contamination in various hospital areas," *Zentralbl. Hyg. Umweltmed.*, **19 4**: 405-18 (1993).
48. Hruszkewycz V, Ruben B, Hypes CM, Bostic GD, Stassszkiewcz J, Band JD, "A cluster of pseudofungemia associated with hospital renovation adjacent to the microbiology laboratory," *Infect. Control Hosp. Epidemiol.*, **1 3**:147-50 (1992).
49. Nichols RL, "Surgical wound infection," *Am. J. Med.*, **9 1(3B)**: 54S-64S (1991).
50. Maurice J, "The Rise and Rise of Food Poisoning," *New Scientist*, Dec 17, 1994, pp 28-33.
51. Kang YJ, Frank JF, "Characteristics of biological aerosols in dairy processing plants," *J. Dairy Sci.*, **7 3**:621-6 (1990).
52. Chandler SW, Trissel LA, Wamsley LM, Lajeunesse JD, Anderson RW, "Evaluation of air quality in a sterile drug preparation area with an electronic particle counter," *Am. J. Hosp. Pharm.*, **5 0**:2330-4, (1993).
53. Hambleton P, Dedonato G, "Protecting researchers from instrument biohazards," *Biotechniques*, **1 3**:450-3 (1992).
54. Fletcher MW, Fiscus DE, Analysis of Airborne Viable Bacteria at Solid Waste Processing Facilities, U. S. Environmental Protection Agency, Contract 68-02-1871, report EPA-600/2-79-131, August, 1979.
55. Seltzer JM, "Biological contaminants," *J. Allerg. Clin Immunol.*,"**9 4**: 318-26 (1994).
56. Burge HA, "Indoor air and infectious disease," *Occup. Med.*, **4**: 713-21 (1989).
57. PR Morey, JC Feeley, JA Otten (eds.), Biological Contaminants in Indoor Environments, Americam Society for Testing Materials, Philadelphia, Penssylvania 1990.
58. Colwell, RR, Brayton, PR, Grimes, DJ, Roszak, DB, Huq, SA, Palmer, LM, "Viable but non-culturable Vibrio Cholerae and related pathogens in the environment: Implications for release of genetically engineered micro-organisms," *Biotechnology* **3**, 817-820 (1985).
59. JA Thomas, LA Myers (eds.), Biotechnology and Safety Assessment, Raven Press, New York, 1993.
60. Berthold F. "Luminescence instrumentation for microbiology," in WF Nelson (ed), Physical Methods of Microorganisms Detection, CRC Press, Boca Raton, 1991, pp. 140-149.
61. Brown RGW, "ATP photon counting using avalanche photodiodes," in PE Stanley, BJ McCarthy, R Smither (eds), ATP Bioluminescence: Rapid Methods in Microbiology, Blackwell Scientific Publications, Oxford, 1989, pp. 257-259.
62. JensenPA, Todd WF, Davis GN, Scarpino PV, "Evaluation of eight bioaerosol samplers challenged with aerosols of free bacteria," Am. Ind. Hyg. Assoc. J., **5 3**: 660-67 (1992).