Joe

Protein Solutions, Inc.

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March 30, 1998

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 third 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).

I would like to request that you review and accept this report and promptly convey your recommendations for additional funding to your financial office. We are a small company and a two week delay in receipt of funds would be 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

033098A

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: 10/01/97 - 3/30/98
Grantee Name: Protein Solutions
P.I. Name: Richard A. Van Wagenen
Grantee Address: P.O. Box 58093
Salt Lake City, UT
U.S.A. 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: J. Andrade **Date:** 3-30-98

P.I. Signature: Richard A. Van Wagenen **Date:** 3-30-98

NSF Form 1372 (STTR 2/96)

A. Reporting Period: From: October 1, 1997 to: March 30, 1998

B. Total Estimated Expenditures for this Reporting Period: $ 76,864

C. Cumulative Estimated Expenditures to Date: $242,271

D. Principal Investigator and Key Personnel:

Name and Title	Estimated Level of Effort
1. Richard A. Van Wagenen, P.I.	3.5 man months
2. C. - Y. Wang, Research Scientist	3.5 man months
3. Robert J. Scheer, Research Scientist	1.0 man months
4. J. D. Andrade, Consulting Scientist	1.0 man months
5. M. Hammer, Lab Technician	1.0 man months

E. Subcontractor(s) Utilized and Services Provided:

During the first 18 months of this Phase II STTR grant a total of $62,159 has been billed 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. The quality and quantity of the recombinant luciferase received to date generally continues to meet our expectations, however, we have, from time to time, experienced some variability in the activity of the recombinant luciferase received.

Changing the firefly luciferase (FFL) expression plasmid antibiotic resistance: Our experience to date with the firefly luciferase expression plasmids has been that occasionally some of the E-*coli* cultures would contain little or no FFL after growth and induction. The first FFL expression plasmid (C.-Y. Wang, et. al. "Specific Immobilization of Firefly Luciferase Through a Biotin Carboxy Carrier Protein Domain", Anal. Biochem., 246: 133-139, 1997) which we used until recently contained the ampicillin resistance gene as a selectable marker. Dr. Russell suspected that the increasingly frequent lack of expression was due to the plasmid sometimes being lost during the growth of the cultures. The product of the ampicillin resistance gene - β-lactamase - is secreted out of the E. *coli* cells and the ampicillin in the media is quickly degraded in a rapidly growing E. *coli* culture. When the ampicillin has been destroyed, the selective pressure for cells containing the expression plasmid is lost. If the protein from the expression plasmid inhibits cell growth, cells that have lost the plasmid through random segregation will have a growth advantage. In the absence of ampicillin, the cells without plasmid can overwhelm the culture. Dr. Russell solved this problem by moving the firefly luciferase gene into an expression vector (pET-24a, Novagen) with a kanamycin resistant gene. Unlike the ampicillin, kanamycin is not degraded in the medium by a secreted gene product and, therefore, the selective pressure for the plasmid is maintained even in cultures with high cell density. The previously employed firefly luciferase vector (pRSET-luc) was digested with NdeI and HinDIII. The resulting firefly luciferase containing fragment was ligated into the pET-24a plasmid, which had been digested with NdeI and HinDIII. The pET24a-luc plasmid was then transformed in E. *coli* strain BL21(DE3) for expression tests. Firefly luciferase was expressed at a much higher and consistent level after induction with IPTG and the several clones tested to date have all expressed luciferase to similar high levels.

F. Consultant(s) Utilized and Services Provided:

1. Dr. Russell Stewart: Dr. Stewart continues to play an active role in this project. He has assisted us in designing experiments and diagnosing problems that occur with the production of the recombinant luciferase. Refer to Subcontract Section B above. During this third semiannual period Dr. Stewart Invoiced Protein Solutions, Inc. $2,700 for consulting effort primarily related to activities designed to identify the source of our lot to lot variability in recombinant luciferase and for review of experimental data.
2. Miscellaneous: Mr. Kent Beck, a Design Engineer has helped us with the PVC strip biosensor designs and Mr. Mark Wood, an Electrical Engineer, has helped us with electrical circuit design assessment for the PIN photodiode work. The aggregate billing from these two individuals was approximately $300 for the semi-annual period.

G. Identification of Equipment Purchased

During this third 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 (such as 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. Minimize reagent activity loss during drying of gel matrix support: Both short and long term stability studies have been conducted on: (1) dried gel matrices and (2) lyophilized reagents. A one year, long term stability study was initiated on the gel based system in September of 1997. The matrix of 10 wt% trehalose and 1 wt% agarose gel served as a support for our standard reagents of luciferin ,luciferase.

Mg^{++}, and buffer. After drying the gels with reagents for four hours in cool, dry, filtered air each of the samples was stored in the dark under one of the following four conditions: (1) 4 °C in dessicated air, (2) 25 °C in dessicated air and (3) 25 °C in moist (about 30% RH) room air. Analysis time periods were: (1) 0 days (wet gel prior to drying represents the 100% bioluminescence reference point), (2) zero days (immediately after drying), (3) one day, (4) one week, (5) four weeks, (6) 16 weeks, and in the future (7) 32 weeks and (8) 52 weeks. The bioluminescent intensity was determined by measurement with our CCD camera after a standard ATP solution was added to each dry gel sample. There was a 10 percent drop in sample luminescence immediately following gel drying. FIGURE 1 illustrates that subsequent storage under any of the conditions evaluated results in severe loss of assay activity towards ATP analyte, over several weeks, i.e., a 65% loss after 14 days in dessicated air at 4 °C. Additional long term stability studies were conducted with N_2 gas at 0°C, 4°C and 25°C rather than room air, but there were no significant differences. Initial air drying, storage temperature and water vapor seem to be the primary determinants in reducing the lifetime of the gel based bioassay.

b. As a result of the findings in a. above, we began to actively pursue an alternative means of reagent preservation. Preliminary studies suggested that lyophilization looked much more promising. A longer term storage stability study was initiated in February of 1998. A preservation mixture of trehalose, polyethylene glycol, DTT (a reducing agent to protect protein thiol groups from oxidation) and BSA was used to stabilized the luciferin and luciferase enzyme mixture during freezing and lyophilization. The samples were first frozen rapidly to -70 °C and then after 12-16 hours they were lyophilized over a 24 hour period. All samples were then removed and stored in sealed containers at 22 - 25 °C in the dark. Bioluminescent signal intensities were then obtained at various times following freezing and lyophilization, i.e., (1) just prior to freezing, (2) immediately after lyophilization, (3) four days, (4) seven days and (5) four weeks following lyophilization. The results are shown in FIGURE 1. It is clear that freezing to -70 °C with the appropriate cyroprotectants and lyophilization is highly superior to the gel approach to preservation, reagent activity towards ATP analyte is maintained at almost 100 % for periods of at least one month. The long term stability and optimization of the lyophilization work will continue during the next six month phase of this grant.

c. Triton X-100 and Co-A enzyme incorporation into sensor: There are two different characteristic signal outputs which can occur during the ATP bioluminescence assay. When the ATP concentration is high, usually above 10^{-6} M, an initial light "flash" occurs which reaches a peak in less than a second and then decays quickly to a low basal level over several minutes. When the ATP concentration is low, usually below 10^{-6} M, there is no initial flash. The bioluminescence intensity rises quite rapidly (over several seconds) to a stable level and remains at or slightly below this level for a long period of time (many tens of minutes). Coenzyme A is effective in slowing the decay rate of the peak bioluminescence decay, however, coenzyme A is unable to increase the peak light intensity "flash". Our results continue to indicate that Coenzyme A is effective in increasing the total light output during the first 30-60

seconds of the bioluminescent reaction for relatively high ATP concentrations. For example there was a 7 fold increase in light for 10^{-4} M ATP, but only a 110% increase for 10^{-6} M ATP, and there was no enhancement effect for ATP below 10^{-7} M, where the original bioluminescence was at low plateau level. Coenzyme A can only increase the assay resolution (the S/N ratio) when analyte ATP exceeds about 10^{-6} Molar. Surfactant Triton X-100 can enhance the peak light intensity of bioluminescence by a factor of two for 10^{-4} M ATP and by a factor of three for 10^{-6} M ATP. When Triton X-100 is combined with coenzyme A, a cooperative enhancement is observed. However, for 10^{-9} M ATP, there was no enhancement. We have concluded that both enhancers are effective for ATP concentrations above 10^{-6} M. Consequently, for ATP assays in the sub micro molar range both reagents are of little value. However, both coenzyme A and Triton X-100 would be of value in ATP assays above 10^{-6} Molar.

d. Optimize gel matrix so that it wets and re-hydrates rapidly: After the agarose gel matrix has been dried it exhibits a hydrophobic nature during the early stages of re-hydration. This hydrophobicity coupled with the thickness of the gel results in a time delay of several minutes required to re-hydrate the gel. As a result of the encouraging preliminary lyophilization approach to reagent preservation we elected to not allocate additional efforts on this task, but rather to focus those efforts on optimizing the amount and kind of cryoprotectants necessary for successful lyophilization. It should also be noted that once the reagents are frozen and lyophilized they re-hydrate very rapidly and result in virtually immediate bioluminescence when the aqueous sample containing ATP is added. We did however, determine that Triton X-100 served as a very important surface wetting agent which may be desirable for sensor designs which employ dried agarose gels.

e. Evaluate any new commercial recombinant luciferase enzymes: No new commercial sources of ATP were identified during the last semiannual period covered by this report.

2. Optimize Detection

a. Film detection interfaced to ATP biosensor: Work continued on optimizing the design of the film holder to maximize both response sensitivity and spatial resolution. The optimal configuration to date involves placing the bioluminescent reaction at the bottom of white plastic wells (96 well microtitre plate). The white well serves to channel (via reflection) and direct the light toward the film which is placed in as close a proximity as possible to the opening of the well. Sample wells can be positioned as close as 9 mm C-C distance from each other and within about 5 mm of the film plane. It will be difficult to minimize these distances further. Work was also done to compare the effectiveness of collection lens imaging versus direct contact exposure of the bioluminescent directly to the film. The direct contact exposure was found to be more efficient in imaging the signal onto film.

b. Evaluation of silicon PIN photodiode and imaging optics for sensor detection: A significant amount of modeling work was done to estimate the anticipated level of photon flux during the first five minutes of the luciferin - ATP luciferase catalyzed reaction. Kinetic studies were done to follow the light emission as a function of time and in turn estimate what fraction of the total photon flux occurred in the first five minutes of the reation for a variety of ATP analyte concentrations and luciferase enzyme concentrations. Generally, about 10 percent of the total of all bioluminescent photons generated by the reaction are emitted in the first five minutes of the reaction. Given the signal levels measured with our PMT based luminometer and our CCD we concluded that a silicon photodiode was very much inferior to both the PMT and CCD as detectors. Generally, at the wavelength of emission (563 nm) a PMT is about 10 times more sensitive than a CCD and a CCD, in turn, is about 100 times more sensitive that a silicon photodiode. Also, the most desirable feature of the CCD is that, like film, it produces a two dimensional image of the bioluminescence distribution. On a cost basis an array of photodiodes would hardly be much less expensive (and certainly less versatile) that a 2-D CCD array.

c. Optimize apyrase removal column for detection of ATP below 10^{-13} moles (10^{-9} Molar ATP): The bioluminescence assay usually contains trace ATP contamination (0.1 to 1 nano Molar), which arises during handling operations or comes from luciferase bound ATP. Apyrase (Enzyme Class:3.6.1.5) catalyzes the hydrolysis of ATP and ADP in the presence of bivalent metal ions. Because of its high efficiency in removing ATP in solution, it has been employed to purify assays designed for ultra-low levels of ATP [R. B. Conn, et. al., "Limit of application of the firefly luminescence ATP assay for the detection of bacteria in clinical specimens," Am. J. Clin. Path., 63, 493-501 (1975)]. An ATP removal column was developed with the immobilization of apyrase on polymeric beads. Emphaze Biosupport Media AB1 (3M Corp.), a hydrophilic bead with azlactone functionality, was chosen as the solid support for apyrase. Emphaze beads were first rehydrated with 0.1 M phosphate buffer pH 7.8. Apyrase was then added and rotated at room temperature for the coupling reaction between apyrase and azlactone groups of Emphaze beads. After 30 min of reaction, the beads were centrifuged down with a micro-centrifuge. The residual solution was analyzed by the BioTech protein assay to determine the amount of apyrase left behind in solution. The beads with immobilized apyrase were washed with a Tris buffer quench solution and the activity of the immobilized apyrase was determined. The immobilization efficiency was determined to be 1.1 mg apyrase/g Emphaze beads. The remaining apyrase activity was less than 10% of the activity in solution. We continue to attempt to improve the amount and activity of bound apyrase. Further optimization is necessary to enhance the activity of immobilized apyrase so that such a column can be used to remove residual ATP from our test solutions below 1.0 n M ATP.

d. Investigate concept of light to photocurrent to electrochemical product reaction: During this last semi-annual period we have been fortunate to have the collaborative assistance of an internationally recognized electrochemist, Dr. Erno Pungor, Professor emeritus, General Director and Minister of the Hungarian Academy of

Science. While it is true that the photon flux from the bioluminescent reaction can be converted into a photon current using a silicon photodiode, we (with Dr. Pungor's guidance) were unable to discover in the literature an interfacial electrochemical reaction that could utilize such a photocurrent to produce a colored reaction product visible by the eye in normal ambient light.

e. Concentrate Bioluminescence in a Small Area and Wave Guiding: During this period several studies were undertaken to evaluate the concept of incorporating the luminescent reagents (luciferase, luciferin, Mg^{++} and buffer) into a geometric configuration where wave guiding of the bioluminescence would be efficiently delivered to the photodetector. This was difficult to do with dehydrated agarose gel-based sensors because a considerable amount of time was required both to dehydrate the gels and to re-hydrate the dried gels with aqueous ATP samples because the gels were placed inside capillary tubes. The use of lyophilized reagents was much more successful. Aqueous reagent mixtures were wicked into both glass capillaries and polyester fiber wicks several mm in diameter. The samples were then frozen to -70 C and lyophilized for 24 hours. The samples were then exposed to aqueous ATP solutions which immediately wicked up into both the capillaries and the polyester fiber wicks. Bioluminescence was observed visually and with the CCD camera at the end of the wicks and capillaries. In the best case, enhancement factors of 5 X were obtained with optical path lengths of 10 X for glass capillaries. The results with the polyester wicks of various lengths was generally negative. While the concept works in classical glass capillaries the geometry of the situation is not attractive due to the rather large size and manufacturing constrains such designs place on a biosensor which should be small and inexpensive.

3. Design, Construct and Evaluate Sensor Platform

a. Finalize the design of the glass substrate based ATP platform: The design of the ATP biosensor platform has not yet been finalized. Very early in this semi-annual phase we elected to move away from glass as a base material for the sensor because it was heavy, expensive and somewhat dangerous for a user to handle. FIGURE 2 illustrates a schematic rendition of the current design now under evaluation. It is basically a PVC strip 0.5 mm thick, 60 mm long and 10 mm wide which has 3 mm holes punched into specific areas. A reagent membrane which supports lyophilized luciferin, luciferase, Mg^{++} and buffer salts is heat sealed onto the bottom of the PVC strip. A filter membrane is then glued or heat sealed over the reagent membrane. The holes provide for discrete viewing windows for visualizing a discrete series of steps in the consumase (apyrase) gradient for the ATP. A printed scale on the top surface allows the user to correlate the inflection of the bioluminescence with the actual concentration of ATP.

b. Detection threshold signals and linearity with film, CCD and eye: Considerable work was done to establish the ultimate detection threshold for analyte ATP using the

eye, film and the CCD camera. It was found that the single biggest factor to improve the minimum detection threshold for ATP was to incorporate recombinant luciferase with as high an activity as possible and to use as much luciferase as possible. To date, the most active luciferase we have used routinely (7×10^8 relative light units per mg enzyme) allows us to detect 1.0 nano Molar ATP in a 100 micro liter volume sample. This is $1x\ 10^{-13}$ moles of ATP. This amount of ATP was detectable visually with five minutes of dark adaptation and photographically with a five minute exposure using 3,000 ASA Polaroid Type 57 sheet film (4" x 5") in our sample-film interface holder. FIGURE 3 and FIGURE 4 shows the CCD linearity data for typical experiments where linearity and minimum detection threshold were determined for the CCD. Note the good linearity and minimum detection threshold in the 1 -2 nanomolar ATP for the 0-20 nanomolar study (100 s of signal integration). All of the CCD linearity studies have shown excellent linearity from 10^{-9} to 10^{-3} molar ATP. Additional studies have been conducted using 5 microliter droplets of ATP sample added to 5 microliter droplets of bioluminescent reagent with much higher luciferase activity (7×10^{10} relative light units per mg enzyme). The 10 μL droplets were placed on white a Teflon surface and observed visually, photographically and with the CCD. The droplets were only detectable with the CCD (100 s of signal integration time) and the detection threshold was 5 nano Molar ATP and 10 μL droplets or 50×10^{-15} moles ATP. This detection threshold for a cooled CCD (-20°C) is within about a factor of 10 of a good PMT detector which utilizes photon counting.

c. Evaluate spatial distribution of light concept using optimized apyrase gradient in terms of CCD, film and human visual response in the dark: This work was not accomplished due to the change in the biosensor design from a glass substrate to a PVC strip concept. It will be emphasized in the last semiannual period of this grant.

I. Research and Development Priorities for the Third Semi-annual Period

1. Maximize Luminescence

a. Complete gel preservation matrix long term stability study.

b. Continue work to optimize the short term stability of the lyophilization process. Extend the lyophilization work to include ATP along with luciferin and luciferase in the reagent cocktail. This is desirable because of the possible need to have a positive control (ATP channel) in the final assay.

c. Conduct a long term stability study of lyophilized reagents.

d. Evaluate the "recycling" of AMP back to ATP via enzyme amplification employing adenylate kinases and pyruvate kinase. Conduct both short term and long term stability studies of the reagent cocktail which contains the optimal recycling

enzymes to ensure that lyophilization can provide a means to preserve all of the necessary components for the assay.

e. Maximize activity and concentration of luciferase in biosensor design.

2. Optimize Detection

a. Evaluate wave guiding in a thin planar film for enhanced signal.

b. Make final recommendations regarding film and visual detection.

c. Optimize design for visual reading.

3. Design, Construct and Evaluate Sensor Platform

a. Finalize ATP sensor platform design.

b. Determine ultimate detection threshold sensitivity for final design via visual and film detection.

c. Evaluate spatial distribution of light concept in final sensor design.

d. Detect micro-organisms.

e. Determine materials cost of disposable sensor design.

f. Initiate long term stability study of sensor platform with all reagents incorporated.

g. Make recommendations regarding packaging of the sensor.

4. Write and Submit Final Report(s)

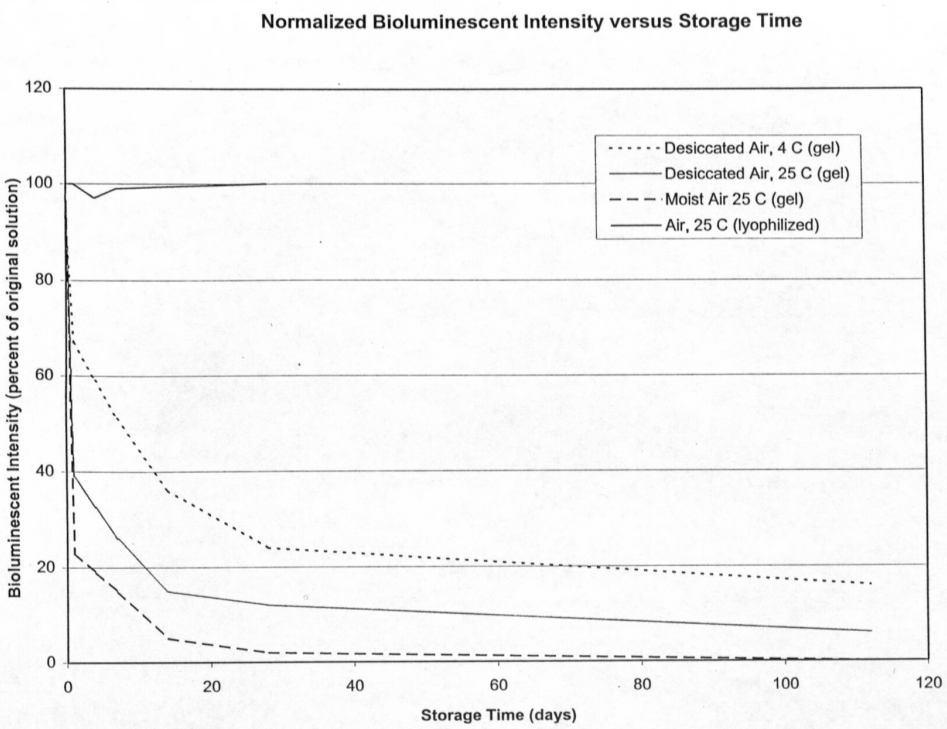

FIGURE 1 Long term storage data for bioluminescent reagents preserved in dried gels and as lyophilized product. Signal has been normalized to bioluminescent signal intensity under standard conditions and presented as a function of storage time under various conditions.

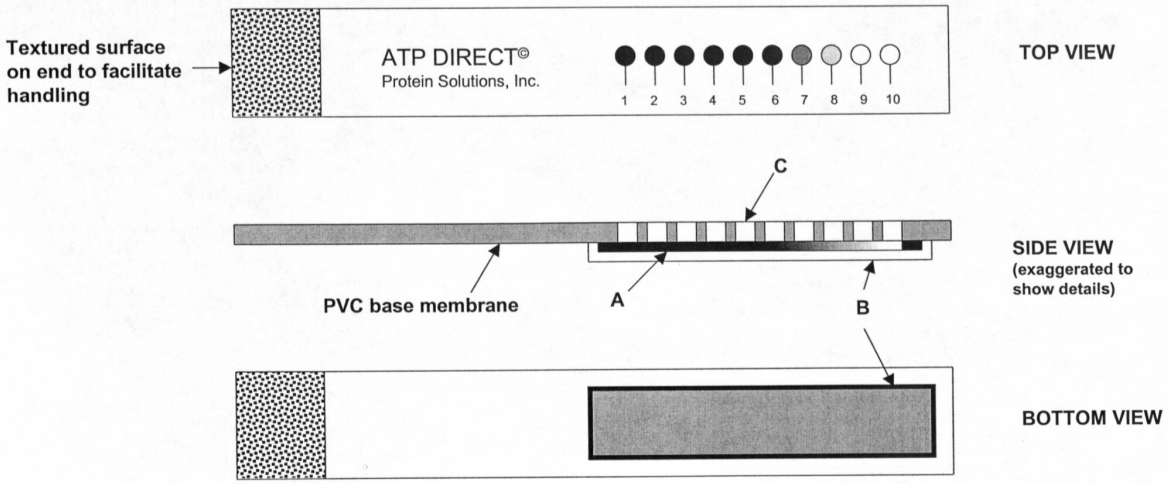

FIGURE 2 Schematic rendition of the ATP biosensor. The base membrane is a 0.5 mm thick, 60 mm long and 10 mm wide strip of PVC. Reagent membrane (A) is heat sealed onto the bottom surface of the PVC and filter membrane (B) is heat sealed over A to the PVC membrane. Three mm diameter holes (C) in the base PVC membrane allow bioluminescence arising in the reagent membrane to escape upward to the observer. The holes provide a window for visualizing a discrete series of steps in the consumase gradient for ATP. A printed scale on the top surface allows the user to correlate the inflection of the luminescence with the actual concentration of ATP.

ATP [n Molar]	Bkg. Corrected CCD Signal (counts/pixel)		
0	9		File: 021398D.XEL
100	359		
200	730		
500	1619		
1000	3336		
	705	Biolink 70 Channel 2	
	3785	Biolink 70 Channel 1	

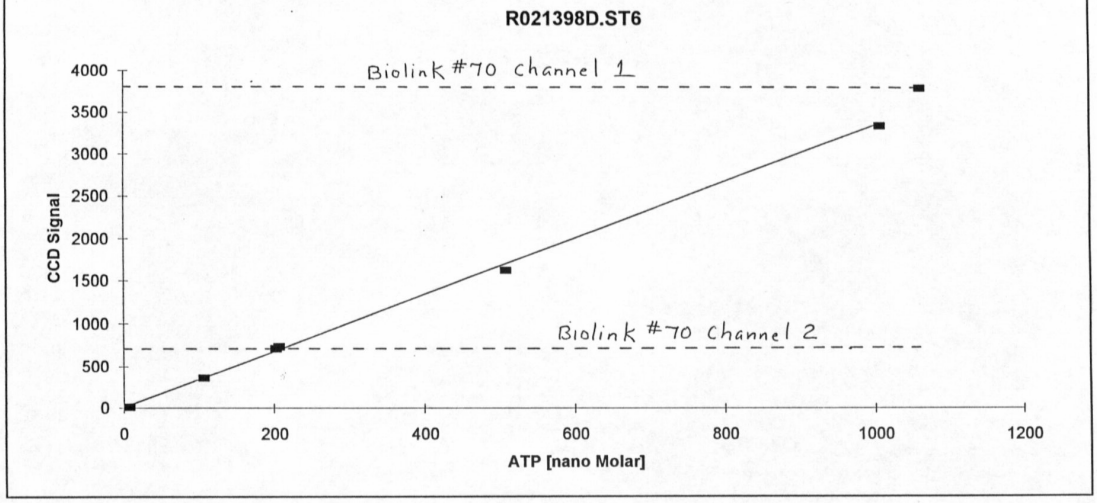

FIGURE 3 CCD linearity study over the range of 0 to 1000 nano Molar ATP concentration. Channels 1 and 2 of the Biolink reference standard # 70 are shown as reference. CCD signals are counts per pixel arising from a 100 second integration of the luminescence from the ATP biosensor.

ATP nano M	Bkg. Corrected CCD Signal (counts/pixel)		
0	155		
1	194		File: 031898A.XEL
2	243		
5	396		
10	622		
20	1,112		
0	717	Biolink 70 Channel 2	
0	82	Biolink 70 Channel 3	

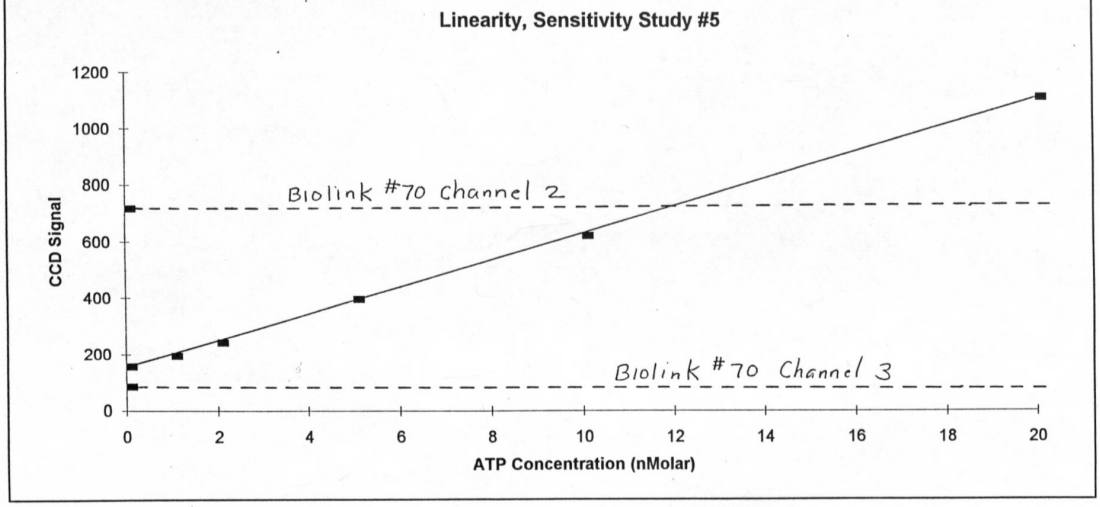

FIGURE 4 CCD linearity study over the range of 0 to 20 nano Molar ATP concentration. Channels 2 and 3 of the Biolink reference standard # 70 are shown as a signal reference. CCD signals are counts per pixel arising from a 100 second integration of the luminescence from the ATP biosensor. Note that the zero ATP signal is not zero. This is due to the residual ATP naturally present in the reagents, buffer and solutions at a level of 0.1 - 1.0 nano Molar.