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13. ABSTRACT (Maximum 200 words)
Report developed under STTR contract for topic "Army02-T016". A plate count method has been traditionally used to determine the effectiveness of anti-microbial agents in decontamination efficacy testing. This method requires large amounts of microbiological growth media and takes several days to produce results. Maxwell Sensors Inc. (MSI) and the Office of Homeland Security at the Southern Research Institute (SRI) propose to develop a rapid quantitative assay and platform based on the cellular ATP-Glow mechanism for determining the efficacy of biological threat agent decontamination. The method not only quantifies the amount of viable organisms after treatment with a decontaminant, but also gives the ratio of live and dead cells in samples. During the Phase I project, Bacillus anthracis spores and eight different microorganisms were used to validate the assay protocol. Decontamination efficacy of suspensions and surface decontamination of B. anthracis spores treated with Lysol™, Clorox™, Micro-Chem Plus™, and 70% ethanol were determined. Phase I results concluded that we have successfully demonstrated feasibility by using an ATP-Glow assay for rapid biological inactivation tests.

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U.S. Army Research Office, STTR Phase I

Contract Number: DAAD19-02-C-0087

ATP-Glow for Biological Decontamination Efficacy Tests

Technical Monitor: Dr. Stephen Lee/Dr. John Weimaster

Project Period: 09/03/02 - 03/02/03

Final Report

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B. RESULT OF THE PHASE I WORK

B.1 SIGNIFICANCE OF THE PROBLEM

Anthrax and biological decontamination is a rapidly evolving field, with new methods and technologies continually being developed as terrorist use of biological warfare agents (BWA) becomes an ever increasing threat [1-7]. Several antimicrobial agents and devices have been used under carefully controlled conditions during recent anthrax cleanups. Common disinfectants / sanitizers include chlorine dioxide, ethylene oxide, bleach, parafomaldehyde, hydrogen peroxide, peroxyacetic acid, methyl bromide, and vaporized hydrogen peroxide, with many others in development. Cleanup plans must reflect the size and type of potentially contaminated areas (e.g. large open rooms, a small office cubicles, electronics, instrumentation, engines, furniture, etc.), and how disinfectants will be dispersed (e.g., liquid, vapor, heat, etc.). Cleanup plans must also take into account the daily activities carried out in a contaminated area.

Methods for rapidly assessing the decontamination efficacy of various antimicrobial agents on bioorganisms at various sites remain underdeveloped. A plate count method has been traditionally used to determine the effectiveness of antimicrobial agents. However this culture method requires large amounts of microbiological growth media and takes several days to produce results. This assay procedure is also very laborious: due to a large number of samples needed for testing 10 disinfectants on 5 different bacteria at 30 different areas would require 1,500 samples, for example. Therefore, any monitoring instrument must have a ***high throughput*** capability and able to handle multiple samples accurately, simultaneously, and quickly.

B.2 THE SOLUTION

In response to the need for faster screening, Maxwell Sensors Inc. **(MSI)** and the Office of Homeland Security at the Southern Research Institute **(SRI),** ***proposed to develop a rapid quantitative assay and platform, based on a cellular ATP-Glow mechanism for determining the efficacy of BWA decontamination. The proposed ATP-Glow testing platform can not only quickly, sensitively, and accurately determine a cell growth-death ratio, but also has a high throughput and is capable of simultaneously handling multiple samples.*** This innovative BWA-ATP glow system combines two technologies:

(1) Cellular ATP (adenosine 5'-triphosphate) bioluminescence measurement. ATP is the primary energy source that living cells use for various processes. The amount of ATP detected, correlates directly to the number of viable cells in the culture system. In addition, this method uses membrane protonphore, and carbonyl cyanide meta-chloro-phenyl hydrazone (CCCP) to quantify ***viable bacteria in an unknown quantity of dead organisms.***

(2) Micro plates (e.g. 96 wells) and microfluidic platforms to automate and simplify the assay process for high throughput operations.

ATP is ubiquitous in all living organisms [8]; ATP assay has been widely used to assess cell viability in the pharmaceutical industry for developing antibiotics and other antimicrobials. The innovation of the proposed BWA ATP-Glow assay technology (see Fig. 1-1), which is different from common cell viability assay, is that BWA-ATP-Glow technology has the ability to measure the exact number of viable organisms in a mixture of dead organisms. Typically the inactivation process does not remove nonviable organisms. Dead cells may still contain residual amounts of ATP, therefore a large number of dead organisms can still contribute to a measurable amount of background luminescence. The CCCP induced ATP decrease can be observed "only in intact and living cells." Therefore, the ATP-Glow (ATP/CCCP) method is able to quantify the amount of viable organisms present after treatment with decontaminants and determine the kinetics associated with their destruction.

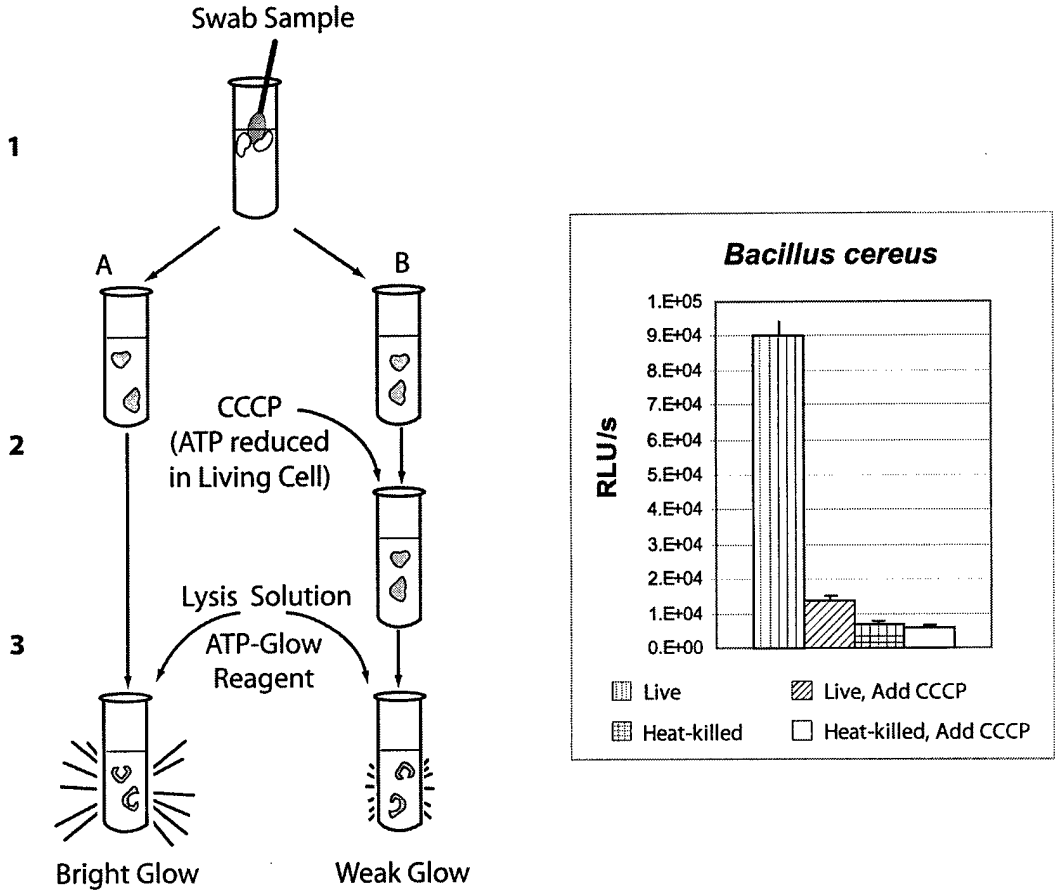


Fig. B-1. ***How does it work?*** The diagram shows the proposed ATP-Glow principle for the rapid & sensitive detection of viable bacterial spores and cells, and assessing the efficacy of decontamination. The assay procedure includes: 1. Add the swab sample (microorganisms) into probe solution; and distribute the solution into two tubes or wells (A & B); 2. Add CCCP solution to well B. 3. Add lysis solution and ATP-Glow solution into both A & B, and measure luminescence. The signal corresponds to the total ATP in the sample, and the decrease in luminescence in tube B is due to the effect of protonophores (CCCP) on live organisms. ***The Phase I Bacillus cereus data shows a significant CCCP effect on live cells, while negligible effect on dead cells (negative control). The decrease in luminescence is corresponds to the number of live cells.*** (see Sec. B.3.2.2 for details)

In summary, the Phase I highlights of BWA-ATP-Glow technology are:

1. **An assay protocol and platform for decontamination efficacy evaluation was developed:** The method offers the ability to accurately quantify the effectiveness of antimicrobial agents. **The whole assay process takes less than 15 minutes on vegetative cells and less than 35 minutes on anthracis spores.**

2. **The ATP-Glow assay for biological pathogen testing was validated:** ***Bacillus anthracis*** **spores and eight different microorganisms and strains** *(Bacillus cereus, Yersinia enterocolitica, Citrobacter freundii, E. coli O157:H7 & K12, Pseudomonas putida, Salmonella typhimurium, and Listeria monocytogenes),* **were used to validate the assay protocol. Without any exception they all showed an ATP signal and expressed a CCCP effect.**

3. **Decontamination efficacy on *B. anthracis* spores with disinfectants was performed:** Suspensions and surface decontamination of **B. anthracis spores in the presence of 10% Lysol™, 10% Clorox™, 10% Micro-Chem Plus™, and 70% ethanol was evaluated.** These studies validated the method for *B. anthracis* after various decontamination treatments.

4. **The high throughput detection system was integrated:**
A prototype system was developed for 96/384 well plate or batch mode formats. The system was characterized by a series of ***Bacillus subtilis*** and ATP experiments. The ATP-Glow system with **sensitivity of 75 *Bacillus subtilis* cells and 10^{-15} - 10^{-16} moles ATP have been achieved in Phase I.**

Phase I results concluded that we have successfully demonstrated feasibility by using an ATP-Glow assay for rapid biological inactivation tests. **A poster was prepared and presented by SRI and MSI at the ASM (American Society of Microbiology) - Biodefense Conference in Baltimore,** MD at (March 9-12). Based on these technical findings we are confident that we can launch into Phase II studies for further development.

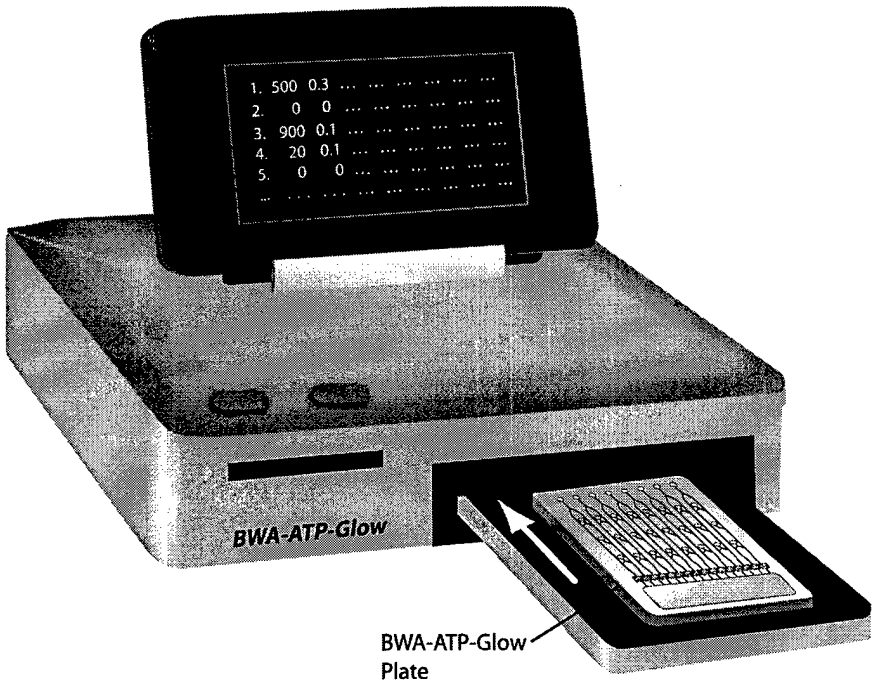


Fig. B-2. The figure shows the MSI's ATP-Glow portable system (11"x8"x4") including a disposable microplates (similar to 96/386 well format) for rapid, and high throughput evaluation of decontamination efficacy for an array of samples. In Phase II, the system will be miniaturized and automated to **simplify operational procedure to a single step.** The ATP-Glow microplate will be fabricated with patterned microchannels and microwells (50 µl - 200 µl) on a plastic cartridge. The system uses microactuator to perform the total automated the procedure and quantify the viable organisms remaining after treatment. (See Sec. B.3.5.1 and D.5 for details)

B.3 PHASE I RESULTS

Phase I Solicitation Topic: Army 02-T016
Period: September 3, 2002 ~ March 02, 2003,

The objective of the Phase I project was to establish and validate the ATP/CCCP assay for determining biological decontamination efficacy. The technical feasibility of the proposed assay and platform for evaluating decontamination effectiveness was successfully demonstrated. During the phase I project the Principal Investigator, Dr. Winston Ho was responsible for the design and construction of the assay platform and optoelectronic system, and collaboration with **STTR subcontractors** at the Southern Research Institutes **(SRI)** and the U.S. Department of Agriculture **(USDA).** SRI (Mr. Matthew Maland, Dr. Thomas Voss, and etc. at Frederick, MD) was responsible for developing anthrax spore viability tests and selecting taminants. USDA (Dr. Shu-I Tu and his team in Wyndmoor, PA) was responsible for developing the assay protocol and performed vegetative cell viability studies. The project was supported by MSI's scientists and optoelectronic engineers during construction and characterizing of the detection system. All of the Phase I objectives below have been achieved.

Objective 1: ***Develop a reliable BWA-ATP-Glow-based assay protocol***
Objective 2: ***Perform standard, calibration, and effects of CCCP assays***
Objective 3: ***Results analysis and technical merit evaluation***
Objective 4: ***Design and construct detection system for bioassay automation***

The work of Phase I is described in detail below.

B.3.1 Development of the Assay Protocol

The Cell Viability Assay is a method for determining the number of viable cells in a culture system, based on the quantification of adenosine 5'-triphosphate (ATP) present. The amount of ATP detected, directly correlates to the number of viable cells in a culture system. The assay provides a sensitive, rapid, high-throughput method that is ideal for automation. In the case of decontamination applications, dead cells may still contain residual amounts of ATP that can still contribute to a measurable amount of background luminescence. Therefore, in order to accurately quantify decontamination efficacy, knowing how many live and dead cells in a sample, CCCP analysis was included, which offers information about background ATP signals.

The membrane of a cell is known to contain an ATP supported H^+-pump (see Fig. B-3). Weak organic acids (e.g. CCCP), can rapidly (~ 3 minutes) shuttle protons across membranes and discharge the protons' electrochemical potential generated by pumping action [9-11]. This results in the increased usage of ATP in a futile attempt to re-establish electrochemical potential. ***Thus, by rapidly shuttling protons across the cell membrane, the overall amount of ATP is immediately exhausted in living cells; in contras, CCCP has no effect on dead cells.***

Bio-organisms are lysed to release ATP that is detected by luciferin-luciferase induced luminescence. A luminescence substrate, such as CellTiter-Glo™ reagent (Promega), is a ready to use cell viability assay formula, which detects the presence of metabolically active cells and generates a "glow-type" luminescent signal. The assay detects as few as 5 cells/well in a 96 well format, or 15 cells/well in a 384 well format [8]. The luminescence has a glow half-life of greater than five hours. The luminescence intensity provides both sensitivity and easiness of optical detection.

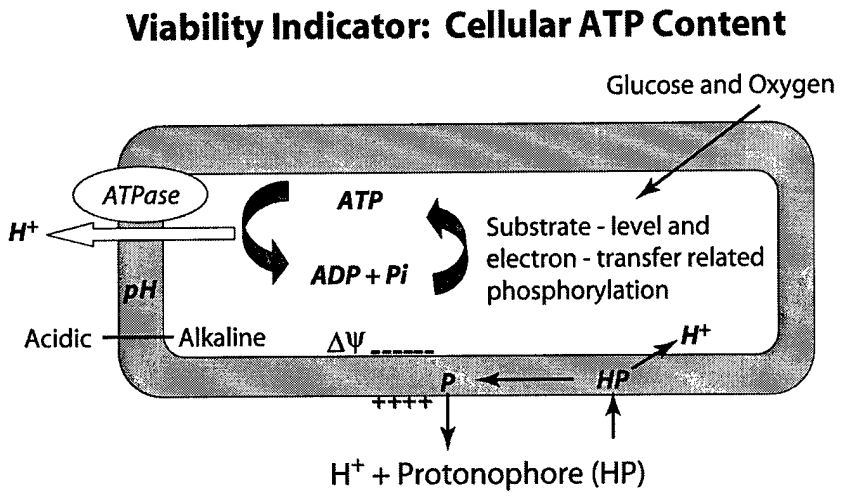


Fig. B-3. The ATP assay principle with CCCP protonophore for cell viability testing.

B.3.2 Validation of the ATP/CCCP Assay for Various Biological Pathogens

The proposed assay protocol should be effective and universal for all biological pathogens. In this task, eight different microorganisms and strains were used to validate the ATP/CCCP assay. These biological pathogens include:

1. Bacillus cereus,
2. Yersinia enterocolitica,
3. Citrobacter freundii,
4. E. coli O157:H7
5. E. coli K12;
6. Pseudomonas putida,
7. Salmonella typhimurium,
8. Listeria monocytogenes

B.3.2.1 Materials and Preparation

All bacterial cultures were grown for 18-24 hr. in 25 ml of media contained in a 50 mL flask. Two, one milliliter aliquots of each bacterial culture were centrifuged at 10 K rpm for 3 min. in microfuge tubes. Each pellet was washed once with buffer (10mM Tris with 2.5mM Mg SO_4 and 150mM NaCl) and resuspended in 1 ml of buffer and diluted 10-fold twice to get 10^7 cells/ml. For heat-killed cells, one tube from each culture was heated for 10min. at 100C in a dry sand bath. To determine CFU/ml, each culture was further diluted in PBS (Sigma) down to 10^3 cells/ml and two-50ul aliquots were plated on the appropriate media, incubated overnight and colonies were counted. Enzyme Luciferase-luciferin (Sigma L 0633) dry powder was dissolved in sterile milli-Q water at 10mg/ml. Carbonyl cyanide 3-chlorophenyhydrazone (CCCP) (Sigma C2759) dry powder was dissolved in ETOH at 1mg/ml. Bacterial Protein Extraction Reagent (B-PER) was obtained from Pierce.

1. E. coli O157:H7, B1409; grew in BHI @ 37C, 160rpm; 18hr
2. Listeria monocytogenes FSL N1-227; grew in BHI @ 37C, 160 rpm; 18 hr
3. Salmonella typhimurium 68430; grew in BHI @ 37C, 160 rpm; 18 hr
4. Bacillus cereus ATCC 49046; grew in NB @ 30C, 160 rpm; 24 hr
5. Citrobacter freundii 33128; grew in NB @ 37C, 160 rpm; 18 hr
6. E. coli K12; grew in BHI @ 37C, 160 rpm; 18 hr
7. Pseudomonas putida KT2442; grew in NB @ 37C, 160 rpm; 18 hr
8. Yersinia enterocolitica):TAC-C; grew in NB @ 37C, 160 rpm; 18 hr

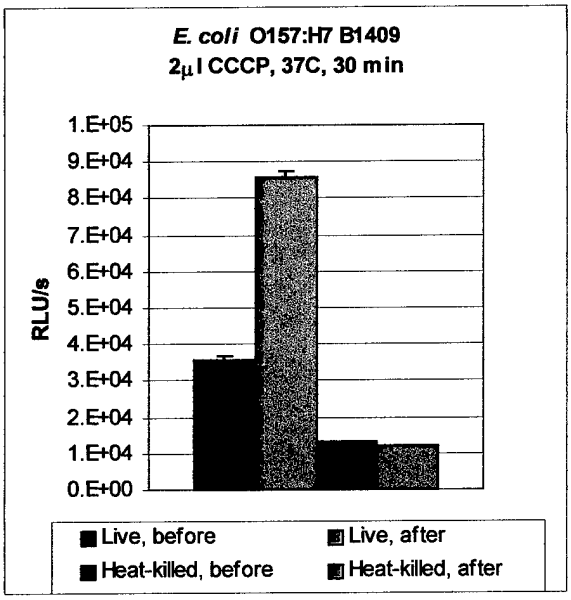
B.3.2.2 The Assay Results of Various Biological Pathogens

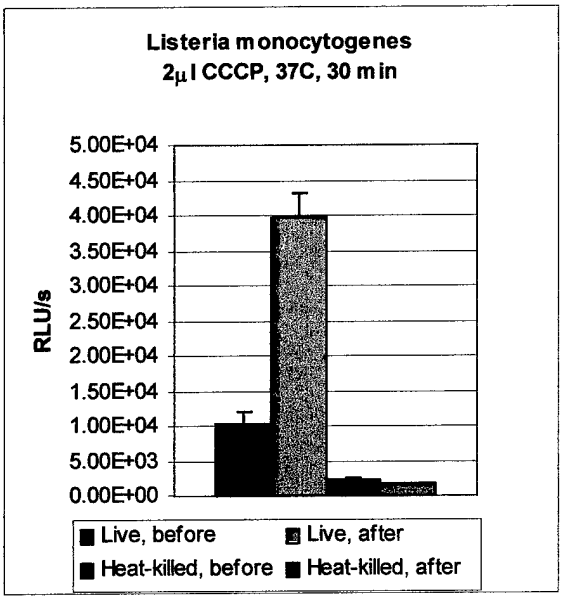
Each bacterial strain was tested for enzyme assay with live or heat killed cells. Each of four microfuge tubes with 100ul of live or heat-killed bacteria were first treated with 2ul of CCCP and incubated at 37C for 30min. At the end of the incubation period, 100ul of B-PER was added into each tube, vortexed and incubated at room temperature (RT) for 10 minutes prior to the enzyme assay of bioluminescence. The luminescence represents the residual ATP content of the live cells after CCCP perturbation to membrane H^+-ATPase.

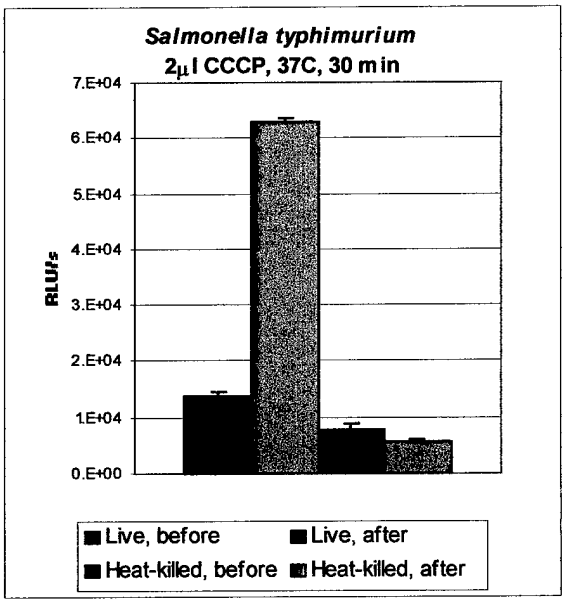
To a second set of four tubes, each with live or heat killed cells, 100ul of B-PER was added, and incubated at room temperature (RT) for 10 min. At the end of the incubation period, 2ul of CCCP was added into each tube, vortexed and incubated for 30min. at 37C prior to enzyme assay. Luminescence represents the total ATP content of live cells. The addition of CCCP after cell breakage by B-PER assures identical sample solution optical properties.

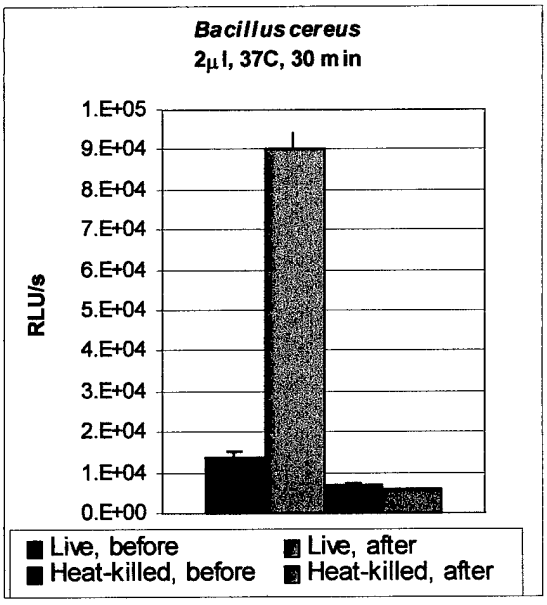
For enzyme assay, 100ul of enzyme was added into each tube, mixed well by vortexing and the tubes were placed in the instrumentation immediately. Relative Luminescent Units (RLU) were read off the instrumentation and exported as Excel files for further analysis. A Berthold FB12 luminometer was used in these experiments.

Fig. B-4 shows the results for 8 different pathogenic bacteria. "Live after" means CCCP was added after cell breakage (by protein extraction reagent (B-PER)). While "Live before" means CCCP was added before cell breakage, thus cell encounters with CCCP exhaust significant amounts of ATP. ***The results show without any exception, large ATP signals and significant signal reduction by CCCP in lived cells; in contrast they show minimal ATP signal and negligible signal reduction by CCCP in dead cells.*** When no CCCP effect is observed, we know that all cells are dead. ***The decrease in luminescence corresponds to the number of live cells.*** The ratio of live after-live before is related to cell growth/death. Since we know the ATP content and luminescent signal from a single live and dead cell, ***we can accurately quantify the number of dead cells and live cells in a mixture.***


E. coli O157:H7 B1409
2μl CCCP, 37C, 30 min
RLU/s
1.E+05
9.E+04
8.E+04
7.E+04
6.E+04
5.E+04
4.E+04
3.E+04
2.E+04
1.E+04
0.E+00
Live, before
Live, after
Heat-killed, before
Heat-killed, after


Listeria monocytogenes
2μl CCCP, 37C, 30 min
RLU/s
5.00E+04
4.50E+04
4.00E+04
3.50E+04
3.00E+04
2.50E+04
2.00E+04
1.50E+04
1.00E+04
5.00E+03
0.00E+00
Live, before
Live, after
Heat-killed, before
Heat-killed, after


Salmonella typhimurium
2μl CCCP, 37C, 30 min
RLU/s
7.E+04
6.E+04
5.E+04
4.E+04
3.E+04
2.E+04
1.E+04
0.E+00
Live, before
Live, after
Heat-killed, before
Heat-killed, after


Bacillus cereus
2μl, 37C, 30 min
RLU/s
1.E+05
9.E+04
8.E+04
7.E+04
6.E+04
5.E+04
4.E+04
3.E+04
2.E+04
1.E+04
0.E+00
Live, before
Live, after
Heat-killed, before
Heat-killed, after

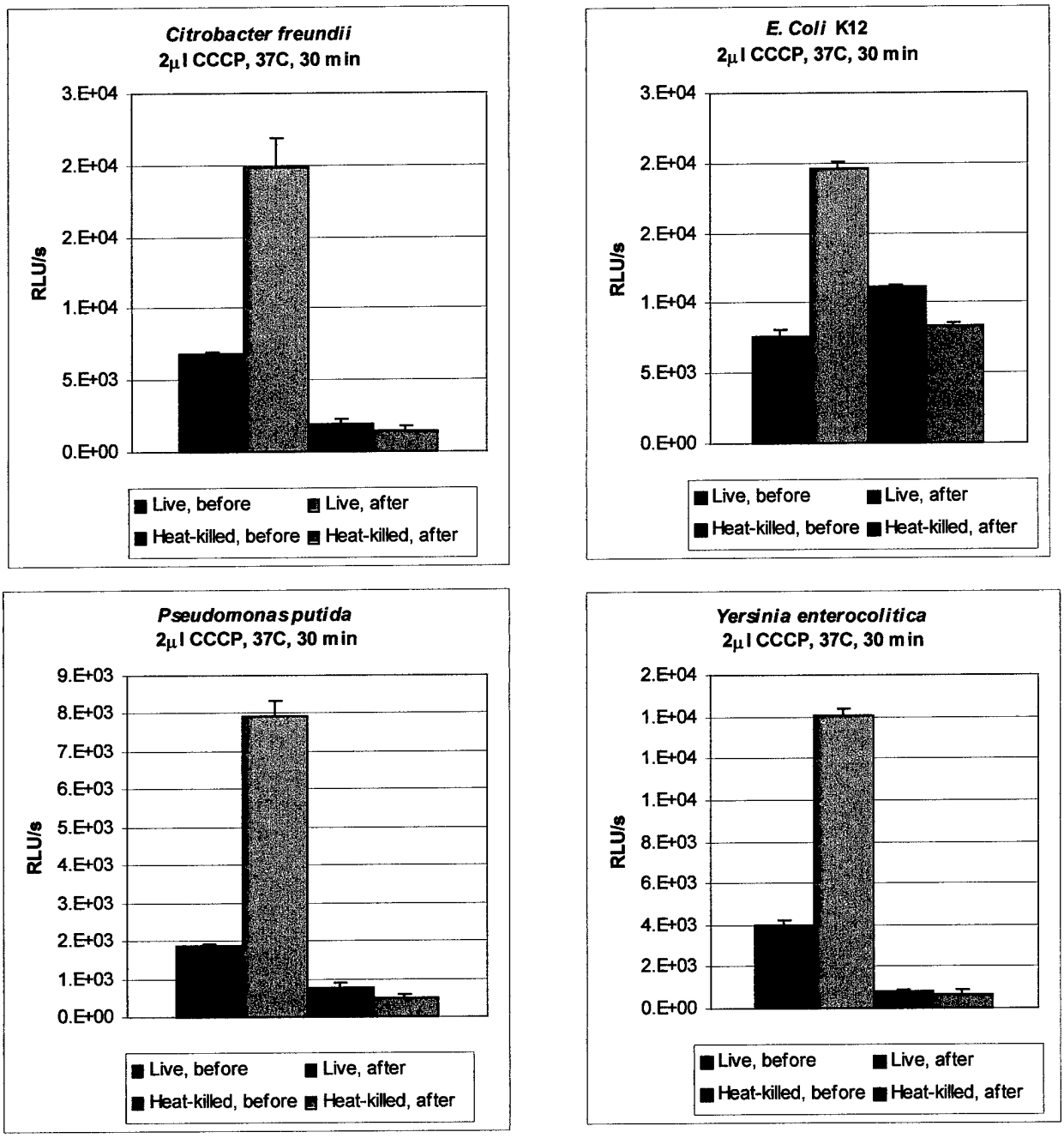


Fig. B-4. The proposed assay protocol proved to be effective and universal to all eight biological pathogens. CCCP significantly reduces ATP content in live cells, and has minimal effect on dead cells.

Legends: **Live, after**: CCCP was added **after** cell breakage (by protein extraction reagent (B-PER)), thus cells still retain ATP.
Live, before: **CCCP was added** before **cells breakage, thus cells encountered the effects of CCCP and consumed significant amounts of ATP.**

B.3.3 ATP/CCCP Assay of *Bacillus Anthracis* Spores

Bacillus Anthracis has been classified as one of the most dangerous BWAs. Since spores of *B. anthracis* are metabolically dormant, it was unclear if intracellular ATP levels were sufficient for detection by this assay. This experiment was to evaluate different media and demonstrate that it is possible to detect ATP content in B. anthracis spores in a short time. CCCP was introduced to investigate the effect and distinguish between live and dead spores.

B.3.3.1 Materials and Preparation

The Southern Research Institute provided spores of a capsule and toxin positive (pathogenic) strain of *Bacillus anthracis*. CellTiter-Glo™ reagents were obtained from Promega. CCCP and ATP disodium salt were obtained from Sigma-Aldrich. 96-well plates for luminometry, Brain Heart Infusion (BHI) media, buffers, and plasticware were obtained from Fisher Scientific.

Suspensions of *B. anthracis* spores were prepared in:

(a) sterile water for injection (WFI), R/T
(b) sterile water for injection (WFI), 37C
(c) sterile water for injection (WFI), 60C
(d) 1% BHI media, R/T
(e) 1% BHI media, 37 C
(f) 10% BHI media, R/T
(g) 10% BHI media, 37 C

The suspensions were prepared at a concentration of approximately 10^7 cells/well. ATP standard solutions were prepared, ranging from 1μM to 10pM, and 100μL of each were added to the same 96-well plate. Negative control wells (media alone) were prepared to obtain a value for background luminescence. The plates were then incubated at different temperatures for 30 minutes. After incubation 100μL of the CellTiter-Glo™ reagent was added to each well and the plate was mixed on an orbital shaker for 2 minutes to induce cell lysis. After an additional 10-minute incubation at room temperature to stabilize the luminescent signal, the plate was read in a luminometer. Luminescence signals of test samples were then compared to those of ATP standards and concentrations of ATP present in the test samples were determined by extrapolation from the standard curve.

B.3.3.2 Assay Results for Detecting ATP in Bacillus Anthracis Spores

These studies show that it is indeed possible to detect ATP luminescence in B. anthracis spores and determine viability (Fig. B-5). Best assay conditions were achieved by using 10% BHI at room temperature. The suspensions of *B. anthracis* spores prepared in sterile water for injection (WFI) produce a minimal signal: this is attributable to the metabolic dormancy of the cells in spore form. ***Spores incubated with either 1% or 10% BHI at room temperature, or 37°C for 30 minutes, appear to utilize available nutrients and rapidly synthesize detectable levels of ATP.***

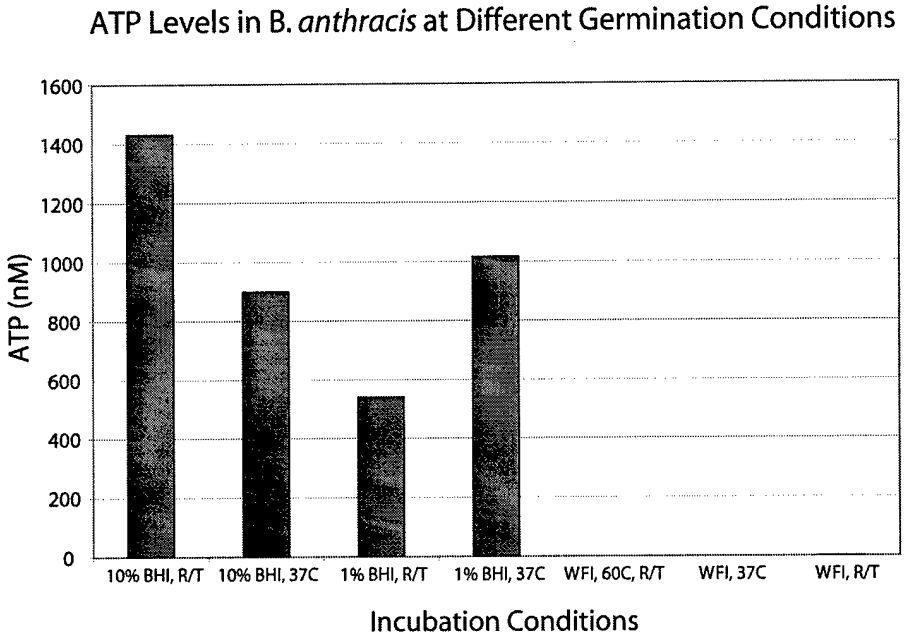


Figure B-5. The data show the concentration of ATP present in **Bacillus anthracis spores** under various incubation conditions.

B.3.3.3 Assay Results for Detecting CCCP Effect on *B. Anthracis Spores*

For this experiment, *B. anthracis spores* were prepared to investigate the effect of CCCP on ATP (Fig. B-6). The spore samples were prepared at 10^7 cells/well in a 96-well opaque walled and incubated with or without CCCP (5-15ug/mL) for 30 minutes at 37°C. The presence of CCCP appears to inhibit ATP synthesis in a dose dependent manner. Treatment with the maximum amount of CCCP (15µg/mL) resulted in an approximate six-fold inhibition of ATP synthesis as compared to spores treated with vehicle alone. Due to solubility problems, the initial stock solution of CCCP was prepared in dimethyl sulfoxide (DMSO), however the final concentration of DMSO in the wells was less than 2% and had no significant effects on the ATP synthesis as shown by the vehicle control. ***The results show that CCCP does have an effect on live spores and significantly reduces the ATP signal***. The data again shows that CCCP assay offers the possibility to quickly distinguishing between live and dead spores.

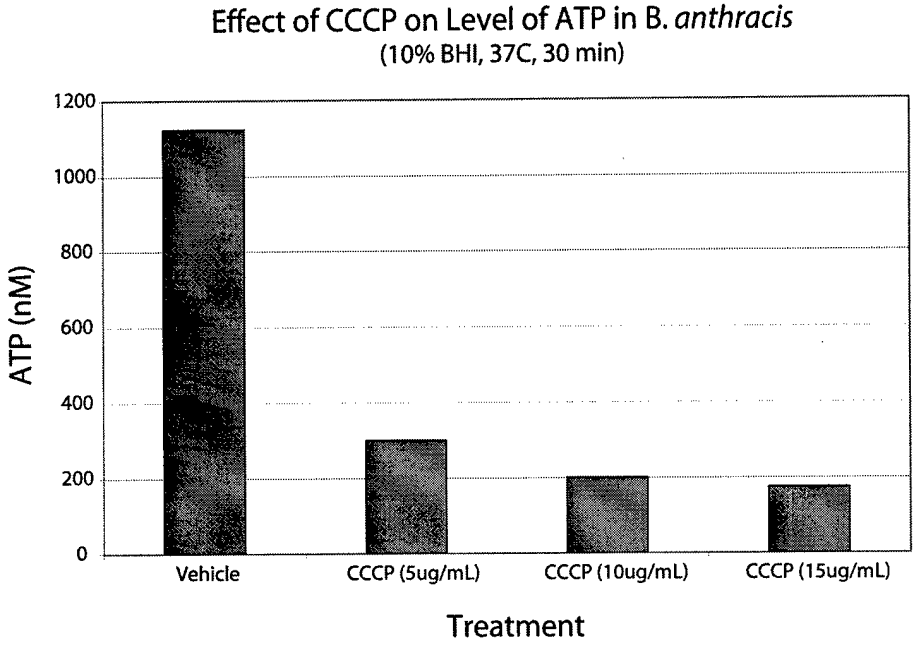


Figure B-6. The data show the concentration of ATP present in spores incubated in the presence or absence of various concentrations of CCCP, in 10% BHI media for 30 minutes at 37ºC.

B.3.4 Antimicrobial Agents Decontamination Efficacy on *B. anthracis Spores*

These studies were designed to further evaluate the ATP-Glow method for detection of viable *B. anthracis* after various decontamination treatments. The spores were inactivated in liquid suspensions and on dried surfaces. The experiment was performed with 96-well plates. Brain Heart Infusion (BHI) media, nutrient agar (NA), buffers, decontaminant solutions and plasticware were obtained from Fisher Scientific. SRI provided spores of a capsule and toxin positive (pathogenic) strain of *Bacillus anthracis*.

B.3.4.1 *B. anthracis* Spores Assay After Suspension Decontamination

Suspensions of B. anthracis spores were prepared in sterile water for injection (WFI) at $1x10^7$ spores/mL in the presence of: antimicrobial agents:

- 10% Lysol™,
- 10% Clorox™ (0.615% hypochlorite),
- 10% Micro-Chem Plus™, and
- 70% ethanol, respectively.

After 0, 1, 5, or 10 minutes of contact time at room temperature, 1mL of each spore-decondecontaminant solution was diluted with 9mL of 10% BHI media (1:10 dilution). This solution was then used to make further 1:5 serial dilutions (i.e. 1:50, 1:250, 1:1250, 1:6250 etc.) in 10% BHI media. ***ATP standard solutions were prepared in 10% BHI containing known amounts of ATP and equivalent amounts of disinfectant present in experimental samples.*** Each sample and standard dilution (100µL) was then used for cell viability assay. All samples were run in triplicate. Figure B-7 shows the concentration of ATP present in spore suspensions decontaminated with various disinfectants. Since it is known that ATP concentration directly relates to numbers of viable cells, it appears that all decontaminants are very effective in killing

liquid suspensions of B. anthracis spores in as little as 1 minute of contact time. However, 70% ethanol is slightly less effective than other decontaminant solutions. Moreover, ***the presence of low concentrations (1-7%) of decontaminant during the ATP detection assay showed no significant adverse effects on the accuracy of the assay as compared to those run in the absence of decontaminant.*** The data show that this method is effective for all disinfectants used in this study.

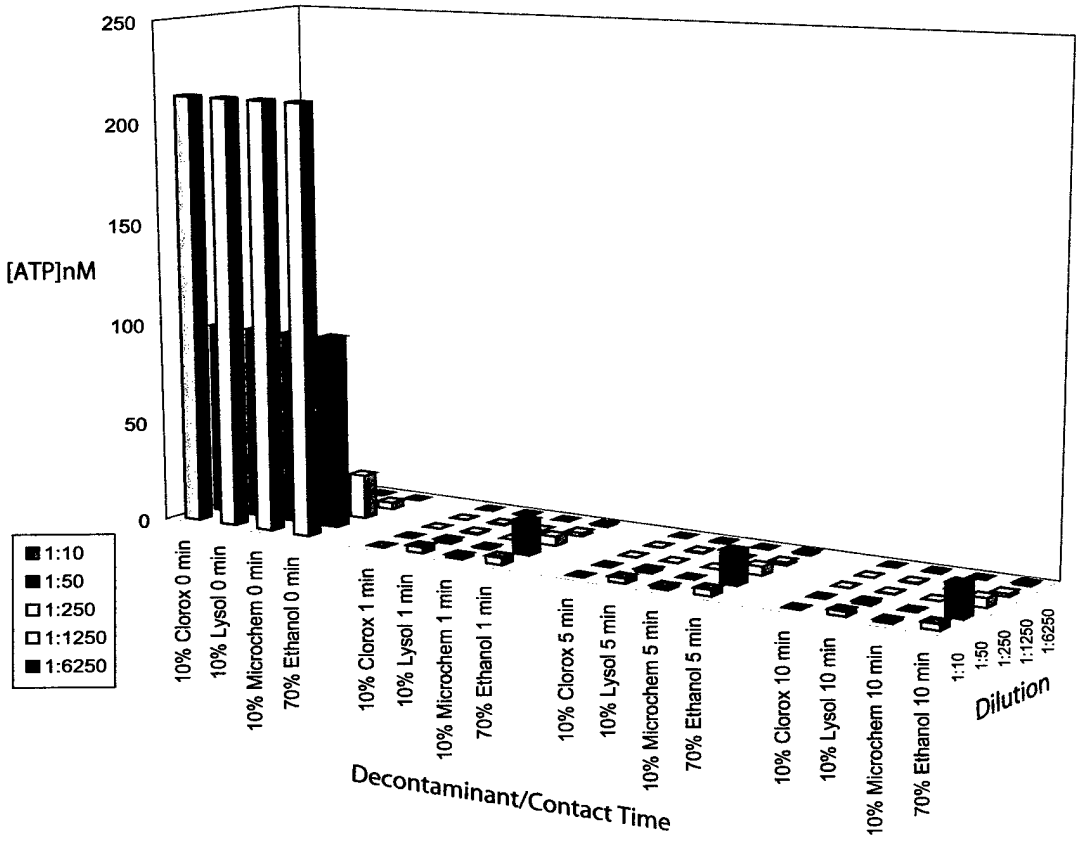


Fig. B-7. *B. anthracis* spore assay after ***suspension decontamination*** by the ATP-Glow method. The ATP measurement of B. anthracis spore suspensions were decontaminated with 10% Lysol™, 10% Clorox™, 10% Micro-Chem Plus™, or 70% ethanol, respectively.

B.3.4.2 *B. anthracis* Spore Assay After Surface (Dried) Decontamination

Individual wells of sterile 96-well plates were spread with 100µL of spore suspension ($1x10^7$ spores per well) and were allowed to ***dry*** under a bio-safety cabinet. Each well contained $1x10^7$ *B. anthracis* spores in sterile water. After drying completely, the plates were incubated in the presence or absence of 1mL of 10% Lysol™, 10% Clorox™, 10% Micro-Chem Plus™, or 70% ethanol respectively. After 0, 1, 5, or 10 min of contact time, the disinfectants were removed and the plates were swabbed with 1mL of sterile 10% BHI media. The samples were then collected off the plates and diluted 1:10 down to 1:100,000,000 with sterile 10% BHI media. Samples and ATP standards were then used for cell viability assay. All samples were run in triplicate and ATP standards were run in duplicate. Fig. B-8 shows the concentration of ATP present in surface samples of spores decontaminated with various disinfectants. Since it is known that ATP concentration directly relates to the numbers of viable cells, it appears that all decontaminants, except for 70% ethanol, are very effective at killing dried B. anthracis spores on

surfaces in as little as 1 minute of contact time. Ethanol appears to be almost totally ineffective at killing B. anthracis spores on surfaces. The result of surface decontamination is similar to the suspension decontamination method, except for 70% ethanol. ***These data prove the technical feasibility of the proposed ATP-Glow method for decontamination efficacy quantification.***

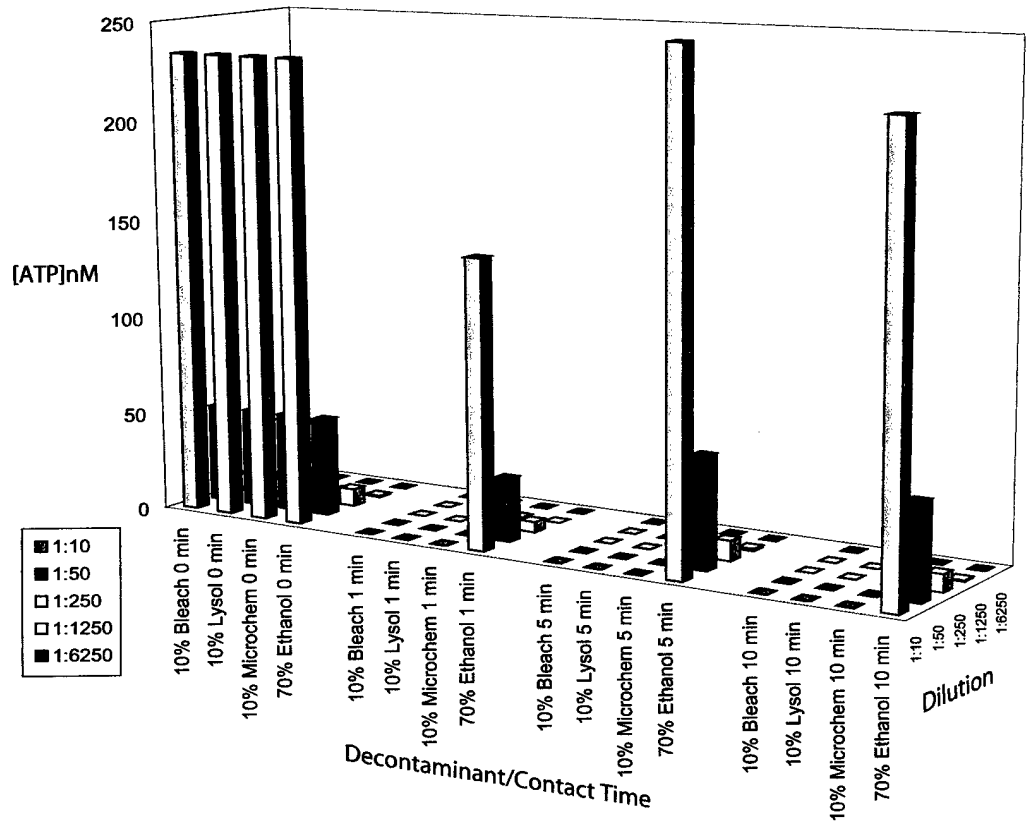


Fig. B-8. *B. anthracis* spore assay after ***surface (dried) decontamination*** by ATP-Glow method. Surface B. anthracis spore samples were inactivated with 10% Lysol™, 10% Clorox™, 10% Micro-Chem Plus™, or 70% ethanol, respectively.

B.3.5 High Throughput Detection System

B.3.5.1 Construct Compact Luminescence Detection Prototype

In order to achieve the Phase I technical objectives for rapid ATP-Glow detection, a readout system with high throughput capability was designed specifically for BWA agent detection. During Phase I of the project, a ATP-Glow readout prototype was constructed (Fig. B-9). The system includes a stepper motor, an intelligent motion controller, a power supply for the motion controller, an Ethernet to serial port converter, and a photon sensor. Development involved:

1. A loading mechanism for a plastic cartridge.
2. Serial protocol sensor/PC development.
3. Serial protocol motion controller/PC development.
4. Software implementation and source code development.
5. Cable, mounting, connectors, switches, etc. assembly.
6. Testing and debugging.

The system is designed for ease of use. After selecting the number of wells, either standard (96 wells) or custom (various multi-well format), hitting "Start" is the only required operation. The system scans all the designated wells and presents the data on the screen in a spreadsheet format (Excel). The signal integration time ranges from 0.01 – 1.0 seconds. The raw data is expressed with optical counts.

Fig. B-9 (a) The photo shows the MSI-ATP-Glow readout optoelectronics and mechanical components. (b) The exterior view of the prototype. The system will be further miniaturized in Phase II.

B.3.5.2 Characterize System Performance and Assay Sensitivity

The Phase I ATP-Glow prototype was characterized and tested based on **direct cell counts and ATP measurements for a purely vegetative culture of *Bacillus subtilis*.** The purpose of this experiment is to determine the detection limit of the assay and platform prototype. The method is to determine the number of viable cells in a culture based on the ATP present. Lyophilized *Bacillus subtilis* (Ehrenberg) Cohn was obtained from ATCC (11774). The bacterial was inoculated into nutrient broth (Difco 0003, Fisher Scientific, S7 1694-4) for 2 days at room temperature with shaking. From this culture, cell suspensions were prepared by 10-fold serial dilution in nutrient broth. From each of these dilutions, 100µl were used for the ATP detection assay and 100µl for live culture CFU determination on NA plates (Fisher Scientific, S716954). Both were performed in triplicate.

Pipette 100µl of broth (blank), diluted bacterial culture, and ATP standards into the black 96 well microplate (Nalge Nunc International) and were then used for cell viability assay. Buffer PBS-6% BSA-pH 7.2 was used to dilute the ATP stock solution. A series of standard ATP dilutions were prepared. The stock solution was rATP, riboprobe, 10 mM, 0.5 ml from Promega.

The system's dynamic range covers five orders of magnitude; however, we are most interested in the low concentration range. Table B-1 and Figure B-10 show the results of cell counts and their corresponding ATP signal for a series of 100µl culture dilutions. **The data shows that the MSI's prototype system has a detection limit of 70 *Bacillus subtilis.*** The luminescent signal for 19 cells was not distinguishable with the background signal from pure broth sample. The detection limit was determined based on the lowest concentration giving a distinguishable assay signal (with >3 times the standard deviation) from a zero sample. Table B-2 and Figure B-11 show the ATP concentration correlated with luminescent outputs. **The data shows the system has a detection limit of 10^{-16} moles for ATP.** According to Promega's literature, 50 Jurkat cells have a content of ~10^{-15} moles ATP. The system appears to be out performs most commercial systems in terms of sensitivity.

Table B-1. The data shows *Bacillus subtilis* concentrations in Nutrient Broth and corresponding luminescence signals.

Bacillus subtilis cell counts	Luminescence Signal (counts)
0, broth only	30
19	31
70	45
162	74
805	163
>2,000	959

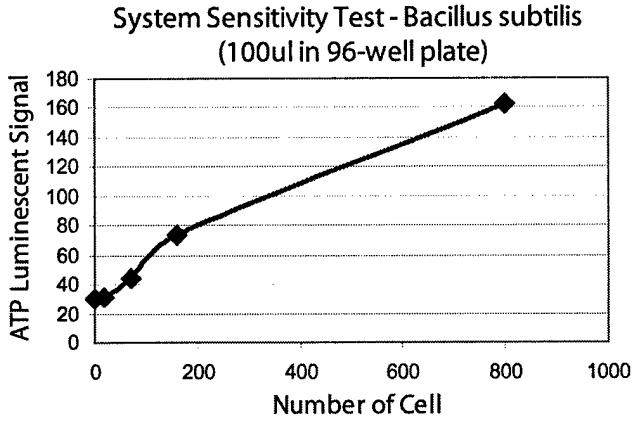


Fig. B-10. The ***Bacillus subtilis*** ATP luminescent signal measured by MSI-ATP-Glow prototype vs. the corresponding cell counts in culture samples. The number of colonies forming units per well was determined by live culture on nutrient agar plates.

Table B-2. The data shows ATP concentrations in Nutrient Broth and corresponding luminescence signals.

ATP Amount	Luminescence Signal (counts)
0, broth only	30
10^{-16} moles	52
10^{-15} moles	86
10^{-14} moles	130
10^{-13} moles	932
10^{-12} moles	9634
10^{-11} moles	98350

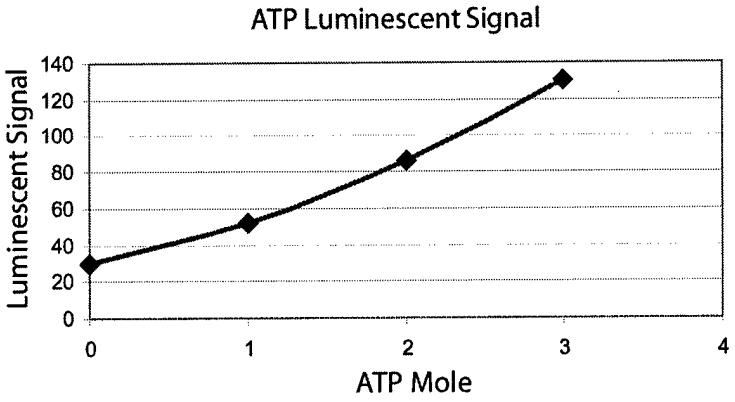


Fig. B-11. The figure shows the luminescence signal obtained by the MSI-ATP-Glow system as a function of ATP content.

B.3.6 Phase I Summary and Conclusion

During Phase I of the project we have:

1. Developed an assay protocol and platform for rapid decontamination efficacy evaluation

The combination of ATP-Glow analysis offers the ability to accurately quantify the effectiveness of antimicrobial agents. This assay provides a ***rapid (15-35 minutes), high-throughput (96 well)*** assay that is ideal for automation. The immuno-magnetic beads (IMB) proposed in Phase I, to capture and concentrate target analytes, were not used. This is due to the fact that microorganism for decontamination test is known and pre-selected.

2. Validated the ATP-Glow assay for a variety of bio-organisms

The assay protocol has proven to be effective for many microorganisms used in Phase I. In addition to B. anthracis spores, eight different microorganisms and strains were used to validate the assay protocol. The pathogens included: Bacillus cereus, Yersinia enterocolitica, Citrobacter freundii, E. coli O157:H7, E. coli K12, Pseudomonas putida, Salmonella typhimurium, and Listeria monocytogenes. ***Without any exception, they all show an ATP signal and express the CCCP effect. CCCP induced ATP decreases can be observed only in intact & viable cells, and shows no effect on broken or killed cells.***

3. Performed ATP-Glow assay using *Bacillus Anthracis* spores

Since spores, such as *B. anthracis,* are metabolically dormant, it was unclear if the intracellular ATP levels were sufficient for detection by this assay. Experiments demonstrated that it is possible to detect ATP content in ***B. anthracis spores*** in a short time. Initial assay conditions were established. Experiments also proved that the CCCP assay can distinguish between live and dead spores.

4. Studied decontamination efficacy on *Bacillus Anthracis spores* with various disinfectants

These studies validated that the ATP-glow method can be used to quantify decontamination efficacy. Suspensions and surface decontamination of B. anthracis spores were prepared in the presence of 10% Lysol™, 10% Clorox™, 10% Micro-Chem Plus™, and 70% ethanol. The assay can be used to ***simultaneously quantify and evaluated multiple decontamination samples in the 96-well high throughput format.***

5. Integrated and characterized a portable high throughput detection system

A prototype system designed for high throughput decontamination efficacy assay was developed. The ***ATP-Glow system shows the system can detect as little as 70 Bacillus subtilis or 10^{-16} moles ATP samples.***

Phase I results concluded that we have successfully demonstrated feasibility by using an ATP-Glow assay with a 96-well platform for rapid biological inactivation tests. The method is sensitive, accurate, fast, easy to use, and can simultaneously test a large number of samples. Based on these technical feasibility findings we are confident that we can launch into Phase II studies for further development.