CRITIQUE OF TEST METHODOLOGIES FOR BIOLOGICAL AGENT DETECTION AND IDENTIFICATION SYSTEMS FOR MILITARY AND FIRST RESPONDERS

David Trudil
New Horizons Diagnostics, Columbia, MD 21045

Currently evaluations of the performance of various BW agent rapid detection or identification systems have primarily focused on defined aerosol samples released under favorable, controlled conditions or controlled samples assayed under laboratory conditions. Although these methods may provide information on the sensitivity, cross-reactivity, and some interferences, they may not provide sufficient information to adequately determine the effectiveness of the technology in the field conditions or when difficult environmental samples are analyzed.

Results with three different technologies currently utilized by various agencies in the field (Colloidal Gold - Hand Held Assays, generic DNA, and Luminescence) have demonstrated significant performance differences when evaluated with common environmental samples. Adulterants or interfering substances, which may be used by a terrorist, have also been tested, demonstrating additional potential problems.

In this study, salt, sugars, detergents, talc, phosphates, cross-reacting organisms (*Bacillus spp*), non-toxic material of biological origin (cereal), as well as very high concentrations of the specific organisms, were used to challenge the technologies. The purpose was to develop methods to determine both interference and potential pro-zoning issues. Results indicated that some of the immunological assays, including Anthrax, can give false positive results when a sample with high pH or ionic strength (salts) were evaluated. Moreover, a very high concentration of the antigen in a positive sample may cause a false negative result.

Utilizing testing challenges similar to those developed by the FDA, USDA, AOAC and other organizations, it is apparent the rapid detection systems developed for the battlefield “air samples” may not be applicable for other environmental samples including terrorist events. Collection and sample processing systems may aid in reducing potential problems with a sample, but the assay format should be evaluated with the specific samples to be tested in ensure reliable results. A modified Luciferase Assay provided an example of a case where minor sample processing significantly reduced interferences. Data as well as improved formats and processing systems will be presented.

INTRODUCTION

Numerous technologies have been considered for the rapid detection of Agents of Biological Origin (ABO). These include Immunoassay (EAI, gold, agglutination), light scattering, fluorescence, luminescence, culture, impedance, chip technology, GCMS, and others. Three of these technologies have been extensively utilized in rapid field tests for detection of ABOs. They have advantages as well as limitations, which have not always been fully appreciated. We have

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performed various evaluations of assays that utilize these formats, delineating certain potential limitations or problem samples that may result in false or unclear results.

The three (3) technologies include:

1. Luminescence (ATP Bioluminescence)
2. Immunoassay (colloidal gold)
3. Fluorescence (DNA)

1. ATP BIOLUMINESCENCE:

Adenosine triphosphate (ATP) bioluminescence is a rapid alternative to the standard plate count for estimating microbial loads. ATP is the energy molecule in all living cells, including insects, plants, animals, bacteria, molds, or yeast. ATP bioluminescence is the technique of measuring ATP based on light emission during a bioluminescence reaction. The underlying premise of ATP bioluminescence is that the amount of ATP in a sample is proportional to the biomass. In the case of bacteria, there exists a strong correlation between cell number and ATP content. ATP can be measured using naturally occurring reagents from the firefly (*Photinus pyralis*) or genetically engineered. The reaction has been utilized with various samples from numerous sources including food, human, or environmental.

In the ATP bioluminescence reaction, luciferin is oxidized by the enzymatic reaction catalyzed in luciferase in the presence of magnesium and ATP. An end product of the reaction is energy in the form of yellow-green visible light (562nm). The emitted light , measured with a luminometer, is directly proportional to the amount of ATP in the reaction mixture. Data can be reported as the actual amount of ATP, however, in most cases it is reported as relative light units (RLU).

It is important to note that when measuring the ATP content of samples, one is measuring an average of the ATP content of the cells at that specific point in time. The ATP is in a constant state of flux and is species dependent. When comparing to culture, one must consider that the APC is growth based, where as the ATP assay measure a metabolite.

The ATP that is from non-microbial sources is termed somatic ATP. Generally, somatic cells have 100 to 1,000 times more ATP than bacteria cells. The ATP from an environmental sample may come from food, animals, plants, bacteria or even free ATP. This technology has become a standard tool for determining the “filth” or hygiene in a food plants. As such, this technique is a staple of many Hazard Analysis of Critical Control Point (HACCP) programs within the food industry worldwide (Cutter et al., 1996). Additionally, the ATP test with Luciferin Luciferase bound to a membrane (Model 4700) was included in the original Biological Integrated Detection System (BIDS) for the U.S. Army. This system detects the total ATP present in an air sample.

Most luminescence methods, however, neither differentiate between bacterial and non-bacterial ATP, nor correlate with standard culture methods. Also, it has been suggested that residual sanitizers as well as other substances may adversely affect the bioluminescence signal (Velazquez et al., 1996) by degrading Luciferin-Luciferase. Thus the presence of chemical residues on sampling sites could present a potential problem in underestimating or overstating the actual ATP signal and consequently affecting the decision tree.

A filtration-based bioluminescence technique which is able to separate bacteria from non-bacterial sources, thus able to detect bacterial ATP only (Siragusa et al., 1995, 1997), has been developed (Model 3550). Additionally, interfering substance were successfully removed. Evaluation studies performed on over 1000 food and environmental samples by researchers of the United States Department of Agriculture, Department of Defense, University of Michigan and others. A good correlation (r = >0.92) between the counts of bacteria on plates and the ATP from the luminometer for the same sample was obtained (Siragusa et al., 1995; 1997; Cutter et al., 1996; Stopa et al, 1998, 1999; Deininger et al, 1999).

To further expand the use of this technology, a unique heat incubation method was developed to identify spore samples within 20 minutes in a field setting. This method was able to reliably detect 10^5 CFU/ml of bacteria (Bartoszcze et al; Stopa et al 1999). A further modification of this Bioluminescence technique included the use of a Phage Associated Enzyme (PAE) for specific lysing and identification of bacteria.

2. COLLOIDAL GOLD TECHNOLOGY:

During the 1990s Hand Held Assays have developed which utilize a colloidal gold particle format to effect sensitive and selective detection of Agents of Biological Origin (ABO). Antibody (described below), specific to the agent of interest, is adsorbed on colloidal gold particles. Colloidal gold particles are discrete, electron-dense, non-fading, red-colored particles, approximately 20nm – 50nm in diameter. A 20nm gold particle adsorbs 20-30 antibody molecules (Figure 1). When antigens are combined with the colloidal gold-antibody conjugate, these complexes are concentrated on solid surfaces, either by capture antibody on nitrocellulose membrane or by immobilization on selective porous capture membrane, a distinct red spot is visualized by the naked eye. Labeled antibodies can be easily lyophilized (freeze dried) and reconstituted without losing activity or specificity which enables the product to be stored at ambient temperature.

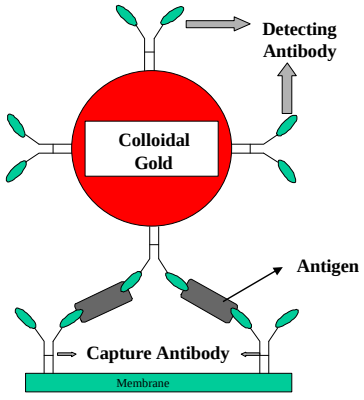


Figure 1. Gold Technology.

The two basic Gold Based assay formats are:

1. Flow Through
2. Lateral Flow

1. FLOW THROUGH COLLOIDAL GOLD PARTICLE CONCENTRATIONS:

Two variations of the Flow-Through Gold Technology utilize a colloidal gold particle concentration immunoassay to achieve sensitive and selective detection of biological materials, as illustrated in FIG. 2A and 2B. These are the Sandwich Assay (bacteria/toxin/virus) and the Charged Complex (spore). Antibodies specific to the agent of interest are conjugated to colloidal gold particles.

Figure 2A: For bacterial/toxin/virus samples, the antibody-antigen (Ab-Ag) complex is filtered and then concentrated onto a Nitrocellulose Membrane with capture-antibody. In the toxin or soluble antigen ticket format, the membrane is coated with a specific capture antibody to elicit binding of the antibody-antigen complexes to the membrane. The Control Spot utilizes antibodies derived from non-immune serum from the species of animal used to prepare the specific, active anti-serum. The complex is immobilized on the surface of the porous capture membrane and can be visually detected as a red spot. If the antigen is not present, the unbound colloidal gold reagents will diffuse through the membrane and will not be visually detectable. This assay does not require a washing step due to minimal interaction between the colloidal gold labeled antibody and the glass fiber capture membrane. However, in some cases, where there is a particularly dirty sample, the reaction area can be washed for better visualization.

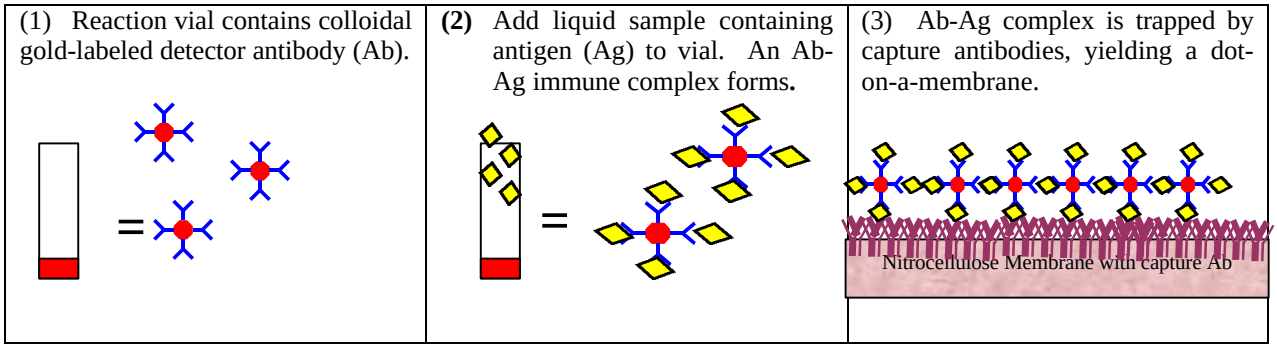


Figure 2A. Bacterial/Toxin/Virus: Colloidal Gold.

Figure 2B: For spores, the antibody-antigen (Ab-Ag) complex is filtered and concentrated onto a glass fiber membrane. The complex is immobilized on the surface of the porous capture membrane due to electrostatic attraction (charge) and can be visually detected as a red spot. If the antigen is not present, the unbound colloidal gold reagents will diffuse through the membrane and will not be visually detectable. This assay does not require a washing step due to minimal interaction between the colloidal gold-labeled antibody and the glass fiber capture membrane. However, in some cases, where there is a particularly dirty sample, the reaction area can be washed for better visualization. There is no Control Spot in the spore assay.

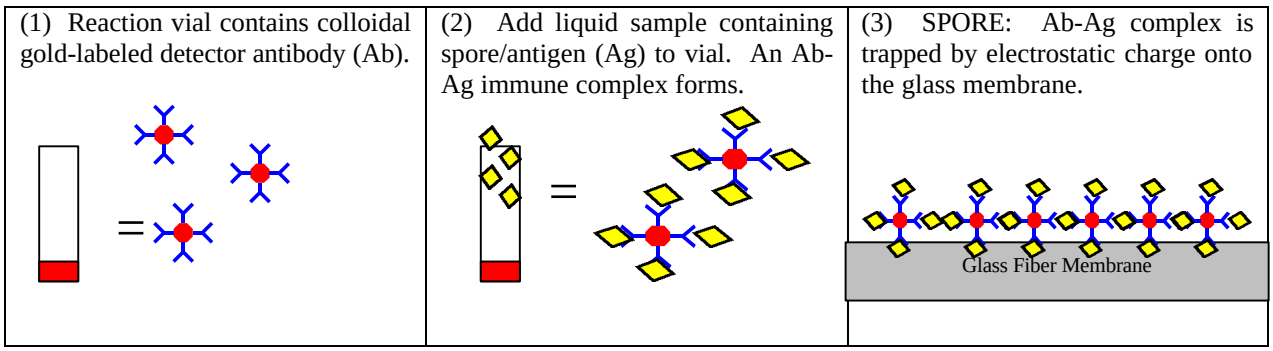
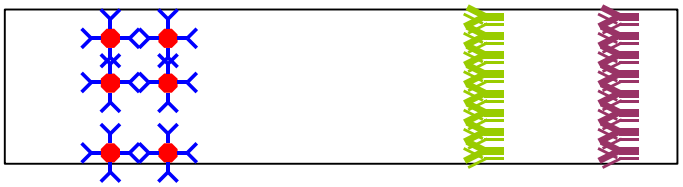


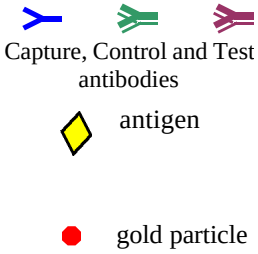
Figure 2B. Spore: Colloidal Gold Concentration Immunoassay.

2. LATERAL FLOW TECHNOLOGY:

In the Lateral Flow format, antibody coated colloidal gold particles are applied to a membrane surface and dried. When a test sample is applied, the gold conjugate reacts with any antigen that is present as it migrates across the length of the membrane to where it encounters a zone of capture antibody. Those antibody-gold conjugates, which have bound to antigen in the test sample, are then bound in the capture antibody zone, presenting a visually detectable line of color and indicating a positive test result (see Figure. 3). Sufficient antibody will be available to permit passage through the capture zone. These particles will then contact an area coated with an appropriate IgG fraction, where they will bind, producing a visible line of color.

1: Dry Strip





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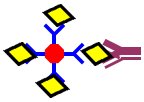
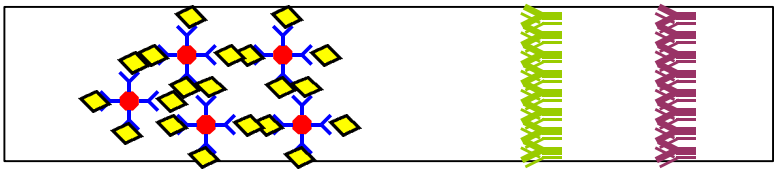


complex

2: Add Sample (with Antigen)

3: Sample flow moves particles; antigen form sandwich



4: Dyed particles form colored lines for Positive test and Control

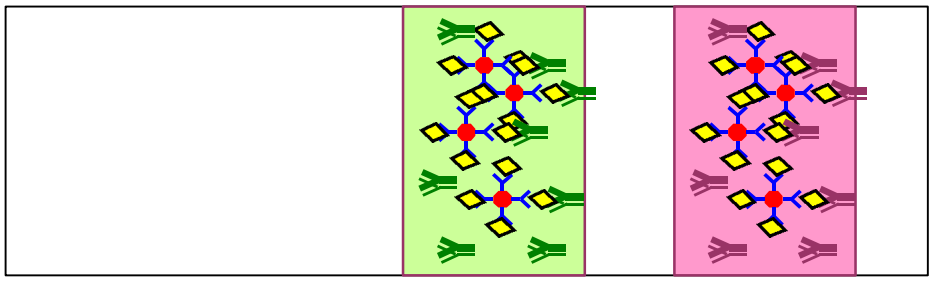


Figure 3. Chromatographic Strip Test (1 – 4).

The first Gold based tests developed and utilized during Desert Storm and subsequently the initial BIDS system employed the Flow Through technology (SMART I). Subsequent Hand Held Assays have been modified for the Lateral Flow technology as well. The lateral flow Technology, due to manufacturing and cost advantages, has become the format of choice for rapid medical, veterinary and environmental use as well.

FLUORESCENCE (DNA):

Fluorescence is a physical property of certain atoms and molecules. It is a molecule's ability to absorb light energy at one wavelength, then instantaneously re-emit light energy of another, usually longer, wavelength,. Each compound that fluoresces has a characteristic excitation wavelength, (the wavelength of light is absorbs) and a characteristic emission wavelength, (the wavelength of light that it emits when the molecules relax and return to their ground state). These excitation and emission wavelengths, (or spectra), are often referred to as the compound's fluorescence signature. Detecting and quantitating small amounts of DNA is important in a wide variety of biological applications. These include standard molecular biology techniques, such as synthesizing cDNA for library production and purifying DNA fragments for subcloning, as well as diagnostic and detection techniques, such as quantitating DNA amplification products and detecting DNA molecules in various samples (Turner Design, 2000).

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260nm. Unfortunately, this absorbance method has limitations including; interference from contaminants found in nucleic acid preparations, inability to distinguish between DNA and RNA, lack of sensitivity and others. The use of a unique dye, PicoGreen ® (Molecular Probes) has resolved many of these problems and, as such, has been successfully utilized for the field detection of ABO (Stopa, et al., 1998).

TECHNOLOGY REVIEW

All three of these technologies (Luminescence, Colloidal Gold, and Fluorescence) have their advantages as well as disadvantages. During the course of development and in the haste to field an assay, there may be tendency to evaluate a test system in pristine laboratory conditions. Consequently, one may determine sensitivity by obtaining a pure sample and diluting the target analyte in a defined, controlled buffer until a negative result is obtained. Additionally, only a limited specificity panel of potential cross-reacting organisms may be performed. Obviously, in the case of human sample testing there are very defined criteria that must be met prior to issuance of an FDA 510(k) or PMA. In lieu of such criteria it is incumbent the potential user be aware of the limitations and capabilities of the assay system in question.

Awareness of the sensitivity, specificity, and known cross reactions is essential to the individual performing and interpreting the test results. These personnel must be familiar with each of these parameters for each assay performed to provide the decision makers/commanders with the appropriate information. In immunological assays, these factors are generally due to the properties of the antisera (polyclonal, monoclonal, avidity, affinity), or antigen from which the reagent is made; and therefore may change with new production materials.

A number of factors may also be of concern in the user of these tests. These include Prozoning and nonspecific cross reactions. Prozoning can occur when there is such an

overwhelming abundance of antigen/analyte that all antibodies sites are "flooded", not allowing for a "sandwich" (antibody-antigen-antibody) binding or allowing for latice formation. In this situation a false negative would be seen from what should have been a very strong positive sample. The non-specific reactions can be due to either a reaction to the chemistry of the test (not the antibody or detection system) or cross-reactions with other organisms.

Obviously, one would like an assay system that detects all positive samples without any false positives. This tends to be a lofty goal that can never realistically be achieved. Recognizing this, as a product is developed the intended use as well as the requirements of the intended user must be of primary importance. As an example, the original colloidal gold hand held assay was designed to detect a specific analyte from an air sample. The sample buffer and all other parameters were very well defined. The goal was to provide as much sensitivity in this defined buffer as possible. There was no requirement for determining interferences from various other environmental or human samples as, therefore, during the developmental process there was less of a need to balance sensitivity vs potential non-bacterial cross reacting substances. A good example would be Anthrax spore. Due to the nature of the spore and the antibody supplied, the most effect format was determined to be the Colloidal Gold Concentration Immunoassay as previously described. This assay system met sensitivity as well as specificity requirements when utilizing a defined air sample. The major drawback, however, was the lack of a negative control. However, as there were several test systems employing different formats, this limitation was incorporated in the decision tree and accounted for. Subsequently, however, attempts were made to utilize this "air sample" assay with various environmental or even hoax samples. As the assay was neither designed for nor tested with such samples, this created a problem fraught with unknowns. The unknown was further compounded by the lack of a negative control.

In another example of a human test system, the typical Limulus amebocyte lysate (LAL) agglutination assay can detect 1,000 gram-negative bacteria per ml. In untreated meningitis, there generally is 100,000 bacteria per ml, but significantly less in treated ones. Thus the sensitivity of the LAL test for untreated patients was 90%, whereas the sensitivity for treated patients was only 65-75%. This system would be intended for untreated patient samples, but would lacking when testing treated patient samples.

As there have been numerous studies of technologies utilizing air or pristine laboratory samples, we have focused primarily on filed samples. This includes both routine samples found in the environment, both microbial and chemical, as well as hoax or artificially dispensed material. Additionally, we have considered the other limitations of the technology employed such as pH, temperature, etc.

Another factor that was considered is the material or solutions utilized to collect the sample prior to performing the assay. The samples considered where primarily environmental, but in one case to emphasize microbial cross reactivity studies, human and clinical laboratory isolates were utilized.

MATERIALS AND METHODS

INTERFERING SUBSTANCES:
Trichloroacetic acid and sodium hypochlorite (bleach) at various concentrations, talc (baby powder), diet cola, plain sugar, ethanol, table salt, garlic powder, cinnamon, coffee creamer,

HCL, maltrin, heparin, sugar water, casein, EDTA, potassium phosphate, sodium phosphate, laboratory salt, trahalose in hepes, Dipel® (Ortho), laundry detergent, dish washer detergent, lawn fertilizer, lemon aide, road dust, soil, silica gel, gelatin, iron, foam cleaner, ammonium compounds, and various other compounds.

RAPID MICROBIAL ATP ASSAY (PROFILE®):

Microluminometer NHD Model 3550i (Fig.1), Filtravette™ (.45u and 5.0u), Somatic Cell Releasing Agent (SRA), Bacteria Cell Releasing Agent (BRA), Luciferin-Luciferase (LL), and cell concentrator with syringe.

STANDARD PROFILE® PROCEDURE:

A sample suspension is transferred to the Filtravette™ (.45u for bacteria). Three (3) drops of Somatic Cell Releasing Agent (SRA) are added. The mixture is pushed through the Filtravette™ by a positive pressure device. Three (3) more drops of SRA are added and pressure-filtered to ensure the removal of interfering substances, free ATP, and somatic cell ATP. The Filtravette™ is then placed into the drawer slide of the Microluminometer (PROFILE®). Two (2) drops of Bacterial Releasing Agent (BRA) are added into the Filtravette™ to extract the microbial ATP. Immediately after the addition of the BRA, 50µl of Luciferin-Luciferase (LL) is added and mixed by aspirating the fluid up and down three (3) times. The drawer slide is closed immediately. Light emission is measured with integration over ten (10) seconds. ATP is reported as Relative Light Units (RLUs), taken directly from the luminometer's digital readout (Fig 2-6).

STANDARD COLLOIDAL GOLD PROCEDURE:

Flow through:

A sample (100ul) is added to the gold conjugate tube. Two drops of buffer is added. Transfer the sample/gold mixture to the test device via a swab (provided). Close device wait 5-15 minutes (maximum 18hours). Open device, red dot is positive.

Lateral flow:

A sample (100ul) is added to the sample well of the test device. Add two (2) drops, wait for 15 minutes. Two red lines are positive. One (1) line in the control area indicates a negative result.

STANDARD FLUORESCENCE (DNA) PROCEDURE:

Add sample (50ul) to cuvette, add solution A (50ul). Add sample (50ul) to another cuvette, add solution B (50ul). Let both stand for 5 minutes. Place cuvette A into instrument and record result. Place cuvette B into instrument and record result. Reading cuvette B – reading of cuvette A = Fluorescence of DNA in sample.

ASSAY WITH INTERFERING SUBSTANCES:

A fifty (50) µL suspension of *S.aureus* and *E.coli* was transferred to the Filtravette™. Fifty (50) µl of substance was then added to the bacterial (.45u) Filtravette™ and the standard PROFILE® procedure was performed. The experiment was repeated with various concentrations and types of sanitizers. The expelled liquid filtrate was then assayed for bacteria utilizing the standard PROFILE® procedure**.**

The Colloidal Gold and Fluorescence assays were performed as per the standard sample protocol.

ASSAY WITH PHAGE ASSOCIATED ENZYME:

A 100ul sample of Group A Streptococci and Group B Streptococci was added to the Filtravette™. The standard SRA washing procedure was followed as per instructions of the PROFILE®. 100ul of PAE was then added in lieu of the standard BRA total lysing reagent. The standard PROFILE® procedure was then followed. To determine background, the PAE was tested with the addition of a sample.

ASSAY WITH SPORES:

The sample of the suspected spore is incubated in an equal volume of Trypticase Soy Broth (TSB) for 15 min at 37°C. The entire sample is removed and filtered through a cell concentrator containing the Filtravette™ and the standard Profile® procedure was then followed.

Table 1.

Efficacy of Filtration-Based
Bioluminescence to Remove Inhibitors

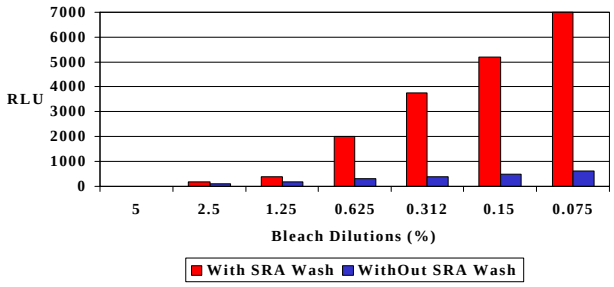


Table 2. Luminescence Interfering Substances.

SUBSTANCE	EFFECT
Ammonium compounds	Increase in signal
Bleach	Decrease in signal
Salt	Decrease in signal
Foam Cleaner	Decrease in signal
Iron	Decrease in signal

Velazquez, et al, JFP 1997

Table 3. Gold Interfering Substances.

Talc (Baby Powder)
Acid (HCl)
Ethanol
Heparin
Salts
Sugar
Detergent

Table 4. Modified Gold Test – Non-Interfering Substances.

Dipel	Baby Powder
Morton Salt	Sugar Water
Coffee Creamer	Sugar (plain)
Diet Coke	Cinnamon
Garlic Powder	Various Salts
Laundry Detergent	

Table 5. Cholera Gold Test Screened for Specificity.

Bacterial Strain	**No. of strains tested**	**% Positive**
V. cholerae O1	30	100
V. cholerae O139 (Bengal)	10	0
V. cholerae non-O1	20	0
V. vulnificus	4	0
V. parahaemolyticus	4	0
V. minicus	10	0
V. alginolyticus	2	0
V. campbelli	2	0
V. fluvialis	3	0
V. damsela	2	0
V. natriegens	2	0
V. pelagius	2	0
V. proteolyticus	2	0
Aeromonas hydrophila	5	0
A. veronii bv. Sobria	2	0
A. caviae	2	0
Shigella dysentariae	2	0
S. boydii	1	0
S. flexneri	1	0
S. sonnei	1	0
Escherichia coli	3	0
Salmonella typhimurium	2	0
S. thompson	1	0
S. hadar	1	0
S. berta	1	0
S. johannesburg	1	0
Klebsiella pneumoniae	2	0
Serratia marcescens	2	0
Citrobacter freundii	2	0
Proteus vulgaris	2	0
Enterobacter aerogenes	2	0
Pseudomonas aeruginosa	2	0
Plesiomonas shigelliodes	2	0

Table 6. Fluorescence (DNA) Interference.

Silica Gel	Dish Detergent
Gelatin	Phosphate
Baby Powder	Sugar
LemonAid	Road Dust
Lawn Fertilizer	

STOPA ET. AL 6TH CBW PROT. SYMP.

Table 7. Effective pH Range.

Luminescence	7.75
Colloidal Gold	6.0 – 8.5
Fluorescence	7.50

Table 8. Effects of Interfering Substances.

INTERFERING SUBSTANCES	**ATP PROTOCOL**	**PROTEIN PROTOCOL**	**DNA PROTOCOL**
Pollens/Mold Spores	N	Y^4	N
HOUSEHOLD/LABORATORY DETERGENTS	N	Y^3	N
HOUSEHOLD FOOD ITEMS	N	Y^2	N
HOUSEHOLD CHEMICALS/DETERGENTS	N	Y^3	Y^3
LABORATORY NUTRIENT MEDIA	N	Y^2	Y^2
LABORATORY CHEMICALS	N	Y^3	N
COLOR PRODUCING SUBSTANCES	N	Y^4	Y^4
LARGE PARTICLES IN SAMPLE	N	Y^4	N

N = No interference seen

Y^2 = Result was high due to a natural presence of substance being analyzed.

Y^3 = Inhibition/enhancement of reaction due to interaction between reagents and interfering substance. High concentration of detergents causes a false positive with the protein test.

Y^4 = Physical interference caused by particles in the sample, discoloring reagents, or mechanically dispersing or reflecting instrument light. *Stopa et al 6th CBW Prot. Symp.*

Table 9. Prozone Effect.

SEB	**50ng**	**100ng**	**500 ng**	**1000 ng**	**5000 ng**
	2+	**4+**	**2+**	**1+**	**Neg.**

RESULTS AND DISCUSSION

LUMINESCENCE (ATP)

It has been widely reported that basic ATP assessment although sensitive is subject to variability and when compared to standard culture methods is not accurate. As previously mentioned, these issues are due to various factors not the least of which is due to the presence of interfering substances, somatic ATP, and free ATP mixed with microbial ATP. It is interesting to note that these factors could cause false positives as well as false negatives. (Cutter et al, 1996; Siragusa et al, 1995, 1997; Velazquez et al, 1997; Stopa et al, 1999).

The filtration-based bioluminescence method was challenged to perform with various samples under pristine, as well as stressed environments. The assay performed well; correlating to culture for bacteria directly from various samples. The filtration method employed also demonstrated satisfactory performance when confronted with high salts, detergents, cleaners, ammonium compounds and metals of the type normally found in the environment or samples presented to a first responder.

Utilizing sodium hypochlorite (bleach) and trichloroacetic acid, a reduction of 94 to 96% in the bioluminescence signal was observed when concentrations higher than 1% were used in conjunction with the standard non-filtered ATP method (Table 1). It was further demonstrated that when the SRA wash filtration step was employed, this quenching effect was significantly reduced or removed expanding the utility of this method. It was also interesting that some compounds (ammonium) actually increased the RLU signal overstating the result, potentially leading to a false positive result if this filtration step was not implemented (Velazques et al,1997) [Table 2]. This method also tends to keep the pH at the optimum 7.75 {Table 7) and minimizes the effect of temperature variability.

Testing with a pure culture of *S. aureus* this method with a Model 3550 luminometer (New Horizons) could readily detect 10^5 CFU/ml with a 50 µl sample size. When the sample size increased to 2 ml, there was a 1 log increase in sensitivity. Subsequent testing has indicated sensitivity can be further improved by increasing the sample size as well as adjusting the voltage setting of the instrument.

The method described detects and enumerates generic bacteria or yeast. Several modifications can be employed that will allow specific identification. These may include antibody coated beads, chemiluminescence, or the use of specific lysing reagents. Recent preliminary data has demonstrated that a specific bacteria phage enzyme (PAE) may be employed in lieu of a generic bacterial releasing agent (BRA) to selectively lyse a target organism. The Group A Streptococci phage enzyme was chosen due to the long history of use and complete definition of the phage and bacteria. This method may be useful not only for the routine identification of various bacteria, but also as a rapid environmental monitoring tool to determine the effectiveness of cleaning or identification of potential problem areas.

As spores are deficient in ATP, an incubation method was developed whereas the spore would convert to the vegetative state. During this phase ATP is produced at sufficient levels to be detected by the model 3550 luminometer. Although spores are detectable after less than 10

minutes, a 15 minute incubation was utilized to insure consistent agreement to standard methods. Data demonstrates a detection limit of 10^5-10^6 can be achieved with a normal sample size of less than 1 ml.

COLLOIDAL GOLD

The standard colloidal gold based assays has gone through dramatic changes since New Horizons introduced the first tests in the late 1980's. Improved membranes, sample pads, conjugate pads and gold chemistries have allowed for continued improvement in the overall performance of this format.

The initial flow through colloidal gold particle concentration technique offered the advantages of ease of use and ambient storage with improved sensitivity compared to the agglutination or rapid EAI tests. The sensitivity improvement came not only as a result of the gold chemistries, but due to the transfer of virtually 100% of the sample/gold complex and focusing this complex at a small point (small hole in the laminant). Sensitivity ranged from 10^4 for Group A strep to $5x10^5$ for many ABO tests. The primary variable, however, was the antibody.

As a answer to the issues of improved ease of use (1 less step), decrease cost of goods, and decreased antibody usage; the lateral flow assays were developed. The primary issue that had to be resolved was sensitivity. With the improvements mentioned above this format was off and running.

The first lateral flow assays were designed for medical applications and as such the samples were fairly consistent. The entry into the environmental and first responder arena, however, precipitated other issues. The standard gold based assays are optimized for a sample with a pH of from 6.0-8.5 (dependent on the test) [Table 7]. Also, various samples can cause the gold to "crash" causing a deposition on the membrane giving the indication of a positive. Alternatively, some sample may cause no or slow flow of the complex leading to an invalid result (Ethanol, trahalose). Some of these interfering substances are listed in Table 3.

To resolve these, the chemistries and membranes of the lateral flow assays were modified. It is interesting to note that typically there is no one fix, but each assay must be developed separately, utilizing a panel of potential interfering substances (Table 4). This is further complicated if the antibody changes in any significant manner - as may be the case with polyclonal antibodies from different sources.

In addition to the various chemical compounds, the assay systems must be tested for potential cross reactions for other organisms. This again, is primarily due to the specificity of the antibody utilized in the test. Table 5 is a sample of some of the specificity data for a gold based Cholera O1 test. As this assay is for human use, this as well as other testing was necessary for FDA 510(k) submission. Here it is importance to have an understanding of which potential organisms may cause cross reactions with the target analyte. As another example, Anthrax spore antibody may also react with (false positive for an Anthrax spore test) other *Bacillus* species such as *Bacillus cereus* and *Bacillus thuringiensis* (BT). This is of critical importance as these are very common in the soil and the BT is common used to kill insects such as the gypsy moth. The commercial form of the BT is sold under the name Dipel® (Ortho). This further complicates

testing of an assay as a test system that may function very well in a desert situation where these organisms are not present, may not be fully usable in the US where they are very common.

Another issue that is known in the human clinical laboratory is the effect of prozoning. In this situation an excess of a specific analyte will cause a false positive. The most striking example of this phenomenon is SEB. I this example, a 5000ng sample reacted as a negative but further dilutions gave a 4+ reaction at 100ng (Table 9).

It is important to verify the assay system will detect all levels of expected ABO. This further underscores the intended use. Clearly for the battlefield, the commanders want immediate answers therefore underscoring the need for sensitivity with minimal concern about sample overload (sampling an air sample). The first responder who may obtain a large packet of powder may be more concerned about a false negative due to prozoning.

FLUORESCENCE (DNA)

The fluorescence assay is a good tool to assist in determining the presence of a virus, however, it has similar limitations as other technologies. Clearly the pH is optimized for the dye utilized (Picogreen® – 7.5). Additionally any substance of biological origin may cause interference. The system is further by other easily obtained substances such as phosphates, detergent, sugar, baby powder , etc. (Table 4). Obviously, these effects must be taken into account when analyzing a sample. The effects of general interfering substances for ATP, DNA as well as protein are seen on Table 8.

INHIBITORY PROPERTIES OF COLLECTION DEVICES

From the studies conducted it is apparent that common compounds such as ammonium, phosphates, salts and various sanitizers may interfere with some assays. Additionally, these may also inhibit the growth or even kill bacteria. Cellulose sponges and even some swabs may contain sulfur, ammonium, and other compounds. These compounds are utilized in the manufacturing process to break down wood fibers from which these sponges are made. Studies have shown that significant bacterial loads could be reduced to zero (0) in a little as 4 hours (Perry and Ballou, 1997). Additionally, it has been demonstrated that bacteria and bacterial antigens tend to be absorbed by the wooden shaft swabs. Also, bacteria are not as freely released from cotton swabs as opposed to dacron or rayon swabs (Becton Dickinson, personal communication).

When expands this further to the collection fluid it becomes apparent that all facets of the system should be verified (collection, processing, detection) . Consequently, a Phosphate Buffered Saline solution should be evaluated for over effects on recovery and detection with assay systems employed.

Recognizing these factors a collection system has been developed for different samples. One for a large surface sample, a second for a powder/small surface sample, a third for a liquid sample and the fourth for an air sample. Additionally a sample processing packet has been designed for cleaning up a “dirty” sample as well as submitting for further testing. All components have been tested with the testing formats described.

CONCLUSION

- Presence of salts, sugars, ammonium compounds, metals or other chemicals could present a potential problem by interfering with some luminescence, colloidal gold (hand held assays), and fluorescence (DNA) tests systems.

- In determining the acceptance of an assay system one should consider the sensitivity (including prozoning), specificity, and potential cross reacting substance based upon the manufacturers intended use as well as the users requirements.

- Employing filtration-based bioluminescence technique, interfering residues could be removed/reduced to a level that does not significantly inhibit the bioluminescence signals. Consequently, the actual bacterial status of a sample is obtained.

- The filtration-based system detects Bacteria in less than two (2) minutes while providing good correlation (>90%) with conventional culture methods which require as long as 4-7 days.

- The major variable with immunological (antibody) based systems is the antibody. Care should be taken to fully evaluate the total system for other potential cross reactants of biological origin as well as method to insure lot to lot reproducibility.

- Evaluations performed on similar assay systems but with different intended use should be reviewed carefully for potential limitations.

- Further advances in this system allow for increased detection limits as well as specific identification including the use of Specific Phage Associated Enzymes (PAE).

- The measurement of the ATP from a sample prior to and after incubation demonstrated the presence of a spore (i.e. *Bacillus spp.*) in less than 15 minutes

- Collection devices and processing buffers should be carefully considered to insure compatibility with assay systems as well as effective recovery of target analyte.

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CONTACT: David Trudil, NEW HORIZONS DIAGNOSTICS CORPORATION,
9110 Red Branch Road, Columbia, MD 21045
410-992-9357 / email: NHDiag@aol.com