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AN EVALUATION OF THE NIDS® ACE™ TEST

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14. ABSTRACT
The current field water test kit--the water quality analysis set-preventive medicine--has a very limited capability to detect TICs. Comprehensive analysis for TICs in water requires off-site analysis, a process that can take at least 10-14 days. Available hand-held, analyte-specific field water tests do not address all TICs. Toxicity sensors considered were devices with biologically-based sensors that can respond to a wide range of toxic chemicals. Development of the Environmental Sentinel Biomonitor (ESB) was initiated to provide a hand-held, short duration field toxicity test for Army drinking water. One component of the ESB the ACE Test) is an enzymatic assay designed to detect neurotoxicants (specifically, organophosphate and carbamate pesticides) utilizing ultraviolet light. This report describes the current capabilities of the ACE Test using the TRL-6 reader. Specific evaluations were conducted in toxicity detection, ultraviolet flashlight use, and temperature/shelf life testing.

15. SUBJECT TERMS
ACE Test, acetylcholinesterase, aquatic toxicity testing, biosensor, carbamate, carboxylesterase, ESB, environmental sentinel biomonitor, MEG, n-methyl isonoate, organophosphate, pesticide, toxicity sensor, TIC, toxic industrial chemical

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1. Introduction

1.1 The Environmental Sentinel Biomonitor

Disease and non-battle injuries are the leading causes of soldier casualties (Department of the Army, 2005). One possible source of adverse health effects is the presence of toxic industrial chemicals (TICs) in drinking water. The current field water test kit – the water quality analysis set-preventive medicine (WQAS-PM) - has a very limited capability to detect TICs. Comprehensive analysis for TICs in water requires off-site analysis, a process that can take at least 10-14 days. Available hand-held, analyte-specific field water tests do not address all TICs. One way to address this capability gap is through the use of toxicity sensors – devices with biologically-based sensors that can respond to a wide range of toxic chemicals. Development of the Environmental Sentinel Biomonitor (ESB) was initiated to provide a hand-held, short duration field toxicity test for Army drinking water.

The goal of the ESB (Figure 1) is to respond rapidly (in an hour or less) to chemicals in water at concentrations that exceed the 7-14 day Military Exposure Guideline (MEG) concentration (assuming 15 liter (L)/day consumption; USACHPPM, 2013) but below the estimated human lethal concentration (HLC) (TERA, 2006); other performance criteria are detailed in the Capability Development Document (CDD) for the ESB (MRMC, 2013). The test set of chemicals used to evaluate the ESB are shown in Table 1. Also tested were interferences: materials found in natural waters that, although not toxic to humans, had the potential to cause a response in a toxicity sensor. Potential interferences are shown in Table 2.

A down-selection process for the ESB that began with 40 candidate technologies (Kooistra et al., 2007) ended with the selection of two technologies for inclusion of the ESB: an electric cell-substrate impedance sensing (ECIS) device from Biosentinel, Inc. (Austin, TX) and the NIDS® ACE™ test from ANP Technologies, Inc. (Newark, DE) (Kooistra and Walther, 2013). The ECIS technology monitors changes in the electrical impedance of a monolayer of rainbow trout gill cells on a Lexan® fluidic chip to indicate toxicity due to chemical contamination (Brennan et al., 2012). The ACE™ Test is an enzymatic assay designed to detect neurotoxicants (specifically, organophosphate (OP) and carbamate pesticides) utilizing the reactions of stabilized carboxyl esterase (CE) and acetylcholinesterase (AChE) and a reporting chemical that fluoresces under ultraviolet (UV) light.

Figure 1. A prototype ESB with electric cell-substrate impedance sensing reader (left), accessories (center) and ACE™ reader (right)

Table 1: Test Chemical Military Exposure Guidelines and Human Lethal Concentration Values		
Test Chemicals[a]	**MEG[b] (mg/L)**	**HLC[c] (mg/L)**
OP[d] and Carbamate Pesticides		
Aldicarb	0.005	0.17
Fenamiphos	0.004	0.56
Methamidophos	0.002	1.4
Methyl parathion	0.15	33.6
Other Chemicals		
Acrylonitrile	0.14	4.2
Ammonia	30	924
Arsenic (sodium arsenite)	0.02	4.5
Azide (sodium azide)	0.12	47
Copper (sulfate)	0.14	71.9
Cyanide (sodium)	2	14
Fluoroacetate (sodium)	0.0009	5.1
Mercury (chloride)	0.001	24.7
Nicotine	0.13	16.8
Paraquat (dichloride)	0.05	4.6
Pentachlorophenate (sodium)	0.14	71.9
Phenol	3	91.5
Thallium (sulfate)	0.003	13.5
Toluene	1	840

[a] More chemical information available in Appendix A

[b] **MEG** – 7 to 14 day Military Exposure Guidelines (15 liter [L]/day), when available, 1 year MEG for copper, fluoroacetate, and strychnine; < 7 day MEG for nicotine; fenamiphos MEG estimated from terbufos (Richards, personal communication)

[c] **HLC –** Human Lethal Concentration (70 kg person, 15 L/day)

[d] **OP** – Organophosphate

Table 2: Interference Chemicals	
Test Chemicals	**Concentration (mg/L)**
Chlorine (total residual chlorine)	10
Chloramines	10
Geosmin	0.0001
Methyl-isoborneol (MIB)	0.0001
Humic / Fulvic Acids (50%/50%)	5 (2.5/2.5)
Blank – Hard Water	250

1.2 Previous Pesticide Assay Evaluations

As part of the down-selection process for the ESB, Battelle (Columbus, OH) tested the OP and carbamate pesticide detection capabilities of several technologies, including an early version of the ACE™ Test (Table 3). Of these technologies, the Abraxis, LLC OP/C Screen detected the most OP and carbamate pesticides within the MEG-HLC range (Buehler, 2008), but the OP/C Screen was not packaged for single use, required refrigerated reagents and multiple sample manipulations, and cost $200 for 3 tests as modified (Trader et al., 2009). The Neogen Agri-Screen® and Hach Pesticide/Nerve Agent Test technologies provided a single use, quick method (< 5 min) with simple operation (less than 3 steps and one volume transfer), and cost under $20 per test (Trader, 2010). However, the Agri-Screen® contained a hazardous chemical (bromine) and could only detect methyl parathion within the MEG-HLC range, while the Pesticide/Nerve Agent Tests did not detect any of the tested OP/C pesticides within the MEG-HLC range (Trader and van der Schalie, 2010). The best of the tested technologies was the Technology Readiness Level (TRL) 5 tethered ACE™ Test reader (Figure 2), which was able to detect 3 of the 4 OP/C pesticides within the MEG-HLC range (aldicarb, fenamiphos and methyl parathion) and one at 2.5 times the HLC (methamidophos at 4 mg/L). These test results, along with additional evaluations of the Neogen Agri-Screen®, Hach Pesticide / Nerve Agent Strips, and the Abraxis Organophosphate/Carbamate Test (Trader and van der Schalie 2010; Trader, et al. 2009), contributed to the selection/confirmation of the ACE™ Test as a component of the ESB, since it had the best combined performance in terms of sensitivity to pesticides as well as utility in a field situation (e.g., temperature stability of reagents, ease of use, size/weight/cube, etc.).

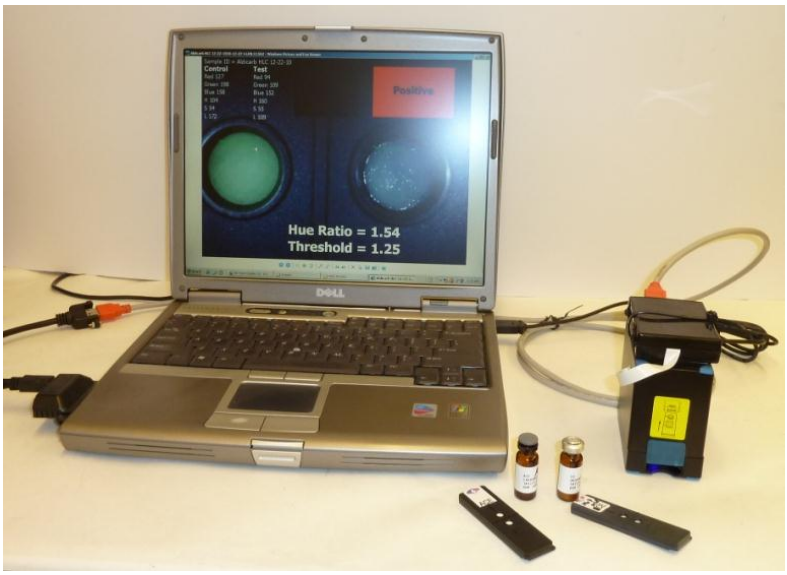

Figure 2. TRL 5 NIDS® ACE™ tethered reader attached to a laptop, with reagents and tickets.

Table 3: Responses of Four Enzyme-based Technologies to Chemicals at the HLC					
Test Chemicals[a]	**HLC[b] mg/L**	***Agri-Screen*[c]**	***Hach Pesticide/Nerve Agent Strips*[c]**	***Abraxis OP/C Test*[d]**	***ACE™ TRL 5 Tethered Reader*[e]**
		Detected? (n=3)[f]	**Detected? (n=1)**	**Detected? (n=16)**	**Detected? (n=16)**
Acrylonitrile	4.2	ND[g]	ND	ND	ND
Aldicarb	0.17	ND	ND (n=3)	Yes 0.02 mg/L	Yes
Ammonia	924	ND	ND	ND	ND
Arsenic (sodium arsenite)	4.5	ND	ND	ND	ND
Azide (sodium azide)	47	ND	ND	ND	ND
Copper (sulfate)	103	Yes (16 of 16)	Yes (1 of 1)	Yes, 52 mg/L	Yes 25 mg/L (3 of 3)
Cyanide (sodium)	14	Yes (1 of 3)	ND	Yes, 3.5 mg/L	ND
Ethylene glycol	3157	ND	ND	ND	---[h]
Fenamiphos	0.56	ND	ND (n=3)	Yes, 0.08 mg/L	Yes
Fluoroacetate (sodium)	3.9	ND	ND	ND	ND
Mercury (chloride)	24.7	ND	ND	Yes, 12.4 mg/L	Yes 12.4 mg/L (3 of 3)
Methamidophos	1.4	ND	ND (n=3)	Yes, 1.25 mg/L	4 mg/L
Methyl parathion	33.6	Yes, 2.3 mg/L (16 of 16)	ND (n=3)	Yes 0.016 mg/L	Yes
Nicotine	16.8	ND	ND	ND	ND
Oxamyl	0.63	Yes (15 of 16)	Yes (n= 3)	Yes, 0.04 (n= 3)	---
Paraquat (dichloride)	4.6	ND	ND	ND	ND
Pentachlorophenate (sodium)	71.9	ND	ND	Yes, 50 mg/L	ND
Phenol	91.5	ND	ND	ND	ND
Strychnine	1.3	ND	ND	ND	---
Thallium (sulfate)	13.5	ND	ND	ND	ND
Toluene	840	ND	ND	ND	ND

[a] More chemical information available in Appendix A
[b] **HLC** – Human Lethal Concentration (70 kg person, 15 L/day)
[c] Trader et al., 2009.
[d] Trader and van der Schalie, 2010.
[e] Trader (unpublished data)
[f] **n** – number of samples
[g] **ND** – not detected
[h] **---** Not tested

	Detected at or below the HLC with every replicate
	Detected above but close to the HLC or partial response at the HLC
	Not detected at the HLC

1.3 ACE™ Test Development

The ACE™ Test was developed under an Army Small Business Innovation Research (SBIR) contract with ANP Technologies to detect organophosphate and carbamate chemicals in the MEG-HLC range, contains reagents that are temperature stable, uses a simple method, and achieves a result using a battery-operated, hand-held device. The need for increased reagent stability drove several test modifications. During the Phase I SBIR, encapsulated acetylcholinesterase enzyme was discarded in favor of a naked enzyme. While in Phase I, the enzyme was on a test ticket and the reporter chemical was in a sample vial, but in Phase II enhanced stability was achieved by reversing the locations. A water sample is added to lyophilized enzyme in an amber vial, and then a sample of the enzyme solution is added to a test ticket containing the fluorescent reporter chemical. The ticket is placed in a reader, which illuminates the ticket with UV light. If the enzyme is not inhibited, the reporter chemical will be cleaved and fluoresce, which is detected by a camera in the reader. Initially, the reporter chemical was Ellman's Reagent (Ellman, 1961); subsequently, it was changed to n-methyl isonoate (NMI).

A subsequent Army SBIR contract further enhanced the ACE™ Test with development of a hand-held TRL-6 reader and improved test tickets, leading to successful completion of a US Environmental Protection Agency-sponsored Technology Testing and Evaluation Program (TTEP) evaluation, where the ACE™ Test was evaluated as part of the ESB. In 2012, the ESB passed Milestone B in the Army acquisition process and moved into advanced development. Production-ready versions of the ACE™ Test are scheduled to be delivered in January 2014. The ACE™ Test is now commercially available (available online since 2011), with sales to water utilities, agriculture, and as well as the military sector (http://anptinc.com).

ACE™ Test readers used for this report (serial numbers 002002 through 002013) had minimal reader-to-reader variability when reading the same test ticket. However, excessive variability in same-ticket readings was found in some readers; correcting this problem is a focus of the advanced development process. The readers used for this report did not require any maintenance and did not drift in readings over the duration of the study.

As with the readers, the ACE™ Test tickets are being improved in advanced development. Testing reported in this document used the April 2013 strip test tickets in combination with May 2013 enzyme formulations. These test results provide benchmarks for evaluation of the final ACE™ Test components to be provided after advanced development is complete.

1.4 ACE™ Test Method

The ACE™ Test contains two sets of two vials of enzyme (AChE and CE) and two tickets. A one mL clean water sample and a one mL test water sample are incubated in each enzyme vial for 30 minutes at room temperature and placed on a respective ticket. After 15 minutes, the reporting molecule (NMI) on the pad will yield a green hue if there

is no pesticide present or a purple/blue hue if a pesticide is present when viewed with a UV light source. Since the ACE™ Test Readers have a UV light source, it is possible a UV flashlight could be used instead of the reader. A user could read a test ticket directly, using a much less expensive UV flashlight. Results of UV flashlight testing are included in this report. A more detailed procedural description is found in Appendix B.

1.4.1 ACE™ Test Method – Temperature Considerations

ESB performance requirements include a minimum shelf life of 9 months at 45 °C in an approved storage device. (Note that 45 °C is the ambient temperature and that the approved storage device can provide temperature control.) ACE™ Test reagents have a shelf life of 12 months at room temperature according to the manufacturer. It was necessary to verify both the shelf life of ACE™ Test reagents at relevant storage temperatures as well as to test the response of test tickets in the ACE™ Test reader at a range of operational temperatures.

1.5 Objectives

This report describes the current capabilities of the ACE™ Test using the TRL-6 reader, April 2013 Strip test tickets, and May 2013 enzyme formulations, unless otherwise specified. Specific evaluations were conducted in three areas:

- Toxicant detection. After testing with clean water blanks established an appropriate threshold for detection, responses to a positive control (copper) and a challenge set of test chemicals (OP and carbamate pesticides, Table 1) and potential interfering substances (Table 2) were determined.
- UV flashlight option. Reading test tickets directly with a UV flashlight instead of the ACE™ Test reader would greatly decrease cost and the size/weight/cube of the ACE™ Test. Testing was conducted to determine how UV flashlight use would affect toxicant sensitivity and variability in test results.
- Temperature testing. Testing determined the shelf life of ACE™ Test reagents at several temperatures relevant to Army field environments.

2. Materials and Methods

2.1 Concept

AChE and CE are enzymes integral to the nervous system of insects, humans, and other animals. Organophosphate and carbamate pesticides disrupt, interfere, or inhibit the operation of these enzymes. The ACE™ Test contains freeze-dried vials of each enzyme, as well as several stabilizing components, for testing for the presence of OP/C pesticides in water. To conduct the test, a one milliliter water sample is placed in an enzyme vial and the enzyme is re-hydrated. If the sample contains a pesticide, the 30 minute incubation period provides an opportunity for the enzyme to be interfered with or inhibited by toxicants in the test sample. After incubation, a 0.1 milliliter volume of the re-hydrated enzyme-sample solution is placed onto the ACE™ Test ticket pad. If there is no pesticide present, and the enzyme is intact, the enzyme will react with the NMI (pre-cast upon the pad) and will fluoresce under UV light as a green color. If a pesticide has altered the enzyme, the pad will generally not fluoresce as a green color under UV light and will produce a blue-purple color when using the ACE™ reader. A color development time of 15 minutes is recommended.

The ACE™ Test reader consists of a touch-screen computer, a light-emitting diode (LED-UV) light ring, and a camera. The ticket is inserted into the side of the reader, and an image is taken. Imaging software locates the center of the control well and test well, and captures red/green/blue (RGB) values for each well. These RGB values are used to calculate hue, luminance, and saturation. Hue was determined to be the best measure of color, by its tendency to yield consistent values with blank sample (negative) reagent. In addition to low negative sample variance, hue offered a serviceable ability to differentiate positive pads from negative pads. The green channel was also considered for its higher dynamic range when comparing a negative pad to a pesticide containing pad, but the negative pad to pad variability was too high. Hue is on a scale of 0-1 and is best represented as a color wheel (See Fig 3).

In the ACE™ Test, the reader calculates a ratio of the control well and the test well (test well hue value /control well hue value). A negative sample would yield a hue ratio of 1.00 (identical test and control well hue values). A pesticide-laden sample would have a higher hue ratio of 1.25 or more (blue-purple hue values are between 0.5 - 0.8, compared to the green hue values of 0.2 - 0.4). A hue ratio of 1.25 or greater indicates that the color is sufficiently different than the control pad and is a "Detect". See Section *3.1.1 Blank Testing and Threshold Determination* for more information.

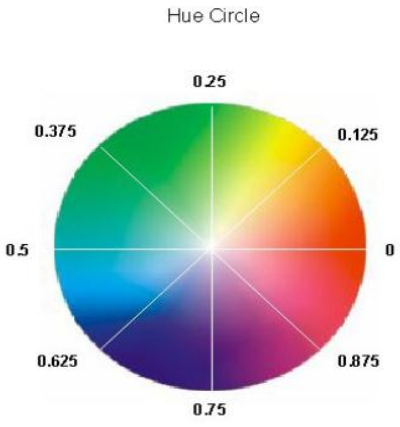

Figure 3. Hue values in relation to a color wheel. Image from TAOS-AMS, Berlien, 2004.

2.2 Materials

2.2.1 Required materials

The ACE™ Test Reagent Pack consists of the following:

A - Enzyme / Ticket Reagents (Figure 4.A):

a. two Reagent A (AChE) amber-colored vials (one for control and one for test)
b. two Reagent B (CE) amber-colored vials (one for control and one for test)
c. two tickets (one for Reagent A and one for Reagent B).

B - Delivery materials (Figure 4.B)

d. two 0.1 mL transfer pipettes (for transferring enzyme/sample solution to ticket)
e. two 1mL transfer pipettes (for transferring sample to vial)

OR

d1. one 0.1 mL calibrated pipettor and two 0.1 mL tips (included in reagent pack)
e1. one 1.0 mL calibrated pipettor and two 1 mL tips (included in reagent pack)

C – Reader (Figure 4.C)

f. NIDS ACE™ Test reader

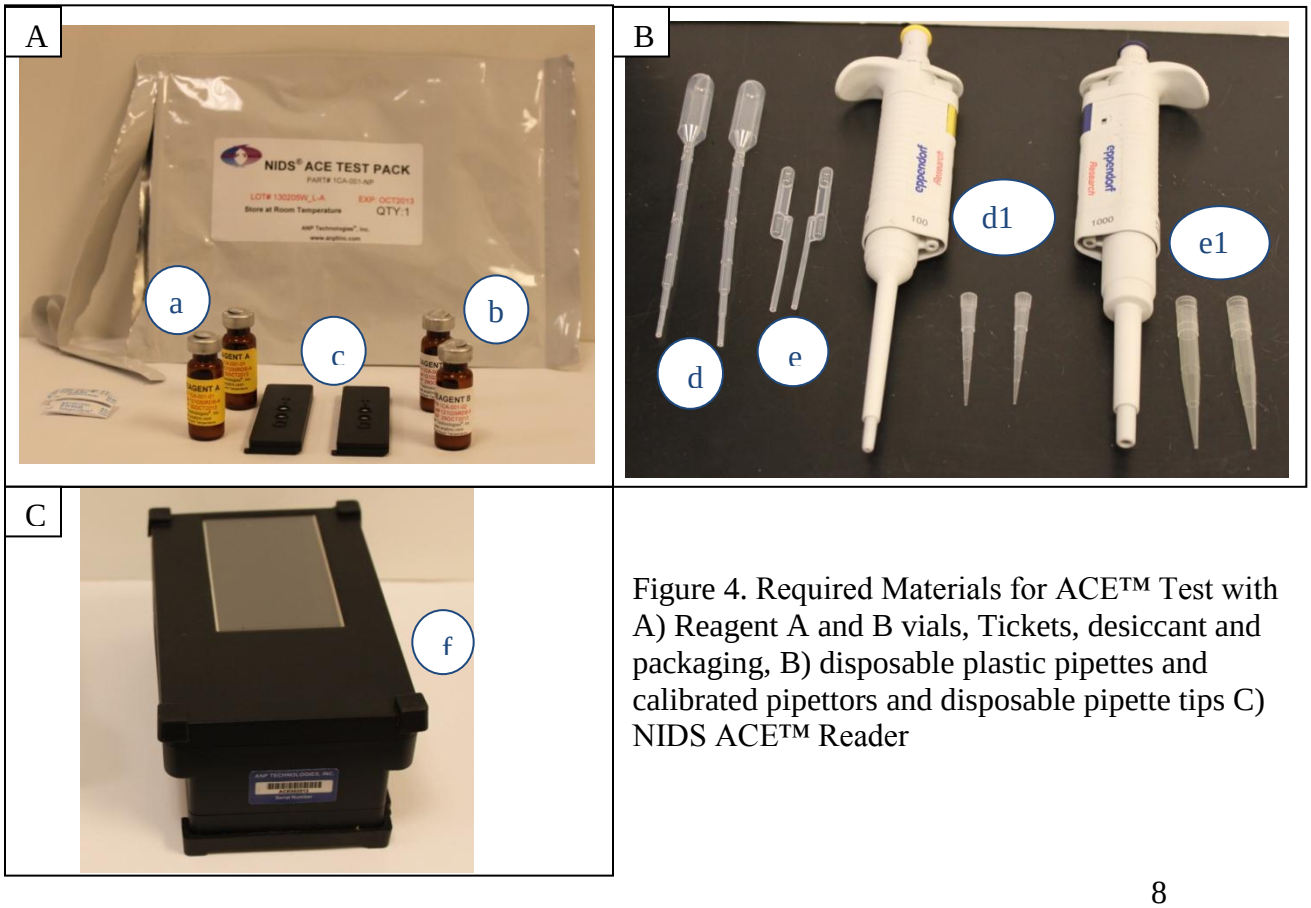

Figure 4. Required Materials for ACE™ Test with A) Reagent A and B vials, Tickets, desiccant and packaging, B) disposable plastic pipettes and calibrated pipettors and disposable pipette tips C) NIDS ACE™ Reader

2.2.2 Accessories

Figure 5 shows useful accessories for the ACE™ Test.

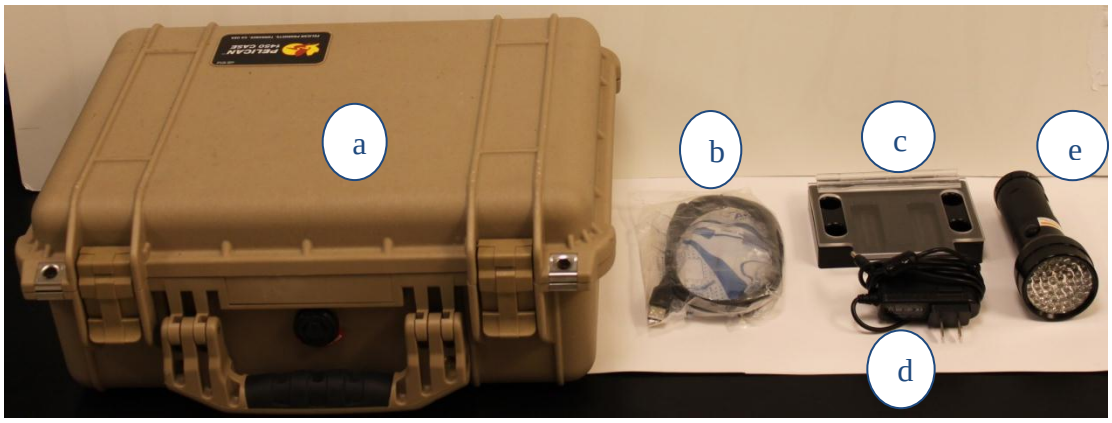

Figure 5. Accessories for the ACE™ Test include a) Pelican™ 1450 Case, b) USB cable, c) Workstation d) Power cable, e) UV flashlight. An ACE™ Test procedure card is shown in Appendix B.

2.3 Specifications

2.3.1 Power

The ACE™ Test reader can operate using 6 AA batteries or 110-120 V AC line power (Figure 6).

Figure 6. AA Battery input (bottom view of reader) and line power input to the ACE™ Test reader (side view of reader with power on).

2.3.2 Software

The internal software of the ACE™ Test reader can store up to 1800 data records without overwriting data (with a warning message appearing at record number 1400). The image, hue ratio, date/time read, and any user-input data (sample description, location etc.) is stored on the reader and can be accessed under the "Review Data" button on the touch screen. Additionally, the images and data can be downloaded to a computer using a

standard USB 2.0 cable. The data storage file (ace.dat) is in a comma separated value format and images are standard JPEG files.

2.3.3 Size

The ACE™ Test reader is 7.5 cm (3") high x 18 cm (7") long x 10 cm (4") wide. The 1450 Pelican™ case with 5 ACE™ Test Packs, a reader and all associated accessories is 17 cm (7") high x 33 cm (13") long x 42 cm (16.5") wide and weighs 4.5 kg (10 lbs). The ACE™ Test Pack is currently 1.5 cm (0.5") high x 23 cm (9") long x 15 cm (6") wide, although these dimensions are likely to change during the advanced development phase of the Army acquisition process.

2.4 Blank Testing and Threshold Determination

Performance requirements for the ACE™ Test require a false positive rate of no more than 1 in 1,000. Blank testing and statistical analysis was conducted to determine how high a hue ratio would be required to meet this goal. Thirty packs (30 Reagent A tickets and 30 Reagent B tickets) were tested with the same blank water sample (Milli-Q® Gradient ultra-pure water). These data (n=30) were used to determine the hue ratio at which no blank sample would register a positive result (threshold). The specific data and methods used in the final threshold determination are described in Appendix C. The variability of the observations for both Reagent A and Reagent B was determined, and false positive thresholds for responses were computed for a range of probabilities (0.05 to 0.00001).

2.5 Test Sample Evaluations

2.5.1. Positive Control Testing with Copper

A positive control chemical was considered essential to allow users to demonstrate that both the reagents and test reader are functional. Copper (as copper sulfate) was selected as the positive control chemical because it was relatively non-toxic to humans, it was stable for long periods over a wide range of temperatures, and it inhibited the activity of both enzymes. An OP or carbamate pesticide, while more relevant to the desired detection capabilities of the device, was more problematic because of hazardous nature of the materials. A test concentration of 25 mg/L was found produce a consistent positive response in the ACE™ Test.

2.5.2. Chemical and Interference Testing

OP and carbamate test chemicals included aldicarb, fenamiphos, methamidophos, and methyl parathion (Table 1). Interferences tested (Table 2) included chemicals commonly used for drinking water disinfection (chlorine and chloramine), byproducts of cyanobacteria blooms (geosmin and 2-methylisoborneol (MIB)) and plant decomposition (humic and fulvic acids) found in certain source waters. Hard water (water high in calcium and magnesium and associated anions) was included because of the potential sensitivity of some biological systems to ionic materials. Test concentrations selected for

interference testing were judged to be the highest likely to be encountered in field testing. Testing on interferences was completed with pre-TTEP evaluation well-style tickets.

2.5.3. Minimum Detection Level (MDL) Testing

Determination of an MDL for each test chemical was based upon the statistical approach used by the Joint Chemical Biological Radiological Agent Water Monitor (JCBRAWM) program. The MDL is the lowest tested concentration at which there is a 90% probability of detection with 80% confidence (Hogan et al., 2007). Based on binomial probabilities, this required that a minimum of 16 of 16 samples be detected at the MDL, with no false negatives.

Minimum detection limits (MDL, lowest concentration at which 16 of 16 samples detected) were determined by first testing the HLC with three samples. If the chemical was detected below the HLC, the MEG was tested with three samples. If the MEG did not respond at 3 of 3 samples, the next concentration tested was the HLC divided by a factor of two. Testing continued until 3 of 3 samples were not detected. The last two concentrations that yielded 3 of 3 detections were tested with three more samples. The lowest concentration that yielded 6 of 6 detections was further tested with ten more samples. Once 16 of 16 samples were detected, the MDL was defined. Sample images and data records were created on the reader prototype and copied onto a laptop via USB and Microsoft ActiveSync software.

2.6 Ultraviolet (UV) UV Flashlight Testing

The ACE™ Test method uses a UV-LED to illuminate the ticket wells and the ticket is inserted into the reader. For test method simplicity and for a significant potential cost savings, the United States Army Center for Environmental Health Research (USACEHR) tested whether the ACE™ Test tickets could be read with a UV-flashlight. Two types of UV flashlights (see Fig. 7) were purchased from LED Wholesalers (Hayward, CA): a 400 nm 9 LED flashlight (Model number: 7301UV400, $7.50) and a 395 nm 25 LED flashlight (Model number: 7202UV395, $15). (Note that the WQAS-PM already includes a 6 watt 365-nm long-wave UV lamp for use with the Colilert™ Coliform bacteria test). Although the 365 nm lamp did illuminate the wells and produce a green color, there was poor distinction between initial blind tests with negative samples and toxic samples, and it is not recommended for use with the ACE™ Test.) Preliminary testing found that the larger 395 nm flashlight produced a stronger visual differentiation between positive and negative samples, so testing reported here was completed using 395 nm flashlight.

Figure 7. The UV flashlights (left: 395 nm 25 –LED; right: 400 nm 9 LED).

The ACE™ Test reader provides an objective output of the hue ratio and a positive/negative result based upon the detection threshold (1.25), but readings from a UV flashlight are more variable and subjective, since they are based upon the person taking the reading. The following procedures were used to compare results from the ACE™ Test reader with manual UV-flashlight readings for the same test tickets. Test samples, including at least two negative controls, were processed and read with the ACE™ Test reader. Then, ticket sample numbers were hidden, and the tickets were placed in a randomized order to be read via UV flashlight by four individuals. The same individuals did all the readings throughout the testing. The users were given simple instructions (see Appendix D) and asked whether the control well was different than the test well. Pictures of typical positive and negative test results were provided as reference. The users could provide written observations on the test results if they wished.

2.7 Temperature Storage Testing

To further evaluate reagent shelf life, an accelerated test method was used. Accelerated testing results have been effective in estimating storage life at a specified temperature using the Arrhenius equation and short-duration, high-temperature data (Anderson and Scott, 1991).

The Arrhenius equation was used to estimate effective storage times for supplies at a specific temperature, when the effectiveness is demonstrated for a specific storage time at a higher temperature. The temperature coefficient (Q_{10}) is used for biological systems and ranges approximately 2-3. For 37 °C, a Q_{10} of 2 is used. Using the Arrhenius equation, as the accelerated temperature is increased; the Q_{10} constant (such as 2.5 for 70 °C) is increased as well. Table 4 estimates the storage times at temperatures of 45°C, 22 °C, 20 °C and 6 °C.

Equation:

$Time_{20°C} = Time_{70°C} \times Q_{10}^{(70\ °C - 20\ °C)/10}$ where $Q_{10} = 2.5$.

Table 4. Arrhenius Equation Time Estimations				
Days Effective at 70 °C	**Estimated Months Effective if Stored at:**			
	45 °C	**22 °C**	**20 °C**	**6 °C**
1	0.3	2.7	3.3	12
2	0.7	5.4	6.5	24
3	1.0	8.1	9.8	36
4	1.3	10.8	13	48
5	1.6	13.6	16.3	60

Ten reagent packs were placed in a 70 °C hybridization oven, tested at days 1-5 (one reagent pack for a negative blank and one for a positive test [aldicarb for reagent A and fenamiphos for reagent B]). Ten corresponding reagent packs were kept at room temperature for testing. Confirmatory testing of all OP/C's was done with the entire pack at the last known day where all three variables were successful in both negative blank and toxicant testing (Table 8). Section 3.3.1 provides accelerated testing results with the ACE™ Test.

It is possible that the ACE™ Test will be operated at temperatures higher than room temperature (from 22 – 45 °C). Therefore, assay packs were stored at temperatures of 25 °C, 37 °C, or 45 °C for 3 days, then tested (operated) in incubators at the same temperatures to determine any differences in toxicant response. Temperatures were verified using a NIST-traceable thermometer. Test results at different operational temperatures are found in Section 3.3.4.

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3. Results and Discussion

3.1 Toxicant Detection

3.1.1 Blank Testing and Threshold Determination

Figure 8 illustrates the blank sampling data. The calculated hue ratio toxicity threshold for a false positive rate of 1 in 1000 for both Reagents A and B was 1.12. However, for this study, a more conservative hue ratio of 1.25 was established and selected as the threshold for determining a toxic effect due to variability experienced between readers. This more conservative threshold corresponds to a false positive ratio of less than 1 in 2,000,000 (Appendix C). Blank testing in conjunction with the TTEP evaluation resulted in no false positives (0/40 tests (80 tickets); Allgeier et al, 2013).

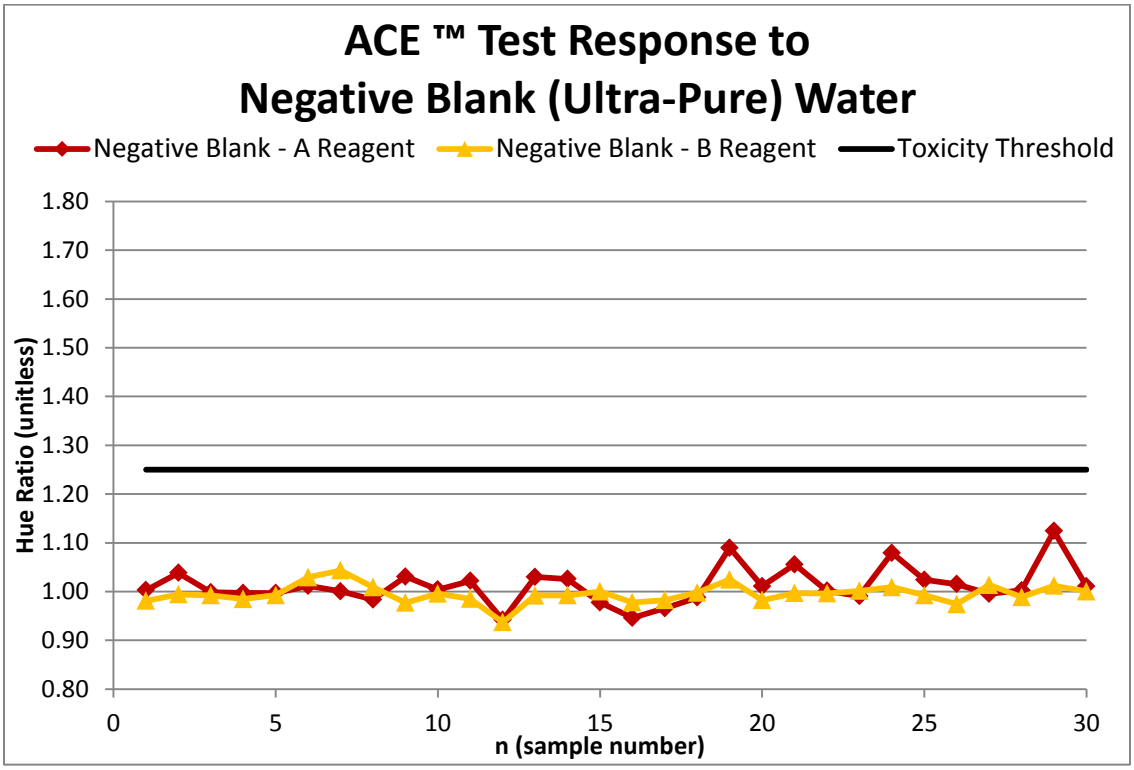

Figure 8. Negative Blank Sampling (n=30 for each reagent) with the ACE™ Test

3.1.2 Positive Control Testing with Copper

Responses to copper at 25 mg/L are shown in Figure 9; all 16 responses were well above the threshold hue ratio of 1.25. It may be possible to use a lower concentration of copper for the positive control; in TTEP testing, copper sulfate at 10 mg/L was positive in all 34 tests conducted (Allgier, 2013).

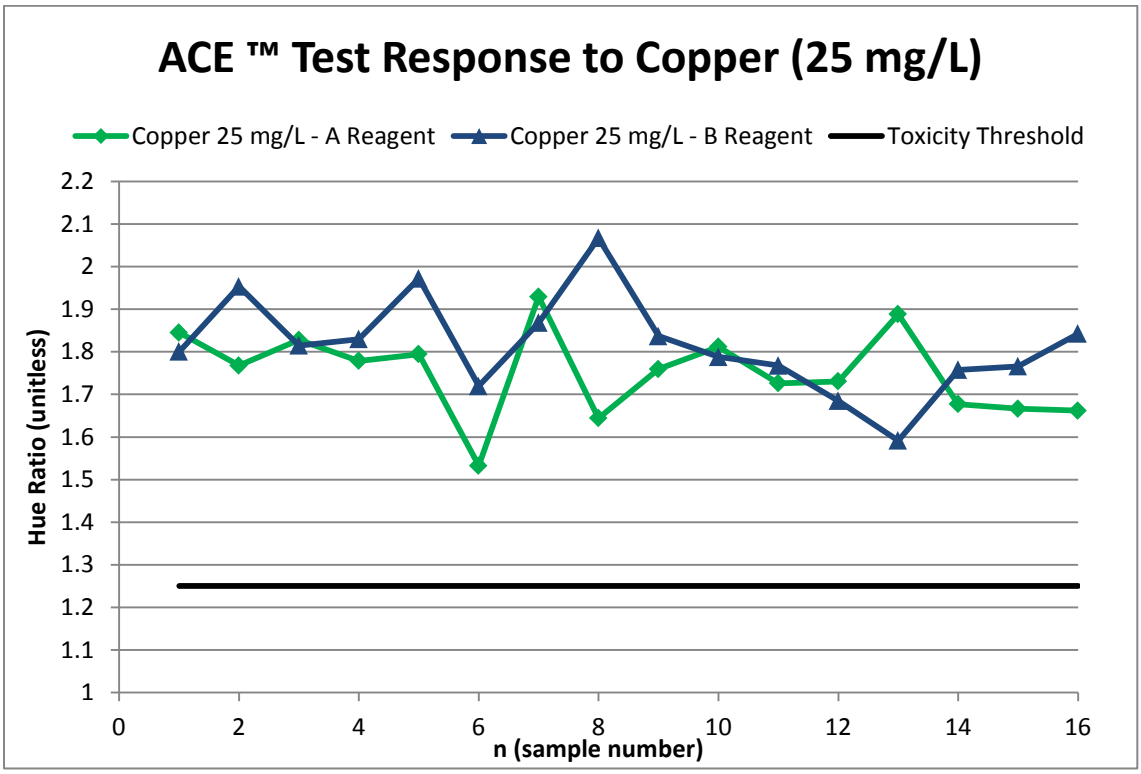

Figure 9. Positive Control Sampling (n=16 for each reagent) with the ACE™ Test

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3.1.3 Definitive Testing

Sample images for aldicarb, fenamiphos, methamidophos, and methyl parathion are shown in Figure 10 and a comparison graph of hue ratios is shown in Figure 11. All 4 OP/C chemicals are detected either at the HLC (aldicarb and methamidophos) or lower (methyl parathion at 5 mg/L and fenamiphos at 0.015 mg/L. Non-OP/C chemicals have been evaluated with an earlier design (well-style tickets, prior to TTEP).

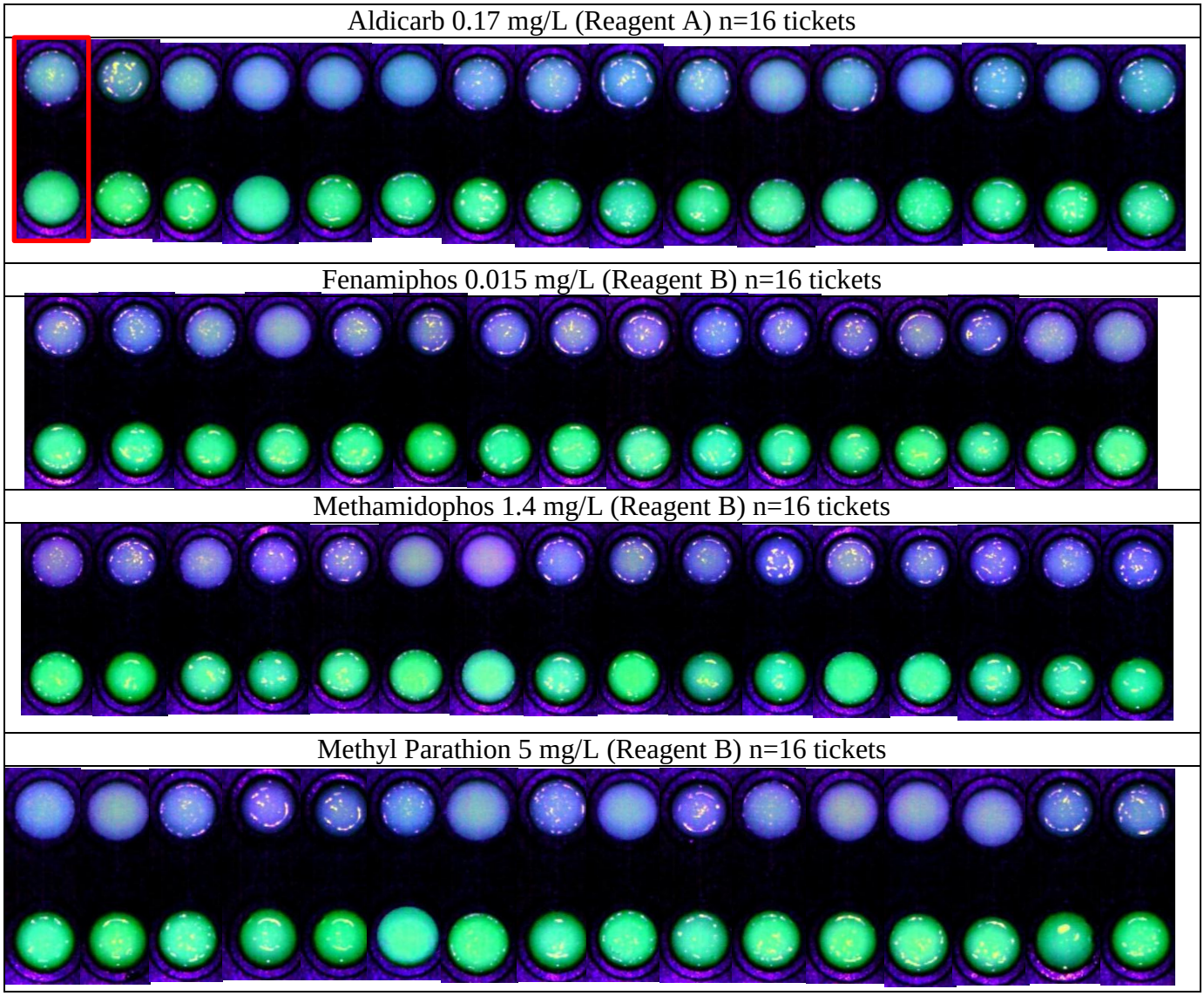

Figure 10. Images from definitive OP/C testing.

One ticket is outlined in red on the upper left. Each ticket has two wells (upper test well and lower control well). The green color indicates a hydrolysis of the NMI substrate by either enzyme (negative) and the bluish-purple color shows the absence of active enzyme. While each test has a Reagent A ticket and Reagent B ticket, carbamate pesticides interfere with the Reagent A (AChE) and organophosphate pesticides interfere with Reagent B (CE). Only the responding reagent is shown here.

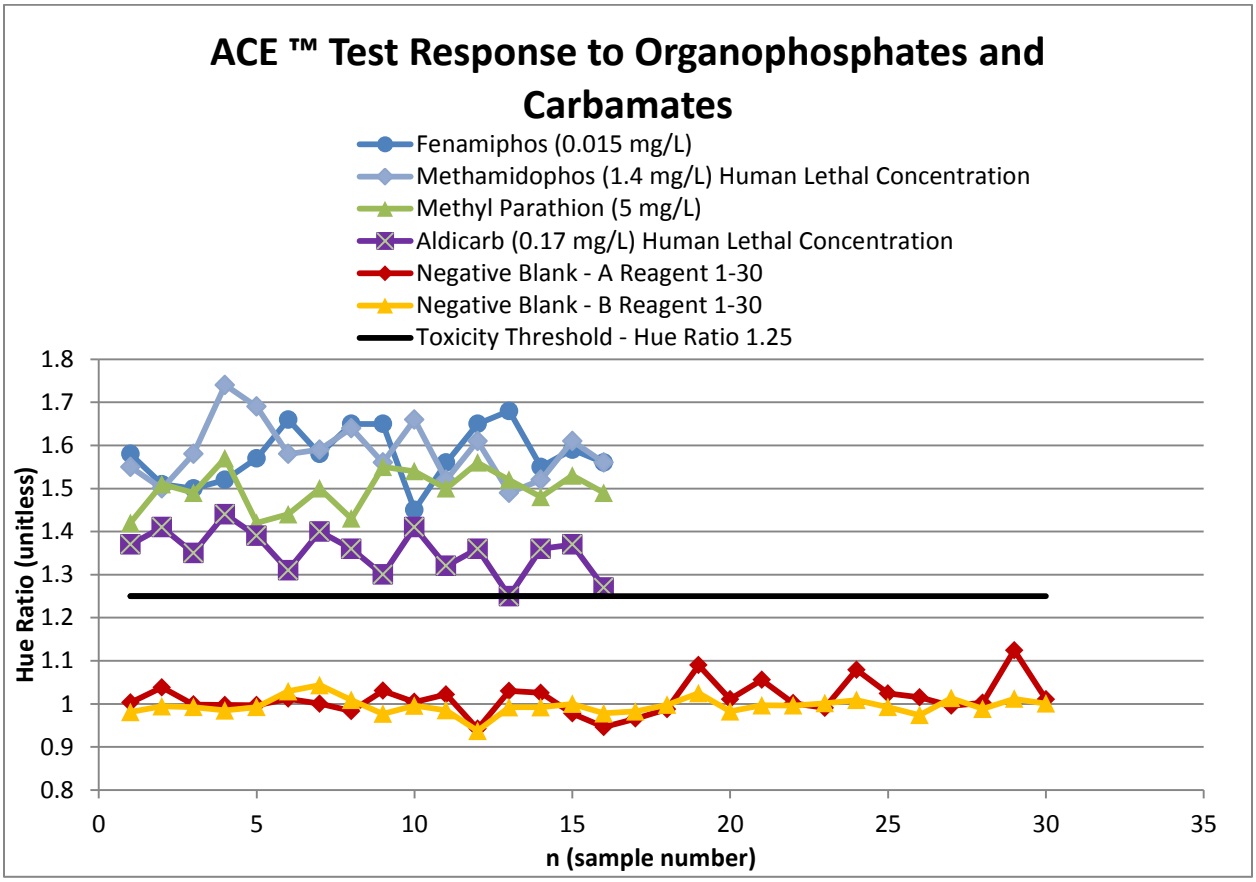

Figure 11. Organophosphate, and carbamate response with negative blank comparison.

3.1.4 Interferences

The following interferences have been evaluated with an earlier design of the ACE™ Test (well-style design) and have been confirmed at TTEP (one discrepancy was noted with chloramines, Table 5).

Table 5. Interference Chemical Responses

Test Chemicals	Concentration[1]	USACEHR Response (n=16)	TTEP Response (n=4)
Chlorine	10	No Response	No Response
Chloramines	10	No Response	1 of 4 Responded
Geosmin	0.0001	No Response	No Response
Methyl-isoborneol (MIB)	0.0001	No Response	No Response
Humic / Fulvic Acids (50%/50%)	5 (2.5/2.5)	No Response	No Response
Blank – Hard Water	250	No Response	No Response

[1] All concentrations reported as mg/L

3.2 UV Flashlight Testing

Table 6. ACE™ Test Reader and UV Flashlight Comparison

		ANP Prototype Reader			UV Flashlight	
Chemical		Concentration (mg/L)	Percent Detected	n	Percent Detected	n[1]
Aldicarb		1.7	100	2	100	8
		0.54	100	16	100	64
	HLC[2], MDL[3]	0.17	100	16	86	64
	MEG[4]	0.0047	0	2	0	8
Fenamiphos	HLC	0.56	100	2	100	8
		0.056	100	16	88	16
		0.042	100	16	75	8
	MDL	0.015	100	2	88	64
	MEG	0.0042	0	2	38	8
Methyl Parathion	HLC	33.6	100	2	100	8
		10	100	16	100	8
	MDL	5	100	16	98	64
		1	0	2	38	8
		0.5	0	2	0	8
	MEG	0.14	0	2	0	8
Methamidophos		14	100	2	100	8
	HLC, MDL	1.4	88	16	100	64
		0.44	0	16	63	16
		0.14	0	2	6	16
	MEG	0.00023	0	2	0	8
Blank Sample (Millipore™ Water)			0	28	2	112

[1] samples were observed by four different observers, yielding four times as many observations as the ANP prototype reader
[2] HLC – Human Lethal Concentration
[3] MDL – Minimum Detection Limit – concentration where 16 of 16 detections occur with the ANP prototype reader
[4] MEG – Military Exposure Guideline concentration

	No samples detected
	Some samples detected
	All samples detected

With the exception of one sample of methamidophos at the HLC, the ANP prototype reader detected all four OP/C pesticides below the HLC (Table 6). UV flashlight users detected fenamiphos, methyl parathion and methamidophos below the HLC, but above the HLC (3.17 times the HLC) for aldicarb. The reader detected fenamiphos, methyl parathion, and aldicarb at a lower concentration than the flashlight users. With the exception of one sample, methamidophos was detected at the same concentration (HLC, MDL) by both the reader and the flashlight users.

Blank samples were determined as negative for 100% of samples with the reader (28 of 28 samples). Flashlight users found 98% of blank samples to be negative (110 of 112 observances). The reader was more absolute in the determination of toxicity, detecting either 100% of the samples at the specified concentration or 0%, with the exception of one sample of methamidophos. In contrast, flashlight users provided more variable responses. 10 of the 24 sets of concentrations (42%) were not consistent (either incomplete detection (1 of 3 or 2 of 3) or with other user responses.

With a projected cost of the ACE™ Test reader of $3,000 to $10,000 (depending on quantity purchased), a UV LED flashlight ($10-20) offers a substantial cost reduction ($2980-$9,980). However, introducing human subjectivity into the response measurement results in an increase in false positive rate (1 in every 50 tests). The ANP reader yields consistent results (16 of 16) and responds at a lower concentration than observers with a UV flashlight. Another issue is the risk of UV exposure to eyes and skin with the UV flashlight that would need an occupational health risk assessment prior to implementation. Based on this information, it is not recommended that the UV flashlight replace the ACE™ Test reader in the ESB system. However, the UV flashlight could be used where a reader is not available, or as a pre-screening/diagnostic tool for ACE™ Test operation.

3.3 Temperature Testing

3.3.1 Accelerated Testing

Table 7 illustrates the data of a negative blank and a representative OP/C for each reagent (aldicarb for reagent A and fenamiphos for reagent B). Based on the Arrhenius Equation (Table 4), successful testing after three days at 70 °C corresponds to a shelf life of 9 months at room temperature and 3 years at 6 °C. The control failure after 4 days of storage was considered to be a failure of the tickets (NMI reagent), not the enzymes. The 9 month shelf life is consistent with Army threshold performance requirements.

Table 7. Accelerated Shelf Life Testing of June 2013 ACE™ Test Reagent Packs (April 2013 Tickets and May 2013 AChE)

Days at 70 °C	Hue Ratios			
	Blank Sample with Reagent A	Blank Sample with Reagent B	Aldicarb 0.17 mg/L with Reagent A	Fenamiphos 0.015 mg/L with Reagent B
3	1.01	1.00	1.36	1.54
4	0.83	.96	CF[1]	1.91

[1] CF - Two control failures were observed with Aldicarb (Reagent A) on Day 4

Previous experience shelf life testing of the ACE™ Test reagents has focused on enzyme shelf life, using NMI tickets stored at room temperature (Table 8). These tests show that the enzymes are consistently predicted to last more than a year at room temperature and in fact even lasted more than a year at 45 °C. These data suggest that the relatively short predicted shelf life of 9 months for the reagent packs at room temperature (Table 7) are due to the NMI on the test tickets, not the enzymes, since the test tickets for the temperature tests shown in Table 8 were not subjected to high temperatures, while the entire reagent packs (enzymes plus test tickets) were tested at high temperature in the most recent test (Table 7). The shorter shelf of life test tickets may be due to NMI oxidation (Y. Vallejo, ANP Technologies, personal communication).

Table 8. Estimated and Actual Shelf Life Determinations of Enzyme Reagents

Enzyme Lot	Sample	Hue Ratio	Shelf Life	Comments
130205 W_L-A	Blank[1] (Reagent A)	1.07	>13.6 months at room temperature (estimated)	Enzyme was stored at 70 °C for over 5 days, using June 2013 lot of Reagent A and B
	Blank (Reagent B)	1.03		
	Aldicarb (Reagent A, 0.17mg/L)	1.79		
	Fenamiphos (Reagent B, 0.015 mg/L)	2.05		
121022 RDS-A	Blank (Reagent A)	0.95	>54 months at room temperature (estimated)	Enzyme was stored at 70 °C for over 20 days
	Blank (Reagent B)	1.01		
	Aldicarb (Reagent A, 0.17mg/L)	1.38		
	Fenamiphos (Reagent B, 0.015 mg/L)	1.41		
14DEC2010 D-M	Aldicarb (Reagent A, 0.17mg/L)	2.27	>15 months at 45 °C (actual)	
	Fenamiphos (Reagent B, 0.56mg/L)	2.38		
	Methamidophos (Reagent B, 1.4 mg/L)	2.17		
	Methyl Parathion (Reagent B, 33.6 mg/L)	2.24		

[1]Blank Sample is Millipore™ Water

3.3.2 Operational Testing at Different Temperatures

Currently, the manufacturer recommends the ACE™ Test be operated at room temperature (20-25 °C) with a read time of 15 minutes (read time extends from placing the enzyme solution on the ticket to pressing the "read" button). However, an operator may be forced to conduct a test at higher temperatures in the field and may not always maintain a consistent read time. Table 9 provides data on the effect of varying read times and operational temperatures on test outcome.

Table 9. ACE ™ Test Response at Different Incubation Temperatures			
Sample	**Average Hue Ratio[1] at 25 °C**	**Average Hue Ratio at 37 °C**	**Average Hue Ratio at 45 °C**
Negative Blank with Reagent A	1.02	1.07	0.89
Negative Blank with Reagent B	1.00	0.89	1.03
Fenamiphos 0.015 mg/L with Reagent B	2.59	2.28	2.22
[1] average hue ratio from n=3 from each temperature, for each enzyme/chemical			

This data shows that at 25 °C, 37 °C, and 45 °C:

- No false positives with blank samples at any temperature with either A or B reagents.
- 3 of 3 samples of fenamiphos at 0.015 mg/L were detected.

3.3.3 Storage Considerations and Recommendations:

- Enzyme vials have shown stability well over 15 months at temperatures of 20-25 °C and 45 °C in real-time. Previous designs of the ticket lots have lasted over 12 months in real time.
- The estimated longevity for the June 2013 lot is 9 months due to ticket life (specifically, the NMI on the ticket pad). The manufacturer's claims are 12 months. With enzyme formulation, NMI and ticket design improvements in advanced development, it is expected that the manufacturer will achieve a 12 month shelf life at 20-25 °C.
- The maximum real-time test length with all reagent pack components (enzyme vials and tickets) is 9 months at 20-25 °C.
- For the range of temperatures tested, the NMI on the ticket is considered to be the most susceptible component to degradation over time.
- The AChE (Reagent A) is less stable than the CE (Reagent B).

3.4 Future Development

Advanced development efforts have been focused on decreasing reader-to-reader variability and ease-of-use improvements. ANP Technologies, Inc. has developed calibration tickets with known hue ratio tolerances. Readers will be screened to ensure that calibration ticket readings fall within the accepted hue ratio range (eg. 0.92 – 1.11) for each reader.

Additionally, ANP Technologies will be adjusting the enzyme formulations to improve toxicant sensitivity, endurance of higher storage temperatures, and achievement of longer shelf-life. The

ACE™ Test produced by the advanced development is likely to yield results that are similar or better than those represented here.

Acknowledgments

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List of Abbreviations and Acronyms

%	Percent
µL	microliter
AChE	acetylcholinesterase
ASTM	American Society of Testing and Materials
°C	degree Celsius
CDD	Capabilities Development Document
CE	carboxylesterase
ECIS	Electric Cell-substrate Impedance Sensing
ESB	Environmental Sentinel Biomonitor
HLC	human lethal concentration
IPT	Integrated Product Team
kg	kilogram
JCBRAWM	Joint Chemical Biological Radiological Agent Water Monitor
L	liter
LED	light-emitting diode
M	Molar
MDL	minimum detection limit
MEG	Military Exposure Guidelines
mg	milligram
min	minute
MIB	methyl iso-borneol
mM	millimolar

MRMC	Medical Research and Materiel Command
ND	no detection
NIDS	Nano-Intelligent Detection System
NMI	n-methyl isonoate
NT	not tested
OP	organophosphate
OP/C	organophosphate and carbamate
P/NA	Pesticide / Nerve Agent
PCP	pentachlorophenate
RGB	red/green/blue
SBIR	Small Business Innovation Research
TEEX	Texas Engineering Extension Services
TICs	Toxic Industrial Chemicals
TTEP	Technology Testing and Evaluation Program
USACEHR	U.S. Army Center for Environmental Health Research
USACHPPM	U.S. Center for Health Promotion and Preventative Medicine
UV	ultraviolet
WQAS-PM	water quality analysis set – preventive medicine

Appendix A: Chemicals Evaluated

Compound [measured analyte]	Chemical Abstracts Service Number[a]	Storage Requirements	Analytical Method	Source	Stability in Deionized Water	Purity %
Acrylonitrile [acrylonitrile]	107-13-1	4º C / dark	HPLC	Chem Service West Chester, PA	<3 hrs - open container;14 days - no head-space vial	99.5
Aldicarb [aldicarb]	116-06-3	4º C / dark	HPLC	Chem Service West Chester, PA	>14 days	99
Ammonium chloride [total ammonia]	12125-02-9	4º C / dark	colorimetric	Sigma-Aldrich St. Louis, MO	>14 days	99.99
Sodium arsenite [As]	7784-46-5	4º C / dark	ICP-MS	Chem Service West Chester, PA	>14 days	98
Sodium azide [azude]	26628-22-8	4º C / dark	Ion Chromatograph	Sigma-Aldrich	>14 days	99.5
Chloramine [monochloramine]	10599-90-3	4º C / dark	amperometric titration	Sigma-Aldrich	24 hrs	NA
Sodium hypochlorite [chlorine residual]	76881-52-9	4º C / dark	amperometric titration	Riedel-de Haën Fine Chemicals Seelze Germany	>14 days	NA
Copper sulfate [Cu]	7758-99-8	4º C / dark	ICP-MS	Sigma-Aldrich	>14 days	99.95
Sodium cyanide [cyanide]	143-33-9	4º C / dark	ion probe	Sigma-Aldrich	>14 days	99.98
Ethylene glycol [c] [ethylene glycol]	107-21-1	4º C / dark	Nominal	Sigma-Aldrich	not measured[b]	99.8
Fenamiphos [fenamiphos]	22224-92-6	room temp / dark	Nominal	Chem Service	>14 days	98.5
Sodium fluoroacetate [fluoroacetate]	62-74-8	4º C / dark	HPLC	Sigma-Aldrich	> 14 days	>90
Geosmin	19700-21-1	4º C / dark	Nominal	Sigma-Aldrich	not measured[b]	98
Humic/fulvic acid mixture (1:1 by weight)	NA	4º C / dark	Nominal	International Humic Substances Society, St. Paul, MN	not measured[b]	NA
Mercuric chloride [Hg]	7487-94-7	room temp / dark	ICP-MS	Sigma-Aldrich	>14 days	99.5
Methamidophos [methamidophos]	10265-92-6	4º C / dark	Nominal	Chem Service West Chester, PA	>14 days	98.8
Methyl parathion [methyl parathion]	298-00-0	4º C / dark	HPLC	Chem Service West Chester, PA	>14 days	99.3
2-methylisoborneol (MIB)	2371-42-8	4º C / dark	Nominal	Sigma-Aldrich	not measured [b]	98
Nicotine [nicotine]	54-11-5	4º C / dark	HPLC	Chem Service West Chester, PA	>14 days	99.4
Oxamyl [c] [oxamyl]	23135-22-0	4º C / dark	HPLC	Chem Service West Chester, PA	> 14 days	99
Paraquat dichloride [paraquat]	1910-42-5	4º C / dark	HPLC	Chem Service	>14 days	99
Sodium pentachlorophenate [pentachlorophenate]	131-52-2	4º C / dark	HPLC	Mallinckrodt Baker Phillipsburg, NJ	>14 days	99
Phenol [phenol]	108-95-2	4º C / dark	HPLC	Sigma-Aldrich	>14 days	99.5
Strychnine [c] [strychnine]	57-24-9	4º C / dark	HPLC	Sigma-Aldrich	> 14 days	98
Thallium sulfate [Tl]	7446-18-6	4º C / dark	ICP-MS	Sigma-Aldrich	> 14 days	99.995
Toluene [toluene]	108-88-3	4º C / dark	HP6890 GC and HP-7694 HS	Sigma-Aldrich	14 days; no-head space vial	99.8

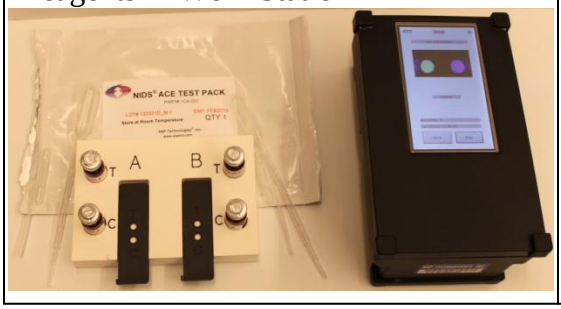
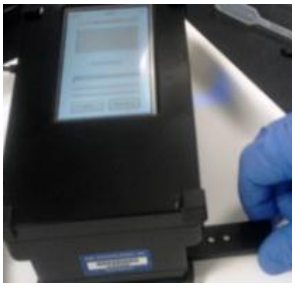
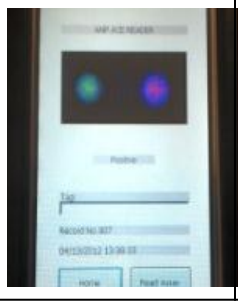
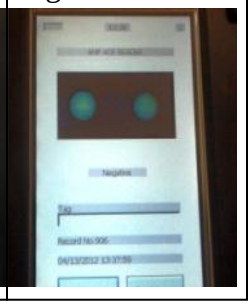
GC = gas chromatography ····· LC-MS = liquid chromatography – mass spectrophotometry ····· ICP-MS = inductively coupled plasma-mass spectrophotometry ·····
HPLC = high performance liquid chromatography ····· HP6890 GC and HP-7694 HS = gas chromatography & head-space sampling ····· NA = Not available
[a] Number for compound [b] Tested within 24 hrs of preparation [c] No longer being tested with TRL6 and above

Appendix B – ACE™ Test Checklist

ANP Technologies, LLC. NIDS ®ACE™ Test Checklist
Sample Tested: _____
Date: _____ Time Start:_____

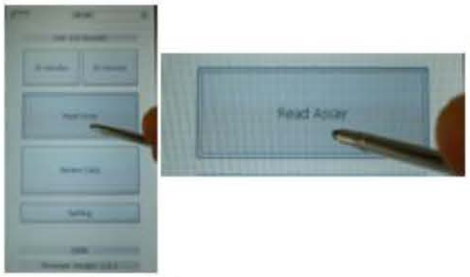
Directions	Check (√)
Open reagent pack	
Open 2 Reagent A vials, place in work station	
Open 2 Reagent B vials, place in work station	
Place tickets in work station, plug reader in and turn "ON"	
Sample transfer (using large plastic pipette): remove vial stoppers	
Place 1 mL **clean** water in A- Control (C) vial	
Place 1 mL **clean** water in B- Control (C) vial	
Place 1 mL **test** water in A- Test (T) vial	
Place 1 mL **test** water in B- Test (T) vial and Replace vial stoppers and invert/shake each vial for 10 seconds	
Wait 30 minutes (press 30 min timer on touch screen), then Sample transfer (using small plastic pipette): remove vial stoppers	
Place 0.1 mL Control vial A- Control (C) well of ticket A	
Place 0.1 mL Control vial B- Control (C) well of ticket B	
Place 0.1 mL Test vial A- Test (T) well of ticket A	
Place 0.1 mL Test vial B- Test (T) well of ticket B	
Wait 15 minutes, (press 15 min timer on touch screen)	

RESULTS	Circle one	Record #	2nd Sample
Place Ticket A into Reader, press "Read Assay", then "Read Assay" again (forcefully slide ticket into slot on right side)	Negative or Positive		
Place Ticket B into Reader, press "Read Assay"	Negative or Positive		
If either Ticket A or Ticket B is positive, the sample is considered contaminated.			

Reagents in Work Station	Inserting ticket	Positive Result	Negative Result
			

Procedure for Reading a Ticket

1. Turn on the reader by sliding the power button to the line ▬ symbol.



2. On the home screen, tap on the "Read Assay" box with stylus.

3. Tap on the "Tag" entry box with stylus. A virtual keyboard will appear.

4. Type in sample ID, then tap the enter arrow on the keyboard.

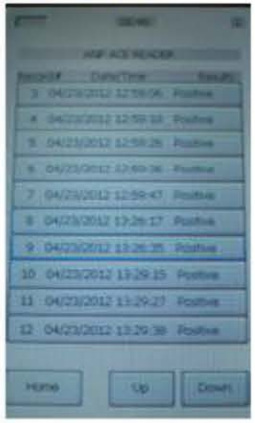
5. Alternatively, the operator can skip steps 3 and 4 and proceed to step 6.

6. Insert ticket. Tap on the " Read Assay" again. Wait several seconds until an image and result appear. The result is automatically stored in the reader memory.

Procedure for Retrieving Results

1. On the home screen, tap on the "Review Data" box.

2. A list of all results will appear on the screen. Scroll to find the specific result of interest by tapping on the up and down arrows at the bottom of the screen. The result can be identified by its sample ID and/or the time it was run.



Appendix C
False Positive Rate assessment for Millipore Hue Ratio test.

Elgin Perry, Ph. D.
eperry@chesapeake.net
410-535-2949

6/11/2013

The study considers negative control data from 30 blank water (Millipore) tickets. Each ticket has two reagents: A and B and was read by one meter and by one technician. Comparisons are made between reagents and detection thresholds are computed by reagent and collectively. The hue ratio data from both Reagent A and Reagent B are reasonably well approximated by the normal distribution (Figures 1 and 2) as indicated by the observed and expected data falling fairly close to a straight line in the Normal Probability plots. Data from both reagents exhibit slight excess Kurtosis (Table 1.) indicating that the distribution is more tightly clustered about the mean than is typical of the normal distribution. This tight clustering of the majority of observations results in some of the remainder appearing as outliers (box-plot display Figures 1 and 2) where a few points for both reagents A and B are identified as outliers (shown by *)

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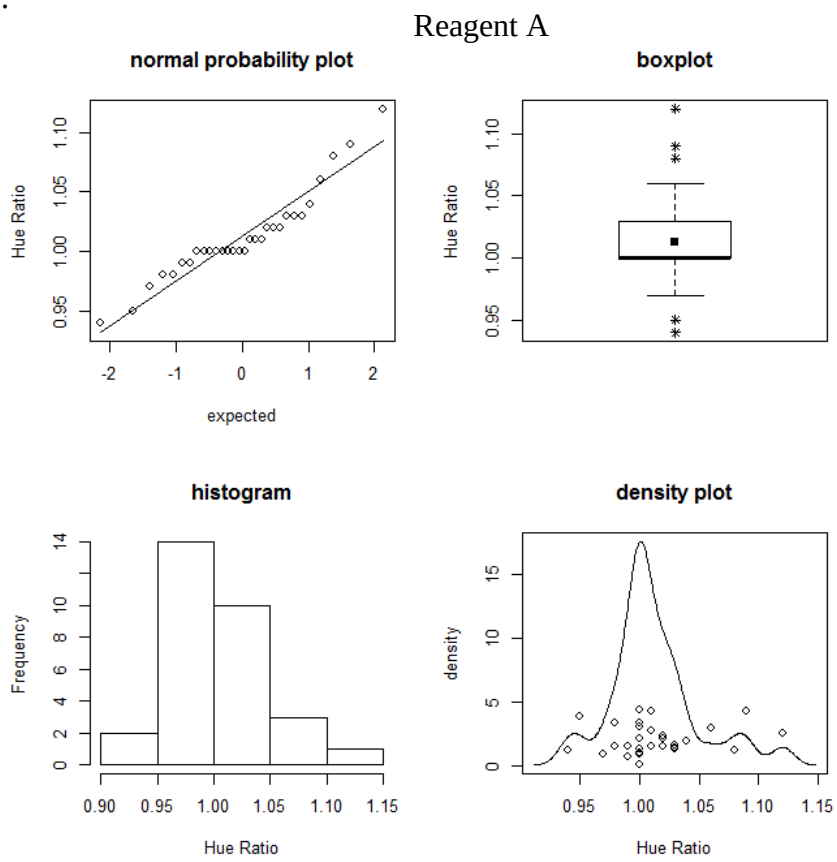


Figure 1. Distribution plots for Hue Ratio Reagent A.

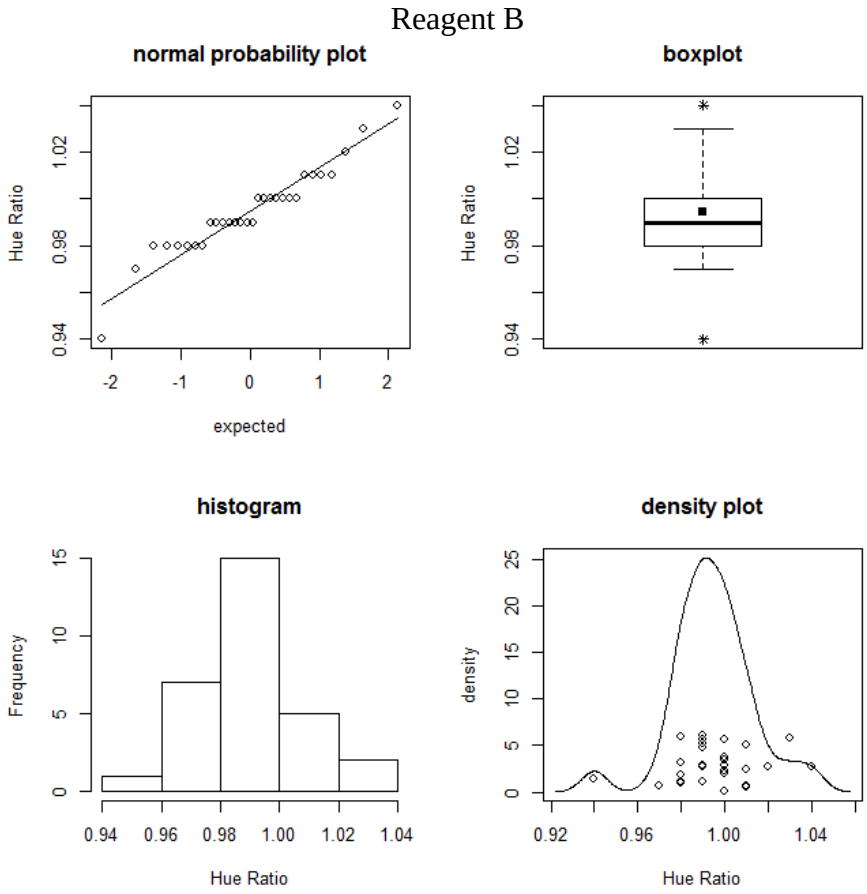


Figure 2. Distribution plots for Hue Ratio Reagent B.

Table 1. Descriptive statistics of hue ratio for negative control responses for Reagents A and B.

Statistic	Reagent A	Reagent B
sample size	30	30
mean	1.0123	0.9947
standard dev	0.0379	0.0187
variance	0.0014	3e-04
skewness	0.8367	-0.1482
excess kurtosis	0.9646	1.3575
minimum	0.94	0.94
q25	1	0.9825
median	1	0.99
q75	1.0275	1
maximum	1.12	1.04

There is a curious shift from reagent A to reagent B (Figure 3) where reagent A tends to produce a hue ratio of 1 or greater while reagent B tends to produce a hue ratio of less than 1. Using an analysis of variance where the data are paired by sample this shift was shown to be statistically significant (p= 0.0096) (Table 2.) The variability for reagent A as measured by the standard deviation of 0.0379 is greater than that for reagent B which is 0.0187 (Table 1, Figure 3). Thus it is expected that reagent A will have more extreme thresholds than reagent B.

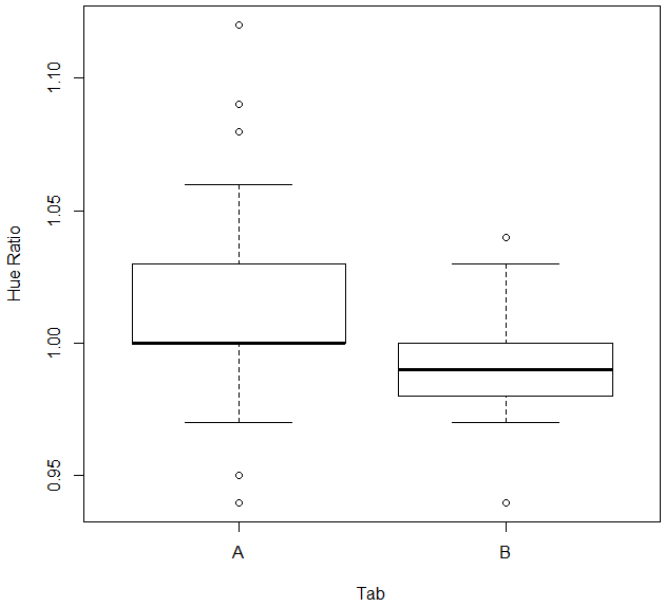


Figure 3. Box and Whisker plots comparing Hue Ratio data between Reagents.

Table 2. Analysis of Variance for Reagent effect.

Source	sum of squares	Df	mean square	F-stat	p-value
as.factor(Reagent)	0.0047	1	0.0047	7.6843	0.0096
as.factor(sample)	0.0342	29	0.0012	1.9365	0.0402
Residuals	0.0177	29	0.0006		
Total	0.0566	59			

Threshold Estimation:

Given variability of these observations, false positive thresholds for responses greater than 1 are computed for a range of probabilities (0.05 to 0.00001) defining the false positive rates for reagent A and reagent B (Table 3 and 4.) These thresholds are computed under the assumption that the hue ratio of negative control observations is centered at 1.0 rather than centered at the observed mean hue ratio for each reagent. In each table, the thresholds for reagents A and B are shown in columns 1 and 2 and the individual reagent false positive probability or reagent-wise false positive rate is shown in column 3. The test-wise false

positive rate, which is the probability of a false positive for Reagent A, Reagent B or both, is given in column 4. The thresholds for the 1 in 10,000 test-wise false probability rate are 1.1476 and 1.0728 for Reagents A and B, respectively.

The test rejection rates for various thresholds are given for Reagents A and B. The rejection rate gives the expected proportion of tests that would be rejected for a given rejection threshold. Given a rejection rate of 1 in 10,000 for rejection by reagent A, reagent B, or both, the lower side thresholds are 0.8524 and 0.9272 for reagents A and B, respectively.

Table 3. Upper side false positive rate thresholds for Reagent A and Reagent B.

Reagent A Threshold	Reagent B Threshold	False Positive Rate A or B	False Positive Rate A and B
1.0624	1.0308	0.05	0.0975
1.0883	1.0435	0.01	0.0199
1.0977	1.0482	0.005	0.009975
1.1172	1.0578	0.001	0.001999
1.1248	1.0616	5e-04	0.00099975
1.1411	1.0696	1e-04	0.00019999
1.1476	1.0728	5e-05	1e-04
1.1618	1.0798	1e-05	2e-05
1.1676	1.0826	5e-06	1e-05
1.1803	1.0889	1e-06	2e-06

Lower side test rejection rate thresholds for Reagent A and Reagent B.

Reagent A Threshold	Reagent B Threshold	Rejection Rate A or B	Rejection Rate A and B
0.9376	0.9692	0.05	0.0975
0.9117	0.9565	0.01	0.0199
0.9023	0.9518	0.005	0.009975
0.8828	0.9422	0.001	0.001999
0.8752	0.9384	5e-04	0.00099975
0.8589	0.9304	1e-04	0.00019999
0.8524	0.9272	5e-05	1e-04
0.8382	0.9202	1e-05	2e-05
0.8324	0.9174	5e-06	1e-05
0.8197	0.9111	1e-06	2e-06

Discussion

Given the range of thresholds observed for the different reagents, it seems that a working toxicity threshold for a 1 in 10,000 false positive rate is about 1.15 which does not appear to improve on previous results. A quick glance at toxicant results shows that this threshold is effective at discriminating toxicants at the concentrations tested.

Data for this study.

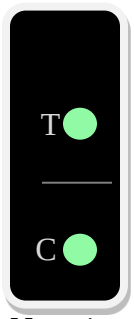
Ticket	Reagent	Sample	Record Number	Hue Ratio
1	A	1A	140	1.00
1	B	1B	141	0.98
2	A	2A	142	1.04
2	B	2B	143	0.99
3	A	3A	144	1.00
3	B	3B	145	0.99
4	A	4A	146	1.00
4	B	4B	147	0.98
5	A	5A	148	1.00
5	B	5B	149	0.99
6	A	6A	150	1.01
6	B	6B	151	1.03
7	A	7A	152	1.00
7	B	7B	153	1.04
8	A	8A	154	0.98
8	B	8B	155	1.01
9	A	9A	156	1.03
9	B	9B	157	0.98
10	A	10A	158	1.00
10	B	10B	159	1.00
11	A	11A	160	1.02
11	B	11B	161	0.99
12	A	12A	162	0.94
12	B	12B	163	0.94
13	A	13A	164	1.03
13	B	13B	165	0.99
14	A	14A	166	1.03
14	B	14B	167	0.99
15	A	15A	168	0.98
15	B	15B	169	1.00
16	A	16A	170	0.95
16	B	16B	171	0.98
17	A	17A	172	0.97
17	B	17B	173	0.98
18	A	18A	174	0.99
18	B	18B	175	1.00

19	A	19A	176	1.09
19	B	19B	177	1.02
20	A	20A	178	1.01
20	B	20B	179	0.98
21	A	21A	180	1.06
21	B	21B	181	1.00
22	A	22A	120	1.00
22	B	22B	121	1.00
23	A	23A	122	0.99
23	B	23B	123	1.00
24	A	24A	124	1.08
24	B	24B	125	1.01
25	A	25A	182	1.02
25	B	25B	183	0.99
26	A	26A	184	1.02
26	B	26B	185	0.97
27	A	27A	186	1.00
27	B	27B	187	1.01
28	A	28A	188	1.00
28	B	28B	189	0.99
29	A	29A	190	1.12
29	B	29B	191	1.01
30	A	30A	192	1.01
30	B	30B	193	1.00

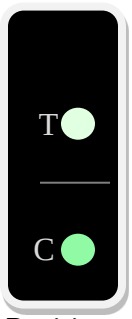
	10 lowest hue ratios
	10 highest hue ratios

Appendix D UV Flashlight Data Sheet

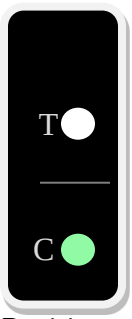
Place the UV flashlight (LED Wholesalers, 7202UV395nm) 3 inches (roughly the width of your hand) above the ticket and turn on the flashlight. Read under normal room/office fluorescent lighting.
Please answer the following question for each ticket: Is the "C" control well different than the "T" test well?



Negative



Positive



Positive

Ticket Number	**Please circle either negative or positive for each ticket**		**After Flashlight Corresponding Information (Chemical-Concentration-Reagent A/B)**	**Randomized Ticket Number**
1	Negative	Positive		
2	Negative	Positive		
3	Negative	Positive		
4	Negative	Positive		
5	Negative	Positive		
6	Negative	Positive		
7	Negative	Positive		
8	Negative	Positive		
9	Negative	Positive		
10	Negative	Positive		
11	Negative	Positive		
12	Negative	Positive		

Comments:_____

_____

_____

_____

Name of Operator: _____ Initials:_____