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Environmental Technology Verification Report

PHARMALEADS
EZYBOT[®] A AND EZYBOT[®] B TEST KITS

Prepared by

Battelle
The Business of Innovation

Under a cooperative agreement with

 **EPA** U.S. Environmental Protection Agency

ETV ✓ ETV ✓ ETV ✓

**THE ENVIRONMENTAL TECHNOLOGY VERIFICATION
PROGRAM**



ETV Joint Verification Statement

TECHNOLOGY TYPE: IMMUNOASSAY TEST KITS

APPLICATION: DETECTING BOTULINUM TOXIN

TECHNOLOGY NAME: EzyBot[®] A and EzyBot[®] B Test Kits

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The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative or improved environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies. Information and ETV documents are available at www.epa.gov/etv.

ETV works in partnership with recognized standards and testing organizations, with stakeholder groups (consisting of buyers, vendor organizations, and permittees), and with individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The Advanced Monitoring Systems (AMS) Center, one of six technology areas under ETV, is operated by Battelle in cooperation with EPA's National Exposure Research Laboratory. The AMS Center evaluated the performance of immunoassay test kits used to detect botulinum toxin in water. This verification statement provides a summary of the test results for the PharmaLeads EzyBot[®] A and B test kits.

VERIFICATION TEST DESCRIPTION

The verification test for the EzyBot[®] test kits was conducted at Battelle between November 2005 and January 2006 according to procedures specified in the *Test/QA Plan for Verification of Immunoassay Test Kits* for the following parameters: contaminant presence/absence; false positive/false negative response to interferents, drinking water (DW) matrix effects, and cross-reactivity; consistency; lowest detectable concentration; field portability; ease of use; and sample throughput. The ability of the EzyBot[®] test kits to detect various concentrations of botulinum toxin was evaluated by analyzing performance test (PT) and DW samples. PT samples included American Society for Testing and Materials Type II deionized (DI) water fortified with the target contaminant, an interferent, both, or only a cross-reactive species. Target analytes were added to DI water at lethal dose concentrations as well as at several concentrations selected based on the vendor-stated limit of detection (LOD). The effect of interferents was evaluated by analyzing two types of interferent solutions. The first type contained both humic and fulvic acids in DI water, and the second type contained magnesium (Mg) and calcium (Ca) in DI water. Both types of interferent solutions were prepared with and without the addition of the contaminants at a single concentration level (10 times the vendor-stated LOD). In addition, specificity was evaluated by exposing the EzyBot[®] test kits to lipopolysaccharide, a potentially cross-reactive compound for botulinum toxin. PT samples were analyzed in triplicate (with the exception of DI water fortified with target analytes at five times the vendor-stated LOD, for which ten replicates were analyzed). DW samples were collected from four water utilities that use a variety of treatment methods. DW samples, both unconcentrated and concentrated by a factor of 400, were analyzed in triplicate both with and without the addition of botulinum toxin A and B at a concentration of 10 times the vendor-stated LOD. The EzyBot[®] A test kit was specific to botulinum toxin A, and the EzyBot[®] B test kit was specific to botulinum toxin B. In addition to the PT and DW samples analyzed, method blank (MB) samples consisting of DI water were analyzed to confirm negative responses in the absence of any contaminant and to ensure that no sources of contamination were introduced during the analysis procedures.

QA oversight of verification testing was provided by Battelle and EPA. Battelle QA staff conducted a technical systems audit and a data quality audit of 10% of the test data. This verification statement, the full report on which it is based, and the test/QA plan for this verification are all available at www.epa.gov/etv/centers/center1.html.

TECHNOLOGY DESCRIPTION

The following description of EzyBot[®] was provided by the vendor and was not verified in this test.

EzyBot[®] test kits provide a means for detecting botulinum toxins A (EzyBot[®] A) and B (EzyBot[®] B) in water. The technology is based on the PharmaLeads internal collision fluorescence quenching technology. A fluorogenic substance and a quenching substance in the substrate bracket an amino-acid sequence that, in the presence of botulinum toxin A or B, is cleaved, generating an intense fluorescence. This fluorescence is measured using either a laboratory or a field fluorimeter. Note that a laboratory fluorimeter is not provided by PharmaLeads with the EzyBot[®] kit; however, a field fluorimeter is available for purchase as part of the field case. The type of fluorimeter used for detection can affect the sensitivity of the analysis obtained with the EzyBot[®] test kit, therefore users may want to contact the vendor for recommended fluorimeters in order to achieve optimal sensitivity with the EzyBot[®] kits. The fluorescence generated by the EzyBot[®] test kit increases in intensity with time and with botulinum toxin concentration. Data can be read from the fluorimeter display or for the PharmaLeads field portable fluorimeter can be transferred to a computer through a cable provided with the fluorimeter. Note that a computer is not provided by PharmaLeads.

EzyBot[®] A and B are available individually in kits of 50 ready-to-use cuvettes containing freeze-dried reagents, which can be used in the laboratory or in the field. The PharmaLeads field case provides a field incubator which can be plugged into the auxiliary power outlet of a car to perform the 1-hour incubation at 37°C in the field. The field case also includes the PharmaLeads field portable fluorimeter. The price of an EzyBot[®] kit depends on the quantity ordered. For large quantities, unit price is approximately \$30 per ready-to-use cuvette. Cost for the field case, including the field fluorimeter, the portable incubator, and 100 cuvettes, is less than \$12,500.

VERIFICATION OF PERFORMANCE

The tables below summarize the performance of the EzyBot[®] test kits in detecting botulinum toxins A and B.

EzyBot[®] A Summary Table

Parameter	Sample Information	Botulinum Toxin A (mg/L)	Lab Bench Scale Fluorimeter ^(a)		Field Portable Fluorimeter ^(a)	
			30 min.	60 min.	30 min.	60 min.
Contaminant-only	DI water	0.01 (vendor-stated limit of detection)	0	0	0	0
		0.05	0	10	0	0
		0.1	0	3	0	0
		0.3 (lethal dose)	3	3	0	3
		0.5	3	3	1	3
Interferent	0.5 and 2.5 mg/L each humic/fulvic acids	0.1	NA	0	NA	NA
	50 and 250 mg/L each Ca/Mg	0.1		3		
DW-all locations	unconcentrated	0.1		3		
DW-California	concentrated	0.1		3		
DW-Florida	concentrated	0.1		3		
DW-New York	concentrated	0.1		0		
DW-Ohio	concentrated	0.1		3		
Lowest Detectable Concentration ^(b) (mg/L)			0.3	0.05	ND	0.3
False positives	There were no false positive results from interferents including a preservative blank, humic and fulvic acids, and Ca and Mg; DW from four locations using different water treatment technologies; or the potentially cross-reactive lipopolysaccharide (0.1 mg/L).					
False negatives	False negatives were obtained in the presence of both 0.5 and 2.5 mg/L each humic and fulvic acids. A false negative was also obtained in New York water which was concentrated by a factor of 400. A total of 3 false negative results were obtained out of the 12 solutions assessed at 60 minutes. The vendor informed Battelle after testing that the lab bench scale fluorimeter provided for testing may have had inconsistent functioning which could have caused the false negative results that were obtained.					
Consistency	Using the lab bench scale fluorimeter, results were consistent in 100% of the samples tested. Using the field portable fluorimeter, results were consistent in 90% of the samples tested.					
Other Performance Factors	Convenient ready-to use cuvettes. Easy to operate in the lab and easy to transport and operate in the field. No formal scientific education would be required to use the kit; however, general lab skills and training on fluorimeter use were helpful. Approximately 12-15 analyses were completed in one hour in the laboratory. Only five samples could be processed in one hour in the field due to size limitation of the field portable incubator. Each Ezybot [®] kit contains 50 ready-to-use cuvettes.					

NA = Not tested. Testing concentration below detection in the contaminant only PT testing.

ND = not detectable at concentrations tested.

^(a) Results out of 3 replicates except for the 0.05 mg/L contaminant only concentration for which results are out of 10 replicates

^(b) The lowest concentration of contaminant-only PT samples to have at least two thirds of the measurements generate positive results.

EzyBot[®] B Summary Table

Parameter	Sample Information	Botulinum Toxin B (mg/L)	Lab Bench Scale Fluorimeter ^(a)		Field Portable Fluorimeter ^(a)	
			30 min.	60 min.	30 min.	60 min.
Contaminant-only	DI water	0.01 (vendor-stated limit of detection)	0	3	0	0
		0.05	7	10	0	0
		0.1	3	3	0	0
		0.3 (lethal dose)	3	3	3	3
		0.5	3	3	0	3
Interferent	0.5 mg/L each humic/fulvic acids	0.1	3	3	NA	
	2.5 mg/L each humic/fulvic acids	0.1	1	3		
	50 and 250 mg/L each Ca/Mg	0.1	0	0		
DW- all but New York	unconcentrated	0.1	0	3		
DW- New York	unconcentrated	0.1	3	3		
DW-California	concentrated	0.1	0	3		
DW-Florida	concentrated	0.1	3	3		
DW-New York	concentrated	0.1	0	3		
DW-Ohio	concentrated	0.1	3	3		
Lowest Detectable Concentration ^(b) (mg/L)			0.05	0.01		
False positives	There were no false positive results from interferents including a preservative blank, humic and fulvic acids, and Ca and Mg; DW from four locations using different water treatment technologies; or the potentially cross-reactive lipopolysaccharide (0.1 mg/L).					
False negatives	False negative results were obtained in the presence of both 50 and 250 mg/L Ca and Mg using both a 30 minute and 60 minute incubation time. The 30 minute incubation time also generated false negative results in unconcentrated water from California, Florida, and Ohio; and in concentrated water from California and New York. A total of 8 false negative results were obtained out of the 12 solutions assessed at 30 minutes. A total of 2 false negative results were obtained out of the 12 solutions assessed at 60 minutes. The vendor informed Battelle after testing that the lab bench scale fluorimeter provided for testing may have had inconsistent functioning which could have caused the false negative results that were obtained.					
Consistency	For the lab bench scale fluorimeter, results were consistent in 97% of the samples tested. With the field portable fluorimeter, results were consistent in 100% of the samples tested.					
Other Performance Factors	Convenient ready-to use cuvettes. Easy to operate in the lab and easy to transport and operate in the field. No formal scientific education would be required to use the kit; however, general lab skills and training on fluorimeter use were helpful. Approximately 12-15 analyses were completed in one hour in the laboratory. Only five samples could be processed in one hour in the field due to size limitation of the field portable incubator. Each Ezybot [®] kit contains 50 ready-to-use cuvettes.					

NA = Not tested. Testing concentration below detection in the contaminant only PT testing.

ND = not detectable at concentrations tested.

^(a) Results out of 3 replicates except for the 0.05 mg/L contaminant only concentration for which results are out of 10 replicates.

^(b) The lowest concentration of contaminant-only PT samples to have at least two thirds of the measurements generate positive results.

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September 2006

Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

PharmaLeads
EzyBot[®] A and EzyBot[®] B Test Kits

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Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, has financially supported and collaborated in the extramural program described here. This document has been peer reviewed by the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use.

Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development provides data and science support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

The Environmental Technology Verification (ETV) Program has been established by the EPA to verify the performance characteristics of innovative environmental technology across all media and to report this objective information to permittees, buyers, and users of the technology, thus substantially accelerating the entrance of new environmental technologies into the marketplace. Verification organizations oversee and report verification activities based on testing and quality assurance protocols developed with input from major stakeholders and customer groups associated with the technology area. ETV consists of six environmental technology centers. Information about each of these centers can be found on the Internet at <http://www.epa.gov/etv/>.

Effective verifications of monitoring technologies are needed to assess environmental quality and to supply cost and performance data to select the most appropriate technology for that assessment. Under a cooperative agreement, Battelle has received EPA funding to plan, coordinate, and conduct such verification tests for "Advanced Monitoring Systems for Air, Water, and Soil" and report the results to the community at large. Information concerning this specific environmental technology area can be found on the Internet at <http://www.epa.gov/etv/centers/center1.html>.

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List of Abbreviations

AMS	Advanced Monitoring Systems
ATEL	Aqua Tech Environmental Laboratories, Inc.
Ca	calcium
CDC	Centers for Disease Control and Prevention
COA	certificate of analysis
DI	deionized
DW	drinking water
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
L	liter
LD	lethal dose
LOD	limit of detection
MB	method blank
Mg	magnesium
mg/L	milligram per liter
mL	milliliter
mM	millimolar
PT	performance test
QA	quality assurance
QC	quality control
QMP	quality management plan
RPD	relative percent difference
TSA	technical systems audit

Chapter 1 Background

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies.

ETV works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, and permittees; and with the full participation of individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The EPA's National Exposure Research Laboratory and its verification organization partner, Battelle, operate the Advanced Monitoring Systems (AMS) Center under ETV. The AMS Center recently evaluated the performance of the PharmaLeads EzyBot[®] A and EzyBot[®] B test kits which operate on principles of an enzymatic reaction between botulinum toxin and a specific substrate. Immunoassay test kits and related technologies which determine the presence or absence of botulinum toxin were identified as a priority technology category for verification through the AMS Center stakeholder process.

Chapter 2 Technology Description

The objective of the ETV AMS Center is to verify the performance characteristics of environmental monitoring technologies for air, water, and soil. This verification report provides results for the verification testing of EzyBot[®] A and EzyBot[®] B test kits. Following is a description of the EzyBot[®] test kits, based on information provided by the vendor. The information provided below was not verified in this test.

Botulinum toxins A and B cause paralysis by destroying neuronal compounds that are necessary for muscle contraction. EzyBot[®] test kits provide a means for detecting botulinum toxins A (EzyBot[®] A) and B (EzyBot[®] B) in water. The technology is based on the PharmaLeads internal collision fluorescence quenching technology. A fluorogenic substance and a quenching substance in the substrate bracket an amino-acid sequence that, in the presence of botulinum toxin A or B, is cleaved, generating an intense fluorescence. This fluorescence is measured using either a laboratory or a field fluorimeter. Note that a laboratory fluorimeter is not provided by PharmaLeads; however, a field fluorimeter is available for purchase as part of the field case. The recorded intensity increases with time and with botulinum toxin concentration. The EzyBot[®] A test kit is shown in Figure 2-1.

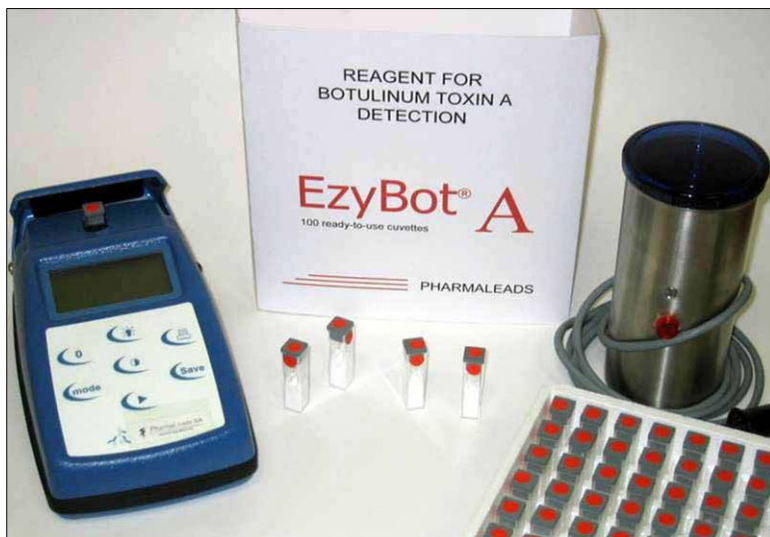


Figure 2-1. PharmaLeads EzyBot[®] A Test Kit

To perform an assay, 2 milliliters (mL) of the water sample are introduced into a cuvette containing freeze-dried reagents. The cuvette is incubated at 37°C for 15 minutes and an initial fluorescence measurement is taken as a baseline. The cuvette is then incubated at 37°C with additional fluorescence measurements taken after a total incubation time of 30 minutes and again after a total incubation time of one hour. If the difference between the baseline

measurement and the 30 minute or 60 minute measurement reaches a pre-set threshold (described in Section 3.2.1), the test is declared positive. Data can be read from the fluorimeter

display and/or transferred to a computer through a cable provided with the fluorimeter. Note that a computer is not provided by PharmaLeads.

EzyBot[®] A and B are available individually in kits containing 50 ready-to-use cuvettes containing freeze-dried reagents, which can be used in the laboratory or in the field. To perform the 1-hour incubation at 37°C in the field, PharmaLeads provides a field incubator to be plugged into the auxiliary power outlet of a car. The fluorescence level can be read on the PharmaLeads field fluorimeter. Both incubator and field fluorimeter are included in the field case.

The price of an EzyBot[®] kit depends on the quantity ordered. For large quantities, unit price is approximately \$30 per ready-to-use cuvette. Cost for the field case, including the field fluorimeter, the portable incubator, and 100 cuvettes, is less than \$12,500.

Chapter 3 Test Design

The objective of this verification test was to evaluate the ability of the EzyBot[®] test kits to detect a specific biological toxin in water samples and to determine whether the test kits are susceptible to interferents in drinking water (DW).

During this verification test, the EzyBot[®] test kits were subjected to various concentrations of botulinum toxin in American Society for Testing and Materials Type II deionized (DI) water. Note that the EzyBot[®] A test kits are specific to botulinum toxin A and were only tested with botulinum toxin A. Similarly, EzyBot[®] B test kits were only tested with botulinum toxin B. Table 3-1 shows the contaminants, the vendor-stated limit of detection (LOD), the lethal dose (LD) concentrations, and the contaminant source. . It should be recognized that there is a wide range of LD concentrations in the literature. In selecting an LD level for use in verification testing, literature oral LD50 values were reviewed and included in the test/QA plan and amendments.⁽¹⁾ In addition to reviewing the LD values in the literature, two factors were taken into consideration in selecting the final LD concentration for use in testing:

- 1) Consistency with the LD concentrations used in the first round of ETV immunoassay technology evaluations.
- 2) Applicability of the LD concentration level to the participating technologies' expected limits of detection.

In some instances this resulted in an LD level being selected that was on the high end of the literature values reported. Given the range of LD concentrations that are available in the literature, it is recommended that all readers evaluate the LD concentrations used for verification testing with respect to their particular LD requirements. The lethal dose concentration was determined using a 250 mL ingestion volume.

The EzyBot[®] test kits also were used to analyze contaminant-fortified DW samples that were collected from four water utilities that use a variety of treatment methods. The effect of interferents was evaluated by analyzing two types of interferent solutions. The first type contained both humic and fulvic acids in DI water and the second type contained magnesium (Mg) and calcium (Ca) in DI water. Both types of interferent solutions were prepared with and without the addition of the contaminants. In addition, specificity was evaluated by exposing the EzyBot[®] test kits to lipopolysaccharide, a potentially cross-reactive compound for botulinum toxin.

Table 3-1. Lethal Dose and Source of Contaminants

Contaminant	Vendor-Stated LOD	Lethal Dose Concentration^(a)	Source of Contaminant
Botulinum toxin A and B	0.01 milligrams/liter (mg/L)	0.3 mg/L ^(a)	Metabiologics, Inc. (Madison, Wisconsin)

^(a) The lethal dose of each contaminant was determined by calculating the concentration at which 250 mL of water would probably cause the death of a 154-pound person, based on human mortality data and as outlined in the Test/QA Plan for Verification of Immunoassay Test Kits Amendment Number 5. ⁽¹⁾

The verification test for the EzyBot[®] test kits was conducted from November 2005 through January 2006, according to procedures specified in the *Test/QA Plan for Verification of Immunoassay Test Kits* including amendments 1-5. ⁽¹⁾ This test was conducted at Battelle in Columbus, Ohio. Aqua Tech Environmental Laboratories, Inc. (ATEL) of Marion, Ohio, performed physicochemical characterization for each DW sample to determine the following parameters: turbidity; concentration of dissolved and total organic carbon; specific conductivity; alkalinity; concentration of Mg and Ca; pH; hardness; and concentration of total organic halides, trihalomethanes, and haloacetic acids. The EzyBot[®] test kits were evaluated for the following parameters:

- Contaminant presence/absence
- False positive/false negative response
 - Interferents
 - DW matrix effects
 - Cross-reactivity
- Consistency
- Lowest detectable concentration
- Other performance factors
 - Field portability
 - Ease of use
 - Sample throughput.

3.1 Test Samples

Tables 3-2 and 3-3 summarize the samples analyzed for each contaminant. The ability of the EzyBot[®] test kits to individually detect various concentrations of botulinum toxin was evaluated by analyzing performance test (PT) and DW samples. PT samples included DI water fortified with either the target contaminant, an interferent, both, or only a cross-reactive species. DW samples were analyzed using the EzyBot[®] test kits with and without the addition of each target contaminant. Note that the EzyBot[®] A test kit was tested with solutions containing only botulinum toxin A and that the EzyBot[®] B test kit was tested with solutions containing only botulinum toxin B.

3.1.1 Performance Test Samples

The contaminant-only and method detection limit PT samples (shown in Table 3-2) were prepared in DI water using certified standards of botulinum toxin. Reference methods were not available for quantitative confirmation of the botulinum toxin test solutions so certificates of analysis (COA) and QA oversight of solution preparation were used to confirm their concentrations.

The interferent PT samples consisted of samples of humic and fulvic acids isolated from Elliott Soil (obtained from the International Humic Substances Society) and Ca and Mg (prepared from their chlorides with concentrations based on metals only), each spiked into DI water at two concentration levels. These solutions were analyzed both with and without the target contaminant. In addition, because the commercially available botulinum toxins contained a preservative (sodium citrate), a preservative blank sample consisting of 0.025 millimolar (mM) sodium citrate was prepared in DI water. This 0.025 mM sodium citrate solution represents the concentration of the preservative that would be found in the most concentrated contaminant solution. This preservative blank was analyzed along with the contaminant solutions to ensure that the preservative would not cause false positive results during testing.

The last type of PT sample was a cross-reactivity check sample to determine whether the EzyBot[®] test kits produced false positive results in response to similar analytes.

Lipopolysaccharide is biologically similar to botulinum toxin. Solutions of lipopolysaccharide were prepared in DI water at concentrations ten times greater than the vendor-stated LOD for botulinum toxin.

Three replicates of each PT sample were analyzed except for the sample concentration five times greater than the vendor-stated LOD (0.05 mg/L) for which a total of ten replicates were analyzed. The results provided information about how well the EzyBot[®] test kits detected the presence of each contaminant at several concentration levels, the consistency of its responses, and its susceptibility to interferents.

Table 3-2. Performance Test Samples

Type of PT Sample	Sample Characteristics	Botulinum toxin A or B ^(a) Concentrations
Contaminant	Botulinum toxin A or B in DI water	0.01 to 0.5 mg/L
Interferent	Botulinum toxin A or B in 50 mg/L Ca and 50 mg/L Mg	0.1 mg/L
	Botulinum toxin A or B in 250 mg/L Ca and 250 mg/L Mg	0.1 mg/L
	Botulinum toxin A or B in 0.5 mg/L humic acid and 0.5 mg/L fulvic acid	0.1 mg/L
	Botulinum toxin A or B in 2.5 mg/L humic acid and 2.5 mg/L fulvic acids	0.1 mg/L
	Preservative Blank: 0.025 mM sodium citrate	NA
Cross-reactive species	Lipopolysaccharide (botulinum toxin analogue): 0.1 mg/L in DI water	NA

NA = not applicable

^(a) = EzyBot[®] A test kits were tested with botulinum toxin A only, EzyBot[®] B test kits were tested with botulinum toxin B only.

Table 3-3. Drinking Water Samples

Drinking Water Sample Description				Approximate Contaminant Concentrations
Water Utility	Water Treatment	Source Type	Conc. / Unconc.	Botulinum Toxin (mg/L)
Metropolitan Water District of Southern California (CA)	Filtered chloraminated	surface	both	unspiked 0.1 (Type B) 0.1 (Type A)
New York City, New York (NY)	Unfiltered chlorinated	surface	both	
Columbus, Ohio (OH)	Filtered chlorinated	surface	both	
Orlando, Florida (FL)	filtered chlorinated	ground	both	

3.1.2 Drinking Water Samples

The DW samples were collected from four geographically distributed municipal sources (Table 3-3). These samples were unique in terms of their source, treatment, and disinfection process. All collected samples were finished DW either ready for the distribution system or from within the distribution system.

Approximately 175 liters (L) of each of the DW samples were collected in pre-cleaned low-density polyethylene containers. One hundred twenty-five liters of each DW sample were shipped to the Metropolitan Water District of Southern California and dechlorinated with sodium thiosulfate. Out of this, 100 L was concentrated using ultra-filtration techniques to a final volume of 250 mL. This concentration factor was selected because it is the goal of an EPA on-site ultra-filtration sample concentration method that is being developed to increase the concentration of insoluble microbiological species in a water sample so they may be detected by available detection technologies. Concentrated water samples were included in the test/QA plan due to stakeholder interest in this technique and because the large concentration factor could affect the amount of potential interferences in various types of water compared to testing only with unconcentrated water. Twenty-five liters of each water sample was shipped to ATEL for water quality analysis. The remaining 25 L of each sample was shipped to Battelle where the sample was dechlorinated with sodium thiosulfate. Each DW sample (unconcentrated and concentrated) was analyzed without adding any contaminant, as well as after fortification with individual contaminants at a single concentration level.

3.1.3 Quality Control Samples

In addition to the PT and DW samples analyzed, method blank (MB) samples consisting of DI water were analyzed to confirm negative responses in the absence of any contaminant and to ensure that no sources of contamination were introduced during the analysis procedures. Method blanks were to be analyzed at a frequency of at least 10% of all samples. A positive control consisting of a metabolite solution provided by PharmaLeads was analyzed to ensure that the fluorimeter was operating properly. The positive control was to be analyzed at a frequency of at least 5% of all samples.

3.2 Test Procedures

3.2.1 Laboratory Testing

Each day, fresh samples were prepared from standards or stock solutions in either DI water, an interferent matrix, or a DW matrix. Each sample was prepared in its own container and labeled with a sample identification number that was recorded in a laboratory record book with details of the sample preparation. PharmaLeads provided two fluorimeters for use in this test. One was a laboratory bench scale fluorimeter (Jenway Model 6200), the second was an early model of a field portable fluorimeter under development by PharmaLeads. The EzyBot[®] kits can be used in both a “quick” screen mode where fluorescence is measured after incubating for 30 minutes or in a higher sensitivity mode where fluorescence is measured after incubating for 60 minutes. The

60 minute incubation can also be used to confirm the “quick” screen mode result. Tests were conducted using both the 30 minute and 60 minute incubation times.

To test a liquid sample for the presence of botulinum toxin using the EzyBot[®] kit and the lab bench scale fluorimeter, the following procedure was used. Two milliliters of sample were placed in an EzyBot[®] ready-to-use cuvette which contained freeze-dried reagents. The lid was replaced on the cuvette and the cuvette was shaken until the freeze-dried reagent was completely dissolved. The cuvette was incubated at 37°C for 15 minutes and then a baseline fluorescence reading was taken. The cuvette was incubated at 37°C for an additional 15 minutes and a fluorescence reading was taken, in total, 30 minutes from the time the sample and reagents were first mixed. Per instructions from PharmaLeads, if the difference between the fluorescence reading at 30 minutes and the baseline fluorescence reading was greater than 100, the result was considered positive. The cuvette was incubated for an additional 30 minutes at 37°C and the fluorescence was recorded again at 60 minutes from the time the sample and reagents were first mixed. Similarly, if the difference between the fluorescence reading at 60 minutes and the baseline fluorescence reading was greater than 100, the result was considered positive. For testing with the field portable fluorimeter, samples were prepared in the cuvettes, incubated, and fluorescence readings taken at the same intervals as described above; however, positive results were determined by subtracting the baseline fluorescence reading (at 15 minutes) from the fluorescence reading at 30 minutes and dividing that quantity by the baseline fluorescence reading. A fluorescence measurement that was 20% higher than the baseline was considered positive. The same calculation was carried out for the 60 minute fluorescence measurement; however, a 40% increase in fluorescence at the 60 minute interval was considered a positive result. Data generated using the laboratory bench scale fluorimeter were manually recorded on data sheets and calculations between the 30 or 60 minute measurement and the baseline measurement were hand calculated. Data generated with the field portable fluorimeter were collected on a laptop computer and manually transferred into an electronic spreadsheet for data calculations.

3.2.2 Non-Laboratory Testing

Because of the toxic nature of botulinum toxin, only MB samples and the vendor supplied positive control were analyzed at a non-laboratory location. Because the field portable incubator supplied by PharmaLeads was designed to operate using a car auxiliary power outlet, the non-laboratory testing took place in a parked car. Only the field portable incubator and field portable fluorimeter were tested in this setting. Because the PharmaLeads field fluorimeter did not have a finalized instruction manual at the time of testing and because PharmaLeads intends to provide a customized training session to kit purchasers, this technology was only tested by operators that had received training from PharmaLeads.

3.2.3 Drinking Water Characterization

An aliquot of each DW sample, collected as described in Section 3.1.2, was sent to ATEL to characterize the water samples based on the water quality parameters shown in Table 3-4. The table lists the methods used as well as the characterization data for the four water samples collected as part of this verification test. Water quality parameters were characterized upon sampling in June 2005, while the EzyBot[®] kits were tested with DW in November 2005. The

time delay between collection and testing was due to the fact that the water samples were collected for use during a separate ETV test conducted prior to this one. Because of this, an aliquot of each DW was tested by ATEL again in January 2006 to verify some of the parameters with the most potential to change over time. Note that dissolved organic carbon was not retested as this result was verified by the total organic carbon results, additionally the total organic halides and calcium and magnesium were not verified as there was no reason to expect a change in these parameters. The concentrations of most water quality parameters were similar; however, there was a decrease in levels of volatile compounds such as trihalomethanes and haloacetic acids over this time-period.

Table 3-4. Water Quality Characterization of Drinking Water Samples

Parameter	Method	Columbus, Ohio		Metropolitan Water District of Southern California		New York City, New York		Orlando, Florida	
		2005	2006	2005	2006	2005	2006	2005	2006
Alkalinity (mg/L)	SM 2320 B ⁽²⁾	40	44	71	97	14	12	142	125
Specific conductivity (µmho)	SM 2510 B ⁽²⁾	572	602	807	812	84	78	322	325
Hardness (mg/L)	EPA 130.2 ⁽³⁾	118	107	192	182	20	26	143	130
pH	EPA 150.1 ⁽³⁾	7.6	7.4	8.0	7.9	6.9	6.8	8.5	7.6
Total haloacetic acids (µg/L)	EPA 552.2 ⁽⁵⁾	32.8	<6.0	17.4	<6.0	39.0	<6.0	34.6	<6.0
Total organic carbon (mg/L)	SM 5310 B ⁽²⁾	2.1	2.3	2.5	2.7	1.6	4.1	1.7	2.1
Dissolved organic carbon (mg/L)	SM 5310 B ⁽²⁾	2.1	NA	2.9	NA	1.1	NA	1.6	NA
Total organic halides (µg/L)	SM 5320B ⁽²⁾	220	NA	170	NA	82	NA	300	NA
Total trihalomethanes (µg/L)	EPA 524.2 ⁽⁴⁾	74.9	16.6	39.2	24.1	39.0	23.1	56.4	41.8
Turbidity (NTU)	SM 2130 B ⁽⁷⁾	0.1	0.6	0.1	0.2	1.1	1.3	0.5	0.1
Calcium (mg/L)	EPA 200.7 ⁽⁶⁾	33	NA	45	NA	5.6	NA	8.8	NA
Magnesium (mg/L)	EPA 200.7 ⁽⁶⁾	7.7	NA	20	NA	1.3	NA	43	NA

NTU = nephelometric turbidity unit

NA = not retested

Chapter 4

Quality Assurance Quality Control

Quality assurance/quality control (QC) procedures were performed in accordance with the quality management plan (QMP) for the AMS Center⁽⁸⁾ and the test/QA plan⁽¹⁾ for this verification test.

4.1 Quality Control of Stock Solution Confirmation Methods

The COA for botulinum toxin was provided by the supplier of those contaminants. Because standard reference methods do not exist, the concentration of botulinum toxin was not independently confirmed. The COA stated that both botulinum toxin A and B standards (Metabiologics, Inc., Madison, Wisconsin) had concentrations of 1000 mg/L. Each toxin stock solution was in 50 mM sodium citrate buffer at a pH of 5.5 and had passed Metabiologics' tests for activity, identity and purity. Test samples containing these contaminants were prepared by diluting aliquots of these stock solutions. All records pertaining to stock solution dilutions were reviewed as part of the technical systems audit review. For the interferent samples, the concentration of calcium and magnesium was confirmed by EPA Method 200.7.⁽⁶⁾

4.2 Quality Control of Drinking Water Samples

A method blank sample consisting of DI water was analyzed once for approximately every 7 water samples analyzed for a frequency of approximately 15% of all samples. A positive control sample was analyzed once for approximately every 13 water samples for a frequency of approximately 8% of all samples. While performance limits were not placed on the results of the positive control sample, the vendor informed Battelle that for the lab bench scale fluorimeter (Jenway Model 6200) the positive control should read near the instrument maximum reading of 1999 and for the field portable fluorimeter the positive control should have a significantly higher fluorescence reading than the method blank to indicate that the instrument was functioning properly.

4.3 Technical Systems Audit

The Battelle Quality Manager conducted a technical systems audit (TSA) to ensure that the verification test was performed in accordance with the Test/QA Plan and amendments⁽¹⁾ and the AMS Center QMP.⁽⁸⁾ As part of the audit, the Battelle Quality Manager reviewed the standards and methods used, compared actual test procedures with those specified in the Test/QA Plan and

amendments,⁽¹⁾ and reviewed data acquisition and handling procedures. Observations and findings from this audit were documented and submitted to the Battelle Verification Test Coordinator for response. No findings were documented that required any significant action. The records concerning the TSA are permanently stored with the Battelle Quality Manager.

4.4 Audit of Data Quality

At least 10% of the data acquired during the verification test was audited. Battelle's Quality Manager or designee traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting, to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

4.5 QA/QC Reporting

Both the technical systems and data quality audits were documented in accordance with Sections 3.3.4 and 3.3.5 of the QMP for the ETV AMS Center.⁽⁸⁾ Once an assessment report was prepared for each audit, the Battelle Verification Test Coordinator responded to any findings and implemented any necessary follow-up corrective action. The Battelle Quality Manager ensured that follow-up corrective action was taken. The results of the TSA were sent to the EPA. During this test, all quality assurance findings were minor and had minimal impact on the overall test results.

4.6 Data Review

Data generated during this verification test were reviewed before they were used to calculate, evaluate, or report verification results. Table 4-1 summarizes the types of data recorded. The review was performed by a technical staff member involved in the verification test, but not the staff member who originally generated the record. The person performing the review added his/her initials and the date to a hard copy of the record being reviewed.

Table 4-1. Summary of Data Recording Process

Data to Be Recorded	Responsible Party	Where Recorded	How Often Recorded	Disposition of Data
Dates and times of test events	Battelle	ETV data sheets	Start/end of test, and at each change of a test parameter	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Sample collection and preparation information, including chain-of-custody	Battelle and Water Utilities providing DW samples	Laboratory record books and chain-of-custody forms	At time of sample collection and preparation	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Fluorimeter procedures and sample results	Battelle	ETV data sheets or captured in data acquisition system	Throughout test duration	Manually incorporated in ETV data sheets or transferred to electronic spreadsheets
Reference method procedures and sample results	ATEL	Data acquisition system, as appropriate	Throughout sample analysis process	Transferred to spreadsheets and reported to Battelle

Chapter 5

Statistical Methods and Reported Parameters

The methods presented in this chapter were used to verify the performance parameters listed in Chapter 3. The EzyBot[®] kits produce qualitative results; i.e., the kits indicate only the presence or absence of a contaminant and do not measure the concentration present. Therefore, the data evaluation methods were applied in that context.

5.1 Qualitative Contaminant Presence/Absence

Accuracy of the EzyBot[®] A Kit for botulinum toxin A and EzyBot[®] B Kit for botulinum toxin B was assessed by reporting the number of positive results out of the total number of contaminant-only PT samples tested at each concentration level. A result was considered positive based on the vendor's specifications for a positive result which are described in Section 3.2.1.

5.2 False Positive/Negative Responses

A result was considered a false positive when a DI water or DW sample was spiked with a potential interferent, a cross-reactive compound, or not spiked at all and a positive response was obtained. A result was considered a false negative when any DW or interferent sample was spiked with botulinum toxin at a concentration greater than lowest detectable concentration (as determined during DI water contaminant-only testing) and produced a negative response. Interferent PT samples, cross-reactivity PT samples, and DW samples were included in the analysis. The number of false positive and negative results is reported.

5.3 Consistency

The reproducibility of the results was assessed by calculating the percentage of individual test samples within a set (i.e., within a single concentration level or type of interferent) that produced positive or negative results without variation within replicates.

5.4 Lowest Detectable Concentration

The lowest detectable concentration for each contaminant was determined to be the concentration level at which at least two-thirds of the replicates generated positive responses. In addition, all concentrations greater than that lowest detected level were required to have a

positive responses in at least two-thirds of the replicates. These concentration levels were determined for botulinum toxin A and B in solutions of DI water.

5.5 Other Performance Factors

Aspects of the EzyBot[®] test kit performance such as ease of use, field portability, and sample throughput are discussed in Section 6. Also addressed are qualitative observations of the verification staff pertaining to the performance of the EzyBot[®] test kits.

Chapter 6 Test Results

6.1 Qualitative Contaminant Presence/Absence

The results obtained for the PT samples containing botulinum toxin are given in Tables 6-1a through 6-1d. The EzyBot[®] kits can be used in both a “quick” screen mode where fluorescence is measured after incubating for 30 minutes or in a higher sensitivity mode where fluorescence is measured after incubating for 60 minutes. The 60 minute incubation can also be used to confirm the “quick” screen mode result. During this test, results were obtained using both the 30 and 60 minute incubation times with both a lab bench scale fluorimeter (Jenway Model 6200) and an early model of a small, field portable fluorimeter. It should be noted that the quality of the fluorimeter used for analysis can affect the sensitivity of the results obtained with the EzyBot[®] kits. At the time of verification testing, the vendor provided Battelle with the Jenway Model 6200 for use in testing. Subsequently, the vendor was informed by the Jenway manufacturer that this fluorimeter model is no longer in production due to some discontinuities in its functioning. Therefore, it is important to note that use of this particular fluorimeter may have affected the lab bench scale fluorimeter results determined during verification testing. Similarly, the sensitivity of the field portable fluorimeter used for analysis will affect the sensitivity of results obtained with the EzyBot[®] kits. After the verification test, the vendor informed Battelle that the field portable fluorimeter available from PharmaLeads has undergone modification for improved sensitivity and stability since this verification testing took place and is available as the EzyBot 2[®]. Any subsequent changes to the field portable fluorimeter were not verified as part of this report. Because of the variation in EzyBot[®] test kit sensitivity that can be attributed to the type of fluorimeter used, users may want to contact the vendor for recommendations on fluorimeters to use in order to achieve optimal sensitivity with the EzyBot[®] kits.

The interpretation of results from the lab bench scale and field portable fluorimeters used for this verification test is described in Section 3.2.1, and briefly summarized as follows. Using the lab bench scale fluorimeter, the difference between the fluorescence reading at 30 or 60 minutes and the baseline fluorescence reading needed to be greater than 100 to be considered positive. For the field portable fluorimeter the 30 minute fluorescence reading needed to be 20% higher than the baseline reading and the 60 minute reading needed to be 40% higher than the baseline reading to be considered positive.

As shown in Table 6-1a, with the lab bench scale fluorimeter and the 30 minute incubation time, EzyBot[®] A generated positive results to botulinum toxin A in all replicates at the lethal dose

concentration (0.3 mg/L) and higher. Note that while the 30 minute results for the 0.1 mg/L concentration (10 x LOD) had consistently elevated responses, these responses did not meet the criteria for a positive result. With the lab bench scale fluorimeter and the 60 minute incubation time, EzyBot[®] A generated positive results in all replicates at concentrations five times the vendor-stated LOD (0.05 mg/L) and higher. Table 6-1b shows EzyBot[®] A results using the field portable fluorimeter. With this fluorimeter and the 30 minute incubation, only one of three replicates generated a positive response at the highest concentration tested (0.5 mg/L). With the 60 minute incubation time and the field portable fluorimeter, EzyBot[®] A generated positive results in all replicates at the lethal dose concentration (0.3 mg/L) and higher.

Table 6-1c presents results using EzyBot[®] B and the lab bench scale fluorimeter. With this fluorimeter and the 30 minute incubation time, EzyBot[®] B generated positive results to botulinum toxin B in all replicates at ten times the vendor-stated LOD (0.1 mg/L) and higher and in seven of ten replicates at five times the vendor-stated LOD (0.05 mg/L). A total of ten replicates were analyzed at this concentration level because three replicates were contaminant-only PT samples and seven were included in the test/QA plan as a method detection limit study. Because of the qualitative nature of the EzyBot[®] test kits, the results of all ten analyses are reported as additional contaminant-only PT replicates because a method detection limit cannot be calculated for a technology that reports a presence/absence result. With the lab bench scale fluorimeter and the 60 minute incubation time, EzyBot[®] B generated positive results in all replicates at the vendor-stated LOD (0.01 mg/L) and higher. Table 6-1d shows EzyBot[®] B results using the field portable fluorimeter. With this fluorimeter and the 30 minute incubation, positive results were generated only for the three replicates at the lethal dose concentration (0.3 mg/L). Why the highest concentration (50 times the vendor-stated LOD, 0.5 mg/L) was not detected with the field portable fluorimeter when the 0.3 mg/L concentration was detected is uncertain. With the 60 minute incubation time and the field portable fluorimeter, EzyBot[®] B generated positive results to all replicates at the lethal dose level (0.3 mg/L) and higher.

Table 6-1a. Botulinum Toxin A Contaminant-Only PT Sample Results Using the Lab Bench Scale Fluorimeter-Contaminant Presence/Absence Evaluation

Analyte	Concentration (mg/L)	Testing Level	30 Minute Results ^(a)	No. of Positives (30 min.)	60 Minute Results ^(a)	No. of Positives (60 min.)
Botulinum Toxin A	0.01	LOD	9	0	-7	0
			-29		-18	
			3		30	
	0.05	5 x LOD	61	0	466	10
			-18		172	
			1		261	
			6		941	
			0		758	
			3		742	
			-1		783	
			4		754	
			3		747	
	0.1	10 x LOD	55	0	508	3
			61		466	
			60		408	
	0.3	LD	210	3	>1374	3
			157		>1384	
			198		>1356	
	0.5	50 x LOD	250	3	>1342	3
			262		>1374	
			247		>1321	

LOD = vendor-stated limit of detection.

LD = lethal dose

> indicates that the 60 minute fluorescence reading exceeded the fluorimeter maximum.

^(a) Difference between fluorescence at 30 or 60 minutes and baseline fluorescence at 15 minutes. Result is considered positive if the difference is greater than 100.

Shaded areas highlight positive results.

Table 6-1b. Botulinum Toxin A Contaminant-Only PT Sample Results Using the Field Portable Fluorimeter-Contaminant Presence/Absence Evaluation

Analyte	Concentration (mg/L)	Testing Level	30 Minute Results ^(a)	No. of Positives (30 min.)	60 Minute Results ^(a)	No. of Positives (60 min.)
Botulinum Toxin A	0.01	LOD	16	0	21.1	0
			8.7		2.5	
			2.8		-5.1	
	0.05	5 x LOD	5.3	0	15.6	0
			-4.5		0.9	
			3.6		28.4	
			-3.4		-0.1	
			-1.2		1.9	
			-3.9		-1.6	
			-1.6		-0.6	
			0.9		2.8	
			-3.1		0.1	
	0.1	10 x LOD	8.2	0	9.5	0
			-19.2		-6.4	
			10.3		14.5	
	0.3	LD	9.5	0	67.1	3
			5.0		52.6	
			8.4		65.7	
	0.5	50 x LOD	9.5	1	71.3	3
			15.8		86.0	
			20.5		93.8	

LOD = vendor-stated limit of detection.

LD = lethal dose concentration.

^(a) ((Fluorescence at 30 or 60 minutes – baseline fluorescence at 15 minutes) x 100)/(baseline fluorescence at 15 minutes). Result is considered positive if >20% for 30 minute measurements and >40% for 60 minute measurements. Shaded areas highlight positive results.

Table 6-1c. Botulinum Toxin B Contaminant-Only PT Sample Results Using the Lab Bench Scale Fluorimeter-Contaminant Presence/Absence Evaluation

Analyte	Concentration (mg/L)	Testing Level	30 Minute Results ^(a)	No. of Positives (30 min.)	60 Minute Results ^(a)	No. of Positives (60 min.)
Botulinum Toxin B	0.01	LOD	11	0	172	3
			16		196	
			9		188	
	0.05	5 x LOD	84	7	678	10
			67		563	
			85		731	
			190		1462	
			139		1013	
			127		1041	
			133		1076	
			154		1113	
			121		992	
			131		1237	
	0.1	10 x LOD	253	3	>1655	3
			188		1403	
			160		1150	
	0.3	LD	1369	3	>1393	3
			1070		>1429	
			1046		>1425	
	0.5	50 x LOD	1230	3	>1430	3
			938		>1442	
1075			>1498			

LOD = vendor-stated limit of detection.

LD = lethal dose concentration.

> indicates that the 60 minute fluorescence reading exceeded the fluorimeter maximum.

^(a) Difference between fluorescence at 30 or 60 minutes and baseline fluorescence at 15 minutes. Result is considered positive if the difference is greater than 100. Shaded areas highlight positive results.

Table 6-1d. Botulinum Toxin B Contaminant-Only PT Sample Results Using the Field Portable Fluorimeter-Contaminant Presence/Absence Evaluation

Analyte	Concentration (mg/L)	Testing Level	30 Minute Results ^(a)	No. of Positives (30 min.)	60 Minute Results ^(a)	No. of Positives (60 min.)
Botulinum Toxin B	0.01	LOD	-14.6	0	-25.9	0
			-17.9		-22.8	
			-20.5		-26.6	
	0.05	5 x LOD	-9.0	0	-9.9	0
			-10.9		-10.3	
			0.1		-0.2	
			-13.3		14.7	
			-10.0		-4.7	
			-17.0		-5.0	
			-9.6		16.3	
			-14.1		-4.2	
			16.6		9.5	
	0.1	10 x LOD	-8.9	0	38.4	0
			-14.1		-2.4	
			-4.3		-1.2	
	0.3	LD	26.7	3	209.3	3
			32.9		200.2	
			23.4		160.5	
	0.5	50 x LOD	8.2	0	160.3	3
			14.1		154.9	
			9.7		121.6	

LOD = vendor-stated limit of detection.

LD= lethal dose concentration.

^(a) ((Fluorescence at 30 or 60 minutes – baseline fluorescence at 15 minutes) x 100)/(baseline fluorescence at 15 minutes). Result is considered positive if >20% for 30 minute measurements and >40% for 60 minute measurements. Shaded areas highlight positive results.

6.2 False Positive/Negative Responses

Three types of samples were analyzed to evaluate the susceptibility of the EzyBot[®] kits to false positive and negative results. These included interferent PT samples, made up of DI water fortified with Ca and Mg or with humic and fulvic acids, both with and without the addition of target contaminants; cross-reactivity PT samples made up of DI water fortified with a contaminant biologically similar to botulinum toxin; and DW samples both concentrated and unconcentrated and both with and without the addition of botulinum toxin. In addition, a preservative blank containing sodium citrate, which is used as a preservative in commercially available botulinum toxin, was analyzed to evaluate the potential for false positive results from the preservative. A false positive result was defined as a positive result in the absence of botulinum toxin and a false negative result was defined as a negative result from a sample containing botulinum toxin at ten times the vendor-stated LOD, if that concentration level was detectable in the PT contaminant-only testing. Note that only the lab bench scale fluorimeter was used for the false positive and false negative assessments due to lack of sensitivity of the field portable fluorimeter to the botulinum toxin concentrations used for assessing false negative results.

6.2.1 Interferent PT Samples

The results from the interferent PT samples are given in Table 6-2. Neither EzyBot[®] A nor EzyBot[®] B had false positive results from the PT samples containing possible interferences, but no contaminant. Based on the EzyBot[®] A contaminant-only PT testing described in Section 6.1, the 0.1 mg/L botulinum toxin A solutions were detectable for 60 minute incubation/lab bench scale fluorimeter readings, while the 30 minute incubation time was below the detection limit. Therefore for EzyBot[®] A, only the 60 minute incubation/lab bench scale fluorimeter results were assessed for false negatives in the intereferent testing. While the 0.1 mg/L botulinum toxin A solution was detected in the presence of calcium and magnesium, false negative results were obtained in the presence of both 0.5 mg/L each and 2.5 mg/L each humic and fulvic acids.

As described in Section 6.1, the 0.1 mg/L botulinum toxin B solutions were detectable with EzyBot[®] B for both the 30 and 60 minute incubation times with the lab bench scale fluorimeter. Therefore for interferent testing, both the 30 and 60 minute incubation/lab bench scale fluorimeter results were assessed for false negatives. False negative results were obtained for two of three replicates of 0.1 mg/L botulinum toxin B in the presence of 5 mg/L humic and fulvic acids with the 30 minute incubation time. False negative results were also obtained in the presence of both concentrations of calcium and magnesium at both the 30 and 60 minute incubation times. There were no false negative results for 0.5 mg/L each humic and fulvic acids during the 30 or 60 minute incubation and no false negative results for 2.5 mg/L each humic and fulvic acids during the 60 minute incubation.

The false negative results obtained while analyzing interferent PT samples using the 60 minute incubation time with both the EzyBot[®] A and EzyBot[®] B kits were unexpected given the performance of the EzyBot[®] kits while testing drinking water samples which are presented in Section 6.2.2. Because similar concentrations of total organic carbon, calcium, and magnesium were in the various drinking waters (Table 3-4) as were added to interferent PT samples, similar

results were expected. No definitive reason could be found which would explain this unexpected performance. Possible reasons are that the sources of interferents added to DI water to create the interferent PT samples are not identical to the interferents in the drinking waters and that matrix effects from the various drinking waters enhance the detection capability of the EzyBot[®] kits; however, these possible reasons for differences in performance were not further evaluated during verification testing. Additionally, the vendor informed Battelle after testing that the lab bench scale fluorimeter provided for testing may have had inconsistent functioning which could have affected the results obtained.

6.2.2 DW Samples

The results from the DW samples are given in Tables 6-3a for EzyBot[®] A and 6-3b for EzyBot[®] B. For both EzyBot[®] A and EzyBot[®] B there were no false positive results generated by DW samples without contaminant. EzyBot[®] A was evaluated with DW fortified with 0.1 mg/L botulinum toxin A using only the 60 minute incubation time because no positive results were obtained with the 30 minute incubation when analyzing the contaminant-only PT samples at the 0.1 mg/L concentration. With the 60 minute incubation, positive results were generated for 0.1 mg/L botulinum toxin A in all geographic types of water except the concentrated water from New York, for which a false negative result was obtained. The reason for the false negative result for concentrated New York water is not clear; however, the New York water is from the only source that was not filtered and had the highest turbidity reading; therefore, the high particle content of the New York water may have interfered with EzyBot[®] A. Additionally, as mentioned previously, the vendor informed Battelle after testing that the lab bench scale fluorimeter provided for testing may have had inconsistent functioning which could have affected the results obtained.

EzyBot[®] B results are reported for both the 30 minute and 60 minute incubation times. With the 60 minute incubation, there were no false negative results as botulinum toxin B (0.1 mg/L) was detected in all geographic types of water, both unconcentrated and concentrated. The 30 minute incubation results were not easily interpreted. All of the unconcentrated drinking water samples, except for New York, generated false negative results, while two out of four concentrated water samples (New York and California) generated false negative results.

Table 6-2. Botulinum Toxin Contaminant-Interferent Testing –False Positive/Negative Evaluation

	Botulinum Toxin Conc.	Interferent	Lab Bench Scale Fluorimeter			
			30 Minute Results ^(a)	No. of Positives (30 min.)	60 Minute Results ^(a)	No. of Positives (60 min.)
EzyBot [®] A and EzyBot [®] B	None	0.025 mM sodium citrate	Individual results not listed	0	Individual results not listed	0
		0.5 mg/L each humic/fulvic acids		0		0
		2.5 mg/L each humic/fulvic acids		0		0
		50 mg/L each Ca/Mg		0		0
		250 mg/L each Ca/Mg		0		0
EzyBot [®] A	0.1 mg/L Type A	0.5 mg/L each humic/fulvic acids	NA	NA	26	0
					28	
					62	
		2.5 mg/L each humic/fulvic acids	NA	NA	-18	0
					-103	
					-86	
		50 mg/L each Ca/Mg	NA	NA	301	3
					283	
					238	
		250 mg/L each Ca/Mg	NA	NA	109	3
					138	
					203	
EzyBot [®] B	0.1 mg/L Type B	0.5 mg/L each humic/fulvic acids	198	3	>1708	3
					277	
					286	
		2.5 mg/L each humic/fulvic acids	93	1	793	3
					105	
					83	
		50 mg/L each Ca/Mg	0	0	42	0
					1	
					2	
		250 mg/L each Ca/Mg	-4	0	-2	0
					-23	
					-3	

NA = EzyBot[®] A was not sensitive to the 0.1 mg/L contaminant concentration after 30 minutes in the contaminant-only PT testing and was therefore not included in the interferent testing.

> indicates that the 60 minute fluorescence reading exceeded the fluorimeter maximum.

^(a) Difference between fluorescence at 30 or 60 minutes and baseline fluorescence at 15 minutes. Result is considered positive if the difference is greater than 100. Shaded areas highlight positive results.

Table 6-3a. DW Sample Results Using EzyBot[®] A - False Positive/Negative Evaluation

Botulinum Toxin Conc.	DW	Lab Bench Scale Fluorimeter	
		60 Minute Results ^(a)	No. of Positives (60 min.)
None	California unconcentrated and concentrated	Individual results not listed	0
	Florida unconcentrated and concentrated		0
	New York unconcentrated and concentrated		0
	Ohio unconcentrated and concentrated		0
0.1 mg/L Type A	California unconcentrated	476	3
		465	
		535	
	California concentrated	450	3
		216	
		215	
	Florida unconcentrated	581	3
		294	
		476	
	Florida concentrated	221	3
		317	
		421	
	New York unconcentrated	450	3
		430	
		438	
	New York concentrated	-12	0
		-30	
		-50	
	Ohio unconcentrated	359	3
		445	
		544	
	Ohio concentrated	241	3
		471	
		344	

Note that EzyBot[®] A was not sensitive to the 0.1 mg/L contaminant concentration after 30 minutes in the contaminant-only PT testing. Therefore the 30 minute incubation was not included in the interferent testing.
^(a) Difference between fluorescence at 60 minutes and baseline fluorescence at 15 minutes. Result is considered positive if the difference is greater than 100. Shaded areas highlight positive results.

Table 6-3b. DW Sample Results Using EzyBot® B - False Positive/Negative Evaluation

Botulinum Toxin Conc.	DW	Lab Bench Scale Fluorimeter			
		30 Minute Results ^(a)	No. of Positives (30 min.)	60 Minute Results ^(a)	No. of Positives (60 min.)
None	California unconcentrated and concentrated	Individual results not listed	0	Individual results not listed	0
	Florida unconcentrated and concentrated		0		0
	New York unconcentrated and concentrated		0		0
	Ohio unconcentrated and concentrated		0		0
0.1 mg/L Type B	California unconcentrated	19	0	121	3
		30		155	
		41		239	
	California concentrated	99	0	435	3
		60		352	
		77		360	
	Florida unconcentrated	25	0	163	3
		18		144	
		22		133	
	Florida concentrated	199	3	746	3
		222		762	
		215		714	
	New York unconcentrated	296	3	>1636	3
		135		900	
		176		1236	
	New York concentrated	29	0	115	3
		28		110	
		23		115	
	Ohio unconcentrated	46	0	241	3
		32		212	
		28		141	
	Ohio concentrated	488	3	>1434	3
		436		>1238	
		476		>1417	

^(a) Difference between fluorescence at 30 or 60 minutes and baseline fluorescence at 15 minutes. Result is considered positive if the difference is greater than 100. Shaded areas highlight positive results. > indicates that the 60 minute fluorescence reading exceeded the fluorimeter maximum.

6.2.3 Cross-Reactivity PT Samples

DI water fortified with a chemical similar to the target contaminant was analyzed in the absence of the target contaminant. Lipopolysaccharide at a concentration of 0.1 mg/L (equivalent to ten times the vendor-stated LOD for the target contaminant) was used as the cross-reactivity analyte for botulinum toxin and was analyzed in triplicate with each EzyBot[®] kit. Neither EzyBot[®] A nor EzyBot[®] B had any positive response to the lipopolysaccharide using either the lab bench scale fluorimeter or the field portable fluorimeter with both 30 and 60 minute incubation times.

6.3 Consistency

Using the lab bench scale fluorimeter, EzyBot[®] A results were consistent between replicates in 100% of the samples tested. With the field portable fluorimeter, EzyBot[®] A results were consistent between replicates in four out of the five sets of samples analyzed (90%). The 30 minute reading of 0.5 mg/L botulinum toxin A had a positive response in only one of three replicates with the field portable fluorimeter.

For EzyBot[®] B, using the lab bench scale fluorimeter, results were consistent between replicates for all but 0.1 mg/L botulinum toxin B in the presence of 2.5 mg/L each of humic and fulvic acids (for which only one of three replicates had a positive response) and 0.05 mg/L botulinum toxin B (for which seven out of ten replicates had a positive response). This resulted in a consistent response in 58 of the 60 sample sets analyzed (97%). Using the field portable fluorimeter, EzyBot[®] B results were consistent between replicates in 100% of the samples tested.

6.4 Lowest Detectable Concentration

The lowest detectable concentration of each target contaminant was defined as the lowest concentration of contaminant-only PT sample to have at least two thirds of the measurements generate positive results. In addition, all concentrations greater than the lowest detectable concentration were required to have at least two thirds of the measurements generate positive results. The concentrations in Table 6-4 summarize the results presented in Table 6-1a through 6-1d. Note that while the field portable fluorimeter results for the 30 minute incubation with the EzyBot[®] B kit had 3 positive detects of the 0.3 mg/L botulinum toxin B solution, because there were no detects of the 0.5 mg/L solution, no detectable concentration is reported. The highest sensitivity results for the both the EzyBot[®] A and EzyBot[®] B kits were obtained using the 60 minute incubation time and the lab bench scale fluorimeter resulting in detection of 0.05 mg/L botulinum toxin A with EzyBot[®] A and 0.01 mg/L botulinum toxin B with EzyBot[®] B.

Table 6-4. Lowest Detectable Concentrations

	Lab Bench Scale Fluorimeter		Field Portable Fluorimeter	
	30 minutes	60 minutes	30 minutes	60 minutes
EzyBot [®] A	0.3 mg/L	0.05 mg/L	ND	0.3 mg/L
EzyBot [®] B	0.05 mg/L	0.01 mg/L	ND	0.3 mg/L

ND = Not detectable at concentrations tested.

6.5 Other Performance Factors

6.5.1 Ease of Use

Both kits contained clearly written and informative instructions for use with the lab bench scale fluorimeter. At the time of testing, instructions for the field portable fluorimeter were still being finalized so testing staff relied on the training provided by PharmaLeads. Contents of the kit were clearly labeled. Storage requirements were marked on the outer container and in the instruction manual. Overall, all packaging was easy to open. Ready-to-use cuvettes required no reagent preparation. The sample was added to the cuvettes, mixed and incubated at 37°C. Readings were taken at 15, 30, and 60 minutes. Prior to use, cuvettes were required to be stored at 4° C. Expiration dates were clearly printed on the outer container.

All equipment was supplied with the kit except for pipettes with tips needed to dispense 2 mL of sample into the cuvettes, the incubator, and a fluorimeter. Laboratory scale fluorimeters and incubators are available commercially. A field portable incubator and fluorimeter are available from PharmaLeads in a field case. The field case also contains pipettes necessary for dispensing sample into the cuvettes. A laptop computer (not included with the kit or field case) was needed to acquire data using the field portable fluorimeter. The lab bench scale fluorimeter used for this test required manually recording a 3-4 digit fluorescence measurement. The field portable fluorimeter acquired multiple readings of a 6-7 digit fluorescence measurement on a laptop computer. These readings were averaged and transferred into a spreadsheet for further calculations. With the lab bench scale fluorimeter, because relatively few digits were involved in each measurement and because a positive response only involved assessing whether the 30 or 60 minute fluorescence measurement differed by 100 units from the baseline 15 minute measurement, a positive result was easily determined. With the field portable fluorimeter, a positive or negative result required more extensive data manipulation (determining the percentage change in fluorescence between the baseline measurement and the 30 or 60 minute measurement) with each measurement involving more digits. The vendor states that since the verification testing took place, the field portable fluorimeter has been revised so that its results are determined by measuring a difference of 100 units, similar to the measurement with the lab bench scale fluorimeter and now also includes a warning sound emitted when a positive result is obtained. The revised field portable fluorimeter (EzyBot[®] 2) was not evaluated during this ETV test. The lab bench scale fluorimeter required calibration prior to use using a standard provided in the EzyBot[®] kit. The field portable fluorimeter had fluorimeter and software settings that

needed to be set prior to use, but did not require calibration. The surfaces of both fluorimeters were easily wiped clean.

No formal scientific education would be required for using the kits, but general good laboratory skills are needed. Verification testing staff were able to conduct tests with the kit after a training session which lasted several hours. In particular the training related to operating the fluorimeters was helpful. The vendor's address and website are included in the instruction manual providing easy access to the vendor's contact information. One cuvette per sample and pipette tips were generated as solid waste. It was not stated in the kit or product literature whether the ready-to-use cuvettes should be considered hazardous waste due to any of the reagents contained within.

6.5.2 Field Portability

Because the field portable incubator supplied by PharmaLeads was designed to operate using a car auxiliary power outlet and because the fluorimeter and laptop computer were battery powered allowing easy portability, the non-laboratory testing took place in a parked car. The incubator only needed to be plugged into the car auxiliary outlet, the car did not need to be turned on and running in order for the incubator to work. Only the field portable incubator and field portable fluorimeter were tested in this setting (and not the laboratory bench scale fluorimeter). The technology was tested with a method blank and the vendor-provided positive control. The fluorimeter, incubator, and necessary supplies were transported in several small boxes, and the laptop for use with the fluorimeter was carried in its case. Two people easily transported all equipment. Once at the field testing location, the equipment was set up and the incubator was warmed to temperature within five minutes. The small size of all the equipment allowed all analyses for this test to be carried out in the back seat of a car (see Figures 6-1 and 6-2). The field portable incubator only holds 5 cuvettes which limits the number of samples that can be processed during the 30 minute or 60 minute incubation time. The vendor stated that the reagent in the cuvettes is stable for 24 hours at 25°C. Therefore, within that timeframe, field deployment could be carried out without concern for reagent storage. Longer-term deployment would require a means of keeping the cuvettes at 4°C. The following items in addition to the kit, incubator, computer, and fluorimeter were needed for field use: a timer, pipettes and tips, and a waste container. Note that with purchase of the PharmaLeads field case, the field portable incubator and fluorimeter, as well as pipettes are included. As mentioned in Section 6.5.1, the field portable fluorimeter generates raw data which needs to be taken through calculations in order to assess whether the result was positive. Such an assessment could not be made by looking at the raw data alone and required additional time to arrive at a result beyond the field analysis time. The vendor states that since the verification testing took place, the field portable fluorimeter has been revised so that its results are determined by measuring a difference of 100 units, similar to the measurement with the lab bench scale fluorimeter and now also includes a warning sound emitted when a positive result is obtained. The revised field portable fluorimeter (EzyBot[®] 2) was not evaluated during this ETV test.

6.5.3 Throughput

Approximately 12-15 sample analyses plus method blanks and controls were completed in one hour in the laboratory where a larger incubator was available. Note that the field portable incubator only held 5 cuvettes so unless multiple portable incubators or a different type of incubator were used only 5 samples could be processed in one hour in the field. Each EzyBot[®] kit contains 50 ready-to-use cuvettes.

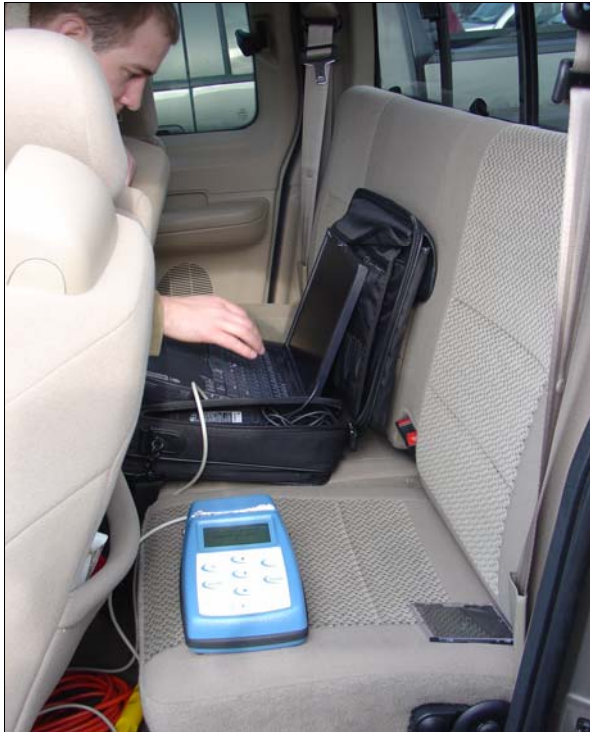


Figure 6-1. Field Use of the PharmaLeads Fluorimeter



Figure 6-2. PharmaLeads' Portable Incubator Operates Using a Car Auxiliary Power Outlet and Fits into a Cup Holder

Chapter 7 Performance Summary

Table 7-1. EzyBot[®] A Summary Table

Parameter	Sample Information	Botulinum Toxin A (mg/L)	Lab Bench Scale Fluorimeter ^(a)		Field Portable Fluorimeter ^(a)	
			30 min.	60 min.	30 min.	60 min.
Contaminant-only	DI water	0.01 (vendor-stated limit of detection)	0	0	0	0
		0.05	0	10	0	0
		0.1	0	3	0	0
		0.3 (lethal dose)	3	3	0	3
		0.5	3	3	1	3
Interferent	0.5 and 2.5 mg/L each humic/fulvic acids	0.1	NA	0	NA	NA
	50 and 250 mg/L each Ca/Mg	0.1		3		
DW-all locations	unconcentrated	0.1		3		
DW-California	concentrated	0.1		3		
DW-Florida	concentrated	0.1		3		
DW-New York	concentrated	0.1		0		
DW-Ohio	concentrated	0.1		3		
Lowest Detectable Concentration ^(b) (mg/L)			0.3	0.05	ND	0.3

NA = Not tested. Testing concentration below detection in the contaminant only PT testing.

ND = not detectable at concentrations tested.

^(a) Results out of 3 replicates except for the 0.05 mg/L contaminant only concentration for which results are out of 10 replicates.

^(b) The lowest concentration of contaminant-only PT samples to have at least two thirds of the measurements generate positive results

Table 7-1. EzyBot[®] A Summary Table (Continued)

False positives	There were no false positive results from interferents including a preservative blank, humic and fulvic acids, and Ca and Mg; DW from four locations using different water treatment technologies; or the potentially cross-reactive lipopolysaccharide (0.1 mg/L).
False negatives	False negatives were obtained in the presence of both 0.5 and 2.5 mg/L each humic and fulvic acids. A false negative was also obtained in New York water which was concentrated by a factor of 400. A total of 3 false negative results were obtained out of the 12 solutions assessed at 60 minutes. The vendor informed Battelle after testing that the lab bench scale fluorimeter provided for testing may have had inconsistent functioning which could have caused the false negative results that were obtained.
Consistency	Using the lab bench scale fluorimeter, results were consistent in 100% of the samples tested. Using the field portable fluorimeter, results were consistent in 90% of the samples tested.
Other Performance Factors	Convenient ready-to use cuvettes. Easy to operate in the lab and easy to transport and operate in the field. No formal scientific education would be required to use the kit; however, general lab skills and training on fluorimeter use were helpful. Approximately 12-15 analyses were completed in one hour in the laboratory. Only five samples could be processed in one hour in the field due to size limitation of the field portable incubator. Each EzyBot [®] kit contains 50 ready-to-use cuvettes.

Table 7-2. EzyBot[®] B Summary Table

Parameter	Sample Information	Botulinum Toxin B (mg/L)	Lab Bench Scale Fluorimeter ^(a)		Field Portable Fluorimeter ^(a)	
			30 min.	60 min.	30 min.	60 min.
Contaminant-only	DI Water	0.01 (vendor-stated limit of detection)	0	3	0	0
		0.05	7	10	0	0
		0.1	3	3	0	0
		0.3 (lethal dose)	3	3	3	3
		0.5	3	3	0	3
Interferent	0.5 mg/L each humic/fulvic acids	0.1	3	3	NA	
	2.5 mg/L each humic/fulvic acids	0.1	1	3		
	50 and 250 mg/L each Ca/Mg	0.1	0	0		
DW- all but New York	unconcentrated	0.1	0	3		
DW- New York	unconcentrated	0.1	3	3		
DW-California	concentrated	0.1	0	3		
DW-Florida	concentrated	0.1	3	3		
DW-New York	concentrated	0.1	0	3		
DW-Ohio	concentrated	0.1	3	3		
Lowest Detectable Concentration ^(b) (mg/L)			0.05	0.01		ND
False positives	There were no false positive results from interferents including a preservative blank, humic and fulvic acids, and Ca and Mg; DW from four locations using different water treatment technologies; or the potentially cross-reactive lipopolysaccharide (0.1 mg/L).					
False negatives	False negative results were obtained in the presence of both 50 and 250 mg/L Ca and Mg using both a 30 minute and 60 minute incubation time. The 30 minute incubation time also generated false negative results in unconcentrated water from California, Florida, and Ohio; and in concentrated water from California and New York. A total of 8 false negative results were obtained out of the 12 solutions assessed at 30 minutes. A total of 2 false negative results were obtained out of the 12 solutions assessed at 60 minutes. The vendor informed Battelle after testing that the lab bench scale fluorimeter provided for testing may have had inconsistent functioning which could have caused the false negative results that were obtained.					
Consistency	For the lab bench scale fluorimeter, results were consistent in 97% of the samples tested. With the field portable fluorimeter, results were consistent in 100% of the samples tested.					
Other Performance Factors	Convenient ready-to use cuvettes. Easy to operate in the lab and easy to transport and operate in the field. No formal scientific education would be required to use the kit; however, general lab skills and training on fluorimeter use were helpful. Approximately 12-15 analyses were completed in one hour in the laboratory. Only five samples could be processed in one hour in the field due to size limitation of the field portable incubator. Each EzyBot [®] kit contains 50 ready-to-use cuvettes.					

NA = Not tested. Testing concentration below detection in the contaminant only PT testing.

ND = not detectable at concentrations tested.

^(a) Results out of 3 replicates except for the 0.05 mg/L contaminant only concentration for which results are out of 10 replicates.

^(b) The lowest concentration of contaminant-only PT samples to have at least two thirds of the measurements generate positive results.

Chapter 8 References

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