**Proposal Subject:** Reveal ASP (Domoic Acid) Test Kit

**Specific NSSP Guide Reference:** 2009 NSSP Section IV Guidance Documents Chapter II. Growing Areas .10 Approved NSSP Laboratory Tests, Table 4 - Type III and Type IV Marine Biotoxin Test Methods

**Text of Proposal/ Requested Action** We request review of the validation study submission for the Reveal ASP (domoic acid) test kit and consideration of the method for approval as a Type IV marine biotoxin screening method for qualitative determination of domoic acid in shellfish. Add Reveal ASP (domoic acid) test to list of approved Type III and Type IV marine biotoxin methods.

**Public Health Significance:** Amnesic shellfish poisoning is caused by the toxin domoic acid, produced by phytoplankton of the genus Pseudonitzschia. It is associated with eating contaminated oysters, clams, mussels, and other shellfish. There have been numerous outbreaks of ASP, and there is evidence that the occurrence of the phytoplankton responsible for ASP is widespread. Current methods for detection of domoic acid consist primarily of instrumental chemistry methods, which are laborious and time-consuming. Methods for rapid screening for domoic acid, in field and laboratory settings, are needed and will assist the industry and public health authorities in responding to this health concern. The Reveal ASP test is a lateral flow immunoassay designed for qualitative determination of domoic acid in shellfish at levels of 10 ppm (mg/kg) and above. The test uses minimal equipment and simple reagents, does not require specialized training, and can provide results in 20 minutes from sample receipt, including sample preparation.

**Cost Information (if available):** Approximately $17.00 per test.

**Action by 2011 Laboratory Methods Review Committee** Recommended referral of Proposal 11-107 to the appropriate committee as determined by the Conference Chairman and further recommended the following guidance on the data needed from the submitter:

- Analysis of samples with naturally incurred residues over a range of toxin concentrations.
- Evaluate extraction recovery by comparison with HPLC.
- Additional replicates of spiked samples of shellfish species.

Eliminate theoretical data regarding dose response curve.

**Action by 2011 Task Force I** Recommended adoption of Laboratory Methods Review Committee recommendations on Proposal 11-107.

**Action by 2011 General Assembly** Adopted recommendation of 2011 Task Force I on Proposal 11-107.

**Action by FDA February 26, 2012** Concurred with Conference action on Proposal 11-107.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

| | |
|---|---|
| **Name of the New Method** | Reveal ASP (Domoic Acid) |
| **Name of the Method Developer** | Neogen Corporation |
| **Developer Contact Information** | Mark Mozola, 517-372-9200, mmozola@neogen.com |

| Checklist | Y/N | Submitter Comments |
|---|---|---|
| **A. Need for the New Method** | | |
| 1. Clearly define the need for which the method has been developed. | | There is a need for a simple, rapid screening method for domoic acid in shellfish, one that can be used in the field as well as in a laboratory setting. |
| 2. What is the intended purpose of the method? | | The method is designed for rapid qualitative screening of shellfish for domoic acid. |
| 3. Is there an acknowledged need for this method in the NSSP? | | Simply assays that provide rapid results are needed. |
| 4. What type of method? i.e. chemical, molecular, culture, etc. | | Lateral flow immunoassay in dipstick format. |
| **B. Method Documentation** | | |
| 1. Method documentation includes the following information: | | |
| Method Title | | Reveal ASP (Domoic Acid) |
| Method Scope | | Qualitative detection of domoic acid in oysters, clams, and mussels. |
| References | | Study report and kit insert included in this submission. |
| Principle | | Competitive lateral flow immunoassay in dipstick format. Water extraction of analyte from homogenized shellfish tissue. |
| Any Proprietary Aspects | | Yes, commercial test kit. |
| Equipment Required | | Extraction containers with lids (40 mL capacity), timer, bag roller, sample cup rack, pipettes (0.1 mL), result interpretation card. |
| Reagents Required | | Reveal ASP test devices, extraction bags with mesh filter, sample cups, distilled water. |
| Sample Collection, Preservation and Storage Requirements | | Shellfish should be collected according to standard industry practices and stored at 2-8$^{o}$C before testing. |
| Safety Requirements | | Used test devices, extraction bags, sample cups, and pipettes should be treated as if contaminated with domoic acid and handled accordingly. Gloves and lab coats should be worn while performing the test. |
| Clear and Easy to Follow Step-by-Step Procedure | | Step-by-step procedure in kit insert and study report. |
| Quality Control Steps Specific for this Method | | Test device contains an internal positive control that confirms that it is functioning properly. A domoic acid solution in buffer at a concentration $\geq$ 10 mg/kg can be used as an external positive control if desired. |

| | | |
|---|---|---|
| **C. Validation Criteria** | | |
| 1. Accuracy / Trueness | | 95.9% overall for oysters, clams, and mussels |
| 2. Measurement Uncertainty | | Not applicable. |
| 3. Precision Characteristics (repeatability and reproducibility) | | Not applicable. |
| 4. Recovery | | Not applicable. |
| 5. Specificity | | 100%. No impact on test results by potentially interfering compounds – okadaic acid, glutamic acid, glutamine, saxitoxin. No false-positive results on unpiked samples. |
| 6. Working and Linear Ranges | | Not applicable. |
| 7. Limit of Detection | | ≥ 10 ppm |
| 8. Limit of Quantitation / Sensitivity | | Not applicable. |
| 9. Ruggedness | | No statistically significant differences in results using 2 kit lots and +/- 2 min. variation in test incubation time. |
| 10. Matrix Effects | | None observed. |
| 11. Comparability (if intended as a substitute for an established method accepted by the NSSP) | | Agreement with LC-UV reference method in testing of mussel tissue samples with incurred domoic acid. |
| **D. Other Information** | | |
| 1. Cost of the Method | | Approx. $17.00 per test. |
| 2. Special Technical Skills Required to Perform the Method | | None |
| 3. Special Equipment Required and Associated Cost | | None |
| 4. Abbreviations and Acronyms Defined | | ppm = parts per million, equivalent to mg/kg |
| 5. Details of Turn Around Times (time involved to complete the method) | | The test can be performed in approximately 20 minutes including sample preparation. |
| 6. Provide Brief Overview of the Quality Systems Used in the Lab | | |

| | |
|---|---|
| Submitters Signature  | Date: June 3, 2011 |
| Submission of Validation Data and Draft Method to Committee | Date: |
| Reviewing Members | Date: |
| Accepted | Date: |
| Recommendations for Further Work | Date: |
| Comments: | |

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix – The component or substrate of a test sample.
12. Method Validation – The process of verifying that a method is fit for purpose.[1]
13. Precision – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity – the ability of a method to measure only what it is intended to measure.[1]
18. Working Range – the range of analyte or measurand concentration over which the method is applied.

REFERENCES:


7. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
8. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
9. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
10. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
11. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
12. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

# Validation Study of the Reveal® ASP Test for the Qualitative Detection of Domoic Acid in Shellfish

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## Introduction

Domoic acid, produced by certain species of the diatom *Pseudonitzschia*, is the primary toxin responsible for amnesic shellfish poisoning (ASP) associated with consumption of contaminated shellfish including oysters, clams, and mussels. Current methodologies for detection of domoic acid in shellfish are laborious and time-consuming, consisting primarily of LC-UV, LC-MS, and immunoassay procedures. LC-UV methods [1, 2] have been accepted as quantitative reference methods in many parts of the world. Assays facilitating more rapid determination of domoic acid with simplified procedures are needed by the shellfish industry and regulatory authorities.

In this report, we describe results of a validation study of the Reveal® ASP test for qualitative detection of domoic acid in shellfish. Reveal ASP is a lateral flow immunoassay designed for rapid determination of domoic acid at a level of approximately 10 ppm or greater (one-half the regulatory limit in many countries). The test is easy to use and results can be obtained in less than 20 minutes, including sample preparation.

## Principle of the Method

Reveal ASP is a single-step, lateral flow immunochromatographic assay based on the principle of competitive immunoassay. Following a simple distilled water extraction of domoic acid from homogenized shellfish tissue, the dipstick-format Reveal device is placed into the extract. The extract is wicked through a reagent zone containing antibodies specific for domoic acid conjugated to colloidal gold particles. If domoic acid is present, it will be captured by the labeled antibody. Migration of the sample continues through a membrane, which contains a zone of domoic acid conjugated to a protein carrier. This zone captures any unbound antibody- gold conjugate, resulting in a visible line. With increasing amounts of domoic acid in the test sample, less unbound conjugate is available for binding to the test line. Thus, intensity of the test line is inversely proportional to the amount of domoic acid in the sample. The test device also incorporates a control conjugate and which binds to a second line. The control line will form regardless of the amount of domoic acid present in the sample, ensuring that the test device is functioning properly. Test results are interpreted as positive or negative by scoring the intensity of the test line using an interpretation card supplied with the test kit.

## Intended Use

For the qualitative detection (at greater than or equal to 10 ppm [mg/kg]) of domoic acid in shellfish, including oysters, clams, and mussels.

## Reveal ASP Method

The kit insert is included as Appendix I.

***Materials Provided***

Starter Kit (Neogen #9563), contains:

- Sample cup rack
- Roller

Reveal ASP kit (Neogen #9560), contains:

- 25 lateral flow test strips
- 25 sample cups
- 25 filter extraction bags
- 50 100 µL disposable pipettes
- Interpretation card

***Materials Required but not Supplied (available from Neogen Corp. and other sources)***

- Blender and blender jar
- Scale, capable of weighing 0.5-400 g $\pm$ 0.1 g
- Timer
- 50-mL graduated cylinder or bottle-top liquid dispenser
- Distilled water
- Leakproof container with lid, 40 mL capacity

***Storage Requirements***

Store Reveal ASP kit components at controlled room temperature (18-30$^{o}$C, 64-86$^{o}$F). Do not freeze.
Test strips should remain in their original sample tubes until use to maintain shelf life and ensure optimal performance.

***Precautions***

Do not use test kits beyond their expiration date.
Treat all liquids, including sample extract, and used components as if contaminated with toxin. Gloves and other protective apparel should be worn at all times.
To avoid cross-contamination, use clean pipettes, extraction bags, and fresh extraction solution for each sample.
A Material Safety Data Sheet (MSDS) is available from Neogen Corp.

**Sample Preparation and Extraction**

Samples should be collected according to accepted sampling techniques.

1. Obtain a representative sample and shell the sample.
2. Thoroughly rinse with cold water.
3. Homogenize in a high-speed blender.
4. Weigh 1.0 $\pm$ 0.1 g of homogenized sample, preferably in a leak-proof container capable of holding 40 mL of liquid.
5. Add 20 mL distilled water to the container with sample.
6. Shake the container vigorously by hand for 30 seconds until all shellfish tissue is in solution (a cloudy appearance and/or bubbles are normal).
7. Number one side of the extraction bag “1” and the other side “2”.
8. Pour the sample extract into side 1 of the extraction bag. The extraction bag contains a mesh filter which allows for partial filtration of the sample extract.

9. Seal the extraction bag by positioning the green straw approximately 2-3 inches down from the top of the bag, fold the upper edge of the bag so that it covers the green straw, and firmly clip on the white bag clip. This prevents leakage of the sample extract.

10. Press the roller firmly on the extraction bag, pushing the roller back and forth for 30 seconds to aid in obtaining a homogenous sample extract.

11. Slide out the green straw and remove the white bag clip.

12. Pinch the top of the bag and carefully pour all the bag contents from side 2 back into the original sample container (there may be small pieces of shellfish remaining on side 1 of the bag). Discard the used extraction bag.

13. Shake container vigorously by hand for 30 seconds.

14. Remove 100 µL of the sample extract using the disposable pipette* provided (alternatively by use of a standard pipette), and add to a fresh container containing 20 mL distilled water.

    * To use the disposable pipettes provided, firmly press the top bulb of the pipette, insert the tip into the sample extract, and slowly release the top bulb to draw up the sample extract. Excess volume (above 100 µL) will overflow into the lower bulb, ensuring that 100 µL is available to dispense. Press the top bulb firmly and slowly release the top bulb to dispense the liquid into the container with distilled water. Discard the used pipette.

***Assay Procedure***

All steps should be performed at controlled room temperature (18-30$^{o}$C, 64-86$^{o}$C).

1. Remove the appropriate number of sample cups and place in the sample cup rack.

2. Shake the extracted sample prepared above vigorously by hand for 30 seconds.

3. Remove 100 uL using a fresh pipette and add 100 uL to the sample cup.

4. Remove the required number of Reveal ASP test strips from the container and immediately close the container.

5. Place the Reveal test strip with the sample end down (Neogen logo on top) into the sample cup.

6. Allow the test strip to develop in the sample cup for 10 minutes.

7. Remove the test strip and interpret the results as described below.

***Interpretation of Results***

Test strips should be interpreted immediately following completion of the 10 minute incubation.
Using the interpretation card provided, score the test line intensity to determine if the sample contains less than 10 ppm or greater than or equal to 10 ppm domoic acid.
*Note*: The control line should always be present and will be darker than the test line. If no control line is visible, this indicates an invalid result and the sample should be retested using another Reveal device.

**Single-Laboratory Validation Study**

A single-laboratory validation study was conducted to measure accuracy/trueness, specificity, and ruggedness of the Reveal ASP method, as well as effects of potential interfering compounds. In addition, Reveal ASP results were compared to those of an accepted LC-UV reference method [1]. Matrices tested were oysters, clams, and mussels.

***I. Accuracy/trueness and specificity***

*Methods*
Fresh oysters, clams, and mussels were obtained from a local retail market that receives fresh shellfish by air shipment daily. Shellfish were held at 2-8°C before use. Shellfish were shucked and approximately 12-15 animals were combined and homogenized in a blender to produce a bulk sample. The bulk samples were separated into 10 portions of 1 g each. Five served as unspiked controls. One each of the remaining 5 samples was spiked separately at 5, 10, 15, 20 and 40 ppm domoic acid. Certified reference material (CRM-DA-f), obtained from the National Research Council, Canada- Institute for Marine Biosciences (NRC- IMB), was used as the spiking material. The CRM consisted of 101.8 µg/mL domoic acid extracted from contaminated cultured blue mussels and dissolved in a solution of 5% acetonitrile/95% water.
Each sample was then prepared according to the procedures in **Sample Preparation and Extraction** above, and tested with the Reveal ASP assay. Ten replicates of each extracted spiked sample and three replicates of each extracted unspiked sample were tested with the Reveal ASP assay.

Accuracy rates were calculated for each shellfish matrix separately and in combination. A dose-response curve was constructed using the combined data.

*Results*
Results of the accuracy study are shown in Table 1. Accuracy is defined as the level of agreement between the assay and the expected test results based on the domoic acid spike level.
For oysters, accuracy of the Reveal ASP method was 95.4%. Seven of ten tests at 10 ppm domoic acid were positive. All tests at higher levels of domoic acid were positive. All tests at 5 ppm were negative. There were no false-positive results on unspiked control samples.
For clams, accuracy of the assay was 92.3%. All tests at 10 ppm domoic acid and higher were positive. Five of ten tests at 5 ppm domoic acid were also positive. There were no false positive results on unspiked control samples.
For mussels, accuracy of the assay was 100%. All tests at 10 ppm domoic acid and higher were positive. All tests at 5 ppm were negative. There were no false-positive results on unspiked control samples.

Overall accuracy of the Reveal ASP test was 95.9%. A dose-response curve was constructed using combined data from all three shellfish matrices and is shown in Fig. 1. Based on the dose-response curve, performance of the Reveal ASP test can be characterized as follows:

| | | |
|---|---|---|
| Zone 1 | Positive < 5% of the time | < 2 ppm domoic acid |
| Zone 2 | Positive 5-50% of the time | 2-7 ppm domoic acid |
| Zone 3 | Positive 51-95% of the time | 8-11 ppm domoic acid |
| Zone 4 | Positive > 95% of the time | > 11 ppm domoic acid |

**II. Interfering compounds**

*Methods*
Fresh oysters, clams, and mussels were obtained as described above. Approximately 12-15 animals were combined and homogenized in a blender a produce a bulk sample. The bulk samples were separated into 12 portions of 1 g each. The 12 portions were separated into 4 groups each containing three 1-g samples. Samples in each group were spiked individually with one of the following potentially interfering compounds: okadaic acid, 10 ppm; glutamic acid, 100 ppm; glutamine, 100 ppm; or saxitoxin, 5 ppm. One sample in each group was spiked with 10 ppm domoic acid, one sample was spiked with 40 ppm domoic acid, and one sample was left unspiked. All interfering compounds were obtained from Sigma, except saxitoxin which was obtained from NRC-IMB. Domoic acid CRM, described above, was used as the spiking material.
Sample preparation and testing were performed as described above. Five replicates of each extracted sample were tested with the Reveal ASP assay.

*Results*

Results of testing for effects of potentially interfering compounds on performance of the Reveal ASP assay are shown in Table 2. There was no evidence of interference by okadaic acid, glutamic acid, glutamine, or saxitoxin on assay performance in any of the three shellfish types. All tests produced expected results at levels of 0, 10, and 40 ppm domoic acid.

***III. Ruggedness***

*Methods*
Fresh oysters, clams, and mussels were obtained as described above. Approximately 12-15 animals were combined and homogenized in a blender a produce a bulk sample. The bulk samples were separated into 3 portions of 1 g each. One portion was spiked at 10 ppm, one at 40 ppm, and the remaining sample left unspiked. Domoic acid CRM, described above, was used as the spiking material.

Sample preparation and testing were performed as described above. Ten replicates of each extracted sample were tested with the Reveal ASP assay. Each replicate was tested using devices from two different test kit lots (LFD-001 and LFD-002). The devices were interpreted after 8, 10 and 12 minutes to measure potential differences in results at different test incubation times. For each shellfish type, this trial was performed twice, on separate days, by two operators each day.
For each shellfish matrix, results from the two days of testing were pooled. Chi-square analysis (McNemar's test, [3]) was performed to determine if results were significantly different for the two kit lots or three test incubation times evaluated.

*Results*
Results of assay ruggedness trials with respect to Reveal ASP kit lot and assay incubation period are shown in Tables 3 and 4, respectively. In the trials measuring the effect of kit lot, there were no significant differences in the number of positives obtained with kit lots 1 and 2 at any spike level in any shellfish matrix, as determined by chi-square analysis at $p < 0.05$ (Table 3). Similarly, in the trials measuring the effect of variation in test incubation time, there were no significant differences in the number of positives obtained at incubation times of 8, 10 and 12 minutes at any spike level in any shellfish matrix (Table 4).

***IV. Comparison with Reference Method***

*Methods*
Fresh mussels were obtained as described above. Approximately 12-15 animals were combined and homogenized in a blender a produce a bulk sample. Incurred CRM consisting of a thermally stabilized homogenate of mussel tissue containing domoic acid at a concentration of 41 µg/g (ppm) was purchased from NRC-IMB (CRM-ASP-Mus-c). The incurred material was blended 1:1 with clean mussel tissue to obtain a domoic acid level of approximately 20 ppm. From the blended material, 20 samples of 1 g each were prepared. Ten samples were retained and tested in triplicate using the Reveal ASP test. The remaining 10 samples were sent to NRC-IMB for testing by the LC-UV method.

*Results*
Results of testing of samples of mussel tissue with incurred domoic acid by both the Reveal ASP assay and a reference LC-UV quantitative method are shown in Table 5. All 10 samples tested with the Reveal ASP method produced positive results. Results obtained with the LC-UV method were also positive for all 10 samples, ranging from 11.9 to 16.4 ppm.

**Quality Control Testing**
Quality control testing of manufactured lots of the Reveal ASP assay is performed at both in-process and finished product stages. In-process testing consists of balancing the antibody-colloidal gold conjugate for optimal test and control line intensity, and testing the device membrane for proper test and control line placement by running negative samples.

For finished product testing, samples are produced by diluting domoic acid (certified reference material CRM-DA-f, NRC-IMB) to concentrations of 2, 10, and 40 ppm in buffer. An unspiked sample is also prepared. Ten Reveal devices, randomly selected from the lot, are run at each concentration. For acceptance of the lot, all tests at 0 and 2 ppm must be negative and all tests at 10 and 40 ppm must be positive.

**Discussion**

Results of the validation study showed that the Reveal ASP test is an effective procedure for qualitative determination of domoic acid in oysters, clams, and mussels. In the accuracy study, all tests at the accepted action level of 20 ppm were positive. There were no false-positive results on unspiked control samples. The dose-response curve indicates that the test produces a positive result greater than 95% of the time at a concentration above 11 ppm, 51-95% of the time at a concentration of 8-11 ppm, and less frequently at levels below 8 ppm.

Four compounds, okadaic acid, glutamic acid, glutamine, and saxitoxin, were tested for potential interference with the Reveal ASP assay. None was noted, as all samples produced the expected results at 0, 10, and 40 ppm domoic acid.

Results of ruggedness trials indicated that there was no statistically significant difference in performance between two Reveal ASP kit lots, nor was there any significant difference in performance in assays conducted with variation of +/- 2 minutes around the specified incubation time of 10 minutes.

Results of testing of mussel tissue samples containing incurred domoic acid showed agreement between the Reveal ASP and reference LC-UV methods, with all 10 samples testing positive by Reveal and LC-UV producing results in the range of 11.9-16.4 ppm.

Reveal ASP can be used as an accurate screening test for the rapid determination of domoic acid in shellfish. The test requires little equipment, uses water for sample extraction, and can be performed by personnel with minimal training. The test can be used in a field or laboratory setting, with results available within 20 minutes of sample receipt.

It is recommended that the Reveal ASP test be approved by the Interstate Shellfish Sanitation Conference as a screening method for qualitative determination of domoic acid in oysters, clams, and mussels.

**References**

1. Quilliam, M.A., Xie, M., & Hardstaff, W.R. (1995) Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood. *J. AOAC Intl.* **78**,543-554.

2. Lawrence, J.F., Charbonneau, C.F., & Menard, C. (1991) Liquid chromatographic determination of domoic acid in mussels, using AOAC paralytic shellfish poison extraction procedure: collaborative study. *J. Assoc. Off. Anal. Chem*. **74**,68-72.

3. Siegel, S. (1956) *Nonparametric Statistics for the Behavioral Sciences*, McGraw-Hill Book Co., New York, NY.

**Acknowledgements**

We thank Dr. Michael Quilliam and Kelley Reeves of the National Research Council, Institute for Marine Biosciences, Halifax, Nova Scotia, Canada for performing the LC-UV analyses.

Table 1. Results of accuracy study of the Reveal ASP test.

| Sample Type | Level Domoic Acid (ppm) | Number Tests | Number Positive |
|---|---|---|---|
| Oysters | 0 | 15 | 0 |
| | 5 | 10 | 0 |
| | 10 | 10 | 7 |
| | 15 | 10 | 10 |
| | 20 | 10 | 10 |
| | 40 | 10 | 10 |
| Clams | 0 | 15 | 0 |
| | 5 | 10 | 5 |
| | 10 | 10 | 10 |
| | 15 | 10 | 10 |
| | 20 | 10 | 10 |
| | 40 | 10 | 10 |
| Mussels | 0 | 15 | 0 |
| | 5 | 10 | 0 |
| | 10 | 10 | 10 |
| | 15 | 10 | 10 |
| | 20 | 10 | 10 |
| | 40 | 10 | 10 |
| All Data | 0 | 45 | 0 |
| | 5 | 30 | 5 |
| | 10 | 30 | 27 |
| | 15 | 30 | 30 |
| | 20 | 30 | 30 |
| | 40 | 30 | 30 |

Table 2. Results of interference study for the Reveal ASP test.

| Sample Type | Interfering Compound and Level | Level Domoic Acid (ppm) | Number Tests | Number Positive |
|---|---|---|---|---|
| Oysters | Okadaic acid 10 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Glutamic acid 100 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Glutamine 100 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Saxitoxin 5 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| Clams | Okadaic acid 10 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Glutamic acid 100 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Glutamine 100 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Saxitoxin 5 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| Mussels | Okadaic acid 10 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Glutamic acid 100 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Glutamine 100 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |
| | Saxitoxin 5 ppm | 0 | 5 | 0 |
| | | 10 | 5 | 5 |
| | | 40 | 5 | 5 |

Table 3. Results of assay ruggedness trials for the Reveal ASP test – effect of kit lot.

| Sample Type | Level domoic acid (ppm) | Number Tests | Number Positive Lot 1 | Number Positive Lot 2 | $\chi^{2a}$ |
|---|---|---|---|---|---|
| Oysters | 0 | 20 | 0 | 0 | -[b] |
| | 10 | 20 | 15 | 15 | - |
| | 40 | 20 | 20 | 20 | - |
| Clams | 0 | 20 | 0 | 0 | - |
| | 10 | 20 | 18 | 20 | 0.50 |
| | 40 | 20 | 20 | 20 | - |
| Mussels | 0 | 20 | 0 | 0 | - |
| | 10 | 20 | 15 | 14 | 0.00 |
| | 40 | 20 | 20 | 20 | - |
| All Data | 0 | 60 | 0 | 0 | - |
| | 10 | 60 | 48 | 49 | 0.00 |
| | 40 | 60 | 60 | 60 | - |

[a] $\chi^2 > 3.84$ indicates a significant difference at $\rho < 0.05$.
[b] $\chi^2$ not applicable since all results were in agreement.

Table 4. Results of assay ruggedness trials for the Reveal ASP test – effect of incubation time.

| Sample Type | Level domoic acid (ppm) | Number Tests | Number Positive 8 min. | Number Positive 10 min. | Number Positive 12 min. | $\chi^{2a}$ 8 vs. 10 min. | $\chi^2$ 12 vs. 10 min. |
|---|---|---|---|---|---|---|---|
| Oysters | 0 | 40 | 0 | 0 | 0 | -[b] | - |
| | 10 | 40 | 36 | 35 | 35 | 0.00 | - |
| | 40 | 40 | 40 | 40 | 40 | - | - |
| Clams | 0 | 40 | 0 | 0 | 0 | - | - |
| | 10 | 40 | 34 | 37 | 38 | 0.44 | 0.00 |
| | 40 | 40 | 40 | 40 | 40 | - | - |
| Mussels | 0 | 40 | 0 | 0 | 0 | - | - |
| | 10 | 40 | 30 | 29 | 29 | 0.00 | - |
| | 40 | 40 | 40 | 40 | 40 | - | - |
| All Data | 0 | 120 | 0 | 0 | 0 | - | - |
| | 10 | 120 | 100 | 101 | 102 | 0.00 | 0.00 |
| | 40 | 120 | 120 | 120 | 120 | - | - |

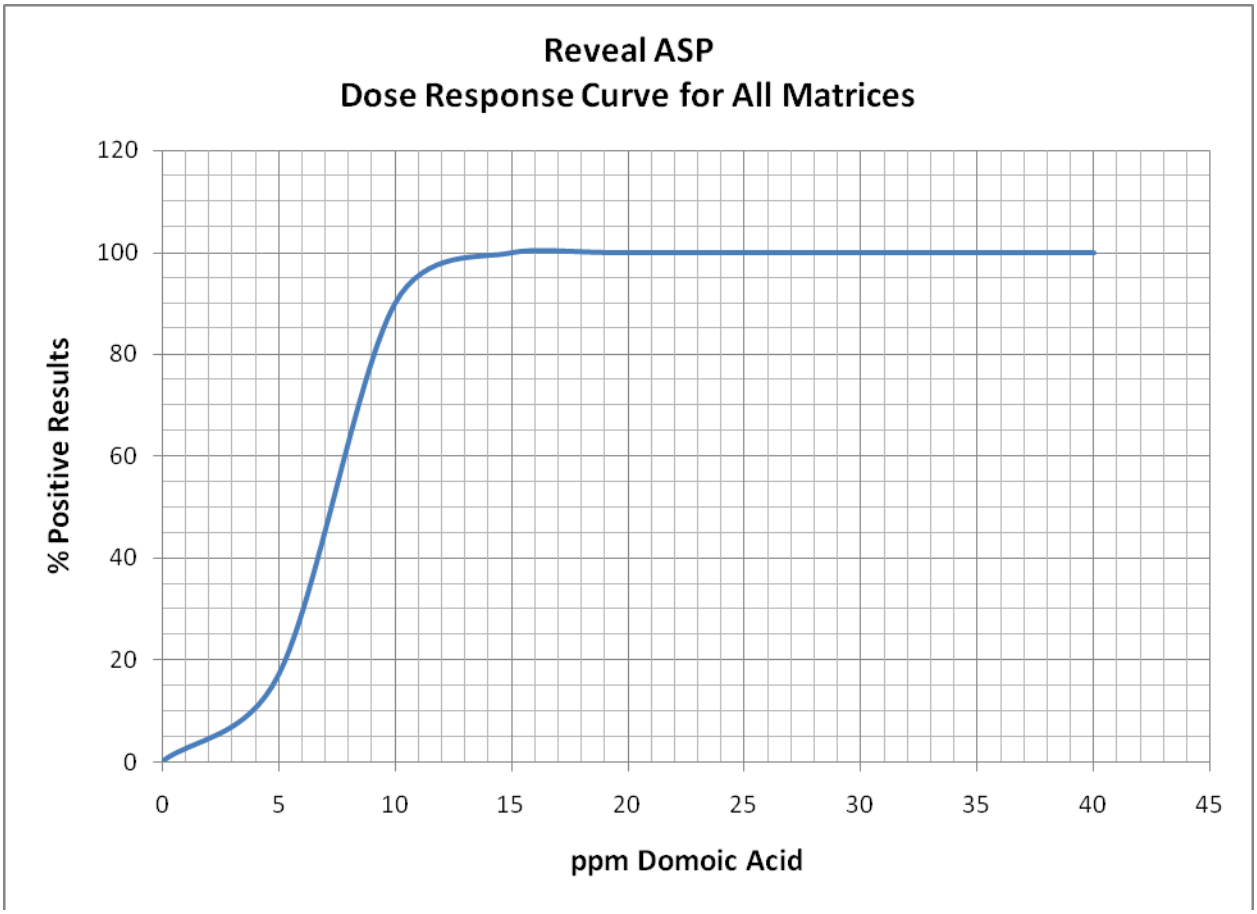
[a] $\chi^2 > 3.84$ indicates a significant difference at $\rho < 0.05$.
[b] $\chi^2$ not applicable since all results were in agreement.

Table 5. Results of testing of domoic acid containing mussel tissue with Reveal ASP and a LC-UV reference method.

| Sample No. | LC-UV Method Result ppm Domoic Acid (mean ± SD)[a] | Reveal ASP Result |
|---|---|---|
| 1 | 16.4 ± 0.3 | Positive |
| 2 | 15.4 ± 0.1 | Positive |
| 3 | 14.5 ± 0.1 | Positive |
| 4 | 15.7 ± 0.2 | Positive |
| 5 | 15.06 ± 0.04 | Positive |
| 6 | 14.60 ± 0.03 | Positive |
| 7 | 13.65 ± 0.06 | Positive |
| 8 | 15.17 ± 0.08 | Positive |
| 9 | 14.0 ± 0.1 | Positive |
| 10 | 11.92 ± 0.05 | Positive |

[a] Mean of 3 determinations.

Fig. 1. Dose-response curve for the Reveal ASP test.




**Appendix I. Reveal ASP kit insert**

Product #9560

*Read instructions carefully before starting test*

# Reveal®
# ASP
# (Domoic acid)

**THE TOXIN**

Amnesic shellfish poisoning (ASP) causing toxins are produced mainly by the toxigenic diatom strain *Pseudo-nitzchia multiseries*. The ASP toxins include primarily domoic acid (DA).

In addition to contamination of seafood, these marine biotoxins can result in human and marine wildlife mortality. The clinical toxicological effects attributed to DA can include: permanent loss of short-term memory, nausea, vomiting, headache, disorientation, and loss of balance.

Action limits for DA were established soon after the 1987 domoic acid/mussel crisis in Canada in which over 150 people became ill and four deaths resulted. Many countries have currently established a maximum permitted level of 20 mg DA per kg in whole shellfish (20 ppm).

**INTENDED USE/USER**

Reveal for ASP is intended for the qualitative screening of shellfish for DA, with the cut-off value for a positive result at approximately 10 ppm (i.e., half of the regulatory limit in many countries). The test kit is designed for use by personnel with an interest in the rapid screening of shellfish samples (further to obtaining a sample homogenate, it takes less than 20 minutes to carry out the sample extraction and obtain the result).

**ASSAY PRINCIPLES**

Reveal for ASP is a single-step lateral flow immunochromatographic assay based on a competitive immunoassay format. The extract is wicked through a reagent zone, which contains antibodies specific for DA conjugated to coloured particles. These DA-antibody-particle complexes result in the visible signal. If DA is present, it will be captured by the particle-antibody complex. The particle-antibody complex is then wicked onto a membrane, which contains a zone of DA conjugated to a protein carrier. This zone captures any uncomplexed DA antibody, allowing particles to concentrate and form a visible line. As the level of DA in the sample increases, free DA will complex with the particle-antibody complex. This, in turn, allows less particle-antibody complexes to be captured in the test zone. Therefore, as the concentration of DA in the sample increases, the test line decreases. The membrane also contains a control zone where an immune complex present in the reagent zone is captured by the antibody, forming a visible line. The control line will always form regardless of the level of DA, ensuring the strip is functioning properly.

**STORAGE REQUIREMENTS**

Store kit components at room temperature (18-30°C, 64–86°F) to assure full shelf life. Test strips should remain capped in their original sample tubes until used to assure optimal performance.

**PRECAUTIONS**

1. The test strips must remain inside the stay-dry tube before use.
2. Store test kit at room temperature (18–30°C, 64–86°F) when not in use, do not freeze.
3. Do not use kit contents beyond expiration date.
4. Treat all liquids, including sample extract, and used components as if contaminated with toxin. Gloves and other protective apparel should be worn at all times.
5. To avoid cross-contamination- use clean pipettors, extraction bags and fresh extraction solutions for each sample.

**MATERIALS PROVIDED**

| **9563 - Starter Kit - Reveal ASP (Domoic Acid)** | **9560 - Reveal ASP (Domoic Acid)** |
|---|---|
| 1 Reveal sample cup rack | 25 ASP lateral flow test strips |
| 1 Roller | 25 sample cups |
| 1 Bag-clip (white clip and green straw) | 25 filter extraction bags |
| | 50 exact volume pipettors |

**MATERIALS RECOMMENDED BUT NOT PROVIDED**

1. Distilled water
2. 40 mL leakproof container including lids
3. Blender, Oster (Neogen item #9493)
4. Blender Jar, MINI, with blade & cap, 250 mL (Neogen item #9477)
5. Blender Jar, Stainless Steel 1 L (Neogen item #9495)
4. Scale capable of weighing 0.5–400 g ± 0.1 g (Neogen item #9427)
5. Timer (Neogen item #9452)
6. 50 mL Graduated cylinder (Neogen item #9367)
7. Bottle-top dispenser (Neogen item #9448)

**EXTRACTION SOLUTION PREPARATION**

The required extraction solution for the test is distilled water. A total volume of 40 ml of the solution is required per each sample tested. Fill 2 bottles with 20 mL each of distilled water, and label as SOLUTION 1 and SOLUTION 2, respectively.

***Please note:*** *The solution containers should be capable of holding a volume of 40 mL to effectively carry out the procedure.*

**SAMPLE PREPARATION AND EXTRACTION**

The sample to be tested should be collected according to accepted sampling techniques.

1. Obtain a representative sample. Shell the samples.
2. Thoroughly rinse with cold water.
3. Homogenise† the shellfish in a high-speed blender.
   **IMPORTANT: A good homogenate is essential in order to obtain an accurate result.**
4. Weigh 1g (± 0.1g) of homogenized sample, ideally in leak-proof bottle capable of holding ~40 mL of liquid.
5. Pour entire contents of one bottle containing 20 mL of SOLUTION 1 into bottle containing one sample.
6. Shake the sample bottle vigorously by hand for 30 seconds, until all shellfish tissue is in solution (a cloudy appearance or bubbles may form, which do not affect the running of the test).
7. Number both sides of the extraction bag using a marker, so that there is a side labeled "1" and the other side labeled "2". Pour solution/sample mixture into one extraction bag (side 1). The extraction bag contains a mesh filter which allows for partial filtration of the sample.

8. To seal the bag, position and hold the green straw approximately 2–3 inches down from the top of the bag, fold the upper edge of the bag so that it covers the green straw and firmly clip on the white bag-clip. This prevents leakage of the sample.
9. Press the roller firmly on the sample extraction bag, pushing the roller back and forth for 30 seconds to aid in obtaining a homogenous sample extract.
10. Slide out the green straw and remove the white bag-clip.
11. Pinch the top of the bag and carefully pour all the bag contents from side 2 back into the original sample container (there may be small pieces of shellfish remaining on side "1" of the bag). **Discard the used extraction bag** (***Note:*** *pinching the top of the bag to create a sharp edge allows easy pouring of the sample, preventing any spillage*).
12. Shake bottle with sample extract vigorously by hand for 30 seconds.
13. Remove 100ul of the sample extract using the exact-volume pipettors* provided (alternatively by use of a standard pipettor), and add into a fresh bottle containing SOLUTION 2.

    **To use the exact-volume pipettors, firmly press the top bulb of the pipettor, insert the tip into the sample, slowly release the top bulb to draw up the sample extract. Excess volume (i.e. above 100 µL) will overflow into the lower bulb, ensuring 100µl is ready to dispense. Press the top bulb firmly and slowly release the top bulb to dispense 100 µL into the bottle containing SOLUTION 2. Discard the used pipettor.*

    †Homegenise – to blend or to puree

**TEST PROCEDURE**

1. Remove the appropriate number of sample cups and place in the sample cup tray.
2. Shake the SOLUTION 2 bottle (which contains 100 µL of the sample extract) vigorously by hand for 30 seconds.
3. Remove 100 µL from the SOLUTION 2 bottle using a fresh pipettor and add 100 µL per sample cup.
4. Remove the required number of strips from the lateral flow device container and immediately close the container.
5. Place the new ASP strip with the sample end down (Neogen logo on top) into the sample cup.
6. Allow the strip to develop in the sample cup for 10 minutes.
7. Remove strip and interpret the results (as described below).

**INTERPRETATION OF RESULTS (VISUAL)**

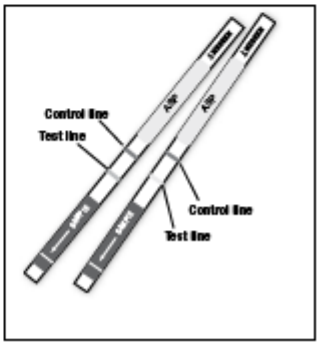
Test strips should be interpreted directly following completion of the 10 minute incubation.

Use the provided interpretation card to determine the level of the toxin present in the sample.

***Note:*** *The control line should always be present and will always be darker than the test line. If there is no control line development, this indicates an invalid result and the sample should be retested using another device.*

**MSDS INFORMATION AVAILABLE**

Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's Food Safety test kits, at www.neogeneurope.com.



**VALIDATED MATRICES**

Mussels, scallops, oysters, clams and cockles. Contact your Neogen representative concerning additional commodities.

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