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

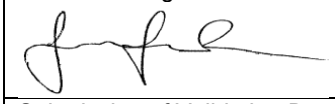
The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the 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 2.0 DSP
Name of the Method Developer	Neogen Corporation
Developer Contact Information	Jennifer Rice 517-372-9200 Jrice@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 okadaic acid (OA) and dinophysistoxins (DTX) 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 OA group of toxins (OA and DTXs).
3. Is there an acknowledged need for this method in the NSSP?		Simple assays that provide rapid and accurate 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 2.0 DSP
Method Scope		Qualitative detection of OA and DTXs in mussels, oysters, clams and scallops.
References		Study report and kit insert included in this submission.
Principle		Competitive lateral flow immunoassay in dipstick format. Solvent extraction of analyte from homogenized shellfish tissue.
Any Proprietary Aspects		Yes, commercial test kit.
Equipment Required		Timer, bag roller, microwell holder, pipettes (1.0, 0.1 mL), heatblock, reader
Reagents Required (consumables)		Reveal DSP test devices, extraction bags with mesh filter, filter syringes, microwells, methanol, screwcap glass vials, sodium hydroxide and hydrochloric acid
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, microwells, and pipettes should be treated as if contaminated with OA group toxins 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 control (control line) that confirms that the device is functioning properly. An okadaic acid solution in buffer at a concentration 5.34

		ng/mL can be used as an external positive control, if desired. This is the equivalent of a shellfish samples containing OA at a level of approx. 320 ug/kg (320 ppb).
C. Validation Criteria		
1. Accuracy / Trueness		No false negatives with incurred samples containing at or above 160 ppb OA eqs (various toxin profiles and species). No false positives with incurred samples containing at or below approx. 80 ppb OA eqs.
2. Measurement Uncertainty		Not applicable.
3. Precision Characteristics (repeatability and reproducibility)		Not applicable.
4. Recovery		Not applicable.
5. Specificity (cross-reactivity)		Approx. cross reactivity profiles at cut-off (based on QC): OA: 100% DTX1: 89% DTX2: 47% No impact on test results by potentially interfering compounds – Domoic acid (incurred samples).
6. Working and Linear Ranges		Not applicable.
7. Limit of Detection		Approximate cut off for positive results (based on QC): OA: 125 ppb OA eqs. DTX1: 140 ppb OA eqs DTX2: 160 ppb OA eqs
8. Limit of Quantitation / Sensitivity		Not applicable.
9. Ruggedness		No impacts on performance in results using 3 kit lots, +/- 2 min variation in test incubation time or +/- 10 uL in sample volume.
10. Matrix Effects		None observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		The assay was found comparable to LC-MS/MS reference methods in testing naturally incurred OA/DTX samples
D. Other Information		
1. Cost of the Method		Approximately $17.00 per test (list price)
2. Special Technical Skills Required to Perform the Method		None
3. Special Equipment Required and Associated Cost		Reader (list price approximately $1,995)
4. Abbreviations and Acronyms Defined		ppb = parts per billion, equivalent to ug/kg
5. Details of Turn Around Times (time involved to complete the method)		The test can be fully performed in less than 30 min including sample preparation. If hydrolysis is necessary, this adds approximately 40 min to the procedure.
6. Provide Brief Overview of the Quality Systems Used in the Lab		

Submitters Signature 	Date: June 27, 2013
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:

1. 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.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. 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.

Laboratory Evaluation Checklist – Reveal 2.0 DSP

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
SHELLFISH PROGRAM IMPLEMENTATION BRANCH
SHELLFISH SAFETY TEAM
5100 PAINT BRANCH PARKWAY
COLLEGE PARK, MD 20740-3835
TEL. 301-436-2151/2147 FAX 301-436-2672

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**
LABORATORY REPRESENTED BY:	**TITLE:**	
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:** **REGION:**	
OTHER OFFICIALS PRESENT:	**TITLE:**	

Items which do not conform are noted by:

C - Critical **K** - Key **O** - Other **NA** - Not Applicable Conformity is noted by a **"√"**

PART I – QUALITY ASSURANCE		
Code		**Item Description**
		1.1 Quality Assurance (QA) Plan
K		1. Written Plan adequately covers all the following: (check √ those that apply) a. ☐ Organization of the laboratory. b. ☐ Staff training requirements. c. ☐ Standard operating procedures. d. ☐ Internal quality control measures for equipment, calibration, maintenance, repair and performance. e. ☐ Laboratory safety. f. ☐ Quality assessment. g. ☐ Proper animal care.
C		2. QA plan implemented.
		1.2 Work Area
O		1. Adequate for workload and storage.
O		2. Clean and well lighted.
O		3. Adequate temperature control.
O		4. All work surfaces are nonporous and easily cleaned.
C		5. A separate, quiet area with adequate temperature control for mice acclimation and injection is maintained.
		1.3 Laboratory Equipment
C		1. The balance provides a sensitivity of at least 0.1g at a load of 150 grams.
K		2. The balance calibration is checked monthly using NIST Class S or ASTM Class 1 or 2 weights or equivalent. Records maintained.
C		3. Refrigerator temperature is maintained between 0 and 4°C.
K		4. Refrigerator temperature is monitored at least once daily. Records maintained.
C		5. Freezer temperature is maintained at -20°C or below.
K		6. Freezer temperature is monitored at least once daily. Record maintained.
C		7. All glassware/plastic used with the high speed blender for homogenization is cleaned with water after each use.
C		8. Accuscan Pro Reader is calibrated before use
C		9. The correct QR code is scanned in the reader for the lot of strips that will be used
C		10. The heat block (or waterbath) is set at 76^{o}C (+/- 2 ^{o}C)
		1.4 Reagent and Reference Solution Preparation and Storage
C		1. Buffers are stored in plastic screw top vials at room temperature.
K		2. Buffers are within expiration date.
C		3. Analytical grade methanol, 2.5M HCL and 2.5M NaOH are used for extracton
		1.5 Collection and Transportation of Samples
K		1. Shellstock are collected in clean, waterproof, puncture resistant containers.
K		2. Samples are appropriately labeled with the collector's name, harvest area and time and date of collection.
K		3. Immediately after collection, shellstock samples are placed in dry storage for transport (e.g. cooler) which is maintained between 0 and 10°C. Upon receipt at the lab, samples are placed under refrigeration.
K		4. The time from collection to completion of the assay does not exceed 48 hours if stored refrigerated. However, if there are significant transportation delays, then shellstock samples are processed immediately as follows *(circle the appropriate choice):* a. Washed, shucked, drained, frozen until extracted; b. Washed, shucked, drained, homogenized and frozen; c. The laboratory has an appropriate contingency plan in place to handle samples which can't be analyzed within 24 hours due to transportation issues.
K		5. Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.

PART II – EXAMINATION OF SHELLFISH FOR DSP TOXIN		
		2.1 Preparation of Sample
C		1. At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.
O		2. The outside of the shell is thoroughly cleaned with fresh water.
O		3. Shellstock are opened by cutting adductor muscles.
O		4. The inside of the shell is rinsed with fresh water to remove sand or other foreign material.
O		5. Shellfish meats are removed from the shell by separating adductor muscles and tissue connecting at the hinge.
K		6. Damage to the body of the mollusk is minimized in the process of opening.
O		7. Shucked shellfish are drained on a #10 mesh sieve (or equivalent) without layering for 5 minutes.
K		8. Pieces of shell and drainage are discarded.
C		9. Drained meats or thawed homogenates are blended at high speed until a homogenous sample is obtained (time required is species dependent).
		2.2 Extraction Procedure – Rapid (to detect OA, DTX1, DTX2)
C		1. The extraction bag is numbered on both sides using a marker, so that one side is labelled "1" and the opposite side labelled "2".
C		2. 2 g (± 0.1 g) of homogenized sample is weighed into the bottom of extraction bag on side "1"
C		3. 8 mL of methanol is added to side "1" of the extraction bag containing the sample.
K		4. The green straw is placed approximately half-way down from the top of the bag and the upper edge of the bag is folded over the green straw ensuring that the sample and methanol remain in the lower half of the bag.
K		5. The white clip is firmly applied to the bag to prevent sample leakage.
C		6. The extraction bag is placed firmly on a surface and the roller is pressed firmly on the sample extraction bag pushing the roller back and forth for 30 seconds.
K		7. The green straw and white clip are removed and the bag contents from side "2" are removed (with pipette or poured) into a clean container.
K		8. The extraction bag is discarded as biohazardous waste.
C		9. The sample extract is poured into the barrel of a filter syringe until the syringe is approximately half full. The plunger is placed on top and approximately 1 mL of the sample is filtered into a collection tube.
C		10. The filtered solution is transparent and not cloudy. If cloudy, then the solution is refiltered through a fresh syringe filter.
C		11. 100 µl of the sample extract is removed using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and added into DSP buffer B vial.
		2.3 Extraction Procedure – Hydrolysis (to detect OA, DTX1, DTX2, DTX3)
C		1. The Heater block is turned on to 76°C
C		2. 800 µL of the sample prepared in step 9 is transferred into a screw cap glass vial.
C		3. 100 µL of NaOH (2.5 M) is added to the vial and the vial is capped tightly.
C		4. The sample is thoroughly mixed using a vortex on high speed for 30 seconds.
C		5. The vial is heated in the heater block at 76°C for 40 mins
K		6. After 40 mins, the vial is removed from the heater block and allowed to cool to room temperature or the sample vial is placed on ice to cool to room temperature.
C		7. 100 µL of HCl (2.5 M) is added to the glass vial and mixed using a vortex on high speed for 30 seconds.
C		8. 100 µl of the sample extract is removed using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and added into a DSP buffer A vial.

		2.4Assay Procedure
K		1. The appropriate number of microwells are removed and place into the microwell holder.
C		2. The DSP buffer vial (containing diluted sample) from either extraction process, is shaken vigorously by hand for 30 seconds.
C		3. 100 µL of diluted sample is transferred into each microwell using a new disposable pipette
K		4. The required number of test strips are removed from the lateral flow device container and the container is immediately closed.
C		5. The DSP test strips with the sample end down (Neogen logo on top) are placed into the microwells.
C		6. The strip is allowed to develop in the microwell for 15 minutes.
C		7. After 15 minute run time, the test strip is immediately removed and read using the AccuScan® Pro reader
		2.5 Reading Test Results
C		1. Test strips are read within 1 minute of completion of the 15 minute incubation.
C		2. The Reveal 2.0 DSP test strip is fully inserted into the black cartridge "R" adapter with the sample end first and results facing out.
K		3. The cartridge with test strip side up is inserted in the AccuScan® Pro. The reader automatically begins analysis of the cartridge. The cartridge is not removed until the reader has completed the analysis.
O		4. Results are displayed on the AccuScan® Pro reader and stored by the reader.
O		5. The reader reports Positive (160ppb or greater OA EQS) or Negative (<160ppb OA EQS).

LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Obersvation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
DSP COMPONENT: PARTS I and II	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key and Other (O) nonconformities	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the DSP Component

1. **Does Not Conform Status** The DSP component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is $\geq$ 3 **or**
   b. The total # of Key nonconformities is $\geq$ 6 **or**
   c. The total # of Critical, Key and Other is $\geq$ 10

2. **Provisionally Conforms Status**: The PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is $\geq$ 1 but < 3

C. Laboratory Status *(circle appropriate)*
Does Not Conform - Provisionally Conforms – Conforms

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NEOGEN® CORPORATION

June 27, 2013

Laboratory Methods Review & Quality Assurance Committee
Interstate Shellfish Sanitation Conference
209-2 Dawson Road
Columbia, SC 29223-1740

Dear Members of the Committee:

Please find enclosed a validation study report and other supporting documentation for the Reveal 2.0 DSP test kit. We respectfully request your review of this submission and consideration of the test for acceptance as an ISSC approved method for qualitative determination of okadaic acid group of toxins (OA, DTX1, DTX2, DTX3) in molluscan shellfish. We believe that the test provides significant advantages in terms of time-to-result and ease of use, and we feel that acceptance of the method by ISSC will be of benefit to the shellfish industry and public health authorities.

I would be pleased to answer any questions or provide any further information that you may require. Thank you very much for your consideration.

Sincerely,

Jennifer Rice, DVM, MSc, Ph.D, MBA
Vice President and Senior Research Director
Neogen Corporation

Enclosures:
- Validation study report
- Reveal 2.0 DSP Kit insert
- Hydrolysis Pack Kit insert
- Reveal 2.0 DSP Hydrolysis Pack MSDS
- Reveal 2.0 DSP MSDS
- Single lab validation checklist
- 2013 ISSC conference proposal
- Laboratory Evaluation Checklist - DSP

620 Lesher Place • Lansing, MI 48912-1595
(517) 372-9200 • (800) 234-5333 • Fax: (517) 367-0514
www.neogen.com • e-mail: jrice@neogen.com

Validation Study of the Reveal ® 2.0 DSP Test for the Qualitative Detection of the Okadaic Acid group of toxins in Shellfish

Waqass Jawaid, Karrie Melville, Mark Hooper, Paul Muirhead, Frank Klein and Jennifer Rice*
Neogen Corporation
620 Lesher Place, Lansing, MI 48912 USA
*corresponding author: phone 517-372-9200, email jrice@neogen.com
Submitted June 2013

1. Introduction

Toxins that cause diarrhetic shellfish poisoning (DSP) include the okadaic acid (OA) group of toxins. OA is produced by marine dinoflagellates such as *Dinophysis*, and has structural analogues referred to as the dinophysistoxins (DTXs). The established European Union maximum permitted levels are 160 µg OA equivalents (OA, DTX1, DTX2, DTX3, pectenotoxins) per kg shellfish meat (160 ppb OA eqs). The U.S. Food and Drug Administration action limits are 160 ppb OA equivalents (OA, DTX1, DTX2, DTX3) in shellfish.

LC-MS/MS methods [1] have been accepted as quantitative reference methods in many parts of the world. Assays facilitating more rapid determination of OA toxins 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 2.0 DSP test for qualitative detection of the OA group (OA, DTXs 1 - 3) in shellfish. Reveal 2.0 DSP is a lateral flow immunoassay designed for rapid determination of OA-group toxins at or greater than 160 ppb OA eqs. The test is easy to use and results can be obtained in less than 70 mins, including sample preparation. A preliminary screening result for free toxins (OA, DTX1, DTX2) is also possible to obtain within the first 25 mins, including sample preparation.

2. Principle of the Method

Reveal 2.0 DSP is a single-step, lateral flow device based on a competitive immunoassay format. In summary, the shellfish extract is wicked through a reagent zone, containing antibodies specific for OA-group toxins that have been conjugated to coloured particles. If the toxins are present in the sample, the toxin will be captured by the particle-antibody complex. The complex is then wicked onto a membrane which contains a stationary capture zone of a toxin-protein conjugate. This zone captures any uncomplexed toxin particle-antibody. Therefore, as the concentration of toxins in the sample increases, the test line intensity decreases. The membrane also contains a stationary control zone which always will form regardless of the level of toxins. Results are analyzed qualitatively as either positive or negative using Neogen's AccuScan® Pro Reader.

3. Intended Use

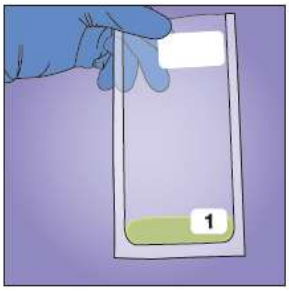
Reveal 2.0 DSP is a simple to use assay intended for the qualitative screening of shellfish for OA-group toxins at levels of 160 ppb or above. The test kit is designed for use by quality control personnel and other personnel who may be involved with handling shellfish possibly contaminated by OA toxins.

4. Reveal 2.0 DSP Method

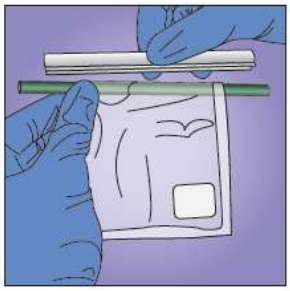
The kit insert is included as Appendix I which describes the rapid extraction method and the hydrolysis method. An overview of the rapid extraction method is shown below.

Rapid Screen Procedure

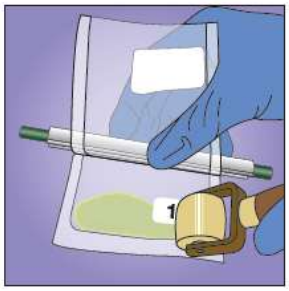
1. Number both sides of the extraction bag, one side is labelled **1** and opposite side labelled **2**



2. Weigh out 2 g (± 0.1 g) of homogenised sample and add to bottom of the extraction bag on side **1**. Add 8ml of analytical grade methanol into side **1** of extraction bag.



3. Fold the upper edge of the bag over the green straw and clip the white clip to prevent leakage of the sample.

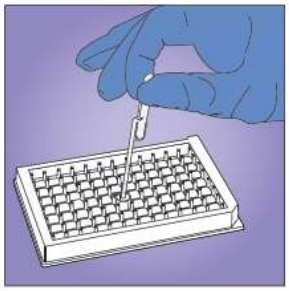


4. Press the roller firmly on the sample extraction bag, pushing the roller back and forth for **30 seconds to aid in obtaining a homogenous sample extract.**

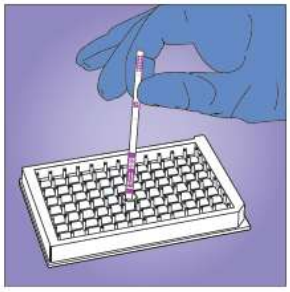
5. Remove straw and clip. Pour all bag contents from side 2 into a suitable container. Discard the used extraction bag.

6. Shake sample cup vigorously by hand for 30 seconds. Filter 1–2 mL of the sample into the collection test tube.

7. Remove 100 uL of the sample extract using a disposable pipettor, add into DSP buffer B vial and mix.



8. Transfer 100 uL of the diluted sample to a microwell.



9. Place the DSP strip into the well. Set a timer for **15 minutes.**

10. Remove promptly at 15 minutes and interpret results using the AccuScan® Pro Reader.

Reveal® 2.0 for DSP, an easy to use, one-step rapid test. Usable in the field as well as in the lab, with consistent interpretation of results using AccuScan® Pro Reader.

4.1. MATERIALS PROVIDED

Reveal 2.0 DSP (Neogen item 9561)

1. 24 Reveal 2.0 DSP lateral flow test strips
2. 24 microwells
3. 24 vials of DSP buffer A (for hydrolysis extraction protocol)
4. 24 vials of DSP buffer B (for rapid extraction protocol)
5. 25 extraction bags
6. 48 disposable exact volume (100 µl) pipettors

4.2. MATERIALS RECOMMENDED BUT NOT PROVIDED

1. Marine biotoxins starter kit (Neogen item 9563)
   - Microwell holder
   - 1 roller
   - 1 bag clip (white clip with green straw)
2. Filter syringes (Neogen item 9420)
3. Sample collection cups with lids (Neogen items 9428, 9428B)
4. Blender (Neogen items 9493, 9477 or 9495)
5. Scale capable of weighing 0.5 – 400 g ± 0.1g (Neogen item 9427)
6. Timer (Neogen item 9452)
7. Graduated cylinder, 10 mL
8. AccuScan® Pro reader (Neogen item 9565)

4.3. MATERIALS REQUIRED IF PERFORMING THE HYDROLYSIS EXTRACTION

1. Reveal 2.0 DSP hydrolysis accessory pack (Neogen item 9561), which contains
   - 2.5 M sodium hydroxide (NaOH) solution (5 mL)
   - 2.5 M hydrochloric acid (HCl) solution (5 mL)
2. Methanol (analytical grade recommended)
3. Pipettor, 100-1000 µL and pipette tips
4. Heater block capable of holding 76 ± 2 °C (recommended Stuart item SBH130D)
5. Glass sample vials (recommended Water's item 600000751CV)
6. Vortex (Capable of speeds of approximately 3000 rpm. E.g. Fisher Scientific item 11726477)

4.4. PRECAUTIONS

1. The test strips must remain inside the stay-dry tube before use.
2. Do not use kit contents beyond expiration date.
3. 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.
4. To avoid cross-contamination, use clean pipettes, extraction bags and fresh extraction solutions for each sample.
5. A Material Safety Data Sheet (MSDS) is available from Neogen Corp.

4.5. Storage Requirements

Store 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.

4.6. AccuScan® Pro Reader Set up

1. Enter the lot-specific QR code by selecting the QR code icon on the reader. Place the QR code into the cartridge and insert the cartridge into the reader.
2. Return to the home screen and select the test strip icon. Touch the Marine Biotoxins category, and then select the DSP test type.

4.7. Sample Preparation and Preliminary 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 the samples with distilled or deionized water, and allow any excess water to drain.
3. Homogenize the shellfish in a high-speed blender.
   NOTE: A good homogenate is essential in order to obtain an accurate result.
4. Number both sides of an extraction bag using a marker, so that one side is labelled “1” and the opposite side labelled “2”. Note: The extraction bag contains a mesh filter which allows for partial filtration of the sample. All samples should only ever be added to side “1”
5. Weigh 2 g (± 0.1 g) of homogenized sample in the bottom of the extraction bag on side “1”
6. Add 8 mL of methanol to side “1” of the extraction bag containing the sample.
7. Ensuring that the sample and methanol remain in the lower half of the extraction bag, position and hold the green straw approximately half-way down from te top of the bag. Fold the upper edge of the bag over the green straw. Firmly clip on the white clip to prevent leakage of the sample.
8. Place the extraction bag on a firm surface and 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.
9. Slide out the green straw and remove the white bag clip
10. Remove the bag contents from side “2” into a suitable container. Discard the used extraction bag.
11. Pour the sample extract into the barrel of a filter syringe until it is approximately half full. Place the plunger on top and filter approximately 1 mL of the sample into a collection tube. IMPORTANT: The filtered solution should be transparent and not cloudy. Should the filter syringe block or should the filtered extract not be clear, pour the unfiltered contents into a fresh syringe filter to ensure a clear solution.

Proceed to the extraction procedure(s) of interest (i.e. Rapid and/or Hydrolysis)

4.8. Extraction Procedure – Rapid (to detect OA, DTX1, DTX2)

1. Remove 100 µl of the sample extract using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and add into a DSP buffer B vial.

4.9. Extraction Procedure – Hydrolysis (to detect OA, DTX1, DTX2, DTX3)

1. Switch on heater block to 76 °C
2. Ensuring that the filtered extract is well-mixed, transfer 800 µL into a glass vial.
3. Add 100 µL of NaOH (2.5 M) and cap tightly. Mix using a vortex on full speed for 30 seconds.
4. Heat the vial in the heater block at 76°C for 40 mins
5. After 40 mins, remove the vial from the heater block and allow it to cool to room temperature. Alternatively, the sample vial can be placed in ice to cool faster,
6. Add 100 µL of HCl (2.5 M) and mix using a vortex on full speed for 30 seconds.
7. Remove 100 µl of the sample extract using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and add into a DSP buffer A vial.

4.10. Test Procedure

1. Remove the appropriate number of microwells and place into the microwell holder.
2. Shake the tube containing sample extract and buffer vigorously by hand or with a vortex on full speed for 30 seconds.
3. Remove 100 µL of the buffered sample extract and add to microwell.
4. Remove the required number of DSP strips from the lateral flow stay dry tube and immediately close the tube
5. Place the DSP strip with the sample end down into the well containing the filtered extract solution. Set a timer for 15 mins to allow the lines on the strip to develop.
6. After 15 mins, remove the test strip and immediately interpret the results using the AccuScan Pro reader.

4.11. Reading Test Results

1. Test strips should be read within 1 min of completion of the 15 min incubation. Refer to AccuScan® Pro Reader Set Up for test selection and set up information.
2. Fully insert the Reveal 2.0 DSP test strip into the black cartridge adapter with the sample end first and results facing out.
3. Insert the cartridge with test strip side up in the AccuScan® Pro. The reader will automatically begin analyzing the cartridge. CAUTION: Removing cartridge prior to completion can result in invalid readings.
4. The AccuScan® Pro reader will analyze the test strip and results will be displayed and stored in the reader. Results will be displayed on the screen of the AccuScan Pro as either positive or negative.

   Notes:

   Ensure device is fully inserted into cartridge.

   The strips must be read using Neogen's AccuScan® Pro reader.

5. Development Aims

The Reveal 2.0 DSP device and extraction methodology has been developed to ensure detection of OA, DTX1, DTX2 and DTX3 toxins. The method for extracting these toxins for the Reveal 2.0 DSP strip is based on the EU Harmonized Standard Operating Procedure [1]. During development, the rationale was to retain key steps from the accepted analytical method, whist aiming to ensure a relatively simple extraction procedure without the need for expensive equipment nor a high degree of expertise.

DTX2 is less toxic than OA and DTX1 and therefore is assigned a toxicity equivalence factor (TEF) of 0.6, as opposed to 1.0 (OA, DTX1). The theoretical maximum permitted level of DTX2 in a sample is therefore approximately 267 ppb. The perfect cross-reactivity to ensure the assay is fit for purpose for regulatory monitoring should detect samples containing 160 ppb OA/DTX1 and 267 ppb DTX2. In reality, however, there are no reported cases of DTX2 occurring in isolation to the authors' knowledge. DTX3 cannot be detected directly via analytical, enzyme-inhibition or immunoassays. The hydrolysis step was therefore required to ensure detection of DTX3 esters which may be present in samples. This alkaline hydrolysis converts DTX3 esters back into parent toxin forms (OA, DTX1 or DTX2), which can be detected.

6. Materials

Fresh shellfish (common mussels, king scallops, pacific oysters, surf clams) were obtained locally. Shellfish were shucked and tissue combined and homogenized in a blender. Shellfish were held refrigerated (2 - 8 ^{o}C) before use; or frozen (- 20 ^{o}C) if not used on the same day.

LC-MS/MS verifications were carried out on in-house preparations of shellfish homogenates to ensure samples were free of OA-group toxins, prior to spiking. Certified negative common mussel tissue, CRM-Zero-Mus (NRCC), was also used as the negative materials for spiked matrix studies.

Naturally contaminated / incurred shellfish homogenates were obtained from different countries (US, Canada, Europe).

Shellfish homogenates were aliquoted into 2.0 g amounts into filter extraction bags prior to spiking and/or extractions. Certified Reference Materials (CRMs) were obtained from the National Research Council, Canada (NRCC) for all spiked buffer and matrix studies. These included CRM-OA-c (17 µM), CRM-DTX1 (18.5 µM) and CRM-DTX2 (9.7 µM).

To ensure spiking and extraction efficiencies, the accepted analytical methodology (LC-MS/MS) has also been used to determine DSP toxin level for key studies (spiked or incurred). Unless otherwise stated, the analytical procedure employed followed the EU Harmonised Standard Operating Procedure for determination of OA-Group toxins by LC-MS/MS [1]

7. Methods Validation

7.1. Extraction efficiency of toxins

Accepted extraction methods require centrifugation and usually 0.2/0.45 uM filtration, which are not desired for a rapid LFD assay. Therefore studies were conducted to simplify accepted extraction methods without sacrificing the extraction recoveries of key toxins.

1. Study A (Oysters containing OA and DTX3 esters)

Four oyster samples known to contain high levels of DTX3 esters (> 90%) of OA were employed for the study. Sample extracts were quantified using analytical methods. Key changes of the LFD extraction is the absence of centrifugation and a lower sample:solvent ratio.

Results are shown in Table 1 and demonstrate good equivalence of results with a mean recovery of 102.2% (n = 4), indicating no clear impacts on recovery of OA/DTX3 from samples. The large standard deviations are most likely due to several reasons, including sample homogeneity and also that the LC-MS/MS and LFD extractions were carried out in two different laboratories in 2008 and 2012, respectively.

2. Study B (Mussels and Clams containing DTX1 and DTX3 esters)

Samples known to contain primarily DTX1 and DTX3 esters of DTX1 were employed for the study (mussels, clams). Extracts prepared using LC-MS/MS methodologies were compared to those prepared via the LFD extraction method (n=2 extractions per each condition). All extractions were carried out at the same site.

Results are shown in Table 1 and demonstrate a high level of equivalence with a mean LFD recovery of 114% (n = 5 samples). The mean LFD recovery for the three samples within the standard working range of LCMS was 105%.

Table 1. The extraction efficiencies of LFD extracts in comparison to the LCMS extracts.

Study	Sample	LCMS extract (ppb)	LFD extract (ppb)	Recovery (%)	Mean recovery (%)
Study A	**Sample 1**	190	130	68.4%	**102.2%**
	Sample 2	280	350	125.0%	
	Sample 3	410	230	56.1%	
	Sample 4	440	700	159.1%	
Study B	**Sample 5**	483	513	106.2%	**113.8%**
	Sample 6	822	866	105.4%	
	Sample 7	540	556	103.0%	
	Sample 8*	1382	1863	134.8%	
	Sample 9*	1983	2372	119.6%	
* outwith LCMS standard working range					

7.2. Sensitivity and cross-reactivity

7.2.1. Spiked Matrix

A spiked matrix study was conducted to confirm the sensitivity and cross-reactivity in a variety of key matrices (mussels, scallops, oysters and clams). Based on previous validations (Reveal DSP version 1) it was not expected to encounter any matrix effects due to the dilutions of shellfish employed. Shellfish were spiked at various levels of toxins (OA, DTX1, DTX2) ranging from 0 – 533 ppb.

Results

Results indicated a cross-reactivity profile deemed acceptable for regulatory screening and monitoring purposes with OA and DTX1 data indicating 100% accuracy at action limit levels, with DTX2 data indicating 100% accuracy containing approximately 1.5 times the action limits.

Okadaic acid (Table 2): 100% accuracy at correctly identifying negative samples spiked at 0 and 40 ppb was obtained (n=40). 95% accuracy at correctly identifying 60 ppb samples as negative was observed. At 60 ppb one out of five replicates for the scallop sample gave a positive response, all other conditions obtained a negative result. Samples containing 120 ppb OA generated a mix of positive and negative results (85% positive results). All matrices spiked at ≥ 140 ppb obtained a positive result.

DTX1 (Table 3): 100% accuracy at correctly identifying negative samples spiked at 0 and 40 ppb was obtained (n=40). At 100 ppb, the accuracy to correctly generate a negative result was 95% as one device obtained a positive result (scallops). 50% accuracy was observed at 120 ppb, with 95% accuracy to generate positive results at 140 ppb. All matrices containing ≥ 160 ppb DTX1 obtained a positive result.

DTX2 (Table 4): All blank samples provided a negative result (n=20). At 133 ppb, the accuracy to detect negatives was 95%, as one from five replicate Reveal 2.0 DSP devices generated a positive response (scallops). The overall accuracy to detect 267 ppb was 50%. All matrices evaluated at ≥ 400 ppb obtained a positive result.

Table 2: Dose response of Reveal 2.0 DSP with OA

	Species				
Level (ppb OA)	**Mussels**	**Oysters**	**Clams**	**Scallops**	**% positive**
0, 40	-	-	-	-	0
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
60	-	-	-	-	5
	-	-	-	-	
	-	-	-	+	
	-	-	-	-	
	-	-	-	-	
120	+	+	+	+	85
	+	-	+	+	
	+	-	+	+	
	+	-	+	+	
	+	+	+	+	
140, 160, 320	+	+	+	+	100
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	

Table 3: Dose response of Reveal 2.0 DSP with DTX1

	Species				
Level (ppb)	**Mussels**	**Oysters**	**Clams**	**Scallops**	**Overall % positive**
0, 40	-	-	-	-	0
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
100	-	-	-	-	5
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	+	
120	-	-	-	-	50
	+	+	-	-	
	+	+	-	+	
	+	+	-	+	
	-	+	-	+	
140	+	+	+	+	95
	+	+	+	+	
	+	+	+	+	
	-	+	+	+	
	+	+	+	+	
160, 320	+	+	+	+	100
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	

Table 4: Dose response of Reveal 2.0 DSP with DTX2

	Species				
Level (ppb)	**Mussels**	**Oysters**	**Clams**	**Scallops**	**Overall % positive**
0	-	-	-	-	0
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
133	-	-	-	-	5
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	+	
267	-	+	-	-	50
	-	+	-	+	
	+	+	+	+	
	+	-	-	+	
	-	-	-	+	
400, 533	+	+	+	+	100
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	

*Note: no data on clams at 400 ppb

7.3. Robustness

Methods

A single-laboratory validation study was conducted to measure robustness of the Reveal 2.0 DSP method. Bulk mussel homogenate was separated into 180 portions of 2.0 g each into extraction bags and spiked to prepare samples at levels ranging from 0 to 534 ppb (table 5).

Table 5: Sample preparation to fulfill Methods Robustness Protocol

Toxin	Level (ppb)	Number of samples prepared
Blank	0	18
OA	40	18
	120	18
	160 (action limits)	18
DTX1	40	18
	120	18
	160 (action limits)	18
DTX2	133	18
	267 (action limits)	18
	534 (2 * action limits)	18

Spiked samples were also sent for LC-MS/MS extraction and analysis to ensure levels of toxins were acceptable using the reference method. The results are shown in Table 6. LC-MS analysis of samples indicated that DTX1 (160 ppb) and OA (160 ppb) may be present at lower levels than intended with recoveries of 87% (139 ppb ± 36 ppb) and 77% (123 ppb ± 32 ppb), respectively. Based on the spiked matrix studies in the section above, the possibility of a low level of negative results with the LFD method were expected on these '160 ppb' samples.

Samples were randomized, with each operator provided 20 samples each day consisting of:

- 2 x blank
- 2 x OA at 40 ppb, 2 x OA at 120 ppb and 2 x OA at 160 ppb
- 2 x DTX1 at 40 ppb, 2 x DTX1 at 120 ppb and 2 x DTX1 at 160 ppb
- 2 x DTX2 at 133 ppb, 2 x DTX2 at 267 ppb and 2 x DTX2 at 534 ppb

Each sample was extracted according to the LFD hydrolysis procedure. Each extract was tested with one device each from three lots of Reveal 2.0 DSP LFDs. 60 test results were to be collected from each operator. In total, there were 3 operators testing over three days, which equated to a total of 540 test strips. However, due to errors in extraction (mainly operator 3, day 1), data from 12 sample extracts were not obtained.

Table 6: Results of assay method robustness for the Reveal 2.0 DSP test

					Number of positives (n = 3 lateral flows, one from each of three batches)			Total Positives (%)
Toxin	**Level ppb**	**LC-MS/MS analysis ppb (uncertainty at 26%)**	**Extraction**	**Operator**	**Day1**	**Day2**	**Day3**	
Blank	0	< LOD (< 25 ppb)	1	1	0	0	0	0
			2		0	0	0	
			1	2	0	0	0	
			2		0	0	0	
			1	3	0	0	0	
			2		No data	0	0	
OA	40	35	1	1	0	1	0	13
			2		0	1	0	
			1	2	1	0	0	
			2		2	0	0	
			1	3	No data	0	0	
			2		1	0	1	
	120	95	1	1	3	1	1	67
			2		1	0	2	
			1	2	3	0	3	
			2		2	3	3	
			1	3	No data	2	3	
			2		No data	2	3	
	160	123	1	1	3	3	3	98
			2		2	3	3	
			1	2	3	3	3	
			2		2	1	3	
			1	3	No data	3	3	
			2		No data	3	3	

Table 6: Results of assay method robustness for the Reveal 2.0 DSP test

					Number of positives (n = 3 lateral flows, one from each of three batches)			Total Positives (%)
Toxin	**Level ppb**	**LC-MS/MS analysis ppb (uncertainty at 26%)**	**Extraction**	**Operator**	**Day1**	**Day2**	**Day3**	
DTX1	40	36	1	1	0	0	0	6
			2		0	0	0	
			1	2	0	0	0	
			2		2	0	0	
			1	3	2	0	0	
			2		1	0	0	
	120	117	1	1	0	1	0	51
			2		0	1	0	
			1	2	3	3	3	
			2		3	3	2	
			1	3	No data	1	No data	
			2		No data	0	3	
	160	139	1	1	3	No data	1	94
			2		3	2	3	
			1	2	3	3	3	
			2		3	3	3	
			1	3	3	3	3	
			2		3	3	3	
DTX2	133	95	1	1	2	0	0	31
			2		1	0	0	
			1	2	3	2	1	
			2		2	0	2	
			1	3	No data	0	0	
			2		1	1	1	
	267	296	1	1	2	2	2	90
			2		3	3	3	
			1	2	3	3	3	
			2		3	3	3	
			1	3	No data	3	2	
			2		3	2	3	
	534	571	1	1	3	3	3	100
			2		3	3	3	
			1	2	3	3	3	
			2		3	3	3	
			1	3	3	3	3	
			2		No data	3	3	

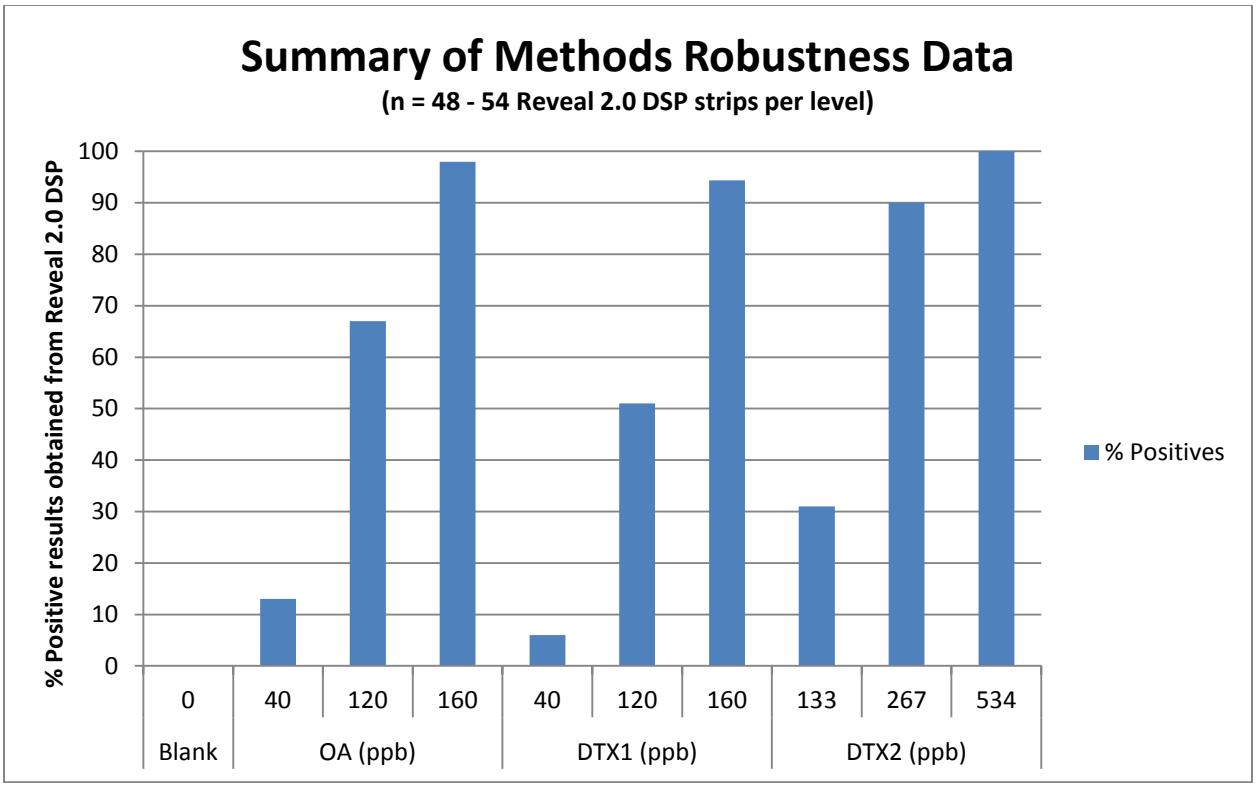


Figure 1. Summary of Reveal 2.0 DSP single laboratory robustness study

Results

Figure 1 shows a summary of the results. The study demonstrated that results were consistent across LFD lots, operators and days. The accuracy of the method to generate negative results from blank samples and positive results from samples containing 160 ppb (OA and DTX1) and 534 ppb (DTX2) was 98%. The accuracy of the assay to generate positive results with samples containing 267 ppb DTX2 was 90%. As expected, at 120 ppb OA eqs, both positive and negative results were obtained. The accuracy of samples containing 40 ppb of DTX1 and OA to generate negative results was 94% and 87%, respectively.

7.4. Post-robustness follow-up experiment

A follow-up experiment was carried out to gather more data at 40, 160 and 175 ppb OA or DTX1. The aim of this experiment was two-fold: (1) to determine whether the few false results obtained during methods robustness could be recreated when using certified negative material, and (2) to determine assay performance at toxin levels higher than the current regulatory limit. CRM-Zero-Mus samples were employed for the spiked matrix portion of this study. All samples were extracted and hydrolysed following the LFD protocol; and each extract was tested using three lateral flow devices. In addition, four naturally contaminated mussel samples were also evaluated with known levels of OA/DTX3 (around the regulatory limits), as characterized and quantified via a national reference laboratory (NRL) in the UK.

Table 7. Post-robustness study single operator study

Sample (Mussels)	Level (ppb OA eqs)	LFD results - positives (n = 3)
OA	40	0
	160	3
	175	3
	200	3
DTX1	40	0
	160	3
	175	3
	200	3
Incurred Sample A (OA/DTX3)*	80	2
Incurred Sample B (OA/DTX3)*	122	2
Incurred Sample C (OA/DTX3)	125	3
Incurred Sample D (OA/DTX3)	170	3

*Samples A and B negative when diluted 1:2 using negative mussel extracts

Results

Results are shown in Table 7. From the spiking data, at 40 ppb OA equivalents, all Reveal 2.0 DSP devices obtained a negative outcome; at 160 ppb OA equivalents and above, all devices were positive. Correlation between Reveal 2.0 DSP test results and levels of toxins measured in the naturally contaminated samples was good.

7.5. Naturally Contaminated / Incurred Samples (Neogen)

A total of 29 naturally contaminated samples were evaluated. These consisted of common mussel samples from the UK; native oysters (*Crassostrea virginica)*, clams and common mussels from the US; and common mussels and king scallop samples from Scotland (UK).

Samples were extracted and run according to hydrolysis LFD procedures. Analytical data was obtained via accepted analytical methods for most samples, as well as by PP2A*.

Results

Results are shown in Table 8. Samples containing OA equivalents at concentrations less than the limit of quantification by analytical means were all negative on the Reveal 2.0 DSP device. Samples quantified to contain ≤ 122 ppb OA equivalents obtained a negative result on the lateral flow device. All the remaining samples (≥ 125 ppb OA equivalents) were positive on Reveal 2.0 DSP.

* PP2A assay: OA is well known as a protein serine/threonine phosphatase inhibitor. Within a certain range, the inhibition of serine/threonine protein phosphatase type 2A (PP2A) is proportional to the concentration of OA and DTXs in solution [3]. Inhibition of this enzyme by OA standards of known concentration allows generation of a standard curve against which OA and DTXs in extracts from shellfish samples can be quantified in total OA equivalents. This method is based on the protein phosphatase inhibition assay using fluorescence substrates (FFPIA) first described by Vieytes *et. al.* (1997) [2]. The method incorporates a chemical clean-up procedure using solid phase extraction.

Table 8. Test of Naturally Incurred Samples

Sample Group	Sample Number	Quantitation Laboratory (LC-MS/MS unless otherwise stated)	Analytical Results (ppb OA equivalents)	Reveal 2.0 DSP Result + positive - negative
Mussels (UK) Various toxin profiles including OA, DTX1, DTX2 and DTX3 esters at different levels	Sample 1	EXTERNAL LAB 1	< LOD	-
	Sample 2	EXTERNAL LAB 1	81	-
	Sample 3	EXTERNAL LAB 1	122	-
	Sample 4	EXTERNAL LAB 1	125	+
	Sample 5	EXTERNAL LAB 1	170	+
	Sample 6	EXTERNAL LAB 1	269	+
	Sample 7	EXTERNAL LAB 1	336	+
	Sample 8	EXTERNAL LAB 1	386	+
	Sample 9	EXTERNAL LAB 1	415	+
	Sample 10	EXTERNAL LAB 1	569	+
	Sample 11	EXTERNAL LAB 1	569	+
	Sample 12	EXTERNAL LAB 1	618	+
	Sample 13	EXTERNAL LAB 1	< LOD	-
Oysters (US) Toxin profiles consisting of primarily OA, most of which as DTX3 esters (>90%).	Sample 14	EXTERNAL LAB 3	< LOD	-
	Sample 15	EXTERNAL LAB 3	190	+
	Sample 16	EXTERNAL LAB 3	376	+
	Sample 17	EXTERNAL LAB 3	410	+
	Sample 18	EXTERNAL LAB 3	< LOD	-
	Sample 19	EXTERNAL LAB 3	440	+
	Sample 20	EXTERNAL LAB 3	450	+
	Sample 21	EXTERNAL LAB 3	470	+
	Sample 22	EXTERNAL LAB 3	< LOD	-
Mussels and Clams (US) Toxin profiles consisting primarily of DTX1 and DTX3.	Sample 23 (clams)	Neogen	< LOD	-
	Sample 24 (clams)	EXTERNAL LAB 2	515	+
	Sample 25 (clams)	Neogen	< LOD	-
	Sample 26 (mussels)	EXTERNAL LAB 1	< LOD	-
	Sample 27 (mussels)	EXTERNAL LAB 2	524	+
	Sample 28 (mussels)	EXTERNAL LAB 2	944	+
	Sample 29 (mussels)	EXTERNAL LAB 2	~1700	+
	Sample 30 (mussels)	EXTERNAL LAB 2	~2400	+
Mussels and Scallops (Scotland)	Sample 31 (mussels)	Veromara (PP2A)	83-132	-
	Sample 32 (scallops)	Veromara (PP2A)	41-66	-
	Sample 33* (scallops)	Neogen	200	+
	Sample 34 (scallops)	Neogen	No detection	-

* Sample 33 contained OA & DTX2 (70% DTX3) as quantified by LCMS

7.6. Naturally Contaminated / Incurred Samples (External Lab)

A total of 40 naturally contaminated samples (mussels, scallops or oysters) were evaluated in a blind study. Samples were extracted and run according to both free toxin (unhydrolysed) and total toxin (hydrolysis) LFD procedures. Quantitative confirmatory data was obtained via accepted LCMS analytical methods for samples and/or by MBA.

Results

Results are shown in Table 9. Samples containing OA equivalents at concentrations less than the limit of quantification by analytical means were all negative. There were no false negative results with the hydrolysis procedure. The LFD generated two false positive results with respects to the action limits; samples 9 and 29, which were quantified to contain 130 and 83.7 ppb OA eqs, respectively. Sample 13 was negative by the free toxin LFD procedure and MBA, but positive with the hydrolysed LFD procedure and LCMS at 177 ppb OA eqs.
In addition, all positive results by the free toxin protocol were positive further to hydrolysis, as intended. This indicates that a screen can be completed effectively using the rapid extraction protocol with samples containing free toxins at levels significant to the action limits (without the requirement of testing hydrolysed extracts).

Table 9. LFD results in comparison to confirmatory reference methods (laboratory 2)

Sample	Sample Type	LFD Result (free toxins)	LFD Result (hydrolysis)	Confirmatory Result	Confirmatory Result (LCMS, where quantitation provided)
1	Mussel	Negative	Negative	Negative	151 ppb OA eqs
2	Mussel	Negative	Negative	Negative	149 ppb OA eqs
3	Mussel	Positive	Positive	Positive	180 ppb OA eqs
4	Mussel	Positive	Positive	Positive	189 ppb OA eqs
5	Mussel	Positive	Positive	Positive	176 ppb OA eqs
6	Mussel	Negative	Negative	Negative	Negative by LCMS
7	Mussel	Positive	Positive	Positive	181 ppb OA eqs
8	Mussel	Positive	Positive	Positive	176 ppb OA eqs
9	Mussel	Negative	Positive	Negative	130 ppb OA eqs
10	Mussel	Positive	Positive	Positive	176 ppb OA eqs
11	Mussel	Negative	Negative	Negative	61 ppb OA eqs
12	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
13	Scallops	Negative	Positive	Negative	DSP Negative by MBA ; ASP Positive LCMS: 177 ppb OA eqs OA eqs
14	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
15	Oyster	Negative	Negative	Negative	DSP Negative by MBA
16	Oyster	Negative	Negative	Negative	DSP Negative by MBA
17	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
18	Scallops	Negative	Negative	Negative	DSP Negative by MBA
19	Scallops	Negative	Negative	Negative	DSP Negative by MBA
20	Scallops	Negative	Negative	Negative	DSP Negative by MBA
21	Scallops	Negative	Negative	Negative	DSP Negative by MBA
22	Scallops	Negative	Negative	Negative	DSP Negative by MBA
23	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
24	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
25	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
26	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
27	Scallops	Negative	Negative	Negative	DSP Negative by MBA
28	Mussel	Positive	Positive	Negative	139.4 ppb OA eqs
29	Mussel	Negative	Negative	Negative	83.7 ppb OA eqs
30	Mussel	Positive	Positive	Positive	850 ppb OA eqs
31	Mussel	Positive	Positive	Positive	1141 ppb OA eqs
32	Mussel	Negative	Positive	Positive	215 ppb OA eqs
33	Mussel	Positive	Positive	Positive	2796 ppb OA eqs
34	Mussel	Positive	Positive	Positive	365 ppb OA eqs
35	Mussel	Positive	Positive	Positive	695 ppb OA eqs
36	Mussel	Negative	Positive	Positive	452 ppb OA eqs
37	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive
38	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive
39	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive
40	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive

7.7. Inter-laboratory evaluations

Three sites were provided six mussel samples (*Mytilus edulis*) randomized blind and carried out extractions in duplicate. Results are shown in table 10. Accuracy to generate negative results with samples containing < 40 ppb OA eqs and positive results with samples containing 170 ppb was 100%.

Table 10. Summary of inter-laboratory data

Sample	Level (OA eqs)	Expected result	LFD	Actual LFD result		
				Site 1	Site 2	Site 3
A	120 ppb	Neg or Pos	Test 1	Positive	Positive	Positive
			Test 2	Positive	Positive	Positive
B			Test 3	Positive	Negative	Negative
			Test 4	Positive	Positive	Negative
C	< 40 ppb	Negative	Test 5	Negative	Negative	Negative
			Test 6	Negative	Negative	Negative
D			Test 7	Negative	Negative	Negative
			Test 8	Negative	Negative	Negative
E	170 ppb	Positive	Test 9	Positive	Positive	Positive
			Test 10	Positive	Positive	Positive
F			Test 11	Positive	Positive	Positive
			Test 12	Positive	Positive	Positive

7.8. LFD lot comparability

A spiked buffer study was conducted as a means to determine accuracy and lot comparability. The following levels were tested across multiple cards of three LFD lots; buffer only (representing 0 ppb), OA and DTX1 at 40, 80, 120 and 160 ppb and DTX2 at 134, 267, 400 and 534 ppb (n = 9 LFDs per sample).

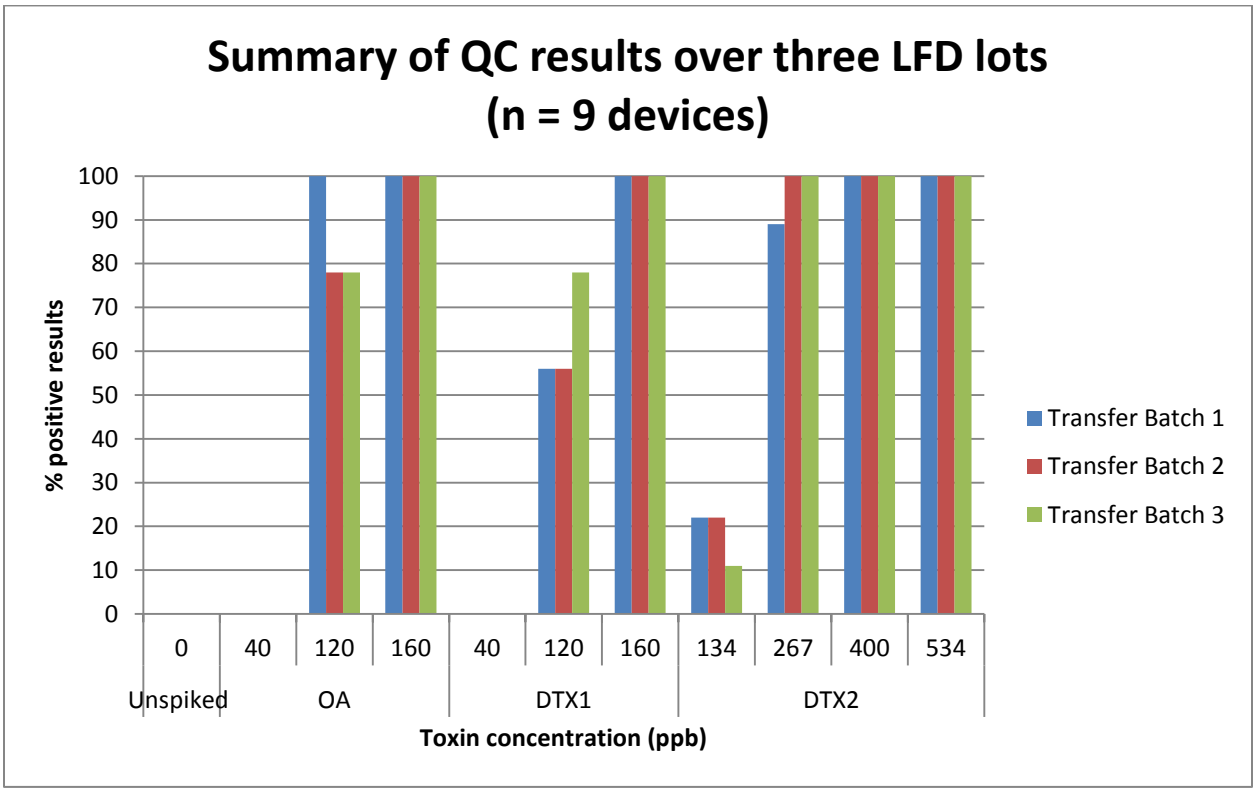


Figure 2. Summary of LFD lot comparability (n = 3 lots)

Results

Results are shown in Figure 2. The overall accuracy to generate negative results at 0 and 40 ppb OA eqs, and positive results at 160 ppb OA eqs (OA/DTX1) as well as 400/533 ppb DTX2; was 100% (n = 162) across all three lots. The overall accuracy to detect DTX2 at 267 ppb was 96% (n = 27), as one test produced a borderline negative result with lot 1 devices.

7.9. Reveal 2.0 DSP Stability

An 18 month real time stability trial of the Reveal 2.0 DSP assay is currently in process. Briefly, test strips from three lots were evaluated at different conditions including room temperature (22 – 25 °C) and incubated (37°C). Room temperature strips were tested at weeks 0, 1, 2, 3, 4, 8, 12,16, 20 and 26 (month 6). Further testing time points are planned for months 8, 10, 12, 14, 16 and 18. The accuracy of the device to date to generate negative results at 0 ppb and positive results at 160 ppb (OA/DTX1) and 534 ppb (DTX2) is 100% across all three lots.

Using the Arrhenius equation* to predict the shelf life of an assay using accelerated data, the predicted shelf life at room temperature of Reveal 2.0 DSP is currently at least 8 months. Stability trials of all lots will continue until a final shelf life can be assigned.

*Arrhenius equation:
For every 10^{o}C temperature rise above the normal storage temperature, the stability at the higher temperature is multiplied x 2^{x} to give the predicted stability where x is the number of 10^{o}C increments above the planned storage temperature. Therefore if the planned storage temperature is 25°C, accelerated testing at 45^{o}C allows a predicted shelf life of x 2^{2} = 4.

7.10. Ruggedness

The following three parameters were evaluated using spiked mussel samples (hydrolysed and rapid extracts) using LFDs from the robustness study lots of devices (n = 3):

- Extract stability (RT) post extraction (up to approximately 240 mins)
- Sample volume in microwell (100 uL ± 10 uL)
- Run time (15 mins ± 2 mins)

Results
No impacts on accuracy (to generate negative results at 0 and 40 ppb and positive results at 160 ppb OA) were observed with any of the parameters evaluated.

7.12. Quality Control Testing

Quality control (QC) testing of manufactured lots of the Reveal 2.0 DSP assay is performed at both in-process and finished product stages. In-process QC consists of testing every card with negative buffers A and B (n= 2) to check for line intensity, line position and intra-lot variability between cards on both the test and control lines. For finished product testing, strips from various cards representative of the batch are selected for positive testing. The following positive samples are tested: OA (40, 160 ppb), DTX1 (40, 160 ppb) and DTX2 (267 and 534 ppb). To ensure maximal performance of batches all tests containing 40 ppb must produce a negative result and all tests containing 160 ppb must produce a positive result. It is acceptable for DTX2 at 267 ppb to produce borderline negative results, although the majority of results are positive. This is due to the fact the DTX2 always co-occurs with OA. The final stage of testing involves testing using shellfish extracts containing approximately 40 ppb OA and 160 ppb OA (both rapid and hydrolysis extracts) to ensure negative and positive results are obtained, respectively.

8. Discussion and Summary

The use of LFDs has been previously exploited for detection of marine biotoxins. However, two major weaknesses for end-users have been the cross reactivity profile and the subjective nature of the interpretation of results. Reveal 2.0 DSP was designed as a reader-based assay to eliminate any subjective element, with a positive cut off for OA group toxins in shellfish relevant to regulatory limits of 160 ppb OA eqs. Sample homogenates could be screened in 20 min for free toxins (including extraction and assay time) while waiting for hydrolysis. If positive, then the hydrolysed extract would not need to be screened. If negative, the hydrolysed extract would be screened post-hydrolysis to determine presence of esters. Substantial validation data demonstrated the occurrence of no false negative results with naturally contaminated samples (various toxin profiles and matrices) containing 160 ppb OA eqs or greater and no false positives with incurred samples containing up to approximately 80 ppb OA eqs.

A robust and rapid LFD has been reported which demonstrates practical, simple and accurate screening for shellfish contaminated with OA group toxins. The assay can be utilised as a potential early warning detection system for use within the shellfish industry including shipboard or remote locations, providing added protection of shellfish consumers. Results demonstrate a rapid single-step assay, which requires minimal materials, that provides the simplest OA extraction and detection system reported to date. It allows either/both free toxins and total toxins to be rapidly screened from the same original sample extracts. More importantly, the assay has demonstrated high performance characteristics with respect to accuracy, recovery, cross-reactivity, specificity, matrix effects, robustness, ruggedness, reproducibility and stability. There is also a significant potential for this method to be used in regulatory laboratories to replace the costly LC-MS/MS or MBA based tests that are routinely employed as part of national monitoring programmes.

References

1. Community Reference Laboratory for Marine biotoxins (CRLMB)., Agencia Española de Seguridad Alimentaria y Nutrición (AESAN). (2009). EU Harmonised Standard Operating Procedure for determination of OA-Group Toxins by LC-MS/MS. Version 1. http://www.aesan.msps.es/en/CRLMB/web/procedimientos_crlmb/crlmb_standard_operating_procedures.shtml
2. Vieytes, M. R., Fontal, O. I., Leira, F., Baptista de Sousa, J. M. V., and Botana, L. M. (1997) A Fluorescent Microplate Assay for Diarrheic Shellfish Toxins. *Anal. Biochem.* **248,** 258-264.

Acknowledgements
We thank Nate Banner, Sharon Graham, James Clarke, Steve Schadler and Frank Klein of Neogen for all their help throughout this study.

Proposal No. 13-113

SAMPLE PREPARATION AND PRELIMINARY 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 the samples with distilled or deionized water, and allow any excess water to drain.
3. Homogenize (e.g., blend, puree) the shellfish in a high-speed blender.
   NOTE: A good homogenate is essential in order to obtain an accurate result.
4. Number both sides of an extraction bag using a marker, so that one side is labeled "1" and the opposite side labeled "2."
   NOTE: The extraction bag contains a mesh filter which allows for partial filtration of the sample. All samples/solution should only ever be added to side "1".
5. Weigh out 2 g (± 0.1 g) of homogenized sample in the bottom of the extraction bag on side "1."
   IMPORTANT: Ensure the entire sample is at the bottom of the bag prior to next step.
6. Add 8 mL of analytical grade methanol to side "1" of the extraction bag containing the sample.
7. Ensuring that the sample and methanol remain in the lower half of the extraction bag, position and hold the green straw approximately half-way down from the top of the bag. Fold the upper edge of the bag over the green straw. Firmly clip on the white clip to prevent leakage of the sample.
8. Place the extraction bag on a firm surface and 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.
9. Slide out the green straw and remove the white bag clip.
10. Remove the bag contents from side "2" into a suitable container (there may be small pieces of shellfish remaining on side "1"). Discard the used extraction bag.
11. Ensuring the sample extract is well mixed, pour into the barrel of a Neogen filter syringe until it is almost half full. Place the plunger on top, and filter sample extract into a collection tube.
    IMPORTANT: The filtered solution should be transparent/clear and not cloudy. Should the filter syringe block or should the filtered extract not be clear, pour the unfiltered contents into a fresh syringe filter to ensure a clear solution.

NOTE: Use **Procedure 1 – Rapid screen (to detect OA, DTX-1 and DTX-2**) or **Procedure 2 – Hydrolysis (to detect OA, DTX-1, DTX-2 and DTX-3)**, prior to proceeding to the test procedure.

EXTRACTION PROCEDURE 1 – RAPID SCREEN (TO DETECT OA, DTX-1, DTX-2)

1. Remove 100 µL of the sample extract using a disposable pipettor* provided (or alternatively, by use of a standard pipettor), and add into a vial of DSP buffer B (white cap).
   *To use the disposable pipettors, firmly press the top bulb of the pipettor, insert the tip into the solution, slowly release the top bulb to draw up the sample extract. Excess volume (e.g., above 100 µL) will overflow into the lower bulb, ensuring 100 µL is ready to dispense. Press the top bulb firmly and release slowly to dispense. Discard the used pipettor.
2. Proceed to **Test procedure**.

EXTRACTION PROCEDURE 2 – HYDROLYSIS (TO DETECT OA, DTX-1, DTX-2, DTX-3)

1. Switch on heater block to 76°C.
2. Ensuring that the filtered extract is well-mixed, transfer 800 µL into a glass vial.
3. Add 100 µL of NaOH (2.5M) and cap tightly. Mix using a vortex on full speed for **30 seconds**.
4. Heat the vial in the heater block at 76°C for **40 minutes**.
5. After 40 minutes, remove the vial from the heater block and allow it to cool to room temperature. Alternatively, the sample vial can be placed in ice to cool faster.
6. Add 100 µL of HCl (2.5M) and mix using a vortex on full speed for **30 seconds**.
7. Remove 100 µL of the sample extract and add into a vial of DSP buffer A (gray cap).
8. Proceed to **Test procedure**.

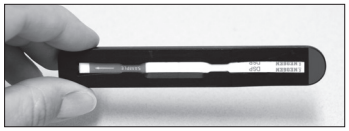
TEST PROCEDURE

1. Remove the appropriate number of microwells and place into the microwell holder.
2. Shake the vial containing sample extract and buffer vigorously by hand or with a vortex on full speed for **30 seconds**.
3. Remove 100 µL of the diluted sample extract and add to a microwell.
4. Remove the required number of DSP strips from the lateral flow stay dry tube and immediately close the tube.
5. Place the DSP strip with the sample end down into the well containing the filtered extract solution. Set a timer for **15 minutes** to allow the lines on the strip to develop.
6. After 15 minutes, remove the test strip and immediately interpret the results using AccuScan Pro reader.

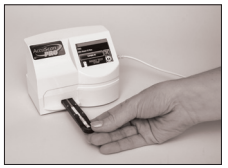
READING TEST RESULTS

Test strips should be read within **1 minute** of completion of the 15 minute incubation. Refer to **AccuScan Pro Reader Set Up** for test selection and set up information.

1. Fully insert the Reveal 2.0 for DSP test strip into the black R cartridge adapter with the sample end first and results facing out.



2. Insert the cartridge with test strip side up in the AccuScan Pro. The reader will automatically begin analyzing the cartridge. **CAUTION:** Removing cartridge prior to completion can result in invalid readings.
3. The AccuScan Pro reader will analyze the test strip and results will be displayed and stored in the reader.



NOTES

1. Ensure device is fully inserted into cartridge.
2. Readings should be made between **15–16 minutes**. Readings after 16 minutes may be inaccurate due to overdevelopment of the device.
3. The strips must be read using Neogen's AccuScan Pro reader.

PERFORMANCE CHARACTERISTICS

1. Reveal DSP is designed to screen for OA group toxins (OA and DTXs) in shellfish.

VALIDATED MATRICES

Mussels, scallops, oysters, clams and cockles.

NOTE: Neogen continues to validate new commodities. Please contact a representative for the latest validated commodity list.

CUSTOMER SERVICE

Neogen Customer Assistance and Technical Services can be reached by using the contact information on the back of this booklet. Training on this product, and all Neogen test kits, is available.

MSDS INFORMATION AVAILABLE

Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's website at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY

Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

NEOGEN® CORPORATION

North America
Neogen Headquarters
620 Lesher Place, Lansing, MI 48912 USA
800/234-5333 (USA/Canada) or 517/372-9200
Fax: 517/372-2006 • foodsafety@neogen.com
www.neogen.com

Mexico
Neogen Latinoamérica
Darwin No. 83, Col. Anzures, México, 11590 D.F.
(55) 5254-8235, (55) 5203-0111, (55) 5531-2837
Fax: (55) 5531-1647
informacion@neogenlac.com • www.neogen.com

Europe, Middle East and Africa
Neogen Europe
The Dairy School, Auchincruive, Ayr
KA6 5HW Scotland, UK
+ 44 (0) 1292 525 600
Fax: + 44 (0) 1292 525 601
info_uk@neogeneurope.com
www.neogeneurope.com

Brazil
Neogen do Brasil
Rua: Alberto Guizo 760, Distrito Industrial João Narezzi, Indaiatuba – SP Brasil, Cep: 13.347-402
Tel: +55 19 3935.3727
info@neogendobrasil.com.br • www.neogen.com

NE1590 R2.0_DSP_0113

Product 9561

Read instructions carefully before starting test

Reveal® 2.0 for DSP

For use with the Reveal AccuScan® Pro reader

Store at 18–30°C (64–86°F) • Do not freeze.

THE TOXINS

Toxins that cause diarrhetic shellfish poisoning (DSP) include the okadaic acid (OA) group of toxins. OA is produced by marine dinoflagellates such as *Dinophysis*, and has structural analogs referred to as the dinophysistoxins (DTXs). Clinical toxicological effects attributed to DSP following consumption of contaminated seafood includes diarrhea, nausea and vomiting. Human cases have been reported since the early 1960s in Norway and elsewhere on a global scale. The established European Union maximum permitted levels are 160 µg OA equivalents (OA, DTXs, pectenotoxins) per kg shellfish meat (160 ppb). The U.S. Food and Drug Administration action limits are 160 µg (160 ppb) OA equivalents (OA, DTXs) in shellfish.

INTENDED USE

Reveal 2.0 for DSP is an immunochromatographic lateral flow assay used for the rapid and practical qualitative analysis of shellfish possibly contaminated by OA group toxins (OA, DTX-1, DTX-2, and DTX-3). The test can detect as little as 160 ppb of OA equivalents in shellfish samples.

INTENDED USER

The test kit is designed for use by quality control personnel and other personnel familiar with handling shellfish possibly contaminated by OA toxins.

ASSAY PRINCIPLES

Reveal 2.0 for DSP is a single-step lateral flow device based on a competitive immunoassay format. In summary, the shellfish extract is wicked through a reagent zone, containing antibodies specific for OA-group toxins that have been conjugated to colored particles. If the toxins are present in the sample, the toxin will be captured by the particle-antibody complex. The complex then is wicked onto a membrane, which contains a stationary capture zone of a toxin-protein conjugate. This zone captures any uncomplexed toxin particle-antibody. Therefore, as the concentration of toxins in the sample increases, the test line intensity decreases. The membrane also contains a stationary control zone which always will form regardless of the level of toxins.

STORAGE REQUIREMENTS

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

MATERIALS PROVIDED

Reveal 2.0 for DSP (Neogen item 9561)

1. 24 Reveal 2.0 for DSP lateral flow test strips
2. 24 wells
3. 24 vials of DSP buffer A (gray cap)
4. 24 vials of DSP buffer B (white cap)
5. 25 extraction bags
6. 48 disposable 100 µL pipettors

MATERIALS RECOMMENDED BUT NOT PROVIDED

1. Marine Biotoxins Starter Kit (Neogen item 9563)
   - Microwell holder
   - 1 roller
   - 1 bag clip (white clip and green straw)
2. Distilled water
3. Methanol (analytical grade recommended, VWR 20864.320)
4. Filter syringes (Neogen item 9420)
5. Sample collection cups with lids (Neogen items 9428, 9428B)
6. Blender (Neogen items 9493, 9477 or 9495)
7. Scale capable of weighing 0.5–400 g ± 0.1 g (Neogen item 9427)
8. Timer (Neogen item 9452)
9. Graduated cylinder, 50 mL (Neogen item 9447)
10. Sample collection tubes with caps, 5 mL (Neogen item 9421, 9421B)
11. AccuScan Pro reader (Neogen item 9565)

MATERIALS REQUIRED IF PERFORMING A HYDROLYSIS EXTRACTION

1. DSP Hydrolysis Pack (Neogen item 9554)
   - 2.5 M sodium hydroxide (NaOH) solution (5 mL)
   - 2.5 M hydrochloric acid (HCl) solution (5 mL)
2. Pipettor, 100–1000 mL, and pipette tips (Neogen item 9463, 9464)
3. Heater block (capable of holding 76 ± 2°C (recommended Stuart, SBH130D)
4. Glass sample vials (Recommended Water's P/N 600000751CV)
5. 2.5 M NaOH and 2.5M HCL solutions (Neogen item 9561)
6. Vortex (~ 3000 rpm, recommended Fisher Scientific 11726477)

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.

ACCUSCAN PRO READER SET UP

1. Enter the lot-specific QR code by selecting the QR code icon on the reader. Place the QR code into the cartridge and insert the cartridge into the reader. **NOTE**: For instructions on manually entering sample IDs, see the AccuScan Pro user manual.
2. Return to the home screen and select the test strip icon. Touch the **Marine Biotoxins** category, then select the **DSP** test type.

MATERIAL SAFETY DATA SHEET

Section 1. Company Identification and Product Information			
Product Name or Identity:	**Reveal® 2.0 for DSP**		
Manufacturer's Name:	**Neogen Europe, Ltd.**	**Fax No.:**	UK, 01292 525 601 International: ++44 (0) 1292 525 601
	The Dairy School	**Phone No.:**	UK, 01292 525 600 International: ++44 (0) 1292 525 600
	Auchincruive, Ayr, KA6 5HW, Scotland, UK	**e-mail:**	info@neogeneurope.com
Date Prepared or Revised: February 2013		**Chemtrec: (800) 424-9300** **Outside US and Canada: (703) 527-3887**	

Section 2. Composition / Information on Hazardous Ingredients			
This product is a mixture of the substances listed below with the addition of nonhazardous materials.			
Hazardous Components Specific Chemical Identity:	**CAS-No.**	**%**	**Hazard Symbol**
This product contains no hazardous constituents, or the concentration of all chemical constituents are below the regulatory threshold limits described by Occupational Safety Health Administration Hazard Communication Standard 29 CFR 1910.1200 and the European Directive 91/155/EEC, and 93/112/EC.	NA	NA	NA

Section 3. Health Hazard Identification	
Health Hazards: *(Acute and Chronic)*	**Information pertaining to particular dangers for man and environment.** When used and handled according to specifications, the product does not have harmful effects according to the information provided to us. May cause minor irritation of the eyes and skin.

Section 4. First Aid Measures	
Emergency / First Aid Procedures:	**Ingestion:** If swallowed, seek medical attention immediately. Wash out mouth with water, provided person is conscious. Show physician product label.
	Inhalation: If inhaled, supply fresh air or oxygen. Seek medical attention if breathing is labored or becomes difficult. If not breathing, apply artificial respiration.
	Eye Contact: Rinse opened eye for at least 15 minutes under running water, lifting lower and upper eyelids occasionally. Seek medical attention.
	Skin Contact: Remove contaminated clothing. Immediately wash with plenty of soap and water for at least 15 minutes. Seek medical attention if irritation develops. Wash clothing before reuse.

Section 5. Fire and Explosion Hazard Data	
Flash Point (Method Used): N/A	**Flammable Limits**: LEL – N/A UEL – N/A
Extinguishing Media: Use alcohol foam, dry chemical, or carbon dioxide. Water may be ineffective.	
Protective Equipment: Firefighters should wear protective equipment and self-contained breathing apparatus.	
Unusual Fire and Explosion Hazards: During heating or in case of fire, poisonous gases are produced. Fine dust dispersed in air in sufficient concentrations, and in the presence of an ignition source, is a potential dust explosion hazard.	

Section 6. Accidental Release Measures

Personal Precautions: Wear self-containing breathing apparatus, rubber boots, and heavy rubber gloves. Place contaminated material in a chemical waste container.

Environmental Precautions: Prevent dispersion of material. Wipe up with damp sponge or mop.

Clean-up Methods: Contact safety officer if questions arise and ventilate area.

Refer to Section 7 for Handling Information.

Refer to Section 8 for Person Protection Equipment.

Refer to Section 13 for Disposal Information.

Section 7. Handling and Storage

Handling: Protect against physical damage. Ensure good ventilation / exhaustion and do not breathe vapor. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

Storage: Keep container tightly closed. Keep away from heat, sparks, flame and incompatible material. Storage area should be cool, dry, and away from incompatible materials. Containers of this material may be hazardous when empty since they retain product residues. Store at 18 - 30˚C.

Other Precautions: N/A

Section 8. Exposure Controls / Personal Protection

Components with limit values that require monitoring: Not Applicable

OSHA–PEL: N/A	**TLV:** N/A

Additional Information: Personal Protection listed below are general requirements for laboratory personnel. Follow the usual precautionary measures for handling chemicals / powder. Avoid contact with eyes, skin, and clothing.

Personal Protective Equipment:
Keep away from food, beverages, and feed.
Wash hands before and after entering laboratory.

Breathing Equipment: In case of brief exposure, use a chemical fume hood or a NIOSH/MSHA-approved respiratory.

Hand Protection: Use chemical resistant gloves.

Eye Protection: Wear safety glasses.

Body Protection: Wear lab coat or other protective work clothing.

Section 9. Physical and Chemical Properties

Appearance and Odor: N/A

Boiling Point: Not determined

Melting Point: Not determined

Density: Not determined

Section 10. Stability and Reactivity

Stability:	Unstable		
	Stable	X	Conditions to Avoid: Stable under normal storage conditions.

Incompatibility *(Materials to Avoid)***:** None known.

Hazardous Decomposition or Byproducts: Carbon dioxide (CO_2), Carbon monoxide (CO), or Nitrogen oxides (NOx).

Hazardous Polymerization:	May Occur		Conditions to Avoid: No dangerous reactions known.
	Will Not Occur	X	

NEOGEN CORPORATION

Section 11. Toxicological Information

LD/LC50 values that are relevant:
Acute Toxicity: When used and handled according to specifications and according to information provided for us, this product is not known to be toxic or hazardous at use concentrations.
Carcinogenicity Classification: Not Applicable
IARC (International Agency for Research on Cancer) – Not Listed
NTP (National Toxicology Program) Not Listed

Chronic: Prolonged or repeated skin contact may cause dermatitis.
Additional toxicological information: Any toxin(s) present in this kit are at concentration levels below the regulatory threshold limits which require registration under the Select Agent Program in as detailed in 42 CFR Part 73, 9 CFR Part 121, and 7 CFR Part 331.

Section 12. Ecological Information

Ecotoxicity Tests: The ecological effects have not been thoroughly investigated, but currently none have been identified.

Section 13. Disposal Considerations

Waste Disposal Method: Dispose in accordance with all applicable federal, state, and local environmental regulations.
RCRA P-Series: None listed.
RCRA U-Series: None listed.
Contact a licensed professional waste disposal service to dispose of this material if questions arise.

Container Information: Do not remove labels from containers until they have been cleaned.

Section 14. Transport Information

DOT Regulations: Not Regulated

Land Transport ADR/RID (cross-border): Not Regulated

Maritime Transport IMDG: Not Regulated

Air Transport ICAO-TI and IATA-DGR: Not Regulated

Section 15. Regulatory Information

EU Regulations, Hazard Symbol(s): N/A

Section 16. Other Information

This document is believed to be correct, but does not purport to be all inclusive and shall be used only as a guide. Neogen Corporation shall not be held liable for any damage resulting from handling or from contact with the above product. These suggestions should not be confused with state, municipal or insurance requirements, and constitute NO WARRANTY.

Read full Reveal 2.0 for DSP (Neogen item 9561) kit instructions carefully before starting test

Reveal® 2.0

DSP Hydrolysis Pack

INTENDED USE
DSP Hydrolysis Pack is intended to be used as an accessory to the Reveal 2.0 for DSP test kit for the extraction of OA and DTXs in shellfish samples.

MATERIALS PROVIDED

DSP Hydrolysis Pack (Neogen item 9554)

1. 2.5 M sodium hydroxide (NaOH) solution (5 mL)
2. 2.5 M hydrochloric acid (HCl) solution (5 mL)

STORAGE
DSP Hydrolysis Pack may be stored at room temperature but should not be frozen or exposed to high temperatures (> 90°F) for more than two weeks. For best results, do not store in direct sunlight.

DISPOSAL
Dispose in accordance with all applicable federal, state and local environmental regulations.

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Services can be reached by using the contact information on the back of this booklet. Training on this product, and all Neogen test kits, is available.

Proposal No. 13-113
Product 9554

Read full Reveal 2.0 for DSP (Neogen item 9561) kit instructions carefully before starting test

Reveal® 2.0

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DSP Hydrolysis Pack (Neogen item 9554)

1. 2.5 M sodium hydroxide (NaOH) solution (5 mL)
2. 2.5 M hydrochloric acid (HCl) solution (5 mL)

STORAGE
DSP Hydrolysis Pack may be stored at room temperature but should not be frozen or exposed to high temperatures (> 90°F) for more than two weeks. For best results, do not store in direct sunlight.

DISPOSAL
Dispose in accordance with all applicable federal, state and local environmental regulations.

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Services can be reached by using the contact information on the back of this booklet. Training on this product, and all Neogen test kits, is available.

MSDS INFORMATION AVAILABLE

Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's website at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY

Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

North America
Neogen Headquarters
620 Lesher Place, Lansing, MI 48912 USA
800/234-5333 (USA/Canada) or 517/372-9200
Fax: 517/372-2006 • foodsafety@neogen.com
www.neogen.com

Europe, Middle East and Africa
Neogen Europe
The Dairy School, Auchincruive, Ayr
KA6 5HW Scotland, UK
+ 44 (0) 1292 525 600
Fax: + 44 (0) 1292 525 601
info_uk@neogeneurope.com
www.neogeneurope.com

Mexico
Neogen Latinoamérica
Darwin No. 83, Col. Anzures, México, 11590 D.F.
+52 (55) 5254-8235, +52 (55) 5203-0111,
+52 (55) 5531-2837
Fax: +52 (55) 5531-1647
informacion@neogenlac.com • www.neogen.com

Brazil
Neogen do Brasil
Rua: Alberto Guizo 760, Distrito Industrial João
Narezzi, Indaiatuba – SP Brasil, Cep: 13.347-402
Tel: +55 19 3935.3727
info@neogendobrasil.com.br • www.neogen.com

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Europe, Middle East and Africa
Neogen Europe
The Dairy School, Auchincruive, Ayr
KA6 5HW Scotland, UK
+ 44 (0) 1292 525 600
Fax: + 44 (0) 1292 525 601
info_uk@neogeneurope.com
www.neogeneurope.com

Mexico
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+52 (55) 5254-8235, +52 (55) 5203-0111,
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Tel: +55 19 3935.3727
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NE1809 DSP Hydrolysis Pack_0113

SAFETY DATA SHEET

Section 1. Identification of the Substance/Mixture and of the Company/Undertaking			
1.1 Product Identifiers:	**DSP Hydrolysis Pack** Product #9554		
1.2 Relevant Identified Uses of the Substance or Mixture and Uses Advised Against		Identified Uses: Laboratory chemicals	
1.3 Details of the Supplier of the Safety Data Sheet	**Neogen Europe, Ltd.**	**Fax No.:**	UK, 01292 525 602 International: ++44 (0) 1292 525 601
	The Dairy School	**Phone No.:**	UK, 01292 525 610 International: ++44 (0) 1292 525 600
	Auchincruive, Ayr, KA6 5HW, Scotland, UK	**e-mail:**	info@neogeneurope.com
1.4 Emergency Telephone Number			
Date Prepared or Revised: February 2013		**Chemtrec (US): (800) 424-9300** **Outside US and Canada: (703) 527-3887** **Poison Control (UK): 08454 24 24 24**	

Section 2. Hazards Identification		
2.1 Classification of the Substance or Mixture		
Specific Chemical Identity:	**Regulation (EC) No 1272/2008**	**EU Directives 67/548/EEC or 1999/45/EC**
Sodium Hydroxide Solution	-Skin corrosion (Category 1A)	R35: Causes severe burns
Hydrochloric Acid Solution	-Skin corrosion/Irritation (Category 2) -Serious eye damage/Eye irritation (Category 2) -Specific target organ systemic toxicity (single exposure) (Category 3)	R36/37/38: Irritating to the eyes, respiratory system and skin.
2.2 Label Elements	**Labelling according to Regulation (EC) No 1272/2008 [CLP]**	
Pictogram		
Signal Word	Danger	
Hazard Statements	H314: Causes severe skin burns and eye damage H335: May cause respiratory irritation	
Precautionary Statements	P280: Wear protective gloves/protective clothing/eye protection/face protection. P305/351/338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P310: Immediately call a POISON CENTER or doctor/physician.	
Other Hazards	None known	

Section 3. Composition/Information on Ingredients		
3.2 Mixtures:	*(For the full text of the H-Statements and R-phrases mentioned in this Section, see Section 16.)*	
Components		**Classification**
Sodium hydroxide solution Concentration: 62%	CAS No. 1310-73-2 EC No. 215-185-5 Index No. 011-002-00-6	Skin Corr. 1A; H314 C; R35
Hydrochloric acid solution Concentration: 50%	CAS No. 7647-01-0 EC No. 231-595-7 Index No. 017-002-01-X	Skin Corr. 1B; H314; STOT SE 3; H335 C; R34; X1; R37

Section 4. First Aid Measures	
4.1 Description of First Aid:	**Ingestion:** DO NOT INDUCE VOMITING. If swallowed, seek medical attention immediately. Wash out mouth with water, provided person is conscious. Show physician product label.
	Inhalation: If inhaled, supply fresh air or oxygen. Seek medical attention if breathing is laboured or becomes difficult. If not breathing, apply artificial respiration.
	Eye Contact: Rinse opened eye for at least 15 minutes under running water, lifting lower and upper eyelids occasionally. Immediate medical attention is required.
	Skin Contact: Remove contaminated clothing. Immediately wash with plenty of soap and water for at least 15 minutes. Seek medical attention immediately. Wash clothing before reuse.
4.2 Most Important Symptoms and Effects, Both Acute and Delayed	Burning sensation, cough, wheezing, laryngitis, shortness of breath, spasm, inflammation and oedema of the larynx, spasm, inflammation and oedema of the bronchi, pneumonitis, pulmonary oedema. Material is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes and skin.
4.3 Indication of Any Immediate Medical Attention and Special Treatment Needed	No data available

Section 5. Firefighting Measures
5.1 Extinguishing Media Suitable extinguishing media: Use alcohol-resistant foam, dry chemical, or carbon dioxide. Unsuitable extinguishing media: None known
5.2 Special Hazards Arising from the Substance or Mixture: Contact with metals may evolve flammable hydrogen gas. Thermal decomposition can lead to release of irritating gases and vapours.
5.3 Advice for Firefighters: Firefighters should wear protective equipment and self-contained breathing apparatus.

Section 6. Accidental Release Measures
6.1 Personal Precautions, Protective Equipment and Emergency Procedures: Use personal protective equipment. Place contaminated material in a chemical waste container. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas.
6.2 Environmental Precautions: Prevent dispersion of material. Do not let produce enter drains.
6.3 Methods and Materials for Containment and Cleaning Up: Soak up with inert absorbent material and dispose of as hazardous waste. Keep in suitable, closed containers for disposal. If questions arise contact safety officer.
6.4 Reference to Other Sections: For disposal see section 13.

Section 7. Handling and Storage
7.1 Precautions for Safe Handling: Wear appropriate personal protective equipment. Ensure good ventilation / exhaustion and do not breathe vapour or mist. Avoid contact with eyes, skin, and clothing.
7.2 Conditions for Safe Storage, Including Any Incompatibilities: Keep container tightly closed in a cool, well-ventilated area. Do not store in metal containers.
7.3 Specific End Uses: No data available

Section 8. Exposure Controls / Personal Protection
Components with limit values that require monitoring: Not Applicable
8.1 Control Parameters (Exposure Limits)
Sodium hydroxide solution: STEL: 2 mg/m^3 (UK EH40 WEL – Workplace Exposure Limits)
Hydrochloric acid solution: STEL: 8 mg/m^3 (UK EH40 WEL – Workplace Exposure Limits)
8.2 Exposure Controls **Appropriate Engineering Controls:** Personal Protection listed below are general requirements for laboratory personnel. Follow the usual precautionary measures for handling chemicals / liquid. Avoid contact with eyes, skin, and clothing. Wash hands before breaks and at the end of the workday. Ensure eyewash stations and safety showers are close to the workstation location.
Personal Protective Equipment: **Eye/Face Protection:** Tightly fitting safety goggles or face shield. Use equipment for eye protection tested and approved under appropriate government standards such as EN 166. **Skin and Body Protection:** Protective gloves that satisfy the specifications of EU Directive 89/686/EEC and the standard EN 374 derived from it. Use proper glove removal techniques to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands. Wear long-sleeved clothing. Wash contaminated clothing before reuse. **Respiratory Protection:** When workers are facing concentration above the exposure limit, they must use appropriate certified respirators.

Section 9. Physical and Chemical Properties		
9.1 Information on Basic Physical and Chemical Properties		
a) Appearance	Colourless liquid	
b) Odour	Hydrochloric Acid: Sharp, disagreeable odour	
c) Odour threshold		
	Sodium hydroxide solution	**Hydrochloric acid solution**
d) pH:	14.0	0.1
e) Freezing point:	-12 - 10°C	-17°C
f) Boiling point:	105 - 140°C	81. - 110°C
g) Flash point:	Not applicable, not flammable	Not applicable, not flammable
h) Evaporation rate	No information available	No information available
i) Flammability (solid, gas)	Not applicable, liquid	Not applicable, liquid
j) LEL/UEL	Not flammable	Not flammable
k) Vapour pressure	< 24 hPa at 20°C	160 mm Hg at 20°C
l) Vapour density	1.38 (Air = 1.0)	1.26 (Air = 1.0)
m) Relative density	1.327	1.05-1.12
n) Water solubility	Completely miscible, soluble	Soluble

Section 9. Physical and Chemical Properties *(cont'd)*		
9.1 Information on Basic Physical and Chemical Properties *(cont'd)*		
	Sodium hydroxide solution *(cont'd)*	**Hydrochloric acid solution** *(cont'd)*
o) Partition coefficient	No information available	No information available
p) Autoignition temperature	No information available	No information available
q) Decomposition temperature	No information available	No information available
r) Viscosity	No information available	No information available
s) Explosive properties	No information available	No information available
t) Oxidizing properties	No information available	No information available
9.2 Other information: No information available		

Section 10. Stability and Reactivity
10.1 Reactivity: No data available
10.2 Chemical Stability: Stable under normal conditions
10.3 Possibility of Hazardous Reactions: May react with metals and lead to form flammable hydrogen gas
10.4 Conditions to Avoid: Excess heat; incompatible substances
10.5 Incompatible Materials: Acids, aldehydes, aluminium, bases, chlorinated solvents, metals, organic materials, oxidizing agents, phosphorus, reducing agents, tin/tin oxide, zinc
Hazardous Decomposition Products: Hydrogen chloride gas

Section 11. Toxicological Information		
11.1 Information on Toxicological Effects		
	Sodium hydroxide solution	**Hydrochloric acid solution**
a) Acute toxicity	Oral, rat LD_{50}: >90 mL/kg	Oral, rat LD_{50}: 700 mg/kg
b) Skin corrosion/irritation	Skin, rabbit: 500 mg/24hr severe	No data available
c) Serious eye damage/irritation	Eye, rabbit: 1 mg/24hr severe	No data available
d) Respiratory or skin sensitisation	No data available	No data available
e) Germ cell mutagenicity	No data available	Mutagenic effects have occurred in experimental animals
f) Carcinogenicity	Not listed (IARC, ACGIH, NTP)	IARC Group 3
g) Reproductive toxicity	No data available	Experiments have shown reproductive toxicity effects on laboratory animals
h) STOT-single exposure	No data available	Skin, eyes
i) STOT-repeated exposure	No data available	Respiratory system, skin, eyes, gastrointestinal tract (GI), liver, kidney, blood, teeth
j) Aspiration hazard	No data available	No data available

Potential health effects

Inhalation: May be harmful if inhaled. Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract.

Ingestion: May be harmful if swallowed. Causes burns.

Skin: May be harmful if absorbed through the skin. Causes skin burns.

Eyes: Causes eye burns.

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Section 11. Toxicological Information *(cont'd)*
11.1 Information on Toxicological Effects *(cont'd)*
Signs and symptoms of exposure Burning sensation, cough, wheezing, laryngitis, shortness of breath, spasm, inflammation and oedema of the larynx, spasm, inflammation and oedema of the bronchi, pneumonitis, pulmonary oedema. Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract.

Section 12. Ecological Information
12.1 Toxicity: Hydrochloric Acid: Fresh water fish 282 mg/L LC_{50} 96h Sodium Hydroxide: No data available
12.2 Persistence and Degradability: No data available
12.3 Bioaccumulative Potential: No data available
12.4 Mobility in Soil: No data available
12.5 Results of PBT and vPvB Assessment: No data available
12.6 Other Adverse Effects: None known

Section 13. Disposal Considerations
13.1 Waste Treatment Methods: Product should be disposed of with a licensed waste disposal company, while following all regional, local and national regulations.

Section 14. Transport Information			
	ADR/RID	**IMDG**	**IATA**
14.1 UN Number	1760	1760	1760
14.2 UN Proper Shipping Name	Corrosive, liquid, n.o.s.	Corrosive, liquid, n.o.s.	Corrosive, liquid, n.o.s.
14.3 Transport Hazard Class	8	8	8
14.4 Packing Group	II	II	II
14.5 Environmental Hazards	No	Marine pollutant: No	No
14.6 Special Precautions for User	No information available		

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Section 15. Regulatory Information

15.1 Safety, Health and Environmental Regulations/Legislation Specific for the Substance or Mixture

International Inventories:

Hydrochloric Acid:

EINECS	ELINCS	TSCA	DSL	NDSL	PICCS	ENCS	CHINA	AICS	KECL
231-595-7	-	T	X	-	X	X	X	X	KE-20189

Legend:

EINECS/ELINCS – European Inventory Lists

TSCA – United States Toxic Substances Control Act Inventory, Section 8(b)

DSL/NDSL – Canadian Domestic Substances List/Non-Domestic Substances List

PICCS – Philippines Inventory of Chemicals and Chemical Substances

ENCS – Japan Existing and New Chemical Substances

CHINA – China Inventory of Existing Chemical Substances

AICS – Inventory of Chemical Substances

KECL – Existing and Evaluated Chemical Substances

15.2 Chemical Safety Assessment

No data available

Section 16. Other Information

H314: Causes severe skin burns and eye damage
H335: May cause respiratory irritation

R35: Causes severe burns
R37: Irritating to respiratory system
R36/37/38: Irritating to eyes, respiratory system and skin.

This safety data sheet complies with the requirements of Regulation (EC) No. 1907/2006.

This document is believed to be correct, but does not purport to be all inclusive and shall be used only as a guide. Neogen Corporation shall not be held liable for any damage resulting from handling or from contact with the above product. These suggestions should not be confused with state, municipal or insurance requirements, and constitute NO WARRANTY.