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# RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID

R. WAYNE LITAKER,[1]* THOMAS N. STEWART,[2] BICH-THUY L. EBERHART,[3] JOHN C. WEKELL,[3] VERA L. TRAINER,[3] RAPHAEL M. KUDELA,[4] PETER E. MILLER,[4] ALICE ROBERTS,[4] CASSANDRA HERTZ,[4] TYLER A. JOHNSON,[4] GREG FRANKFURTER,[5] G. JASON SMITH,[5] ASTRID SCHNETZER,[6] JOE SCHUMACKER,[7] JONNETTE L. BASTIAN,[7] ANTHONY ODELL,[8] PATRICK GENTIEN,[9] DOMINIQUE LE GAL,[10] D. RANSOM HARDISON[1] AND PATRICIA A. TESTER[1]

[1]*National Ocean Service, NOAA, 101 Pivers Island Road, Beaufort, North Carolina 28516;*
[2]*Mercury Science Inc., 2801 Blue Ridge Road, Suite G-70, Raleigh, North Carolina 27607;*
[3]*Northwest Fisheries Science Center, NOAA, 2725 Montlake Boulevard East, Seattle, Washington 98112;* [4]*Ocean Sciences & Institute for Marine Sciences, University of California Santa Cruz, 1156 High Street, Santa Cruz, California 95064;* [5]*Moss Landing Marine Laboratories, 8272 Moss Landing Rd, Moss Landing, California 95039;* [6]*Department of Biological Sciences, University of Southern California, 3616 Trousdale Parkway, AHF 301, Los Angeles, California 90089;* [7]*Quinault Indian Nation, Division of Natural Resources, Department of Fisheries, 1214 Aalis Drive Bldg C, Taholah, Washington. 98587;*
[8]*Olympic Region Harmful Algal Bloom Partnership, 14 North Shore Place, Hoquiam, Washington 98550;*
[9]*IFREMER- Centre de Brest, DYNECO, Pointe du Diable, BP 70, 29280 Plouzane, France;*
[10]*IFREMER, 13 Rue Kerose, 29900 Concarneau, France*

***ABSTRACT*** Domoic acid (DA) is a potent toxin produced by bloom-forming phytoplankton in the genus *Pseudo-nitzschia*, which is responsible for causing amnesic shellfish poisoning (ASP) in humans. ASP symptoms include vomiting, diarrhea, and in more severe cases confusion, loss of memory, disorientation, and even coma or death. This paper describes the development and validation of a rapid, sensitive, enzyme linked immunosorbent assay test kit for detecting DA using a monoclonal antibody. The assay gives equivalent results to those obtained using standard high performance liquid chromatography, fluorenylmethoxycarbonyl high performance liquid chromatography, or liquid chromatography—mass spectrometry methods. It has a linear range from 0.1–3 ppb and was used successfully to measure DA in razor clams, mussels, scallops, and phytoplankton. The assay requires approximately 1.5 h to complete and has a standard 96-well format where each strip of eight wells is removable and can be stored at 4°C until needed. The first two wells of each strip serve as an internal control eliminating the need to run a standard curve. This allows as few as 3 or as many as 36 duplicate samples to be run at a time enabling real-time sample processing and limiting degradation of DA, which can occur during storage. There was minimal cross-reactivity in this assay with glutamine, glutamic acid, kainic acid, epi- or iso-DA. This accurate, rapid, cost-effective, assay offers environmental managers and public health officials an effective tool for monitoring DA concentrations in environment samples.

***KEY WORDS:*** ASP, domoic acid poisoning, ELISA, mussels, scallops, razor clams, test kit

## INTRODUCTION

Domoic acid (DA) is a potent toxin produced by bloom-forming phytoplankton in the genus *Pseudo-nitzschia* (Fig. 1). It is a glutamate analog, which acts as a potent excitatory neurotransmitter and causes amnesic shellfish poisoning (ASP) in humans (Quilliam & Wright 1989, Quilliam et al. 1989b, Wright et al. 1989). Symptoms include vomiting, diarrhea, and in more severe cases confusion, loss of memory, disorientation, and even death. As a tricarboxylic acid, fully ionized at seawater pH, DA can behave as a potent trace metal ligand (Rue & Bruland 2001, Wells et al. 2005). DA can bioaccumulate and rapidly transvectors throughout the food chain *via* clams, mussels, crabs, filter feeding fish, and other organisms (Horner & Postel 1993, Scallet et al. 2005, Vigilant & Silver 2007). DA poisoning was first recognized after a lethal event on Prince Edward Island, Canada in 1987 (Wright et al. 1989). Since that time, a number of toxic events have occurred on the United States west coast where DA has been shown to commonly accumulate in the edible parts of razor clams (*Siliqua patula*), mussels (*Mytilus californianus* or *edulis*), and Dungeness crabs (*Cancer magister*) (Wekell et al. 1994, Horner et al. 1997). High levels of DA in razor clams in Oregon and Washington are responsible for beach closures that can last for more than a year. Losses of more than $20 million annually result from these closures caused by lost tourism and reduced recreational and commercial and tribal clam harvests (Adams et al. 2000). DA has also been implicated in the death and illness of brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) (Fritz et al. 1992, Work et al. 1993), California sea lions (*Zalophus californianus*) (Scholin et al. 2000, Trainer et al. 2000, Brodie et al. 2006), sea otters (*Enhydra lutris*) (Kreuder et al. 2003), and possibly whales (Lefebvre et al. 2002).

The regulatory method for DA detection sanctioned by the Interstate Shellfish Sanitation Conference (Quilliam et al. 1989a, Quilliam et al. 1995) is a high performance liquid chromatography (HPLC) assay (Quilliam et al. 1991, Hatfield et al. 1994). Though accurate, these analyses are generally run

*Corresponding author. E-mail: wayne.litaker@noaa.gov

by centralized state facilities with results typically not available for 3–14 days after the samples are collected. In more remote communities, many of which depend heavily on subsistence clam harvests, these long delays and the costs of sample analysis are causes for public health concern. The average cost of approximately $100 per sample limits the number of samples that can be analyzed (Harold Rourk, WA State Department of Health, pers. comm.). Resource managers in coastal communities have expressed their desire for a cost-effective method for rapid and accurate determination of DA concentrations in shellfish and phytoplankton samples. This paper describes the development and optimization of a robust monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) test kit for DA that will meet management needs for rapid detection of DA in environmental samples.

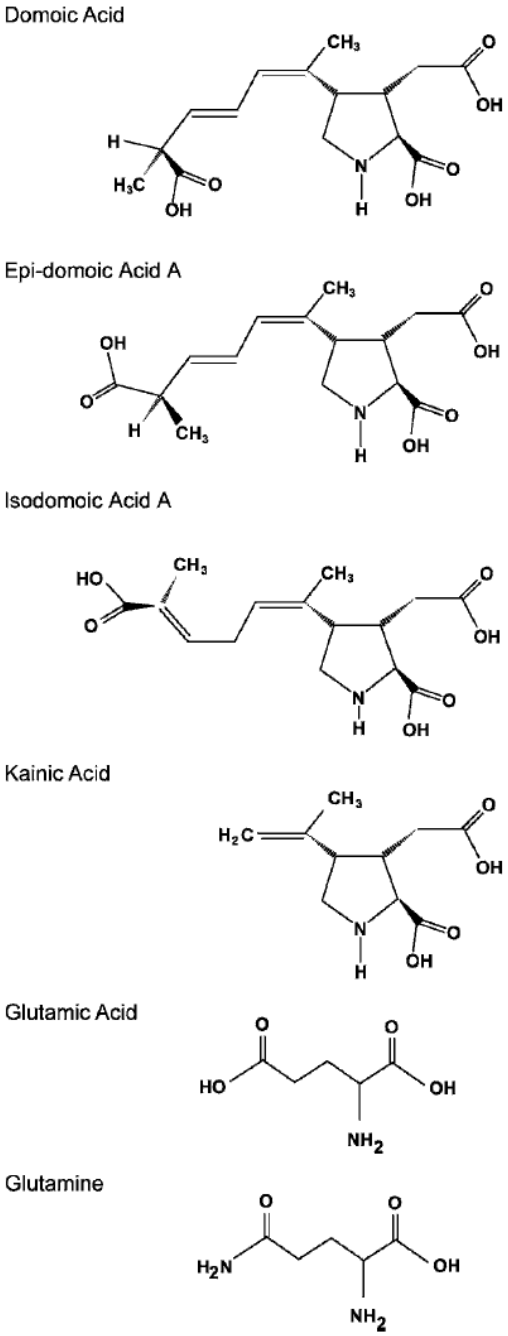


**Figure 1. Structure of domoic acid, the isomers epi-domoic acid, isodomoic acid, and two analogues kainic acid and glutamic acid.**

## MATERIALS AND METHODS

### *Assay Kit Overview*

The DA assay kit was developed jointly by NOAA's National Centers for Coastal Ocean Science, National Ocean Service, and the Northwest Fisheries Science Center, together with an industry partner Mercury Science, Inc., Durham, NC (NOAA/MSI). It was designed as a sequential competitive enzyme linked immunosorbent assay (ELISA) utilizing a high avidity monoclonal antibody (mAb) to DA to ensure assay specificity and consistency across production lots. In the current format, a fixed number of anti-DA mAb binding sites are incubated with dissolved DA in the sample followed by the addition of a DA—horseradish peroxidase (HRP) conjugate. As these binding events occur, the anti-DA mAb molecules are simultaneously captured by antimouse antibodies affixed to the surface of the microtiter plate wells. Subsequent HRP derived color development, readable on standard microplate readers, was inversely proportional to the concentration of DA in the sample matrix. The assay reagents were titrated so that the amount of mAb and the DA–HRP conjugate added produced a maximal absorbance signal of 3 absorbance units when no DA was present. The implementation of this ELISA system required the development and validation of two essential reagents, a high avidity monoclonal antibody to DA and a stable DA-HRP conjugate recognized by the same mAb.

### *Production of the Anti-Domoic Acid Antibody*

Domoic acid (Sigma-Aldrich, St. Louis, MO), was conjugated with bovine serum albumin (BSA) using dicyclohexyl carbodiimide and N-hydroxysuccinimide by a two-step synthetic pathway (Adamczyk et al. 1994). Ten mice were immunized with the DA-BSA immunogen. Serum titers were determined five days after each boost. A fusion was performed on the three mice that showed the greatest response. Hybridoma cell lines and monoclonal antibody production was performed according to the method of Fenderson et al. (1984). The 10 clones with highest affinity mAbs were selected for further growth and their affinity to DA was compared. The most sensitive clone was ultimately selected as the primary mAb for use in the assay development.

### *DA-HRP Conjugate*

Domoic acid (Sigma) was cross-linked to horseradish peroxidase (HRP) using the procedure of Yoon et al. (1993). The reagent was tested for stability and was used to screen for high affinity mAbs after the fusion and for assay development.

*Domoic Acid Standards*

The DA standards used to calibrate the assay were purchased from the Certified Reference Materials Program at the National Research Council of Canada Institute for Marine Biosciences (Halifax, Nova Scotia, Canada).

*Assay Calibration*

A series of dose response curves using varying amounts of antibody and DA-HRP were performed to optimize the assay sensitivity. The optimal assay conditions were found to have an effective linear range from approximately 0.1–3.0 ppb. These conditions were used in all the subsequent phases of assay development. The antibody was also tested for cross-reactivity with varying concentrations of kainic acid, glutamine and glutamic acid. These compounds are structurally similar to various portions of DA molecule and have the potential to cross-react with anti-DA mAbs. Glutamine and glutamic acid, in particular, are common in animal tissues, including shellfish.

*Calculation of the Parameters Needed to Construct an Internal Domoic Acid Standard for Each Well Strip*

Using the optimized DA assay, multiple dose response curves were made using the NRC standards diluted to between 0 and 10 ppb (1–10 ng $mL^{-1}$) in the assay reaction buffer. The average response derived from each of the individual response curves was calculated and a dose response curve was generated using a four parameter logit-log curve fitting analysis (Ritchie et al. 1981; Fig. 2). Four parameters were derived from this analysis. This first was $B_o$, the maximal signal, which occurred when no sample DA was present (Fig. 3A). The second was B, the signal produced by a known amount of sample DA. The third was the slope of the logistic transformed data [proportional to the linear portion of the sigmoidal curve describing the relationship between the ln sample DA concentration versus signal (B)]. And the fourth was $ED_{50}$, the DA concentration at the mid point of the slope curve where half the available anti-DA mAbs in the well are bound to DA-HRP (Fig. 3A). Because the concentration ratio of anti-DA antibody and DA–HRP conjugates are standardized within reagent lots, the kinetics of the reaction were fixed between assay runs (assuming constant temperature), such that the slope and $ED_{50}$ values remain constant. This made it possible to calculate DA concentrations using the four parameter model.

$$\text{DA concentration} = ED_{50}[(B_o/B) - 1]^{-\text{slope}}$$

Because the slope and $ED_{50}$ are constants, all that was needed to calculate the DA concentrations was an accurate $B_o$ and the B estimates from individual samples. In the assay, the mean value for $B_o$ for each strip of wells was determined by adding sample dilution buffer lacking DA to the first two wells in that strip. Duplicate aliquots from each of three extracted samples diluted with sample buffer were then added to the six remaining wells to obtain the B values. Duplicates were run to ensure assay replicability. It should be noted that $B_o$ (the maximal value with no DA added) can have noticeable variation between assays depending on differences in temperature and development time as shown in Figure 2A. However, when the B values for each strip are divided by $B_o$, the kinetics of the curve become normalized (i.e., replicable between strips and between runs) (see Fig. 2B). In this way the average $B_o$ values serves as an internal standard that can be used in place of a standard curve provided the variation in the $B_o$ is not above or below certain limits, which are specified in the calculation software described later.

*Domoic Acid ELISA Test Kit Procedure*

The 96-well assay tray used in the assay contained 12 strips. Each strip of 8 wells could be removed and stored until it was needed. The first two wells of each strip were used as a control (no DA added). The remaining six wells were used to analyze three samples in duplicate. This format provided the flexibility of running anywhere from 3–36 duplicate samples at a time. For unknown sample analysis, extracts were diluted to a final concentration ranging from 0.3–3 to ppb using the sample buffer (phosphate salt solution, pH 7.8, containing casein). For

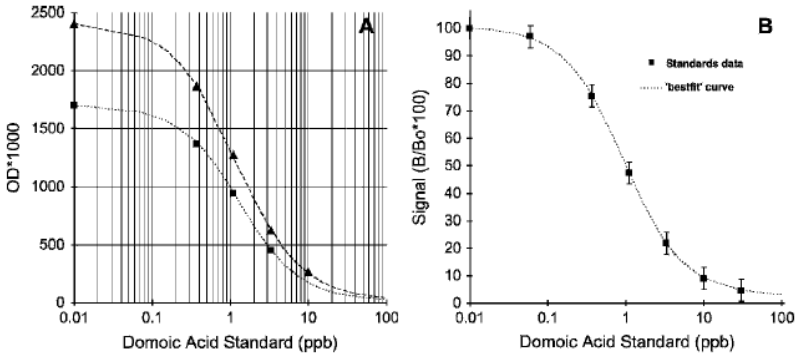


**Figure 2. (A) Representative dose response curves for domoic acid analyzed on different days. It should be noted that $B_o$ (the average of the maximal 450 nm absorbance values from the first two wells of a strip to which no DA is added) can vary noticeably between assays depending on differences in ambient temperature and development time. (B) The mean and SD in signal from eight normalized domoic acid dose response curves carried out over the course of several weeks. These data were specifically normalized by dividing each of the resultant absorbance values by $B_o$. The result of this normalization process, given that the concentrations of antidomoic acid antibody and HRP-domoic acid conjugate are fixed, is that the resultant curves are replicable between rows and between assays done on different days. The black squares and error bars indicate the mean value at each given domoic acid concentration ±1 SD.**

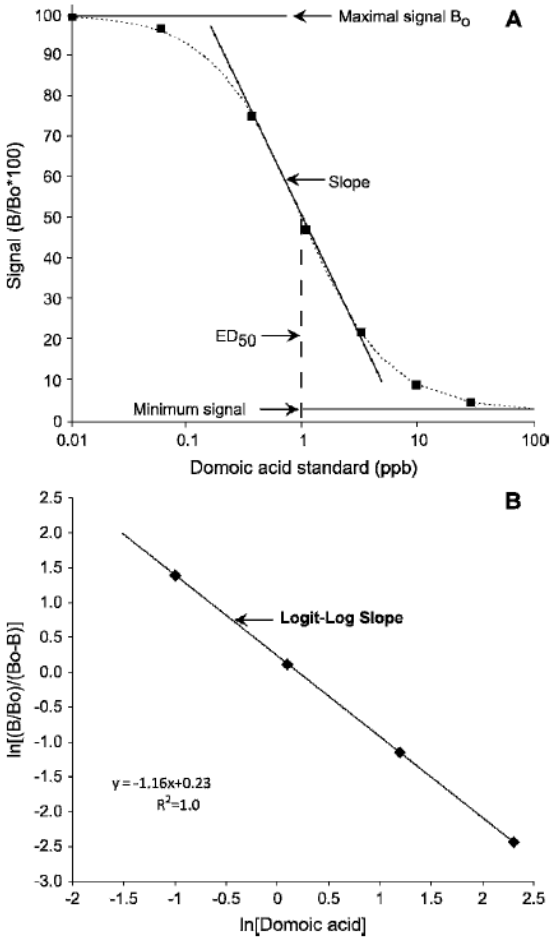


**Figure 3. (A) DA concentrations versus the corresponding ELISA absorbance values, which were normalized by dividing by maximal ($B_o$) absorbance value. (B) Log-logit transform of the data shown in Fig. 3A. From this analysis it was possible to calculate the parameters needed to accurately calculate domoic acid concentrations using the ELISA assay. These parameters include $B_o$, the maximal absorbance value at 450 nm obtained from the first two wells of a strip to which no free domoic acid is added and B, the 450 nm absorbance value for a given sample, slope of the logit-log transformed data, which were proportional to the linear portion of the sigmoidal curve describing the relationship between the ln DA concentration versus signal (B), and $ED_{50}$, the mid point of the slope curve where half the available anti-DA mAbs are bound to DA.**

clam tissues containing DA, sample dilutions of 1:50 and 1:1000 were typically used. Preliminary tests with razor clam extracts showed that a 25-fold dilution in sample dilution buffer eliminated matrix effects in ELISA analysis.

The assay was initiated by adding 50 µL of the anti-DA antibody to each well using a multi channel pipettor. Next, 50 µL of the control solution (sample buffer without DA) was added to the first two wells in each row. Duplicate 50 µL aliquots from the diluted DA extracts were then added to the remaining wells in each strip and the plate incubated at room temperature for 30 min on an orbital shaker set to vigorously mix the solution in each well (PerkinElmer Waltham, MA 1296–004 DELFIA Plateshake set on high). Vigorous mixing is key to obtaining replicable results from one run to the next. In this step, the bulk of the native DA will bind to available mAbs in proportion to the DA concentration. At the end of the incubation, 50 µL of DA HRP conjugate was added to each well and the plate incubated a second time for 30 min at room temperature on an orbital shaker. The DA-HRP will then bind to remaining available mAb sites. After the incubation, the plate was washed three times with wash solution [Tris-HCl buffered salt solution (pH 7.8) containing Tween 20 and sodium azide as a preservative] using a commercial plate washer, making certain the fluid was completely aspirated from all the wells. Alternatively, these washes can be done manually by adding wash solution to wells using a multichannel pipettor and then flicking all fluid from the wells. The manual method may result in slightly higher variability. Next, 100 µL of K-Blue TMB substrate (5.5′-tetramethylbenzidine, Neogen Corporation, Lexington, KY) was added to each well. The plate was placed on an orbital shaker for no more than 5 min, or until adequate color development was observed. Color development was terminated by adding 100 µL stop solution (1N hydrochloric acid) to each well. The absorbance in each well was measured at 450 nm using a Thermo Ascent MultiSkan plate reader (Thermo Scientific, Waltham, MA). The DA concentrations were determined using the sample (B) and control ($B_o$) absorbances, the original tissue weights, and the volume of 20% or 50% methanol used to extract each sample. The actual calculations were made using a Microsoft Excel work sheet (Microsoft Corporation, Redmond, WA), which incorporates the constants for the four parameter model described above. This worksheet can be downloaded from Stewart (2008). Processing time for this assay was ~1.5 h.

### *Routine Tissue Extraction*

In the case of razor clams and scallops, pooled samples of 10–12 individual shellfish were cleaned, and ground to a smooth and uniform homogenate in a commercial blender (Waring model HGBSS56, Torrington, CT). Clams were pooled because previous studies of DA in razor clams from the Washington coast indicated that the coefficient of variation for DA between clams in a population exceeded 100% (Wekell et al. 2002). If the homogenate appeared to be forming a gel caused by unusually high lipid content, an equal weight of water was added and the dilution noted. Approximately 2 g of homogenized tissue were added to a tared 50 mL conical tube and the weight recorded to the nearest 0.01 g. Next, 18 mL of 50% methanol were added and the samples mixed at high speed on a vortex mixer for 2 min. Once the extraction was completed the tubes were spun in a table top centrifuge for 20 min at 10,000× g or until a tight pellet and clear supernatant were obtained. If the samples did not clear despite the spinning at high speed, the supernatant was poured into a syringe, then passed through a 0.45 µm Millex HA syringe filter (Millipore, Billerica, MA) to remove proteins and other compounds that can form micelles, whereas soluble DA remained in the filtrate. At this point the homogenate was ready for analysis by ELISA and HPLC. If necessary, the sample was stored at 4°C for up to 24 h in an explosion proof refrigerator prior to analysis.

*Phytoplankton Extraction*

Approximately 0.1–1.0 L of cultured cells or sea water samples were filtered onto a GF/F filter, which was immediately frozen at –80°C until the filter could be processed. For processing, the filter was placed in a 5 mL conical BD Falcon Tube (Becton Dickinson, Franklin Lakes, NJ) and 3 mL of 20% methanol were added. The samples were then sonicated using a Thermo Fisher Scientific Model 100 Sonic Dismembrator with a 1/8 inch probe (model 15-338-80, Fisher Scientific, Waltham, MA) until the filter was completely homogenized. Care was taken to prevent the probe from rupturing the tube. The sonicator probe was cleaned very carefully with 20% methanol between samples to prevent cross-contamination. Next the homogenate was centrifuged at 3000 *g* for 10 min. The supernatant was then passed through a disposable Whatman GD/X 0.2 µm syringe filter (Florham Park, NJ) into a 5 mL tube. At this point the sample was split for analysis using both the ELISA and HPLC assays.

*HPLC Validation of DA concentration from Razor Clam Tissues*

HPLC is the accepted standard method for measuring DA and is the basis of the current official method for regulatory action in the U.S. (AOAC Official Method 991.26). The lower detection level for the standard assay is ~0.5 ppm. This technique was used to validate the DA concentration in the razor clams in this study. Briefly, 10–15 mL of the clarified supernatant prepared as described above was transferred into a 25 mL disposable plastic syringe and filtered through 0.45 micron HA Millipore filter (Bedford, MA) into a labeled scintillation vial. Salt clean-up was done with solid phase extraction columns (Hatfield et al. 1994). Strong anion exchange (SAX) solid phase extraction (SPE) cartridges (Whatman, Florham Park, NJ) were conditioned by washing successively with 6 mL of methanol, 6 mL of deionized water, and 6 mL of 50% methanol. The SPE clean up also removes tryptophan, which is a major source of false positives in HPLC-UV detection of DA because it coelutes with DA. Each sample was then drawn through a conditioned SAX SPE cartridge at a rate of 1 drop per second using a vacuum manifold. Flow was stopped when the meniscus was just above the top of the column. The columns were washed with 5 mL of 0.1 M NaCl in 10% aqueous acetonitrile (10% acetonitrile: 90% deionized water). The columns were immediately moved to a new row in the vacuum manifold and the DA eluted from the SPE cartridge using 5 mL of 0.5 M NaCl in aqueous 10% acetonitrile (10:90, acetonitrile:deionized water) and collected in 5 mL graduated centrifuge tubes. Flow was stopped when eluant reached 4.9 mL in the graduated centrifuge tube. The graduated centrifuge tube was removed from the manifold and the actual volume recorded. The graduated centrifuge tubes were capped and the eluant immediately mixed by shaking the tube vigorously 5–10 times. Tissues from the other invertebrate species examined (Table 1) were processed similarly, except that the extracts were filtered through Nanospec MF GHP 0.45 µm centrifugal filters (Pall, Ann Arbor, MI) instead of SPE columns before HPLC analysis. Eluted samples were transferred to HPLC analysis vials. The HPLC conditions were as follows: Vydac TP210 column (Grace, Deerfield, IL), 2.1 by 250 mm, 40°C, elution of DA in 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Twenty µl of each sample were injected into the column and eluted isocratically at 0.3 mL per min. The retention time for the DA peak was about 6–8 min depending on the column. Canadian NRC DACS standards at concentration of 1 ppm in 10% acetonitrile solution were run simultaneously (Hardstaff et al. 1990).

*HPLC Detection of Domoic Acid in Phytoplankton Using Fluorenylmethoxycarbonyl (FMOC) Derivatization*

A more sensitive fluorescent fluorenylmethoxycarbonyl chloride (FMOC) derivatization method (Pocklington et al. 1990) was used to determine particulate DA concentrations in phytoplankton samples, which typically contained less DA than shellfish tissues. The samples were processed on a Hewlett-Packard 1090 HPLC using a Vydac 201TP, 5 µm, 25 cm column, HP 1046A fluorescence detector, and column heater set to 40°C with the following modification. In our analysis, solvents A (HPLC Water with 0.1% v/v TFA) and B (acetonitrile with 0.1% v/v TFA) were pumped at 0.2 mL/min and the linear gradient elution was changed allowing for increased separation and resolution of the domoic acid peak. The initial gradient went from 70% A and 30% B at time of injection to

**TABLE 1.**

**Intertidal invertebrates sampled from several locations around Monterey Bay in November 2006. HPLC-UV analysis detected significant levels of compounds comigrating with iso- and epi-domoic acid standards. These crude methanolic extracts were used to challenge the NOAA and Biosense ELISAs. The goal was to establish the extent to which the ELISA assays are confounded by the presence of coeluting compounds called as the domoic acid isomers epi- and iso-domoic acid by HPLC-UV assay. Nondetect samples are represented as 0 values.**

| Organism | Combined epi and iso-DA by HPLC (ppb) | DA Concentration by NOAA ELISA (ppb) | % Total DA Detected by NOAA ELISA | DA Concentration by Biosennse ELISA (ppb) | % Total DA Detected by Biosense ELISA |
|---|---|---|---|---|---|
| *Chthamalus fissus/dalli* | 281.7 | 0.00 | 0.00 | 0.02 | 0.01 |
| *Chthamalus fissus/dalli* | 1,137.1 | 15.41 | 1.36 | 1.53 | 0.13 |
| *Littorina scutulata* | 198.7 | 10.57 | 5.32 | 3.02 | 1.52 |
| *Littorina scutulata* | 682.0 | 15.98 | 2.34 | 1.02 | 0.15 |
| *Littorina scutulata* | 119.5 | 0.00 | 0.00 | 0.17 | 0.14 |
| *Lottia digitalis* | 236.7 | 0.00 | 0.00 | 0.10 | 0.04 |
| *Lottia digitalis* | 477.9 | 13.91 | 2.91 | 0.09 | 0.02 |
| *Lottia digitalis* | 390.6 | 10.31 | 2.64 | 0.78 | 0.20 |

60% A and 40% B over 0–10 min, then held constant for 10 min; adjusted to 0% A and 100% B from 20–30 min, held isocratic for 2 min; adjusted from 0% A and 100% B to 70% A and 30% B over 2 min, and then held constant at these (initial) conditions until the end of the run at 45 min. Dihydrokainic acid was used as an internal standard, as described by Pocklington et al. (1990).

A subset of phytoplankton samples was validated to confirm the presence of DA (by mass) using liquid chromatography-mass spectrometry (LC-MS) on a ThermoFinnigan Quantum Discovery Max TSQ ESI Mass Spectrometer coupled to a HP 1100 series binary pump HPLC, following the general protocol of Quilliam et al. (1989a). Samples for LC-MS were prepared as for HPLC, but were then dried down under vacuum and redissolved in 100% methanol prior to injection. The HPLC conditions for the reverse phase were programmed for a linear gradient elution of 10:90% acetonitrile:deionized water (both containing 0.1% formic acid) up to 0:100% water:acetonitrile over 30 min.

### *Testing Cross-Reactivity of the ELISA Against Glutamine, Kainic Acid and Putative Isomers Epi-DA and Iso-DA*

Domoic acid is structurally similar to glutamine, glutamic acid and kainic acid, all of which can potentially co-occur with DA in sample extracts (Fig. 1). To test for potential cross-reactivity with these compounds, the NOAA/MSI ELISA kit was run using concentrations of glutamine, glutamic acid and kainic acid ranging from 10 ppb to 5 ppm. The $ED_{50}$ for each compound was calculated and then divided by $ED_{50}$ for DA and multiplied by 100 to determine percent cross-reactivity (Table 2). A majority of DA in razor clams and phytoplankton is in the form shown at the top of Figure 1. However, samples sometimes contain a larger quantity of compounds closely eluting with DA on standard HPLC runs that have been identified as the DA conformers epi- and iso-DA (Wright et al. 1990, Kotaki et al. 2005). To determine if the mAb used in this assay could detect these DA isomers, and the extent of interference by such coeluting compounds present in crude extracts of intertidal barnacle, limpet, and snail samples, crude methanolic extracts of these tissues were assays using HPLC-UV and both the NOAA/MSI and Biosense (Biosense Laboratories, Bergen, Norway) ELISA methods. These intertidal invertebrate extracts exhibited high levels of the putative epi-DA and iso-DA isomers as called by comigration on HPLC chromatograms. These compounds are generally near detection limits in razor clams, crabs, and to a lesser extent in mussels, and therefore these extracts provided novel matrices for evaluating the accuracy of NOAA/MSI ELISA.

**TABLE 2.**

**Cross-reactivity of the NOAA/MSI ELISA with kainic acid. glutamine, and glutamic acid.**

| Analyte | % Reactivity in the Domoic Acid Assay |
|---|---|
| Domoic acid | 100 |
| Kainic acid | 0.3 |
| Glutamine | <0.1 |
| Glutamic acid | <0.1 |

### *Data Analyses*

Analytical results for DA concentrations determined from razor clams, mussels, scallops and phytoplankton cells determined by HPLC, FMOC-HPLC, LC-MS and the NOAA/MSI ELISA were compared using linear regression analysis (Sokal & Rohlf 1995). The performance of the NOAA/MSI and Biosense ELISA kits was also compared using a subset of the phytoplankton samples. This comparison involved simultaneously analyzing phytoplankton extracts using the two kits and comparing the results with those obtained using FMOC-HPLC. All samples were run within a 24 h period to prevent differential degradation of DA, which may occur in some samples. Data were compared using linear regression analysis.

## RESULTS AND DISCUSSION

The NOAA/MSI ELISA accurately measured NRC standard DA concentrations (Fig. 4) and gave equivalent results for razor clam (Fig. 5), mussel (Fig. 6), scallop (Fig. 7), and phytoplankton extracts (Fig. 8) as obtained when using HPLC, FMOC-HPLC, or LC-MS methods. When the variability in the NOAA/MSI ELISA and FMOC-HPLC method were compared using replicate phytoplankton extracts they were found to be comparable (Fig. 9). The primary advantage of the NOAA/MSI ELISA over HPLC methods, besides a significantly lower cost per sample was much higher throughput. As many as 36 samples can be completed in <1.5 h after tissue extraction.

The NOAA/MSI format was also flexible. An internal control was incorporated into each strip, which eliminated the necessity of running a standard curve each time the assay was performed. Any unused strips could be removed and stored in a desiccator pouch at 4°C for at least six months without compromising assay performance. This allowed as few as 3 samples to be run in real time thereby avoiding the degradation of DA that can occur during storage, particularly once the samples have been extracted (Smith et al. 2006). For example, when phytoplankton samples were run within 24 h using the Biosense ELISA kit, which has been validated by an international collaborative study, and is officially approved by the AOAC International for regulatory detection of DA in shellfish,

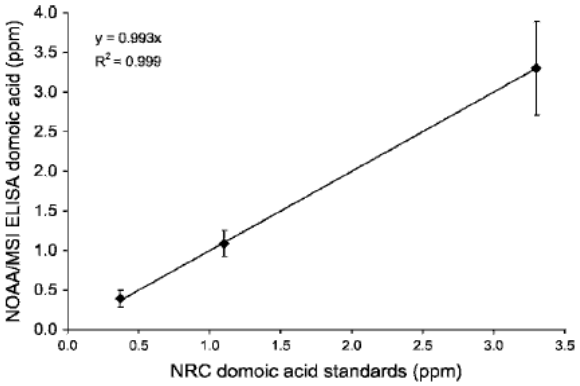


**Figure 4. Relationship between various concentrations of National Research Council of Canada (NRC) domoic acid standards and the resultant NOAA/Mercury Science (NOAA/MSI) ELISA values determined using 10 different plates.**

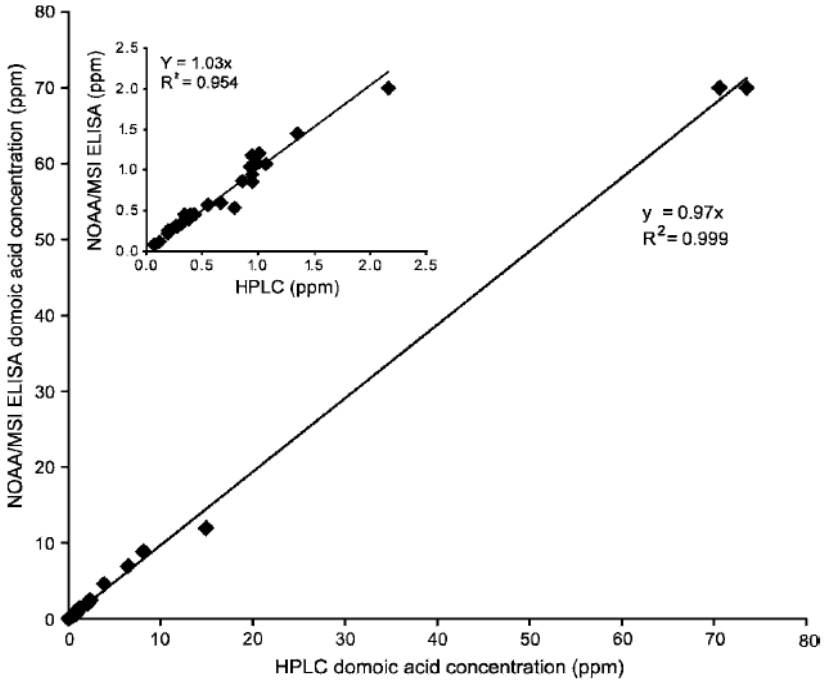


**Figure 5. Domoic acid concentrations in razor clam tissues determined from replicate tissue extracts analyzed using HPLC and NOAA/Mercury Science (NOAA/MSI) ELISA. The inset shows an expanded version of the regression analysis for sample containing less than 2.5 ppm domoic acid.**

and the NOAA/MSI ELISA kit, equivalent results were obtained (Fig. 10, $r^2 = 0.97$). In contrast, when samples were run two weeks apart the correlation dropped to $r^2 = 0.79$, indicating DA degradation.

The ability to efficiently run a small number of samples in real time was not incorporated into other DA ELISA formats. For example, the Biosense DA ELISA kit includes reagents for only two standard curves (product insert), therefore, only two batches of samples can be run per kit. This means that when small numbers of samples are being collected, they may have to be stored until a sufficient number of samples have been accumulated to maximize the number of samples per kit. This could lead to sample degradation and a critical delay in reporting when samples surpass the regulatory limit of 20 ppm.

Another advantage of the NOAA/MSI assay is that it could be run in either a quantitative or screening mode when assaying shellfish tissues. For quantitative analysis, several dilutions were assayed simultaneously to obtain an accurate DA concentration.

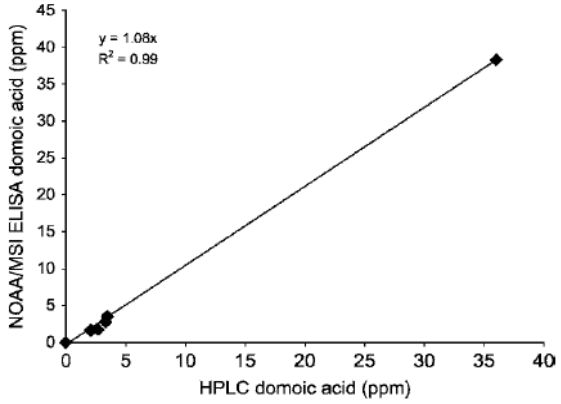


**Figure 6. Domoic acid concentrations in mussel tissues determined using HPLC and the NOAA/Mercury Science (NOAA/MSI) ELISA. Aliquots from each sample were run simultaneously.**

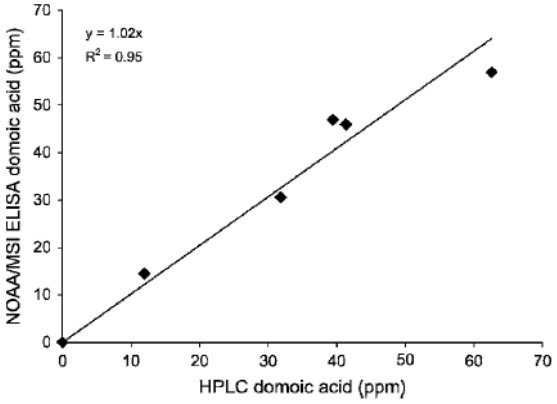


**Figure 7. Concentration of domoic acid in scallop tissues extracted from the scallop (*Pecten maximus*) using the standard NOAA/Mercury Science (NOAA/MSI) protocol.**

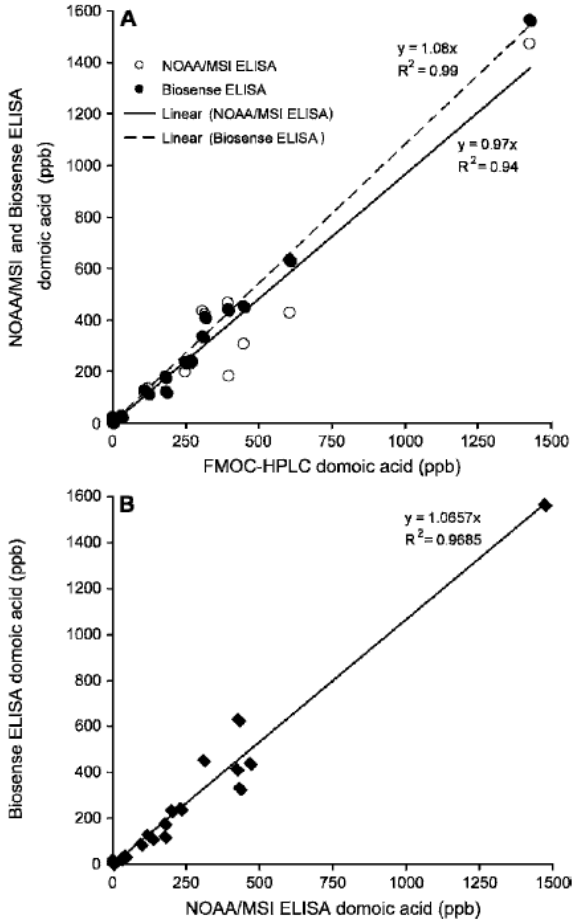


**Figure 8. (A) Domoic acid concentrations measured from split phytoplankton sample extracts, which were measured within 24 h by FMOC-HPLC and either the NOAA/Mercury Science (NOAA/MSI) or Biosense ELISAs. (B) Comparison of domoic acid concentrations measured in split samples by either HPLC or ELISA.**

Alternatively, to rapidly screen for DA concentrations of concern, the sample extracts were diluted 1:1,000 before running the assay. Taking into account the 1:10 dilution that occurred during the extraction process, the 1:1,000 dilution reduced samples in the 20 ppm DA range to ~2 ppb in the diluted sample. This concentration was within the linear range of the assay (0.1–3 ppb). Tissue samples with 5–10 fold less DA, and far below levels of concern, would show no detectible DA at this dilution. Tissues containing initial DA concentrations >30 ppm would be off scale and indicate a significant DA concentration requiring action. Any samples from this rapid screening that were of concern could then be diluted and run again to obtain an accurate concentration. The NOAA/MSI ELISA test kit also comes with a simple Excel spreadsheet, which allowed the toxin concentrations to be quickly and easily calculated in either a quantitative or rapid screening mode. All that had to be entered was the $B_o$ (no DA added) and sample absorbance data from each strip, the weight of the extracted tissue samples, and the extraction volumes.

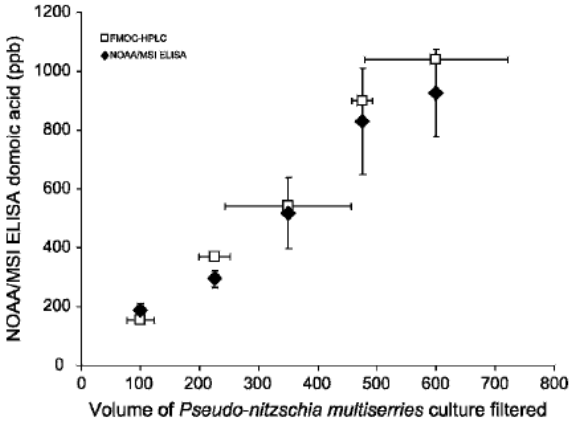


**Figure 9. Comparison of the variability between phytoplankton extracts measured using FMOC-HPLC (□) and the NOAA/Mercury Science ELISA (♦). Error bars indicate ±1 SD. Because the error bars largely overlap, for clarity, the standard deviation for the FMOC-HPLC is plotted in the horizontal direction and the NOAA ELISA in the vertical direction.**

The NOAA/MSI and Biosense ELISA kits were tested against crude methanolic extracts of several intertidal invertebrates, which HPLC identified as containing >100 ppb levels of epi-DA and iso-DA. These compounds are reported to be less toxic DA congeners based on receptor binding assays (Sawant et al. 2007). Results from both ELISA kits revealed the presence of only trace amounts of DA equivalents in the extracts. The NOAA/MSI ELISA cross-reactivity with these compounds ranged from 0% to 5.3% and the Biosense ELISA cross-reactivity from 0.01% to 1.5% (Table 1) indicating that the ELISA assays are relatively insensitive to cogener interference. It should also be noted that the regulatory methods for assessing human safety are currently based on measuring DA alone, not the combination of DA, iso-DA and epi-DA. These results indicated that both the NOAA/MSI and Biosense

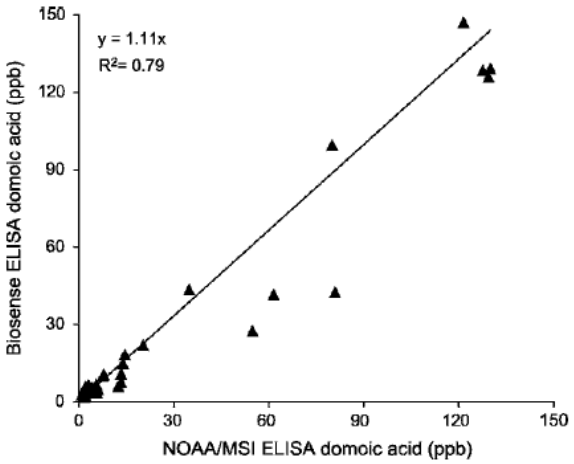


**Figure 10. NOAA/MSI ELISA versus Biosense ELISA for phytoplankton samples when the two assays were run several weeks apart showing the increased variability, caused by differential degradation or absorption rates, when samples are not measured within the same 24 h period.**

ELISA methods provide DA values comparable to the HPLC values currently used as a basis for regulatory decisions.

Measuring low concentrations of DA in real time is particularly important because the presence or absence of DA contamination is frequently patchy and associated with variable onshore transport of toxic phytoplankton blooms (e.g., Trainer et al. 2002). Depending on prevailing winds and currents, one harvest area can become highly contaminated over a short period whereas adjacent regions remain uncontaminated (Trainer et al. 2000). These differentially affected regions frequently include areas where significant commercial and recreational clam harvests occur. This variability complicates monitoring programs designed to protect human health. The current standard practice involves shipping shellfish samples to a centralized facility for HPLC analyses, introducing delays between 3–14 days from the date of sample collection to reporting results. This turnaround time is too slow to adequately protect subsistence shellfish harvesters who rely on clams consumed within a day or two of harvest. The cost of HPLC analysis is also relatively high per sample and requires a substantially higher capital investment compared with the NOAA/MSI ELISA method. Having an economical technique for better assessing the degree of contamination locally, and in real time, is of great value for local resource managers and public health officials.

The ability to detect DA in phytoplankton using the NOAA/MSI kits would further benefit environmental monitoring programs designed to detect the early onset of toxic *Pseudo-nitzschia* blooms. It is known that increases in the *Pseudo-nitzschia* capable of producing DA often precedes the contamination of shellfish and other filter feeders by a week or two (Trainer & Suddleson 2005). A combination of cell counts and direct toxicity measurements should provide timely predictions for marine resource managers and public health officials. The kit is now commercially available with MSI authorized to market, manufacture and distribute the 96-well plate format test kits. We anticipate completing the necessary validation procedures to qualify the 96 well plate format for regulatory use by public health officials. We are also developing a field test kit that can be used to detect DA levels in shellfish tissues above or below 20 ppm within 10 min after extraction. The test will require no laboratory equipment other than a homogenizer and can be used directly in the field by non-technical personnel, including shellfish harvesters and members of citizen monitoring groups and local volunteers.

In summary, the NOAA/MSI ELISA test kit provides an accurate, flexible and cost effective method for measuring DA in clam, mussel and scallop tissues, as well as in phytoplankton samples. The assay yields concentrations for DA that are indistinguishable from those obtained by HPLC. With further validation, the NOAA/MSI ELISA kit is expected to be approved as a regulatory method for making decisions concerning public health. The short assay (1.5-h) processing time, and relatively low cost, compared with HPLC analysis, mean that the ELISA can be used in more remote locations by environmental managers and public health officials to provide near real-time monitoring capacities.

## ACKNOWLEDGMENTS

The authors thank Mitch Lesoing and Mel Moon of the Quileute Tribe whose initial request for development of a domoic test kit and constant encouragement were key to the success of the test kit. This work was supported by a competitive grant awarded by the MERHAB program specifically for assay development as well as a NOAA NCCOS Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) grant GAD# R83-1705) and two Monitoring and Event Response for Harmful Algal Blooms (MERHAB) grants: NA04NOS4780239-03 and NA05NOS4781228. J. Bastion and A. Odell's participation was funded by a surcharge to the Washington State shellfish license provided or ORHAB. Jonathan Deeds provided helpful edits and suggestions.

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Doc. 2007A

# Domoic Acid Screening Test Kit

Colorimetric Immunoassay
for the detection of
Domoic Acid
in environmental samples

## Instructions and User Guide

**FOR SCIENTIFIC RESEARCH USE**

Manufactured by
Mercury Science Inc.
Tel: (866) 861-5836

# Domoic Acid Screening Test Kit

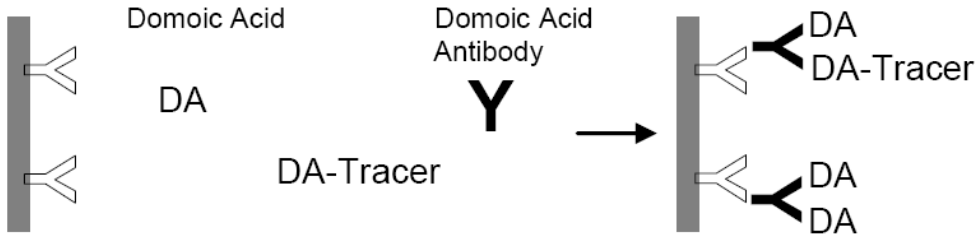
**For Scientific Research Use Only.**
**This product is not to be used for In Vitro or In Vivo Diagnosis.**

## PRINCIPLES OF THE ASSAY

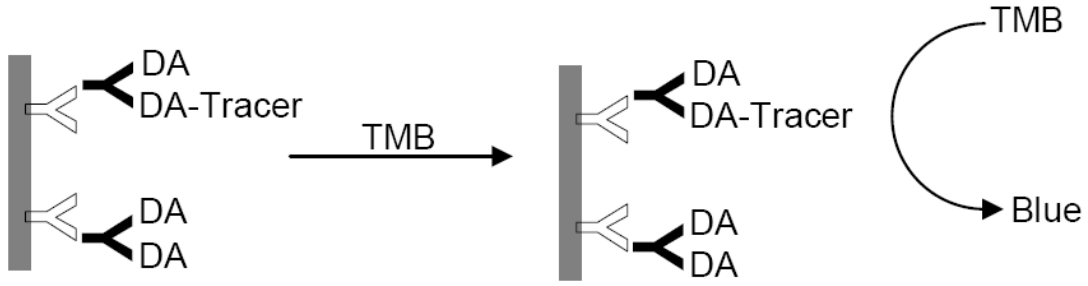
This product contains an antibody (Ab) that binds Domoic Acid and has been developed for the semi-quantitative detection of Domoic Acid in sample extracts. The signal of samples and a control are compared to determine the amount of Domoic Acid present.

The Domoic Acid assay is a solid phase colorimetric immunoassay, based on competition between Domoic Acid and enzyme-labelled Domoic Acid (DA-Tracer) for anti-Domoic Acid antibody. Samples containing Domoic Acid inhibit the binding of the DA-Tracer to the antibody molecules. Both the Ab-Domoic Acid and Ab-DA-Tracer complexes are captured on the surface of the microtiter plate wells.

Following a wash step, the addition of an enzyme substrate (TMB) forms a color proportional to the amount of DA-Tracer in the well. The amount of color measured is inversely proportional to the concentration of Domoic Acid in the sample.




Solid phase
anti-mouse IgG




**TEST KIT CONTENTS** Each Domoic Acid test kit contains reagents for testing a maximum of 36 samples in duplicate.
The expiry date of the test kit is stated on the outer label.
Store the kit between 2°C and 8°C.

## Reagents Store the reagents between 2°C and 8°C when not in use.

| Component | Quantity | |
|---|---|---|
| **Control Solution** | 1 vial | 2 mL |
| The control is a phosphate-buffered salt solution with casein. Contains sodium azide as a preservative. | | |
| **Sample Dilution Buffer** | 1 bottle | 50 mL |
| Ready-to-use phosphate buffered (pH 7.8) salt solution with casein. Contains sodium azide as a preservative. | | |
| **Domoic Acid- Tracer** | 1 vial | 7.5 mL |
| The tracer is in a MOPS-buffered solution containing bovine protein as a stabilizer and methylisothiazolone, bromonitrodioxane, and Proclin 300 as preservatives. | | |
| **Domoic Acid Antibody** | 1 vial | 7.5 mL |
| The antibody is in phosphate-buffered salt solution with casein. Contains sodium azide as a preservative. | | |
| **Wash Concentrate** | 1 bottle | 40 mL |
| A 25-fold concentration of Tris-HCl buffered (pH 7.8) salt solution with Tween 20. Contains sodium azide as a preservative. Prepare for use by mixing entire contents with 960 mL of distilled water and placing in platewasher WASH Bottle. | | |
| **Substrate Solution** | 1 bottle | 15 mL |
| Tetramethylbenzidine and $H_2O_2$ | Keep away from direct sunlight. | |
| **Stop Solution** | 1 bottle | 15 mL |
| 1 N Hydrochloric Acid | | |
| **Anti-Mouse IgG Microtitration Strips** | 1 plate (12 x 8 wells) | |

**WARNINGS AND PRECAUTIONS**

For research use only. Handle all samples as potentially hazardous.
Disposal of all waste should be in accordance with local regulations.

**SCREENING ASSAY PROCEDURE**

Perform each determination in duplicate for the Control and unknowns. All sample extracts should be filtered prior to analysis. All reagents and samples should be brought to room temperature prior to use. Use only the number of strips needed. Keep unused strips stored in their aluminum foil pouch with the included desiccant until needed.

1. Pipet 50 uL of the diluted Domoic Acid Antibody solution into each well.

2. Pipet 50 uL of each Control or sample into a well using the sequence shown in the table below. **Always use wells A and B on each strip as Controls.** Always perform duplicate analyses of samples. Three samples can be tested per strip. The example below shows the testing of eight samples.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Con-trol | Con-trol | Con-trol | | | | | | | | | |
| **B** | Con-trol | Con-trol | Con-trol | | | | | | | | | |
| **C** | 1st Unk | 4th Unk | 7th Unk | | | | | | | | | |
| **D** | 1st Unk | 4th Unk | 7th Unk | | | | | | | | | |
| **E** | 2nd Unk | 5th Unk | 8th Unk | | | | | | | | | |
| **F** | 2nd Unk | 5th Unk | 8th Unk | | | | | | | | | |
| **G** | 3rd Unk | 6th Unk | | | | | | | | | | |
| **H** | 3rd Unk | 6th Unk | | | | | | | | | | |

3. Shake the wells for 30 minutes.

4. Pipet 50 uL of the Domoic Acid Tracer solution into each well.

5. Shake the wells for 30 minutes.

6. Wash the strips 3 times on the platewasher. Tap the strips upside-down firmly on a paper towel to blot away any excess wash solution that may remain in the wells.

7. Add 100 uL of Substrate Solution to each well. Shake the plate for five minutes.

8. Add 100 uL of Stop Solution to each well. Shake the plate briefly.

9. Measure the absorbance in each well. Note: If Control absorbance is greater than 3.0 AU, remove 50 uL from ALL WELLS and measure absorbance.

**10.** The data can be analyzed using the Excel worksheet available at the following link:

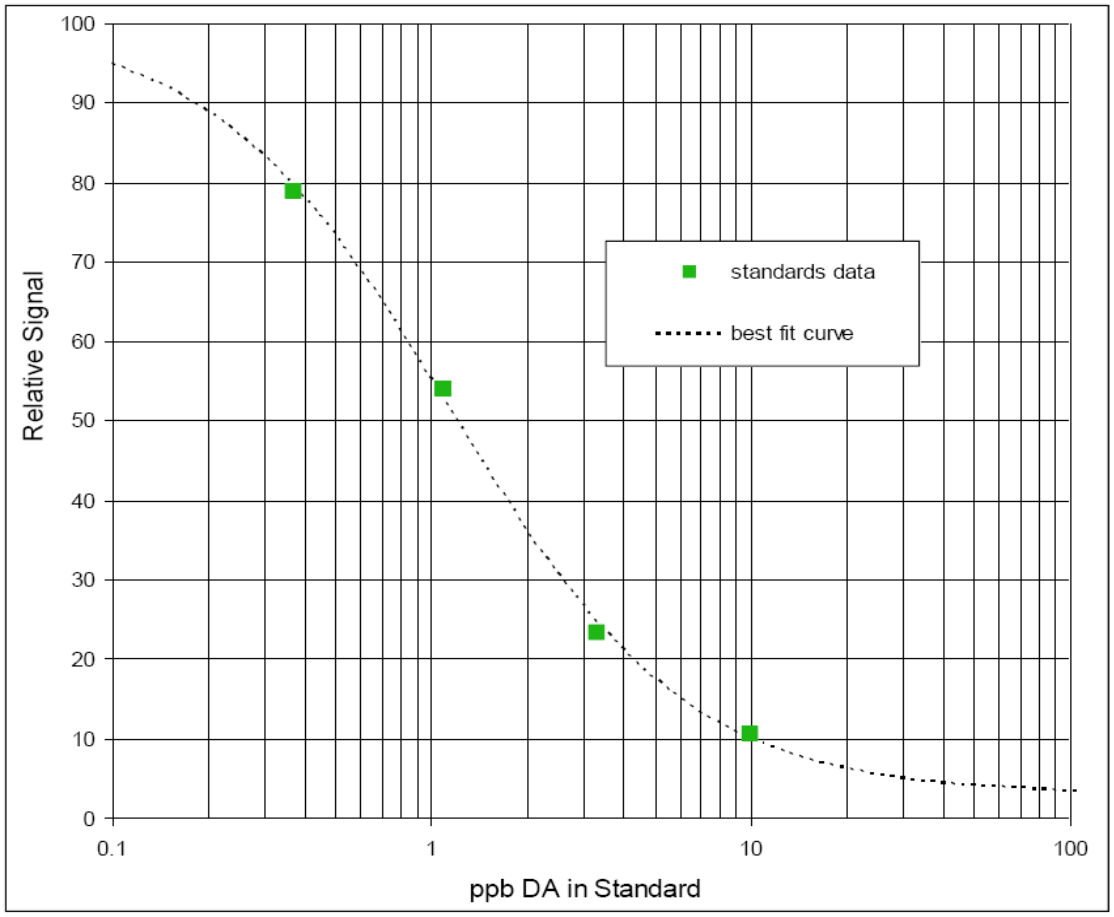
**http://mercuryscience.com/Domoic Acid Quantitation 8Well Strip.xls**

## PERFORMANCE CHARACTERISTICS

### Reproducibility

### Inter-Assay Standard Curve

The average values and standard deviation of 5 separate standard curves is shown below.




### Intra-assay Signal Precision

Analysis of 12 replicates for five different samples

| | A | B | C | D | E |
|---|---|---|---|---|---|
| Signal (% of Control) | 99.5 | 76.5 | 47.5 | 23.5 | 10.4 |
| Standard Deviation | 1.4 | 1.2 | 2.0 | 2.3 | 1.1 |
| % Coeff. Var. | 1.4 | 1.6 | 4.2 | 9.8 | 10.9 |

### Intra-assay Concentration Precision

Analysis of 3 different samples measured in 6 separate quantitative assays.

| | A | B | C |
|---|---|---|---|
| Average Conc. (ppb) | 0.56 | 1.54 | 3.66 |
| Standard Deviation (ppb) | 0.01 | 0.13 | 0.19 |
| % Coeff. Var. | 2.1 | 8.6 | 5.3 |

## PERFORMANCE CHARACTERISTICS (Cont.)

### Detection Limit

The detection limit is defined as the minimum concentration of Domoic Acid that can be distinguished from a blank standard with 95% confidence. A detection limit of 0.1 ppb Domoic Acid in extraction buffer has been demonstrated with this assay.

### Cross Reactivity

This assay is specific for the detection of domoic acid. The ability of the assay to detect structurally related compounds is shown in the following table.

| Analyte | % Reactivity |
|---|---|
| Domoic Acid | 100 |
| Kainic Acid | 0.3 |
| Glutamic Acid | less than 0.1 |
| Glutamine | less than 0.1 |

## PROCEDURAL NOTES

Please read all instructions thoroughly before using this kit. Do not mix reagents from kits having different lot numbers. Do not use kits after the expiration date printed on the kit label.

Reagents should be at room temperature when used.

During washing steps, check that each well is completely filled during wash solution additions. After washing is complete, invert the wells and tap them gently against a paper towel to remove excess liquid.

The platewasher should be rinsed with distilled water at the end of each day of use to prevent clogging of the dispensing and aspirating ports. Prime the platewasher with wash solution before the first wash each day.

Care must be taken during each step to prevent contamination of reagents and equipment Do not use the same pipet tip in two different reagents.

**For Technical Assistance, contact Mercury Science Inc: (866) 861-5836.**

**Additional Information**

**MATERIALS REQUIRED BUT NOT SUPPLIED WITH THE KIT**

The Domoic Acid test kit is part of a complete system of immunodiagnostic reagents and instrumentation. The system requires the following equipment.

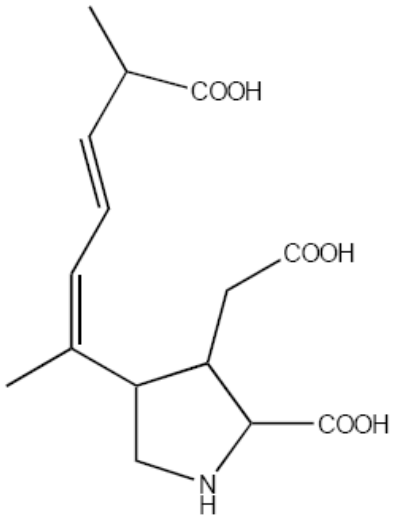
1. Microtiterplate Reader able to measure Absorbance at 450 nm
2. Platewasher
3. Plate Shaker
4. 8 Channel pipet
5. Pipetmen (P10, P200 and P1000)

**Other Notes:**

- Perform each Control and Sample in duplicate wells.
- All sample extracts should be filtered prior to analysis.
- All reagents and samples should be brought to room temperature prior to use.
- Use only the number of strips needed.
- Keep unused strips stored in their aluminum foil pouch with the included desiccant until needed.
- If Control absorbance is greater than 3.0 AU, remove 100 uL from ALL WELLS and repeat absorbance measurement.

An Excel worksheet has been developed to analyze results and quantitate the amount of domoic acid in extracts. Send your request for the "Domoic Acid Quantitation Worksheet - DAK-36" to: info@mercuryscience.com

Structure of Domoic Acid

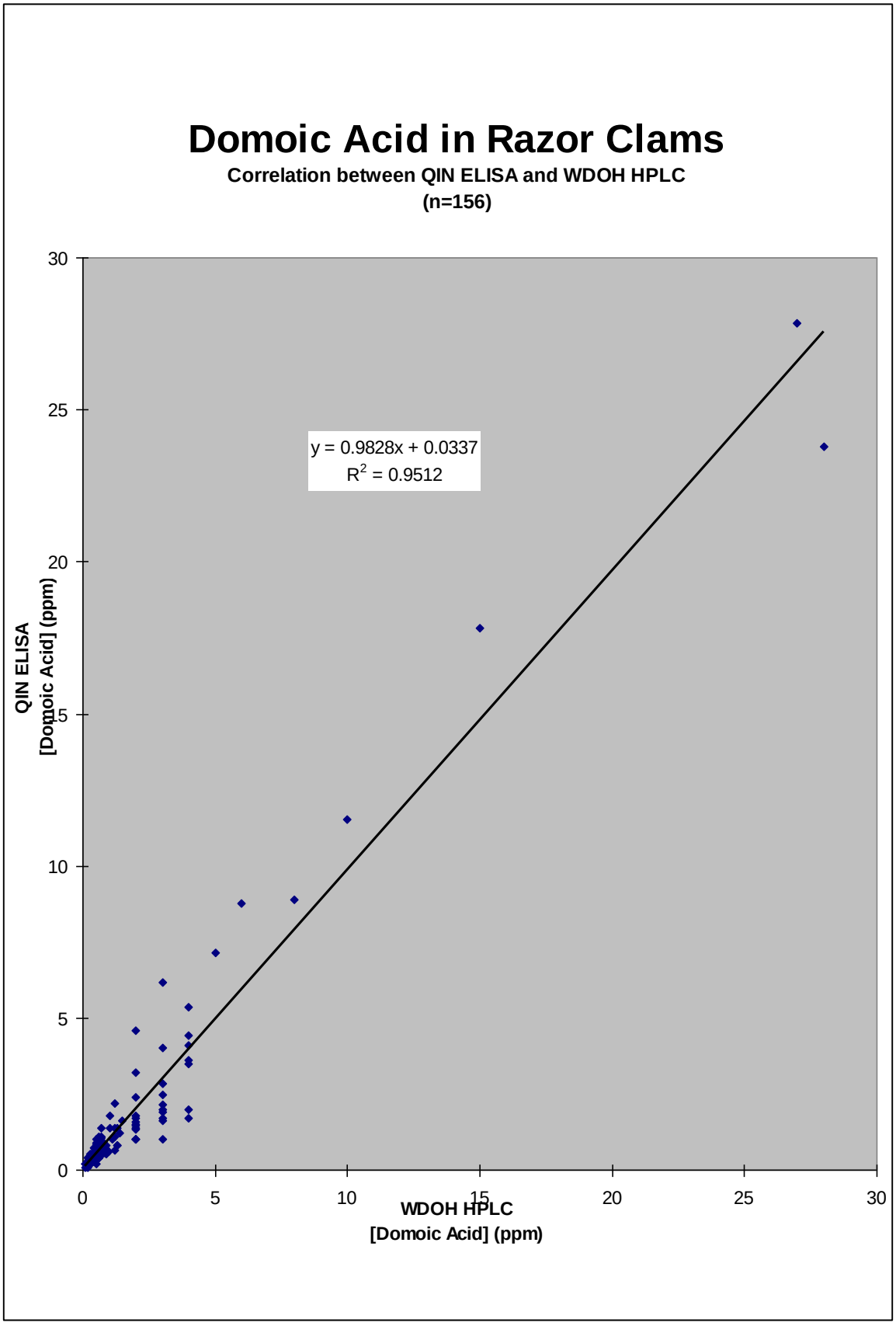


# Domoic Acid Test Kit

## Summary Protocol Sheet

| | |
|---|---|
| Add Antibody | 50 uL |
| Add Control and Samples | 50 uL |
| Incubate | Shake for 30 minutes |
| Add Tracer | 50 uL |
| Incubate | Shake for 30 minutes |
| Wash | "3 WASHES" program |
| TMB | Add 100 uL, shake for 5 minutes |
| Stop | Add 100uL |
| Measure | Absorbance at 450 nm |

**Note:** If Control absorbance is greater than 3.0 AU, remove 100 uL from ALL WELLS and repeat absorbance measurement.


Domoic Acid in Razor Clams
Correlation between QIN ELISA and WDOH HPLC
(n=156)
y = 0.9828x + 0.0337
R2 = 0.9512
QIN ELISA
[Domoic Acid] (ppm)
WDOH HPLC
[Domoic Acid] (ppm)
0
5
10
15
20
25
30

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

(http://www.issc.org/client_resources/lmr%20documents/i.%20issc%20lab%20method%20application%20checklist.pdf)

**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** | DOMOIC ACID RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY – 96 Well Format |
| **Name of the Method Developer** | Mercury Science Inc. and the National Oceanic and Atmospheric Administration |
| **Developer Contact Information** | Attn: Tom Stewart<br>4802 Glendarion Dr.<br>Durham, NC 27713<br>Phone: (866) 861-5836 |

| Checklist | Y/N | Submitter Comments |
|---|---|---|
| **A. Need for the New Method** | | |
| 1. Clearly define the need for which the method has been developed. | Y | Faster, more affordable DA analysis |
| 2. What is the intended purpose of the method? | Y | Monitoring shellfish and water samples for DA |
| 3. Is there an acknowledged need for this method in the NSSP? | Y | Faster analysis decreases public health risks |
| 4. What type of method? i.e. chemical, molecular, culture, etc. | Y | Enzyme-Linked Immunosorbent Assay (ELISA) |
| **B. Method Documentation** | | |
| 1. Method documentation includes the following information: | | |
| Method Title | Y | DOMOIC ACID RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY – 96 Well Format |
| Method Scope | Y | For the analysis of food, phytoplankton, and water |
| References | Y | Peer Reviewed Publication, Independent Correlation Study |
| Principle | Y | Competitive ELISA |
| Any Proprietary Aspects | Y | Unique Antibody and Enzyme Conjugate |
| Equipment Required | Y | Equipment is listed for this method |
| Reagents Required | Y | Reagents are listed for this method |
| Sample Collection, Preservation and Storage Requirements | Y | Requirements are described for this method |
| Safety Requirements | Y | Normal Good Lab Practices |

| | | |
|---|---|---|
| Clear and Easy to Follow Step-by-Step Procedure | Y | See User Guide supplied with DA Test kit. |
| Quality Control Steps Specific for this Method | Y | Described below |
| **C. Validation Criteria** | | |
| 1. Accuracy / Trueness | | SLV Testing in Progress – see preliminary results using oysters |
| 2. Measurement Uncertainty | | SLV Testing in Progress– see preliminary results using oysters |
| 3. Precision Characteristics (repeatability and reproducibility) | | SLV Testing in Progress– see preliminary results using oysters |
| 4. Recovery | | SLV Testing in Progress– see preliminary results using oysters |
| 5. Specificity | | SLV Testing in Progress |
| 6. Working and Linear Ranges | | See publication Dec 2008 issue Journal Shellfish Research - 0.3 to 3 ppb |
| 7. Limit of Detection | | Linear range |
| 8. Limit of Quantitation / Sensitivity | | SLV Testing in Progress |
| 9. Ruggedness | | SLV Testing in Progress |
| 10. Matrix Effects | | SLV Testing in Progress |
| 11. Comparability (if intended as a substitute for an established method accepted by the NSSP) | | Results from one independent study are included |
| **D. Other Information** | | |
| 1. Cost of the Method | Y | $200 per 36 duplicate samples |
| 2. Special Technical Skills Required to Perform the Method | Y | Some ELISA experience or training required |
| 3. Special Equipment Required and Associated Cost | Y | See list |
| 4. Abbreviations and Acronyms Defined | Y | See list |
| 5. Details of Turn Around Times (time involved to complete the method) | Y | 90 minutes |
| 6. Provide Brief Overview of the Quality Systems Used in the Lab | Y | See attached |
| | | |
| Submitters Signature  | Date: June 18, 2009 | |
| Submission of Validation Data and Draft Method to Committee | Date: | |
| Reviewing Members | Date: | |
| Accepted | Date: | |
| Recommendations for Further Work | Date: | |

## II. 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.


## III. Single Laboratory Validation (SLV) Protocol For Submission to the Interstate Shellfish Sanitation Conference (ISSC) For Method Approval

**Single Laboratory Validation (SLV) Protocol**
**For Submission to the Interstate Shellfish Sanitation Conference (ISSC)**
**For Method Approval**

**Information:** Applicants shall attach all procedures, with materials, methods, calibrations and interpretations of data with the request for review and potential approval by the ISSC. The ISSC also recommends that submitters include peer-reviewed articles of the procedure (or similar procedures from which the submitting procedure has been derived) published in technical journals with their submittals. Methods submitted to the ISSC LMR committee for acceptance will require, at a minimum, 6 months for review from the date of submission.

**Note:** The applicant should provide all information and data identified above as well as the following material, if applicable:

**Justification for New Method**

- Name of the New Method.

  DOMOIC ACID RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY – 96 Well Format
  (Marketed by Mercury Science Inc. as Product # DAK-36 Domoic Acid Test Kit.)

- Specify the Type of Method (e.g., Chemical, Molecular, or Culture).

  Enzyme linked immunosorbent assay (ELISA) using an anti-domoic acid monoclonal antibody

- Name of Method Developer.

  The DA assay kit was developed jointly by NOAA's National Centers for Coastal Ocean Science, National Ocean Service, and the Northwest Fisheries Science Center, together with an industry partner Mercury Science, Inc., Durham, North Carolina

- Developer Contact Information [e.g., Address and Phone Number(s)].

  Mercury Science Inc.
  Attn: Tom Stewart
  4802 Glendarion Dr.
  Durham, NC 27713

Phone: (866) 861-5836

• Date of Submission.

June 18, 2009

• Purpose and Intended Use of the Method.

The method is an accurate, rapid, cost-effective tool for use by environmental managers and public health officials to monitor Domoic Acid concentrations in environment samples.

• Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods.

The regulatory method for DA detection sanctioned by the Interstate Shellfish Sanitation Conference is a high performance liquid chromatography (HPLC) assay. Though accurate, these analyses are generally run by centralized state facilities with results typically not available for 3 to 14 days after the samples are collected. In more remote communities, many of which depend heavily on subsistence clam harvests, these long delays and the costs of sample analysis are causes for public health concern. The average cost of approximately $100 per sample limits the number of samples that can be analyzed (Harold Rourk, Washington State Department of Health, personal communication). Resource managers in coastal communities have expressed their desire for a cost-effective method for rapid and accurate determination of DA concentrations in shellfish and phytoplankton samples. The high throughput capacity of the assay also allows for much faster response times when domoic acid events occur. The relatively low cost of the assay means that significantly more sampling is also possible on the same or smaller budget.

• Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types.

This ELISA is sensitive to organic solvents such as methanol. Sample extracts that contain methanol can be diluted with Sample Dilution Buffer (provided in the kit) to reduce methanol concentrations to less than 1%.

• Other Comments.

The implementation of this ELISA system required the development and validation of two essential reagents, a high avidity monoclonal antibody to DA and a stable DA-HRP conjugate recognized by the same monoclonal antibody.

**Method Documentation**

• Method Title.

Domoic Acid Rapid Enzyme-Linked ImmunoSorbent Assay (ELISA) – 96 Well Format

• Method Scope.

The method is a sequential competitive enzyme linked immunosorbent assay (ELISA) utilizing a high avidity monoclonal antibody (mAb) to DA to ensure assay specificity and consistency across production lots. The assay is specific for Domoic Acid and can be used for the analysis of tissue extracts, phytoplankton samples, and water samples.

• References.

RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID, Journal of Shellfish Research, Vol. 27, No. 5, 1301–1310, 2008.
Available online at: http://mercuryscience.com/LitakerStewartDec2008.pdf

User Guide Available Online at: http://www.mercuryscience.com/DA User Guide 2007A.pdf

• Principle.

A fixed number of anti-DA mAb binding sites are incubated with dissolved DA in the sample followed by the addition of a DA – horseradish peroxidase (HRP) conjugate. As these binding events occur, the anti-DA mAb molecules are simultaneously captured by anti-mouse antibodies affixed to the surface of the microtiter plate wells. Following a wash step, subsequent HRP derived color development, readable on standard microplate readers, was inversely proportional to the concentration of DA in the sample matrix. The assay reagents were titrated so that the amount of mAb and the DA–HRP conjugate added produced a maximal absorbance signal of approximately 2.5 absorbance units when no DA was present.

• Analytes/Measurands.

Domoic Acid

• Proprietary Aspects.

The assay uses a unique monoclonal antibody and enzyme conjugate developed by Mercury Science Inc.

• Equipment.

Microtiterplate orbital shaker
Automated microtiterplate washer
Multichannel pipette
Pipetman (P20, P200, P1000) or equivalent
Microtiterplate reader (capable of reading at 450nm)

• Reagents.

1. anti-DA antibody
2. DA-HRP conjugate
3. Assay Buffer
4. Control Solution
5. Wash solution
6. TMB substrate
7. Stop solution

• Media.

Tissue samples are extracted using a solvent of Methanol:Water (50:50, v:v)
Extracts are diluted into an aqueous sample buffer prior to analysis by the ELISA.

Water samples are filtered and buffered prior to analysis by the ELISA.

Phytoplankton samples are ruptured by appropriate methods in aqueous sample buffer prior to analysis by the ELISA.

• Matrix or Matrices of Interest.

Butter clam (*Saxidomus giganteus*), blue mussel (*Mytilus edulis*), geoduck (*Panopea abrupta*), manila clam (*Venerupis japonica*), oyster (*Crassostrea virginica*), quahog (*Mercenaria mercenaria*) and razor clam (*Siliqua patula*) tissues, as well as phytoplankton and water samples

• Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.

**Shellfish preparation**: In the case of shellfish, pooled samples of 10-12 individuals are cleaned, and ground to a smooth and uniform homogenate in a commercial blender. Approximately 2 g of homogenized tissue are added to a tared 50 mL conical tube and the weight recorded to the nearest 0.01g. Next, 18 mL of 50% methanol are added and the samples mixed at high speed on a vortex mixer for 2 min. Once the extraction is complete, the tubes are spun in a table top centrifuge for 20 min at 10,000xg or until a tight pellet and clear supernatant are obtained. If the samples do not clear despite the spinning at high speed, the supernatant is passed through a 0.45 µm syringe filter. The extract is then diluted 1:100 or 1:1000 into Sample Dilution Buffer and is ready for analysis by ELISA. If necessary, the sample may be stored at 4ºC for up to 24 h in a refrigerator prior to analysis.

**Phytoplankton preparation**: Approximately 0.1 to 1.0 L of cultured cells or sea water samples are filtered onto a GF/F filter which can be immediately frozen at -80$^{o}$C until the filter can be processed or processed immediately. For processing, filters are placed in a 5mL conical tube and 3 mL of 20% methanol are added. The samples are sonicated until the filter is completely homogenized. Care is needed to prevent the probe from rupturing the tube. The sonicator probe is cleaned carefully with 20% methanol between samples to prevent cross-contamination. Next the homogenate is centrifuged at 3000xg for 10 minutes. The supernatant is passed through a 0.2 µm syringe filter. The extract is then diluted into Sample Dilution Buffer and is ready for analysis by ELISA.

**Storage of test kit**: Any unused strips can be removed and stored in a desiccator pouch at 4$^{o}$C for at least six months without compromising assay performance

• Safety Requirements.

General Good Laboratory Practices should be followed at all times.
Safety Glasses should be worn at all times.
The Stop solution in the assay contains 1 M hydrochloric acid. Care must be taken to avoid skin or eye contact with the Stop solution.

• Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]).

Cost of the Method: The DAK 36 Domoic Acid Test Kit costs $200 and contains sufficient assay reagents to perform 36 sample analyses (less than $6 per sample)

Special Technical Skills Required to Perform the Method: It is recommended that users have prior experience performing ELISA assays or receive training from Mercury Science Inc.

Special Equipment Required and Associated Cost (estimated):

- Microtiterplate orbital shaker $500
- Automated microtiterplate washer $5,000
- Multichannel pipette $700
- Pipetmen (P20, P200, P1000) (or equivalent) $1,500
- Microtiterplate reader (capable of reading at 450nm) $6,500

This equipment is commonly available in most state laboratories.

Abbreviations and Acronyms Defined:

ELISA – Enzyme-Linked Immunosorbent Assay
HRP – Horseradish Peroxidase
TMB – Tetramethylbenzidine
DA – Domoic Acid
mAb – monoclonal Antibody

Details of Turn Around Times: As many as 36 sample extracts can be analyzed in <1.5 hours.

• Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures and indicate critical steps.).

The 96 well assay tray used in the assay contains 12 strips. Each strip of 8 wells can be removed and stored until it is needed. The first two wells of each strip are used as a control (no DA added). The remaining six wells are used to analyze 3 samples in duplicate. This format provided the flexibility of running anywhere from 3 to 36 duplicate samples at a time.

1. For unknown sample analysis, extracts are diluted to a final concentration ranging from 0.3 to 3 to ppb using the Sample Dilution Buffer [phosphate salt solution, pH 7.8, containing casein]. For clam tissues containing DA, sample dilutions of 1:100 and 1:1000 are typically used. (Preliminary tests with razor clam extracts showed that a 25-fold dilution in sample dilution buffer eliminates matrix effects in ELISA analysis.)
2. The immunoassay is started by adding 50 µl of the anti-DA antibody reagent to each well using a multi-channel pipette.
3. Next, 50 µl of the Control solution (sample buffer without DA) is added to the first two wells in each strip.
4. Duplicate 50 ul aliquots from the diluted DA extracts are then added to the remaining wells in each strip and the plate is shaken at room temperature for 30 minutes on an orbital shaker set to vigorously mix the solution in each well. **Vigorous mixing is key to reaching equilibrium in the allotted time and obtaining replicable results from one run to the next.** In this step, DA in the sample binds to available mAb in proportion to [DA].
5. At the end of the incubation, 50 µl of DA HRP conjugate is added to each well and the plate is shaken a second time for 30 min at room temperature on an orbital shaker. The DA-HRP will bind to available mAb sites.
6. Following Step 5, the plate is washed three times with wash solution [Tris-HCl buffered salt solution (pH 7.8) containing Tween 20 and sodium azide as a preservative] using a commercial plate washer, making certain the fluid is completely aspirated from all the wells. *Alternatively, these washes can be done manually by adding wash solution to wells using a multichannel pipettor and then flicking all fluid from the wells*. The manual method may result in slightly higher variability.
7. Next, 100 µL of SureBlue TMB substrate (5,5'-tetramethylbenzidine, kpl.com) is added to each well.
8. The plate is placed on an orbital shaker for no more than 5 minutes, or until adequate color development is observed.
9. Color development is terminated by adding 100 µL stop solution (1N hydrochloric acid) to each well.
10. The absorbance in each well is measured at 450 nm using a plate reader.

11. The DA concentrations are determined using the sample (B) and control ($B_o$) absorbances, the original tissue weights, and the volume of 20% or 50% methanol used to extract each sample.
    The actual calculations are made using a Microsoft Excel work sheet that incorporates the constants for a four parameter model (DA concentration $=ED_{50}(B_o/B\ -1)^{-slope}$). This worksheet can be downloaded from:

    http://www.mercuryscience.com/Domoic%20Acid%20Quantitation%208Well%20Strip.xls

Processing time for this assay is approximately 1.5 hours.

- Quality Control (Provide Specific Steps.).

  Bo signals should be greater than 1.5 AU and less than 3.0 AU. When Bo values are greater than 3.0, the user can remove 50 ul of the yellow solution from ALL wells on that strip and re-read the signal.

  Duplicate signals should be within 10% of their average value. For example: Two duplicate wells having AU values of 1.500 and 1.600 are acceptable because the difference between the values and their average (1.550) is less than 10%. If two duplicate wells have AU values of 1.000 and 1.400, this result is invalid and should be retested because the variation between the values is too great because: (1.200 - 1.000)/1.000 = 20%

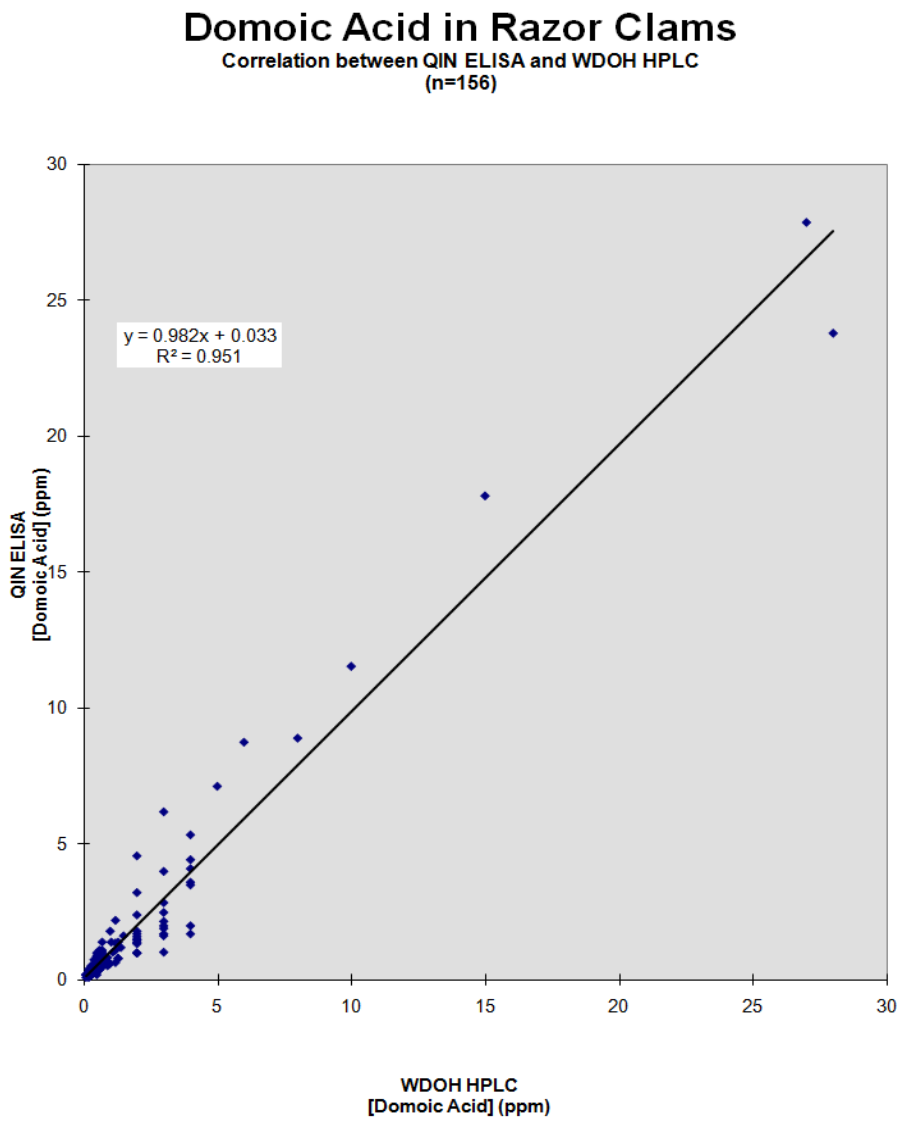
  Domoic Acid standard solutions can be run as needed to QC the accuracy of the assay. QC protocols can be developed on a case-by-case basis with assistance provided by Mercury Science Inc.

- Validation Criteria (Include Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).

  A preliminary validation study using oyster tissue has been completed and provided to the committee for feedback. Oysters were selected because they were locally available and could be run prior to the submission date. These data should be considered preliminary. In addition, an informal validation study was conducted by the Quinault Tribe and the Washington Department of Health and included below. The remaining validation studies are will be done in the latter part of the summer and fall 2009. Results will be provided to the LRM committee as they become available.

  During internal validation studies at Mercury Science, the assay was found to have an effective quantitative range from approximately 0.3 to 3.0 ppb using domoic acid standard solutions.

- Comparability: The graph below shows the results of a year-long study done by the Quinault Indian Nation (QIN) and the Washington Department of Health (WDOH) comparing razor clam analysis performed by the Domoic Acid Test Kit versus HPLC analysis. One hundred fifty six samples were compared. This independent study was planned and performed without any input from Mercury Science or NOAA.




Additional correlation studies are reported in the following research paper:

RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID, Journal of Shellfish Research, Vol. 27, No. 5, 1301–1310, 2008.
Available online at: http://mercuryscience.com/LitakerStewartDec2008.pdf

- Data and Statistical Analyses Performed for Each Validation Criterion Tested (Be Specific and Provide Clear Easy-to-Follow Step-by-Step Procedures.). Preliminary study presented for feedback from the committee

- Calculations and Formulas Used for Each Validation Criterion Tested. Testing in Progress

- Results for Each Validation Criterion Tested. Testing in Progress

- Discussion of Each Validation Criterion Tested. Testing in Progress

- Summary of Results. Testing in Progress

**Additional Requirement**

If a laboratory method is found acceptable for use in the National Shellfish Sanitation Program and adopted by the Interstate Shellfish Sanitation Conference, the method submitter will draft a laboratory checklist that can be used to evaluate laboratories performing their procedure. The checklist will be submitted to the ISSC and reviewed by the Laboratory Quality Assurance Committee for Conference approval.

(For guidance: refer to the checklists in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2003, Guidance Documents, Chapter II – Growing Areas, .11 Evaluation of Laboratories by State Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.)

VII. SLV Documents for Marine Biotoxin and Non-MPN Based Microbiological Methods (http://www.issc.org/lmrforms.aspx)

**VII. #1 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Accuracy/Trueness & Measurement Uncertainty**

**VALIDATION CRITERIA**

**Accuracy/Trueness** is the closeness of agreement between test results and the accepted reference value. To determine method accuracy/trueness, the concentration of the targeted analyte/measurand/organism of interest as measured by the analytical method under study is compared to a reference concentration.

**Measurement uncertainty** is 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.

**Procedure:** This procedure is applicable for use with either growing waters or shellfish tissues. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots of either the homogenate or growing water sample appropriately sized for your work and spike one(1) of the two (2) aliquots with a suitable known concentration of the target analyte/measurand/organism of interest. Do not spike the second aliquot. This is the sample blank. For microbiological methods determine the concentration of the target organism of interest used to spike each sample by plating on/in appropriate agar. Process both aliquots of sample as usual to determine the method concentration for the target analyte/measurand/organism of interest. For growing waters do twenty (20) samples collected from a variety of growing areas. For shellfish do twenty (20) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. **Use a variety of concentrations spanning the range of concentrations of importance in the application of the method to spike sample homogenates or growing water samples**. Both the low and high level spike concentrations must yield determinate values when analyzed by the method under study.

**Data:**

Working Range _The working range is 0.3 to 3.0 ppb and samples are diluted into the effective range so the working range is 0 to over 100 ppm

Sample Type _Shellfish Tissue__

Agar used to determine spike concentration ___Not applicable__

Organism used for spiking Oyster (*Crassostrea virginica*)

Sample Spike conc/plate count Sample blank conc Spiked sample conc from analysis

The regulatory limit for DA is 20 ppm in shellfish tissue and the dynamic range of the assay was tested using oyster tissues spiked with 2.3 to 35.5 ppm domoic acid. The standard spikes of domoic acid were calibrated using the Canadian NRC standards. The following procedure was used.

Extraction:

1. Live oysters were shucked on 3/30 and 3/31/2009 and homogenized 12 at a time in a blender and stored in 50mL tubes in -80C freezer
2. Samples thawed just prior to use
3. 2 g oyster weighed out in 50mL tube and exact weigh recorded to nearest mg
4. 18mL 50% MeOH added to tube
5. DA added to the homogenate so that the final concentrations in 20 mL were 0.25, .5, 1, 2, 4 ppm. This is equivalent to 2.5,5,10,20 or 40ppm in 2g oyster that is subsequently extracted into the total 20 ml volume.
6. Each tube vortexed for 1 min

ELISA

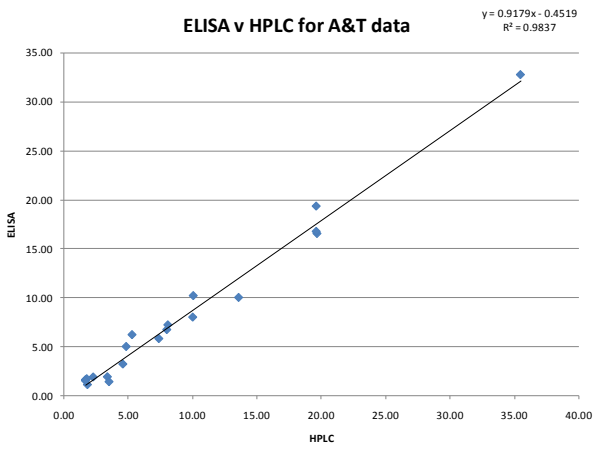
1. ~1.4mL from each tube were transferred into a 2mL microfuge tube
2. Samples in microfuge tubes centrifuged at 14,000 rpm for 5 min
3. Aliquots of the resulting supernatant were diluted with ELISA kit sample dilution buffer with a 2 step dilution series so each extract contained ~2ppb
4. Diluted extracts processed on ELISA following kit instructions

HPLC was used to determine initial spike concentration using the following procedure:

1. Spiked 50mL tubes centrifuged at 3000rpm for 20 min
2. Supernatant filtered with 25mm GF/F filter first, and then filtered with .45um syringe tip filter with 30mL syringe
3. SPE tubes pre-conditioned with 6mL MilliQ water, then 3mL 100% MeOH, then 50% MeOH
4. 5mL of extract though SPE tube, 1 drop per second
5. Washed with 5mL .1M NaCl
6. Eluted/ collected with 5 mL .5M NaCL in 15mL tube
7. ~1mL pipetted with 9 inch glass Pasteur pipette into clear HPLC vial
8. Run through HPLC- 20uL injection, .3mL/min, 15 min/sample….
9. Area and time of peak recorded
10. The DA concentration in each oyster extract was estimated using the previously determined standard curve where peak area =15.704 x DA concentration, $R^2$=0.9977.

Results

| Sample # | Sample Spike conc (HPLC) | Sample blank conc | Spiked sample conc. from analysis (ELISA) |
|---|---|---|---|
| 1 | 5.32 | 0.00 | 6.20 |
| 2 | 10.07 | 0.00 | 10.18 |
| 3 | 19.69 | 0.00 | 16.53 |
| 4 | 35.50 | 0.00 | 32.74 |
| 5 | 8.02 | 0.00 | 6.72 |
| 6 | 2.30 | 0.00 | 1.88 |
| 7 | 4.60 | 0.00 | 3.20 |
| 8 | 1.70 | 0.00 | 1.60 |
| 9 | 8.10 | 0.00 | 7.20 |
| 10 | 1.80 | 0.00 | 1.70 |
| 11 | 3.40 | 0.00 | 1.90 |
| 12 | 7.40 | 0.00 | 5.80 |
| 13 | 13.60 | 0.00 | 10.00 |
| 14 | 19.63 | 0.00 | 16.74 |
| 15 | 1.85 | 0.00 | 1.10 |
| 16 | 3.53 | 0.00 | 1.40 |
| 17 | 4.86 | 0.00 | 4.99 |
| 18 | 1.70 | 0.00 | 1.50 |
| 19 | 10.03 | 0.00 | 7.99 |
| 20 | 19.63 | 0.00 | 19.32 |
| Average | 9.14 | 0.00 | 7.93 |




The results of this preliminary study showed an excellent correlation between the HPLC and the ELISA assay, but with a slope of 0.92 instead of 1.0. This means the ELISA assay consistently underestimated the HPLC validated DA concentrations by ~10%. Preliminary tests using other shellfish tissues have shown a slope of approximately 1.0 (Litaker et al. 2008). I will do additional tests to determine whether or not the lower slope is due to matrix effects unique to oysters.

A consequence of this underestimation is that some of the statistical analyses below will show a significant difference between the spike concentration and the ELISA results. Given that this is the first time I have run through the calibration assay procedures I would request that the committee to wait for additional data before making any judgments concerning the robustness of the assay. Instead, I would like to use the preliminary oyster data to get the committee's feedback on whether I have adequately completed the necessary statistical analyses correctly and to obtain further clarifications concerning several of the analyses. The feedback will then be used for finalizing the subsequent analyses done in my laboratory and by the NOAA CCFHR laboratory.

**For shellfish samples, repeat for each tissue type of interest.**

## DATA HANDLING

### Accuracy/Trueness

The accuracy/trueness of a method consists of two distinct components, the portion due to the method itself regardless of the laboratory performing it and the portion contributed by the laboratory's performance. In a single laboratory method validation, it is impossible to distinguish the contribution of each to the overall accuracy/trueness of the method. Consequently, what is being estimated is the accuracy/trueness of the method as implemented by the laboratory performing the analysis. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work. Poor accuracy/trueness on the other hand indicates the potential unsuitability of the method and/or the laboratory's performance of it for the intended work.

Accuracy /trueness will be determined by calculating the closeness of agreement between the test results and either a known reference value or a reference value obtained by plate count for microbiological methods.

### Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations

between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

**Data Summary:**
Calculated % accuracy/trueness ___86.84____

Again, the reason for the lower than expected accuracy is the fact that the slope of the relationship was 0.92 between the ELISA and HPLC for this first set of oyster samples.

| Sample # | Sample Spike conc (HPLC) | Spiked sample conc. from analysis (ELISA) | Difference (ppm) |
|---|---|---|---|
| 1 | 5.32 | 6.2 | -0.88 |
| 2 | 10.07 | 10.18 | -0.11 |
| 3 | 19.69 | 16.53 | 3.16 |
| 4 | 35.5 | 32.74 | 2.76 |
| 5 | 8.02 | 6.72 | 1.3 |
| 6 | 2.3 | 1.88 | 0.42 |
| 7 | 4.6 | 3.2 | 1.4 |
| 8 | 1.7 | 1.6 | 0.1 |
| 9 | 8.1 | 7.2 | 0.9 |
| 10 | 1.8 | 1.7 | 0.1 |
| 11 | 3.4 | 1.9 | 1.5 |
| 12 | 7.4 | 5.8 | 1.6 |
| 13 | 13.6 | 10 | 3.6 |
| 14 | 19.63 | 16.74 | 2.89 |
| 15 | 1.85 | 1.1 | 0.75 |
| 16 | 3.53 | 1.4 | 2.13 |
| 17 | 4.86 | 4.99 | -0.13 |
| 18 | 1.7 | 1.5 | 0.2 |
| 19 | 10.03 | 7.99 | 2.04 |
| 20 | 19.63 | 19.32 | 0.31 |
| Average | 9.14 | 7.93 | 1.21 |
| stdev | | | 1.21832223 |
| 95% confidence interval | | | 0.53393371 |

Calculated measurement uncertainty __0.5 ppm___

**VII. #2 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Ruggedness**

## VALIDATION CRITERIA

**Ruggedness** is 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.

**Procedure:** This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine method concentration for the target analyte/measurand/organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the method's intended application to spike the sample aliquots. However both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days.

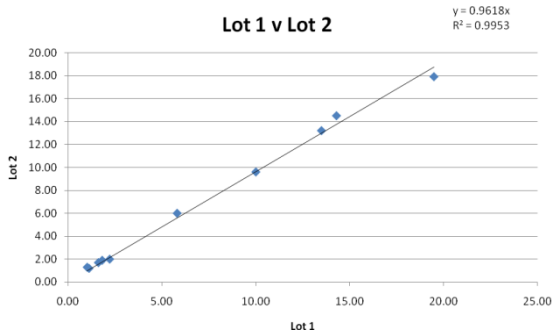
**Data:**

Sample type ___Oyster tissue_____
Sample Conc "Batch or Lot 1" Conc "Batch or Lot 2"
Media and/or Reagents Media and/or Reagents

Procedure:
Samples were spiked and extracted as listed in section VII. #1 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Accuracy/Trueness & Measurement Uncertainty. After the sample was diluted in the 2-step dilution series, the sample was processed on two different ELISA kits with different lot numbers. Samples were processed between 5/19/09 and 5/27/09.

| Sample # | Lot 1 | Lot 2 |
|---|---|---|
| 1 | 1.60 | 1.70 |
| 2 | 13.50 | 13.20 |
| 3 | 2.20 | 2.00 |
| 4 | 14.30 | 14.50 |
| 5 | 1.80 | 1.90 |
| 6 | 5.80 | 6.00 |
| 7 | 10.00 | 9.60 |
| 8 | 19.50 | 17.90 |
| 9 | 1.10 | 1.20 |
| 10 | 1.00 | 1.30 |

The $R^2$ between the results for the two batches was 0.995 and the slope was y=0.96




**For shellfish samples, repeat for each tissue type of interest.**

**DATA HANDLING**

**Ruggedness**

In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

**Procedure:** To determine whether the method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distribution produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis based on the following considerations.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the analysis.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis.
   - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range of -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

**Paired T-test results** – assumption that the variances are equal

| Sample # | Lot 1 | Lot 2 |
|---|---|---|
| 1 | 1.6 | 1.7 |
| 2 | 13.5 | 13.2 |
| 3 | 2.2 | 2 |
| 4 | 14.3 | 14.5 |
| 5 | 1.8 | 1.9 |
| 6 | 5.8 | 6 |
| 7 | 10 | 9.6 |
| 8 | 19.5 | 17.9 |
| 9 | 1.1 | 1.2 |
| 10 | 1 | 1.3 |
| mean | 7.08 | 6.93 |
| stdev | 6.7677 | 6.3808 |
| t | | 0.0504 |
| df | | 18 |
| Significantly different | | no |

**Welch's t-test**

The t-value assuming unequal variance was 0.9599.
DF = 18
Two-tailed probability 0.3498, NS

**Data Summary:**
Value for the test of symmetry of the distribution of batch/lot 1 data _Not determined__
Value for the test of symmetry of the distribution of batch/lot 2 data _Not determined__
Variance of batch/lot 1 data _6.767701_____
Variance of batch/lot 2 data __6.380883_____
Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 _1.0606__
Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples ____N__

Neither the paired or Welch's t-test estimates showed a significant difference between batches

**VII. #3 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Precision & Recovery**

**VALIDATION CRITERIA**
**Precision** is the closeness of agreement between independent test results obtained under stipulated conditions.
**Recovery** is the fraction or percentage of an analyte/measurand/organism of interest recovered following sample analysis.
**Procedure:** This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take four (4) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work. Spike one of the four aliquots with a low (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Spike the second aliquot of the growing water sample or shellfish homogenate with a medium concentration of the target analyte/measurand/organism of interest. Spike the third aliquot of the growing water

sample or shellfish homogenate with a high (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Do not spike the fourth aliquot of the growing water sample or shellfish homogenate. This is the sample blank. Spiking levels must cover the range in concentrations important to the application of the method (working range). For microbiological methods determine the concentration of the target organism of interest used to spike each aliquot by plating in/on appropriate agar. Process each aliquot including the sample blank as usual to determine the method concentration for the target analyte/measurand/organism of interest. Do two (2) replicates for each of the three (3) spiked aliquots. Replicate analysis is unnecessary for the sample blank. Do only one sample blank per sample. For growing waters, do ten (10) samples collected from a variety of growing areas. For shellfish, do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed in this exercise (i.e. $10^1$, $10^3$ and $10^5$).

**Data:**

Working Range _The working range is 0.3 to 3.0 ppb and samples are diluted into the effective range so the working range is 0 to over 100 ppm

Sample Type _Shellfish Tissue__

Agar used to determine spike concentration ___Not applicable__

Organism used for spiking Oyster (*Crassostrea virginica*)

Procedure: Samples were spiked and extracted as listed in section VII. #1 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Accuracy/Trueness & Measurand Uncertainty. Each sample was spiked with a low, medium and high concentration of approximately 2.5, 20, and 40ppm in the tissue sample. HPLC was used to determine actual spike concentration.

Sample Spike conc/Plate count/Conc of blank Conc in spiked sample from analysis

| | Aliquot 1 | Aliquot 2 | | | Aliquot 3 | | | Aliquot 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample # | Blank | L spike | $L_a$ | $L_b$ | M spike | $M_a$ | $M_b$ | H spike | $H_a$ | $H_b$ |
| 1 | 0.00 | 2.60 | 3.00 | 2.50 | 20.14 | 20.50 | 19.40 | 39.93 | 33.70 | 38.50 |
| 2 | 0.00 | 2.71 | 2.85 | 2.96 | 19.10 | 19.17 | 19.90 | 39.28 | 31.66 | 33.55 |
| 3 | 0.00 | 2.26 | 2.11 | 2.19 | 19.64 | 23.42 | 22.29 | 39.84 | 29.32 | 30.24 |
| 4 | 0.00 | 2.50 | 1.48 | 1.86 | 19.21 | 16.09 | 16.57 | 35.50 | 32.74 | 30.30 |
| 5 | 0.00 | 2.62 | 2.08 | 1.87 | 19.11 | 14.01 | 15.92 | 36.56 | 30.95 | 30.84 |
| 6 | 0.00 | 2.45 | 2.00 | 2.70 | 15.89 | 17.11 | 13.72 | 34.97 | 26.14 | 27.82 |
| 7 | 0.00 | 1.99 | 2.06 | 2.31 | 16.42 | 13.00 | 12.36 | 35.32 | 25.44 | 27.08 |
| 8 | 0.00 | 1.70 | 1.60 | 1.70 | 14.77 | 13.50 | 13.16 | 27.30 | 19.50 | 19.40 |
| 9 | 0.00 | 2.14 | 1.80 | 1.70 | 14.60 | 12.50 | 12.40 | 29.48 | 27.40 | 27.70 |
| 10 | 0.00 | 1.80 | 1.70 | 1.80 | 14.84 | 12.90 | 12.20 | 30.49 | 26.80 | 30.60 |

~~1L 1L~~ ~~a~~
~~1L~~ ~~b~~
~~1M 1M~~ ~~a~~
~~1M~~ ~~b~~
~~1H 1H~~ ~~a~~
~~1H~~ ~~b~~
~~1B~~
~~2L 2L~~ ~~a~~
~~2 L~~ ~~b~~
~~2M 2M~~ ~~a~~
~~2M~~ ~~b~~
~~2H 2H~~ ~~a~~
~~2H~~ ~~b~~
~~2B~~
~~“ “~~
~~“ “~~
~~“ “~~
~~“ “~~
~~10L 10L~~ ~~a~~
~~10L~~ ~~b~~
~~10M 10M~~ ~~a~~
~~10M~~ ~~b~~
~~10H 10H~~ ~~a~~
~~10H~~ ~~b~~
~~10B~~

L, M and H refer to low, medium and high concentrations respectively. $L_a$, $L_b$, $M_a$, $M_b$, $H_a$ and $H_b$ refer to the replicate determinations of the sample aliquots spiked with low (L), medium (M) and high (H) concentrations of the target analyte/measurand/organism of interest. B refers to the sample blank.

**For shellfish samples, repeat for each tissue type of interest.**

## DATA HANDLING

### Precision

To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

| | | **Low** | | | | | **Medium** | | | | | **High** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L spike | La | (La)^2 | Lb | (Lb)^2 | M spike | Ma | (Ma)^2 | Mb | (Mb)^2 | H spike | Ha | (Ha)^2 | Hb | (Hb)^2 | |
| | | 2.6 | 3 | 9 | 2.5 | 6.25 | 20.14 | 20.5 | 420.25 | 19.4 | 376.36 | 39.93 | 33.7 | 1135.69 | 38.5 | 1482.25 | |
| | | 2.71 | 2.85 | 8.1225 | 2.96 | 8.7616 | 19.1 | 19.17 | 367.489 | 19.9 | 396.01 | 39.28 | 31.66 | 1002.36 | 33.55 | 1125.6 | |
| | | 2.26 | 2.11 | 4.4521 | 2.19 | 4.7961 | 19.64 | 23.42 | 548.496 | 22.29 | 496.844 | 39.84 | 29.32 | 859.662 | 30.24 | 914.458 | |
| | | 2.5 | 1.48 | 2.1904 | 1.86 | 3.4596 | 19.21 | 16.09 | 258.888 | 16.57 | 274.565 | 35.5 | 32.74 | 1071.91 | 30.3 | 918.09 | |
| | | 2.62 | 2.08 | 4.3264 | 1.87 | 3.4969 | 19.11 | 14.01 | 196.28 | 15.92 | 253.446 | 36.56 | 30.95 | 957.903 | 30.84 | 951.106 | |
| | | 2.45 | 2 | 4 | 2.7 | 7.29 | 15.89 | 17.11 | 292.752 | 13.72 | 188.238 | 34.97 | 26.14 | 683.3 | 27.82 | 773.952 | |
| | | 1.99 | 2.06 | 4.2436 | 2.31 | 5.3361 | 16.42 | 13 | 169 | 12.36 | 152.77 | 35.32 | 25.44 | 647.194 | 27.08 | 733.326 | |
| | | 1.7 | 1.6 | 2.56 | 1.7 | 2.89 | 14.77 | 13.5 | 182.25 | 13.16 | 173.186 | 27.3 | 19.5 | 380.25 | 19.4 | 376.36 | |
| | | 2.14 | 1.8 | 3.24 | 1.7 | 2.89 | 14.6 | 12.5 | 156.25 | 12.4 | 153.76 | 29.48 | 27.4 | 750.76 | 27.7 | 767.29 | |
| | | 1.8 | 1.7 | 2.89 | 1.8 | 3.24 | 14.84 | 12.9 | 166.41 | 12.2 | 148.84 | 30.49 | 26.8 | 718.24 | 30.6 | 936.36 | |
| Subgroup sample number | n(I, j, l) | | 10 | | 10 | | | 10 | | 10 | | | 10 | | 10 | | |
| Subgroup sum | Sum (i, j, l) | | 20.68 | | 21.59 | | | 162.2 | | 157.92 | | | 283.65 | | 296.03 | | Sum |
| Subgroup variance | [(Sum (i, j, l))^2]/n(I, j, l) | | 42.77 | | 46.61 | | | 2630.88 | | 2493.87 | | | 8045.73 | | 8763.38 | | 22023.24 |

| | | | | | |
|---|---|---|---|---|---|
| Group sample number | n(i) | 20 | 20 | 20 | 60 |
| Group sum | Group sum | 42.27 | 320.12 | 579.68 | 942.07 |
| Group mean | Xhat (i) | 2.17 | 16.46 | 30.95 | |
| Group variance | [(Xhat (i))^2]/n(i) | 89.3376 | 5123.84 | 16801.4 | 22014.62 |

| | |
|---|---|
| C | 14791.59808 |
| Total SS | 7859.977618 |
| Among all subgroups SS | 7231.65 |
| error SS | 628.33 |
| Groups SS | 7223.025403 |
| Subgroups SS | 8.62 |
| Total DF | 59 |
| Groups DF | 2 |
| Among all subgroups DF | 5 |
| Subgroups DF | 3 |
| Error DF | 54 |

| Source of Variation | SS | DF | MS |
|---|---|---|---|
| Total | 7859.98 | 59 | |
| Among all subgroups | 7231.65 | 5 | |
| Groups | 7223.03 | 2 | 3611.52 |
| Subgroups | 8.62 | 3 | 2.87 |
| Error | 628.33 | 54 | 11.64 |

Ho: There is no significant difference among the replicates (a,b) in affecting domoic acid concentration.
HA: There is a significant difference among replicates (a,b) in affecting domoic acid concentration.

$F = 2.87/11.64 = 0.25$ $F_{0.05(1),3,54} = 2.79$ $F < F_{0.05(1),3,54}$ Do not reject Ho.

**The replicates are NOT significantly different**

Ho: There is no difference in Domoic Acid concentration among the three concentrations (L, M, H).
HA: The three concentrations (L, M, H) are significantly different.

$F = 3611.52/2.87 = 1258.37$ $F_{0.05(1),2,3} = 9.55$ $F > F_{0.05(1),2,3}$ Reject H0

**The concentrations are significantly different.**

**Source of variation Degrees of freedom Sum of Squares Mean Square**
Samples 9
Concentrations in samples 20
Determinations within concentrations 30
Total 59

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations. If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

Per the ISSC instructions, I used F = Concentrations in samples/determinations within concentrations = 2.87/3611.52 = 0.00079

$F_{0.05(1),2,3} = 9.55$ F <<< $F_{0.05(1),2,3}$ Accept H0.

**So, there is no significant difference in precision among each of the three concentrations (L,M,H)**

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.

   Avg. concentration of Domoic acid in the spiked samples

   | | |
   |---|---|
   | Low | 2.17 |
   | Med | 16.46 |
   | High | 34.867 |

2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA variance component, **Total**.

   Standard deviation of spiked sample data

   | | SD |
   |---|---|
   | Low | 0.43 |
   | Med | 3.25 |
   | High | 5.23 |

3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and

   | | |
   |---|---|
   | Low | 0.20 |
   | Med | 0.20 |
   | High | 0.17 |

4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

   | | |
   |---|---|
   | Low | 20 |
   | Med | 20 |
   | High | 17 |

**Recovery**

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.

4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.

| Sample | Spike | Average ELISA | Spike-ELISA |
|---|---|---|---|
| 8L | 1.7 | 1.65 | 0.05 |
| 10L | 1.8 | 1.75 | 0.05 |
| 7L | 1.99 | 2.18 | -0.19 |
| 9L | 2.14 | 1.75 | 0.39 |
| 3L | 2.26 | 2.15 | 0.11 |
| 6L | 2.45 | 2.35 | 0.1 |
| 4L | 2.5 | 1.67 | 0.83 |
| 1L | 2.6 | 2.75 | -0.15 |
| 5L | 2.62 | 1.97 | 0.65 |
| 2L | 2.71 | 2.91 | -0.2 |
| 9M | 14.6 | 12.45 | 2.15 |
| 8M | 14.77 | 13.33 | 1.44 |
| 10M | 14.84 | 12.55 | 2.29 |
| 6M | 15.89 | 15.41 | 0.47 |
| 7M | 16.42 | 12.68 | 3.74 |
| 2M | 19.1 | 19.53 | -0.43 |
| 5M | 19.11 | 14.96 | 4.15 |
| 4M | 19.21 | 16.33 | 2.88 |
| 3M | 19.64 | 22.86 | -3.22 |
| 1M | 20.14 | 19.95 | 0.19 |
| 8H | 27.3 | 19.45 | 7.85 |
| 9H | 29.48 | 27.55 | 1.93 |
| 10H | 30.49 | 28.7 | 1.79 |
| 6H | 34.97 | 26.98 | 7.99 |
| 7H | 35.32 | 26.26 | 9.05 |
| 4H | 35.5 | 31.52 | 3.98 |
| 5H | 36.56 | 30.9 | 5.67 |
| 2H | 39.28 | 32.61 | 6.68 |

5. Perform a one way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

**Source of variation Degrees of freedom Sum of Squares Mean Square**
Concentration 2
Error 27
Total 29

| Source of Variation | Sum of Squares | d.f. | Mean Squares | F |
|---|---|---|---|---|
| Between | 181.9 | 2 | 90.93 | 20.22 |
| Error | 121.4 | 27 | 4.496 | |
| Total | 303.2 | 29 | | |

Group A (low): Number of items= 10
Mean = 0.16400
95% confidence interval for Mean: -1.212 thru 1.540
Standard Deviation = 0.353
High = 0.8300 Low = -0.2000

Median = 7.5000E-02
Average Absolute Deviation from Median = 0.252

---

Group B (medium): Number of items= 10
Mean = 1.3660
95% confidence interval for Mean: -9.8640E-03 thru 2.742
Standard Deviation = 2.20
High = 4.150 Low = -3.220
Median = 1.795
Average Absolute Deviation from Median = 1.68

---

Group C (high): Number of items= 10
Mean = 5.8830
95% confidence interval for Mean: 4.507 thru 7.259
Standard Deviation = 2.92
High = 10.06 Low = 1.790
Median = 6.175
Average Absolute Deviation from Median = 2.44

The probability of this result, assuming the null hypothesis, is less than 0.0001. The highest spikes had greater variability. Those in regulatory range (Low and Medium) were less variable.

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   F= 90.93/4.496 = 20.22
   Numerator degrees of freedom = 2
   Denominator degrees of freedom = 27
   Probability Value: 0.000004

   This confirms greater variability in recovery at the higher spike concentrations

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

   To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

   1. Use log transformed data for microbiological methods.
   2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
   3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.
      18.17
   4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.
      15.7

5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.
86.4%

**Data Summary:**

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant? **Y**
- If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **NA/_____%**
- Is the one way analysis of variance to determine the consistency of recovery of the method under study significant? **Y**
- At what concentrations is the one way analysis of variance significant? **NA/___?_____**
- What is the overall percent recovery of the MPN based method under study? **NA/__86.4___%**

**VII. #4 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Specificity**

**VALIDATION CRITERIA**

**Specificity** is the ability of the method to measure only what it is intended to measure. To determine method specificity samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/targeted organism of interest.

**Procedure:** This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the targeted analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method under study) of the targeted analyte/measurand/ organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank, the aliquot spiked with the targeted analyte/measurand/organism of interest and the aliquot spiked with the targeted analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method concentration for the targeted analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

**Data:**

Glutamine and Glutamic are structurally related to domoic acid and present in shellfish tissues. Hence they represent potentially important competitors. These compounds were therefore tested to determine if high concentrations would interfere with the DA ELISA.

Name of suspected interfering organism/compound/toxin #1 _____ Glutamine _____
Sample type ____Shellfish Tissue _____
Sample blank concentration for the targeted analyte/measurand/organism of interest __0.0__
Concentration of aliquot spiked with targeted analyte/measurand/ with targeted analyte/measured: see below
Organism of interest organism: oyster

Procedure:

1. 2000 ppm solutions of Glutamine and Glutamic acid were made by mixing 26.7mg Glutamine in 13.35mL $dH_2O$ and 26.8 mg Glutamic Acid in 13.4 mL dH2O
2. 2 g thawed oyster sample weighed into 50 mL tube
3. 17mL 50% MeOH added to tube
4. 3.34 µL 90% 1670ppm DA added to make 2.5ppm DA spike
5. Sample vortexed
6. Sample split into two 15mL tubes
7. 500 µL 50% MeOH added to DA-only tube
8. For tube spiked with interfering compound, 250mL 50% MeOH added + 250 µL 2000ppm Gulatime/Glutamic Acid for an ~55ppm spike in shellfish tissue
9. Samples then processed by ELISA and HPLC as described previously.

| Replicate | Conc. of spike | Conc. of Spike Glutamine |
|---|---|---|
| 1 | 1.70 | 1.70 |
| 2 | 1.60 | 1.70 |
| 3 | 1.70 | 1.60 |
| 4 | 1.90 | 2.10 |
| 5 | 1.70 | 2.20 |
| Avg | 1.72 | 1.86 |
| mean | 1.7 | 1.9 |
| Standard deviation | 0.1 | 0.2 |
| SIavg | 0.925 | |

Name of suspected interfering organism/compound/toxin #2 _____ Glutamic Acid _____
Sample type ____Shellfish Tissue _____
Sample blank concentration for the targeted analyte/measurand/organisn of interest __0.0__
Concentration of aliquot spiked with targeted analyte/measurand/ with targeted analyte/measured: see below
Organism of interest organism: oyster

| Replicate | Conc of spike | Conc of Spike Glutamic Acid |
|---|---|---|
| 1 | 1.90 | 1.80 |
| 2 | 1.60 | 1.80 |
| 3 | 1.50 | 1.40 |
| 4 | 1.30 | 1.50 |
| 5 | 1.90 | 1.50 |
| Avg | 1.64 | 1.60 |
| Standard deviation | 0.2 | 0.2 |
| SIavg | 1.025 | |

**Repeat for each suspected interfering organism tested.**

**DATA HANDLING**

The **Specificity index** will be used to test the specificity of the method in the presence of suspected interfering organisms/compounds/toxins. The **Specificity index (SI)** is calculated as indicated below:

**Specificity index (SI) = Sample spiked with target of interest only**
**Sample spiked with both target and suspected interferences**

All microbiological count data must be converted to logs before analysis. Samples spiked with both the targeted analyte/measurand/organism of interest and the targeted anaalyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any corrections that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index should equal one (1) in the absence of interferences. To test the significance of a Specificity index other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test is used. For each suspected interfering organism/compound/toxin calculate the average **Specificity Index (SI)** for the 5 replicatesanalyzed for each sample by obtaining the average concentration for both the aliquot containing the targeted analyte/measurand/organism of interest only and the aliquot containing the targeted analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

**$SI_{avg}$ = Avg concentration of sample spiked with target of interest only**
**Avg concentration of sample spiked with both target and suspected interferences**

Perform a two-sided t-test at the .05 significance level to determine if the average Specificity index (SI) obtained from the 5 replicates of each analysis differs from one (1).

Repeat for all interfering organisms/compounds/toxins tested.

**Data Summary:**

Interfering organism/compound/toxin #1 _____Glutamine_____ $SI_{avg}$_0.925_____

Significant difference from 1 _____

Interfering organism/compound/toxin #2 ____Glutamic Acid_____ $SI_{avg}$___1.025____

Significant difference from 1 _____

Glutamine Two tailed T-test 95% confidence level

T=2.0
DF=8
Confidence Level 91.95%
Not Significant

Glutamic Acid

T=0.3162
DF=8
Confidence Level 24.01%
Not Significant

VII. #5 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – **Linear Range, Limit of Detection, Limit of Quantitation/Sensitivity**

**VALIDATION CRITERIA**

**Linear Range** is the range within the working range where the results are proportional to the concentration of the analyte/measurand/organism of interest present in the sample.

**Limit of Detection** is the minimum concentration at which the analyte/measurand/organism of interest can be identified.

**Limit of Quantitation/Sensitivity** is the minimum concentration of the analyte/measurand/organism of interest that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

**Procedure:** This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take at least six (6) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work and spike five (5) of the six (6) aliquots with five (5) different concentrations (i.e. $10^a$, $10^b \ldots 10^n$) of the target analyte/measurand/organism of interest spanning 50 – 150% of the working range/range of interest for the method under study. Do not spike the sixth or last aliquot of each sample. This is the sample blank. For microbiological methods determine the concentration of the target analyte/measurand/organism of interest used to spike each aliquot of each sample by plating in/on appropriate agar. Do not use aliquots of the same master solution/culture to spike all the samples in this exercise. A separate master solution /culture should be used for each sample. Process each aliquot including the sample blank as usual to determine method concentration for the target analyte/measurand/organism of interest. Do three (3) replicates for each aliquot excluding the sample blank. Do only one blank per sample. For growing waters do ten (10) samples collected from a variety of growing areas. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed ($10^a$, $10^b \ldots 10^n$).

This is a section where I could use guidance by the committee. The assay has a wide dynamic range because samples are diluted into the 0.3 to 3 ppb linear range of the assay. It is this aspect of the assay which makes it difficult to implement the instructions provided above. The actual linear range was determined as by diluting the standards to various levels and testing the assay multiple times. This was a necessary step in developing the critical parameters needed by the data analysis software provided with the kit to back calculate DA values from the B and Bo values (see article published in the December 2008 issue of the Journal of Shellfish Research for details). I need to know if the data presented in the published article are sufficient to meet the committee's requirements for determining the linear range and limits of detection. If not, please recommend what procedure should be followed considering that the samples must be diluted. This is similarly true for determining the dynamic range of the assay.

**Data: Testing in progress**

Sample type _____
Working range/Range of interest _____
Range in spiking levels used _____
Agar used to determine spike concentration _____
Organism used for spiking _____
**Aliquot 0* 1 2 3 4 5**
**Sample 1**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Aliquot 0* 1 2 3 4 5**
**Sample 2**

Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Sample 3**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Sample 4**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Sample 5**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Sample 6**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Sample 7**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Sample 8**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Aliquot 0* 1 2 3 4 5**
**Sample 9**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
**Sample 10**
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
*** Unspiked sample blank**

Response is the signal data (absorbance, florescence, Ct value), colonies, plaques, etc resulting from the analysis.
For shellfish samples repeat for each tissue type of interest**.**

## DATA HANDLING

### Linear Range

To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it.
6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response
7. Multiply the value obtained in step 5 by 0.95 and 1.05.
8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects either of the lines obtained above.
10. The linear range of the method as implemented by the laboratory is comprised of the range in concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

### Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organisn of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of "blank + 3σ" and "blank + 10σ" generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

**Data Summary:**

Linear range of the method as implemented _____

The limit of detection of the method as implemented _____

The limit of quantitation/sensitivity of the method as implemented _____

**IX.** SLV Documents for New or Modified Methods as Alternatives to NSSP Methods http://www.issc.org/client_resources/lmr%20documents/ix%20%20_1%20new%20or%20modified%20methods%20as%20alternatives.pdf

**IX. #1 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Comparing Methods**

**VALIDATION CRITERIA**

**Comparability** is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

**Comparison of Methods:**

New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

**Procedure to compare the new or modified method to the officially recognized method:** This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

**Data:**

Sample type ____Shellfish tissue- oyster_____

Date Sample/Station # Conc. Recognized method Conc. Alternative Method

Data still being gathered to answer this question.

~~1~~
~~2~~
~~3~~
~~4~~
~~5~~
~~6~~
~~7~~
~~8~~
~~9~~
~~10~~
~~n~~
~~n is the last sample in the comparison~~

**For shellfish samples, repeat for each tissue type of interest**

**Data handling to compare the new or modified method to the officially recognized**

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level ($\alpha$) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each method and their respective

variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

**Data summary for the comparison of the new or modified method to the officially recognized method:**

Value for the test of symmetry for the distribution of the data generated by the officially recognized method _____

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method _____

Variance of the data generated from the officially recognized analytical method _____

Variance of the data generated from the proposed alternative analytical method _____

Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____

Is there a significant difference between the analytical methods Y/N