Single Laboratory Validation (SLV) Submission to the Interstate Shellfish Sanitation Conference (ISSC) in support of Method Approval as an Approved NSSP Method

Justification for New Method

For: Domoic Acid (ASP) Plate Kit, Cat. # 20-0249
Type of Method: Enzyme Linked Immunosorbent Assay (ELISA) utilizing a polyclonal antibody for detection of the ASP Biotoxin, Domoic Acid.
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Purpose of Intended Use of the Method. The purpose and intended use of this method is to provide a laboratory method for quickly establishing a quantified level of the ASP biotoxin, Domoic Acid, in *Mytilus edulis* (Blue Mussel) tissue as required for closing and opening of shellfish growing areas.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods. It appears there is a need for additional approved methods as an alternative to HPLC for the Biotoxin type: Amnesic Shellfish Poisoning (ASP). An ELISA method would enable monitoring laboratories to become proficient in operating a quantification method for ASP toxins levels at a lower equipment and reagent cost using a method that requires less training than HPLC to operate. This proposal demonstrates the equivalency of the HPLC and ELISA when using the same sample extract. This offers an additional benefit in that any confirmation testing could be completed using the same sample extract.

Method Limitations: This proposal offers supporting data for use of the method with *Mytilus edulis (blue mussel)* tissue only.

Method Documentation
Method Title: Domoic Acid (ASP) Plate Kit, Cat. # 20-0249
Method Scope: The method is a competitive enzyme linked immunosorbent assay (ELISA) for the quantification of domoic acid (DA) residues in *Mytilus edulis* shellfish tissue. Domoic acid is produced by some species of the diatom Pseudo-nitzschia which is the primary toxin associated with amnesic shellfish poisoning (ASP). Current legislation in the NSSP limits the amount of DA allowed in harvested shellfish to 2 mg/100 g (20 ppm) and will close shellfish growing areas to shellfish harvesting to protect consumers from exposure to the toxin. The test kit provides a tool to close and open shellfish growing areas by rapidly monitoring toxin levels as levels can quickly rise and fall.

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References: (For HPLC Method) M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Sample. NRC Institute for Marine Biosciences, Technical Report #64 National Research Council Canada #33001.

ELISA Principle- The kit is a competitive ELISA method utilizing a plate coated with Rabbit polyclonal antibodies specific to the analyte Domoic Acid. The Domoic Acid–HRP (Horse Radish Peroxidase) Enzyme Conjugate competes with any toxin from the shellfish sample extract for binding with the antibody on the plate. After and incubation period of 30 min, the plate is washed with water to remove excess material and an enzyme substrate is added to react with any HRP Enzyme Conjugate bound to the plate. The amount of bound Conjugate is inversely proportional to the amount of DA toxin in the sample extract and can be visualized by development of a blue color. The ELISA is stopped at 30 min. with a 0.1 N HCl solution, and evaluated by reading the absorbance (OD) at 450 nm wavelength in a plate reader. The OD of the sample is compared to the Calibration Curve and multiplied by the total dilution factor of 4000 to obtain the concentration of toxin in the shellfish tissue sample.

Shellfish Sample Preparation: Fresh shellfish are externally washed and removed from the shell, approximately 15 single animals are combined. Composite sample is washed, drained then homogenized for ~20 seconds using a Waring blender with 16 oz. Mason jar fitted with ice crusher blade. Samples are aliquoted and can be frozen at -20^{o}C until use.

Shellfish Sample Extract Preparation: Composite mussel samples are extracted using a 4 X ratio of 50% methanol/water to tissue weight and mixed using a vortex mixer for 3 minutes. A sample of approximately 1 ml was aliquoted into a 1.5 ml Eppendorf tube and centrifuged for 5 min. at 12,000 rcf. Supernatants were diluted 1:1000 (as directed in the Test Kit Product Insert) into 10% acetonitrile/water (Sample Dilution Buffer). Diluted samples are used in the ELISA. The extraction and dilution procedure results in a total dilution factor of 4000 to be used in calculation of DA residues present in the original tissue sample.

Proprietary Aspects. Beacon Analytical Systems has developed the kit including antibodies and HRP enzyme conjugate.

Equipment: Microplate Reader with a filter for reading at 450 nm wavelength. Sample Preparation-blender, scale, extraction container with lid (10-20 ml), vortex mixer, microcentrifuge (12,000 rcf), disposable 1.5 ml centrifuge tubes, calibrated variable pipettes 1.0 ml and 0.010 ml with disposable tips, timer, and wash bottle.

ELISA Kit Reagents.

- **Plate** – (1) containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.
- **Domoic Acid Calibrators–** (4) vials each containing 2 ml with a concentration of 0, 0.5, 5, and 50 µg/L (ppb) Domoic Acid
- **Domoic Acid HRP Enzyme Conjugate** – (1) vial containing 12 ml
- **Substrate** – (1) vial containing 14 ml
- **Stop Solution** – (1) vial containing 14 ml (Caution! Contains 1N HCl. Handle with care.)
- **Product Insert** containing instructions for use.
- **Certificate of Conformity** (Specific to each Kit Lot#).

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ELISA Test Procedures:

1. Allow reagents and sample extracts to reach RT prior to running the test
2. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
3. Using a pipette with disposable tips, dispense **100 µl** of the **Calibrator or sample extract** into the appropriate test wells. Please use a clean pipette tip for each sample addition.
4. Dispense **100 µl** of the **HRP Enzyme Conjugate** into each well.
5. Shake the plate gently for 30 seconds using a back and forth motion. Then incubate the wells for **30 minutes** at RT.
6. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with laboratory grade water and then decant. Repeat four times for a total of five washes.
7. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the water.
8. Dispense **100 µl** of the **Substrate** into each well. Shake the plate gently for 30 seconds using a back and forth motion.
9. Incubate the wells for **30 minutes** at RT.
10. Dispense **100 µl of the Stop Solution** into each well.
11. Measure and record the absorbance (Optical Density; OD) of the wells at 450 nm using a strip or plate reader. The OD correlates to a concentration of DA (ppb) based upon the Calibration Curve run with each set of samples.
12. To obtain the concentration of Domoic acid in the sample multiply the concentration results by the Total Dilution Factor of 4000.

Note: If the sample absorbance is higher or lower than the 0.5 or 50 ppb Calibrator results, the tissue levels should be expressed as less than or greater than the corresponding tissue levels (<2ppm or >200ppm DA). The sample dilution can be modified appropriately and retested along with another set of Calibrators.

Note: Running Calibrators and samples in duplicate will provide optimal assay precision and accuracy.

Quality Control:

Beacon Analytical Systems is ISO 9001:2015 approved for their quality systems for immunochemical test kit development, manufacturing and supporting activities.

Overview of Kit Quality Control - Each kit is tested following the ELISA procedure in the product insert. During manufacturing operation duplicates of the Kit Calibrator Reagents are run in order to meet established criteria prior to shipment.

Each Domoic Acid (DA) Calibrator's absorbance (OD) and binding characteristics (% B/B0) must be within a set of ranges. Ranges have been established for the Domoic Acid Plate Kit from historical data.

All plate components are tested for precision prior to using them in kits. The tolerance for variation within one lot of plates is less than or equal to 5%.

DA Calibration solutions are prepared using certified reference standard material purchased from the Canadian National Resource Council and are tested to be within 2% of the previous lot of control.

The R^2 correlation of the DA Kit Calibration Curve should be 0.99 or above.

All CV's must be less than or equal to 5%.

All QC data is kept electronically and backed up with hard copies at our manufacturing plant.

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Single Laboratory Validation Criteria and Results
Section # 1 - Accuracy/ Trueness & Measurement Uncertainty
Section # 2 – Ruggedness
Section # 3 - Precision & Recovery
Section # 4 - Specificity
Section # 5 - Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity,
Section # 6 - Comparability (If intended as a substitute for an established method accepted by the NSSP).

Materials and Methods

Blank Mussel Tissue: Five different bags of mussels were purchased locally and screened on the ELISA for DA content. The ELISA screening did not find DA residues in these samples. They were used in spiking experiments (Table 1).

Table 1: Sample Type - Mussel (Blank) DA Blank Mussel Tissue used in validation.

ID	Harvest Date	Location	Type	Commercial Name	DA ELISA Screening Results*
A.	10/24/2016	Chebeague Island, ME	Aquaculture	Bangs Island Mussels	0 ppm
58	01/30/2017	Addison, ME	Natural	Moosabec Mussels	0 ppm
59	02/02/2017	Chebeague Island, ME	Aquaculture	Bangs Island Mussels	0 ppm
F.	04/18/2017	P.E.I., Canada	Natural	Cape Cod Shellfish Co.	0 ppm
E.	04/24/2017	Addison, ME	Natural	Moosabec Mussels	0 ppm

*All Blank tissue screening results were below the DA ELISA Level of Quantitation (LOQ).

Domoic Acid Standards

Certified Reference Material (CRM) – Certified Calibration Solution for Domoic Acid (CRM-DA-g, Lot# 20140730) purchased from National Research Council Canada. The certified concentration values and associated uncertainties for Domoic Acid and *epi*-Domoic Acid in this solution is 103.3 $\pm$ 3.4 ug/ml (at 20 ^{o}C). This is used in the manufacturing of Calibrator Solutions supplied with test kit.

Domoic Acid (DA) Standard used for sample spiking at ppm levels - Domoic Acid from *Mytilus edulis*, Calbiochem Catalog # 324378, Lot #2879693, 1 mg/vial. Reconstituted Solution adjusted for purity (98%).

Equivalency of CRM and DA Standards using HPLC Analysis: HPLC was used to confirm the concentration and equivalency of this standard to the reference material prior to the use in spiking experiments. The Calbiochem standard was diluted 1:40 into 10% acetonitrile/DI water (ELISA sample dilution buffer) or 50% methanol/50% DI water (Sample extraction solution) and each run in triplicate on HPLC. The concentration of the Calbiochem solution used for tissue spiking was assigned using the average of 6 replicates as 1.873 mg/ml.

Shellfish Sample Extract Preparation: Composite mussel samples are extracted using a 4 X ratio of 50% methanol/water to tissue weight. Samples were spiked with Calbiochem DA Standard at this time (if

required) and mixed using a vortex mixer for 3 minutes. A sample of approximately 1 ml was aliquoted into a 1.5 ml Eppendorf tube and centrifuged for 5 min. at 12,000 rcf. Supernatants were diluted 1:1000 (as directed in the Test Kit Product Insert) into 10% acetonitrile/water (Sample Dilution Buffer). Diluted samples were used in the ELISA resulting in a total dilution factor from the sample preparation of 4000.

Mussel Tissue- Certified Reference Material for Domoic Acid in Mussel tissue. - Certified Reference Material for Domoic Acid (CRM-ASP-MUS-d, Lot# 201112) purchased from National Research Council Canada. The concentration of DA and epi-DA is reported to be 49 $\pm$ 3 ug/g.

Extraction efficiency using Mussel Tissue CRM. Evaluation of Test method extraction and recovery was evaluated using this reference material (Table 2). Sample A was prepared by addition of CRM Reference mussel tissue to blank mussel tissue (Table 1) at a 1:1 ratio resulting in a DA tissue concentration of 24.5 ug/g, Sample F was prepared by blending the 4 gm standard with 9 gm of blank mussel tissue to obtain a tissue concentration of 15 ug/g. Both samples were extracted as described above and tested by ELISA. Recovery percentages at two different spiking levels were found to be 107 and 109 percent in mussel tissue by ELISA.

Table 2. – Mussel CRM extraction recovery results.

Sample Blank Mussel Spiked with DA Mussel CRM	DA ELISA Result (ppm)	% Recovery
Sample A spiked at 24.5 ppm DA	26.2 ppm	107 %
Sample F spiked at 15.0 ppm DA	16.3 ppm	109 %

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Section 1: Accuracy/Trueness & Measurement Uncertainty (Table 3)

Working Range – Twenty samples of DA Blank Mussel Tissue were spiked with a low level (10 ppm), and twenty samples at a high level-20 ppm using the Calbiochem standard and extracted and evaluated by ELISA. Data and results are shown in Table 3.

Data Summary- Accuracy/Trueness

% Accuracy 10 ppm spike = 96.0 %

% Accuracy 20 ppm spike = 95.9 %

Data Summary – Measurement Uncertainty

Measurement uncertainty determined using a two-sided, 95% Confidence interval calculation

10 ppm spike = 0.662

20 ppm spike = 1.224

Table 3. Results of Accuracy/Trueness Testing of Blank and Spiked Mussel Tissue

Sample	Spiked Mussel (10 ppm)	% Accuracy (10 ppm)		Sample	Spiked Mussel (20 ppm)	% Accuracy (20 ppm)
1	10.70	107.0		1	21.06	105.3
2	8.06	80.6		2	23.97	119.8
3	8.07	80.7		3	16.87	84.3
4	10.29	102.9		4	19.07	95.3
5	11.15	111.5		5	16.27	81.4
6	8.18	81.8		6	17.18	85.9
7	8.43	84.3		7	16.80	84.0
8	11.26	112.6		8	18.62	93.1
9	11.42	114.2		9	16.31	81.5
10	8.81	88.1		10	22.74	113.7
11	8.91	89.1		11	20.10	100.5
12	9.51	95.1		12	18.06	90.3
13	97.7	97.7		13	21.85	109.3
14	10.60	106.0		14	17.25	86.2
15	8.63	86.3		15	17.55	87.8
16	12.20	122.0		16	22.39	111.9
17	9.42	94.2		17	19.16	95.8
18	8.39	83.9		18	17.06	85.3
19	8.02	80.2		19	22.27	111.4
20	10.18	101.8		20	18.96	94.8
Average	9.60	96.0 %			19.18	95.9 %
+/- SD	1.31				2.42	
Measurement Uncertainty @ 95% CI		0.662			Measurement Uncertainty @ 95% CI	1.224

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Section 2: Ruggedness

Method: Composite mussel samples were spiked at 10 and 20 ppm, extracted with 50% methanol/water for 3 minutes, centrifuged and diluted in 10% acetonitrile/water with a total dilution factor of 4000. The diluted sample extract was evaluated on two different manufactured test kit lots. The data and results can be found in Table 4.

Data Summary:

Value for the test of symmetry of the distribution of Kit Lot 1	**0.153**
Value for the test of symmetry of the distribution of Kit Lot 2	**0.563**
Variance of kit Lot 1	**26.07**
Variance of kit Lot 2	**49.53**
Ratio of the larger to smaller variance of Lot 1 & Lot 2	**1.89**
Significant Difference between Lot 1 & Lot 2 based upon paired t-test	**No**

Table 4

Time of Analysis	Sample	Kit Lot 1	Kit Lot 2
Day 1	1A	8.43	8.81
	1B	6.94	8.91
	2A	11.26	9.51
	2B	11.42	9.77
Day 2	3A	9.51	9.25
	3B	10.6	9.42
	4A	8.63	8.39
	4B	12.2	7.86
Day 3	5A	10.48	8.77
	5B	10.18	9.99
Day 1	6A	17.18	22.74
	6B	16.8	29.36
	7A	18.62	23.97
	7B	16.31	25.41
Day 2	8A	22.39	16.87
	8B	19.16	19.07
Day 3	9A	21.06	16.27
	9B	17.23	24.37
	10A	20.77	17.4
	10B	22.27	19.1
Skewness		0.153	0.563

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Variance	**26.07**	**49.53**
Ratio of variances	**1.89**	
P-Value (Paired t-test)	**0.546**	
Significant Difference	**No**	

Results: The data summary indicates the values of symmetry for kit lot 1 and 2 are within the range of -2 to +2, a non-significant degree of skewness in the distribution. The ratio of the variances between lot 1 & 2 is less than 2 indicating homogeneity of variance. A paired t-test used for data analysis results in a p-value of 0.546 which indicates there is no significant difference between Kit 1 and Kit 2.

Ruggedness continued - ELISA parameters

Method: Composite mussel samples were spiked at 10 and 20 ppm, extracted with 50% methanol/water for 3 minutes, centrifuged and diluted in 10% acetonitrile/water with a total dilution factor of 4000. The ELISA Standard Operating Procedure (SOP) parameters were modified in the ELISA then tested for an evaluation of the critical steps in procedure (Table 5).

1. Incubation time for the initial step of the ELISA is set at 30 min. The incubation time was modified to be a total of 15 minutes or 45 minutes. The spiked sample data was evaluated by Welsh's t-test and found not to be significant at either time point tested.
2. A wash step is required in the ELISA to remove unbound materials. The SOP wash is repeated 5 times with water. This was changed to a 4 time wash. The spiked sample data was evaluated by Welsh's t-test and found to be significant from the SOP data.
3. The SOP for test incubation temperature is that the ELISA should be run at RT (20-28 °C). The incubation temperature was modified to be 4 °C or 30 °C. The spiked sample data was evaluated by a paired t-test and found not to be significant at either temperature tested.
4. The kit reagents should be equilibrated to RT prior to running the ELISA. All kit reagents were removed directly from a 4 °C refrigerator and run in comparison to RT reagents. A t-test on the resulting data indicated no significant difference in the results.

Table 5

	ELISA Standard Operating Procedure	Definition of ELISA SOP		Variation Factor	Significantly different to SOP by t-test.		Variation Factor	Significantly different to SOP by t-test.
1.	**Primary Incubation Time is 30 min.**	**Incubation time for HRP Enzyme Conjugate, Sample Extract or Calibrator on plate**		**Incubation time changed to 15 min.**	**No**		**Incubation time changed to 45 min.**	**No**
2.	**Plate water Wash Step is Repeated 5 times.**	**Water wash step to remove unbound materials prior to**		**Wash Step is changed to repeat 4 times.**	**Yes**			

		Substrate addition						
3.	**Incubation Temperature done at room temperature**	**ELISA incubation steps run at RT (20-28 °C).**		**ELISA Incubation at 4 °C.**	**No**		**ELISA Incubation at 30 °C.**	**No**
4.	**Reagent Temperature**	**Kit reagents are equilibrated to RT prior to running test.**		**Reagent Temperature is cold (4 °C).**	**No**			

Section 3: Precision & Recovery

Precision

Method: Evaluation of mussel tissue spiked with a low (10 ppm), medium (20ppm) and high level (40 ppm) of DA was completed using the method of extract preparation and ELISA analysis previously outlined, to evaluate the method consistency over a range of concentrations.

Data Summary- Precision -The F value obtained in the evaluation between groups was less than the critical value of 2.39 (for 9 and 20 degrees of freedom) at 0.05 significance level indicating the mean values from the samples are not significantly different.

The F value obtained in the evaluation of different concentrations (subgroups within groups) is greater than the critical value of 1.93 (for 20 and 30 degrees of freedom) at the 0.05 significance level indicating the mean values of each concentration are significantly different. This is an expected result since there were three sample concentration used to generate the data (10, 20 and 40 ppm) which are quite different.

From this ANOVA analysis (Table 6) we can conclude that the precision of the method is consistent over the range of sample concentrations tested.

Table 6. **Fully nested/hierarchical random analysis of variance (ANOVA)**

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	30.651802	9	3.405756
Between Subgroups within Groups	9,583.973276	20	479.198664
Residual	323.251852	30	10.775062
Total	9,937.87693	59	

F (VR between groups) = 0.316078 P = 0.9633

F (using group/subgroup msqr) = 0.007107 P > 0.9999

F (VR between subgroups within groups) = 44.472939 P < 0.0001

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Recovery

Method: Evaluation of mussel tissue spiked with a low, medium and high level of DA was done using the method outlined, to evaluate the method consistency over a range of concentrations. The results are found in Table 7.

Data summary

The variance ratio for the component of concentration in sample is not significant at 95% CI.
Recovery Percentage over the average data set (10, 20 & 40 ppm) using spiked mussel tissue is 99.55%.

Table 7 RECOVERY

	Low Spike 10 ppm		Medium Spike 20 ppm		High Spike 40 ppm	
Sample	Average	Spike minus Average	Average	Spike minus Average	Average	Spike minus Average
1	8.62	1.38	19.96	0.04	37.00	3.00
2	7.93	2.08	23.08	-3.08	39.42	0.58
3	10.39	-0.39	21.30	-1.30	40.02	-0.02
4	10.60	-0.59	20.86	-0.86	37.39	2.61
5	9.38	0.62	19.63	0.37	39.00	1.00
6	10.01	-0.01	19.12	0.88	40.41	-0.41
7	8.51	1.49	18.67	1.34	43.41	-3.41
8	10.03	-0.03	20.80	-0.80	36.52	3.48
9	9.63	0.38	19.09	0.91	43.80	-3.80
10	10.09	-0.09	20.69	-0.69	41.52	-1.52

Anova: Single Factor

Source of variation	*df*	*SS*	*MS*	*F*	*P-value*	*F crit*
Concentration	2	3.24	1.62	0.55	0.59	3.35
Error	27	80.24	2.97			
Total	29	83.48				

Section 4: Specificity

Method: Four compounds were evaluated to challenge the specificity of the ELISA, three were included due to their similarity of structure to DA, glutamine & glutamic acid at 100 ppm and kainic acid at 20 ppm. Saxitoxin (20 ppm) was evaluated due to the potential that it may be present in the shellfish at the same time as DA. Shellfish extracts containing DA from extracted tissue levels of 0 (blank), 10 and 20 ppm DA were run on the ELISA. These same extracts were run in the presence of the suspected interfering compound to evaluate any significant change in the ELISA result.

Data Summary:
Using a two sided t-test at a 0.05 significance level it was determined that the average Specificity index (SI_{avg}) for the four compounds tested did not differ from 1 (Table 8).
Table 8

Interfering Compound	Conc. (ppm)	SI_{avg}	Significantly different from control by t-test.
Glutamine	100	1.15	No
Glutamic Acid	100	0.89	No
Saxitoxin	20	1.26	No
Kainic Acid	20	1.15	No

Section #5 Linear Range/Limit of Detection/Limit of Quantitation/Sensitivity
Method: Multiple blank mussel tissue samples were spiked with Calbiochem DA standard at the following levels: 2, 3, 5, 10, 15, 20, 30, 40 ug/g then extracted and evaluated by ELISA. To establish the linear range of response the data was evaluated and expressed in Fig. 1. The line of response falls within the bracketed 0.95-1.05 range with data from tissue concentrations from 3 ug/g to 40 ug/g. The data for 2 ug/g falls outside and is not considered within the linear range of the ELISA. The range of assay detection from 3-40 ppm is inclusive of the current NSSP criteria for closing of shellfish beds at 2 mg DA per 100 grams shellfish tissue.
Figure 2 plots the coefficient of variation for each concentration within the linear range which are all under 10%. We can calculate the limit of detection (LOD) of the method and the limit of quantitation (LOQ) shown below using this data.
Data Summary
Linear range of the method as implemented is 3-40 ppm DA in tissue (Fig.1).
The limit of detection (LOD) of the method as implemented is 0.91 ppm DA in tissue.
The limit of quantitation (LOQ) of the method as implemented is 3.0 ppm DA in tissue.
Linear Range Plot – Figure 1
The linear range of the ELISA is established to be from 3 to 40 ppm DA in mussel tissue (Figure 1).

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Figure 1: Linearity of Beacon DA ELISA

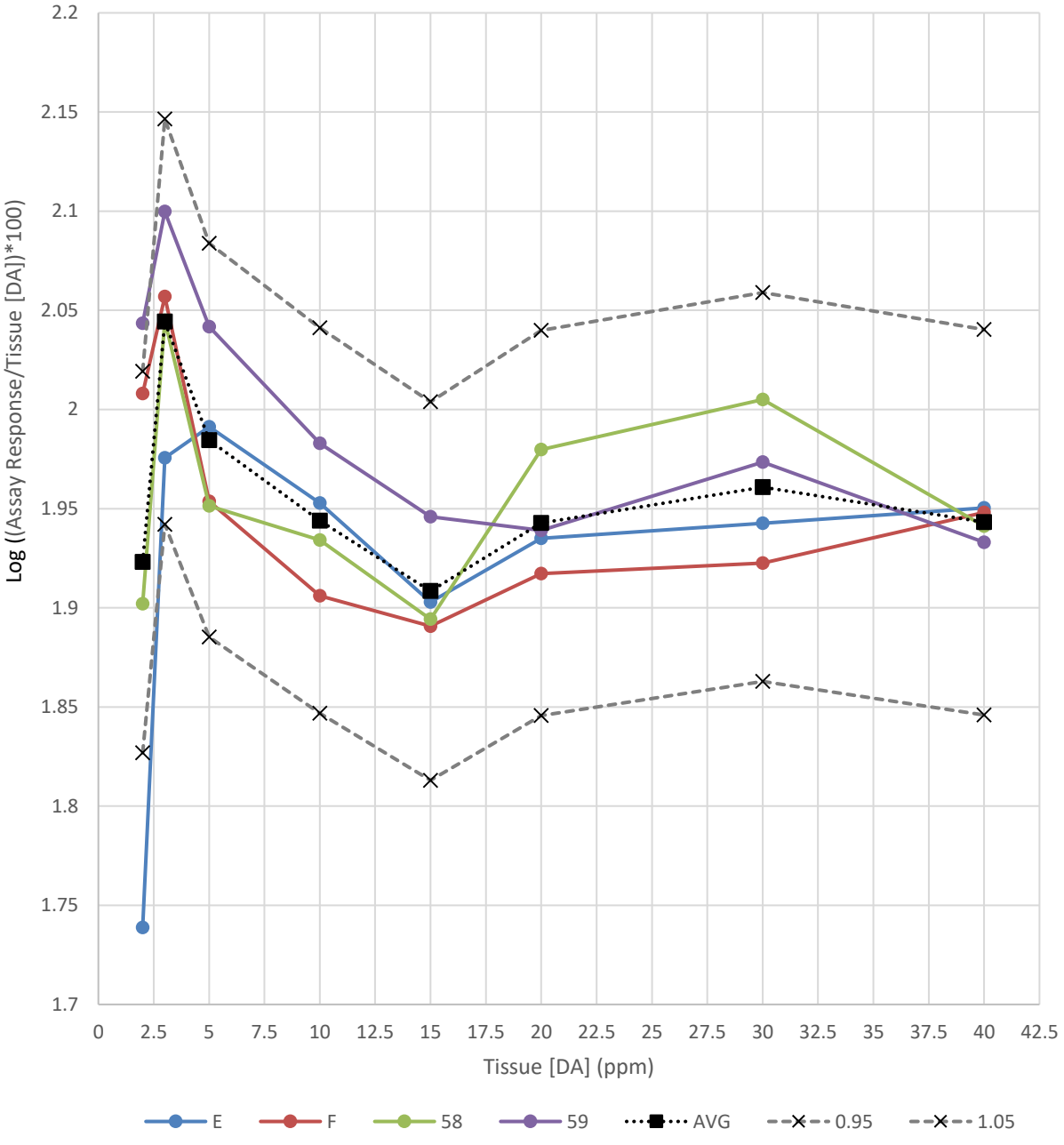
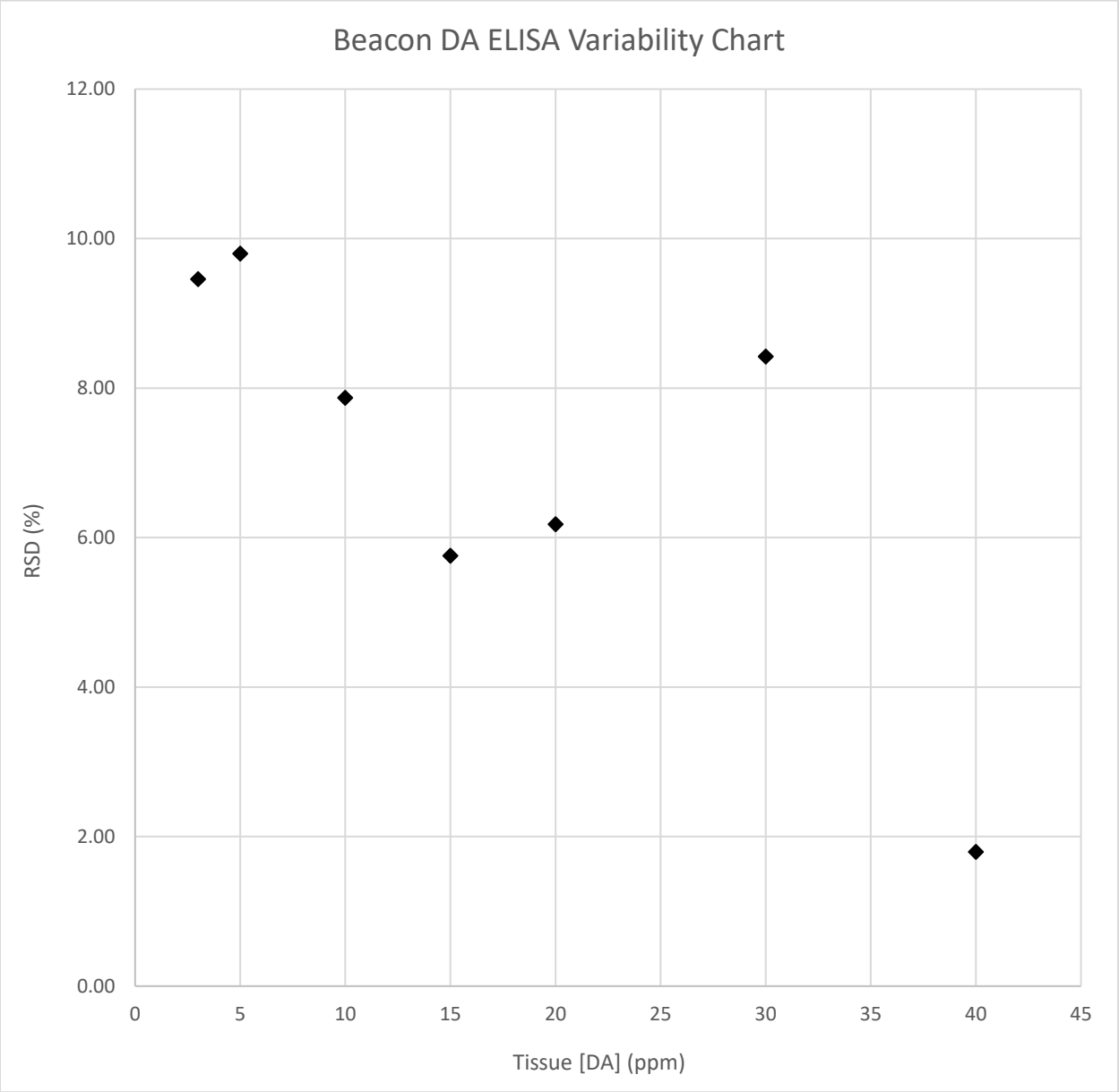


Figure 2: Limit of Detection & Limit of Quantitation/Sensitivity



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Section # 6 - Comparability to NSSP Approved Method for Biotoxin Testing –HPLC

Reference from NSSP Guide for the control of Molluscan Shellfish 2015 Revision.
Table 2- Approved Methods for Marine Biotoxin Testing for ASP: M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Sample. NRC Institute for Marine Biosciences, Technical Report #64 National Research Council Canada #33001.
Method: The HPLC uses a C-18 reverse phase chromatography column with a mobile phase of 10% acetonitrile and 0.1% trifluroacetic acid. The mussel tissue extracts have been prepared using the sample extraction procedure for the ELISA which are then diluted 1:5 with 10% acetonitrile prior to injection into the HPLC system. The 2 procedures use the same sample extracts and the results are compared in Table 9. There were 14 mussel tissue samples tested of which 50% were naturally incurred samples and 50% were spiked with Calbiochem DA standard.

Data Summary for the comparison of the new method to the officially recognized method:
Value for the test of symmetry for the data by HPLC reference method: 0.93
Value for the test of symmetry for the data by the DA ELISA method: 1.45
Symmetry is within the range of -2 to +2 and is not a significant degree of skewness.

Variance of data generated by the HPLC reference method: 166.90
Variance of the data generated by the DA ELISA method: 675.73
Ratio of the larger to smaller of the variances: 4.05
This value indicates a lack of homogeneity of variance and indicates the use of a Welch's t-test for further data analysis to determine if there is a difference between the data means.
Based upon the Welch's t-test there no significant difference between these two analytical methods.

Table 9

Sample	Collection Date	HPLC Data	DA ELISA
Mytilus edulis		DA (ppm)	DA (ppm)
1	9/6/16	9.48	9.50
2	8/30/16	4.78	4.2
3	8/30/16	16.14	19.80
4	01/30/17	4.42	4.80
5	01/30/17	8.77	8.70
6	01/30/17	15.78	22.80
7	01/30/17	28.49	26.20
8	9/20/16	10.64	21.30
9	10/3/16	27.04	51.40
10	9/20/16	1.60	6.90
11	9/28/16	43.11	91.80
12	9/19/16	17.80	36.70
13	9/26/16	39.79	68.70
14	10/3/16	12.10	22.70

	SKEW	0.93	1.45
	VARIANCE	166.90	675.73
	Ratio of Variance		4.05
	Welch's T-test		-1.43
	df =		(19)
	T =		2.09
Conclusion: Means are not different between the 2 methods of analysis.			

Discussion and Summary

The results of this single laboratory validation demonstrate that the Beacon Domoic Acid (ASP) Plate Kit is an effective procedure for quantitative determination of DA residues in *Mytilus edulis* shellfish tissue. Data presented for ELISA performance meets the validation criteria for accuracy/trueness, measurement uncertainty, ruggedness, precision and recovery. The specificity of the test kit was challenged with four compounds of potential interference and was found to perform properly at DA levels of interest. The linear range of the ELISA was determined to be 3 to 40 ppm which brackets the NSSP established criteria of 20 ppm for the ASP biotoxin in shellfish beds. This linear range would allow for the continued use of the method should a lower criteria be established. The comparative data from the ELISA and the officially recognized HPLC method demonstrate good correlative performance.
The ability to use the same sample extract on the ELISA and HPLC confers ease of use for confirmatory testing. The sample throughput is high, while cost and training requirements are minimal. The Beacon Domoic Acid (ASP) Plate Kit is an appropriate tool for quantification of DA residues for use in biotoxin monitoring programs as it allows rapid sample analysis and turnaround time.

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 (ASP) Plate Kit**
Name of the Method Developer	**Dr. Titan Fan**
Developer Contact Information	**Beacon Analytical Systems, Inc.** **82 Industrial Park Road** **Saco, ME 04072** **Tel. (207) 571-4302** **Fax (207) 602 6502** **Email: titan@beaconkits.com**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for an inexpensive laboratory method with the ability to process multiple samples rapidly and quantify the domoic acid levels in mussel tissue.
2. What is the intended purpose of the method?		The method can be used in shellfish bed monitoring programs to document the quantifiable levels of domoic acid in mussel tissue.
3. Is there an acknowledged need for this method in the NSSP?		The method offers higher sample thruput and quantifiable results to monitor increasing or decreasing levels of domoic acid.
4. What type of method? i.e. chemical, molecular, culture, etc.		It is an immunochemical method utilizing an antibody specific to the toxin in an enzyme linked immunosorbent assay (ELISA).
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Domoic Acid (ASP) Plate Kit
Method Scope		Quantitative Analysis of Domoic Acid in mussel tissue
References		Performance Validation Report and Product Insert included with this submission.
Principle		Composite mussel samples are extracted and run on an ELISA Test Kit which quantifies Domoic Acid residues by comparison to an internally run calibration curve.
Any Proprietary Aspects		Yes, this is a commercial test kit.
Equipment Required		Sample Preparation- Blender, scale, extraction container with lid (10-20 ml), vortex mixer, centrifuge (12,000 rcf), Disposable centrifuge tubes. Calibrated variable pipettes 1.0 ml and 0.010 ml with disposable tips. Plate Reader, timer, wash bottle.
Reagents Required		Methanol and laboratory grade water in a 1:1 ratio. 10% Acetonitrile in laboratory grade water.
Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2-8°C before testing.
Safety Requirements		Protective safety items are indicated such as safety glasses, gloves and lab coat. Kit reagents including

		calibrators, conjugates and extracts should be handled with caution since they contain a toxic substance. The kit Stop Solution is a strong acidic solution (1 N Hydrochloric Acid) and needs to be safely handled and disposed of appropriately.
Clear and Easy to Follow Step-by-Step Procedure		Product Insert is included in each test kit and included in this submission package.
Quality Control Steps Specific for this Method		The Certificate of Conformity included with each kit documents the performance characteristics of the Test Kit Lot Reagents. This provides the test operators a reference to evaluate the results generated in their laboratory.
C. Validation Criteria		
1. Accuracy / Trueness		SLV - Section 1
2. Measurement Uncertainty		SLV - Section 1
3. Precision Characteristics (repeatability and reproducibility)		SLV - Section 3
4. Recovery		SLV- Section 3
5. Specificity		SLV- Section 4
6. Working and Linear Ranges		SLV - Section 5
7. Limit of Detection		SLV - Section 5
8. Limit of Quantitation / Sensitivity		SLV - Section 5
9. Ruggedness		SLV- Section 2
10. Matrix Effects		None observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		SLV - Section 6
D. Other Information		
1. Cost of the Method		The price per sample is eight to nine dollars dependent upon the number of samples tested during one ELISA run, and/or the volume of kits purchased.
2. Special Technical Skills Required to Perform the Method		Knowledge of GMP laboratory skills as well as proper pipetting technique, and safe handling of solvents.
3. Special Equipment Required and Associated Cost		An ELISA Plate Reader is required which can range in price from a low cost unit at approximately $2,600 to a higher cost of $15,000 USD unit depending upon complexity.
4. Abbreviations and Acronyms Defined		ASP-amnesic shellfish poisoning. DA-domoic acid. ELISA-Enzyme Linked Immunosorbent Assay. OD-Optical Density (Reader Output). HRP-horse radish peroxidase enzyme. % B/B0- percent of measured bound fraction in a test sample divided by the total bound from a blank Calibrator. ppm = parts per million, equivalent to mg/kg, ppb –parts per billion.
5. Details of Turn Around Times (time involved to complete the method)		One assay can be completed in under 90 minutes including sample preparation (12 samples). One plate can be used to test a maximum of 36 samples.
6. Provide Brief Overview of the Quality Systems Used in the Lab		Beacon Analytical Systems is ISO 9001:2015 approved for their quality systems for Immunochemical test kit development, manufacturing and supporting activities. Overview of Kit Quality Control - Each kit is tested following the ELISA procedure in the product insert. During manufacturing operation duplicates of the Kit Calibrator Reagents are run in order to meet established criteria prior to shipment. Each Domoic Acid (DA) Calibrator's absorbance (OD) and binding characteristics (% B/B0) must be within a set of ranges. Ranges have been established for the Domoic Acid Plate Kit from historical data. All plate components are tested for precision prior to using them in kits. The tolerance for variation within one lot of plates is less than or equal to 5%. -DA Calibration solutions are prepared using certified reference standard material purchased from the Canadian National Resource Council and are tested to be within 2% of the previous lot of control. - The R^2 correlation of the DA Kit Calibration Curve should be 0.99 or above. - All CV's must be less than or equal to 5%. - All QC data is kept electronically and backed up with hard copies at our manufacturing plant.

Submitters Signature 	Date: 06/30/2017
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:

Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.

14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery** - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** - the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range** - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

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

Proposal No. 17-108

Beacon Analytical Systems Inc.®

Domoic Acid (ASP)

Plate Kit

Cat. # 20-0249
Product Insert

PLEASE READ COMPLETELY BEFORE USE

INTENDED USE

The Beacon Domoic Acid (ASP) Plate Kit is a competitive ELISA for the quantitative analysis of domoic acid in shellfish samples.

USE PRINCIPLES

The Beacon Domoic Acid (ASP) plate kit is a competitive enzyme-labeled immunoassay for the biotoxin which causes Amnesic Shellfish Poisoning (ASP). Shellfish sample extract(s) or calibrator solution(s) are pipetted into a test well followed by Domoic Acid HRP enzyme conjugate to initiate the reaction. During a 30 minute incubation period, domoic acid from the sample and domoic acid HRP enzyme conjugate compete for binding to the domoic acid antibody coated on the plate wells. Following this incubation, the wells are washed to remove any unbound domoic acid and HRP enzyme conjugate. After washing, a colorless substrate is added to the wells and any bound enzyme conjugate will convert the substrate to a blue color. Following another 30 minute incubation, the reaction is stopped with the addition of stop solution and the amount of color in each well is measured. The color of the unknown sample is compared to the color of the calibrators and the domoic acid concentration of the sample is derived. The color intensity is inversely proportional to the amount of domoic acid present.

MATERIALS PROVIDED

The kit in its original packaging can be used until the end of the month indicated on the box label when stored at 2 to 8 ºC.

- **Plate** – (1) containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.
- **Domoic Acid Calibrators–** (4) vials each containing 2 ml with a concentration of 0, 0.5, 5, and 50 µg/L (ppb) Domoic Acid
- **Domoic Acid HRP Enzyme Conjugate** – (1) vial containing 12 ml
- **Substrate** – (1) vial containing 14 ml
- **Stop Solution** – (1) vial containing 14 ml (Caution! Contains 1N HCl. Handle with care.)
- **Product Insert** containing instructions for use.
- **Certificate of Conformity** (Specific to each Kit Lot#).

MATERIALS REQUIRED BUT NOT PROVIDED

Acetonitrile,& Methanol (ACS grade)	Timer
Laboratory quality distilled or deionized water	Wash bottle
Variable volume pipettes with disposable tips capable of dispensing 10-100 microliters (µl), and up to 1000 µl.	Vortex mixer
Multi-channel pipette; 8 channel capable of dispensing 100 µl	Paper towels or equivalent absorbent material
Microwell plate or strip reader with 450 nm filter	Disposable micro centrifuge tubes
Microcentrifuge capable of a speed of 12,000 rcf. (x g)	Kitchen Blender for sample homogenization

SPECIFICITY

Domoic Acid (DA) is an amino acid similar in structure to kainic acid which naturally occurs in some seaweed. The % cross reactivity of several compounds relative to DA is shown in the table below.

Compound	% CR	Compound	% CR
Domoic acid	**100 %**	**Saxitoxin**	**< 0.1 %**
Glutamine	**< 0.1 %**	**Kainic acid**	**0.005 %**
Glutamic acid	**< 0.1 %**		

KIT HANDLING NOTES and PRECAUTIONS

- Store all kit components at 4 °C to 8 °C (39 °F to 46 °F) when not in use.
- Each reagent is optimized for use in the Beacon Domoic Acid (ASP) Plate Kit. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other Beacon Plate Kits with different lot numbers.
- Dilution or adulteration of reagents or samples not called for in the procedure may result in inaccurate results.
- Do not use reagents after expiration date.
- Reagents should be brought to room temperature (RT), 20 to 28 ºC (62 to 82 ºF) prior to use. Avoid prolonged (> 24 hours) storage at room temperature.
- Domoic acid calibrators contain 10% acetonitrile and should be kept tightly capped to minimize evaporation.
- The Stop Solution is 1N hydrochloric acid, which is corrosive and an irritant. Avoid contact with skin and mucous membranes. Immediately clean up any spills and wash area with copious amounts of water. If contact should occur, immediately flush with copious amounts of water.
- Precise transfer of samples and reagents by using an appropriate and calibrated pipette is critical to obtain proper assay results. Please pipette carefully.
- If running more than two strips at once, the use of a multichannel pipette is required.
- In one assay a maximum of four strips (32 wells) is recommended, for example –4 calibrators in duplicate (8 wells), and 12 sample extracts in duplicate (24 wells).

SAMPLE DILUTION BUFFER PREPARATION- 10% ACETONITRILE/Water

- Mix 1 part ACS grade acetonitrile with 9 parts distilled or deionized (DI) water to make the Sample Dilution Buffer for the shellfish samples.
- Transfer to a clean glass container with tight-fitting lid and swirl to mix. Store tightly sealed to minimize evaporation.

SAMPLE EXTRACTION BUFFER PREPARATION- 50% METHANOL/Water

- Mix 1 part ACS grade methanol with 1 part distilled or deionized (DI) water to make the Sample Extration Buffer.
- Transfer to a clean glass container with tight-fitting lid and swirl to mix. Store tightly sealed to minimize evaporation.

SAMPLE PREPARATION - Shellfish Tissue Extract –Mytilus edulis (Blue Mussel)

1. Remove shellfish tissue (12-15 animals) from shell, wash, drain dry and homogenize using a kitchen blender.
2. Weigh 2 g of homogenized tissue and add 8 ml of a 50% Methanol/Water solution.
3. Mix for 3 minutes using Vortex mixer (4 X dilution)
4. Transfer 1 ml into a microcentrifuge tube and centrifuge at 12,000 x g for 5 minutes. Extracts can be stored at -20°C.
5. Prepare a 1:1000 dilution of the supernatant with Sample Dilution Buffer using the following procedure:

A. 1:10 dilution - 50 microliters of supernatant layer avoiding any particulates, into 450 microliters Sample Dilution Buffer, Mix.

B. 1:100 dilution – 10 microliters of dilution A. into 990 microliters Sample Dilution Buffer, Mix,

6. Use B. in ELISA. – Total Dilution Factor (TDF) = 4000

Shellfish Analysis:

- ✓ EU Screening Level = 20 ppm (20 mg/kg) Assay Dilution Factors are set to detect 20 ppm Domoic Acid,

Extraction of Shellfish Tissue and Preparation for ELISA	
Dilution of shellfish homogenate in water (2 g homogenized tissue with 8 ml 50% MEOH/DI H_2O)	**4 X** Dilution
*Secondary Dilution into Sample Dilution Buffer	**1000 X Dilution**
Total Dilution Factor (TDF) to obtain Tissue Levels of Domoic Acid	**4000 X**
✓ Assay Range of Detection in Tissue	2 mg / kg to 200 mg / kg
Domoic Acid Plate Kit Calibrators ug / L (ppb)	Predicted Tissue Levels (X 4000 TDF)
Negative Control (Blank)	0
0.5	2 ppm
5.0	20 ppm
50.0	200 ppm

ASSAY PROCEDURE

(Note: Running Calibrators and samples in duplicate will provide optimal assay precision and accuracy.)

1. Allow reagents and sample extracts to reach RT prior to running the test.
2. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
3. Using a pipette with disposable tips, dispense **100 µl** of the **Calibrator or sample extract** into the appropriate test wells. Please use a clean pipette tip for each sample addition.
4. Dispense **100 µl** of the **HRP Enzyme Conjugate** into each well.
5. Shake the plate gently for 30 seconds using a back and forth motion. Then incubate the wells for **30 minutes** at RT.
6. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with laboratory grade water and then decant. Repeat four times for a total of five washes.
7. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the water.
8. Dispense **100 µl** of the **Substrate** into each well. Shake the plate gently for 30 seconds using a back and forth motion.
9. Incubate the wells for **30 minutes** at RT.
10. Dispense **100 µl of the Stop Solution** into each well.
11. Measure and record the absorbance (Optical Density; OD) of the wells at 450 nm using a strip or plate reader.
12. To obtain the concentration of Domoic acid in the sample multiply the results by the Total Dilution Factor of 4000.

Note: If the sample absorbance is higher or lower than the 0.5 or 50 ppb Calibrator results, the tissue levels should be expressed as less than or greater than the corresponding tissue levels (<2ppm or >200ppm DA). The sample dilution can be modified appropriately and retested along with another set of Calibrators.

CALCULATE RESULTS

1. Semi-quantitative results can be derived visually by simple comparison of the sample color to the color of the Calibrator wells. Samples containing less color than a Calibrator will have a concentration of Domoic Acid greater than the tissue correlated concentration of the Calibrator. Samples containing more color than a Calibrator will have a concentration less than the tissue correlated concentration of the Calibrator.
2. It is preferred for quantitative results to be determined using commercially available software for ELISA evaluation such using a 4-Parameter curve fit. Alternatively, a semi-log curve fit can be used if 4-Parameter software is not available. Samples with OD's greater than the lowest calibrator, or lower than the highest calibrator will need to be diluted accordingly and repeated with and calibrators in an additional run.
3. Beacon can supply a spreadsheet template which can be used for data reduction. Please contact Beacon for further details.

SAMPLE CALCULATIONS

Well Contents	OD	Average OD ± SD*	%RSD	%B/Bo**
Negative Control	2.033 1.994	2.014 ± 0.027	1.4	100
0.5 ppb Calibrator	1.610 1.671	1.640 ± 0.043	2.7	81
5 ppb Calibrator	1.095 1.155	1.125 ± 0.042	3.8	56
50 ppb Calibrator	0.501 0.482	0.492 ± 0.013	2.7	24

Actual values may vary; this data is for example purposes only.

* Standard deviation

**B/Bo% equals the average sample absorbance divided by the average 0 ppb Calibrator absorbance multiplied by 100.

TECHNICAL ASSISTANCE

For questions regarding this kit or for additional information about Beacon products, call (207) 571-4302 or contact us at info@beaconkits.com.

Safety- To receive complete safety information on this product, contact Beacon Analytical Systems, Inc. and request Safety Data Sheets. Stop Solution is 1N hydrochloric acid. Handle with care.

General Limited Warranty

Beacon Analytical Systems, Inc. ("Beacon") warrants the products manufactured by it against defects in materials and workmanship when used in accordance with the applicable instructions for a period not to extend beyond a product's printed expiration date. BEACON MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. The warranty provided herein and the data, specifications and descriptions of Beacon products appearing in published catalogues and product literature may not be altered except by express written agreement signed by an officer of Beacon. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and, if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Beacon's sole obligation shall be to repair or replace, at its option, any product or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies Beacon promptly of any such defect. The exclusive remedy provided herein shall not be deemed to have failed of its essential purpose so long as Beacon is willing and able to repair or replace any nonconforming Beacon product or part. Beacon shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by a customer from the use of its products. However, in some states the purchaser may have rights under state law in addition to those provided by this warranty.

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