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ASP ELISA KIT FOR QUANTITATIVE DETERMINATION OF DOMOIC ACID

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AOAC® *Official Method℠* 2006.02

CONTENTS

A. INTRODUCTION

Domoic acid (DA) and DA derivatives are water-soluble neurotoxins produced by a number of marine algae, in particular by the microalgae of the genus *Pseudo-nitzschia* spp (Fig. 1). Blooms of *Pseudo-nitzschia* may lead to the accumulation of DA in shellfish filter feeders and other marine species [Scholin *et al*., 2000]. Ingestion of DA contaminated shellfish may lead to amnesic shellfish poisoning (ASP) by affecting the central nervous system, and has caused the death of both animal and human consumers in severe cases [Wright *et al*., 1989]. The European commision Directive 2002/226/EC implemented a maximum permitted level (MPL) of 20 mg DA equivalents/kg shellfish intended for human consumption. This MPL is adopted by most food safety authorities.

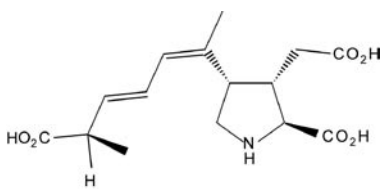


FIGURE 1:
DOMOIC ACID STRUCTURE

Enzyme Linked Immunosorbent Assay (ELISA) has proved to be a sensitive and rapid method for detection of DA in the marine environment [Garthwaite *et al*., 2001]. This quantitative DA ELISA was developed by AgResearch (Hamilton, New Zealand) for the detection of DA in water samples, shellfish and algal extracts, and is based on antibodies described by Garthwaite *et al*., 1998. The assay is specific for DA, with no cross-reactivity to non-toxic, structural analogues like kainic acid, L-glutamic acid, L-glutamine, formimino-L-glutamic acid, proline or g-aminobutyric acid (GABA). The assay is primarily intended for use in routine monitoring of DA levels in bivalve molluscs to comply with the regulatory MPL, but is also applicable for DA quantification in other marine matrixes like algal samples, seawater and body fluids of marine mammals. The assay has been subjected to comprehensive validation studies and is approved AOAC® *Official Method*℠ 2006.02 [*AOAC INTERNATIONAL*, 2006].

Assay principle

The ASP ELISA assay is in a direct competition format, where free DA in the sample competes with DA-conjugated protein coated on plastic wells for binding to anti-DA antibodies free in the solution (Fig. 2). The polyclonal ovine anti-DA antibodies are conjugated to horseradish peroxidase (HRP). Sample diluted in buffer is incubated in the wells with the anti-DA-antibody-HRP conjugate. After washing, the amount of conjugate remaining bound to the well is measured by incubation with a substrate that gives a blue product upon reaction with the HRP enzyme. Addition of acid stops the reaction and changes the product colour from blue to yellow. The colour intensity is measured spectrophotometrically on a plate-reader at 450 nm, and is inversely proportional to the concentration of DA in the sample solution. The assay is calibrated using dilutions of a DA calibration solution supplied with the kit. The calibrated range of the assay ($I_{20} - I_{80}$) is approximately 10 to 300 pg/mL of DA. The ASP ELISA is offered in a 8x12 strip well format. The ASP ELISA kit can be used in 2 separate rounds to analyze 12 samples each time, or the full plate can be used to analyze 36 samples in one round of analysis. The working range for ASP toxins in shellfish is 0.01mg/kg up to at least 250 mg/kg.

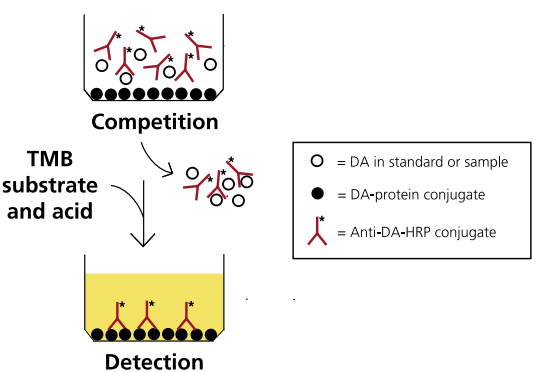
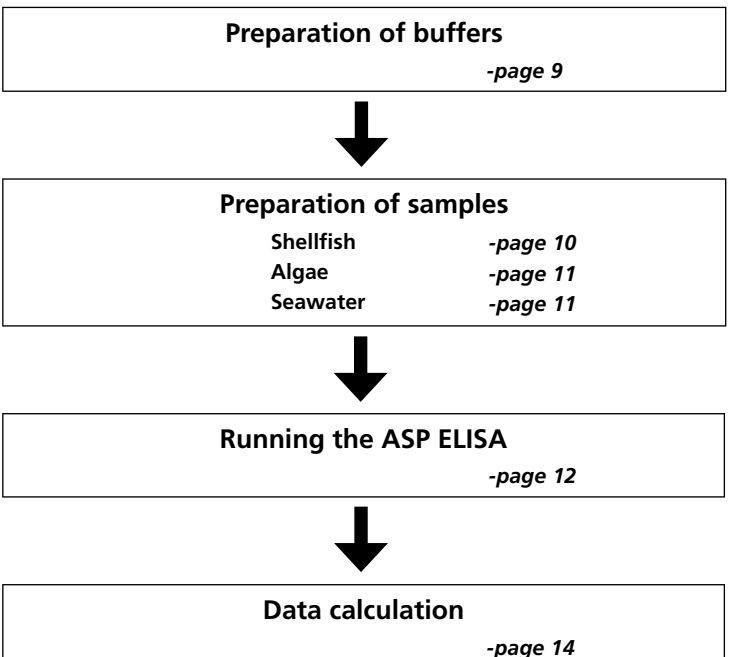


FIGURE 2: ASSAY FORMAT FOR THE COMPETITIVE ASP ELISA

METHOD OVERVIEW



B. SAFETY INSTRUCTIONS

As all chemicals should be considered potentially hazardous, always wear suitable protective clothing during handling of the kit.

CAUTION: Domoic acid is a neurotoxin that is harmful by inhalation and ingestion. Avoid contact with skin, eyes and clothing. Wash hands thoroughly after handling.

Beware of the hazardous nature of methanol and sulfuric acid. Please refer to the manufacturers Material Safety Data Sheet for these reagents.

C. STORAGE AND STABILITY

Store the kit at 2-8°C upon arrival. Do not freeze. See expiry date on the kit box for stability of the kit.

D. WARRANTY AND LIMITATION OF REMEDY

Biosense Laboratories AS (hereafter: Biosense) makes no warranty of any kind, expressed or implied, including, but not limited to, the warranties of fitness for a particular purpose and merchantability, which extends beyond the description of the chemicals on the face hereof, except that the material will meet our specifications at the time of delivery.
Buyer's exclusive remedy and Biosense´s sole liability hereunder shall be limited to refund of the purchase price of, or at Biosense´s option, the replacement of, all material that does not meet our specifications. Biosense shall not be liable otherwise or for incidental or consequential damages, including, but not limited to, the costs of handling.
Said refund or replacement is conditioned on Buyer giving written notice to Biosense within thirty (30) days after arrival of the material at its destination, and Buyer treating the material as outlined in the product data sheet and/or kit insert after arrival. Failure of Buyer to give said notice within said thirty (30) days, or failure of Buyer in treating the material as outlined in the product data sheet and/or kit insert shall constitute a waiver by Buyer of all claims hereunder with respect to said material.
The responsibility of all patent considerations in the use of our products rests solely with the user.

E. KIT CONTENTS

		Number:
A)	12-well microplate strip modules (Precoated with DA-protein conjugate)	2 sealed pouches - 4 strips each
B)	Plate sealers	2
C)	PBS/Tween tablets	2
D)	Domoic Acid standard, 100 ng/mL (derived from NRC CRM-DA-e)	2 vials
E)	Anti-DA-HRP conjugate (6x concentrated)	2 vials
F)	Ovalbumin	2 vials á 60 mg
G)	TMB peroxidase substrate	2 vials

F. ADDITIONAL REAGENTS AND EQUIPMENT REQUIRED

In addition to the reagents supplied with the kit, the following reagents and equipment are required and/or recommended to perform the assay:

- Microplate spectrophotometer equipped with a 450 nm filter.
- Water; distilled and deionised (e.g. Milli-Q water, Millipore).
- Methanol (analytical grade).
- 0.3 M H_2SO_4.
- Vortex mixer.
- Micropipettes.
- Centrifuge.
- Kitchen blender.*

* For homogenizing shellfish samples.

G. IMPORTANT NOTES

1. Read the complete procedure before starting the assay.
2. Protect vials and microwell strips containing DA standard dilutions and samples from direct light during incubations.
3. The plate sealers are used to seal the strips during incubation and care must be taken when removing them from the strips.
4. Positive displacement pipettes (50 µL) are recommended for dispensing methanolic extracts.
5. As in every quantitative ELISA, consistent and precise pipetting at each step in the procedure is essential in order to obtain reliable results.
6. Reproducibility in any ELISA is also dependent upon consistent washing of the microwells.
7. After each wash, the wells are emptied by inverting the strips over a sink and then tap dry the wells against a pile of paper towels to remove all of the remaining liquid.
8. Avoid prolonged intervals between the working steps of the procedure, and do not allow the microwells to dry out totally during the assay procedure.

Definitions

Blank wells: Background absorbance of the TMB peroxidase substrate; approximately 0.05 A.U. (Absorbance Units).

A_{max} wells: Maximum absorbance; no standard or sample is added to these wells allowing maximum binding of the anti-DA-HRP conjugate to the plate-coated DA-conjugate; approximately 1.0 A.U. (Absorbance Units).

H. PREPARATIONS BEFORE THE ANALYSIS

a) Preparation of buffers and reagents

1. Washing buffer (PBS-T; 0.05% Tween 20 in PBS):
   Dissolve one tablet (C) in distilled water and dilute to 500 mL. May be stored at 4°C for one week.
2. Extraction solution (50% methanol in water):
   Prepare sufficient solution for the required number of samples by mixing equal volumes of methanol and distilled water. Prepare fresh each day.
3. Standard/Sample buffer (10% methanol in PBS-T):
   Mix 5 mL of methanol with 45 mL of Washing buffer. May be stored for 2-3 days at room temperature.
4. Antibody-HRP ovalbumin buffer (1% ovalbumin in PBS-T):
   Add 6 mL of Washing buffer to 60 mg of ovalbumin (vial F). Prepare fresh for each assay.

b) Preparation of Domoic acid calibration solutions

The 10-point calibration curve is ***freshly*** prepared using standard dilutions in the range of 10 000 – 0.16 pg DA/mL:

1. Prepare one Eppendorf tube containing 450 µL Standard/Sample buffer (10% methanol in PBS-T) - "tube 1", and 9 Eppendorf tubes containing 300 µL Standard/Sample buffer - "tubes 2-10".
2. Add 50 µL of the DA standard (100 ng/mL, vial D) to tube 1 and vortex, to obtain a 10 ng/mL DA solution.
3. Transfer 125 µL of the 10 ng/mL solution (tube 1) to tube 2 and vortex.
4. Complete the 3.4-fold dilution series by transferring 125 µL from tube 2 to tube 3 and vortex. Repeat this step for all tubes 3-10 (see Fig. 3).

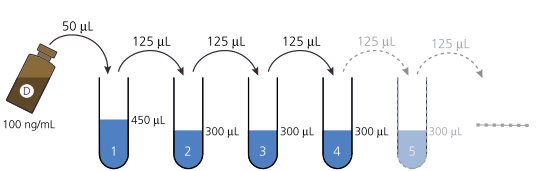


FIGURE 3. DOMOIC ACID STANDARD DILUTION SEQUENCE

I. PREPARATION OF SHELLFISH SAMPLES

a) Extraction of DA from shellfish samples

Shellfish flesh should be prepared as a finely blended homogenate. Preferably analyse fresh, but it may be stored frozen at -20°C for up to 14 days before use.

1. Prepare shellfish homogenate in a high speed blender (kitchen blender), from no less than 50 g shellfish flesh.
2. Accurately weigh 4 g into a 50 mL centrifuge tube.
3. Add 16 mL of Extraction solution (50% methanol).
4. Mix well by vigorous shaking on vortex for 1 min.
5. Centrifuge at 3000x*g* for 10 minutes at room temperature.
6. Retain the supernatant for further dilution prior to analysis. The extracts can be stored at -20°C for up to 14 days, although with a possible reduction in DA content.

b) Dilution of shellfish sample extracts

7. Prepare dilutions of the shellfish extract in Standard/Sample buffer (10% methanol in PBS-T) as follows:

1:20 dilution:	*50 µL shellfish extract*	*+ 950 µL buffer*
1:200 dilution:	*50 µL of the 1:20 dilution*	*+ 450 µL buffer*
1:2000 dilution:	*50 µL of the 1:200 dilution*	*+ 450 µL buffer*
1:20 000 dilution:	*50 µL of the 1:2000 dilution*	*+ 450 µL buffer*
1:200 000 dilution:	*50 µL of the 1:2000 dilution*	*+ 450 µL buffer*

Cap and *vortex* each dilution before proceeding to the next dilution step.

8. Analyze the sample dilutions according to the DA concentration range of interest (see Table 1), to give absorbance values within the calibration curve working range. It is recommended to analyze shellfish extracts diluted at 1:20 000 dilutions to comply with EC directive 2002/226/EC, for the quantification of DA up to the maximum permitted level at 20 mg/kg.

TABLE 1: SHELLFISH EXTRACT DILUTION FOR QUANTIFICATION OF DA

DA concentration range of interest [mg/kg]	Corresponding Sample Extract dilution to be analyzed
0.01 - 0.25	1:200 dilution (minimum dilution)
0.1 - 2.5	1:2000 dilution
1.0 - 25	1:20 000 dilution
10 - 250	1:200 000 dilution

J. PREPARATION OF SAMPLES FROM ALGAL CULTURE AND SEAWATER

The analysis of samples from algal culture and seawater will depend on the amount of algae (cells/mL) and the amount of DA present in the algae and in the seawater or culture medium. The recommended procedure for preparation of samples is derived from Fehling *et al*., 2004.

1. Count the amount of algae (cells/mL) in your sample. If you want to analyze *total DA* and *extracellular DA* (DA released into the medium or seawater), divide each sample in duplicates with exact volumes.

2. *Total DA*: Sonicate the sample for 2 minutes (on ice) to disrupt the cells. Then filter the sample through a 0.2 µm disposable filter (surfactant free cellulose acetate membrane) to remove cell debris. Dilute the *total DA* filtrate in Standard/Sample buffer (see paragraph 3) before analysis . The sample result will be given as DA concentration in pg/mL, and DA per cell can be calculated by dividing the DA content with the cell numbers. The filtrate can be frozen at -20˚C for up to two weeks prior to analysis.

Extracellular DA: Gently filter the duplicate sample under low vacuum onto glass-fiber filters. Be carefull not to disrupt cells. Dilute the *extracellular DA* filtrate in Standard/Sample buffer (see paragraph 3) before analysis. The sample result will be given as DA concentration in pg/mL, and DA per cell can be calculated by dividing the DA content with the cell numbers. The filtrate can be frozen at -20˚C for up to two weeks prior to analysis.

Intracellular DA: Calculate the intracellular DA content by subtracting the *extracellular DA* content from the *total DA* content after analysis. The sample result will be given as DA concentration in pg/mL, and DA per cell can be calculated by dividing the DA content with the cell numbers.

3. Before analysis, dilute the *total DA* and *extracellular DA* filtrates in Standard/Sample buffer. For cell densities up to 100 000 cells/mL in culture medium or seawater, a minimum dilution of 1:25 in Standard/Sample buffer is required to avoid matrix effects.

K. ASSAY PROCEDURE

a) Incubation of standards and samples with antibody

Equilibrate pre-coated plate strips and all reagents to room temperature before use (1 hour max). See Figure 4 for a recommended plate layout for either using 4 strips in 2 rounds of analysis (4A), or all 8 strips at once (4B).

1. Open the packet(s) with pre-coated plate strips gently and place the strips in the strip frame. Label each strip *e.g.* A, B, C and D etc.
2. Add 300 µL Washing buffer to each well. Pre-soak the wells for 5-10 minutes.
3. Remove the Washing buffer by inverting the strips over a sink and tap against a pile of paper towels to remove all the remaining liquid.
4. Add 50 µL Standard/Sample buffer (10% methanol in PBS-T) to each of the duplicate Amax and Blank wells.
5. Add 50 µL Antibody-HRP ovalbumin buffer (1% ovalbumin) to the Blank wells.
6. Add 50 µL of each DA standard dilution to each of two wells.
7. Add 50 µL of each sample dilution to each of two wells.
8. Shake vial E briefly, and tap the vial gently on a hard surface to ensure that all the content is in the bottom of the vial. Transfer ***0.5 mL (for 4 strip assay)*** or ***1.0 mL (for 8 strip assay)*** from vial E (concentrated Anti-DA-HRP) to a Falcon type tube containing ***2.5 mL (for 4 strip assay)*** or ***5.0 mL (for 8 strip assay)*** Antibody-HRP ovalbumin buffer (prepared vial F). Vortex briefly.
9. Add 50 µL of the diluted Anti-DA-HRP conjugate to all wells ***except*** the Blank wells.
10. Seal the strips with the plate sealer (B) and incubate at room temperature (20-25°C) for 1 hour. Protect from light (*e.g.* cover with aluminium foil or place in a drawer).

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 000 pg/ml	2941 pg/ml	865 pg/ml	254 pg/ml	75 pg/ml	22 pg/ml	6,5 pg/ml	1,9 pg/ml	0,56 pg/ml	0,16 pg/ml	Amax	Blank
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	S1 1:20 000	S2 1:20 000	S3 1:20 000	S4 1:20 000	S5 1:20 000	S6 1:20 000	S7 1:20 000	S8 1:20 000	S9 1:20 000	S10 1:20 000	S11 1:20 000	S12 1:20 000
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

FIGURE 4A: SUGGESTED PLATE LAYOUT, USING 4 STRIPS, FOR THE QUANTIFICATION OF DA IN 12 SHELLFISH SAMPLES IN 2 SEPARATE ROUNDS. S1 = SAMPLE 1, S2 = SAMPLE 2, ETC.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 000 pg/ml	2941 pg/ml	865 pg/ml	254 pg/ml	75 pg/ml	22 pg/ml	6,5 pg/ml	1,9 pg/ml	0,56 pg/ml	0,16 pg/ml	Amax	Blank
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	S1 1:20 000	S2 1:20 000	S3 1:20 000	S4 1:20 000	S5 1:20 000	S6 1:20 000	S7 1:20 000	S8 1:20 000	S9 1:20 000	S10 1:20 000	S11 1:20 000	S12 1:20 000
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	S13 1:20 000	S14 1:20 000	S15 1:20 000	S16 1:20 000	S17 1:20 000	S18 1:20 000	S19 1:20 000	S20 1:20 000	S21 1:20 000	S22 1:20 000	S23 1:20 000	S24 1:20 000
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	S25 1:20 000	S26 1:20 000	S27 1:20 000	S28 1:20 000	S29 1:20 000	S30 1:20 000	S31 1:20 000	S32 1:20 000	S33 1:20 000	S34 1:20 000	S35 1:20 000	S36 1:20 000
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

FIGURE 4B: SUGGESTED PLATE LAYOUT, USING ALL 8 STRIPS, FOR THE QUANTIFICATION OF DA IN 36 SHELLFISH SAMPLES. S1 = SAMPLE 1, S2 = SAMPLE 2, ETC.

b) Developing and reading the microplate strips

11. Carefully remove the plate sealer. Remove all the contents by inverting the strips over a sink and tap to remove remaining liquid. Wash the wells 4 times with 300 µL Washing buffer per well.

12. Add 100 µL of TMB peroxidase substrate (vial G) to all wells. Incubate at room temperature (20-25°C) for 15 minutes. Protect from light.

13. Stop the reaction by adding 100 µL 0.3 M H_2SO_4 to all wells.

14. After 2-5 minutes, read the absorbance in a microplate spectrophotometer using a 450 nm filter.

L. CALCULATION OF RESULTS

a) Calibration using the four-parameter logistic curve fit model

When the measured absorbance values of the standard dilutions are plotted on a linear scale (y axis) against the DA-concentrations of the standard dilutions on a logarithmic scale (x axis), a sigmoid (S-shaped) curve is obtained (see Fig. 5).
The non-linear 4-parameter logistic curve-fit model is extensively used for sigmoid curves, in order to get accurate quantification of samples and a good fit at the extremes of the curve. The following equation is given for a 4-parameter fitted curve:

$$y = (a-d)/[1+(x/c)^b]+d$$

where:

***x** is the concentration of DA in the standard/sample*

***y** is the absorbance of the standard/sample*

***a** is the y-value of the upper asymptote (A_{max})*

***b** is the relative slope of the curve at its center*

***c** is the x-value at the midpoint of the curve (I_{50})*

***d** is the y-value of the lower asymptote (Blank/A_{min})*

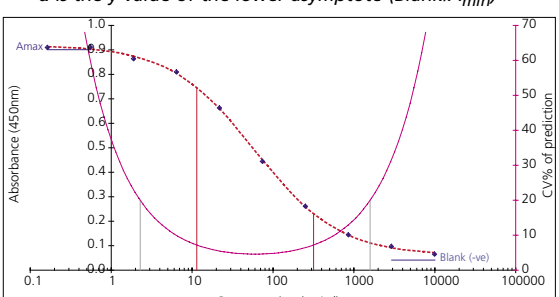


FIGURE 5. NON-LINEAR CALIBRATION CURVE PREPARED BY 4-PARAMETER LOGISTIC CURVE FIT.

b) Calculation formula

The following formula is used to convert ELISA results in pg/mL to shellfish concentrations in mg/kg:

$$\text{mg DA/kg} = \mu\text{g DA/g} = (\text{pgDA/mL}) \cdot D \cdot V \cdot \frac{1\ \mu g}{1\ 000\ 000\ pg} \Big/ M$$

where:

***pg DA/mL** is the concentration of DA in the diluted extract*

***D** is the dilution factor of the diluted extract*

***V** is the volume of the methanolic extract (16 mL plus 4 g of homogenate giving nominal 20 mL total volume).*

***M** is the mass of the shellfish homogenate (4 g).*

c) Excel macro EMA31 calculation of DA concentration in shellfish samples

For calculation of assay results, a spreadsheet has been developed implementing the calibration function and the conversion formula from pg/mL in the extract to mg DA/kg shellfish.

1. Open the provided Excel Macro EMA31, enable macros and install the Solver as described in the "Instructions" sheet of the Macro.
2. Copy the measured absorbance values (to at least 3 significant figures, *e.g.* 0.682) from the plate reading software result/report sheet and paste the values in the Excel Macro EMA31 "Data Entry" sheet.
3. Enter the correct dilution factor used for the samples, in the corresponding duplicate well windows.
4. Run the macro according to the instructions.
5. Go to the "Results" sheet. The results from the column "Shellfish sample DA eqv. (mg/kg)" give the concentration of DA in the shellfish samples.
6. Sample concentrations should only be calculated from datapoints that are within the valid working range of the standard curve as defined by the Excel macro. If more than one sample dilution hit the working range of the standard curve, we recommend that the dilution closest to the I_{50} value of the standard is used.

   Alternatively; another data analysis software (e.g. the software provided with the plate reader) may be used as long as it supports the 4-parameter logistic curve fit model.

d) Excel macro EMA31 calculation of DA concentration in Algal samples

1. Use the provided Excel macro EMA31 as described in the previous section.
2. Enter the correct dilution factor used for the algal samples, in the corresponding duplicate well windows.
3. The results from the column "Sample extract/solution (pg/mL)" will provide the DA concentration of the algae extracts as pg/mL.
4. If *Pseudo-nitzschia* cell counts are available for the filtered water sample, the results can be converted to pg DA/cell, taking into account the volume of water filtered and the extraction volume.

e) Excel macro calculation of DA concentration in seawater samples

1. Use the provided Excel macro EMA31 as described in the previous section.
2. Enter the correct dilution factor used for the seawater samples, in the corresponding duplicate well windows.
3. The results from the column "Sample extract/solution (pg/mL)" will provide the DA concentration of the seawater samples as pg/mL.

M. QUALITY ASSURANCE MEASURES FOR VALID ANALYSIS

- In order to qualify as a valid calibration curve suitable for accurate quantification of DA in samples, the requirements listed in Table 2 must be fulfilled.
- Sample concentrations should only be calculated from datapoints that are within the valid working range of the calibration curve as defined by the Excel macro.
- The estimated curve fit (%CV of prediction) for the calibration curve should be <20%, as indicated in the "Results" sheet of the Excel Macro EMA31.
- The concentration difference should not be more 15% between two duplicate wells for a given sample.

TABLE 2: QUALITY ASSURANCE REQUIREMENTS FOR VALID CALIBRATION CURVE

Calibration curve Parameter	Requirement
Maximum absorbance (A_{max})	> 0.8 A.U.
Blank/A_{min}	< 0.1 A.U.
Calibration curve I_{20} value	6-20 pg/mL
Calibration curve I_{50} value	35-80 pg/mL
Calibration curve I_{80} value	180-450 pg/mL

N. REFERENCES

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O. QUICK GUIDE

1. Prepare dilutions of standard and samples.

2. Pre-soak the wells for 5-10 minutes with 300 µl Washing buffer. Empty before use.

3. Add 50 µL Standard/Sample buffer to the A_{max} and Blank wells.

4. Add 50 µl Antibody-HRP buffer to the Blank wells.

5. Transfer 50 µL of diluted standards and samples (in duplicate) to the plate.

6. Dilute the concentrated antibody-HRP conjugate and add 50 µL to all wells except the Blank wells.

7. Seal the plate and incubate at room temperature for 1 hour (keep dark).

8. Wash the wells.

9. Add 100 µL TMB peroxidase substrate to all wells.

10. Incubate at room temperature for 15 minutes (keep dark).

11. Add 100 µL of 0.3 M H_2SO_4 to all wells to stop the reaction.

12. Read the absorbance at 450 nm.

13. Calculate the concentrations using the Excel Macro EMA31.

NOTES...

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