$$x = EXP((y - b)/a)$$

Where ***x*** is the OA concentration in the sample (Cs) and ***y*** the absorbance of the sample.

Note: An Excel worksheet to calculate results is available upon request.

3.- Calculate the diarrheic shellfish toxins concentration in tissue (Ct) as follows:

$$Ct\ (\mu g/kg) = \frac{(Cs\ (nM) \times FD \times MW\ (g/mol) \times Ve\ (L))}{Mt\ (g)}$$

Ct: toxins concentration in tissue, expressed as equivalents of OA; **Cs**: toxins concentration in sample; **FD**: Methanolic extract dilution factor (i.e. 640 µL/20 mL → x 31.25); MW: Okadaic acid molecular weight = 805; **Ve**: Methanolic extract volume (0.025L); **Mt**: Tissue weight (5g).

Example: for OA concentration of 1.5 nM: 1.5 nM x 31.25 x 805 g/mol x 0.025L / 5g = 189 µg OA q/kg.
For samples with OA concentration falling outside the working range (< 0.5 nM or > 2.8 nM), results will be reported as < 0.5 nM (or < 63 µg/Kg) or > 2.8 nM (or > 352 µg/kg), respectively.

F. Importance of Okadaic Acid Determination

Okadaic Acid is one of the "diarrheic shellfish poisons" (DSP) produced by the dinoflagellate species *Dinophysis* and *Prorocentrum*. Contamination of shellfish with okadaic acid has been associated with harmful algal blooms throughout the world.

In man, DSP causes dose-dependent symptoms of diarrhea, nausea, and vomiting. The action level established by the FDA is 0.2ppm. The EU has established a level of 160ug OA eq (OA, DTXs, PTXs)/kg.

The Okadaic Acid Phosphatase assay allows the determination of 40 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in less than 1 hour.

G. REFERENCES

1. A Fluorescent Microplate Assay for Diarrheic Shellfish Toxins. Vieytes M. R. et al. Analytical Biochemistry 248, 258-264 (1997).
2. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Mounfort D. O. et al. Toxicon 39, 383-390 (2001).
3. Inter-laboratory validation of the fluorescent protein phosphatase inhibition assay to determine diarrheic shellfish toxins: intercomparison with liquid chromatography and mouse bioassay. Gonzalez J. C., et al. Analytica Chimica Acta 466, 233-246 (2002).

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Date these instructions are effective : 03/18/2025

Version: 01

Kit reagents are manufactured by Zeu-Immunotec

Okadaic Acid (PP2A) Microtiter Plate

Test for the Detection of Okadaic Acid-toxins group

Product No. 520025

1. General Description

This protocol specifies a method for the quantitative determination of Okadaic Acid (OA) and other carboxylic toxins of the OA group including DTX1, DTX2 and DTX3 by a colorimetric phosphatase inhibition assay. This method is applicable to shellfish species such as mussels, clams, oysters and scallops.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Okadaic Acid in solution. Avoid contact of standard and stopping solutions with skin and mucous membranes. If these reagents come in contact with the skin, wash with water. Recommended: Polypropylene material should be avoided throughout sample collection, conservation and treatment, since loss of toxins has been shown to occur.

3. Storage and Stability

The Okadaic Acid-PP2A Kit should to be stored in the refrigerator (2-8°C) prior to use and protected from light. The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the last day of the month as indicated by the expiration date on the box.

4. Test Principle

Test based on the phosphatase activity inhibition by OA-toxins group, responsible for diarrheic shellfish poisoning (DSP). Phosphatase enzyme PP2A is able to hydrolyse a specific substrate, yielding a product that can be detected at 405 nm. Samples containing toxins from the okadaic acid group will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. The concentration of toxin in the sample can be calculated using a standard curve.

5. Limitations of the Okadaic Phosphatase Assay, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in water samples, test interferences caused by matrix effects cannot be completely excluded.

Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, wrong pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the assay and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 40°C). The assay procedure should be performed away from direct sun light.

As with any analytical technique (GC, HPLC, mouse bioassay, etc...) positive results requiring some action should be confirmed by an alternative method.

6. Working Instructions

A. Materials Provided

1. Microtiter plate
2. Phosphatase, 4 vials
3. Standards Okadaic Acid (5): 0.5, 0.8, 1.2, 1.8, and 2.8 nM
4. Chromogenic Substrate, 1 vial
5. Phosphatase Dilution Buffer, 1 vial
6. Stock Buffer Solution, 1 vial
7. Stop Solution, 1 vial
8. Adhesive Film

B. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (50-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate reader (wave length 405 nm)
4. Timer
5. Tape or Parafilm
6. Glass vials with Teflon-lined caps
7. Distilled or deionized water
8. Vortex mixer
9. Heater at 30 +/- 2 °C
10. Water bath at 76 +/- 2 °C
11. Methanol (analytical grade)
12. NaOH, 2.5 N (analytical grade)
13. HCl, 2.5N (analytical grade)
14. Deionized water (grade 2, ISO3696)
15. Graded 50 mL centrifuge tubes with screw caps
16. Tube shaker

C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the assay buffer, substrate and stop solutions in order to equalize the incubations periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

SOLUTIONS

1.- **Okadaic Acid Standards:** to make sure these solutions are homogeneous, it is very important to mix well using a vortex, before applying to the plate.

2.- **Chromogenic Substrate solution:** The solution contains stabilization resin. Make sure this resin is not added to the microwells. To assure that, it is recommended to transfer the volume needed into a transparent labware (i.e.: test tube or eppendorf) and take the solution from that container to add into the wells. *Note:* Do not use this solution if the absorbance of 90 µL of this solution at 405 nm is over 0.6.

3.- **Phosphatase solution:** Add 2.0 mL of phosphatase dilution buffer ***(Phosphatase Dilution Buffer)*** to one of the phosphatase vials ***(Phosphatase)*** and dissolve by mixing gently for 1 hour ± 5 minutes at room temperature (22 ± 2 ºC) to ensure that the enzyme is fully hydrated. **Do not use the tube shaker at any moment**. This solution must be stored under refrigeration if not in use immediately after preparation. Do not use the phosphatase solution for following days. Each enzyme vial contains enough volume for 24 wells. If more than one vial is used in the assay, dissolve each vial as described above, make a pool with the content of the vials and mix gently, by inversion, before use.
*__Attention__: this reagent is blue and becomes brownish when dissolved. If brownish color is noticed before hydration, discard this reagent as it could be damaged.

4.- **Buffer solution x1:** dilute the ***Stock Buffer Solution*** included in the kit by mixing 1 volume with 9 volumes of deionized water. Use buffer solution x1 only freshly made, and store under refrigeration if not in use immediately after preparation.

5.- **2.5 N NaOH:** weigh 100 g of NaOH and add to 500 mL of water and dissolve. Transfer to a volumetric flask and add deionized water up to a final volume of 1000 mL.

6.- **2.5 N HCl:** add 205 mL of HCl (37 %) to 400 mL of deionized water already contained in a volumetric flask. Make the volume up to 1000 mL with deionized water.

D. Assay Procedure

Warning:

The volume of some reagents used in this assay is small and special attention must be paid when added to the wells:

- Make sure the pipettes are calibrated before running the assay.
- Use pipettes according to the volumes to be dispensed. Use pipettes with a maximum pipette volume of 100 or 200 µL.
- Be sure that the incubator's temperature is stabilized before use.

It is recommended to run samples and standards in duplicate.

1.- Add 50 µL of samples or standards.
2.- Add 70 µL of the Phosphatase Solution to each well. Mix well by gentle tapping on the side of the plate.
3.- Cover the plate with the adhesive film provided and incubate for 20 ± 0.5 minutes at 30 ± 2 ºC.
4.- Remove the adhesive film and add 90 µL of Chromogenic Substrate to each well. Mix well by gently tapping on the side of the plate.
5.- Cover the plate with the adhesive film and incubate 30 ± 0.5 minutes at 30 ± 2 ºC.
6.- Remove the adhesive film and add 70 µL of Stop Solution to each well.
7.- Read absorbance of samples and standards at 405 nm.

E. Sample Preparation

The method described below includes a hydrolysis step to detect all toxins forms of okadaic acid (okadaic acid and dinophysistoxins).

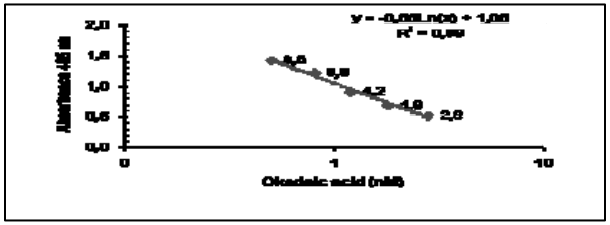
1.- Clean the shell thoroughly using water
2.- Open the shellfish by cutting the abductor muscles.
3.- Wash inside the shell thoroughly to get rid of any dirt.
4.- Remove the tissue inside the shell by cutting all the muscles attached to the shell.
5.- Place the shellfish tissue in a filter paper for few minutes to remove water in excess.

It is recommended to use graded 50 mL centrifuge tubes with screw caps during the following steps of hydrolysis in order to prevent loses due to labware changes.

6.- Mash the shellfish tissue to obtain a representative sample and weigh 5 g. Add 25 mL of Methanol and homogenize the mixture for 2 minutes using a tube shaker.
7.- Centrifuge at 2000 g for 10 min at 4 ºC. The supernatant (*methanolic extract*) is poured into a centrifuge tube.
8.- Take 640 µL of *methanolic extract* and pour into another centrifuge tube.
9.- Add 100 µL of 2.5 N NaOH.
10. - Seal and heat at 76 ± 2 ºC for 40 minutes.
11. - Add 80 µL of 2.5 N HCl (the sample does not need to be cooled down previously).
12. - Add up to 20 mL of **Buffer solution x1**.

E. Calculations and Graphic Representation of Results

1. - Obtain a standard curve by plotting the absorbance values in a linear *y axis* and the concentration of okadaic acid in a logarithmic *x axis* and use a logarithmic fitting as shown in the graphic next page. R^2 has to be greater than or equal to 0.96.



2. - The OA concentration contained in the sample (Cs) is calculated by interpolation into the calibration curve or using the following equation: