OKADAIC ACID ELISA

A competitive enzyme immunoassay for quantitative analysis of okadaic acid in scallop, mussel and oyster samples.
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11. LITERATURE


12. ORDERING INFORMATION

For ordering the okadaic acid ELISA kit please use cat. Code 5191OKA.
10. INTERPRETATION OF RESULTS

Subtract the mean OD value of the wells A1 and A2 from the individual OD of the wells containing the standards and the samples. The OD values of the six standards and the samples (mean values of the duplicates) are divided by the mean OD value of the zero standard (wells B1 and B2) and multiplied by 100. The zero standard is thus equal to 100% (maximal OD) and the other OD values are quoted in percentages of the maximal OD.

OD standard (or sample) x 100 = % maximal OD

OD zero standard

**Calibration curve:**
The values (% maximal OD) calculated for the standards are plotted (on the linear Y-axis) versus the okadaic acid concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.2 - 10 ng/ml range.

The amount of okadaic acid in the samples is expressed as okadaic acid equivalents. The okadaic acid equivalents in the extracts (ng/ml), corresponding to the % maximal absorbance of each extract, can be read from the calibration curve.

The calculated okadaic acid equivalents have to be multiplied by 200 to obtain the okadaic acid equivalents in the shellfish tissue.

BRIEF INFORMATION

The okadaic acid ELISA is a competitive enzyme immunoassay for the screening and quantitative analysis of the shellfish toxin okadaic acid in various matrices. The test is based on rabbit polyclonal antibodies against okadaic acid. With this ELISA, 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 samples can be analysed. The ELISA kit contains all reagents required to perform the test, including ready-to-use standards. Materials and chemicals necessary for the extraction of okadaic acid from sample material are not included in the test kit.

1. INTRODUCTION

Marine biotoxins, also called phytotoxins or shellfish toxins, are naturally occurring compounds produced by algae and phytoplankton. Under normal conditions these compounds do not cause any problem. However, filter feeders such as clams, mussels, oysters and crustaceans can consume large quantities of these algae when environmental conditions result in harmful algal blooms, also known as ‘Red Tides’. High concentrations of shellfish toxins then accumulate in these animals causing illness amongst people who eat them. There are four syndromes called shellfish poisoning, i.e. paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP) and amnesic shellfish poisoning (ASP). A variety of symptoms are reported to arise from these poisonings, including stomach cramps, abdominal pain, diarrhea, headaches, memory loss, paralysis and in some cases even death.

PSP is caused by saxitoxin and its analogues. DSP is primarily caused by okadaic acid (OA) and several analogues of OA, whereas ASP is caused by domoic acid (DA).

OA, a polyether toxin, is a potent tumor promoter. It was named from the marine sponge *Halichondria okadai*, from which it was first isolated. The real producers of OA belong to the algae group of dinoflagellates. OA and its analogues, the dinophysistoxins (DTX1, DTX2, and DTX3), together form the group of OA-toxins. These toxins are lipophilic and heat-stable, and can be found in various species of shellfish. Inhibition of serine/threonine phosphoprotein phosphatases is assumed to constitute the mode of action of OA-group toxins.
In the European Union, Regulation (EC) no 853/2004 stipulates that live bivalve mollusks must not contain OA in total quantity (measured in the whole body or any part edible separately) that exceeds a limit of 160 µg OA equivalents (OA, dinophysistoxins, and pectenotoxins together) per kilogram.

2. PRINCIPLE OF THE OKADAIC ACID ELISA

The microtitre plate based ELISA kit consists of 12 strips, each containing 8 wells, precoated with sheep antibodies to rabbit IgG. Antibodies (rabbit polyclonal anti-okadaic acid antibodies), horseradish peroxidase (HRP) labelled okadaic acid (enzyme conjugated okadaic acid-HRP) as well as okadaic acid standard solutions or samples are added to the precoated wells, followed by a single incubation step. The rabbit anti-okadaic acid antibodies are bound by the immobilized sheep anti-rabbit antibodies and simultaneously the okadaic acid-HRP and okadaic acid present in the standard solution or sample compete for binding to the anti-okadaic acid antibody (competitive enzyme immunoassay). After incubation for 30 minutes, non-bound reagents are removed in a washing step. The amount of bound okadaic acid-HRP is visualised by the addition of a substrate/chromogen (H₂O₂/TMB; TetraMethylBenzidine). The colourless chromogen is converted by the enzyme (HRP) into a blue reaction product. The more okadaic acid present in the standard solution or sample, the less colour is developed. The substrate is stopped by the addition of sulfuric acid. In the acidic environment the blue colour changes into a yellow colour. The obtained colour intensity is measured photometrically at 450 nm and is inversely proportional to the okadaic acid concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

For this okadaic acid ELISA a specific antibody is used, obtained by immunisation of rabbits with a okadaic acid protein conjugate. This antibody showed no cross-reactivity to any of the following common PSP shellfish toxins such as: saxitoxin, neosaxitoxin, dc-STX, gonyautoxins-1/4, gonyautoxins-2/3, B-2, B-1, C-1/2 and domoic acid.

The reactivity pattern of the antibody as tested in buffer is:
- Okadaic acid 100 %
- Dinophysistoxins DTX-1 78 %
- Dinophysistoxins DTX-2 2.6%

The detection capability (CCß) is defined as the smallest concentration of analyte that can be identified in a sample with an error probability of less or equal to 5%.

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9. ASSAY PROCEDURE

Rinsing protocol
In ELISA’s, between each immunological incubation step, un-bound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results. Basically, manual rinsing or rinsing with automatic plate wash equipment can be done as follows:

Manual rinsing
1. Empty the contents of each well by turning the microtiter plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out for at least 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate washing equipment
When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is correctly dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute at least three rinsing cycles.

Assay Protocol
1. Prepare samples according to Chapter 7 (Sample treatment) and prepare reagents according to Chapter 8. Microtiter plate is ready-to-use.
2. Pipette 100 µl of zero standard in duplicate (well A1, A2).
3. Pipette 50 µl of zero standard in duplicate (well B1, B2).
4. Pipette 50 µl of each standard dilution in duplicate (well C1,2 to H1,2, i.e. 0.2, 0.5, 1, 2, 5 and 10 ng/ml).
5. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).
6. Add 25 µl of conjugate (HRP) to all wells, except wells A1 and A2.
7. Add 25 µl antibody to all wells, except wells A1 and A2.
8. Seal the microtiter plate and shake the plate for 1 minute.
9. Incubate for 30 minutes at room temperature (20°C – 25°C).
10. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
11. Pipette 100 µl of ready to use peroxide/TMB into each well. Mix thoroughly and incubate for 15 min. at room temperature in the dark (20°C - 25°C).
12. Read the absorbance values immediately at 450 nm.
8. PREPARATION OF REAGENTS

Before starting the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2°C to +8°C. For longer storage see Chapter 4 (Handling and storage).

The reagents included in the test-kit are sufficient to carry out at least 96 analyses (including standard analyses). Each standard and sample is analysed in duplicate.

**Microtiter plate**

Return unused strips into the resealable zip lock bag with desiccant and store at +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

**Rinsing buffer**

The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

**Substrate solution**

The substrate solution (ready-to-use) precipitates at 4°C. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.

**Dilution buffer (10x concentrated)**

The sample dilution buffer is 10 times concentrated. Before dilution (10 ml buffer + 90 ml distilled water) the concentrated buffer should be at room temperature and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water. The 10 times diluted buffer can be stored in a refrigerator (+2°C to +8°C) until the expiry date stated on the kit label.

**Okadaic acid enzyme conjugate**

The okadaic acid enzyme conjugate is provided in concentrated form. Spin down the conjugate in the vial by a short centrifugation (1 minute, 1000 x g). The conjugate is diluted 100x in dilution buffer (e.g. 5 µl conjugate + 495 µl of dilution buffer). Per 2x8 wells 400 µl of diluted conjugate is required. Store the unused conjugate immediately in the dark at +2°C to +8°C.

**Okadaic acid antibody**

The okadaic acid antibody is provided lyophilised. Reconstitute the lyophilised antibodies with 4 ml dilution buffer, mix thoroughly. Store the unused antibody immediately in the dark at +2°C to +8°C.

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The limit of detection (LOD) is calculated as: Xn + 3SD (n > 20). For extraction procedures the LOD is calculated reflecting the respective concentration steps. The LOD is determined under optimal conditions. Cut-off values need critical consideration.

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The calibration curve should be virtually linear in the range of 0.2 to 10 ng/ml. The concentration of residue necessary to cause 50% inhibition of antibody binding is estimated between 1 and 4 ng/ml.

4. HANDLING AND STORAGE

- Store the kit at +2°C to +8°C in a dark place. DO NOT FREEZE.
- Return any unused microwells to their original bag and reseal them together.
- The quality of the product can not be assured after the expiry date.
- Do not use partially used strips.
- Reconstitute or dilute the kit components immediately before use, but after the components are at room temperature.
- Any direct action of light on the substrate/chromogen solution should be avoided.
- Any coloration of the chromogen solution is indicative of deterioration. The reagent should be discarded.
- The stopping reagent contains sulfuric acid. Avoid contact with skin.
- After the expiry date, dilution or adulteration of reagents may result in a loss of sensitivity.
- A value of less than 0.6 absorbance units for the zero standard may indicate deterioration of reagents.
- Avoid introducing air bubbles into solutions in the wells during assay. Before reading a plate on a plate reader, ensure that no air bubbles are present in the wells as they may interfere with OD readings.
- Okadaic acid is subject to degradation when exposed to the light or warmth.
5. KIT CONTENTS

Manual

1 sealed microtiter plate (12 strips, 8 wells each), coated with purified sheep anti-rabbit IgG

Position of the reagents in the kit. For preparation of the reagents see Chapter 8.

6. SAFETY PRECAUTIONS

- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiry date and do not intermix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate, which might crystallise at 4°C.
- Optimal results will be obtained by strict adherence to this protocol.
- Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy.

7. SAMPLE TREATMENT

This test can be used for measuring okadaic acid into different matrixes. We have provided you information on different techniques but alternative methods may be used.

Shellfish tissue

- To 1 g of homogenised shellfish tissue, 1 ml of water is added.
- Mix (vortex) for 1 minute.
- Add 2 ml of methanol 100%.
- Mix (vortex) for 1 minute.
- Centrifuge at 2000 x g for 10 minutes.
- Take the clear supernatant and filter through a 0.45 µm filter (Millex HV, Millipore).
- Dilute the samples in sample dilution buffer* (1:50). (Pipette 10 µl of sample and add 490 µl of sample dilution buffer).
- Mix thoroughly.
- Use 50 µl per well.

Remark: A fine particulate material often appears after cooling the extract. Filtration is therefore suggested prior to preparing the dilutions.

- Sample dilution buffer
  9.2 ml dilution buffer ready-to-use + 0.8 ml 100% methanol