DSP Rapid Kit

DSP: Diarrhetic Shellfish Poisoning
(A colorimetric phosphatase inhibition assay)

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Notice

The PP2A Stock solution of this kit might contain a small amount of recombinant Baculovirus because of manufacturing method.

Introduction

Diarrhetic Shellfish Poisoning (DSP) is caused by the ingestion of shellfish contaminated by toxic dinoflagellates. DSP has been observed worldwide and caused problems to public health and the shellfish industry. To detect DSP toxins, a colorimetric phosphatase inhibition assay was developed using a highly purified recombinant human PP2A C-subunit. The assay is very sensitive, fast, easy, accurate, reproducible and inexpensive to detect DSP toxins (OA group) in the shellfish. It does not require expensive equipment or a high level of skills. The lowest detectable concentration of OA is 0.1 μg/g in shellfish digestive glands, which comprise about 10-30% of all the soft tissue of the shellfish by weight.

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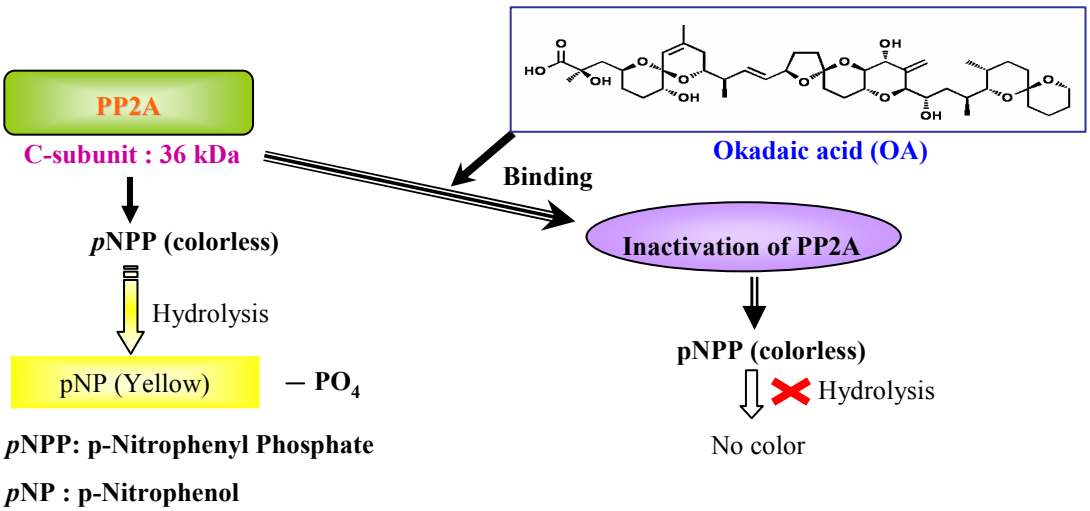
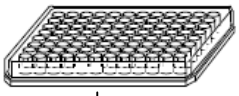
Assay Principle

The assay is based on the inhibition of the protein phosphatase (PP2A) by DSP toxins (OA and DTXs). PP2A can hydrolyze a colorless artificial substrate, *p*-nitrophenyl phosphate (*p*-NPP), and produces the yellow color of *p*-nitrophenol (*p*-NP) in the alkaline solution. The intensity of the color is proportional to the enzyme activity and the absorbance is measured at 405 nm. The concentration of DSP toxins in the sample is calculated from the standard curve produced using known concentrations of OA.



Summary of Assay



96 Well plate

← Add 50 μl of sample and **OA Reference Solution**

← Add 100 μl of **Substrate Working Solution**

← Add 100 μl of **PP2A Working Solution**

Mix for 1 min by a microplate mixer

Incubate for 30 min at 36℃

Measure absorbance at 405 nm against 492nm as reference.

Kit Components

	Item	Amount	Storage
1	Okadaic Acid (OA) Reference Solutions (0, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 10.0 ng/ml)	8 x 0.5 ml	-20°C
2	Sample Buffer	40 ml	-20°C
3	1.25 N NaOH Solution	1.5 ml	Room temperature
4	1.25 N HCl Solution	1.5 ml	Room temperature
5	PP2A Buffer A	11 ml	-20°C
6	PP2A Buffer B	1 ml	-20°C
7	PP2A Stock Solution (0.5 ml tube)	80 µl	-20°C
8	Substrate Buffer	12 ml	-20°C
9	Substrate Tablet (*p*-NPP)	20 mg tablet	-20°C
10	96 Well plate	1	Room temperature
11	Adhesive Film	1	Room temperature

Caution: This kit contains strong alkali and acid solutions, and toxin (Okadaic acid). Wear disposable gloves, eye protection and protective clothing when preparing and handling reagents and samples.

Additional Materials Required

- Microplate reader capable of measuring absorbance at 405 nm.
- Microplate mixer
- Incubator for use at 36°C
- Heating apparatus: water bath or heating block
- Vortex mixer
- Reagent reservoirs
- Micro pipettes (20-200 ml and 200-1000 ml) with disposable tips.
- Multichannel pipette
- 15 ml and 50 ml Polyethylene tubes
- 1.5 ml Microcentrifuge tubes
- 2 ml Screw capped tubes
- Deionized or distilled water

Sample Preparation

Sample for Free OAs (OA, DTX1, and DTX2)

Collect the digestive gland (DG) from more than 10 shellfish.

↓ Remove excess water by placing on filter papers.

Mince the DG and weigh 2 g into a 50 ml centrifuge tube.

↓ ← Add 18 ml of 90% methanol.

Homogenize for 1 min with Polytron homogenizer or an equivalent (any other capable homogenizer) at room temperature.

↓ Centrifuge at 2,500 g for 10min.

Transfer the supernatant into a 15 ml tube.
(the supernatant is designated **Extract Supernatant**)

↓ Keep the Extract Supernatant at 4℃.

Pipette 50 μl of Extract Supernatant into a 1.5 ml microcentrifuge tube and add 950 μl of the Sample Buffer **(Sample for Free OAs)**.

Note: Sample Buffer may contain precipitates that can be dissolved by shaking at room temperature.

Sample for Total OAs (OA, DTX1, DTX2 and DTX3)

Pipette 500 μl of the **Extract Supernatant** into a screw-capped 2 ml tube.

↓ Add 100 μl of 1.25 N NaOH solution, tighten the cap and mix.

Heat at 100℃ for 20 min or at 80℃ for 40 min.

↓

Cool the tube to room temperature and add 100 μl of 1.25 N HCl to neutralize the solution.

↓

Pipette 50 μl of the neutralized solution into a 1.5-ml microcentrifuge tube and add 950 ml of the Sample Buffer **(Sample for Total OAs)**.

Preparation of Working Solution

1. **Substrate Working Solution:** Dissolve the Substrate tablet with 12 ml of Substrate Buffer, wrap the tube with aluminium foil and keep at 4°C until used. Use it within 1 week of preparation.
2. **PP2A Working Solution:** First, prepare the PP2A dilution buffer first by mixing gently PP2A Buffer A (11 ml) and PP2A Buffer B (1 ml), and add 70 µl of PP2A Stock Solution (Spin-down before use) to the dilution buffer. The working solution should be used within 2 hours when kept at 4°C or within 30 minutes when kept at room temperature.

Assay Procedure

Note: Allow all the components, except PP2A Solutions, to come to room temperature before use.

All assays should be carried out in triplicate.

An example for placing OA Reference Solutions and test samples in the 96-well microplate is shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		OA R1	OA R2	OA R3	OA R4	OA R5	OA R6	OA R7	OA R8			
C		OA R1	OA R2	OA R3	OA R4	OA R5	OA R6	OA R7	OA R8			
D		OA R1	OA R2	OA R3	OA R4	OA R5	OA R6	OA R7	OA R8			
E		SF1	SF2	SF3	SF4	SF5	ST1	ST2	ST3	ST4	ST5	
F		SF1	SF2	SF3	SF4	SF5	ST1	ST2	ST3	ST4	ST5	
G		SF1	SF2	SF3	SF4	SF5	ST1	ST2	ST3	ST4	ST5	
H												

OA Reference Solution	R1	R2	R3	R4	R5	R6	R7	R8
Concentration (ng/ml)	0	0.5	1.0	1.5	2.0	2.5	5.0	10.0

SF: Sample for Free OAs
ST: Sample for Total OAs

Assay Procedure

1. Add 50 μl of the Reference OA Solutions , Sample for Free OAs and Sample for Total OAs to each well as illustrated in the example of placing samples. We recommend not to use the most outer wells because of their inconsistency.
2. Add 100 μl of Substrate Working Solution. It is recommended to use a multichannel pipette.
3. Add 100 μl of PP2A Working Solution.
4. Seal the plate with the Adhesive Film provided and mix for 1 minute with a microplate mixer.
5. Incubate the plate for 30 minutes at 36℃ and get the microplate reader ready.
6. After incubation, carefully remove the Adhesive Film and measure the color within 10 minutes with a microplate reader at 405 nm against 492 nm as reference. The absorbance value of the OA-R1 should reach 0.4 or over. If the measurement can not be started within 10 minutes, the plate can be kept at 4℃ for the maximum of 30 minutes.

∗ Detection range: 0.01~0.1 μg/gDG

Data Calculation

The data calculation is performed semi-automatically on a Microsoft Excel spreadsheet (File name “CALC.xls”)

The Excel spreadsheet is on the homepage (www.ttc.co.jp **).**

1. Input the net-absorbance values obtained for OA Reference Solutions into cells colored light blue in the Table placed at the top of the sheet. The numerals shown on the cells indicate the OA concentrations converted to that in the 1g tissue.
2. Check the relative standard deviation values. If the value exceeds 10%, a possible outlier should be excluded.
3. The standard curves for the net-absorbance values and percent of residual enzyme activity are drawn at the middle left and right of the sheet, respectively.
4. Input the net-absorbance values obtained for the sample solutions to the corresponding cells which are tabulated at the bottom of the sheet and colored light green. The sample numbers with prime denote hydrolyzed samples.
5. Check the relative standard deviation values. If the value exceeds 10%, a possible outlier should be excluded by erasing the cell for the outlier.
6. The calculation results expressed in terms of OA-equivalents are shown in the right end column in μg/g. Values less than 0.1 μg (OA-equivalent)/g tissues are expressed as <0.1 μg/g. For calculation of the total OA-equivalent, a factor “1.4” is used to calibrate the value increase due to added alkali and acid solutions.

Quality Assurance

Quality of the performed assay is assured by the following criteria.

1. The absorbance value for OA-R1 should reach 0.4 or over.
2. The absorbance value for OA-R8 should be smaller than 0.15.
3. The relative error* must be smaller than 10%, if a possible outlier is excluded from the three run data in the assay.
   *(data A – data B)/(data A + data B), where data A > data B.

References

1. Ikehara T, Shinjo F, Ikehara S, Imamura S, Yasumoto T.: Baculovirus expression, purification, and characterization of human protein phosphatase 2A catalytic subunits alpha and beta. Protein Expr Purif. 45(1):150-6, 2006.
2. Tubaro A, Florio C, Luxich E, Sosa S, Della Loggia R, Yasumoto T.: A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon 34(7):743-52, 1996.
3. Takai A, Mieskes G.: Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases. Biochem J. 275:233-9, 1991

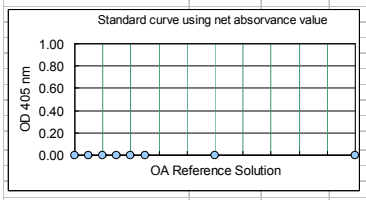
Example of Data Calculation Sheet

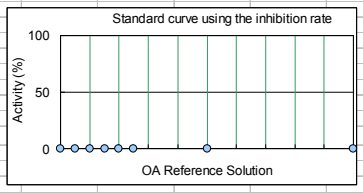
DATE

＜Data calculation sheet＞ Laboratory:

Net absorbance values

OA Reference Solutions	R1	R2	R3	R4	R5	R6	R7	R8
OA concentration (μg/g of DG)	0	0.1	0.2	0.3	0.4	0.5	1	2
run 1								
run 2								
run 3								
Average	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
relative standard deviation	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Inhibition rate (%)	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!





sample name	Hydrolysis*1	run 1	run 2	run 3	average	relative standard deviation(%)	result (μg/g)*2
1					#DIV/0!	#DIV/0!	#DIV/0!
2					#DIV/0!	#DIV/0!	#DIV/0!
3					#DIV/0!	#DIV/0!	#DIV/0!
4					#DIV/0!	#DIV/0!	#DIV/0!
5					#DIV/0!	#DIV/0!	#DIV/0!
6					#DIV/0!	#DIV/0!	#DIV/0!
1'	h				#DIV/0!	#DIV/0!	#DIV/0!
2'	h				#DIV/0!	#DIV/0!	#DIV/0!
3'	h				#DIV/0!	#DIV/0!	#DIV/0!
4'	h				#DIV/0!	#DIV/0!	#DIV/0!
5'	h				#DIV/0!	#DIV/0!	#DIV/0!
6'	h				#DIV/0!	#DIV/0!	#DIV/0!

*1 For hydrolysis sample input "h" in the column indicated "hydrolysis" to enable automatically calibrate the result.

*2 The result is expressed as the concentration in the sample in μg/g.

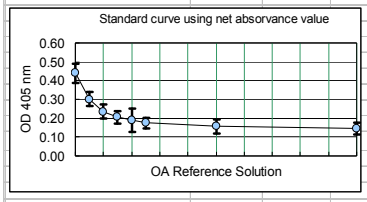
Calculation Example

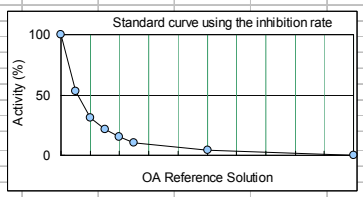
DATE

＜Data calculation sheet＞ Laboratory:

Net absorbance values

OA Reference Solutions	R1	R2	R3	R4	R5	R6	R7	R8
OA concentration (μg/g of DG)	0	0.1	0.2	0.3	0.4	0.5	1	2
run 1	0.465	0.315	0.244	0.214	0.202	0.181	0.164	0.149
run 2	0.422	0.296	0.234	0.202	0.183	0.174	0.153	0.140
run 3	0.430	0.294	0.227	0.203	0.181	0.171	0.154	0.144
Average	0.4390	0.3017	0.2350	0.2063	0.1887	0.1753	0.1570	0.1443
relative standard deviation	5.2%	3.8%	3.6%	3.2%	6.1%	2.9%	3.9%	3.1%
Inhibition rate (%)	100.0	53.4	30.8	21.0	15.0	10.5	4.3	0.0





sample name	Hydrolysis*1	run 1	run 2	run 3	average	relative standard deviation(%)	result (μg/g)*2
1		0.163	0.162	0.167	0.1640	1.6133	0.8091
2		0.274	0.266	0.276	0.2720	1.9454	0.1445
3		0.414	0.412	0.418	0.4147	0.7367	0.0177
4		0.225	0.224	0.233	0.2273	2.1699	0.2267
5					#DIV/0!	#DIV/0!	#DIV/0!
6					#DIV/0!	#DIV/0!	#DIV/0!
1'	h				#DIV/0!	#DIV/0!	#DIV/0!
2'	h				#DIV/0!	#DIV/0!	#DIV/0!
3'	h				#DIV/0!	#DIV/0!	#DIV/0!
4'	h				#DIV/0!	#DIV/0!	#DIV/0!
5'	h				#DIV/0!	#DIV/0!	#DIV/0!
6'	h				#DIV/0!	#DIV/0!	#DIV/0!

*1 For hydrolysis sample input "h" in the column indicated "hydrolysis" to enable automatically calibrate the result.

*2 The result is expressed as the concentration in the sample in μg/g.